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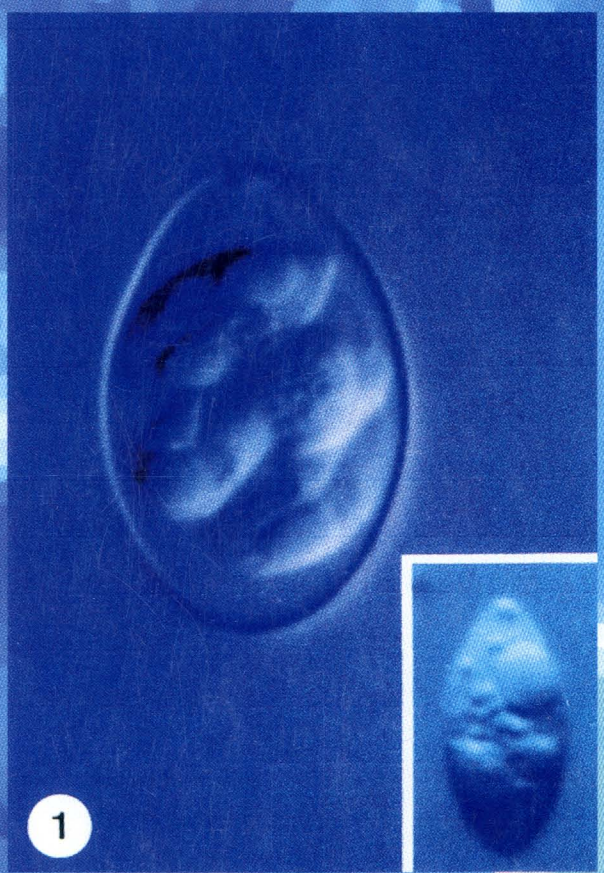
BIOTECHNOLOGY
Guidelines on techniques in coccidiosis research



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Report EUR 16602 EN

Cover photograph

Sporulated oocytes of *Eimeria intestinalis* from the rabbit (magnification x 2 300)
(D. Licois and P. Coudert; micrographs: D. Licois)

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Guidelines on techniques in coccidiosis research

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Preface

In 1989/90, a European program entitled „**Basic Research on Coccidiosis of Poultry and Farm Animals and Development of Vaccines using Biotechnological Procedures**" was initiated. This program was coordinated by the Commission of the European Communities (now European Union) in the framework of the COST Action No.89 and supported by the Bridge Program. At the end of 1993 about 100 scientists from 13 European countries were listed as participants of the Action which had significantly stimulated contacts and cooperation within Europe and also with some groups abroad (Australia, Israel, USA). The research programmes included several genera of coccidia, namely *Eimeria*, *Isospora*, *Sarcocystis* and *Cryptosporidium*, and covered different aspects of parasite biology, immunology and molecular biology. Many species of the coccidia are causative agents of economically important intestinal diseases of poultry and other farm animals in all regions of the world. The main aim of COST 89 was to elaborate basic data and concepts towards the development of vaccines against coccidiosis in various animal species as an alternative approach to the existing hygienic and chemoprophylactic control measures.

The international cooperation between otherwise independent groups of scientists in several European countries stimulated the preparation of „**Guidelines on Techniques in Coccidiosis Research**". These guidelines have several purposes: On the one hand they should contribute to a better knowledge of research techniques used in various laboratories and they should support the necessary development of uniform or even standardised techniques which are the basis for research according to regulations of „Good Laboratory Practice". On the other hand the guidelines should facilitate the establishment and use of certain research techniques in the daily laboratory work. Not a complete review of the existing techniques was the aim, but a summary of practical experiences with a rather broad array of techniques. COST 89 offered the unique opportunity that 41 authors from 8 countries contributed their knowledge and practical experience for the guidelines. Still it was not possible to cover all aspects and fields. We hope, however, that the guidelines will be accepted as a first step towards a better exchange of technical information within the international community of scientists, and that they may be beneficial and helpful to scientists working in the fascinating and important field of coccidiosis research.

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June 1995

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Abbreviations

A: Absorbance

Ab: Antibody

bp: base pair

BSA: Bovine serum albumin

b.w.: body weight

CAM: Chorio-allantois membrane

CH: Switzerland

COST: European Cooperation in the Field of Science and Technology

CR: Czeck Republic

d: day

D: Germany

Da: Dalton

DEAE-cellulose: Diethylaminoethyl cellulose

DK: Denmark

DMSO: Dimethylsulfoxide

DNA: Deoxyribonucleic acid

EDTA: Ethylene diamine tetra acetic acid

ELISA: Enzyme-linked immunoabsorbent assay

F: France

FCS: Fetal Calf Serum

FITC: Fluorescein isothiocyanate conjugate

g: gram

x g: gravitational field (in centrifuging)

G: gauge

Gy: Gray

h: hour

HBSS: Hank's Balanced Salt Solution

IAH: Institute of Animal Health, Compton

ID_{50}: Infection dose 50 %

IFAT: Immunofluorescent antibody test

IgA, IgD, IgE, IgG, IgM: Immunoglobulins

i.m.: intramuscular

i.p.: intraperitoneal

i.v.: intravenous

i.u.: international unit

kbp: kilobase pair

MAB: Monoclonal antibody

MEM: Minimum Essential Medium

MIFC: Merthiolate Iodine Formalin Concentration Technique

min: minute

mOsm: milli osmoles

mRNA: messenger RNA

OD: Optical density

Opg: Oocysts per gram

PBS: Phosphate Buffered Saline

PCR: Polymerase Chain Reaction

p.i. post infection

rpm: revolutions per minute

s.c.: subcutaneous

SDS-PAGE: Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis

sec: second

SEM: Scanning Electron Microscopy

SPF: Specific Pathogen Free

sp., spp.: species (singular and plural)

TEM: Transmission Electron Microscopy

TMB: Tetra methyl benzidine

U: Enzyme unit (international)

UK: United Kingdom

vol.: volume

wt.: weight

w/v: weight in volume

Part I. *Eimeria* and *Isospora*

1. Maintenance in animal hosts

1.1. *Eimeria* species and strains of chickens

M. W. Shirley

The husbandry procedures described below refer predominantly to those used at the former Houghton Laboratory of the Institute for Animal Health (IAH).
Included are descriptions of cages and the more secure accommodation (isolators) that were used for the maintenance of Light Sussex chickens kept for studies with the Houghton reference strains (and field isolates) of *Eimeria*. Isolators can provide very flexible accommodation (making possible the passage of several strains at one time within the same room) and are especially useful if the regular passage of many pure strains is required but the number of individual animal rooms is limited.

1.1.1. Host animals

Different breeds/strains of chickens may be expected to vary with respect to their susceptibility to infection with *Eimeria spp.* and initial studies are usually necessary to determine the doses of individual species/strains that give the maximum yields of oocysts.

It is not necessary to use birds from an SPF flock (young broilers or chicks of commercial egg-laying breeds may be acceptable), but they should be all reared coccidia-free.

1.1.2. Maintenance of host animals

For the continuing propagation of reference laboratory strains of *Eimeria* species it is essential to use animals maintained under strict isolation. If, however, the new culture will not be used for further passage (for example if the oocysts are being propagated for an experiment) it is possible that strict isolation may not be relevant and that the culture can be passaged in chickens kept within open cages in non-secure rooms.

Prospects for contamination between and within rooms can be reduced by ensuring that the floors of all rooms are kept as dry as possible and that staff change their outer clothing, shoes and wear disposable gloves when entering different rooms.
Purpose-built isolators can provide an absolute barrier to coccidial oocysts and it is therefore feasible to keep a number within the same room so that several different species/strains of *Eimeria* may be passaged concurrently.

- **Cages**

If large numbers of cages are to be used, it is helpful to have them mounted on wheeled frames so that they can be transferred easily between different accommodation and/or the washing facilities, etc.

Tab. 1: Chicken cages

Type of cage	Height (cm)	Length (cm)	Width (cm)
Single bird (in mobile units of 12)	41	18	37
"Medium" - sized (stands of 9)	44	46	51
"Large " - sized (stands of 6)	51	69	71

*) **Note:** Types and sizes used in various countries may vary according to the national animal welfare regulations.

This arrangement and number of cages can, for example, be used to accommodate individual groups of 5 birds up to 3-5 weeks of age (in medium and large cages) or groups comprising up to 12 birds housed singly in single bird cages.

Cages fitted with a wire mesh floor enable the daily collection of faeces onto plastic sheets placed on a removable tray.

- **Isolators**

Tab. 2: Isolators for chickens

Type of isolator	Height (cm)	Length (cm)	Width (cm)
Single bird	61	78	31
"Medium" - sized (stands of 9)	61	84	47
"Large " - sized (stands of 6)	69	213	86

*) **Note**: Types and sizes used in various countries may vary according to the national animal welfare regulations

Isolators may be constructed in stainless steel or fibre glass and can be fitted with positive or negative filtration systems. For positive-pressure isolators, incoming air can be drawn throuth a 40-pore polyurethane foam pre-filter and then through a high efficiency particulate (HEPA) air filter (efficiency of 99.95% at 0.3 mm). Outgoing air may be drawn through another pre-filter in the extract box and another 0.3 mm filter to ensure that any coccidial oocysts are contained.

After use, the soiled cages and/or isolators may be fumigated (if it is possible to use ammonia solutions or a noxious gas such as methylbromide) within the rooms prior to removal and being thoroughly washed, cleaned and, if possible, sterilised by steam. Cleaned rooms can be fumigated once again prior to restocking.

Note: Methylbromide is highly toxic and its use needs strict precautions for safe handling.

- **Food and water**

Food and water should be available to the animals *ad libitum* at all times.

Food may be fumigated with methylbromide gas if required, although with rations bought from suppliers of high quality diets for laboratory animals, the risk of them

being contaminated with coccidial oocysts should be minimal. Water can be obtained directly from the mains, and there is no need to take any special action.

1.1.3. Infection of animals

Many of the conditions for the successful propagation of *Eimeria* species in chickens will have to be determined empirically by the investigator. For example, key factors are the strain or type of host animal being used and the strain or population of the parasite being passaged. However, a few guidelines can be given and some recommendations on doses for infection and times for oocyst collection are summarised in Tab. 3.

Tab. 3: Doses of sporulated oocysts for infection of chickens and days for oocyst collection

Species	Dose of oocysts ($x10^2$) per bird	Time after inoculation of oocysts for collection of faeces (days)
Eimeria acervulina	10-100	4-6* or 5-7**
Eimeria brunetti	5-20	6-8
Eimeria maxima	5-20	6-8
Eimeria mitis	50-100	4-6* or 5-7**
Eimeria necatrix	60-80	6-9
Eimeria praecox	5-10	4-5* or 5-7**
Eimeria tenella	5-20	6-7

Note: Day 0 = day when parasites were given. Therefore, day 7 is one week later.
*) If birds are dosed early a.m.; **) If birds are dosed late p.m.

Oocysts of *E. tenella* and *E. necatrix* can be recovered more conveniently from the caeca on days 7 and 8 after infection, respectively.

The times for the collection of oocysts given above relate to the following prepatent times (i.e. time after inoculation of oocysts when the first of the new progeny oocysts are passed in the faeces) (Tab. 4).

Tab. 4: Prepatent periods and site of development of *Eimeria* species (Houghton strains) from chickens (see also Fig. 3)

Species	Prepatent period (hours)	Site of development
Eimeria acervulina	89	small intestine
Eimeria brunetti	120	small intestine, caeca, rectum
Eimeria maxima	120	small intestine
Eimeria mitis	91	small intestine, caeca, rectum
Eimeria necatrix	138	small intestine
Eimeria praecox	84	small intestine
Eimeria tenella	132	caeca

1.1.4. Preparation of doses of oocysts

Doses of oocysts are prepared for either passage or for experimental studies. The latter clearly require more care and accuracy in their preparation, especially if only small numbers of oocysts (perhaps 100) are to be be given and the intention is to estimate the numbers of progeny oocysts excreted in the faeces.

For the more critical doses it is recommended that the oocysts are cleaned with sodium hypochlorite (available chlorine 8%, Fisons Scientific Equipment) both to prevent clumping and to assess sporulation more accurately.

- **Procedure**

The following procedure describes the handling of relatively small numbers of oocysts (up to 10 - 15 millions) but it can be scaled up with suitable modification.

- Remove potassium dichromate (see section 1.1.6.) by repeated centrifugation in water. Constantly reduce the size of the centrifuge bottle/tube being used as the procedure permits. Washes can be started in 1 l buckets or 100 ml glass tubes, and thereafter 50 and 15 ml glass tubes can be used.

- Add sodium hypochlorite to a final concentration of 10% v/v and keep in ice bath for 5-10 minutes.

- Centrifuge, discard supernatant and mix pellet with a small volume of saturated sodium chloride solution. Fill tube to within 2 cm of top with more salt and overlay with distilled water. Centrifuge at 1500 rpm for 5 minutes.

- Collect clean, bacteria-free oocysts (at interface of salt and water) into sterile distilled water. Centrifuge as before.

- **Relatively large doses (typically 5,000-100,000 oocysts) for passage, lesion scores, weight gains, etc.**

  - Dilute oocyst suspension in distilled water or phosphate buffered saline, transfer to a conical flask and count using, for example, a Fuchs-Rosenthal counting chamber. Further dilute suspension of oocysts if it is obvious that they are far too concentrated.

  - Make full use of the ruled area that is available within a counting chamber (useful for doses at the lower end of the scale but remember to make appropriate calculations afterwards!) and, once an appropriate concentration has been achieved, count up to 10 samples for each dose. Adjust concentration of oocysts as necessary (at this stage major dilutions should not be required - perhaps no more than 1:5). Check accuracy of final dose. If the birds are of a reasonable size the required dose of oocysts can be given in 1 ml by crop intubation.

- **Relatively low doses of oocysts for infection/immunity experiments, etc.**

  - Dilute oocyst suspension in distilled water or phosphate buffered saline, transfer to a conical flask and count using, for example, a Fuchs-Rosenthal counting chamber. Further dilute suspension of oocysts if it is obvious that they are far too concentrated.

  - Using a calibrated glass Pasteur pipette (these are typically around 30 drops/ml) remove 6-10 samples from the oocyst culture with the same Pasteur pipette (swirling

the flask each time a sample is taken) and place a drop from each onto a clean glass slide.

- Count sporulated oocysts in each drop, calculate mean and multiply by the appropriate factor to give the concentration of oocysts/ml. If the birds are of a reasonable size the required dose of oocysts can be given in 1 ml by crop intubation.

- **Infections with single *Eimeria* stages**

Infections have been established with single oocysts, sporocysts, sporozoites or merozoites that were isolated by micromanipulation. At the IAH an assembly comprising a De Fonbrune pump attached to a (replaceable) micropipette is used and, although use of the procedure is straightforward, it does require practice. Typical success rates are 50-100% for single oocyst infections and 30-80% for single sporocysts. Infections with single sporozoites are more difficult to establish (about 10%) and surgery may be necessary in order to introduce the parasites into the intestine.
Other authors have embedded oocysts within gelatine and, following inspection by microscopy, have then removed plugs that contain single parasites.

1.1.5. Recovery of oocysts

Ideally, laboratory rooms that can be sealed and then fumigated with ammonia (or another effective fumigant) should be available.

Each culture of oocysts should be collected in a previously fumigated room that contains equipment sterilised in a boiling water bath, glassware and plasticware sterilised by autoclaving, and centrifuges etc. that, prior to room fumigation, were thoroughly cleaned and swabbed with ammonia solution (10% solution).

- **Recovery of oocysts from faeces**

  - Transfer 24 - 48 h faecal samples collected onto plastic sheets into a sterile plastic beaker (1-5 l), add tap water and homogenise with a suitable heavy duty laboratory mixer (e.g. Silverson Machines, Chesham, England) until the mixture is homogeneous.

- Filter homogenate through 2 thicknesses of butter muslin and centrifuge filtrate (GF-8 at 1,500 - 2,000 rpm for 10 min) or leave filtrate to stand overnight (discard muslin and solid contents). If the sample is left overnight it is advisable to add potassium dichromate to a final concentration of between 0.5 and 1.0% w/v and leave at room temperature. This procedure will aid the sporulation of the oocysts.

- Discard supernatant and resuspend deposit in saturated NaCl solution, mix well and centrifuge as above.

- Collect oocysts, using a syringe fitted with a canula or a long needle, from top of the liquid into distilled water and wash further by centrifugation. If, after the first collection, the yield is low and further oocysts are required, the deposit and overlaying salt solution can be re-shaken with more salt solution and the process of centrifugation, etc., repeated. To recover most of the oocysts of *E.maxima* from the faeces, it is usual that the process may have to be repeated up to five, or even more, times.

- Wash oocysts free from salt by repeated centrifugation, taking care to check on each occasion that the oocysts have indeed pelleted and are not present in the supernatant. Finally, resuspend the oocysts in potassium dichromate (2% w/v final concentration) at a concentration of about 0.25×10^6 oocysts per ml in a conical flask.

  Note: the suspension should not be excessively deep within a flask and for example, a 5 litre should not contain more than 2 litres of liquid.

  Incubate culture at 28-30°C for 48 hours with forced aeration using a suitable pump. Take care to ensure that any airlines from the pump into the culture have been properly sterilised by boiling before use. If there is a shortage of water baths, sporulation can normally be achieved at room temperature by stirring the culture with a large magnetic bar with forced aeration for a period longer than 48 hours.

  Cultures that have sporulated satisfactorily can be transferred to a more suitable vessel for storage at 4°C.

  It is advisable that cultures of *Eimeria* species from the chicken are passaged at intervals of no more than 6 months.

- **Recovery of oocysts of *E. tenella* (or *E.necatrix*) from the caeca**

Using sterile scissors, remove caeca from chickens given oocysts of *E. tenella* or *E. necatrix* 7 or 8 days previously, respectively, cut into transverse sections and homogenise using appropriate commercial blenders (e.g. "Atomix"). An alternative procedure is to cut the caeca longitudinally and then scrape and recover contents, down to the caecal wall, using a glass slide.

- **Enzymatic degradation of tissue**

  - To pieces of tissue (or caecal scrapings) add phosphate buffered distilled water (not necessary to make this solution isotonic) at pH 8.0 and homogenise for 2-3 min.

  - Add trypsin (Difco 1:250 powder) to a final concentration of about 1.5% w/v, incubate at 41°C for about 30 min and strain through two thicknesses of muslin.
  - Centrifuge at 1500 rpm for 10 min and discard supernatant. Add saturated salt solution, mix well and centrifuge as above. Collect oocysts from the top of the salt, wash repeatedly and sporulate as described above.

    Note: if oocysts from small volumes of faeces are being collected it is possible that the salt centrifugation step may be done in small tubes, in which case the saturated salt solution can be overlayed with water and the oocysts collected from the interface after centrifugation; see above.

- **Chemical degradation of tissue**

It is possible to recover oocysts from the caeca of chickens by the use of sodium hypochlorite, but the subsequent sporulation of oocysts is sometimes abnormal. At the IAH the sporulation of oocysts treated with sodium hypochlorite varies from strain to strain and whilst some of the laboratory strains may sporulate very well after this treatment, we have found that strains isolated from the field may sporulate very poorly.

- To tissue pieces add tap water and homogenise in blender for 2-3 min.
- Strain homogenate through 2 thicknesses of muslin.

- Add 10-20% v/v final concentration of sodium hypochlorite, mix and leave in an ice bath for 10-15 min. Continue from step 4 above.

1.1.6. Storage of sporulated oocysts

Cultures should be stored in 2% potassium dichromate at 4°C for periods not greater than 6 months before they are re-passaged. Whilst most laboratory strains are serially passaged, it is good laboratory practice to go back to a frozen master stock of sporocysts or sporozoites at regular intervals to prevent any undesired genetic drift in the population that is being studied.

1.1.7. Isolation and purification of sporozoites

- **Isolation of sporozoites**

  Sporozoites should be recovered from oocysts that have been surfaced sterilised after treatment with sodium hypochlorite (see above).

  - For small-scale preparations, resuspend purified oocysts in about 0.5 ml of the appropriate medium, which can be Hanks BSS or PBS pH 7.6 in a round-bottomed glass centrifuge tube.

  - Add 0.5 mm diameter glass balls (number 8, Jencons) so they comprise about half of the total resulting volume and place the tube on a Whirlimix (Fisons Scientific Equipment) turned to maximum speed and agitate the contents until most of the oocysts have been mechanically fractured to release their sporocysts. For large-scale preparations, use 50 ml tubes and increase volumes accordingly.

    Note: The "toughness" of the oocyst wall varies with different species and some care is required during the cracking procedure to ensure that not too much damage is done. For example, oocysts of *E. acervulina* are very robust and 30-40 'strokes' (one stroke = holding the tube on a Whirlimix for a count of 1 second) of the Whirlimix may be necessary to damage most of the oocysts. In contrast, 10-12 bursts of *E. tenella* may be perfectly adequate. Always check the progress of the cracking process by examining under a microscope, a very small sample

at very regular intervals, perhaps every 5 or so strokes until some experience is gained in the procedure.

If too many strokes are used, a large proportion of the sporocysts will also be damaged. As a rule, as soon as sporozoites are seen in the 'soup' then STOP!

- Recover sporocysts from the glass balls with repeated additions of Hanks BSS or PBS pH 7.6 and resuspend the sporocysts in an appropriate volume, e.g. 20 ml for 5 x 10^6 sporocysts or 200 ml for 400 x 10^6 sporocysts.

- Add trypsin (2.5 g/l) and bile salts (up to 5 g/l) (both are available from Difco Laboratories) and incubate at 41°C until most of the sporozoites have excysted.

  Care: At the IAH we have found that sporozoites lyse in some batches of bile salts used at a concentration of 5 g/l (which was established as the optimum some years ago), and each batch should therefore be titrated for efficacy/toxicity. An alternative (safer) is to use taurocholic acid from Fluka Chemicals at 10 g/l.

- Wash sporozoites twice in medium or PBS pH 7.6 and resuspend in an appropriate volume of the same medium.

  Note: phosphate buffered saline at pH 7.6 has been used routinely in the IAH, but more recently been replaced by Hanks balanced salt solution supplemented with 5 mM $MgCl_2$ for many studies. However, for the excystation of sporozoites of egg-adapted lines, the use of Hanks balanced salt solution is recommended as sporozoites of, at least the egg adapted line of *E. tenella*, appear to be comparatively labile and will not survive for long periods in PBS pH 7.6.

- **Purification of sporozoites**

Several methods for the purification of sporozoites have been described and most have been used with success at the IAH. All are variations on the theme of separating sporozoites from oocysts and sporocysts by passage through a column.

Filtration through a simple column comprising glass balls (size 8, Jencons) [1] was used at the IAH for several years although the yields of purified sporozoites could be relatively low. However, if only small numbers of purified sporozoites are required, the method is perfectly satisfactory.

Most recently, a column comprising the barrel of a 50 ml syringe and filled with a 15 - 20 ml layer of "Leukopak Fibres" (Fenwal Laboratories) under a similar volume of DE-52 (Whatman, England) (see Fig. 1) has been used routinely for the recovery of up to 1 x 10^9 sporozoites of *E. tenella*.

This method is a "hybrid" of two methods comprising a layer of Leukopak Fibres [2] and DE-52 anion exchange chromatography [3].

- **Purification of *Eimeria* sporozoites by passage through columns of nylon wool and DE-52.**

- **Preparation of DE-52 cellulose**

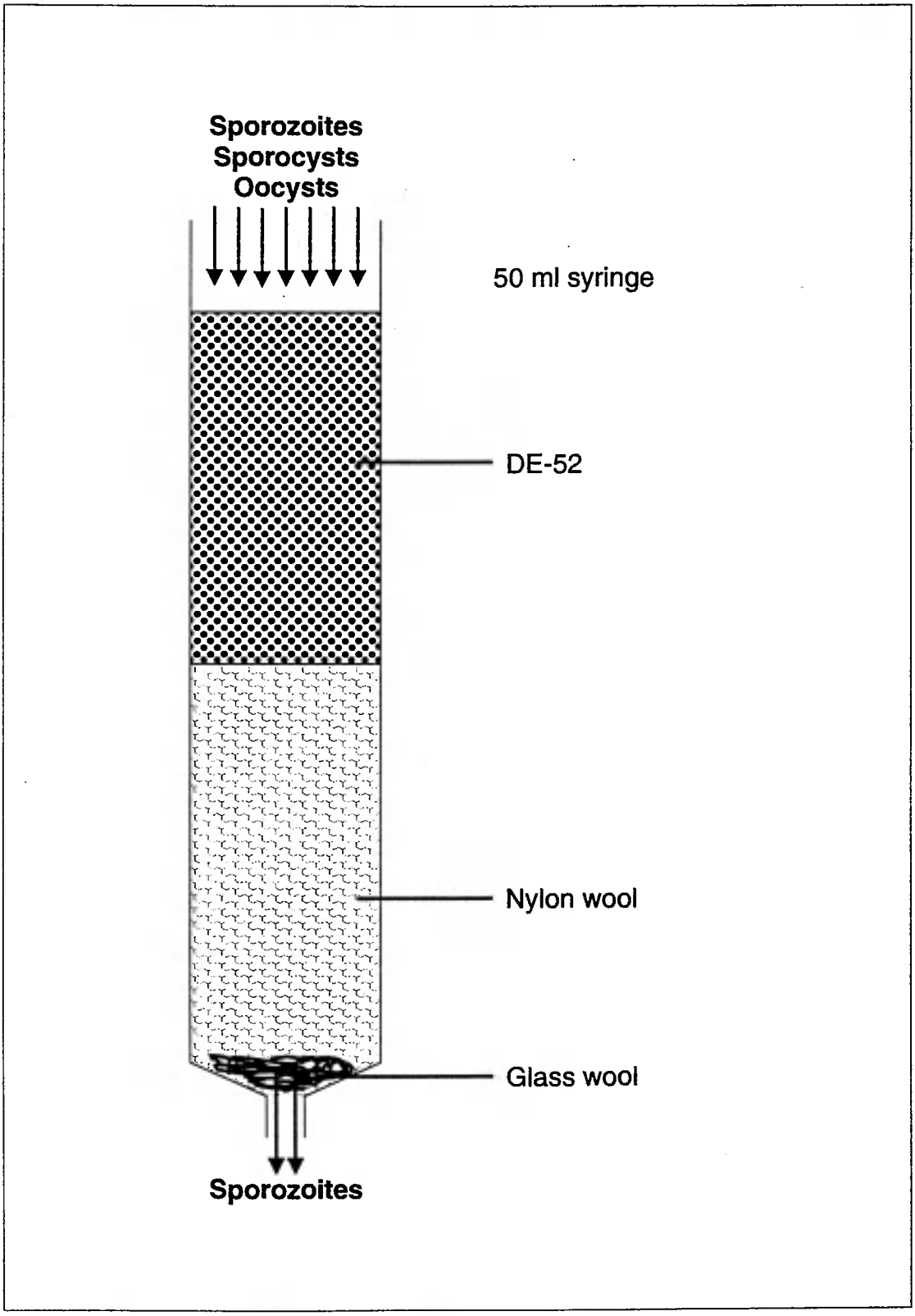
DE-52 (Diethylaminoethyl cellulose - Whatman pre-swollen microgranular anion exchange; Catalogue No: 4057-050).

- Mix DE-52 with 1 x PBS buffer and allow to settle for 1 h.
- Remove supernatant and discard.
- Add more buffer and repeat washing.
- Add more buffer, mix and adjust to pH 8.0 with 5% w/v solution of orthophosphoric acid (H_3PO_4). Stir, allow DE-52 to settle overnight in refrigerator.
- Remove supernatant leaving a small amount of buffer on top of the DE-52.

PBS buffer x 2

Na_2HPO_4	26.96 g/litre
NaH_2PO_4	1.56 g
NaCl	8.5

Fig. 1: Purification of ***Eimeria*** sporozoites from sporocysts and oocysts



- **Preparation of column**

  - Press a small amount of glass wool into the nozzle of a 20 or 50 ml syringe and place syringe in retort stand.
  - Add nylon to a height of about 3 cm and then wet with PBS.
  - Pour on DE-52 into the syringe to a height of 6 cm.
  - Allow the buffer to drain and add more DE-52 to maintain the same level.
  - Wash the column with 1 x PBS supplemented with 1% glucose and ensure that column does not dry.
  - Allow to drain before adding sporozoites.

- **Purification of sporozoites**

  - Wash hatched sporozoites and resuspend in about 10 ml of 1 x PBS supplemented with glucose.
  - Place suspension of sporozoites on the column. Add further buffer and monitor the eluate for the presence of sporozoites.
  - Collect sporozoites and continue to wash column with more buffer.
    When only few sporozoites leave the column, more can be forced through if required by fitting the plunger of the syringe. However, the use of force will inevitably result in the exit of proportionally more oocysts and sporocysts.

A method of centrifugal elutriation has been described [4] but has not been used widely.

1.1.8. Isolation and purification of schizonts and merozoites

Most methods described above for the recovery of sporozoites have also been used with success for the isolation of merozoites recovered from infected gut (chickens) or chorio-allantoic membranes (CAM) (embryonating eggs).

A method for the recovery of merozoites has been described [5] and about 2.6×10^7 and 1.5×10^6 purified merozoites of *E. tenella* were obtained from each infected caecum or CAM, respectively.

- **Method for merozoites of *E. tenella* [5]**

  - Infect chickens with 7.5 x 10^5 oocysts of *E. tenella* or inoculate embryos with 2.5 x 10^4 sporozoites.
  - Remove infected tissues five days later (cut caeca longitudinally), rinse with phosphate buffered Ringer's solution at pH 7.4 containing 2000 units each of penicillin and streptomycin and cut into 1 cm^2 sections.
  - Place sections in 10 volumes of solution containing 120 mM NaCl; 20 mM Tris-Cl (pH 7.4); 3mM K_2HPO_4; 1mM $CaCl_2$ and 1 mg/ml BSA. Add hyaluronidase to 1mg/ml and incubate at 37°C with moderate agitation for 30 min.
  - Centrifuge supernatant at 800 x g for 10 min. Wash pellet once in Ringer's solution.
  - Resuspend pellet in 10 volumes of a solution comprising 7.5% ficoll and 10% hypaque in Ringer's solution and centrifuge at 400 x g for 10 min. Repeat three times and pool supernatants. Dilute supernatants 1:3 with Ringer's solution and centrifuge at 400 x g for 10 min.
  - Wash pellet once and resuspend in an equal volume of Ringer's solution.
  - Filter through a small column (5 cm high x 2 cm diameter) of glass balls at a flow rate of about 2 ml/min.

The method of [6] for *E. tenella* is given below as it describes the recovery of both merozoites and schizonts from a common protocol; (see Fig. 2, reproduced with permission).

- **Method for merozoites and schizonts of *E. tenella* [6]**

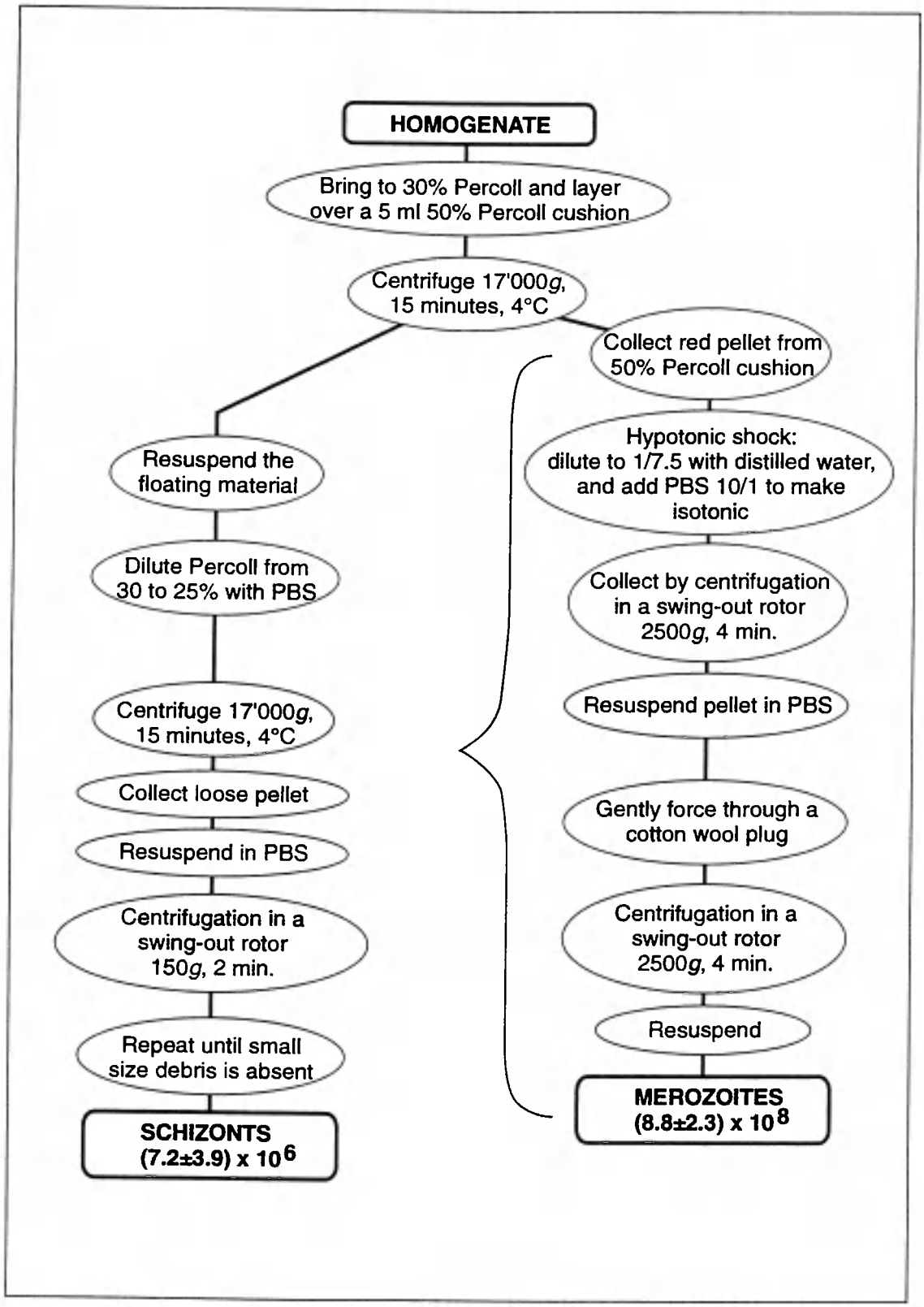
  - **Initial procedure for schizonts and merozoites**

  - Infect chickens with 100,000 oocysts of *E. tenella* and collect caeca 112 h post inoculation.

  - Cut caeca, remove contents and wash twice by swirling in ice-cold PBS.

  - Perform all steps on ice, except where stated.

Fig. 2: Method for recovery of schizonts and merozoites



- Scrape mucosa into PBS and adjust volume to 35 ml and homogenise in pre-cooled Dual Kontess 24 potter homogeniser with a teflon pestle (625 rpm, 10 strokes). An alternative is to shake mucosa in presence of 10 ml of 5 mm glass pearls.

- Filter homogenate (35 ml) over 8 layers of nylon gauze (tights cleaned with 5% Dettol, followed by rinsing in water, ethanol, and drying, pre-wetted in PBS) to remove connective tissue and villi.

- Process as per flow chart and monitor each step microscopically.

- Add 15 ml 100% Percoll-PBS to the filtered homogenate (to make 30% Percoll-PBS)and layer over a 5 ml 50% Percoll-PBS cushion in 2, 40 ml tubes and centrifuge at 17,00 x g for 15 min. (Sorvall SS-34 rotor)(**).

- **Merozoites**

- Collect "red" pellet (containing merozoites and erythrocytes) from the 50% Percoll-PBS cushion (see above last step (**) of "Initial procedure") and resuspend in 7.5 ml PBS.

- While shaking, lower buffer tonicity by adding ice-cold distilled water to a final volume of 45 ml, and within 30 sec add 5 ml of 10xPBS (at room temperature) to restore the original tonicity of the buffer. (It is essential that hypotonic shock is completed within this time.)

- Microscopically inspect erythrocytes for lysis, and then centrifuge merozoites at 2,500 x g for 4 min. (Beckman GLC-1, swinging bucket rotor). Discard supernatant, resuspend pellet in 20 ml PBS by vigorously shaking.

- Gently force cell suspension through 5 ml of compressed cotton wool in a 50 ml syringe and recentrifuge to collect cells.

- Resuspend cells in RPMI-HEPES buffer and store on ice.

- **Schizonts**

- Use material from last step (**) of "Initial procedure" (see above).

- Resuspend the floating material from step 7 in the supernatant fraction by vigorous shaking and add 5 ml PBS to each tube to make 25% Percoll-PBS.

- Centrifuge at 17,00 x g for 15 min (Sorvall SS-34 rotor) and collect a loose white pellet (that contains schizonts, nuclei and small-sized debris).

- Resuspend in 50 ml PBS.

- **Method of N. Smith for merozoites [7]**

  - Infect chickens with 200,000 or more oocysts of *E. tenella* or *E. maxima*.

  - Kill birds at 96 h for *E. maxima* or 112 h for *E. tenella*, remove intestines/caeca, slit open and wash (two times) in cold PBS pH 7.0. Store at 4°C until all animals have been processed.

  - Cut intestine/caeca into smaller pieces (do not scrape mucosa) and stir at 41°C, checking (at the most) 15 min later to monitor the release of parasites.
    Generally the maximum number of parasites is released after 30 min. A longer incubation results in destruction of the merozoites, especially those of *E. maxima*.
    Incubation medium is Hanks BS + 10mM $MgCl_2$ + 0.25% trypsin + 1% taurocholic acid (crude from Fluka).

  - Filter through gauze to remove large debris.

  - Centrifuge at 800 x g for 10 min and replace excystation medium with PBS (for *E. maxima*) or DE-52 equilibration buffer [3 and see above] for *E. tenella*. Repeat two times.

  - Disrupt *E. maxima* pellet as much as possible (but gently) with a pasteur pipette - a globular mass remains after centrifugation and this contains the merozoites.

  - Pass *E. maxima* merozoites through a 7 mm then a 10 mm silk/cloth filter - merozoites should be very clean.

  - Pass *E. tenella* merozoites through a DE-52 column pre-equilibrated with buffer.

- Centrifuge clean merozoites at 800 x g for 10 min, replacing DE-52 buffer with PBS. Repeat two or more times.

This method was used for the first time by the author during the preparation of the manuscript and, because it was so successful, it has become the method of choice. The yields of merozoites with *E. tenella* are very high and if the parasites are washed several times by centrifugation, they are so clean and free from extraneous cellular debris that further purification is unnecessary for many purposes.

1.1.9. Recovery and purification of gametocytes

The procedure below is based on that described for *Eimeria maxima* [8]:

- **Procedure for *E. maxima***

  - Infect chickens with 10,00 oocysts of *E. maxima* per os.

  - Euthanise animals 136-138 h after inoculation, remove intestines and immediately wash in ice-cold buffer containing 170 mM NaCl, 10 mM Tris-HCl pH 7.0, 10 mM glucose, 5 mM $CaCl_2$, 1 mM PMSF, 1 mg/ml BSA (SAC).

  - Tie off one end of intestine with a string and fill intestine with 0.5 mg/ml hyaluronidase in SAC.

  - Tie off other end of the intestine and place in warm (37°C) PBS and incubate in a shaking water bath for 20 min.

  - Remove intestines from PBS, cut open lengthwise and discard the contents.

  - Place opened intestines on a 17 mm polymon filter and wash intestinal mucosa with SAC at room temperature.

  - Discard material left on the filter.

  - Pass the flowthrough through a 10 mm polymon filter. Wash the gametocytes that have accumulated on the filter once with SAC and collect them by centrifugation at 800 x g for 5 min.

1.1.10. Techniques for measuring disease impact on host animals (lesion scores)

Descriptions of the scoring scale are based upon those given by [9].

Infection with most species of *Eimeria* results in observable gross changes to the intestine, the severity of which in the fully susceptible host is related to the numbers of parasites ingested.

In considering the effects of infection with *Eimeria* from the domestic chicken, the distribution of lesions within the gut is a distinctive feature of each species, although overlaps in the preferred sites of development do occur (see Fig. 3). Moreover, the extent of "spreading " within the intestine increases with increasing dose, and gross lesions of *E. necatrix*, for example, can be observed from the duodenum to the cloaca. The nature and severity of gross intestinal lesions can be used in the diagnosis of coccidial infection and as a simple, but subjective, assessment of the severity of infection.

There are limits to the usefulness of this approach, however, as infections with two of the species from the chicken (*E. mitis* and *E. praecox*) do not cause detectable changes in the gross appearance of the gut, and at least two other species (*E.necatrix* and *E.maxima*) induce lesions that, depending on the numbers of oocysts ingested and the virulence of the individual strains, can be similar (see below).

A subjective assessment of the severity of gross lesions is usually easiest to undertake in laboratory experiments (as compared to field cases) because the conditions of infection (parasite, dose, time after inoculation, etc.) are known. At least 5-10 birds are required for each group in laboratory experiments where only one, or few species, are given.

It is usual practice to assign a score between 0 (no discernible gross lesions) through to 4 (death of the animal or very severe pathological changes; depending upon preference of the investigator).

A description of the types of pathological changes typically seen with species of *Eimeria* from the chicken and a score that might be assigned, is given below (Tab. 5).

The scoring of coccidial lesions is a task that requires considerable practice. When only relatively small numbers of chickens are to be examined it is recommended that the intestines are removed from all of the birds, placed side-by-side and then arranged

in a hierarchical order of severity. Assessments of grades 0 and 4 in chickens given *E. necatrix* or *E. tenella* are particularly obvious and the remainder of the intestines can be graded using these two extremes. When attempting lesion scoring for the first time it is helpful to have a microscope to hand so that the presence, type and number of life cycle stages can be confirmed and/or considered.

Fig. 3: Differential characteristics for 6 ***Eimeria*** species of chickens

E. acervulina	***E. brunetti***	***E. maxima***
		
Can cause mortality. Infected birds become anorexic, body weight is depressed.	Severe infections cause anorexia and great loss in body weight. Birds are often thin and dehydrated.	Cause of high morbidity; mortality can reach 15-20%.
E. mitis	***E. necatrix***	***E. tenella***
		
Deaths unlikely, body weight depressed.	Can cause high mortality. Extremely pathogenic.	Can cause high mortality. Highly pathogenic.

Tab. 5: Severity of lesions and description of pathological changes in chickens infected with *Eimeria* species

Eimeria species and sites of development	Pathological changes: Grade 1	Grade 2	Grade 3	Grade 4
E. acervulina Small intestine (upper)	Scattered, white plaque-like lesions containing developing oocysts confined to the duodenum. These lesions are elongated with the longer axis transversely oriented on the intestinal walls like the rungs of a ladder. They may be seen from either the serosal or mucosal intestinal surfaces.	Lesions are much closer together, but not coalescent, lesions may extend below duodenum in young birds. The intestinal walls are not thickened and the gut contents are normal.	Lesions are numerous enough to cause coalescence with reduction in lesion size and to give the intestine a coated appearance. Lesions may extend as far as the yolk sac diverticulum.	The mucosal wall is greyish with colonies completely coalesced. In extremely heavy infections the entire mucosa may be bright red in colour. Individual lesions may be indistinguishable in the upper intestine. Typical ladder-like lesions appear in the middle part of the intestine. The intestinal wall is very much thickened, and the intestine is filled with a creamy exudate which may contain large numbers of oocysts.
E. brunetti Small intestine (lower), Rectum, caeca	Gross lesions are very indistinct with some greying or reddening of the mucosal surfaces.	Intestinal wall may appear grey in colour. The lower portion may be thickened and flecks of salmon coloured material sloughed from the intestine may be present.	Intestinal wall thickened and a blood tinged catarrhal exudate present. Transverse red streaks may be present in lower rectum and lesions occur in the caecal tonsils.	Extensive coagulation necrosis of the mucosal surface of the lower intestine maybe present. In some birds a dry necrotic membrane may line the intestine and caseous cores may plug the caeca. Lesions may extend into the middle or upper intestine.
E. maxima Small intestine (middle)	Small red petechiae may appear on the serosal side of the mid-intestine. There is no ballooning or thickening of the intestine, although small amounts of orange mucus may be present.	Serosal surface may be speckled with numerous red petechiae; intestine may be filled with orange mucus, little or no ballooning of the intestine; thickening of the wall.	Intestinal wall is ballooned and thickened. The mucosal surface is roughened; intestinal contents filled with pinpoint blood clots and mucus.	The intestinal wall may be ballooned for most of its length, contains numerous blood clots and digested red blood cells giving a characteristic colour and putrid odour, the wall is greatly thickened.
E. necatrix Small intestine (middle)	The presence of small scattered petechiae and white spots visible from the serosal surface.	Numerous petechiae on the serosal surface and some slight ballooning of the intestine.	Extensive haemorrhage into the lumen and the presence of red or brown mucus, extensive petechiae on the serosal surface, marked ballooning of the intestine and the absence of normal intestinal contents.	Ballooning may be extensive and haemorrhage may give an intensive dark colour to intestine contents.
E. tenella Caeca	Very few scattered petechiae on the caecal wall, no thickening of the caecal walls, normal caecal contents.	Lesions more numerous with noticeable blood in the caecal contents, caecal wall is somewhat thickened, normal caecal contents.	Large amounts of blood or caecal cores present, caecal walls greatly thickened, little, if any, faecal contents in caeca.	Caecal walls greatly distended with blood or large caseous cores, faecal debris lacking included in cores.

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1.2. *Eimeria* species of sheep

M. Taylor, J. Catchpole, R. Marshall, C.C. Norton and J. Green

1.2.1. Production of coccidia-free lambs

Pure strains of *Eimeria spp.* of sheep are maintained by periodic passage in coccidia-free lambs which are removed from their mothers at birth to isolation facilities. Adult sheep are infected and the environment is contaminated with coccidia and therefore considerable care and attention to detail is required to produce lambs free of infection.

- **Synchronisation of lambing**

Sheep are seasonally polyoestrus animals with an oestrus cycle of approximately 16 days and a gestation period of about 147 days. With most breeds of sheep oestrus commences in the late summer to autumn which effectively means lambs are born in northern latitudes during the period December to May. Finnish Landrace and Dorset Horn breeds, and their crosses, are frequently used to produce "out-of-season" lambs and thus extend the seasonal availability. The lambing period can be shortened by synchronising the ewes using intravaginal sponges and/or vasectomised rams. Dates of lambing can then be accurately predicted for animals holding to first service or subsequent returns.

The production of coccidia-free sheep has therefore to be planned many months in advance and requires a good working knowledge of sheep management and reproduction.

- **Pre-mating**

Ewes are placed on a rising plane of nutrition for approximately 4-6 weeks prior to mating to stimulate multiple ovulation. Vasectomised rams can be grazed with ewes during this period to start ewes cycling and synchronise their oestrus cycles. As an alternative or adjunct, intravaginal progesterone sponges (e.g. Chronogest®, Intervet) can be used. These are inserted into ewes approximately 14 days prior to the planned matings. After 12 days sponges are removed and pregnant mare serum gonadotropin (PMSG) administered (usually between 250 and 550 i.u.). Ewes are

usually mated under supervision ensuring sufficient rams are introduced for the numbers of ewes to be mated (minimum of 1 ram per 10 ewes). Management of rams should include the use of ram raddles with different coloured marker crayons for each ram, which should be changed at fortnightly intervals. Good management and record keeping at this time assists in the organisation of the lambing period.

- **Pre-lambing**

Ultrasound pregnancy diagnosis scanners can be used between 60 and 90 days of gestation, to identify ewes carring twins or triplets. Such ewes can be separated from those carrying singles, and provided with additional feed during the six week period prior to lambing. Preferably, ewes with two or more lambs should be used to provide coccidia-free lambs.

- **Lambing**

Twenty four hour a day supervision of lambing is required so that each birth can be assisted. This is labour intensive especially if lambing of the flock is extended over a period of time. Supervision of lambing should commence 20 weeks from the first recorded matings. The majority of ewes holding to first service will lamb within the next seven days. Ewes which failed to hold to first service (returns) will normally produce their lambs two to three weeks later.

Lambs to be reared coccidia-free should have minimum contact with the ewe or the environment. At the start of signs of second stage labour, the vulva of the parturient ewe should be cleaned, disinfected, and subsequent births assisted. Lambs should be delivered onto clean sheets placed on the floor of the lambing pen and once resuscitated, transferred to a suitable clean transport box, weighed, and those with a minimum of 3.5 kg birth weight, moved to the isolation facility. (Lambs can also be removed by caesarian section although this is not usually necessary). Ewes giving birth to twins or triplets, should not be left with single lambs as this can predispose to mastitis. In such situations it is preferable to remove all lambs before they suckle and allow the ewes to dry off. Lambs are therefore withheld from taking colostrum to prevent contamination from the udder and also transfer of maternal antibody.

- **Rearing of coccidia-free lambs**

Lambs are removed from the mothers at birth before suckling, placed in a sterile box and transferred to an isolation unit, where they are reared coccidia-free in isolation from other sheep.

- **Accommodation for lambs**

Coccidia-free lambs are reared in an isolation disease-free building. The lambs are housed in rooms in individual pens on wire mesh floors approximately one square metre in area, but within sight of each other. Each pen has a tray system, directly under the mesh floor, on which paper can be placed for collection of faeces.

Each pen is fitted with a teat feeding system consisting of a bucket for milk on the outside of the pen, connected to a teat via a long tube which reaches to the bottom of the bucket. A trough for grass nuts is also fitted.

No more than 10 lambs should be housed in the same room, thus reducing the risk of cross infection between lambs.

Great care must be exercised in preventing extraneous infections entering the accommodation!

All pens and equipment are sterilised in a hot air oven, assembled in the animal room which is then fumigated with formaldehyde vapour followed by ammonia vapour before use. Solid formaldehyde (PRILS) is added at a rate of 1 kg/300 m^3 air space to water and boiled for 30 min to give approx. 500 g of gas. Ammonia is added at a rate of 500 ml/25 m^3 to equal volume of water and boiled in an open kettle.

All food should be supplied in clean sealed bags from the manufacturer, which together with sufficient stocks of all consumables to last for the duration of the rearing period, should be placed in the animal accommodation before fumigation.

Staff should wear protective clothing inside the animal room, boots dipped before entering and hands washed before handling animals or equipment.

Attendant staff must have no contact with other sheep during the rearing period!

- **Maintenance of lambs**

On arrival in the isolation unit the lambs are taken straight to the animal room, leaving the transport-box outside. The lambs are given approximately 300 ml of warmed goat colostrum by bottle, as soon as they can stand. They are trained onto the teat and bucket system to self-feed on milk during the first 24 h of life. All milk is fed at ambient room temperature (22°C). The milk is reconstituted from a commercial milk powder formulated for sheep, initially at 150g/l, offered at a rate of 3 l/day.

After 2 weeks the milk powder level is gradually reduced but the volume offered remains the same; by 8 weeks the lambs are receiving water only.

Concurrently, the lambs are offered dried grass nuts, 50 g initially, increasing to a maximun of 500 g/day by about 8 weeks.

From 10 days of age, faecal samples are monitored three times weekly to ensure lambs are not infected with coccidia.

1.2.2. Infection of lambs for oocyst production

Information on prepatent periods for each *Eimeria* species of sheep is variable as few studies have been undertaken with coccidia-free lambs.

Tab. 6: Prepatent periods and organ localisation of *Eimeria* species in sheep

Species	Prepatent Periods (days)	Organ localisation
Eimeria ahsata	18 - 30	small intestine
Eimeria bakuensis	18 - 29	small intestine
Eimeria crandallis	15 - 20	small intestine, caecum, colon
Eimeria faurei	13 - 15	small and large intestine
Eimeria granulosa	?	unknown
Eimeria intricata	23 - 27	small intestine
Eimeria marsica	14 - 16	unknown

Species	Prepatent Periods (days)	Organ localisation
Eimeria ovinoidalis	12 - 15	small intestine, caecum, colon
Eimeria pallida	?	unknown
Eimeria parva	12 - 14	small intestine, caecum, colon
Eimeria weybridgensis	23 - 33	small intestine

- **Infection**

Lambs are reared coccidia-free (sheep colostrum withheld but given goat colostrum) from birth in isolation from other sheep (see page 28).

At three to four weeks old, individual lambs are moved to separate sterile accommodation (pens etc. as described for rearing). Each lamb is infected with an oral suspension of sporulated oocysts in water, using a ball-ended needle on a 2 ml syringe (see Fig. 4, page 30).

Staff attending infected lambs should have no contact with other lambs and follow isolation procedures as for the rearing period!

- **Oocyst collection**

Faecal samples are collected daily from below the pens to check for any adventitious coccidial infection.

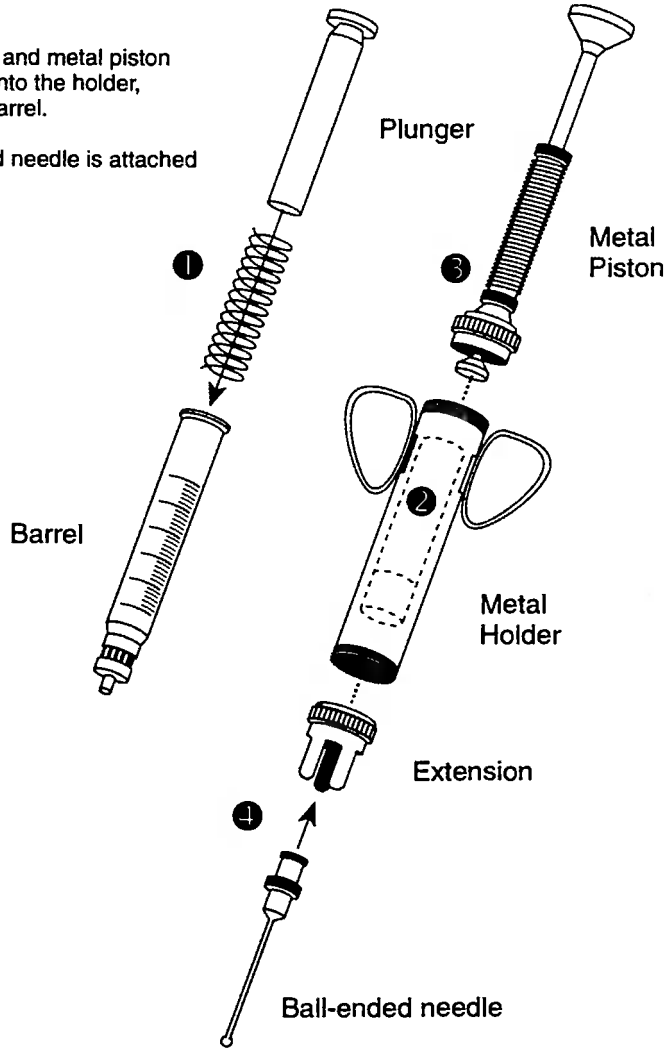
Once patency is established, the total daily faecal output of infected animals is collected from beneath the wire-floored pens. The collection period varies according to species (see Tab. 7).

With the dose rates indicated in Tab. 7, no clinical signs should be observed in lambs used for oocyst production.

Fig. 4: Syringe for inoculation of lambs with oocysts

How to assemble a 2.0 ml syringe:

1. Plunger passes through spring into barrel.
2. This assembly is placed within metal holder.
3. The extension and metal piston are screwed onto the holder, securing the barrel.
4. The ball-ended needle is attached to the barrel



Tab. 7: Oocyst collection in lambs infected with standard doses of oocysts

Species	Dose of oocysts per animal	Time after innoculation of oocysts for collection of faeces (days)
Eimeria ahsata	10,000	23 - 27
Eimeria bakuensis	10,000	23 - 27
Eimeria crandallis	500	18 - 22
Eimeria faurei	10,000	15 - 19
Eimeria granulosa	?	?
Eimeria intricata	?	?
Eimeria marsica	10,000	16 - 20
Eimeria ovinoidalis	250	15 - 19
Eimeria pallida	?	?
Eimeria parva	5,000	12 - 16
Eimeria weybridgensis	5,000	25 - 29

1.2.3. Examination of faeces for coccidia

A. Qualitative Examination for presence of oocysts.

Used for monitoring coccidia free stock, and during the pre-patent period of animal experiments.

- Make a suspension of faeces in water. Take care to obtain an even mixture when examining pooled samples. Use a pestle and mortar to break up hard sheep pellets.
- Squeeze through 15 cm muslin square, held in a 7.5 cm plastic funnel, and into an unchipped glass centrifuge tube.
- Centrifuge at 1500 rpm (200 x g) for 2 min.
- Pour off supernatent, break up sediment and fill to within 5mm of the top with saturated salt solution (NaCl, specific gravity 1.2). Invert several times and place in centrifuge.

- Top up with saturated salt solution until meniscus is just convex, remove bubbles and place an 18 x 18mm No. 1 coverslip on top of the tube. Centrifuge at 1000 rpm (100 x g) for 2 min.

- Remove coverslip vertically, place on slide and examine.

B. Modified MacMaster method for oocyst counts per gramme of faeces. Used for experiments and diagnosis.

- Weigh 3 g of faeces and mix with 42 ml of water in a shaker bottle containing 7 mm glass balls.

- Pour faecal suspension through a wire mesh screen of aperture 0.15 mm, collecting filtrate in a clean, dry bowl.

- Mix the filtrate thoroughly to ensure that there is a uniform suspension of faecal material and transfer an aliquot to a centrifuge tube filling to within 5 mm of the top. (If required the remainder of the fluid in the bowl may be mixed with an equal volume of 4% potassium dichromate and put in a shallow dish at 27°C to allow the oocysts to sporulate).

- Centrifuge at 1500 rpm (200 x g).

- Discard the supernatant and emulsify the packed sediment.

- Add saturated salt solution (NaCl), until the volume equals that of the initial aliquot of filtrate. At this stage further dilution to 1/10 or 1/100 in saturated salt may be necessary if oocyst numbers are large.

- Invert the tube several times until the sediment is evenly suspended and fill 3 chambers (each has a volume of 0.15 ml) of a McMaster slide using a clean Pasteur pipette. McMaster slides with six vertical columns facilitate scanning under the microscope.

- Count all the oocysts (= N) within the ruled area (1 cm^2) of each chamber using the 10x objective and 10x eyepiece.

- Calculation of oocyst numbers per gramme:

Oocysts per gramme of faeces (Opg):

$$Opg = \frac{N \times 100}{3}$$

N = total number of oocysts in 3 chambers of McMaster slide

100 = dilution factor (total volume of 3 chambers: 3 x 0.15 ml = 0.45 ml; correction to original volume: x 100 = 45 ml).

3 = correction to 1 g of faeces.

* If less than 3 g faeces are available, use the following table to preserve the same proportion of faeces to water, so that the calculation becomes N x 100 x multiplication factor (see Tab. 8).

Tab. 8: Relations of faeces and water and multiplication factors for use of < 3 g of faecal material

Faeces g	Water ml	Multiplication factor
0.1	22.5	15
0.2	24	8
0.3	22	5
0.4	23.5	4
0.5	22	3
0.6	26.5	3
0.7	31	3
0.8	23	2
0.9	26	2
1.0	29	2
1.5	21	1
2.0	28	1
2.5	35	1

1.2.4. Mass production, counting, purification and incubation of oocysts

- Collect faeces from experimentally infected animals onto trays on days according to species, prepatent period (Tab. 6) and appropriate for collection of faeces (Tab. 7).

- Break up faeces in polythene bag, then transfer to a 2 l beaker adding sufficient water to form an even consistency.

- Screen faeces through a series of screens starting with a coarse screen (710 µm mesh diameter), followed by a 100 mesh (150 µm) and 300 mesh (53 µm) screen and collect filtrate into a large bowl. At each stage the faeces are washed gently with a liberal amount of tap water.

- Transfer filtrate to beakers and collect final volume in a large container (20 l).

- Mix liquids in container thoroughly making sure to resuspend sediment, and remove 3 x 1 ml samples to test tubes. Make each of these up to 5 ml (1/5 dilution) with water and from each sample count 2 haemocytometer chambers (Fuchs - Rosenthal), a total of 6 counts each of 5 mm².

Calculation Total No. of oocysts (NT) = $\frac{X}{6x5} \; x \; 5 \; x \; \frac{5}{1} \; x \; 1000 \; xy$

Where X = total oocysts counted in 6 chambers
6 x 5 = six chambers each of 5 mm^2
5 = depth of chamber is 1/5 mm. Multiplying by 5 converts to mm^3
5/1 = dilution of 1/5 in test tube
1000 = conversion of mm^3 to ml
y = total volume

- Transfer filtrate to centrifuge bottles and centrifuge at 1500 rpm (200 x g) for 5 minutes. Pour off supernatant

- Resuspend in tap water and repeat the step twice.

- Resuspend sediment into saturated salt solution (NaCl, specific gravity 1.2) and centrifuge in 100 ml centrifuge tubes at 1000 rpm (100 x g) for 5 min.

- "Flip off" scum on top of supernatant into a 1 litre beaker (retain the rest of the supernatant and sediment in case only a small proportion of the oocysts have been recovered).

- Dilute 'flipped off' scum at least 1/10 with tap water and centrifuge at 2000 rpm (1000 x g) for 10 min.

- Syphon off supernatant carefully and transfer sediment from each centrifuge tube to a 250 ml measuring cylinder. Make up volume to 250 ml with distilled water and transfer to 1000 ml culture flask and add an equal volume of 4% potassium dichromate (maximum volume 500 ml per flask).

- If necessary dilute to a maximum of 1 x 10^6 oocysts/ml and put to sporulate in 27°C incubator.

- Aerate culture by constantly bubbling air through using aeration pump and check sporulation daily.

- As soon as sporulation appears complete (usually 7 days), remove culture from incubator, make up to original volume with distilled water and store in refrigerator at 4°C.

Ensure the sterility of all equipment by suitable methods i.e. hot air oven, autoclave, boiling or filtration.

1.2.5. Storage of sporulated and unsporulated oocysts

- Sporulated oocysts, free from faecal material, can be stored at 4°C in 2% potassium dichromate for up to six months with only slight loss of viability.

- Unsporulated oocysts, collected on ice and cleaned by the method described using chilled solutions, can be stored unsporulated at 4°C in 2% potassium dichromate for up to six months.

For long-term storage sporocysts or sporozoites can be preserved in liquid nitrogen.

1.2.6. Standardised technique for the excystation of sporozoites

- Take the required number of freshly sporulated oocysts and wash to remove the potassium dichromate by centrifugation at 1500 rpm (500 x g) for 3 min and re-suspending the oocysts in distilled water. This may need repeating about three times. (Dirty cultures may require salt flotation.)

- Resuspend the oocysts in 2 ml sodium hypochlorite (specific gravity 1.075) for 20 min (about 32% solution, check with hydrometer).

- Wash with distilled water by repeated centrifugation until no odour of chlorine can be detected.

- Resuspend in 1.4% sodium bicarbonate containing 0.0025% methyl red. Saturate solution with carbon dioxide by bubbling gas through until solution turns brick orange. Stopper tightly and leave in 37°C incubator overnight.

- Wash to remove sodium bicarbonate.

- Resuspend in 4 ml phosphate buffered saline (PBS) pH 7.6 in universal container (or similar with approximately 2 cm diameter), add 1 mm glass balls to depth of 1 cm. Liquid should just cover balls.

- Shake gently to release the sporocysts, examine drops at intervals until most sporocysts are released. This should take about 5 minutes. The presence of empty sporocysts indicates too vigorous shaking. The percentage release of sporocysts in relation to intact oocysts should be calculated.

$$\% \text{ Excystation} = \frac{\text{No. of free sporocysts}}{\text{No. of oocysts} + \text{No. of free sporocysts}} \times 100$$

- Recover the sporocysts by repeated washings in PBS and concentrate in conical tube.

- Prepare fresh trypsin solution as follows: To 20 ml PBS add 50 mg trypsin and 100 mg bile salts.

- To the sedimented sporocysts add 2 ml of trypsin solution and incubate in a water bath at 41°C.

- Examine samples at 15 min intervals, count intact sporocysts and free sporozoites and evaluate percentage of excystation of sporozoites.

1.2.7. Technique for the isolation and purification of merozoites

- Infect coccidia-free lambs with 1 x 10^7 oocysts *(Eimeria ovinoidalis, E. crandallis)*.

- Euthanise lambs 10 days later with an intravenous pentobarbitone injection into the jugular vein.

- Remove lower part of small intestine and trim off any adhering mesentery and fat.

- Cut intestine into 20 cm lengths and open pieces of gut longitudinally. Wash twice in cold PBS. Schizonts are usually visible as white spots in the mucosa.

- Add pieces of intestine to a beaker containing cold PBS and agitate gently for 10 min using a Vibro Mixer(*, see below).

- Decant fluid and pour through several layers of muslin into a large measuring cylinder(**).

- Repeat the previous two steps (*/**) twice.

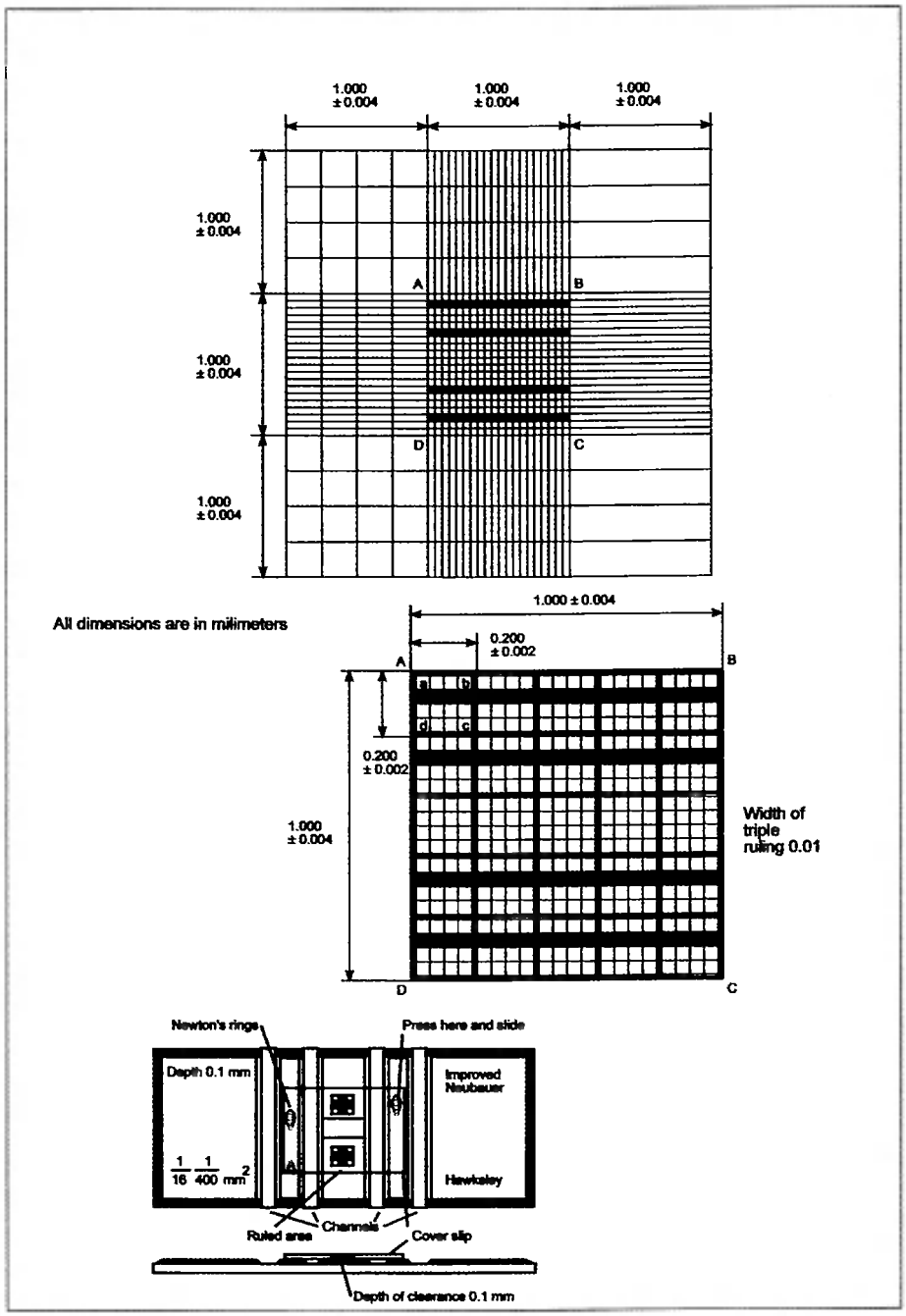
- Centrifuge filtrate at 2000 rpm (1,100 x g) for 10 min (***).

- Discard supernatant and lyse sediment with cold distilled water for 1 min. Resuspend in 5 volumes of PBS and repeat the previous step (***).

- Discard supernatant. Resuspend sediment in PBS and remove 0.1 ml of the suspension and make up to 10 ml (1/100 dilution).

- Estimate the average number of merozoites present in 80 small squares (each 0.0025 mm^2) on a Neubauer haemocytometer (Fig. 5).

Fig. 5: Diagram of Neubauer haemocytometer



Total No. of merozoites = $\bar{x}$ x 5 x 1,000 x 10 x y x 100

$\bar{x}$ = mean count in 80 squares of 0.0025 mm²
5 = correction to 400 squares of 80 counted
10 = depth 0.1 mm
1,000 = conversion to ml
y = volume of suspension
100 = dilution (may vary according to dilution)

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1.3. *Eimeria* species of cattle

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1.3.1. Host animals

Male Holstein Friesian calves are used and are bought from a local cattle dealer at one or two weeks of age. The calves have to be clinically healthy at purchase, but it is not neccessary for them to have been reared under specific pathogen free (SPF) conditions.

Note: If available, calves from isolation units or born by caesarean section should be preferred.

1.3.2. Maintenance of host animals

- **Stables and disinfection**

  Under the given circumstances (Institute of Parasitology, D-Giessen) , the calves are reared in the animal house of the Institute under conditions aiming at prevention of accidental coccidial (and other) infections. Two different floors are used for rearing animals and for the production of oocysts. The floors or walls of the used rooms are only partially tiled.

  The calves are kept in stainless steel metabolic cages (Woetho GmbH, D-Teningen,), which are fixed to the wall or can be installed as freestanding units with sterilized straw (sterilization see feeding) on the slatted floor of the cages during the rearing period. No bedding is offered during those periods of the experiment in which oocyst shedding is expected. Cages are meticulously cleaned with hot water every day. Three times a week (Monday, Wednesday, Friday) they are high-pressure cleaned (150°C, 135 bar operating efficiency, high-pressure cleanser HDS 1250, Alfred Kärcher GmbH & Co., D-Winnenden). After each experiment the cages are taken apart and sterilized (sterilization see feeding). The rooms are high-pressure cleaned twice a week and disinfected with a 5% (v/v) solution of P-chlor-m-cresol and perchlorethylene in water (P3-incicoc®, Henkel AG, D-Düsseldorf).

The staff have to wear separate protective clothes (rubber boots, coat, rubber apron and medical gloves) on each of the different floors. If possible, calves are handled by different personnel during phases of oocyst excretion. Disinfection mats soaked in (P3-incicoc®, see room disinfection) are placed at each entrance.

- **Handling**

On arrival of the animals a clinical examination is performed. Each calf is given one prophylactic dose of Toltrazuril (20 mg/kg b.w., Baycox®, Bayer AG, D-Leverkusen) per os, Flumethrin 1% (1 ml/10 kg b.w., Bayticol®, Bayer AG, D-Leverkusen) pour on and a mixture of vitamins AD_3E (5 ml Vitamin AD_3E-50®, Albrecht GmbH+Co, D-Aulendorf, i.e. 50,000 units vitamine A palmitate, 25,000 units cholecalciferole [vitamin D_3] and 20 mg α-MD_3 tocopherole [vitamine E] i.m.

To ensure that the animals are in good health and maintained free from accidental infections, they are handled throughout their lives as follows: The body temperature is measured every day. Faecal samples are taken on the first three days and then 3 times each week (Monday, Wednesday, Friday) and examined for cryptosporidia (carbolfuchsin stain), other coccidia (flotation with NaCl-$ZnCl_2$ solution, specific gravity =1.3), and (once a week) *Giardia* (MIFC-technique) [1]. Further samples are taken for microbiological (qualitative bacteriological and virological) examinations on the day of arrival, one day before infection and once during the patent period if the calves are used for oocyst production, or the day before slaughtering if the calves are used to provide merozoites.

Note: It has not been possible to rear all calves free from accidental coccidial infections. Accidental infections during the rearing period (before experimental infection) are immediately treated with a single dose of toltrazuril (see above). The earliest date for experimental infection of a calf having received medications (see above) is 3 weeks after treatment. After experimental infection treatment is not possible any longer. In addition, *Giardia* infections could not be prevented with certainty.

- **Feeding**

The calves are first fed with milk-replacer (200 g/meal Hemofac a milk-replacer for the feeding of young animals, Hemo Mischfutterwerke, D-Scheden) and a plant preparation with trace elements against diarrhoea (30 g/meal, Stullmisan®, Pharma Stulln GmbH, D-Stulln/Nabburg) suspended in pre-cooked black tea twice a day and sterilized hay (105°C, 1.2 bar, 180 min, Hochdruck-Dampfsterilisator KS9/6/9-2ED, Kaspar & Co., D-Aachen) ad libitum. Commercial concentrates (supplementary feed for young animals, Raiffeisen Ringfutter, Raiffeisen Hauptgenossenschaft AG, D-Frankfurt/M.) are given ad libitum from the second week onwards. The ration of milk-replacer is increased weekly (50 g milk-replacer and 250 ml tea). After four weeks, tea and Stullmisan® are abandoned.

1.3.3. Infection regimes of calves with *Eimeria bovis*

Two different regimes of infection are used in order to harvest either oocysts or first-stage merozoites.

- **Infection for oocyst production**

Calves for oocyst production are given one dose of 7.0×10^4 sporulated oocysts of *Eimeria bovis*, strain H, suspended in 100 ml tap water per os (approximately 1,000 oocysts per kg b.w.) at an age of approximately eight weeks. The infection dose is given independently of the feeding time. The calves remain in the rearing rooms until day 16 post infection (p.i.) when they are then transferred into metabolic cages without straw bedding in the rooms for oocyst production. Urine aprons (MEDVET Ludwig Bertram GmbH, D-Hannover) are fixed to the abdomen of the calves in order to prevent contamination of the faeces by urine. All faeces are collected and samples are taken for oocyst counts (McMaster) after thorough mixing. The first oocysts are usually found between days 18 and 20 p.i.

- **Infection for merozoite production**

  Calves for merozoite production receive a single dose of $1.5x10^6$ sporulated oocysts of *Eimeria bovis*, strain H, per os (handling see above) about six weeks after purchase and are slaughtered exactly 15.5 days [6]. They remain in the rearing rooms all the time as no contamination by shed oocysts is expected.

  Note: Clinical diarrhoea of unpredictable severity (from softening of faeces to haemorrhagic diarrhoea) for approximately 1 week has to be expected in calves used for oocyst production during patency. Clinical symptoms are not observed in calves used for merozoite production.

1.3.4. Mass production, purification, incubation, and storage of oocysts

- Faeces containing oocysts (>1,000 opg) are washed using a water spray gun through a set of three sieves (300 µm, 150 µm, 80 µm pore) into a large tube. The content of the tube is then filled into 10 l buckets for sedimentation.

- After 2 hours, the supernatant is decanted and the sediment is mixed with saturated sucrose solution (specific gravity of 1.35, 3.2 kg sucrose in 2.0 l tap water) until a final specific gravity of 1.15 is reached in the suspension (densimeter).

- For isolation of oocysts, this suspension is transferred into flat plastic bowls (34 x 24 x 4 cm) up to the brim. The horizontally adjusted bowls are covered with clean, defatted glass plates, which have to be carefully placed in position such that they are in contact with the whole surface of the suspension (avoid air bubbles!) (**, see below).

- After 2 hours the glass plates are carefully removed and the oocysts adhering to the low side of the plate are washed - using a water spray bottle - into another bowl.

- The original suspension in the plastic bowls (see above: **) is stirred up and the flotation process is repeated 2-3 times, until only a few oocysts are left as indicated by microscopical examination.

- The collected washing fluid with the oocysts is mixed with an equal volume of a 4% (w/v) potassium bichromate solution, filled into flat plastic bowls (see above)

for oocyst sporulation, and kept at room temperature for one week. Air is carefully blown into the fluid every day and the progress of the sporulation is controlled microscopically.

- After sporulation the oocyst suspension is centrifuged (10 min, 350 x g), the supernatant is discarded, the sediment containing the oocysts suspended in fresh 2% (w/v) potassium bichromate solution and transferred into 500 ml cell culture flasks (Nunc, DK-Roskilde).

- The oocysts are then stored at 8°C until use. The column of solution on top of the sediment should not be higher than 2 cm and the screw cap of the flask must not be sealed tightly.

Note: *E. bovis* oocysts can be kept alive and infectious this way for more than a year.

1.3.5. Isolation and purification of sporozoites of *Eimeria bovis*

- **Purification and pretreatment of oocysts**

- *Eimeria bovis* oocysts stored in a 2% (w/v) potassium bichromate solution are purified by continuous density gradient centrifugation.

- The potassium bichromate is removed from the oocysts by centrifugation (10 min at 400 x g). The pellet is resuspended in sodium hypochlorite solution (4% free chlorine) and incubated on ice for 20 min under permanent stirring. The solution is diluted with distilled water and the oocysts are centrifuged as above. The supernatant is discarded.

Note: The supernatant should be checked for oocysts before it is discarded. When insufficiently diluted, oocysts may form a band within the solution or even float on the surface. In these cases they can be aspirated directly with a pasteur pipette and washed into distilled water (10 min at 400 x g).

- Two Percoll stock solutions are prepared: Mixing 6 volumes of solution No. 1 (consisting of 9 volumes Percoll [Sigma No. P-1644, Sigma, D-Deisenhofen] and 1 volume 1.5 M NaCl solution) with 4 volumes of solution No. 2 (0.15 M NaCl solution) results in a 60% Percoll solution. Equal amounts of solutions 1 and 2 are used

to prepare the 50% Percoll solution. The gradient is built up by centrifuging 10 ml of the respective Percoll mixture in a 15 ml centrifuge tube at 30,000 g for 20 min in a fixed angle rotor (rotor JA20.1, Beckman centrifuge J2-21, Beckman Instruments, D-München).

- The oocyst pellet is resuspended in a small amount of distilled water. The oocyst suspension (approximately 2 ml per centrifuge tube) is layered on top of the preformed 60% Percoll gradient and centrifuged at 400 x g for 20 min. The visible oocyst band is removed and transferred onto the 50% gradient and centrifuged as above. This time several bands will form. The oocyst bands are transferred into a centrifuge tube and diluted with distilled water (at least 2 parts of water, 3 parts of oocyst suspension, otherwise the oocysts will not form a pellet). They are centrifuged for 10 min at 400 x g. The supernatant is discarded.

Note: The gradients should not be overloaded. Depending on the amounts of oocysts and debris in the potassium bichromate solution 10 or more centrifuge tubes of the 50% and the 60% gradient are prepared for approximately 40 ml of oocyst stock suspension with a total of approximately 10^7 oocysts.

- The oocyst pellet is resuspended in a sterile 0.02 M L-cysteinHCl (Serva No. 17769, Serva, D-Heidelberg) /0.2 M $NaHCO_3$ solution (approximately 20 ml for up to 10^7 oocysts) and this suspension is incubated in a 100% CO_2 atmosphere at 37°C for 20 h. It is then centrifuged for 10 min at 500 x g to harvest the oocysts [5].

- **Excystation of sporozoites**

The pretreated oocysts (see above) are resuspended in excysting medium (sterile filtered and prewarmed to 37°C) using 5 to 10 ml per 10^6 oocysts; insufficient amounts of medium will reduce the rate of excystation.

Two different excysting media may be employed:

- **Excystation medium A [3]**
  0,25% (w/v) trypsin (Sigma No. T-0646, Sigma, D-Deisenhofen)
  4% (w/v) sodium taurodeoxycholate (Calbiochem No. 580221, Calbiochem, D-Bad Soden)
  in Hank's balanced salt solution (HBSS) (Sigma No. H-2387).

Advantage: excystation much more rapid (1 h) at a higher rate.
Disadvantage: cost of taurodeoxycholate [3].

- **Excystation medium B [2]**
  0,4% (w/v) trypsin (Sigma No. T-0646)
  8% (v/v) sterile bovine bile (can be stored at -20°C)
  in HBSS (see above).
  Advantage: fresh bile is cheap.
  Disadvantage: long excystation.

The oocysts are incubated at 37°C in a 5% CO_2 atmosphere in small sterile petri dishes or cell culture flasks for 30 to 90 min in excysting medium A or for 4 (max. 5) hours in excysting medium B.

The process of excystation is checked regularly under the inverted microscope and incubation is abbreviated or extended accordingly.

Caution: Prolonged incubation will negatively influence the viability and probably the infectivity of sporozoites.

The mixture of oocysts, sporocysts, sporozoites, and oocyst walls is transferred into a centrifuge tube and diluted with sterile PBS (13.48 g Na_2HPO_4; 0.78 g NaH_2PO_4; 4.25 g NaCl per liter distilled water; pH 8.0) and centrifuged for 10 min at 200 g. The supernatant is removed and discarded, the pellet used for further purification.

- **Purification of sporozoites**

A DE-52-column [7] is prepared 2-3 hours before excystation is terminated, i.e. before medium A or while medium B oocysts are incubated in excysting medium. 2 g DE-52 (Whatman, Maidstone, UK-Kent) are suspended in PBS (see above), allowed to sediment. The supernatant is decanted. This procedure is repeated 3-4 times until pH 8.0 is reached.
Small pieces of glass wool and cotton gauze are washed several times in distilled water.

The following equipment is autoclaved (45 min at 1 bar):

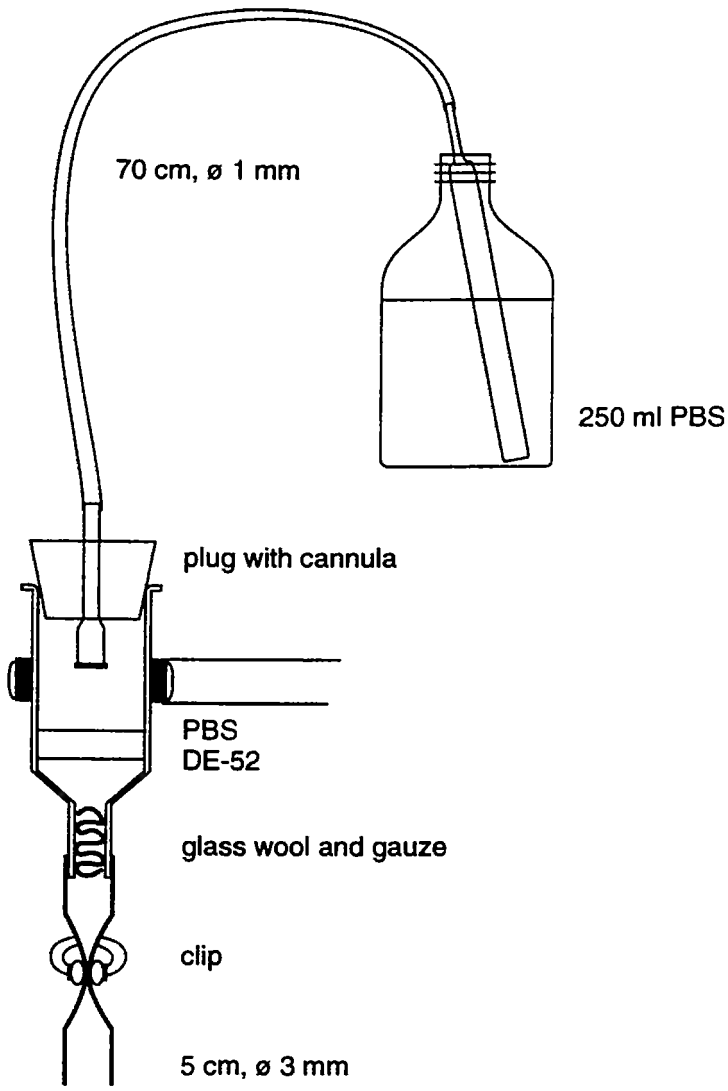
- DE-52
- glass wool and gauze
- 5 ml glass syringe
- rubber tubing: diameter 3 mm, length 5 cm
- diameter 1 mm, length 70 cm
- KECK tube clips
- rubber plug, fitting into the wide opening of the syringe
- Luer lock cannula, 18 G x 1 1/2.
- pasteur pipettes
- 2-3 pairs of forceps (long and slender enough to pack glass wool into tip of syringe).

- The DE-52 column is prepared as follows (Fig.6): The 3 mm tubing is fitted onto the tip of the syringe and closed with a clip. After the conus is packed with glass wool and gauze, the syringe is mounted on a stand. Sterile DE-52 is filled into the syringe until the column material reaches 2 cm in height. A bottle with sterile PBS is positioned approximately 70 cm above the syringe.

- To provide PBS buffer to the column a plug is perforated with the cannula (cone inside the syringe). One end of the 1 mm tube is attached to the needle and the opposite end to the tip of a pasteur pipette which is then immerged into PBS. The PBS is aspirated with a sterile syringe through the cannula in the plug which is then attached to the glass syringe. When the clip is opened PBS should drop into the syringe at the same flow rate as it is coming out of the tube at the bottom. The DE-52 is allowed to equilibrate with approximately 100 ml of PBS.

- The excysting material (resuspended in 1 ml PBS) is layered on top of the column and allowed to flow into the matrix. The column is then washed with PBS. Collection of the fluid starts immediately after the sample has been applied. The majority of the sporozoites will be eluted with the first 20 ml of PBS, but collecting up to 150 ml can still be rewarding. Therefore, fractions are checked microscopically for sporozoite content. Centrifugation of the eluate is performed at 200 x g for 10 min to harvest viable sporozoites for cell culture experiments or at 500 x g for 15 min when viability is not of primary importance.

Fig. 6: Equipment for the purification of ***Eimeria bovis*** sporozoites



Note: The long purification process using the DE-52 column can lead to reduced motility and infectivity of the sporozoites. If sporozoites are needed for *in vitro* infection trials one may try sporozoites with cyst wall debris, in case the infection rate of purified sporozoites is unsatisfactory.

1.3.6. Isolation and purification of schizonts and merozoites of *Eimeria bovis*

First generation schizonts of *E. bovis* are harvested from calves infected with 1.5 x 10^6 sporulated oocysts exactly 15.5 days after the infection according to the technique of Reduker and Speer [6] (see above).

- **Procedure**

  - The calf is euthanized and its gut removed immediately.

  - The small intestine is slit open in total length and the content washed off the mucosa with tap water. Macroschizonts of *Eimeria bovis* can be seen in the ileum and a few meters of the jejunum with the naked eye or by means of a magnifying lens as small white spots.

  - The schizont-containing portions of the intestine are separated and cut into pieces of 10-20 cm length. These strips are washed three times in sterile PBS (pH 7.4) and vigorously stirred in 1.0 mM dithiothreitol (Sigma, D-Deisenhofen) in sterile HBSS (Gibco, D-Eggenstein) for 10 min.

  - The schizonts are removed from each intestinal strip by scraping them gently off with a glass slide. The schizonts are rinsed with PBS through two layers of gauze into 1000 ml beakers. This suspension is transferred into two or three 500 ml glasses with tapered bases.

  - After sedimentation for 5-10 min when the schizonts are visible at the bottom of the glass the supernatant is decanted, the schizonts resuspended in PBS, and allowed to settle again. Sedimentation is repeated 3-5 times. The schizonts are finally resuspended in 15 ml HBSS and disrupted with 6 to 8 strokes with a teflon-coated Dounce homogenizer.

- The HBSS containing free merozoites is pipetted into 50 ml centrifuge tubes (Nunc, Roskilde, Denmark) and the volume is adjusted to 20 ml with sterile HBSS. The samples are vortexed and centrifuged for exactly 1 min at 200 x g. The "1 min" supernatant is further centrifuged at 200 x g for 10 min.

- The resulting "10 min" pellet represents the first batch of purified merozoites which is resuspended in 10 ml HBSS for further use. As the "1 min" sediment still contains 40-50% of the original merozoites mixed with host cells, it is vortexed in 20 ml HBSS and centrifuged again for 1 min.

- In case that batches of merozoites are visibly contaminated with host cells, Nylon wool is used for a final purification step [4]. Nylon wool is washed in 100 ml of 0.5 N HCl, rinsed 20 times in 100 ml of distilled water and packed up to a volume of 5 ml into 10 ml glass syringes (the glass syringes are fitted with plastic tubes of 3 mm diameter, which are clamped with tube clamps and autoclaved). The Nylon wool columns are rinsed with 25 ml HBSS and then filled to the 5 ml mark. The suspension containing merozoites (10 ml) is layered on top of the column. Then each column is rinsed with 40 ml sterile HBSS. The eluate is collected in 50 ml centrifuge tubes (Nunc) and spun for 10 min at 200 x g. The supernatant is discarded and the sediment resuspended in 10 ml HBSS.

Note: Only those parts of the jejunum which contain many schizonts should be selected and the scraping of schizonts has to be done very carefully to avoid contamination with too many host cells. Nylon wool purification seems to be necessary, in case there are many host cells or aggregates of host cells after centrifugation.

Up to 1-7 x 10^9 purified merozoites have been obtained from a single calf which has been infected with 1.5 x 10^6 sporulated oocysts, though less merozoites have been harvested from individual calves.

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1.4. *Eimeria* species and strains of rabbits

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The techniques described are used at the Station de Pathologie Aviaire et de Parasitologie (PAP) of the Institut National de la Recherche Agronomique (INRA - FRANCE).

1.4.1. Host animals

The strains of rabbits used at the PAP are those which have been selected elsewhere for the production of rabbit meat. They are derived from White New Zealand (strain INRA - 1077) or Californian (strain INRA - 1066) rabbits. Other strains (Burgundy Fawn) are also used and no differences have been observed concerning the propagation of *Eimeria*. In other conditions, if the animals have been raised commmercially, it is essential to check that they are really coccidia-free at the age of 30-40 days because rabbits rapidly become immunised, even with small doses of oocysts.

1.4.2. Maintenance of host animals

1.4.2.1. Maintenance of host animals for propagation of reference strains of *Eimeria spp.*

It is essential to use coccidia-free rabbits reared under strict isolation in order to propagate pure strains. Moreover, in order to avoid contamination of strains with infectious agents, we use SPF animals produced at the PAP and particularly animals free from *Escherichia coli* belonging to known virulent serogroups (O103, O15, O128, O132, O109), *Clostridium spiroforme* and *Pasteurella multocida* [3].

Two methods can be used to obtain young coccidia-free rabbits:

Surgical method: the rabbits are obtained by hysterectomy on the 30th day of gestation and raised by artificial feeding in an isolator. For various reasons (unsuitable milk, feeding difficulties, heteroxenic intestinal microflora, etc.) the number of survivors is low.

Chemotherapeutic method [3]: the females constantly receive a coccidiostat in their food and a sulfonamide regularly in their drinking water. The coccidiostat is changed every three weeks and always used in high doses. The young rabbits are separated from the mother except for the 5 min which are necessary for natural feeding each day. Weaning must take place very early (18-20 days) because the coccidia do not develop while the young rabbits are being fed by their mother. This method is convenient and reliable. The real difficulty is to maintain coccidia-free breeding over a long period. Among other things, we recommend the constant use of feed with coccidiostats in order to reduce the infection risk of coccidia-free breeding. This food should be replaced at least five days before inoculation with coccidia.

- **Isolators**

Very few animals are needed to propagate strains (1 rabbit produces 1 x 10^8 to 1 x 10^9 oocysts). We use small isolators comprising three autoclavable polypropylene boxes which are normally sold for rats or guinea pigs and are suitable for 4- to 6-week-old rabbits. These micro-isolators are fitted with feeding and drinking receptacles and the whole unit is autoclaved before use. It is recommended that strain propagation takes place apart from the laboratory and the wearing of gloves is essential for handling animals, materials and food.

- **Food and water**

Rabbits are given feed and water *ad libitum*. We use standard commercial food and contamination has never been observed. Nevertheless, the outside of the food bag is always considered to be contaminated. In addition, it is difficult to find standard food without traces of coccidiostats or anticoccidial drugs that may sometimes account for unsuccessful propagation of a strain.

1.4.2.2. Maintenance of host animals for experimental studies (experiments on pathogenicity, immunogenicity, etc.)

Note: Influence of caecotrophy on the prepatent and patent periods of oocyst excretion in the rabbit. Hard faeces begin to be excreted at the beginning of the afternoon. This excretion stops in the early morning (around 6 a.m.). During the morning soft faeces (caecotrophes) are excreted but directly ingested from the anus. These caecotrophes stay in the stomach for most of the day (diurnal period). Consequently the

prepatent period shoud be defined by the first oocysts found in hard faeces or in soft faeces (in the stomach).

Moreover, because of this particular physiology of the rabbit and the fact that the non-sporulated oocysts are not destroyed during this second passage through the intestine, the patent period is artificially prolonged.

- **Animals**

The number of animals necessary for experimental studies may be variable and the following advice is only an example.

If the available animals are homogeneous and in very good health the results obtained are reliable if 10-12 animals per experimental group divided among 4-6 cages are used. Several conditions must be respected. The young rabbits must be over 4 weeks of age and must have been weaned at least 4 days before the infection experiment. Bear in mind that up to the 30th day sucklings are not or only slightly susceptible to coccidia. Sex and weight are variables which are of little importance for a given age. However, the litter of origin may have a considerable role (also in SPF animals). Randomisation of young rabbits into different experimental groups must take this factor into account.
Having only one animal per cage is to be avoided in view of the gregarious nature of rabbits and in order to prevent psychological stress which might be harmful to the experiment. We usually have two or three young rabbits per cage and the cage must be considered as the experimental unit; three cages of three animals from different litters make up the smallest experimental group to obtain reliable results.

It is advisable to wait 5 to 7 days after setting up the groups before beginning the experiment because adaptation reactions to the environment vary from animal to animal.

- **Cages and equipment**

The most reliable cages for disinfection are those made entirely of wire. All the equipment inside the cage must resist a disinfection heat of 120°C. Drinking receptacles and supply tubes must be autoclavable because these are among the most common sources of contamination from one experiment to another.
After each experiment the rooms and equipment must **first** be washed and **then** disinfected by high-pressure steam at 120°C. A second disinfection should be carried out

with formalin gas. When the rooms are intensively used, a second formalin disinfection should be performed before each experiment.

- **Experimental rooms**

Experimental rooms are separated by about 300 meters of forest from the SPF rabbit production unit [2]. At the PAP we have three identical rooms each equipped with 36 cages (72-108 animals, a maximum of 12 simultaneous groups per room). We also have two smaller rooms equipped with 12 larger cages which can receive 1 adult rabbit or 4 to 5 weanlings.
This number of rooms allows us to have a sanitary isolation of several weeks between experiments, thus permitting more effective disinfection.
For experiments on pregnant females or on suckling rabbits we have a nursery unit with 72 appropriately equipped cages.
The rooms are ventilated by either positive or negative pressure and air is filtered at 10 µm (European Norm: EU 4).

- **Legal requirements**

The types and sizes of cages used vary according to European animal welfare regulations. All dead animals and excreta are incinerated.
All people handling the animals (scientists and technicians) have received special training and legal authorisation from the French authorities.

1.4.3. Infection of animals

1.4.3.1. Infection of animals for propagation of reference strains of *Eimeria* spp.

- **Isolation of pure strains and precocious lines**

Starting generally from a mixture of different species it has been necessary at the PAP to isolate pure strains for each species. The general following procedure has been used:
The first step is to enrich the mixture (over 50%) with the species to be isolated. For this purpose, different methods based on physical or biological aspects of *Eimeria* can be used: size of oocysts (filtration), duration of the prepatent period (to separate the species whose endogenous development is shorter), sporulation time (to separate the

species whose sporulation time is shorter), and/or immunisation against a species different from the one we want to isolate.

The second step is to isolate single oocysts by observing micro-drops under a microscope. When a valid oocyst is detected, it is enclosed in a drop of gelatine, wrapped in a small piece of humidified paper and then inoculated with an insulin syringe [4]. Of a given species 6 to 10 lines obtained in this way are mixed to get a strain representative of the original population.

The isolation of precocious lines from the rabbit has been described in detail [12, 15]. It is based on the first publication of Jeffers [7] who obtained a precocious line of *E. tenella* by selection for early development of oocysts and successive passages in animals. The precocious lines are characterised by a shortened prepatent period, reduced pathogenicity and a multiplication rate which is much lower than that of the parent strain. In addition the morphology of the oocyst of the precocious lines from the rabbit is considerably altered (see chapter 3.1.).

- **Doses of sporulated oocysts for propagation of strains**

The doses used depend on the quality of sporulation and the age of the strain. There is no advantage in doses which are too high because there is a risk of death or diarrhoea which **reduces the amount of oocysts recovered**. The dose of freshly sporulated oocysts indicated in Tab. 9 are those which we use for coccidia-free rabbits.

When unsuccessful, it is necessary to confirm that the food did not contain any coccidiostat. It is also necessary to check if the rabbits were really coccidia-free because they can easily become immunised with very low doses. When rabbits whose mothers are not coccidia-free are used, it is necessary to give the females a feed containing a strong coccidiostat (Salinomycine 20 ppm), to wean early (25 days) and to inoculate the young rabbits at 29 days.

Tab. 9: Doses of sporulated oocysts administered for propagation of reference strains

(P. Coudert and D. Licois)

Species	Dose of oocysts per rabbit ($x10^2$) [1]	Time after inoculation of oocysts for removing caecal and stomach content [2] (day)	Number of oocysts recovered from the caecum and stomach ($x\ 10^8$)
E.intestinalis	10-20	10	10
E. vejdovskyi	20-50	11	5
E. piriformis	100	10	1
E. coecicola	5-10	10	1
E. irresidua	3-5	10	1
E. flavescens	3-5	10	1
E. exigua	20-50	8	1
E. magna	3-5	8	1
E. perforans	3-5	6	1
E. media	3-5	6	1
E. stiedai	1000	21 (**liver**)	100

1) For the precocious strains obtained to date (*E.media, E.magna, E.intestinalis, E.coecicola)* the dose administered must be multiplied by 500 to 1000. The collection time is the prepatent period + 1 day. The number of oocysts recovered is identical to that of the original strain.

2) In view of **morning** caecotrophy between 06.00 h and 12.00 h, the stomach contains essentially caecotrophes, including oocysts.

- **Recovery and purification of oocysts** (propagation of reference isolates and strains)

Rabbits should have feed and water *ad libitum* but solid food should be stopped 24 h before removing caecum and stomach contents. This avoids faecal loss of oocysts and eliminates large vegetable particles.

(a) **Recovery from faeces**

If faeces are used, they must be passed sequentially through sieves with various mesh sizes (400 µm, 300 µm, 200 µm and 100 µm) to eliminate the large non-digested vegetable particles. In this case, the handling takes longer and thus there is a greater risk of contamination. In addition, many oocysts can be lost and this method has little value.

(b) **Recovery from caecum and stomach**

It is preferable to recover oocysts from caecum and stomach contents (minimal risk of external contamination and easier to do because of lack of large plant particles).

- The contents of the stomach and caecum are diluted in water (200 to 300 ml) containing a few drops of wetting agent (Teepol, liquid soap). This mixture is filtered (sieve: 100-150 µm) 1 h later. The residue on the wire gauze sieve is again diluted and refiltered.
- The filtrate (600-800 ml) is centrifuged (800-1,000 x g) for 3 to 5 min. The pellet is then diluted with a 1.14 density solution of magnesium sulphate which is less destructive to oocysts than sodium chloride.

- After centrifugation at 1,000 x g for 10 min the supernatant is collected and washed the first time in fresh water and then treated for 5 min with sodium hypochlorite (12.5% active chlorine) to kill undesirable bacteria and both to prevent clumping and to assess sporulation more accurately. Nevertheless it is necessary to bear in mind that treatment with sodium hypochlorite **makes subsequent identification of species more difficult**. Sodium hypochlorite must be removed **with three or four washes, taking care that the oocysts are not in the supernatant.**

Generally the suspension of oocysts is suitable for most of the animal experiments. If necessary (for the extraction of antigens, molecular biology, etc.) further purification can be carried out using the following procedure:

(c) **Further purification of oocyst suspensions**

- Pass through wire-gauze sieves (100-70-40 µm)

- Centrifuge and resuspend the pellet in one volume of water. Add an equal volume of concentrated sodium hypochlorite (12.5% active chlorine). Incubate for 15 min on ice.

- Add water (9 vol. for 1 vol. of the previous oocyst suspension). Centrifuge for 5 min at 1,000 x g. Discard the supernatant (check that the supernatant contains no oocysts).

- Add 3 vol. of saturated NaCl to 1 vol. of the pellet. Mix vigorously and then add the equivalent of 1.5 cm of water on the surface of the saturated salt solution. Centrifuge for 3 min at 500 x g.

- Collect the oocysts (white layer) at the interface between water and saturated salt solution through the water phase. Transfer into conical flasks and wash four times with water. If a few oocysts are present (without white layer) it is possible to use all the supernatant, but in this case wash once with 9 vol. water for 1 vol. NaCl solution and then wash three more times with water.

Note: Take care not to leave the oocysts in saturated NaCl for a long time, if not they can rapidly become damaged.

- Resuspend the cleaned oocysts at the maximum concentration of $1x10^6$ oocysts per ml in potassium dichromate at the final concentration of 2.5%.

- **Sporulation**

Quality of sporulation is the most essential point to obtain reproducible experimental results. If the sporulation is lower than 80%, unreliable results have to be expected and the isolate should be discarded.

For sporulation oocyst suspensions are incubated in a stirring water bath at 26° C. Temperatures > 28°C are detrimental to oocyst viability. The oocysts are in a 2.5% solution of potassium dichromate and oocyst concentration must not exceed 3×10^6 per ml. (For good oxygenation use for example a 250 Erlenmeyer flask and do not fill with more than 80-90 ml of suspension of oocysts). The sporulation time varies according to the species (Tab. 10) but in practice the duration varies from 24 h for *E. exigua* and *E. perforans*, 72 h for *E. intestinalis, E. coecicola* and *E. piriformis* and 48 h for the other species.

Tab. 10: Prepatent period and sporulation time (hours) at different temperatures
(OS: Original -Parental - Strain, PL: Precocious Line, ND: not done) (D. Licois and P. Coudert)

Species		Prepatent period[1)] (days)	Sporulation time: h 18°C	22°C	26°C
Eimeria perforans		5	50	30	22
Eimeria media	OS	4.5	60	41	30
	PL	2.5	ND	ND	ND
Eimeria exigua		7	ND	23	17
Eimeria magna	OS	6.5	115	80	46
	PL	4.5	ND	ND	ND
Eimeria coecicola	OS	9	120	85	60
	PL	5.5	ND	ND	ND
Eimeria irresidua		9	105	85	50
Eimeria flavescens		9	120	80	48
Eimeria intestinalis	OS	8.5	105	70	60
	PL	5.5	105	70	60
Eimeria piriformis		9	150	90	70
Eimeria vejdovskyi		10	ND	50	35
Eimeria stiedai		14	110	63	57

[1)] The prepatent period is defined as the interval between the inoculation and the observation of the first oocysts excreted in the droppings. Because of the peculiar physiology of the rabbit (caecotrophy) the oocysts in faeces can be detected only during a certain time (afternoon until next morning). **In the caecum and in the stomach**, oocysts can be observed 12 to 24 h before excretion in faeces (see also page 54).

- **Storage of sporulated oocysts**

The cultures are stored in 2.5% potassium dichromate at 4 - 6°C and can be preserved for a long period. Oocysts of most species remain viable for several years (*E.magna* more than 14 years!). *E.piriformis* and *E.exigua* and, to a lesser extent, *E.irresidua* survive less well (1 - 3 years).
Isolates treated in chlorox and those cultures poorly sporulated or left too long in the waterbath during sporulation have reduced survival.

To prevent any undesired genetic drift in the population being studied, oocysts used for each experimental study should be taken from the **oldest available original strain.** The best results are obtained with strains sporulated more than 24 h and less than 1 month prior to the experiment.

- **Recovery of sporocysts and sporozoites for molecular studies**

Freshly sporulated oocysts are used. They are first treated according to the method derived from that of Hosek [6].

- Oocysts are incubated at 4°C in 20% sodium hypochlorite (v/v) for 2 h for *E. intestinalis, E. piriformis* and *E. flavescens* or overnight for the other species, washed several times by centrifugation with water and once with phosphate-buffered saline (PBS, pH 7.4).

- The pellet is resuspended in 20 ml of HAM F10 medium (Sigma) plus 20 µl of 2M L-cysteine (Sigma). Oocysts are then allowed to incubate in this medium under saturated CO_2, at 39°C for 2 h. This time is sufficient for the release of a large number of sporocysts of *E. intestinalis, E. piriformis* or *E. flavescens*. For the other species, because of the resistance of the oocyst wall, the oocysts are washed twice in PBS and the sporocysts are obtained after disruption with a Potter homogeniser (taking care not to use too many strokes, in order to avoid sporocyst damage). At the PAP, using the Potter, the best results are obtained with a concentration of oocysts of $1x10^3$ to $3x10^3$ per ml.

- The sporocysts washed in PBS are pelleted by centrifugation at 2,500 x g for 10 min.

- For all species, excystation is performed by incubation for 3 min to 3 min 30 sec, at 39°C in 1% biliary salt (Prolabo, France) and 0.4% trypsin (Difco).

  Note: Do not exceed this time, otherwise the sporozoites will be destroyed. It is recommended to check the evolution of excystation every minute by microscopic observation of small samples. The reaction is stopped by addition of PBS at 4°C.

- The sporozoites, washed twice in PBS and pelleted at 9,000 x g for 6 min, can be used without further purification for genomic DNA extraction and for some techniques such as Random Amplified Polymorphic DNA assays, but must be further purified through a DE-52- column [17] for Pulse Field Gel Electrophoresis.

- **Preparation of oocyst doses for infection of animals**

- Remove potassium dichromate by repeated centrifugation in water. Discard supernatant and dilute pellet with 100 ml of water ("original suspension").

- Count sporulated oocysts with a modified McMaster counting chamber (20 columns) (objective x 20). An aliquot of 1 ml of the original suspension is mixed with 99 ml of saturated salt solution (NaCl or $MgSO_4$).

Note: Because oocysts float quickly in the saturated solution, the suspension should vigorously agitated and the McMaster chamber filled rapidly. Dilute the oocyst suspension further with the saturated salt solution if oocyst concentration is too high.

The best results are obtained when there are 100 to 900 oocysts per McMaster chamber ($1cm^2$). In order to obtain precise oocyst numbers several counts are necessary. It is preferable to perform these on another aliquot rather than performing several counts on the same sample. The counting methods with Thoma or Fuchs-Rosenthal counting-chambers are accurate only if the original suspension is very clean and passed through a 40 µm wire-gauze sieve. In this case many oocysts are lost and the results are not better.

When the concentration of the original suspension is known, remove the approximate quantity of oocysts required for the experiment and adjust the concentration so that the number of oocysts to be inoculated into the animal are contained in 0.2 ml.

- **Inoculation of animals**

The required dose of oocysts should be given in 0.2 ml. We use an Eppendorf pipette, placing the tip just under the tongue. The animal swallows the 0.2 ml rapidly. The animal will spit out part of the dose if the amount is greater than 0.5 ml. The use of an intra-oesophageal catheter is possible but dangerous (lung injury). The amount inoculated by catheter must be low (0.5 - 1 ml) or the majority of the inoculum may be immediately evacuated to the duodenum, and there will be no excystation (the oocysts must remain for a long time in the stomach where the oocyst wall is lysed releasing the sporocysts which will excyst in the duodenum).

The doses used for infection of animals with reference strains are given in Tab. 9, and Tab. 11 contains data dealing with the reproductive potential of the different species.

Tab. 11: Reproductive potential of different *Eimeria* species of rabbits
(OS: Original Strain; PL: Precocious Line) (P. Coudert and D. Licois)

Species		Maximum yield/rabbit (x 10^8)	Minimum number of oocysts for inoculation to obtain maximum yield	Yield for one oocyst inoculated
E. perforans		2 - 4	200	$5x10^6$ - $6x10^6$
E. media	*OS*	2 - 4	200	$1x10^6$ - $2x10^6$
	PL	2 - 4	$1x10^5$	$2x10^3$ - $4x10^3$
E. exigua		1 - 2	$1x10^3$	$1x10^5$ - $2x10^5$
E. magna	*OS*	1.5 - 3	80	$2x10^6$ - $4x10^6$
	PL	1 - 2	$4x10^4$	$2.5x10^4$ - $5x10^4$
E. coecicola		3 - 4	500	$3x10^5$ - $8x10^5$
E. irresidua		1 - 2	100	$1x10^6$ - $2x10^6$
E. flavescens		2 - 5	100	$2x10^6$ - $5x10^6$
E. intestinalis	*OS*	30 - 50	$1x10^3$	$3x10^6$ - $5x10^6$
	PL	30 - 50	$5x10^5$ - $1x10^6$	$3x10^3$ - $1x10^4$
E. piriformis		1.5 - 2.5	$1x10^4$	$1.5x10^4$ - $2.5x10^4$
E. vejdovskyi		10 - 15	$1x10^3$	$x10^6$ - $1.5x10^6$
E. stiedai[1)]		-	-	-

[1)] Because oocysts are trapped in the biliary ducts of the liver it is not possible to determine the characteristics of the reproduction potential for *E. stiedai.*

1.4.3.2. Experimental infection of animals for pathogenicity studies

- **Dose of sporulated oocysts**

The inoculation dose depends on the severity of the disease to be studied. The oocyst numbers proposed in Tab. 12 are approximate doses. Keep in mind that the data obtained will depend on the quality of sporulation, age of the isolate, the immune status of the animal, etc. Animals are inoculated with 0.2 ml of oocyst suspension using an Eppendorf pipette as descibed above.

Tab. 12: Doses of sporulated oocysts administered for pathogenicity studies
(P. Coudert and D. Licois)

Species	Dose of oocysts per rabbit (x 10^3) Mild disease	Dose of oocysts per rabbit (x 10^3) Severe disease
E. perforans	10	>100
E. media	7 - 10	50
E. exigua	40	>100
E. magna	7 - 10	50
E. coecicola	40	>100
E. irresidua	10	50
E. flavescens	2-5	10-13
E. intestinalis	2-5	10-13
E. piriformis	10	100
E. vejdovskyi	10	100
E. stiedai	10	>100

- **Recovery of oocysts for counting** (pathogenicity studies)

In the rabbit several points must be considered:

- Doses > 1,000 oocysts always cause clinical pathology irrespective of the species, and in this case the excretion is always between 1x 10^9 to 4 x 10^9 with *E. intestinalis* and *E. vejdovskyi,* or 1x 10^8 to 4 x 10^8 for all other species [13] (compare Tab. 11). **There is no correlation between oocyst excretion and the severity of the disease** [1].

- In the rabbit, the excretion peak is of short duration (about 48 h) and simultaneously the excretion of faeces is very low (constipation). It has also to be considered that a part of the faeces (caecotrophes) are reingested by the animal and are in the stomach. From a practical point of view, oocyst excretion can be validly interpreted only on excreta collected during the 3 or 4 first days of the patent period (which corresponds in fact to 99% of the total oocyst output). The criterion "number of oocysts per gram of faeces" must only be used in field studies when other methods are not available.

- The hard faeces of the rabbit contain large amounts of vegetable particles and are very dehydrated. To obtain valid oocyst counts, it is necessary to rehydrate before

filtering the faeces; otherwise oocysts are trapped within the plant particles. The following procedure is used at the PAP:

- **Procedure**

- Collect faeces of the 3-4 first days of the patent period in a plastic bag and weigh.

- Transfer to a plastic beaker (volume: 2 -3 l) and mix faecal material.

- Of this faecal material 300 g are mixed 1/6 with tap water (300 g in 1,500 ml of water) and left to rehydrate for 1 h in minimum (overnight is better).

- 40 g of the rehydrated and homogenized material are passed through a 500 µm wire-gauze sieve, and the filtrate is collected in a 100 ml plastic test-tube. Adjust to 100 ml with a saturated solution of magnesium sulfate.

- Mix the filtrate vigorously to ensure that the suspension is homogeneous and immediately transfer an aliquot with a Pasteur pipette into a McMaster counting chamber (20 columns). Repeat this operation with the second, third and fourth chamber. If oocyst numbers in the whole chamber (1 cm^2) exceed 500-1,000, countings are difficult and generally not accurate.

Dilute 1/10, 1/100 or1/1000 if necessary (5 ml of oocyst supension in 45 ml of $MgSO_4$ saturated solution at each dilution).

If the mentioned proportions have been respected, total oocyst output per animal (**N**) can be calculated:

$$\mathbf{N} = \frac{\mathbf{n} \times \mathbf{d} \times \mathbf{100} \times \mathbf{P}}{\mathbf{y}}$$

n = mean of the oocysts counted in 4 McMaster chambers
d = dilution factor (1, 10, 100 or 1000)
P = weight of the excreta collected
y = number of animals per cage
100 = multiplication factor when all the proportions indicated are respected (dilution of 1/6 of the excreta, sample of 40 g adjusted to 100 ml with $MgSO_4$). All these proportions have been calculated to obtain a representative result of the counting.

1.4.4. Development of *Eimeria* species in the host and pathology

Intestinal localisation of the development of *Eimeria* spp.: The specific sites of development for each *Eimeria* species of the rabbit is presented in Fig. 7. Indeed, for some species, some parts of the endogenous development take place in another site of the intestine. Some histological findings on the endogenous development in rabbit have been described elsewhere [5, 14, 16, 18].

The gross lesions within the gut (Fig. 8) are most visible when gamogony occurs, the intensity varying according to the species. Although spectacular, the duration of the detectable lesions do not exceed 3 or 4 days, and their intensity depends also on the dose of oocysts inoculated. Although overlaps in the preferred sites of development occur, macroscopic intestinal lesions can be used, in association with other criteria, in the diagnosis of coccidial infection .

Pathogenicity: Coccidia are specific pathogenic agents. When inoculated into rabbits, they induce the same lesions and the same symptoms (diarrhoea, loss of weight, mortality etc.).

Coccidiosis can be classified according to the severity of the symptoms (Tab. 13; see also Fig. 8)

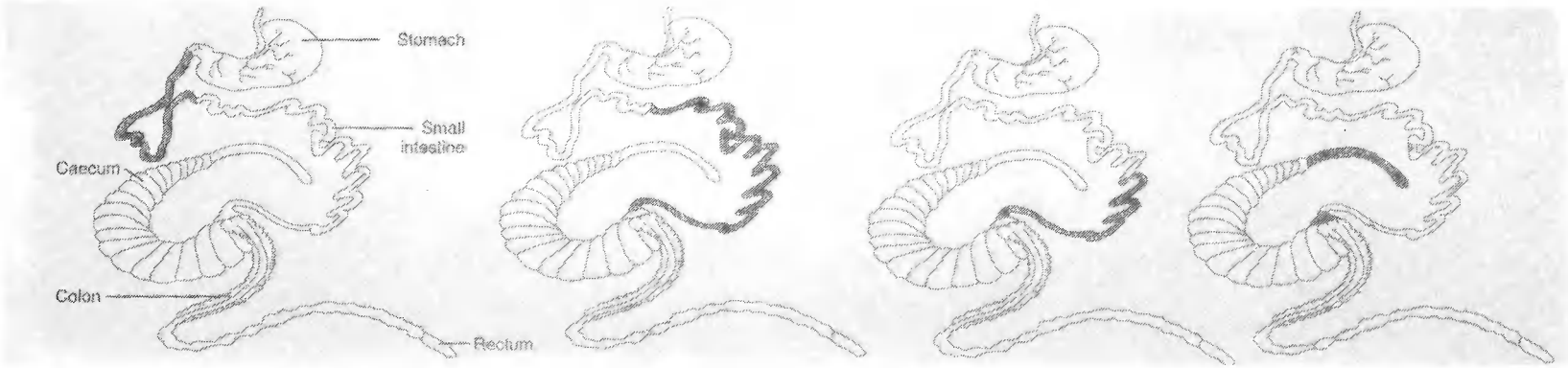
Tab. 13: Pathogenicity of different strains of rabbit coccidia
(P. Coudert, D.Licois, F. Provot, F. Drouet-Viard)

Pathogenicity[1)]	Species	Symptoms
Non pathogenic	*E. coecicola*	No sign of disease.
Slightly pathogenic	*E. perforans* *E. exigua* *E. vejdovskyi*	Slight depression of growth, no diarrhoea, no mortality.
Mildly pathogenic or pathogenic	*E. media* *E. magna*[2)] *E. piriformis* *E. irresidua*	Depression of growth, some cases of diarrhoea, mortality depending on the doses (most important from $1x10^5$ oocysts inoculated).
Highly pathogenic	*E. intestinalis* *E. flavescens*	Severe depression of growth, severe diarrhoea, high mortality (LD_{50}=3,000 to 5,000 oocysts)
Pathogenicity depending on the dose	*E. stiedai*	Slight depression of growth in standard rabbit breeding. Weight loss and mortality with experimental doses > $1x10^5$. Can be more pathogenic under hot climate.

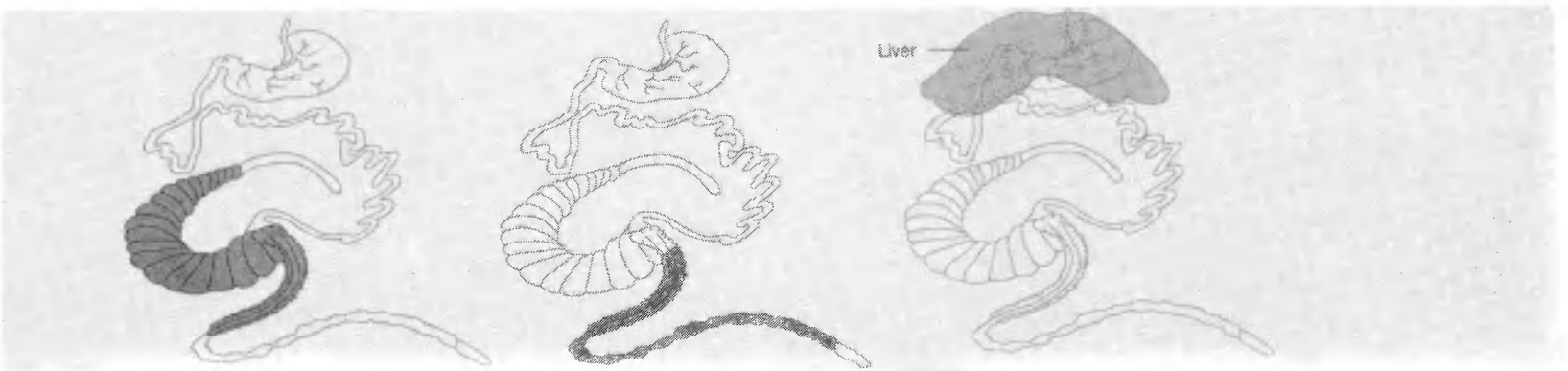
1) The pathogenicity probably may vary for each species, according to the strains.

2) Under field conditions, pathogenicity of *E. magna* (and probably *E. irresidua*) is enhanced by bad hygienic conditions or intercurrent infections (*Escherichia coli*).

Fig. 7: Sites of development of ***Eimeria*** species in rabbits (D. Licois and P. Coudert)

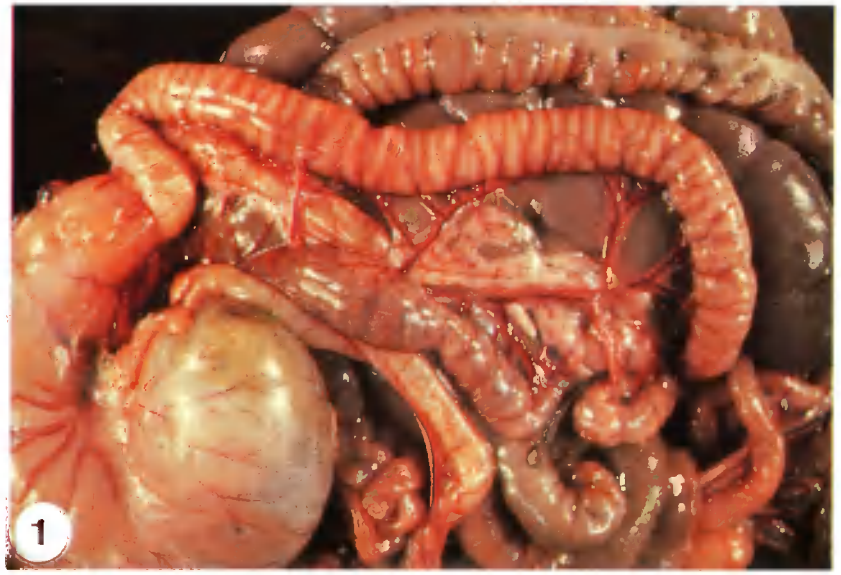


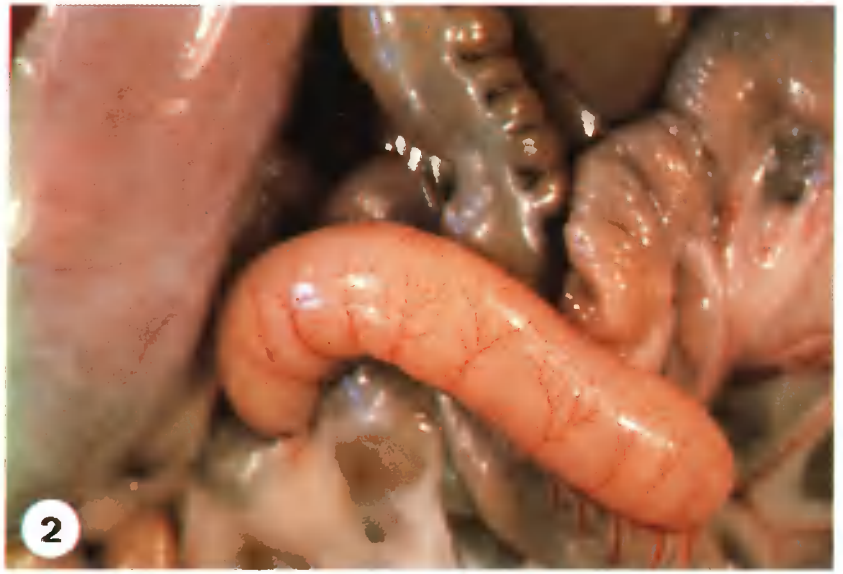
E. perforans | ***E. media, E. magna E. irresidua, E. intestinalis*** | ***E. exigua, E. vejdovskyi*** | ***E. coecicola***

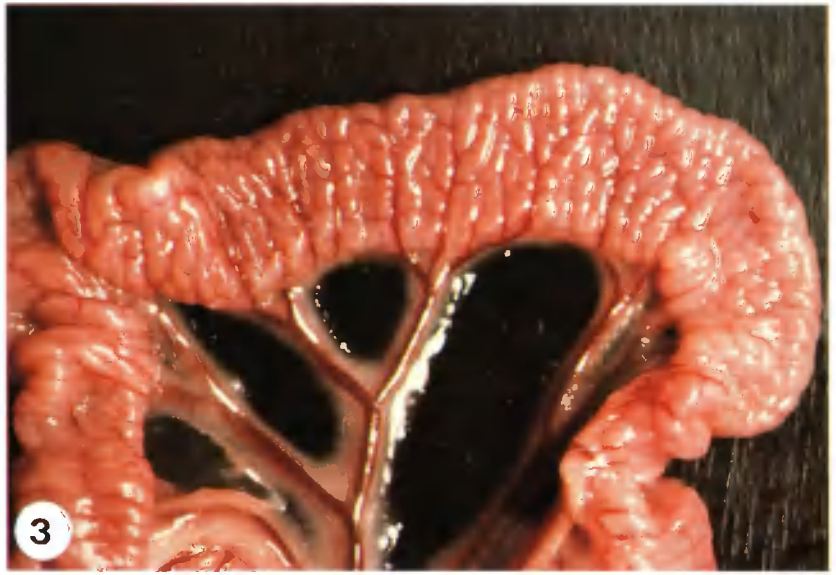


E. flavescens | ***E. piriformis*** | ***E. stiedai***

Fig. 8.1.-8.4.: Explanations see page 70







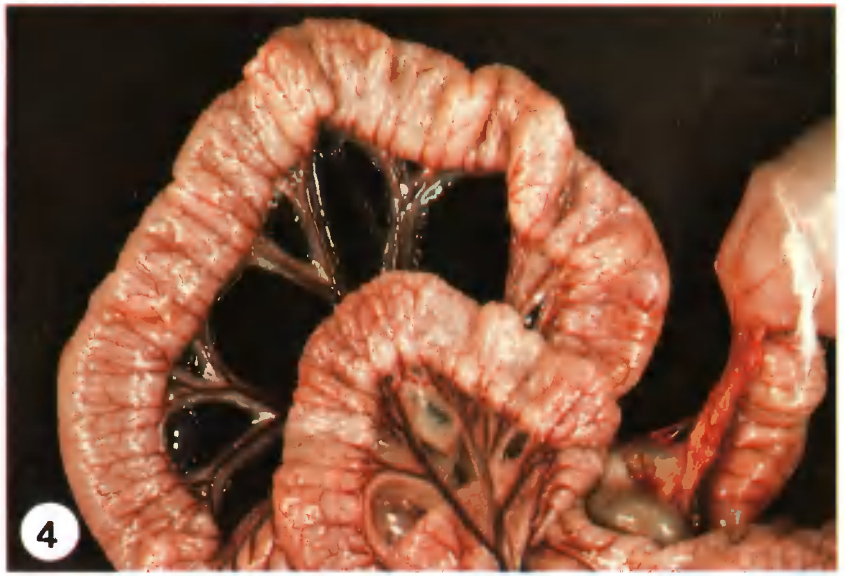
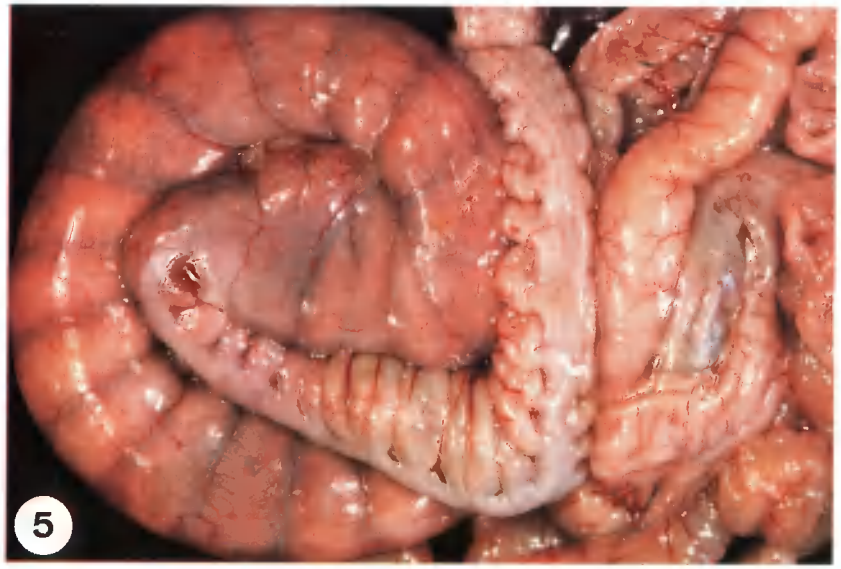
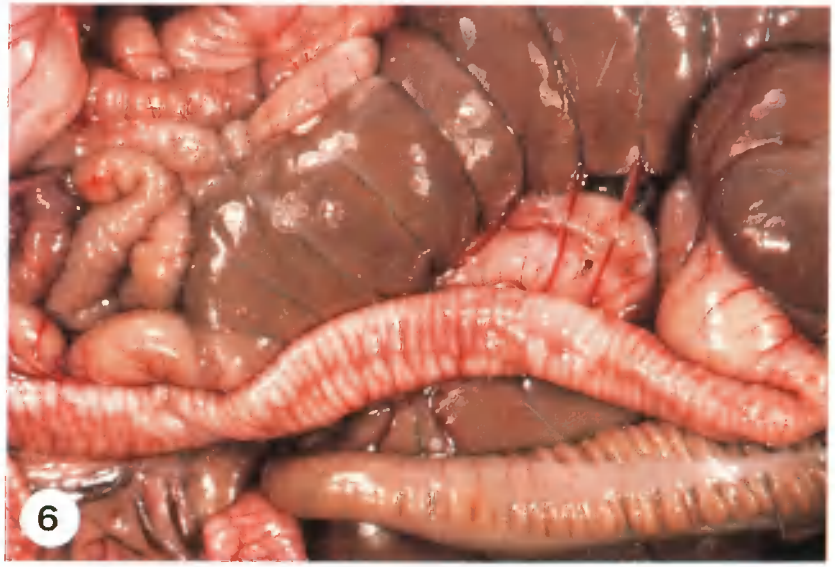
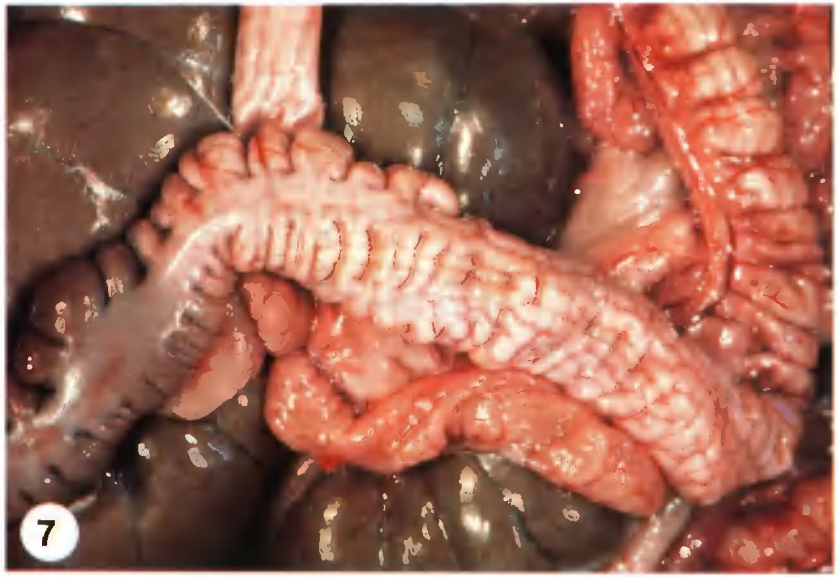
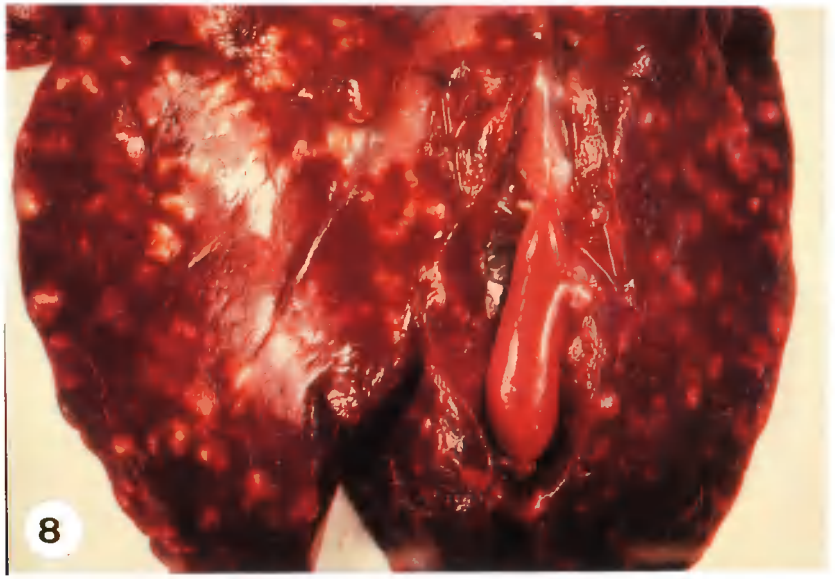


Fig. 8.5.-8.8.: Explanations see page 70









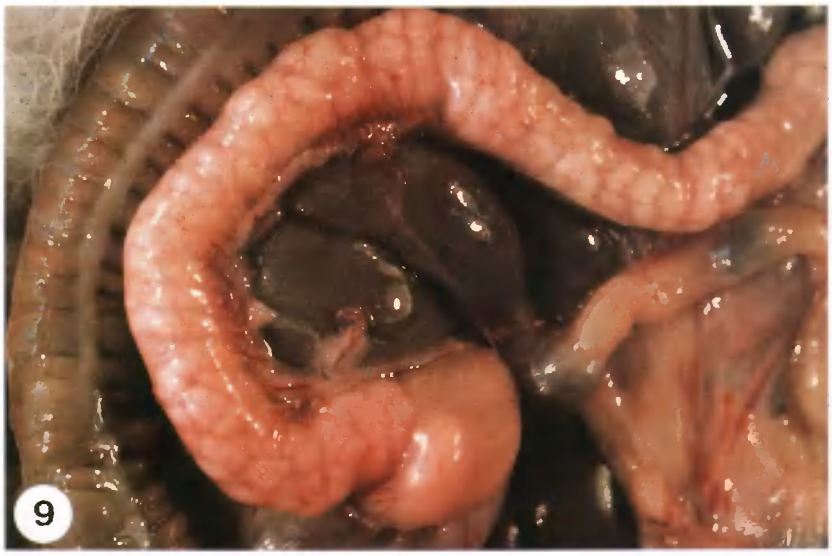


Fig. 8: Macroscopic lesions of digestive tract and liver of rabbits experimentally infected by different *Eimeria* species (photographs: D. Licois)

8.1. ***E. media***: non specific lesions of the duodenum (the endogenous development takes place in the jejunum and the ileum) can be seen after infection with high doses of oocysts (> 10^5 oocysts).

8.2. ***E. coecicola***: this species is non-pathogenic but lesions of the vermiform appendix can be observed in heavy infection.

8.3. ***E. magna***: lesions occur in the jejunum and more pronounced in the ileum, the intensity of the lesions is depending on the dose. The same lesion score can be seen in rabbits given $5x10^4$ to $1x10^5$ oocysts of *E. magna* or $3x10^3$ oocysts of *E. intestinalis*.

8.4. ***E. irresidua***: lesions occur with relatively high doses ($5x10^4$ to $1x10^5$ oocysts) in the jejunum and to a lesser extent in the ileum.

8.5. ***E. flavescens***: this species induces with small doses of oocysts ($2\text{-}3x10^3$ oocysts) marked lesions in the caecum and the colon.

8.6. ***E. intestinalis***: this species induces with small doses of oocysts ($2\text{-}3x10^3$ oocysts) marked lesion in the ileum and in the lower part of the jejunum.

8.7. ***E. piriformis***: only the colon (proximal and distal), except the 6 to 10 cm after the caecocolic junction, is affected by *E. piriformis*.

8.8. ***E. stiedai***: this species induces lesions of the biliary ducts (white nodules) and the liver (hypertrophy).

8.9. ***E. vejdovskyi***: lesions occur only in the ileum and the distal part of the jejunum in rabbits given high doses of oocysts (> $1x10^5$ oocysts).

For all forms of intestinal coccidiosis, depending on the species, the peak of the disease determined by depression of weight gain or loss of weight occurs between the 4th and the 11th day after inoculation. During this time, water and food consumption as well as the faecal excretion is reduced. The faeces are generally dry, but a short period of diarrhoea [faeces more hydrated (*E. intestinalis, E. magna)*, or liquid (*E. flavescens*)] can be observed. Rabbit enteritis following coccidiosis has been studied with reference to calves and human infants in whom diarrhoea is characterised by 3 main phenomena: (a) considerable loss of faecal matter (water and minerals and above all sodium), (b) extracellular dehydration and (c) metabolic acidosis. In fact rabbits suffering from diarrhoea, like other mammals (calves, piglets, infants), certainly have more watery faeces but the faecal excretion is reduced in sick animals when compared to healthy ones. There is no change in the distribution of water in the organism, except that the skin is heavily dehydrated, and no change in the blood pH. The most marked modification of the blood plasma is a severe hypokalemia resulting from faecal losses of potassium [8, 9, 11].

Mortality is observed with constancy only with *E. intestinalis* and *E. flavescens,* between the 9th and the 12th day post inoculation. Except in the case of bacterial complications, the recovery from the disease occurs at the end of the 2nd week for all intestinal *Eimeria* species. In addition, a strong immunity is acquired [4, 10].

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1.5. *Isospora suis* of swine

S.A. Henriksen

1.5.1. Sow units delivering piglets for inoculation

Pure strains of *Isospora suis* are maintained by periodic passage in piglets obtained from sow units free from infections with *I. suis*. The sow units are regularly tested by faecal examination of piglets of the age group of 12 days +/- 2 days (the optimal age group for demonstration of oocysts under field conditions [1].

1.5.2. Handling of piglets in the laboratory

- Piglets to be infected with *I. suis* are transferred from the sow unit to the laboratory when 2-3 days old. At the laboratory the piglets are housed in plastic boxes, breadth 0.8 m, length 1.2 m and height 0.9 m. Such boxes prevent contamination with faecal material from/to the environment. Two or three piglets are housed in each box. The boxes are disinfected with hot water prior to use.

- Heat lamps are placed at an appropriate height to ensure adequate temperature.

- Contact with other pigs or porcine faecal material must be avoided and hand wash employed before the experimental animals are handled.

- Faecal samples should be examined daily to ensure that the piglets are not infected with *I. suis*.

1.5.3. Infection of piglets and sampling of oocysts

The prepatent period of *I. suis* is 5-6 days.

- Piglets at an age of 4-10 days to be infected are inoculated with a suspension of sporulated oocysts in water applied through a stomach tube connected with a 10 ml syringe. Each piglet is infected with 3,000-5,000 sporulated oocysts.

- The piglets are infected on Wednesday or Thursday. Faecal material is collected from rectum by sampling twice daily through Monday - Friday of the following week.

1.5.4. Purification, sporulation, and storing of oocysts

- Faecal material (opg > 10,000) is suspended in tap water (1:5), passed through a 200 µm sieve or gauze (Fig. 9), and subsequently mixed with a 45% v/v sugar solution added with 1% Tween 20 (80 ml faecal suspension in 120 ml sugar/Tween solution).

- Equal amounts of the mixture are transferred to each of four 250 ml centrifuge tubes.

- 5 ml of tap water are carefully deposited upon the surface (of the faecal-sugar-suspension) in each of the tubes.

- Centrifugation for 40 min at 2,800 rpm (1500 x g) at 20°C.

- The upper layer is transferred to a 500 ml beaker, which is subsequently filled with tap water.

- The suspension is divided equally in each of four 250 ml centrifuge tubes.

- Centrifugation for 30 min at 2,800 rpm (1500 x g) at 20°C.

- The supernatant is discarded and the sediment is washed twice in tap water.

- The sediment is suspended in tap water (1:25), and 5% potassium dichromate is added (1:1).

- The suspension is transferred to culture flasks (Erlenmeyer flasks). The depth of the suspension in each flask should not exceed 5 mm.

- The flasks are stored at room temperature for 4 days and the cultures are aerated daily by gently shaking.

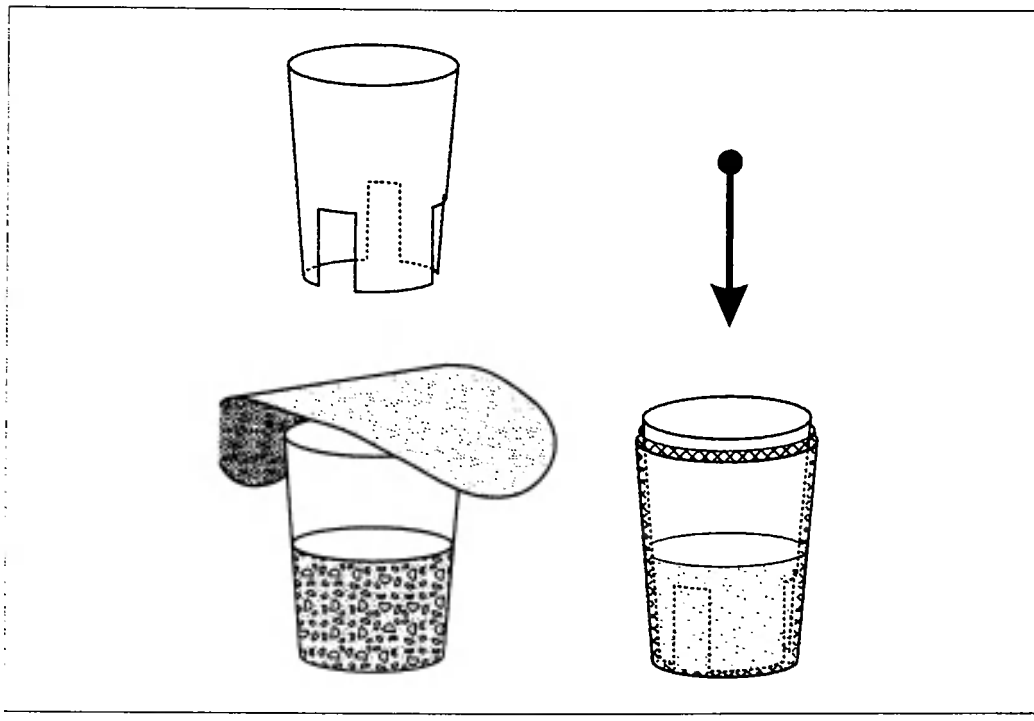

Fig. 9: Device for filtration of faecal suspensions

Filtration of faecal suspensions through gauze by means of two disposable polystyrene cups:

- The faeces sample is mixed thoroughly with water in one of the cups. A circular piece of gauze with a diameter equivalent to twice the height of the cup plus the diameter of its base is placed over the cup.
- By the other cup, with the bottom cut off and slits made in the side, the gauze is pressed down into the intact cup.
- Subsequently, having passed through the gauze, the faecal suspension will appear in the inner cup in a filtered condition [3].

- Following sporulation, the suspensions from all the flasks are collected in one bottle of appropriate volume. The number of oocysts per ml of suspension is determined. Sporulated oocysts will remain infective for at least 2-3 months if stored at 4°C in 2.5% potassium dichromate.

1.5.5. Preparation of dosing material

- 3-5 ml of the oocyst suspension in potassium-dichromate solution is suspended in 40-45 ml of tap water and subsequently centrifuged at 2,000 rpm (680 x g) for 10 min.
- The washing procedure is repeated twice.
- The sediment is suspended in 10 ml of tap water.
- The number of sporulated oocysts is counted (see below).
- The suspension is diluted with tap water to obtain a concentration of 1,000 oocysts/ml for inoculation.

1.5.6. Examination of faeces for *Isopora suis* oocysts

Modificated McMaster technique

- 0.2 g of faeces are suspended in 2.8 ml of flotation medium (saturated sodium chloride with the addition of 50 g sugar per 100 ml, specific gravity 1.27) [2].
- Filtration through gauze (Fig. 9).
- The filtrate is transferred to a McMaster counting slide.
- Count the oocysts in one field of the slide of 1 cm^2 (corresponding to a volume of 0.15 ml).
- Calculate oocyst numbers per gram (opg):

  Opg = N x 100
  N = Number of oocysts counted in one field of 1 cm^2.
  100 = Correction factor to volume and 1 g of faeces.

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2. Cultivation and cryopreservation

2.1. In vitro cultivation of avian *Eimeria* species: *Eimeria tenella*

W. Raether, J. Hofmann and M. Uphoff

In this chapter techniques for cultivation of *Eimeria tenella* are described, including chapters on preparation of parasites (2.1.1.), cryopreservation of sporocysts and sporozoites (2.1.2.), preparation, cultivation and staining of parasitized primary chick kidney (PCK) cells (2.1.3.), *in vitro* assays (2.1.4.), production of polyclonal antibodies (2.1.5.), and a flow cytometric technique (2.1.6.).

2.1.1. Preparation of Parasites

2.1.1.1. Isolation and sporulation of *E. tenella* oocysts (strain Hoechst and Houghton, respectively)

- **Equipment, materials and animals**

- Sterile 2 l glass beakers, wrapped with aluminium foil.
- Sterile 4 % (w/v) potassium dichromate solution (Riedel-de-Haen, D).
- Autoclaved, concentrated NaCl solution (Riedel-de-Haen).
- Sterile distilled water.
- Sterile magnetic stirring bars (Glock, D).
- Magnetic stirrer MR2000 (Heidolph, D) or equivalent.
- Centrifuge bottles with a capacity of 750 ml (Beckmann, USA).
- Beckmann swing out rotor GH-3.7.
- Microwave oven, 650 W (Sharp, UK).
- Vibration sieving machine Vibro (Retsch, D).
- Autoclaved (80°C, 30 min), stackable test sieves (Glock, D) with decreasing porosity of 2 mm, 1 mm, 0.5 mm.
- Centrifuge tubes, polypropylene, 50 ml with screw caps (Greiner, D).
- Rotixa/AP swing out rotor (No. 5096) or equivalent.
- Sterile gauze.
- Sterile screw capped glass bottle, 1 l (Schott, D).
- Membrane air pump WISA (Glock, D).
- Disposable filter holder, 0.45 µm (Schleicher and Schüll, D).
- 1 - 4 week old selected leghorn chickens (LSL Rhein-Main, D-64807 Dieburg).

- **Procedure**

Note: In order to avoid contamination by other *Eimeria* spp. work should be carried out on clean benches.

- Collect faeces from infected birds daily, 5 to 8 days post infection (p.i), in dishes containing 4 % potassium dichromate solution.
- Homogenize 1 l of faeces suspension in a 2 l glass beaker for 15 min using a magnetic stirrer.
- Slowly add 350 g NaCl and agitate suspension for 15 min to dissolve NaCl.
- Remove coarse particles by sieving the faeces suspension through stackable test sieves.
- Rinse the remaining sludge with concentrated NaCl solution and centrifuge sieved suspension in 750 ml centrifuge bottles for 5 min at 2540 x g.
- Aspirate floating oocysts and filter the resulting oocyst suspension through 2 layers of gauze.
  Note: Confirm the identity of aspirated oocysts using a microscope.
- Centrifuge filtered oocyst suspension for 3 min at 2400 x g in a Rotixa swing out rotor.
- Aspirate floating oocysts and dilute 10 ml of recovered oocyst suspension with 40 ml distilled water and centrifuge 3 min at 2400 x g.
- Wash pellet twice with distilled water and resuspend it in 4 % potassium dichromate solution (1 ml should contain 0.5 - 5 x 10^6 unsporulated oocysts).
- Aerate oocyst suspension for 72 h at 25°C.
  Note: Air from membrane pump must be filtered by 0.45 µm filter.
- Determine the number of sporulated oocysts/ml in the well mixed oocyst suspension and store it at 6°C.

2.1.1.2. Sterilisation of sporulated oocysts

Additional materials

- Oocyst suspension in 4 % potassium dichromate solution (about 0.5-5x10^6 oocysts/ml).
- Concentrated sodium hypochlorite solution with 12-13 % active chlorine (Riedel-de-Haen, D).

- Penicillin/streptomycin solution, 10,000 i.u. + 10 µg/ml in physiol. saline (Biochrom, D).
- Fungizone (amphotericin B) solution (Serva, D).

- **Procedure**

  - Centrifuge 50 ml of the oocyst suspension for 3 min at 2400 x g (Rotixa/AP)
  - Resuspend pellet in a final volume of 200-300 ml sodium hypochlorite solution and stir for 20-30 min at 150 rpm on the magnetic stirrer. Check purity of the suspension at 5 min intervals, using a microscope.
    Note: Do not extend treatment above 30 min!
    Subsequent steps have to be performed under sterile conditions.
  - Centrifuge oocyst suspension for 3 min at 2400 x g and aspirate floating oocysts found on top of the sodium hypochlorite solution.
  - Dilute 10 ml oocyst suspension with 40 ml distilled water and wash pellet several times with distilled water until chlorine can no longer be detected (smelling test).
  - Overlay the pellet with 5 ml distilled water, carefully resuspend the upper layers of the pellet by circular movement of the tube and discard the supernatant.
  - Resuspend the remaining pellet in 50 ml distilled water containing 40 units penicillin/ml, 40 µg streptomycin/ml and 1 µg amphotericin B (Fungizone)/ml.
  - Determine number of oocysts/ml and store purified and sterilised oocyst suspension at 6°C.

2.1.1.3. Isolation and purification of sporocysts

Additional materials and equipment

- Oocyst suspension (purified and sterilised) adjusted to 4 x 10^7 oocysts / ml.
- Hanks' balanced salt solution (HBSS) without phenol red (Biochrom).
- Glass tubes, sterile and thick walled (Schott; 16 mm diameter, 160 mm length) filled with glass beads of 710 - 1180 µm diameter (Sigma, USA) up to a height of 15-20 mm.
- Vortexer Reax lR (Heidolph) or equivalent.
- 1.5M and 0.15M NaCl solution (sterile).
- Percoll (sterile), density 1.129 g/ml (Pharmacia, Sweden), high speed centrifuge J2-21M/E (Beckmann) with fixed angle head rotor JA-17.

- High speed centrifuge tubes, polyallomer, 50 ml with stopper (Sarstedt, D).
- Penicillin/streptomycin solution, 10,000 i.u. + 10 µg/ml in physiol. saline (Biochrom).

Procedure

- Centrifuge 50 ml of the purified oocyst suspension for 3 min at 2493 x g (Rotixa/AP).
- Wash pellet twice in HBSS and resuspend pellet in 15 ml HBSS.
- Pour 1.5 to 2 ml of oocyst suspension into tubes containing glass beads and agitate suspension.
  Note: Check disintegration under the microscope.
- Recover sporocysts from glass beads by intensive rinsing of suspension with 3 x 10 ml HBSS. Centrifuge suspension for 3 min at 2400 x g and resuspend pellet in 5 ml HBSS.
- Prepare isoosmotic Percoll solutions as follows: Add 10 ml 1.5M NaCl solution to 90 ml Percoll (=100 % isoosmotic Percoll stock solution). Dilute stock solution with 0.15M NaCl to give a 65 % solution.
- Fill a high speed centrifuge tube with 38 ml of the 65 % isoosmotic Percoll solution and layer 2 ml of the unpurified sporocyst suspension on the top.
- Build up gradient by centrifugation at 39 800 x g for 20 min using rotor JA-17.
- Isolate sporocyst fraction as a distinctly visible, broad, diffuse band in the lower part of the tube and remove Percoll by washing three times with HBSS (3 min, 2500 x g). Control purified sporocyst suspension microscopically.
- Store purified sporocyst suspension in HBSS containing 40 i.u. penicillin/ml and 40 µg streptomycin/ml at 6°C.

2.1.1.4. Isolation and purification of sporozoites

Additional materials and equipment

- Sporocyst suspension (purified) adjusted with HBSS to 4 x 10^7 sporocysts / ml.
- Shaking water bath (Julabo sw-20C, Glock) or equivalent.
- Trypsin, 1: 250 enzymatic activity (Difco, USA).
- Taurodeoxycholic acid, sodium salt (Calbiochem, USA).
- Disposable filter holder, 0.2 µm (Schleicher and Schüll) or equivalent.

- Excystation medium: Dissolve 0.25 g trypsin and 4 g sodium taurodeoxycholate in 100 ml HBSS (prewarmed to 37°C) and sterilised by filtration (0.2 µm).
- Complete Medium 199 (Biochrom) containing 2.5 % fetal calf serum (Serva).
- Thoma chamber (Glock).
- Percoll solutions as described above (2.1.1.3, procedure).

Procedure

- Centrifuge purified sporocyst suspension for 3 min at 2400 x g. Resuspend pellet in excystation medium and incubate for 40 min at 41°C in the water bath. Check excystation rate during incubation period under the microscope.
- Centrifuge the unpurified sporozoite suspension for 3 min at 2400 x g. Wash pellet twice in HBSS and resuspend pellet in 2 ml HBSS.
- Layer 1.5 ml of the sporozoite suspension on 38 ml of 65 % isoosmotic Percoll solution and centrifuge for 15 min at 39 800 x g and 20°C, using fixed angle head rotor JA-17.
- Isolate sporozoite fraction (diffuse band in the lower part of the tube) and remove gradient material by five washes with HBSS.
- Resuspend pellet in 10ml preheated (41°C) complete Medium 199 containing 2.5 % FCS. Determine the number of sporozoites per ml and the purity of the sporozoite suspension.

2.1.2. Cryopreservation of sporocysts and sporozoites

2.1.2.1. Cryopreservation of sporocysts and sporozoites

Additional materials and equipment

- Sporocyst or sporozoite suspension (purified).
- Cryovials (Nalge Comp.,USA) or equivalent.
- Marker, resistant to water and methanol.
- Dulbecco' s MEM (DMEM, Biochrom) containing 15 % FCS (Serva).
- Medium 199 (Biochrom) containing 2.5 % FCS (Serva).
- HBSS plus 10 % (v/v) dimethyl sulfoxide plus 15 % FCS (freezing medium for sporocysts).

- DMEM plus 10 % (v/v) dimethyl sulfoxide plus 15 % FCS (freezing medium for sporozoites).
- Programmable ultracryothermostat KT50S (Colora, D) or equivalent apparatus.

- **Procedure**

  - Centrifuge sporocyst or sporozoite suspension for 3 min at 2400 x g. Add freezing medium (6°C) to achieve cell densities of 5 x 10^5 sporocysts / ml or 1 x 10^6 sporozoites / ml, respectively, and resuspend gently.
  - Immediately place cryovials (containing 1 ml of parasite suspension) into the methanol bath (4°C) of the ultra-cryothermostat KT50S. Cooling rate: 1°C / min (4°C to 50°C).
  - Transfer cryovials directly into liquid nitrogen.
  - **Note:** Reserve an aliquot of suspension prior to and after deep-freezing in order to determine the viability of sporocysts and sporozoites. Viability of parasites correlates with high excystation rates of sporozoites from sporocysts, and high numbers of unstained sporozoites indicate successful cryopreservation (dye exclusion test).

2.1.2.2. Thawing of sporocysts and sporozoites

- Heat the bottom of the cryovial rapidly in a 41°C water bath and resuspend thawed cell suspension by adding 10 - 20 ml HBSS (sporocysts) or Medium 199 plus 2.5% FCS (sporozoites) drop by drop. Discard supernatant after centrifugation for 5 min at 500 x g in order to remove DMSO.
- Adjust sporocysts in HBSS and sporozoites in Medium 199 plus 2.5% FCS, respectively, to desired parasite density and transfer the parasite suspension to chickens (sporocysts) or to a cell culture (sporozoites) by inoculation.

2.1.3. Preparation, cultivation and staining of parasitized primary chick kidney (PCK) cells [1]

- **Materials, equipment and animals**

- 70 % ethanol.
- Hanks' solution (Biochrom, D).
- Penicillin/streptomycin solution 10 000 i.u. + 10 µg / ml in PBS (Serva, D).
- Trypsin-EDTA solution, pH 7.5 (2.5 g trypsin, 1: 250 enzymatic activity (Difco, D).
- 0.2 g EDTA (Serva), made up to 1000 ml with PBS.
- Trypsinizing flask (Wheaton, USA).
- Hotplate with magnetic stirrer (IKA-Werk, D).
- Bar magnet with central protuberance.
- Williams E medium (Flow Laboratories, D).
- Medium 199 (Biochrom).
- Foetal calf serum (FCS; Serva).
- Thoma chamber.
- Sterile distilled water.
- Sterile 0.1M NaOH, sterile 0.1M HCl.
- Sterile gloves.
- Slides (76 x 25 mm).
- Flexiperm units (FLEX; Heraeus, D).
- Optilux petri dishes (Becton Dickinson, USA).
- Gas incubator (Heraeus).
- Trypan blue (Sigma, USA): 0.5 g trypan blue dissolved in 100 ml 0.9 % saline.
- Autoclaved forceps and scissors.
- 1 - 4 week old selected leghorn chickens (LSL Rhein-Main, 64807 Dieburg, D).
- Rotixa RP swing out rotor 5096 (or equivalent).

2.1.3.1. Isolation of PCK cells

Procedure

- Kill chicken by decapitation and exsanguinate it well.
- Place killed chicken on its back on a clean, dry, absorbent paper.
- Wet the plumage and skin with 70 % ethanol .

- Retract the skin, open the abdomen and remove the thorax; tear gently the connective tissue around the reniculi.
- Remove the reniculi and place them in petri dishes containing 3 ml ice-cold Hanks' solution with penicillin / streptomycin compound.
- Remove further fibrous tissue and blood clots. Comminute the organs to pieces which can be pipetted.
- Continue to disperse tissue clumps by drawing up and expelling the suspension several times in 47 ml ice cold Hanks' solution using a 10 ml pipette.
- Centrifuge the suspension (10 min in Rotixa / RP swing out rotor, maximal 200 x g) and discard supernatant. Resuspend cell pellet in 50 ml Hanks' solution and repeat procedure of dispersing tissue (see above) three times.
- Resuspend washed cell pellet in 30 ml preheated (37°C) trypsin-EDTA solution and stir gently in a trypsinizing flask on a heatable magnetic stirrer for 5 min.
- Allow tissue to sediment for 2 - 3 min. Gently remove 20 ml of the supernatant and replace with the same volume of fresh trypsin-EDTA solution. Stir for 5 min and discard a further 20 ml of the supernatant.
- At 2 min intervals collect 10 ml of the supernatant. Immediately inactivate the trypsin by mixing the sample with 10 ml ice cold Williams E medium containing 20 % FCS. Add 10 ml of fresh trypsin-EDTA solution to the trypsinizing flask. Repeat 5 times.
- Centrifuge the collected aliquots for 5 min at a maximum of 100 x g. Resuspend cell pellet in 10 ml heated (41°C) Williams E with 10 % FCS.
- Pool cell suspensions and determine cell viability by a dye exclusion test (100 µl of trypan blue solution plus 100 µl of suspended cell suspension). Count the number of intact (unstained) cells in a Thoma chamber.

2.1.3.2. Preparation of Flexiperm chambers (FLEX)

Procedure

- Submerge FLEX in a beaker containing distilled water and autoclave.
- Dry FLEX in open petri dishes under sterile conditions.
- Immerse microscope slides overnight in 0.1M NaOH, then in 0.1M HCl for 1 h.
- Rinse slides in fresh, sterile distilled water several times and dry them in air.
- Wearing sterile gloves, press the smooth FLEX underside onto the dry slide. The complete adhesion between FLEX and the slide is viewed in reflected light from the underside of slide.

- Until use, store the prepared chambers (in Optilux petri dishes) under sterile conditions.

2.1.3.3. Cultivation of parasitised PCK cells

- Adjust cell density: 7 ml culture medium (Williams E + 10 % FCS) should contain 1 - 2 x 10^6 intact cells.
- Fill each of the four central FLEX chambers with 0.5 ml of the adjusted cell suspension. Incubate FLEX for 24 h at 41°C (5 % CO_2 atmosphere).
  Note: If cell confluence does not reach 50 % after a 24 h incubation period prolong incubation period up to 48 h.
- Remove supernatant, detached and damaged cells from each chamber and gently rinse cell monolayers, each with 0.5 ml Medium 199 + 2.5 % FCS.
- Adjust sporozoite suspension (freshly excysted and highly purified) to 1 - 5 x 10^4 sporozoites / 0.5 ml Medium 199 + 2.5 % FCS
- Replace 0.5 ml of culture medium in each chamber with 0.5 ml of the sporozoite suspension and incubate for 4 h.
- Remove FLEX carefully (sterile gloves) from the slide coated with cell monolayers. Place slide in an Optilux petri dish and immediately add 15 ml of heated (37°C) Hanks' solution. Gently agitate to suspend extracellular sporozoites.
- Discard the rinsing fluid and repeat the rinsing procedure once more.
- Fill the petri dish with 15 - 20 ml of Medium 199 plus 2.5 % FCS and incubate the culture at 41°C and 5 % CO_2 atmosphere.
  Note: Observe the development of parasites and growth of PCK cells twice daily under phase contrast. Do not change medium before 96 h post infection (p.i.) otherwise overgrowth of the host cells and reduction of merozoites I/II may occur. Trophozoites can be observed about 30 h p.i., merozoites of the first generation 45-72 h p.i., merozoites of the second generation 110-144 h p.i., gamonts 144-168 h p.i., and oocysts 144-192 h p.i.

2.1.3.4. Staining of parasitised PCK cells (modifed PAS-AO stain)

Reagents and solutions

- Bouin's fluid: 15 volumes saturated picric acid, 5 volumes 37 % formaldehyde, 1 volume glacial acetic acid. Prepare immediately prior to use!
- 25 % solution of ammonia.
- 1 % sodium tetroxoiodate solution (Riedel de Haen, D); (1 g dissolved in 100 ml distilled water acidified by addition of 0. 5 ml concentrated nitric acid).
- Schiff's reagent: Solution I: 5 g acridine free pararosaniline (Merck, D) dissolved in 150 ml l M HCl. Solution II: 5 g potassium disulphite (Merck) dissolved in 850 ml distilled water. Solutions I and II are mixed becoming a clear and initially deep red solution which lightens gradually over time. After 24 h (store in darkness) add pure active charcoal (Merck) to such an amount that, following agitation and filtering renders the solution water-clear. The solution is now ready for use and must be kept cool and dark.
- 5.1 % sodium disulphite solution (Riedel de Haen): 70 g dissolved in 1400 ml distilled water and with 70 ml l M HCl added.
- Counterstaining solution (stock solution): 2 g orange G (Merck) and 0.5 g aniline blue (Merck) dissolved in 100 ml distilled water with 8 ml glacial acetic acid added.
- Boil solution for 3 min, cool, filter and store it in the dark. Working solution is prepared just prior to use by mixing 1 volume of stock solution with 2 volumes of distilled water.
- Absolute alcohol.
- Xylene.
- Entellan (Merck).

- **Procedure**

  - Rinse cell bearing slides in each of two changes of 15 ml Hanks' solution.
  - Fix monolayers for 24 h in 20 ml Bouin' s fluid.
  - To remove picric acid crystals place slides in 70 % ethanol containing 1 % (v/v) of a 25 % solution of ammonia.
  - Rinse the slides for 2 min in distilled water.
  - Oxidize for 10 min in 1 % sodium tetroxoiodate solution.
  - Rinse slides for 2 min in distilled water.
  - Place slides in Schiff's reagent for 10 min.

- Place slides for 3 min in 5.1 % sodium disulphite solution. Repeat once using fresh disulphite solution.
- Rinse slides under a gently running tap for 10 min.
- Before counterstaining rinse slides in distilled water.
- Place slides in the counterstaining working solution for 5-7 min.
- Rinse slides briefly in distilled water and dehydrate the monolayer (5 min in each of two changes of absolute alcohol).
- Place slides in xylene for 10 min and cover the monolayer in Entellan.
- **Results:** Amylopectin granules are stained red to violet, cytoplasm of host cells and parasites bluish, refractile bodies yellow.

2.1.4. *In vitro* assays

The following assays *in vitro* are suitable for testing anticoccidial or cytotoxic activities of agents or *Eimeria*-specific antibodies.

2.1.4.1. Sporozoite invasion inhibition assay

Procedure

- Prepare Flexiperm chamber units and PCK cell suspensions as described above (2.1.3.2.).
- Fill each of the FLEX chambers with 0.5 ml of cell suspension (2×10^6 cells / 7ml Williams E medium plus 10 % FCS).
- Incubate culture until 100 % cell confluence is achieved (normally after 48 h).
- Prepare drug solutions as follows: Dissolve 10 mg of the agent in 1 ml dimethylsulfoxide (DMSO), add 9 ml of Medium 199 plus 2.5 % FCS and sterilise the well mixed solution by filtration (0.2 µm). Adjust drug concentrations to 100; 10; 1; 0.1 µg/ml by dilution with Medium 199 plus 2.5 % FCS. Solutions containing only DMSO concentrations or culture medium serve as controls.
- Prepare antibody solutions as follows: Add native and heat inactivated (56°C, 30 min) non-immune (control) serum and immune sera to Medium 199 in such a way that serum concentrations come to 10 % and 4 %.
  Note: Sera derived from rabbits, rats and mice are not suitable for an *in vitro* assay. Control = normal sera (native and heat inactivated) from the latter species

show significant inhibition of the sporozoite invasion rate compared to those obtained by using chicken serum.

- Incubate sporozoites (freshly excysted and purified) in treated and untreated medium 199 at 41°C for 1 - 4 h.
- Remove drug, solvent and antibodies with two washes by centrifugation (800 x g, 5 min) in medium 199 plus 2.5 % FCS and adjust each sporozoite suspension to 1 x 10^5 sporozoites / 0.5 ml medium 199 (2.5 % FCS).
- Rinse monolayers as described under 2.1.3.3. and replace 0.5 ml of the culture medium by 0.5 ml of drug or antibody treated sporozoite suspensions. Incubate at 41°C and 5 % CO_2 for 4 - 24 h. Rinse each slide carefully under running phosphate buffered saline.
- Continue with step 2 of the modified PAS-AO stain (2.1.3.4., procedure).
- Count (magnification 400 x) the numbers of intracellular sporozoites in 20 fields of view per chamber. Count only areas showing almost 100 % cell confluence.
  Note: Intracellular sporozoites are located within the parasitophorous vacuole. Calculate inhibition rate by comparison of untreated control and treated samples (average invasion rate).

2.1.4.2. Schizont inhibition assay

Procedure

- Prepare FLEX chamber units and cell suspensions as described under 2.1.3.2./3. but, contrary to the former, adjust cell density to 1 x 10^6 cells / 7 ml Williams E medium (10 % FCS).
- Incubate cells at 41°C and 5 % CO_2 for 24 h, then rinse cells before infection (see 2.1.3.3.).
- Inoculate with fresh sporozoite suspensions adjusted to 1 - 2.5 x 10^4 sporozoites / 0.5 ml Medium 199 + 2.5 % FCS and incubate at 41°C and 5 % CO_2 for 4 h.
  Note: Cell confluence should be 40 - 60 % when parasites are inoculated. If cell confluence is low (e.g. 10 - 30 %) incubate cell culture for further 24 h.
- Remove FLEX chambers and rinse with Hanks' solution to remove extracellular sporozoites (details see 2.1.3.3.). Transfer slides coated with parasitized monolayers to Optilux petri dishes and add 20 ml medicated or unmedicated Medium 199 (1-2.5 % FCS). Drug solutions and immune sera, respectively, are prepared as mentioned under 2.1.4.1. Drug induced cell toxicity can be checked by using uninfected monolayers and comparable drug dilutions.

- Incubate the drug or antibody treated monolayers for 110 - 120 h p.i (41°C and 5 % CO_2). Check the culture daily by microscope!
- Rinse coated slides under running phosphate buffered saline (PBS) and continue with step 2 of the modified PAS-AO staining (see 2.1.3.4., procedure).
- Scan the total cell areas of at least 6 FLEX chambers per sample and count simultaneously PAS positive (deep red) schizonts II (second generation). Stained schizonts II can be differentiated distinctly by lower microscope magnifications (e.g. 250 x).

  Note: It is necessary to count the total cell area/chamber because schizonts II tend to grow in clusters (non random distribution)!
- Calculate schizont II inhibition rate by comparing monolayers with nos. of schizonts of untreated controls.

2.1.5. Production of polyclonal antibodies directed against *E. tenella* sporozoites

Procedure

- Isolate sporozoites (see 2.1.1.4.).
- Immunise 7 day old LSL chickens as indicated in Tab. 14.

Tab. 14: Immunisation schedule for of chickens with *E. tenella* antigens

Time (day)	Mode of application	Antigen dose parasites/chick	Adjuvant (µl)	PBS (µl)	Total volume (µl)
1	subcutaneous	8.0×10^5	100	100	200
3	subcutaneous	1.2×10^6	150	150	300
8	subcutaneous	1.6×10^6	200	200	400
15	intramuscular	2.5×10^6	250	250	500
17	subcutaneous	8.0×10^5	-	100	100
21	intraperitoneal	4.0×10^6	-	500	500
28	Recovery of sera and storage at -20°C				

- Incubate with an aqueous solution of chlorox for 5 min in an ice bath: i.e.: to a pellet of oocysts in a 15 ml tube, add a small volume of water (e.g. 1 ml) and add about 0.5 ml chlorox bleach. Incubate for 5 min in an ice bath.

- Top up tube with water and centrifuge at 1,500 x g for 5 min.

- Resuspend pellet in saturated salt solution and overlay with a layer of about 2 cm distilled water.

- Centrifuge tube at 1,500 x g for 5 min and recover purified oocysts from the salt/water interface.

- Wash several times by centrifugation in water at 1,500 x g for 5 min.

- Place purified oocysts in a 15 ml centrifuge tube and resuspend in about 2 ml of PBS at pH 7.6 or BSS.

- Add a 1 cm layer of 0.5 mm diameter glass balls (Jencons, Number 8).

- Place tube on a Whirlimix (Fisons Ltd.) and agitate the contents until most of the oocysts have been mechanically fractured to release their sporocysts.

  Care must be taken during this cracking procedure to ensure that the oocysts are treated for the optimum length of time as the sporocysts are damaged if cracking is prolonged.

- The sporocysts are recovered from the glass balls and incubated in either PBS at pH 7.6 or Hank's BSS (for example for an egg-adapted line *E. tenella* (TA), which is characterised by relatively labile sporozoites) supplemented with 5 mM $MgCl_2$ containing trypsin (2.5g/l) and taurocholic acid (Fluka) (10 g/l) at 41°C until most of the sporozoites have excysted.

- After two washes in the appropriate medium the sporozoites are counted and then resuspended in an appropriate volume of medium to give the desired inoculum (about 2,000-10,000 sporozoites in 0.1 ml).

- Collect new oocysts 7 days after inoculation of sporozoites for all species.

2.3. Cryopreservation

Various stages of *Eimeria* species can be preserved in a living state by deep-freezing in liquid nitrogen. Some procedures used in various laboratories are described below.

2.3.1. Cryopreservation of avian *Eimeria* stages

M. W. Shirley

The methods are essentially the same as those used for the preservation of *Eimeria spp.* from mammalian hosts (see 2.3.2. and 2.3.3.) and may be done with rudimentary apparatus to control the rate of freezing to about -1° C/min during the critical period down to -70°C.

Freezing procedure

- **Sporocysts**

  - Concentrate *Eimeria* sporocysts by centrifugation, and resuspend in SPGA medium [1, and see below] containing 8% dimethylsulphoxide (DMSO) to a concentration of about 2.5 x 10^6/ml.

  - Leave overnight at room temperature.

  - Dispense sporocysts into plastic, screw-capped ampoules and place in a honey jar or other suitable vessel containing alcohol and leave inside a polystyrene container (or "igloo") in a freezer at -70°C for 1 - 2 hours.

  - Transfer ampoules from the alcohol to a small polystyrene container within the igloo and leave at -70°C for at least 5 h and up to overnight.

  - Transfer ampoules to liquid nitrogen.

- **Sporozoites**

  - Resuspend sporozoites in SPGA containing 8% DMSO at a concentration of about 2.5 x 10^6/ml.

  - Dispense into ampoules and leave at room temperature for about 15 min.

  - Place ampoules in a honey jar containing alcohol inside an "igloo" and transfer to a freezer at -70°C for 1-2 h.

  - Complete freezing as for sporocysts, (see above).

Receipe for SPGA

Chemical	g/litre
Sucrose	74.6210
KH_2PO_4	0.5171
K_2HPO_4	1.2541
L glutamic acid	0.9079
BSA	10.0000

Thawing procedure

Satisfactory results can be obtained by plunging ampoules into a plastic (for safety) beaker of water at 37°C, recovering the thawed parasites and either inoculating them immediately or after a wash in PBS at pH 7.0.

Reference

[1] Bovarnik, M.R., Miller, J.C. Synder, J.C. (1950): The influence of certain salts, amino acids, sugars and proteins on the stability of rickettsiae. J. Bacteriol. 59: 509-512.

2.3.2. Cryopreservation of *Eimeria* stages of sheep

M. Taylor, J. Catchpole, R. Marshall, C.C. Norton, J. Green

Freezing procedure

- **Sporocysts of *Eimeria* stages**

  - Take the required number of freshly sporulated oocysts and wash to remove the potassium dichromate by resuspending the oocysts in distilled water. This may need repeating about three times. (Dirty cultures may require salt flotation.)

  - Resuspend the oocysts in 2 ml sodium hypochlorite (specific gravity 1.075) for 20 min, (about 32% solution, check with hydrometer).

  - Wash with distilled water by repeated centrifugation until no odour of chlorine can be detected.

  - Resuspend in 1.4% sodium bicarbonate containing 0.0025% methyl red. Saturate solution with carbon dioxide by bubbling gas through until solution turns brick orange. Stopper tightly and leave in 37°C incubator overnight.

  - Wash to remove sodium bicarbonate.

  - Resuspend in 4 ml phosphate buffered saline pH 7.6 in universal container (or similar with approximately 2 cm diameter), add 1 mm glass balls to depth of 1 cm. Liquid should just cover balls.

  - Shake gently to release the sporocysts, examine drops at intervals until most sporocysts are released. This should take about 5 min. The presence of empty sporocysts indicates too vigorous shaking.

  - Recover the sporocysts by repeated washings in PBS and concentrate in conical tube by centrifugation (* see below under sporozoites).

  - Resuspend in 5 ml Borate-Ringer with 10% heterologous serum (BRS) (see appendix, page 100).

- Count the free sporocysts using Neubauer haemocytometer counting chamber with a 22 x 22mm No. 0 coverslip under 40 x objective. Count 80 small squares (see Fig. 5, page 38). Count two chambers.

Calculate number of sporocysts/ml: $X \times 5 \times 1{,}000 \times 10x^{z}$

where 5 = 80/400 squares counted = $0.2mm^2$
10 = depth 0.1mm
1'000 = conversion to cm^3
z = dilution

i.e. mean count x 50,000 x dilution.

- Adjust concentration to 1×10^6 sporocysts/ml using BRS.

- Prepare solution of BRS containing 15% dimethyl sulphoxide (DMSO) and slowly (over 2-3 minutes) add equal volume of this to the sporocyst suspension. Leave overnight at room temperature.

- Resuspend and divide between 9 plastic ampoules, labelled with species and date.

- After 19 h equilibrating at room temperature the cooling programme is started.

- The rate of cooling should be about 1°C per min for the first 2.5 h, depending on the freezing apparatus used. When fully frozen the ampoules are attached to metal canes and immersed in the liquid nitrogen.

- **Sporozoites**

  - Until step 8 (see * previous page) as for sporocysts.

  - To the sedimented sporocysts add 2 ml of freshly prepared trypsin solution and incubate in a water bath at 41°C.

- Examine samples at 15 min intervals and count intact sporocysts and free sporozoites.

- Resuspend in 5 ml Borate-Ringer with 10% heterologous serum (BRS).

- Count the free sporozoites using Neubauer haemocytometer counting chamber as described above for sporocysts and calculate the percentage of free sporozoites in relation to sporocysts.

- Adjust concentration to 1 x 10^6 sporocysts/ml using BRS.

- Prepare solution of BRS containing 15% dimethyl sulphoxide (DMSO) and slowly (over 2-3 min) add equal volume of this to the sporozoite suspension. Leave overnight at room temperature.

- Resuspend and divide between 9 plastic ampoules, labelled with species and date.

- After 19 h equilibrating at room temperature the cooling programme is started.

- The rate of cooling should be about 1°C per minute for the first 2.5 h, depending on the freezing apparatus used. When fully frozen the ampoules are attached to metal canes and immersed in the liquid nitrogen.

Thawing procedure

- Remove ampoule from the liquid nitrogen and thaw by immersion in warm water at 37-41°C. (A visor and gloves should be worn as a precaution against liquid nitrogen burns.)

- Transfer contents to sterile small container and slowly add 4 volumes BRS to dilute the DMSO.

- **Appendix**

Borate-Ringers Solution (BRS) [1].

Solution A. Metallic chlorides

NaCl	9	g/l	100 volumes
KCl	11.48	g/l	4 volumes
$MgCl_26H_20$	22.37	g/l	3 volumes
$CaCl_26H_20$	24.1	g/l	1 volume

Solution B. Borate Buffers

$Na_2B_4O_710H_20$	41.96 g/l	2.31 volumes
H_3BO_3	19.71 g/l	7.69 volumes

Dispense 18 ml amounts of solution A into Universal containers and 2 ml amounts of solution B into Bijoux bottles. Autoclave and store in refrigerator. Mix 1 bottle of each solution immediately before use.

Serum. Heterologous serum, inactivated, 10% mixed with Borate-Ringers Solution immediately before use.

2.3.3. Cryopreservation of *Eimeria* stages of cattle

H.-J. Bürger, N. Fiege, A. Gahr, A. Heise and H. Roloff

- **Sporozoites**

  - Sporozoites are harvested, cleaned and excysted as described in section 1.3.5.

  - Immediately after excystation the sporozoites are centrifuged at 200 x g for 10 min and resuspended in cold MEM medium supplemented with 10% (v/v) dimethylsulphoxide (DMSO, Merck, D-Darmstadt), 2.5% (v/v) fetal calf serum (Biochrom KG, D-Berlin) and 1% (w/v) glutamine (Gibco, D-Eggenstein).

  - The final concentration of sporozoites is adjusted to approximately 5 x 10^6 sporozoites per ml. 1 ml aliquots of sporozoites are pipetted into 2 ml cryotubes

(Nunc, DK-Roskilde), incubated for 1 h on ice, frozen in a polystyrene box for 24 h at -80°C and finally stored in liquid nitrogen.

- Sporozoites are thawed as fast as possible by agitating the cryotube gently in a water bath at 37°C. The sporozoites are then diluted in HBSS and centrifuged (10 min; 400 x g) to remove DMSO. This washing step is repeated twice.

- The viability of the sporozoites is estimated by microscopical examination of the integrity and motility of the sporozoites and by trypan blue vital staining: 4% (w/v) trypan blue (Serva, D-Heidelberg) in PBS and sporozoite suspension is mixed at equal volumes - final concentration 2%.

- **Merozoites**

Two different techniques have been used to cryopreserve merozoites of *Eimeria bovis.*

- **Polystyrene box technique**

  - Freshly prepared merozoites (approximately 10^7 per ml) in HBSS (Sigma No. H-2387, pH 7.4) are diluted with fetal calf serum (FCS - final concentration v/v 40% and DMSO (final concentration v/v 10%, vortexed thoroughly, pipetted in 1.0 ml aliquots into 2 ml cryotubes (Nunc), stored in a refrigerator (4°C) for 1 h, frozen in a polystyrene box for at least 1 day at -80°C and finally stored in liquid nitrogen.

  - Thawing: see preservation of sporozoites.

- **Cryostat technique**

  - This technique employs a Colora-Ultra-Kryostat KT50S (Colora, D-Lorch) and methanol as the freezing medium.

  - Merozoites are prepared as described above (about 10^7 merozoites/ml in HBSS, 40% FCS, 10% DMSO).

- Aliquots of 0.5 ml are pipetted into 2 ml cryotubes (Nunc), frozen at a cooling rate of 0.5°C / min from + 4°C to -55°C, and immediately transferred into liquid nitrogen, where they are stored until usage.

- Thawing: see preservation of sporozoites.

References

[1] Lumsden, W.H.R., Cunningham, M.P., Webber W.A.F., van Hoeve, K., Walker, P.J. (1963): A method for the measurement of the infectivity of trypanosome suspensions. Exp. Parasit 14: 269-279.

[2] Norton, C.C., Pout D.D., Joyner L.P. (1968): Freeze preservation of *Eimeria acervulina* Tyzzer 1929. Folia Parasitologica (Praha) 15: 203-211.

3. Identification of *Eimeria* species and strains

3.1. Morphological characteristics of oocysts

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3.1.1. Species identification

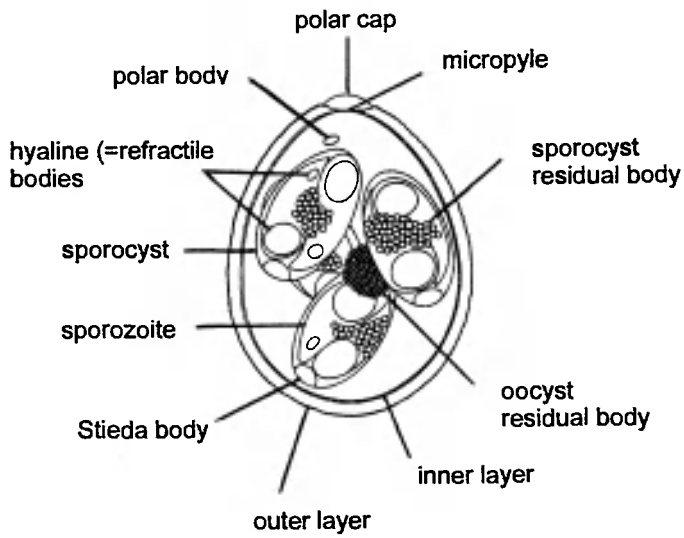
Essentially, identification and differentiation of *Eimeria* and *Isospora* species relies on morphological characters of unsporulated and sporulated oocysts as well as on biological data of development in the environment and in susceptible hosts.

Reliable species identification based on oocyst morphology is possible in most of the *Eimeria* species of cattle, sheep, goat, pig and rabbit and of *Isospora suis* and *Eimeria* species in swine. On the other hand oocyst morphology is only of very limited value for identification of *Eimeria* species of chickens.

Some guidelines for the morphological diagnosis of sporulated oocysts from various animal species are given below. For details consult [1-8].

Oocysts are collected and sporulated as described in chapters 1.2., 1.3., 1.4. and 1.5.

Fig. 10: Scheme of sporulated *Eimeria* oocyst (after Rommel, 1992)



3.1.1.1. Diagnosis of *Eimeria* species from cattle

In Europe, 12 *Eimeria* species are known to occur in cattle. Some morphological characteristics of sporulated oocysts suitable for species identification are presented in Key 1 and Fig. 11. In certain instances specialised literature should be consulted.

Key 1: Key for identification of sporulated *Eimeria* oocysts from cattle (see also Fig. 11)

Abbreviations: OR: Oocyst residuum = residual body; SR: Sporocyst residuum = residual body; ST: Sporulation time; dimensions in parenthesis = average; d = day.

A. Oocysts without micropyle (Fig. 11, A)

Maximum dimension < 15 µm

- oocysts round or subspherical, colourless, without OR and SR, 9-14 x 8-13 µm (11x10.4 µm), ST: 4-5 d. — ***E. subspherica*** (Fig. 11, A/1)

Maximum dimension > 15 µm

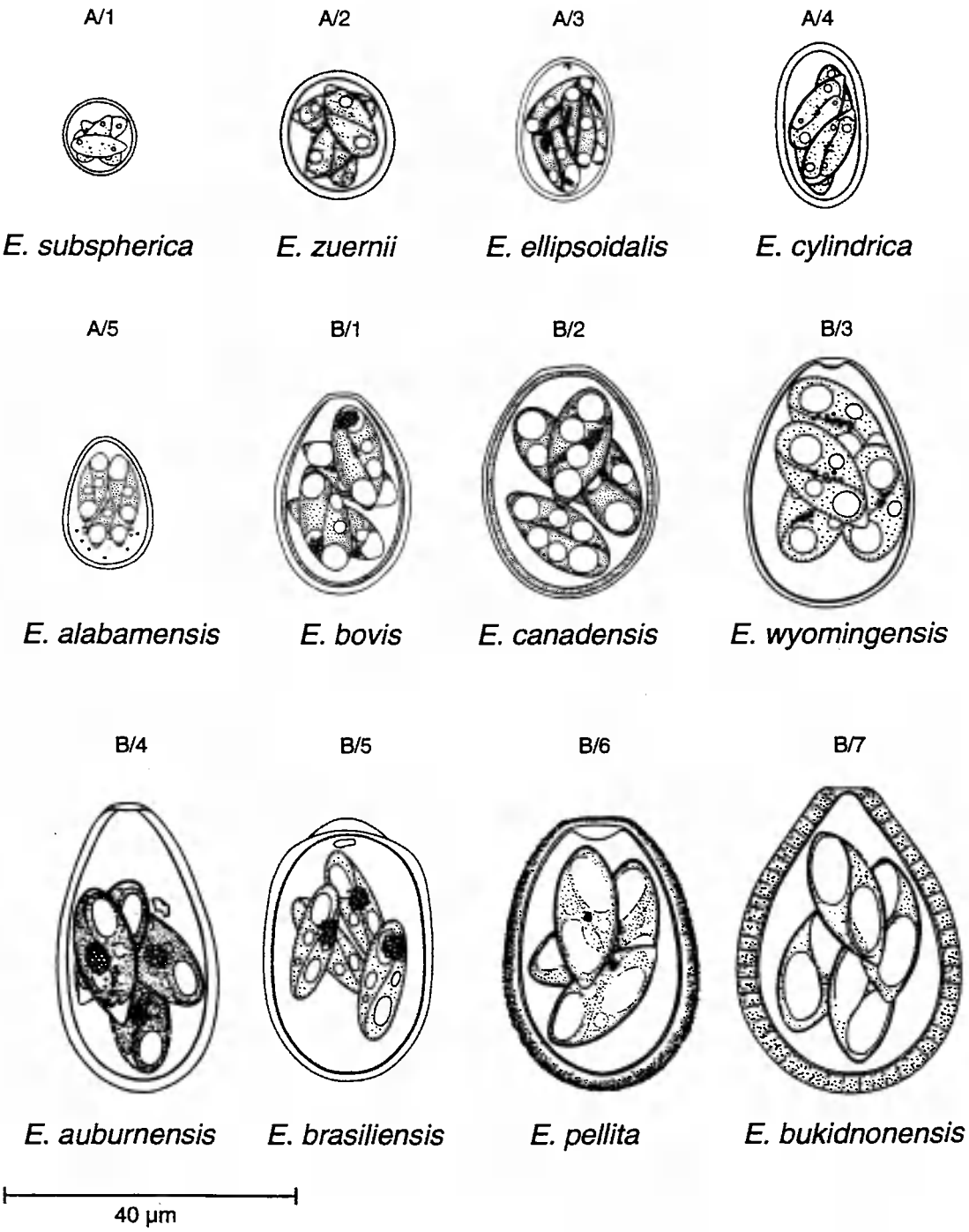
- oocyst subspherical, colourless without OR but with SR, 15-22 x 13-18 µm (17.8x15.6 µm), ST: 2-3 d. — ***E. zuernii*** (Fig. 11, A/2)
- oocyst ellipsoidal, colourless, without OR, with SR, 20-26 x 13-17 µm (23.4x15.9µm), ST: 2-3 d. — ***E. ellipsoidalis*** (Fig. 11, A/3)
- oocyst elongated, cylindrical, colourless, no OR, with SR, 16-27 x 12-15 µm (23.3x12.3µm), ST: 2 d. — ***E. cylindrica*** (Fig. 11 A/4)
- oocyst ovoid or piriform, colourless, without OR or SR, 13-24 x 11-16 µm (18.9x13.4 µm), ST: 5-8 d. — ***E. alabamensis*** (Fig. 11, A/5)

B. Oocysts with micropyle (Fig. 11, B)

Maximum dimension < 45 µm

- oocyst ovoid or subspherical, colourless, micropyle inconspicuous, with OR and SR, 23-34 x 17-23 µm (27.7x 20.3 µm), ST: 2-3 d. — ***E. bovis*** (Fig. 11, B/1)

Fig. 11: ***Eimeria*** species from cattle



- oocyst ovoidal, colourless or pale yellow, no OR, SR composed of a few granules, 28-37 x 20-27 µm (32.5x23.4 µm), ST: 3-4 d. — ***E. canadensis*** (Fig. 11, B/2)
- oocyst ovoid, thick wall, yellowish-brown, without OR or SR (few granules), wide micropyle, 37-45 x 26-31 µm (40.3x28.1 µm), ST: 5-7 d. — ***E. wyomingensis*** (Fig. 11, B/3)
- oocyst elongated ovoid, yellowish-brown, wall smooth or with coarsely granular surface, no OR, with SR, 32-46 x 20-25 µm (38.4x23.1 µm), ST: 2-3 d. — ***E. auburnensis*** (Fig. 11, B/4)
- oocyst ellipsoid, yellowish-brown, micropyle with distinct pole cap, no OR, with SR, 33-43 x 24-30 µm (37x27 µm), ST: 12-14 d. — ***E. brasiliensis*** (Fig. 11, B/5)
- oocyst egg-shaped, very thick brown wall with evenly distributed protuberances, no OR, with small SR, 36-41 x 26-30 µm (40x28 µm), ST: 10-12 d. — ***E. pellita*** (Fig. 11, B/6)

Maximum dimension > 45 µm

- oocyst pear-shaped or oval, tapering at one pole, thick wall with radial striations, yellowish-brown, 47-50 x 33-38 µm (48.6x35.4 µm), ST: 4-7d. — ***E. bukidnonensis*** (Fig. 11, B/7)

3.1.1.2. Diagnosis of *Eimeria* species from sheep

Eleven *Eimeria* species are known to occur in sheep. Most of them can be identified on oocyst morphology (Key 2, Fig. 12).

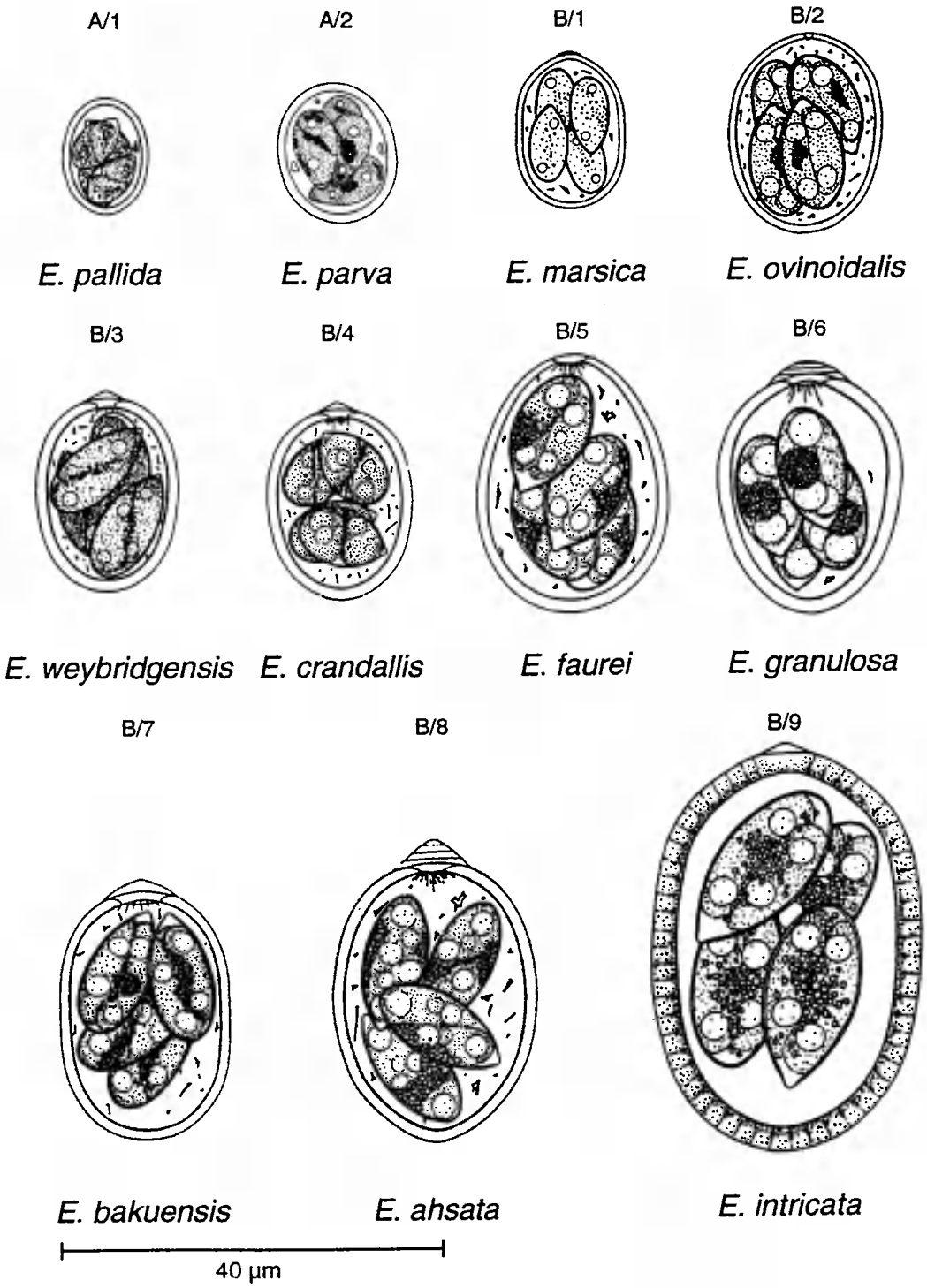
Key 2: Key for identification of sporulated *Eimeria* oocysts from sheep (see also Fig. 12)

Abbreviations: see Key 1.

A. Oocysts without micropyle (Fig. 12, A)

- oocyst ellipsoidal, thin-walled, colourless to pale yellow, without OR, with SR, 12-20 x 8-15 µm (14x10 µm), ST: 1-3 d. — ***E. pallida*** (Fig. 12, A/1)
- oocyst spherical to subspherical, colourless, no OR, SR composed of few granules, 13-22 x 11-13 µm (16.5x14.0 µm), ST: 3-5 d. — ***E. parva*** (Fig. 12, A/2)

Fig. 12: ***Eimeria*** species from sheep



B. Oocysts with micropyle (Fig. 12, B)

Maximum dimension < 35 µm

• oocyst ellipsoidal, with inconspicuous micropyle, colourless or pale yellow, without OR or SR, 15-22 x 11-14 µm (19x13 µm), ST: 3 d..	***E. marsica*** (Fig. 12, B/1)
• oocyst ellipsoidal, indistinct micropyle, colourless or pale yellow, without OR, with SR, 17-25 x 13-20 µm (23x18 µm), ST: 1-3 d.	***E. ovinoidalis*** (Fig. 12, B/2)
• oocyst broadly ellipsoidal or subspherical, micropyle with or without polar cap, without OR or SR, 17-30 x 14-19 µm (24x17 µm), ST: 1-3 d.	***E. weybridgensis*** (Fig. 12, B/3)
• oocyst broadly ellipsoidal or subspherical, with or without polar cap, without OR, sporocysts very broad, with SR, 17-23 x 17-22 µm (21.9x19.4 µm), ST: 1-3 d.	***E. crandallis*** (Fig. 12, B/4)
• oocyst ovoid, pale yellowish-brown, without OR or SR, 28-37 x 21-27 µm (32x23 µm), ST: 1-3 d.	***E. faurei*** (Fig. 12, B/5)
• oocyst urn-shaped with large micropolar cap at broad end, yellowish-brown, without OR, with SR, 22-35 x 17-25 µm (29.4x20.9 µm), ST: 3-4 d.	***E. granulosa*** (Fig. 12, B/6)
• oocyst ellipsoidal, with polar cap, pale yellowish-brown, without OR, with SR, 23-36 x 15-24 µm (31x20 µm), ST: 2-4 d.	***E. bakuensis*** (Fig, 12, B/7)
• oocyst ovoid with distinct polar cap, yellowish-brown, no OR, with SR, 29-37 x 17-28 µm (33.4x22.6 µm), ST: 2-3 d.	***E. ahsata*** (Fig. 12, B/8)

Maximum dimension > 35 µm

• oocyst ellipsoidal, thick and striated wall, brown, no OR, with SR, 40-56 x 30-41 µm (48x34 µm), ST: 3-7 d.	***E. intricata*** (Fig. 12, B/9)

3.1.1.3. Diagnosis of *Eimeria* species from goats

From the goat 9 *Eimeria* species have been described. Previously it was anticipated that sheep and goat have several *Eimeria* species in common but now it appears that most, if not all, are specific to their host.

Key 3: Key for identification of sporulated *Eimeria* oocysts from goats (see also Fig. 13)

Abbreviations: see Key 1.

A. Oocysts without or with inconspicuous micropyle (Fig. 13, A)

• oocyst ovoid or ellipsoidal, colourless or pale yellow, micropyle indistinct, no OR, with SR, 15-23 x 12-22 µm (17x15 µm) , ST: 1-5 d.	***E. alijevi*** (Fig. 13, A/1)
• oocyst ellipsoidal, thin walled, colourless, micropyle absent or indistinct, no OR, with SR, 20-22 x 14-16 µm (20.7x14.8 µm), ST: 1-4 d.	***E. ninakohlyakimovae*** (Fig. 13, A/2)

B. Oocysts with micropyle (Fig. 13, B)

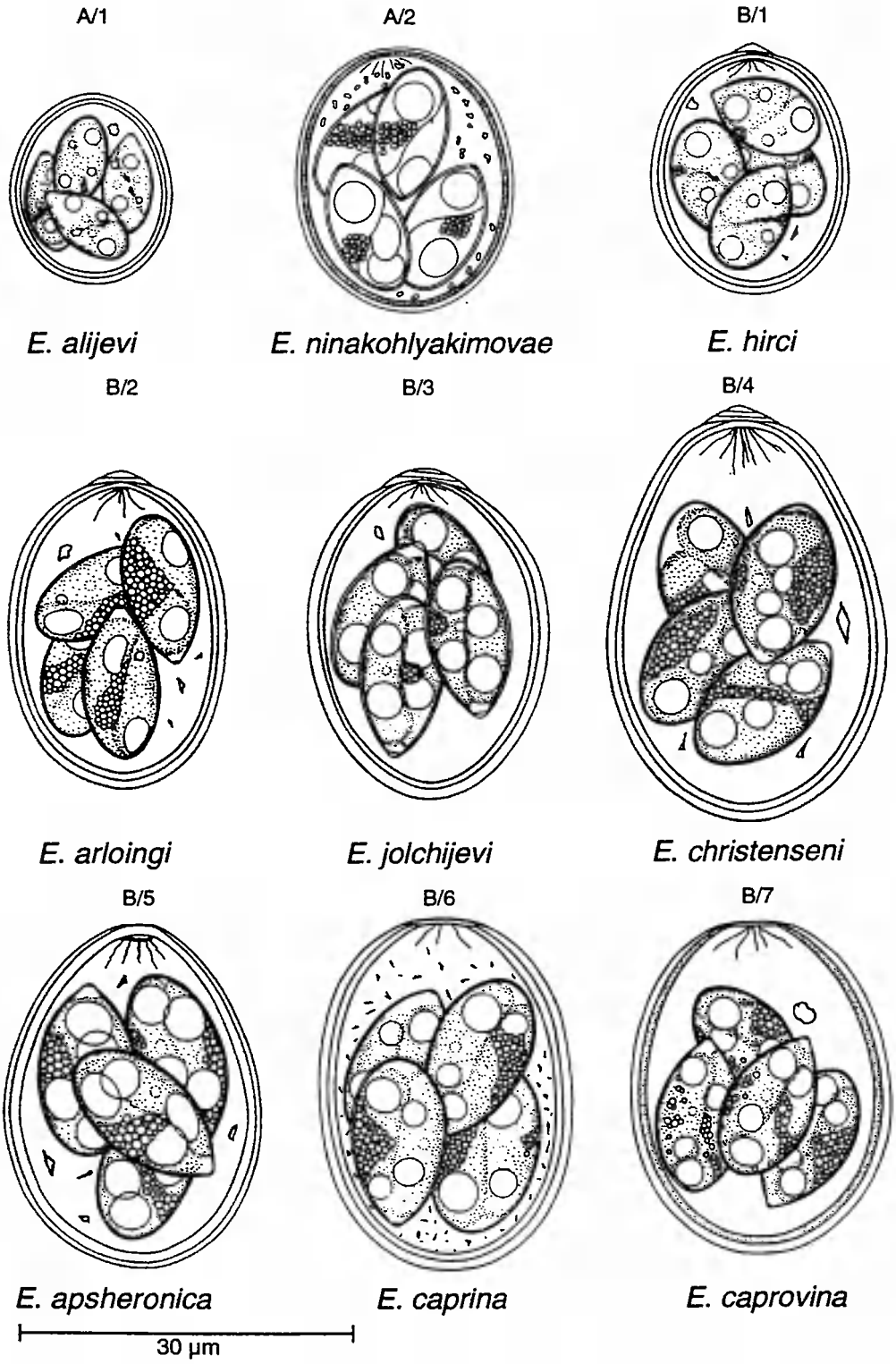
Oocysts with polar cap

• oocyst roundish oval, light yellow, no OR, sporocysts broadly oval with small SR, 18-23 x 14-19 µm (20.7x16.2 µm), ST: 2-3 d.	***E. hirci*** (Fig. 13, B/1)
• oocyst ellipsoid, thick wall, no OR, with SR, 17-42 x 13-27 µm (27x18 µm), ST: 1-2 d.	***E. arloingi*** (Fig. 13, B/2)
• oocyst ellipsoidal or ovoid, pale yellow, without OR, with SR, 26-37 x 18-26 µm (31x22 µm), ST: 2-4 d.	***E. jolchijevi*** (Fig. 13, B/3)
• oocyst ovoid, thick wall, colourless to pale yellow, no OR, with SR, 34-41 x 23-28 µm (38 x 25 µm), ST: 6 d.	***E. christenseni*** (Fig. 13, B/4)

Oocysts without polar cap

• oocyst ovoid, greenish to yellow-brown, no OR, with SR, 24-37 x 18-26 µm (31x23 µm), ST: 1-2 d.	***E. aspheronica*** (Fig. 13, B/5)
• oocyst ellipsoidal, dark brown to brownish yellow, without OR, with SR, 27-40 x 19-26 µm (32x23 µm), ST: 2-3 d.	***E. caprina*** Fig. 13, B/6)
• oocyst ellipsoidal to subspherical, colourless, without OR, with SR, 26-36 x 21-28 µm (30x24 µm), ST: 2-3 d.	***E. caprovina*** (Fig. 13, B/7)

Fig. 13: ***Eimeria*** species from goat



3.1.1.4. Diagnosis of *Eimeria* and *Isospora* species from swine

Among 13 *Eimeria* species from swine 8 are common. In addition *Isospora suis* occurs in swine [7, 8]. *I. suis* can easily be differentiated from *Eimeria* species.

Key 4: Key for identification of sporulated *Eimeria* and *Isospora* oocysts from swine (see also Fig. 14)

Abbreviations: see Key 1.

A. Oocysts with 2 sporocysts with each 4 sporozoites (Fig. 14, A)

- oocyst spherical to subspherical, wall colourless and thin, without OR, with SR, 17-25 x 16-22 µm (20.6x18.1 µm), ST: 1-2 d. — ***I. suis*** (Fig. 14, A/1)

B. Oocysts with 4 sporocysts with each 2 sporozoites (Fig. 14, B)

Oocysts without micropyle

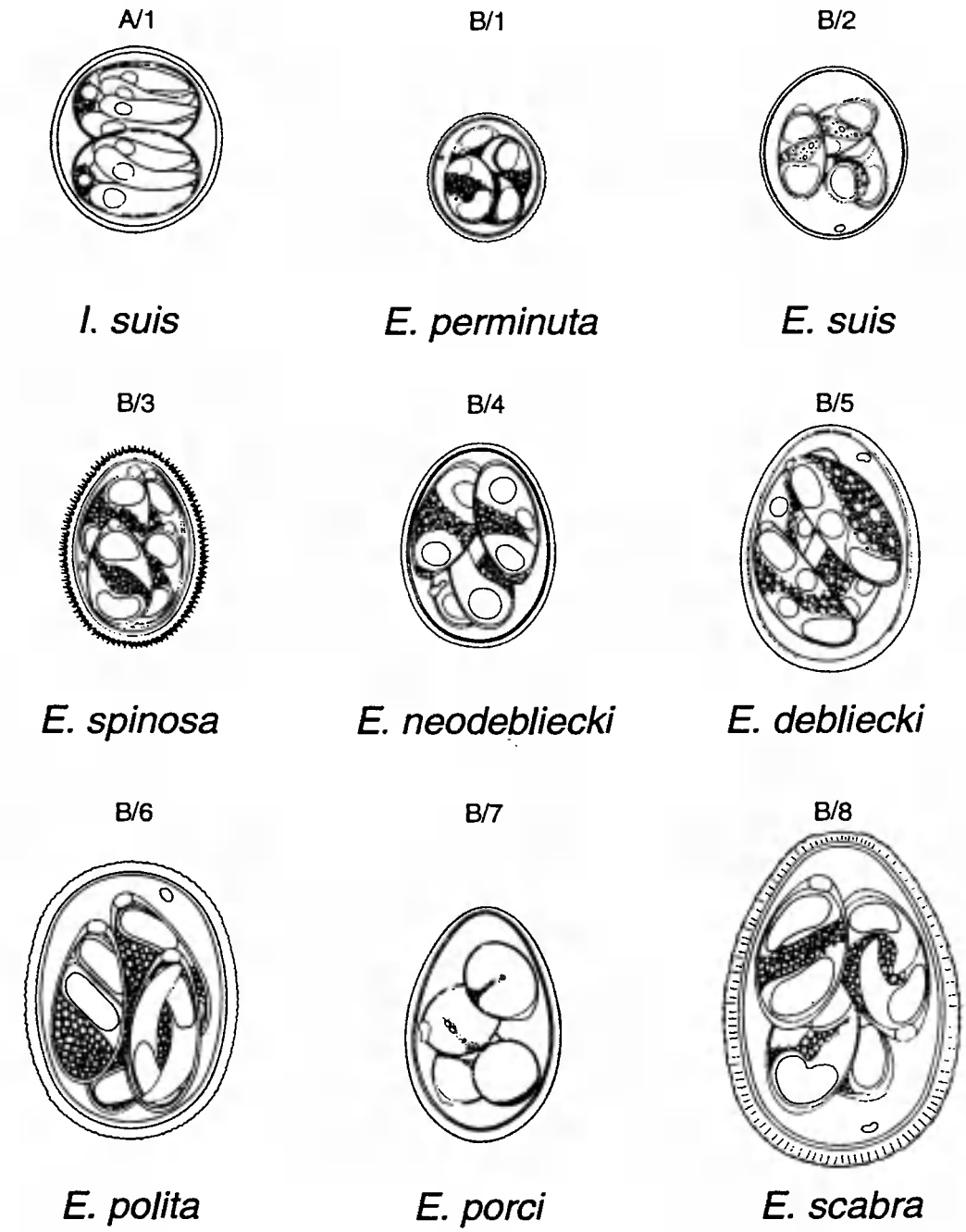
- oocyst ovoid to subspherical, wall with rough surface, no OR, with SR, 12-15 x 10-13 µm (13.3x11.7 µm), ST: 10-12 d. — ***E. perminuta*** (Fig. 14, B1)
- oocyst ellipsoidal, no OR, with SR, 15-23 x 12-18 µm (18.2x14.0 µm), ST: 5-6 d. — ***E. suis*** (Fig. 14, B/2)
- oocyst ovoid, surface with long spines, no OR, with SR, 17-24 x 12-19 µm (20.6x16.2 µm), ST: 9-10 d. — ***E. spinosa*** (Fig. 14, B/3)
- oocyst ellipsoid, wall smooth and colourless, no OR, with SR, 17-26 x 13-20 µm (21.2-15.8 µm), ST: 13 d. — ***E. neodebliecki*** (Fig. 14, B/4)
- oocyst ellipsoid or ovoid, wall smooth and colourless, no OR, with SR, 15-23 x 11-18 µm (18.8x14.3 µm), ST: 5-7 d. — ***E. debliecki*** (Fig. 14, B/5)

  Note: *E. debliecki* is seldom longer than 24 µm, larger oocysts are usually *E. polita.*

- oocyst ellipsoid or broad ovoid, yellowish-brown, no OR, with SR, 20-33 x 14-22 µm (25.9x18.1 µm), ST: 8-9 d. — ***E. polita*** (Fig. 14, B/6)

Fig. 14: ***Isospora*** and ***Eimeria*** species from swine

(modified after Levine [4])



Oocysts with micropyle

- oocyst ovoid, yellowish-brown, smooth, micropyle usually indistinct, no OR, SR composed of fine granules, 18-27 x 13-18 µm (21.6x15.5 µm), ST: 9 d. — ***E. porci*** (Fig. 14, B/7)
- oocyst ovoid or ellipsoid, thick with coarse surface (maybe lacking) no OR, with SR, 24-42 x 20-24 µm (31.9x22.5 µm), ST: 9-12 d. — ***E. scabra*** (Fig. 14, B/8)

3.1.1.5. Diagnosis from *Eimeria* species from rabbits

From the rabbit more than 25 *Eimeria* species have been described [2] but only 11 species were isolated in pure culture and are well characterized [2, 6]. Among these species *E. media* and *E. coecicola* are difficult to identify when in a mixture with other species. Possibly, the name *E. media* covers several species [2].

Key 5: Key for identification of sporulated *Eimeria* oocysts from rabbits (see also Fig. 15)

Abbreviations: see Key 1.

A. Oocysts without or with inconspicuous micropyle (Fig. 15, A)

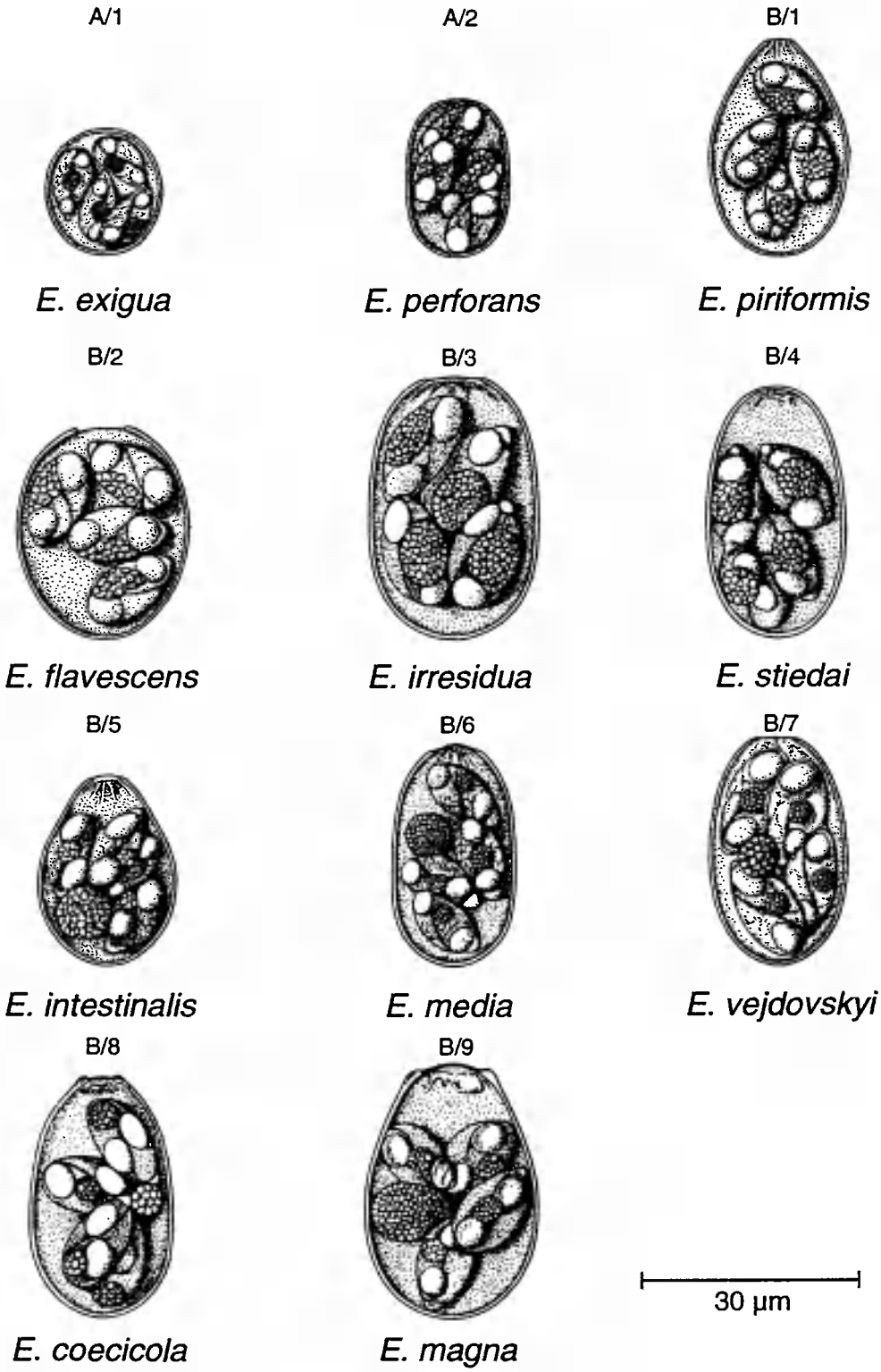
- oocyst spherical or subspherical, colourless, with SR without OR, 10-18 x 11-16 µm (15.1x14.0 µm), ST: 1 d. — ***E. exigua*** (Fig. 15, A/1)

  Note: maybe confused with small *E. perforans*

- oocyst ellipsoid to sub-rectangular, smooth and uniformly thin wall, micropyle very difficult to detect, with SR and small OR, 15-27 x 11-17 µm (22.2x13.9 µm), ST: 1.5 - 2 d. — ***E. perforans*** (Fig. 15, A/2)

  Note: large and medium *E. perforans* maybe confused with small and medium *E. media*.

Fig. 15: ***Eimeria*** species from rabbit



B. Oocysts with micropyle (Fig. 15, B)

Oocysts without oocyst residuum

- oocyst piriform, often asymmetrical, yellowish-brown, prominent micropyle, with SR, 25-33 x 16-21 µm (29.5x18.1 µm), ST: 4 d. — ***E. piriformis*** (Fig. 15, B/1)

  Note: *E. piriformis* is similar to *E. intestinalis* but has no OR.

- oocyst ovoid, yellowish, very large micropyle at broad end, with SR, 25-35 x 18-24 µm (30.0 x 21.0 µm), ST: 4 d. — ***E. flavescens*** (Fig. 15, B/2)

  Note: maybe confused with *E. irresidua.*

- ovoid or barrel-shaped or subrectangular yellowish, micropyle wide, with SR, 31-44 x 20-27 µm (39.2x23.1 µm), ST: 4 d. — ***E. irresidua*** (Fig. 15, B/3)

  Note: maybe confused with *E. stiedai* (micropyle in this species inapparent) or *E. flavescens* (this has micropyle at broad end!).

- oocyst slightly ellipsoid, micropyle almost inapparent, no OR but only a few granules, with SR, 30-41 x 15-24 µm (36.9x19.9 µm), ST: 2-3 d. — ***E. stiedai*** (Fig. 15, B/4)

  Note: maybe confused with *E. irresidua* (but this has distinct micropyle) or *E. coecicola* (is more ellipsoid and has OR).

Oocysts with oocyst residuum

- oocyst piriform, yellowish-brown, large OR (in contrast to *E. piriformis*), with SR, 22-30 x 16-21 µm (26.7x18.9 µm), ST: 3 d. — ***E. intestinalis*** (Fig. 15, B/5)

  Note: see *E. piriformis.*

- oocyst ellipsoid or ovoid, light pink, with medium to large OR and large SR, micropyle with a pyramidal-shaped protuberance. 25-35 x 15-20 µm (31.1x17.0 µm), ST: 2 d. — ***E. media*** (Fig. 15, B/6)

  Note: see *E. perforans, E. coecicola, E. vejdovskyi.*

- oocyst elongate or ovoid, with SR and medium size OR, micropyle present without collarlike protrusion, 25-38 x 16-22 µm (31.5x19.1), ST: 2 d. — ***E. vejdovskyi*** (Fig. 15, B/7)

  Note: This species may be confused with *E. media* (has a micropyle with pyramidal-shaped protuberance) or *E. coecicola* (has collarlike protrusion around micropyle).

- oocyst elongate-ovoid, with OR relatively smaller than in *E. media*, micropyle with a slight collarlike protrusion and SR, 27-40 x 15-22 µm (34.5x19.7 µm), ST: 4 d. — ***E. coecicola*** (Fig. 15, B/8)

  Note: Small *E. coecicola* maybe confused with medium and large *E. media* and with *E. vejdovskyi* (has no collarlike protrusion).

- oocyst ellipsoid ovoid, dark yellow, truncated at micropylar end with marked collarlike thickening around micropyle, with very large OR and with SR, 31-42 x 20-28 µm (36.3x24.1 µm), ST: 2-3 d. — ***E. magna*** (Fig. 15, B/9)

  Note: sometimes *E. magna* may have a more cylindrical shape and can be confused with *E. coecicola;* in *E. magna* the OR is much larger.

3.1.1.6. Diagnosis of *Eimeria* species from chickens

Chickens are hosts of the following 7 *Eimeria* species: *Eimeria acervulina, E. brunetti, E. maxima, E. mitis, E. necatrix, E. praecox and E. tenella* [6].

A reliable species diagnosis based on oocyst morphology is not possible as their dimensions and other features overlap between species. Only *E. maxima* has oocysts with average dimensions exceeding those of other species but even the ranges of these dimensions overlap with other species.

Therefore, species diagnosis has to be based on a combination of various characteristics, including site of development in the intestinal tract of the host, type of macroscopical lesions, size of schizonts in native mucosal smears and some other features. More information is presented in chapter 1.1.

For an exact species diagnosis, pure culture have to be produced by single-oocyst infection of *Eimeria*-free chickens (technique see chapter 1.1.

Because of the limitations and practical problems in species diagnosis using methods described above, the new biochemical and molecular approaches for identification of *Eimeria* species in chickens are of special interest (see chapter 3.2.

3.1.2. Strain identification

Precocious strains ("lines") of *Eimeria* species are characterised by a shorter prepatent period and normally a lower multiplication rate than that of the parent strain. Such strains of chicken *Eimeria* species are used in live vaccines.

In some *Eimeria* species of rabbits (see chapter 1.4.) differences in oocyst morphology have been observed between precocious and parental [2]. For example, the oocysts of the parental strain of *E. intestinalis* have sporozoites which enclose one small refractile body. The precocious strain of the same species differs in that it has two different types of sporocysts in the same oocyst: two of these sporocysts harbour a large refractile body which lies between the sporocysts, whereas in the other two sporocysts such bodies cannot be identified [5]. In the precocious lines of *E. media* [6], *E. magna* and *E. coecicola*, all the sporocysts are similar. They contain only one very large refractile body which is inside the sporocyst but not in a sporozoite.
Oocysts of the parental strain of these 3 species contain sporozoites with a small refractile body.
Some details are depicted in Fig. 16.

Similar morphological differences between precocious and parental strains in *Eimeria* species of other animal hosts than the rabbit have not been observed so far.

For biochemical and molecular techniques of strain identification consult chapter 3.2.

Fig. 16: Oocyst morphology of precocious and parental strains in *Eimeria* species from the rabbit (magnification x 2,300) (D. Licois and P. Coudert; micrographs: D. Licois)

16.1. Parental strain of *E. intestinalis*: the oocysts contains four identical sporocysts each with two sporozoites. Each sporozoite encloses one small refractile body.
16.2. Precocious line of *E. intestinalis*: all oocysts contain two types of sporocysts: two sporocysts harbour each a huge refractile body while in the other two sporocysts no refractile body can be observed. Sporozoites are present in each sporocyst. The large refractile bodies are included inside the sporocysts and not in the sporozoites.
16.3. Parental strain of *E. magna*: classical morphology of sporocysts which contain two sporozoites each with a small refractile body.
16.4. Precocious line of *E. magna*: all the oocysts harbour four identical sporocysts but each sporocyst contain one huge refractile body which is inside the sporocyst and not in a sporozoite. This structure is also observed in other precocious lines of *Eimeria* species obtained until now from the rabbit (*E. media*, *E. coecicola*).

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3.2. Biochemical and molecular techniques for identification of *Eimeria* species and strains

N. Smith, I. Roditi and M. W. Shirley

Several biochemical and molecular techniques have been employed in recent years for the identification of *Eimeria* species and strains. They include isoenzyme electrophoresis, electrophoretic protein characterisation and RNA or DNA analyses. However, none of these techniques are adapted for practical application and still have to be regarded as experimental. Also, many questions of sensitivity and specificity of various test systems are open and require further evaluation.

3.2.1. Isoenzyme electrophoresis

Many species of *Eimeria* are well suited to study by isoenzyme electrophoresis due to the relative ease of isolation and purification of large numbers of oocysts.

- **Sample preparation**

- As a general guide, a minimum concentration of about 5×10^7 oocysts/ml is required.

- The oocysts are disrupted by vortexing with an equal volume (or greater) of 1 mm glass beads for 3-5 min until microscopic analysis confirms total disruption of oocysts, sporocysts and sporozoites.

- Generally, the samples are then centrifuged (for example, < 5 min at 14,000 x g in a microfuge or > 10 min at 1000 x g in a benchtop centrifuge) and the supernatants used for elecrophoresis. However, according to [13], the centrifugation step may be omitted without significantly affecting the electrophoresis.

  Note: Oocyst disruption may be carried out in a variety of buffers and solutions. For example, Shirley [11] simply used 0.9% NaCl, Rollinson et al. [9] used a solution comprised of 1 mM dithiothreitol (DTT), 1 mM Ɛ-aminocaproic acid and 1mM EDTA and Chapman [2] used Tris-citrate buffer (pH 7.6) supplemented with 17.4 mg/l phenylmethylsulphonylfluoride (PMSF) and 200 kallikrein units/ml aprotonin. In our laboratory, good results have been obtained using a solution in-

corporating 0.1% Triton X-100, 0.1% DTT and 10% sucrose. The protein concentrations of the supernatants of oocysts disrupted in this solution may be up to 4 times higher than those after disruption in phosphate buffered saline (PBS). Samples should be used within a few hours of preparation or stored in liquid nitrogen. Other forms of storage sometimes result in the formation of artefactual sub-bands of enzyme activity.

- **Running conditions for horizontal starch and agarose gel electrophoresis**

Thick layer starch gel electrophoresis has been the preferred method for isoenzyme characterisation of *Eimeria* in many laboratories since the studies of Shirley [11].

- **Procedure for starch gels**

- Starch gels are prepared from high quality hydrolysed potato starch (for example, catalogue number S-4501 from Sigma) by heating a mixture of starch with appropriate gel buffer (Tab. 15).

Note: Some examples commonly used in studies involving *Eimeria* are presented in Tab. 15 and many more can be found in excellent manuals [4, 8]. For safety reasons, it is important that conical flasks of toughened glass be used, that the flasks are only filled to half of their capacity and that the mixture be continually shaken or mechanically stirred during heating. Safety glasses and heat-protective gloves should be worn. According to [4] a concentration of 10-12% (w/v) of starch is recommended, however, the manufacturers usually recommend a much lower concentration (6-7%) which is precisely defined for each batch.

- After heating for some time, the starch mixture becomes extremely viscous and then suddenly becomes quite fluid again. At this point heating should be stopped and the air bubbles completely evacuated from the mixture by use of a vacuum pump. The liquid gel can then be poured into a suitable mould on a glass plate to a depth of 4-6 mm. The gels should be allowed to cool and set for several hours before being moved and may be stored, humidified, for up to 48 h at 4°C.

- **Procedure for agarose gels**

Agarose gels are also amenable for isoenzyme electrophoresis of *Eimeria* and may be subjected to similar running conditions and staining procedures as starch gels, producing similar results. However, the preparation of agarose gels is somewhat simpler than that of starch.

- High quality agarose powder (for example, catalogue number 50003-Seakem LE from FMC Bioproducts) is heated in an appropriate gel buffer (see Tab. 15) to boiling point. The agarose is generally present at a concentration of 1% or 2%.

- After boiling, the liquid gel is allowed to cool to about 50°C before pouring on to a level clean glass plate to a depth of 1-2 mm (200-250 ml will neatly cover a plate measuring 200 x 265 mm; surface tension is sufficient to control the spread of the liquid gel).

- After cooling and setting, the gel may be moved and stored, humidified, at 4°C for up to 48 h. Both starch and agarose gels should be left on the glass plates until after electrophoresis is complete.

- For the actual electrophoresis, the gels are placed on top of cooling plates (4-9°C) or in a temperature controlled cold room between the two electrode bridge buffer compartments.

- The buffer is given access to the gels via Whatman 3MM filter paper wicks. Samples are usually applied on small pieces of filter paper which may be inserted into the thick starch gels using fine forceps and a scalpel blade or applied on top of the agarose gels. Samples may also be applied directly onto the agarose gels using removable plastic guides and small scratches made with a scalpel blade to aid penetration.

- Samples are best applied towards the cathode in volumes of 10-20 µl at a protein concentration of 2-4 mg/ml. For starch gels, a voltage of 8-10 V/cm is generally applied for 4 h, though some enzymes require up to 18 h of electrophoresis. For agarose, somewhat higher voltages (20-25 V/cm) are routinely applied. This has the effect of allowing shorter running times (1-3 h). However, some experiments in our laboratory indicate that there is little or no reason that starch cannot also be subjected to similar running conditions.

Tab. 15: Buffers used for electrophoretic analyses

Buffer no.	Gel type	Electrode buffer g/l		Gel buffer g/l		Voltage	Hours
1	Agarose, Starch	Tris	29.00	Tris	2.32	165	4
		Citrate	16.50	Citrate	1.32	or	
			pH 6.7		pH 6.7	400	3
2	Agarose, Starch	Tris	12.11	Tris	1.21		
		Maleate	2.30	Maleate	0.23		
		EDTA	3.72	EDTA	0.37	400	3
		$MgCl_2.6H_20$	2.03	$MgCl_2.6H_20$	0.20		
			pH 7.4		pH 7.4		
3	Agarose	Citrate	38.00	Tris-HC1	1.57		
		NaOH	22.00			400	3
			pH 7.0		pH 7.0		

- **Running conditions: cellulose acetate gels**

Cellulose acetate gels may be obtained commercially as "Cellogel" (Chemtron). This constitutes an important advantage over starch or agarose gels since the quality of the gels is assured. Other notable advantages of cellulose acetate gel electrophoresis include a very short running time and a requirement for substantially smaller samples (< 5 µl at concentrations of 1 - 4 mg protein/ml). Cellulose acetate gels are an excellent choice for isoenzyme analysis because a large suite of enzymes are readily detected using this medium. Thus, good resolution of 19 enzymes of *E. tenella* and *E. acervulina* has been achieved with cellulose acetate [1] compared with 9 for starch [6].

In general, buffers and running conditions (other than running time) for cellulose acetate gels are similar to those used for starch and agarose gels. More details for the electrophoresis of many enzymes may be found in [8]. However, the initial equilibration of the gels is important.

- "Cellogel" strips are normally supplied in sealed packets containing 30% methanol.

- Prior to electrophoresis, the gels are lightly blotted (the cellulose acetate should not be allowed to become completely dry) and then soaked in the appropriate buffer for at least 15-30 min.

- The gels should be handled very carefully and placed in the buffer very gently and gradually. It is advisable to change the buffer at least twice during the equilibration period.

- After equilibration, the gels are once again lightly blotted and carefully placed in the electrophoresis tank so that the ends of the gels actually dip into the electrode buffers. It is usually not necessary to run the gels at low temperatures because a relatively low current is generated.

- **Running conditions: Ampholine polyacrylamide gel (ampholine-PAG) isoelectricfocusing**

A technique which, to date, has received only limited attention with regard to isoenzyme analysis of *Eimeria* is ampholine-PAG isoelectricfocusing. However, ampholine-PAG isoelectricfocusing has several advantages over starch, agarose and cellulose acetate gels. First, ampholine-PAG isoelectricfocusing kits are commercially available (Pharmacia-LKB Biotechnology). These kits include 1 mm thick gels cast on a plastic support film, electrode wicks, sample application pieces, templates and result forms. Also included are detailed, clear instructions regarding all aspects of the procedure including preparation of solutions, sample preparation and application, instrument and gel preparation, running conditions and isoelectric point (pI) determinations.

Ampholine-PAG gels are available in several pH ranges (3.5-9.5, 4.0-5.0, 4.0-6.5, 5.0-6.5, 5.5-8.5), are extremely easy to handle and can accommodate a large number of samples. As with cellulose acetate gels, a relatively small quantity of sample is required and the running time is relatively short due to the high voltages applied (1500-2000 V). Since proteins are separated on the basis of their pI, which can be accurately determined for each protein, comparisons between gels can be made. Gel-to-gel comparisons cannot be made reliably with starch, agarose or cellulose acetate as these media separate proteins on the basis of charge which may vary slightly from gel to gel. Another advantage of ampholine-PAG is that time consuming experiments to determine the most suitable buffer systems and running times and conditions for analysis of different enzymes by

starch, agarose and cellulose acetate are not necessary since the electrode solutions and running conditions are specified for each pH range. Thus, although initial purchase costs for ampholine-PAG are much greater than those for starch or agarose, this outlay is offset by savings in labour time and by reliability and reproduciblity.

Unfortunately, to date, only a handfull of *Eimeria* enzymes have been tested using ampholine-PAG and only using a broad pH range of 3.5-9.5 [12]. Of the enzymes tested (glucose phosphate isomerase, glucose-6-phosphate dehydrogenase, isocitrate dehydrogenase and lactate dehydrogenase) only isoenzymes of lactate dehydrogenase were resolved as sharp bands (although we have recently found that hexokinase, phosphoglucomutase and glucose phophate isomerase also resolve extremely well, both in the broad 3.5-9.5 pH range and in the narrower 4.0-6.5 pH range). The use of gels with other pH ranges may well facilitate the analysis of other enzymes since the narrower the pH range the better the resolution.

A potential drawback of ampholine-PAG is that the kits are designed for use with Pharmacia-LKB electrophoresis units and powerpacks though other systems could probably be used with minor adaptations. There are, of course, certain advantages to using the same manufacturer's equipment and the following brief description of the general procedure for running Pharmacia-LKB ampholine-PAG is written with this in mind.

- **Procedure for ampholine-PAG isoelectricfocusing**

- Gels are removed from their protective coverings and placed in the centre of the cooling plate of the electrophoresis tank (the grid markings on the plate and on the screen print guides supplied in the kit are very helpful). Gloves should always be worn when handling.

- The gels and about 1-2 ml of insulating fluid (kerosene, light paraffin oil or Triton X-100) should be placed between the gel and the cooling plate.

- The electrode strips are soaked in appropriate solutions (about 2-3 ml per strip) and trimmed so that they do not extend beyond the edges of the gel. The electrode strips are placed at opposite ends of the gel.

- Samples are applied to pieces of filter paper (5x10 mm) positioned towards the cathode. (Again, the screen print guides are very helpful in positioning the sample papers). The electrodes are then aligned in the centre of the electrode strips, the safety lid connected and the run commenced.

- Running times and conditions vary from gel to gel, depending on the pH range. Thus, broader pH range gels usually run at 1500 V for 1.5 h whereas narrower pH range gels run at 2000 V for 2.5 - 3 h. At the halfway point of the run, the power is disconnected briefly and the sample application papers removed.

- **Detection procedures**

The manuals [4] and [8] give recipes for the detection of many enzymes. Thus, only a few examples commonly used in coccidiosis research are reproduced here (Tab. 16). The recipes given should be applicable to all gel types, as indicated, but are intended as guides only.

Many enzymes, including those in Tab. 16, are detected by incubating the gels with specific substrates and the dye, methylthiazoldiphenyltetrazolium (MTT), in an NAD- or NADP-linked, phenazinemethosulphate (PMS)-catalysed reaction. The dye, nitrobluetetrazolium (NBT) can be used in place of MTT. Good explanations of the principles and sequence of reactions which enable detection of a wide range of enzymes using MTT are to be found in [4] and so will not be discussed here.

The enzyme detection stains can be applied either as liquid reaction mixes (this is normal for ampholine-PAG) or in hot agar solutions which cool rapidly once in contact with the cold gels and form an overlay for isoenzyme dectection (this method is preferred for starch and agarose gels which are relatively large and would therefore require a large volume of reaction mix which would quickly become an expensive proposition). The preparation of the reaction mixes is straightforward, the only point to note is that the MTT and the PMS should be added immediately prior to staining. Construction of agar overlays is also uncomplicated.

- The reaction buffer mixes and the agar solution are prepared separately. The agar is dissolved by heating until boiling, after which it is allowed to cool slightly before the reaction buffer mix is poured evenly on to the gel.

Tab. 16: Enzymes and reaction mixes

Enzyme	Buffer no.	Reaction mix Ampholine-PAG	Agarose, Starch
Glucose phosphate isomerase (GPI)	1, 3	50 mg Na_2 fructose 6 phosphate, 5 mg NADP, 5 mg MTT, 40 mg $MgCl_2.6H_2O$, 2U glucose 6 phosphate dehydrogenase, 0.5 mg PMS in 35 ml 0.1M Tris-HCl (pH 8)	100 mg Na_2 fructose 6 phosphate- 10 mg NADP, 10 mg MTT, 80 mg $MgCl_2.6H_2O$, 4U glucose 6 phosphate dehydrogenase, 1 mg PMS in 35 ml 0.1M Tris-HCl (pH 8) plus 35 ml 2% agar (in Tris-HCl)
Phosphoglucomutase (PGM)	2	25 mg glucose 1 phosphate, 130 mg $MgCl_2.6H_2O$, 2.5 mg NADP, 2.5 mg MTT, 2U glucose 6 phosphate dehydrogenase, 2.5 mg PMS in 35 ml 0.1M Tris-HCl (pH 8)	50 mg glucose 1 phosphate, 260 mg $MgCl_2.6H_2O$, 5 mg NADP, 5 mg MTT, 4U glucose 6 phosphate dehydrogenase, 5 mg PMS in 35 ml 0.1M Tris-HCl (pH 8) plus 35 ml 2% agar (in Tris-HCl)
Lactate dehydrogenase (LDH)	1	1300 mg lithium lactate, 5 mg NAD, 5 mg MTT, 0.5 mg PMS in 35 ml 0.1M Tris-HCl (pH 8)	600 mg lithium lactate, 10 mg NADP, 10 mg MTT, 1 mg PMS in 35 ml 0.1M Tris-HCl (pH 8) plus 35 ml 2% agar (in Tris-HCl)
Hexokinase (HK)	1	400 mg glucose, 70 mg ATP, 12 mg NADP, 10 mg MTT, 20 mg $MgCl_2.6H_2O$, 5U glucose 6 phosphate dehydrogenase, 2.5 mg PMS in 35 ml 0.1M HEPES (pH 7.5)	800 mg glucose, 140 mg ATP, 24 mg NADP, 20 mg MTT, 40 mg $MgCl_2.6H_2O$, 10U glucose 6 phosphate dehydrogenase, 5 mg PMS in 35ml 0.1M HEPES (pH 7.5) plus 35 ml 2% agar (in HEPES)

- A mould should be used to contain the flow of the overlay. The reactions are then allowed to proceed in the dark at 37°C for 30-60 min (or more in the case of weak reactions). If photography is desired, it should be performed immediately, although

the reaction on ampholine-PAG gels can be stopped and the gels preserved in a 10% glycerol solution. Agarose gels cannot be stored but starch gels can be stored wrapped in plastic after they have been fixed in 10% glycol, 5% acetic acid and then washed several times in water.

3.2.2. Protein characterisation by 2-dimensional (2-D) electrophoresis

A potential alternative to isoenzyme electrophoresis for the biochemical identification of *Eimeria* species and strains is 2-D electrophoresis. This technique first separates proteins on the basis of their pI by isoelectricfocusing (IEF) and then further separates them on the basis of molecular weight by SDS-PAGE (sodium dodecylsulphate-polyacrylamide gel electrophoresis). This produces a "fingerprint" protein profile specific for different species of *Eimeria* [16]. However, the technique is relatively complicated and the fingerprints are often difficult to analyse and interpret. Furthermore, intraspecific strains of various *Eimeria* species could not be reliably differentiated by 2-D electrophoresis and so its usefulness for identification purposes is considered to be limited [16].

- **Sample preparation**

- Oocyst samples are disrupted by vortexing with glass beads in lysis buffer (61.5 mM Tris-HCl, pH 6.8; 2% SDS; 5% 2-mercaptoethanol; 10% DTT; 10% glycerol and 0.0025% bromophenol blue) at about $3x10^7$ oocysts/ml. (Alternatively, oocysts may be disrupted at twice this concentration in saline and then an equal volume of double strength lysis buffer added.)

- The homogenate is stored at -70°C until use, at which time the samples are thawed, refrozen, thawed again and centrifuged to remove debris.

- **Isoelectricfocusing (IFF)**

Isoelectricfocusing may be run using the method described above. After isoelectricfocusing, sample lanes are carefully excised and overlayed onto an SDS-polyacrylamide gel.

- **SDS-PAGE**

The preparation of various concentrations of SDS-polyacrylamide gels is well described [10] and so will not be repeated here.

Precast SDS-polyacrylamide gels suitable for 2-D electrophoresis are also commercially available (for example, from Pharmacia). 10% SDS-polyacrylamide slab gels were used for electrophoresis of *Eimeria* proteins in the second dimension [16].

The gels are usually run at about 40 mA for 3-4 h or until the dye front reaches the bottom of the gel. This gives a total of about 150 "mA h"; thus, one could also run the gels overnight at 9 mA.

- **Protein detection**

  The gels may be stained either with Coomassie Brilliant Blue R250 or with silver nitrate. The latter method is considered to be far more sensitive, however, it is possible to stain a single gel by both methods for optimum detection.

- **Staining with Coomassie Brilliant Blue R250**

- At the completion of the electrophoresis, the electrode wicks are removed from the gel and it is immersed in fixative (10%-20% trichloroacetic acid) for 30 min.

- The gel is then washed 3 times with destaining solution (10% acetic acid, 25% ethanol) and then stained for 15-30 min with 0.1% Coomassie Brilliant Blue R250 in destaining solution (this solution should be heated to 60°C with constant stirring and then filtered before use).

- Thereafter the gel is destained, using several changes of destaining solution, over several hours (preferably overnight) until the background is clear.

Note: Staining and destaining is best carried out using a shaking table.

- **Staining with silver nitrate**

- The gel is fixed in 10% acetic acid in 30% ethanol, then washed 3 times in 30% ethanol followed by 3 washes in distilled water. (If the gel has already been stained with Coomassie Brilliant Blue R250 then the fixation step may be omitted).

- The gel is then immersed in 0.1% silver nitrate for 30 min, then rinsed in distilled water for 1 min. The gel is then immersed in 0.02% formaldehyde in 3% Na_2CO_3 for 10 min and washed 3 times in distilled water.

- The contrast between the protein bands and the background can be improved by carrying out the following additional steps :

- The gel is immersed in Farmer's reducer (1% potassium ferricyanide, 1.6% sodium thiosulphate) for 5 min or until the silver stain disappears, then rinsed in tap water for 1 min and washed at least 5 times in distilled water for 3 min until the yellow colour disappears. The silver staining, developing, stopping and washing steps are then repeated.

- The gels can be preserved in 10% glycerol, though a photographic record (taken after both Coomassie and silver staining) is perhaps preferable.

3.2.3. RNA and DNA analyses

Logically, species and strain differences should ultimately manifest as differences in characteristics of the genetic material, RNA and DNA. This was realised in 1977 by Lee and Fernando [5], who compared the buouyant densities in CsCl of DNA from unsporulated oocysts of two strains of *E. maxima* and one of *E. tenella.* Whereas DNA from *E. maxima* showed peaks of 1.707 and 1.682g/cm^3, DNA from *E. tenella* showed a single peak of 1.711g/cm^3 indicating that this technique could be a useful tool for identification. However, subsequent studies showed that the buouyant densities of DNA from *E. tenella, E. acervulina* and *E. coecicola* were identical [6]. Thus, recent studies have utilised more sophisticated techniques such as pulse field gel electrophoresis for chromosomal characterisations, restriction enzyme digestion and Southern blotting, 5S ribosomal RNA characterisation and polymerase chain reaction (PCR), including random amplified polymorphic DNA (RAPD), analysis. At this stage, these techniques must be regarded as experimental with regard to identification of *Eimeria.*

However, with further study, it is likely that analysis of genetic material will supplant classical and biochemical techniques for identification of *Eimeria* species and strains.

3.2.3.1. Pulse Field Gel Electrophoresis (PFGE)

- **Procedure (see also chapter 4)**

- Purified sporozoites or merozoites are suspended at 0.5-1x10^9/ml in 1% agarose in PBS (pH 7, 37°C).

- Aliquots of this suspension are transferred to a mould containing wells which measure 10x5x1mm and placed on ice to facilitate setting of the agarose.

- The blocks are then removed and placed in NDS buffer (0.5M EDTA at pH 8, 10mM Tris-HCl at pH 9.5, 1% N-lauroylsarcosine) plus 1% proteinase K, incubated at 50°C for 36-48 h with one change of buffer. The blocks are then washed twice with NDS and stored at 4°C until use.

- Agarose gels (0.6%-1.0% in half-strength TBE buffer - 0.5 x 45 mM Tris-borate/1mM EDTA) are cast by pouring 35 ml on to 10.5 cm glass plates.

- Pieces are cut from the agarose blocks containing the parasites and placed into preformed wells along the origin and sealed with 1% low melting point agarose. The running conditions may be varied. For example, for 1% agarose gels, a voltage of 140V is applied for 68 h with a 200 sec pulse time. For 0.65% gels, 200V is applied over 23 h with an 80 sec pulse time and for 0.6% gels, 40V is applied for 268 h with a 1 h pulse time. For *Eimeria,* best results are obtained using the low voltage, long pulse time and running time conditions [14].

- To visualise the chromosomes, the gels are stained with 0.5µg/ml ethidium bromide for 30 min and destained in half-strength TBE for 2-4 h.

3.2.3.2. Restriction enzyme digestion and Southern Blot analysis

Ellis and Bumstead [3] showed that different species of avian *Eimeria* could be distinguished on the basis of hybridisation patterns obtained with ribosomal RNA gene probes. This procedure requires relatively large amounts of high molecular weight DNA (in the microgram range).

- **Procedure**

- DNA of suitable quality can be obtained by lysing up to 10^8 sporozoites/ml in a buffer consisting of 1% sarcosyl, 100µg ml^{-1} proteinase K, 100mM EDTA, 10mM tris-HCl, pH 7.5, and incubating them for a further 30 min at 25°C.

- Proteins are removed by gently extracting the aqueous phase with an equal volume of phenol-chloroform-isoamyl alcohol (PCIA, 25:24:1; see [10]).

- The two phases are separated by centrifugation and the upper, aqueous phase transferred to a fresh tube and extracted with an equal volume of chloroform-isoamyl alcohol (24:1).

- This upper, aqueous phase can be dialysed against 10 mM Tris-HCl (pH 7.0) plus 10 mM EDTA and then separated from cellular RNA by isopycnic banding in caesium chloride gradients [10]. In our experience, however, it is easier to selectively precipate RNA and DNA with lithium chloride.

- This is done by mixing the aqueous phase with 3 volumes of 4M LiCl and incubating it overnight at 4°C. RNA is pelleted by centrifugation for 20 min at 12,000 rpm using either an HB-4 or SS34 rotor (Sorvall). DNA can be precipitated from the supernatant by the addition of 2 volumes absolute ethanol.

- The solution is incubated on ice for > 1 h and the DNA pelleted by centrifugation for 10 min at 12,000 rpm.

- The DNA is dissolved in 10 mM Tris-HCl plus 0.1 mM EDTA and stored at 4°C. Small scale isolation of DNA (from up to 5 x 10^7 sporozoites) can be performed in Eppendorf tubes and the nucleic acid recovered by centrifugation at top speed in an Eppendorf microfuge.

- Digestion with restriction enzymes, separation of the DNA fragments by agarose gel electrophoresis and Southern blotting are all carried out according to standard procedures [10]. For highly repetitive sequences, such as the ribosomal RNA genes, strong signals can be obtained with 0.5-1µg genomic DNA per lane.

This method of species identification has now been largely superceded by methods using the polymerase chain reaction (PCR) (see below).

3.2.3.3. Species identification by PCR - 5S ribosomal RNA genes

A highly sensitive and specific assay has been developed for the detection of *E. tenella* [15]. This method, which makes use of the polymerase chain reaction to amplify a 560 bp intergenic sequence between 5S ribosomal RNA genes, requires as little as 1 pg DNA or <10 oocysts. The assay is specific for *E. tenella* and no amplification products are obtained with *E. acervulina*, *E. maxima*, *E. praecox*, *E. nieschulzi* or *E. intestinalis*. It is also possible to detect *E. tenella* in a mixture of oocysts.

- **Procedure for DNA preparation**

- DNA can be prepared by a variety of methods, however, it can be prepared directly from small numbers of oocysts in the following way:

- Oocysts are diluted in 0.1% $K_2Cr_2O_7$ (positive results are obtained in the range 1 oocyst μl^{-1} up to 2 x 10^3 oocysts μl^{-1}) and 10 µl of the suspension are transferred to an Eppendorf tube.

- An equal volume of phenol-chloroform-isoamyl alcohol (PCIA, 25:24:1; see [10]) is added without mixing the two phases and the sample is incubated at 85°C for 1 h.

- After a brief centrifugation to collect condensed droplets, 100µl of 10mM Tris-HCl plus 0.1mM EDTA (pH 8.0) is added and the mixture extracted by gentle mixing with an equal volume of PCIA.

- The phases are separated by centrifugation for 5 min in an Eppendorf centrifuge and the aqueous (upper) phase is transferred to a tube containing 100 µl chloroform-isoamyl alcohol (CIA, 24:1).

- The two phases are mixed, then separated by centrifugation as above.

- The aqueous (upper) phase is decanted and digested for 2 h with proteinase K (250 µg/ml), then extracted once again with equal volumes of PCIA and CIA.

- Ten micrograms of glycogen are added as a carrier and the nucleic acid is precipated by the addition of 5 µl 2M NaCl and 250 µl EtOH.

- The mixture is incubated on ice for >30 min and the DNA pelleted by centrifugation for 10 min at top speed in an Eppendorf microfuge.

- After thorough removal of the supernatant, the pellet is dissolved in 20µl water and stored at - 20°C until required.

- **Procedure for Polymerase Chain Reaction (PCR)**

- The following oligonucleotides are used for amplification: an antisense primer beginning 14 bases upstream of the 5S gene (5´ TCCCTCAATACACCTCTACT 3´) and a sense primer beginning 2 bases downstream of the gene (5´ TTGTTGGCTTCTCCAACAAC 3´).

- PCR is performed in 100µl reaction buffer (67 mM tris-HCl, 6.7 mM $MgCl_2$, 16.6 mM $(NH_4)_2SO_4$, 10 mM ß-mercaptoethanol, 6.7µM EDTA, pH 8.8) with 50 pmol of each primer, 0.2mM dNTPs and 2.5 units Taq DNA polymerase.

- Samples are overlaid with mineral oil and subjected to 37 cycles of denaturing at 94°C (1 min), annealing at 53°C (2 min) and extension at 74°C (2 min). In each case 20µl of the sample can be analysed by gel electrophoresis on 1% agarose gels and visualised by ethidium bromide staining.

3.2.3.3. Species and strain identification by PCR - Random Amplified Polymorphic DNA (RAPD) Analysis

In this procedure, a single primer with an arbitrary nucleotide sequence can be used to amplify a characteristic set of fragments from genomic DNA. This method requires more genomic DNA (approximately 30 ng) than the method described above, but has the advantage of being able to distinguish between different strains and species. Procunier et al. [7] used primers from a set of random oligonucleotides purchased from Dr J. Carlson (Biotechnology Laboratory and Department of Forest Sciences, University of British Columbia, Vancouver, B. C. Canada). The following primers proved useful in differentiating between species and isolates of avian *Eimeria*:

Primer	Sequence
111	AGT AGA TGA C
114	TGA CCG AGA C
115	TTC CGC GGG C
132	AGG GAT CTC C
134	AAC ACA CGA G

- **Procedure for Polymerase Chain Reaction**

- DNA is amplified from 30 ng genomic DNA in a 25µl reaction mixture containing 10mM tris-HCl (pH 9.0 at 25°C), 50mM KCl, 1.5mM $MgCl_2$, 0.1% (v/v) Triton X-100, 2% formamide, 0.25mM dNTPs, 5 pmol primer and 1 unit Taq polymerase.

- The DNA is first denatured for 3 min at 94°C. 45 cycles of PCR are then performed using the following parameters: denaturing 94°C (1 min), annealing 40°C (1 min) and extension 72°C (2 min). As a final step, extension is carried out for 10 min at 72°C.

- The amplification patterns obtained with the various *Eimeria* isolates and primers are too complex to be described here. No single primer gave positive results for every strain and species, however, and we therefore recommend that a minimum of two reactions are carried out with different primers for unequivocal identification. We also recommend that investigators establish their own reference strains.

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4. Special techniques of molecular biology

R. Braun and M. W. Shirley

In this chapter we describe some of the molecular biology techniques specific to research on *Eimeria spp.*, concentrating largely on how DNA, RNA and proteins are extracted from the various life cycle forms. The further characterisation of macromolecules can then be done by established biochemical procedures.

Standard techniques in work with recombinant DNA technology will not be presented in detail, since there are many excellent compilations of these techniques, such as the one by [30].

4.1. Extraction of macromolecules

Compared to other parasitic protozoa, such as members of the genera *Trypanosoma* or *Plasmodium*, little biochemical and molecular work has been done with *Eimeria*. One significant reason for this lack of data lies in the fact that there is no easy and high yield *in vitro* cultivation system for *Eimeria*. Although large amounts of starting material for experiments can be obtained, this, with the exception of sporozoites, has to be parasites from infected chickens, or, depending on the species to be studied, from other vertebrate hosts (rabbits, rats etc.).

4.1.1. Preparation of DNA

- **DNA from oocysts, sporozoites and merozoites**

Most DNA preparations have been made from oocysts, either unsporulated or sporulated, or from sporozoites. The reason for this preference is that oocysts can relatively easily be isolated from the faeces of infected chickens or other hosts by well established procedures that provide large numbers of very clean, bacteriologically pure parasites.
Sporozoites and merozoites can also be obtained in large amounts. DNA of very high molecular weight can easily be prepared from them, as these parasites have a soft membranous outer envelope and are easily lysed in the absence of shear forces. In

contrast, the harsh homogenization procedures required to break the walls of oocysts can lead to fragmentation of very large DNA molecules.

The methods for parasite isolation are reviewed in chapter 1.1.

- **DNA from gametocytes**

DNA can be isolated from gametocytes in large amounts. However, as gametocytes are located inside host cells (unlike free sporozoites and merozoites) they must be recovered from significant amounts of infected host tissue and this constraint necessarily leads to some contamination with host DNA.

The isolation of gametocytes has been described by [42] and is reported in detail in section 1.1.9.

Genomic DNA has been isolated from gametocytes of *E.maxima* (Wallach, personal communication) using techniques described by [41] for *Plasmodium.*

- **Procedure**

- About 5-10x10^6 gametocytes were resuspended in 5 ml of DNA extraction buffer (10 mM Tris, pH 7.9, 0.1 mM EDTA, 1% SDS, 50 µg per ml RNase A) and the mixture incubated for 1 h at 37° C.

- Proteinase K was added to 100 µg per ml for a 1 h incubation at 50° C.

- The DNA was extracted twice with phenol and once with CIA (chloroform:isoamyl alcohol 24:1).

- After precipitation with ethanol the DNA was spooled and dissolved in TE-buffer (10 mM Tris, pH 7.4, and 1 mM EDTA).

- The DNA was further purified by a CsCl centrifugation at 100,000 x g for 48 h after which it was dialysed against TE, spooled and dissolved in TE buffer. The yield was about 100 µg DNA per 10^6 gametocytes.

- **DNA for species identification by PCR**

The polymerase chain reaction (PCR) makes it possible to identify specific DNA sequences from very small amounts of material [9]. This technology has been applied to many infective agents, including parasitic protozoa, such as *Trypanosoma cruzi* [25] or *Plasmodium falciparum* [32, 39]. For *Eimeria* a PCR method for species identification based on sequences of 5S ribosomal RNA (rRNA) was developed by [38] (see also chapter 3.2.).

- **Procedure**

- DNA was either prepared by conventional methods from large numbers of oocysts (about 10^7) or by a specifically developed technique starting with very small numbers of oocysts.

- For the new method, in which small numbers of oocysts were used, the concentration of oocysts (stored in 1% potassium dichromate) was first determined and the oocysts diluted appropriately. One microliter of oocyst suspension was mixed with 9 µl water.

- 10 µl of phenol:chloroform:isoamylalcohol (øCIA; 25:24:1) were added without mixing the two phases and the sample incubated at 85° C for 1 h.

- After a brief centrifugation, 100 µl of TE were added (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA) and the mixture extracted with an equal volume of øCIA, followed by an extraction with CIA.

- The aqueous phase was removed and digested for 2 h with proteinase K (250 µg/ml) and then extracted once again with equal volumes of øCIA and CIA. As a carrier 10 µg of glycogen were added and the DNA was precipitated with ethanol, dissolved in 20 µl water and then stored at -20° C.

- Clear PCR signals were easily obtained by starting with less than 10 oocysts. *E. tenella* could be distinguished from five other *Eimeria* species.

- **Characterisation of chromosome-size DNA**

Pulse field electrophoresis allows the separation of chromosome-sized DNA molecules of up to about 10 megabase pairs (Mb). Each band in the electrophoretic analysis of *Eimeria* represents the DNA of at least one chromosome. Bands may also come from several chromosomes, if these happen to contain DNA of about the same size. Shirley et al. [35,36] and Fernando and Pasternak [12] obtained species-specific patterns with about 10 - 14 chromosomes ranging in size from roughly 1 Mb to more than 6 Mb.

- **Method used by Fernando and Pasternak [12]**

- Sporulated oocysts were sterilized with hypochlorite solution, washed with water and phosphate-buffered saline (PBS; pH 7.4).

- Cleaned sporulated oocysts were broken with glass beads in a Mickle disintegrator and unbroken oocysts and oocyst walls were removed by filtering through nitex screen printing cloth.

- Sporocysts were excysted by incubation at 41°C with 5% chicken bile and 2.5% trypsin in PBS.

- The resulting sporozoites were purified in 60% or 65% percoll gradients, centrifuging them in a SS34 Sorvall rotor at 30,000 x g for 10 min at 4°C.

- Freshly isolated purified sporozoites, suspended in saline at 2 - 4x10^8 per ml, were mixed immediately at 42°C with an equal volume of 1.2% low melting agarose, liquefied in 10 mM Tris (pH 8.0), 10 mM EDTA and 0.85% NaCl.

- The mixture was poured in plastic moulds and allowed to solidify in small blocks for 20 min on ice.

- The agarose blocks were then removed from the moulds and incubated for 48 h at 46-48° C in 0.5 M EDTA (pH 9.0) and stored in this saine solution at 4° C.

- The electrophoresis was done in a FIGE system (BRL H1) with 1% agarose and buffer containing 90 mM Tris, 90 mM boric acid and 2.5 mM EDTA.

- Electrophoretic separations took up to 12 days running time and gave clear bands, some of which obviously contained multiple molecular species. The resolution is critically dependent on the running conditions. For *E. tenella*, 9 bands were observed with a two-step program of firstly 1.3 V per cm in the forward direction for 3600 sec and 0.43 V per cm for 1440 sec in the reverse direction for a total of 120 h and secondly a forward migration at 1.1 V per cm for 5400 sec and a reverse migration at 0.43 V per cm for 2160 sec for a total of 144 h.

- **Method of Shirley et al. [36] and Shirley [35]**

- Prepare sporozoites and clean by passage through DE-52/nylon wool column.

- Resuspend sporozoites at $1x10^9$/ml in PBS at pH 7.0 or Hank's solution (most preferable).

- Make 2% low gelling temperature agarose (FMC Sea Plaque) in PBS pH 7.0 or 0.125 M EDTA. Cool to 37°C and hold at this temperature.

- Warm cell suspension to 37°C. Mix equal volume of cell suspension in 2% low gelling temperature agarose and, using a syringe and relatively wide-bored needle, quickly dispense into a gel mould. Hold mixture at 37°C while dispensing.

- Place mould on ice to set the agarose plugs, then transfer plugs to a tube containing NDS and 1 mg/ml proteinase K.

- Leave tube at room temperature overnight. Replace NDS and proteinase K with fresh solutions and continue incubation for further 24 h.

- Rinse plugs in 50 mM EDTA, twice for 2 h, to remove most of the proteinase K. Wash in fresh 50 mM EDTA overnight, change buffer and store blocks at 4°C.

- **Recipe for NDS:**

- Add 93 g EDTA to 350 ml DDW.
- Add 0.605 g Tris base and then solid NaOH pellets to get pH above 8.0 in order to dissolve the EDTA (as many as 100 to 200 pellets are needed for this).
- Dissolve 5 g of lauroyl sarcosine in 50 ml DDW. Add this to the above solution. (An alternative is to use 16.5 ml of 30% BDH Sarcosyl).
- Adjust to pH 9.5 using concentrated NaOH. Make volume up to 500 ml.
- Filter, sterilise and store at 4° C.

4.1.2. Preparation of RNA

The main difficulty, generally, in obtaining RNA from cells or tissues is that RNA is easily degraded in a short time by a large variety of endonucleases able to cleave intact RNA internally to smaller fragments. This difficulty is also encountered with *Eimeria*. Extraction methods are required which rapidly inactivate all nucleases. One important point concerns the handling of frozen biological samples from different life cycle stages. Denaturing agents (e.g. phenol) need to be added to the frozen material, without first letting it thaw. During thawing, cellular structures are presumably destroyed and nucleases get access to substrates, from which they are separated in the intact cell.

- **RNA from oocysts and sporozoites**

To study evolutionary relationships, Barta et al. [1] isolated and partially sequenced ribosomal RNA from several species of *Eimeria*.

- Approximately $1x10^9$ oocysts were obtained from chickens experimentally infected with $5x10^5$ oocysts of *E. acervulina* or $5x10^4$ oocysts of *E. tenella*.

- Oocysts from *E. tenella* were collected from caeca, while oocysts from *E. acervulina* were isolated from faeces, 6 to 9 days after infection.

- Oocysts were isolated by a sugar flotation technique and were sporulated for about 7 h.

- RNA was extracted by homogenising in buffer containing 4 M guanidinium isothiocyanate, 0.1 M beta-mercaptoethanol, 10 mM EDTA, 5 mM Na citrate and 0.5% Na sarcosinate.

- The extract was resuspended in 1.2 volumes of caesium trifluoroacetate (density 2.0 g/ml; Pharmacia) and the total RNA was banded by equilibrium centrifugation [47].

- The ribosomal RNA was reverse transcribed using oligonucleotide primers from *Escherichia coli* ribosomal RNA sequences annealed to total *Eimeria* RNA.

- The sequencing protocol of [23] was followed. Samples were heated to 90° C for 2 min prior to separation on a 7 M urea, non-gradient, 5% acrylamide gel operated at about 1200 volts.

- **Production of polyA+ RNA**

- The construction of cDNA libraries usually starts with polyA+ RNA. An attractive procedure to do this was devised by [40]. In addition to the procedure given here, the preparation of RNA for cDNA libraries, starting with merozoites, has been successful with commercial kits, such as one by Invitrogen (Shirley, unpublished).

- $5x10^8$ oocysts of *E. tenella* were sporulated and homogenized for 60 seconds with Jencon's No. 8 glass beads in a buffer containing 10 mM Tris acetate (pH 7.6), 75 mM Na acetate, 1% SDS, 2 mM EDTA, 0.2% proteinase K and 10 mM vanadyl ribonucleoside complexes.

- The aqueous phase was extracted twice with phenol and twice with phenol:chloroform:isoamyl alcohol (øCIA, 25:24:1) and the nucleic acids were precipitated with ethanol.

- The polyA+ RNA was selected by standard oligo(dT)-cellulose chromatography [30].

- **RNA from merozoites**

In one particular procedure RNA was isolated from merozoites obtained from chicken gut 90 h after infection [18]. The merozoites of *E. acervulina* were purified using chromatography over a DE52 ion exchanger as described by [17]. The polyA+ RNA

for the generation of cDNA libraries was made according to [45]. For Southern and Northern blot hybridizations DNA and RNA were purified by ultracentrifugation in caesium trifluoroacetate [47]. The method described in the previous section on oocysts has also been applied to prepare RNA from merozoites [4].

- **RNA from gametocytes**

Gametocytes were isolated as follows: chickens were infected with 10^4 oocysts of *E. maxima* and killed 134 to 136 h later. Their intestines were removed and immediately washed with ice-cold SAC (170 mM NaCl, 10 mM Tris-HCl, pH 7.0, 10 mM glucose, 5 mM $CaCl_2$, 1 mM PMSF, 1 mg/ml BSA). One end of the organs was tied with a string and the intestines were filled with SAC containing 0.5 mg/ml hyaluronidase (Type III, Sigma). The other end was also tied and the filled intestine incubated for 20 min with shaking at 37°C in PBS. After incubation, the contents of the intestine and the PBS were discarded and the intestine slit open lengthwise. The open organ was placed on top of a 17 µm Polymon filter (Swiss Silk Bolting Cloth Manufacturing Company, Zürich) and the mucosa was washed with SAC at room temperature. The material left on the filter was discarded and the flowtrough containing the gametocytes was filtered through a 10 µm Polymon filter. The gametocytes, which accumulated on the second filter, were washed with SAC and collected by centrifugation at 800 x g for 5 min. They were counted on a microscope grid: the yield was from 0.5 to $2x10^6$ per infected intestine [42].

- Purified gametocytes were washed twice with SAC (containing PMSF, but not BSA) and the RNA extracted by a modification of the guanidinium-CsCl procedure of [5].

- The gametocytes were solubilized with guanidinium thiocyanide, homogenized in a Wheaton glass tissue grinder and the RNA was pelleted through a cushion of CsCl-EDTA by ultracentrifugation.

- After centrifugation, the pelleted RNA was dissolved in 3 ml 0.5% Na sarcosine and ethanol precipitated. The pelleted RNA was dissolved in 2 ml Hepes, pH 7.0, 2 ml 4 M LiCl was added and the mixture was chilled at 4° C for 2 h to precipitate the RNA and leave the DNA in the supernatant.

- The RNA was pelleted by centrifugation (12,000 rpm, 20 min, 4°C), dissolved in 3ml HEPES buffer and 0.66 ml 10% K acetate was added to precipitate the RNA with ethanol.

- The RNA was recovered by centrifugation, redissolved in buffer and precipitated a second time with ethanol. The use of large volumes of Hepes (minimum 3 ml) was found to improve the yield of RNA and its translational efficiency [24].

4.1.3. Preparation and characterisation of proteins

Crude extracts of proteins have been obtained from different life cycle stages of several *Eimeria* species as part of studies to isolate antigens that might be potential vaccine candidates. Such extracts were mostly mixtures of very different components. Only few proteins have been purified and extensively characterised.

Degradation of proteins is likely to be a problem as much with *Eimeria* as with other organisms. Of the many proteinase inhibitors available, only PMSF has been used extensively. As noted above, many genes of *Eimeria* have GCA repeats. These will lead to repeats of a small group of amino acids (ala, gln, ser, cys, leu), with alanine being theoretically the most frequent. In practice glutamine appears to be an unexpectedly abundant translation product, when GCA repeats have been found in coding regions.

- **Proteins from oocysts and sporozoites**

Several authors including [34] and [37] have separated crude extracts of oocysts, sporozoites or merozoites by starch gel electrophoresis or isoelectric focusing in order to characterise electrophoretic variation of several enzymes in different species and strains (see also chapter 3.2.).

From the results of numerous studies, the preferred method that evolved at the Institute of Animal Health, was very straightforward.

- **Procedure**

- Homogenise a pellet of approximately $6x10^6$ sporulated oocysts in a small volume of sterile high quality water with an almost equal volume of glass balls (Jencons No.8) until no parasites are intact. A fresh sample of oocysts (up to about one month old) was used routinely.

- Centrifuge homogenate in a microfuge, and use the protein extract without further purification. Protein stabilisers were not used.

- Do not store surplus extract, prepare fresh material for each analysis.

- A simple method for preparing proteins from sporozoites is based on freezing and thawing (N.Smith, personal communication).

- **Procedure**

- Pelleted sporozoites were resuspended at a concentration of 10^7 in 100 µl PBS.

- They were then frozen in liquid nitrogen and thawed again at 40°C. This procedure was repeated another two times.

- The suspension was then sonicated for 20 sec in a Branson 250 sonifier at 20 watts and 4°C.

- After measuring the protein contents, 50 µl aliquots were stored at 20°C. For ELISAs and Western blots the protein suspensions were then centrifuged in a microfuge for 2 min.

By conventional protein purification techniques [20] fractionated *E.tenella* proteins had to be used as potential antigens. The sonicated sporulated oocysts (10^9 per experiment) were in a buffer with zwittergent and were fractionated by chromatography first on a column of S-200 and G-75.

- **Proteins from merozoites**

Danforth and McAndrew [6] prepared proteins from purified merozoites of *E. tenella.*

- **Procedure**

- Merozoites were obtained from caeca of chickens infected 105 h earlier with *E. tenella* oocysts.

- Caeca were slit open and washed briefly with Dulbecco minimal essential medium (DMEM) to remove debris.

- The cut caeca were then placed in 500 ml DMEM and stirred for 30 min at room temperature to disrupt tissue and release merozoites.

- Tissue fragments were removed by a 3 min centrifugation at 100 x g and merozoites were pelleted thereafter by centrifugation for 10 min at 500 x g.

- The merozoites were suspended in Tris (pH 8.0) and purified over a Leukopak column [46]. The purified merozoites were collected by centrifugation (100 min; 500 g) and resuspended in DMEM.

- For the preparation of total proteins, about 2 x 10^7 merozoites were disrupted with glass beads (0.1 mm diameter) by vortexing for 4 min in electrophoresis sample buffer [22]. The material was centrifuged for 1.5 min at 11,600 x g and used for SDS gel electrophoresis and Western blots.

Proteins from *E. acervulina* merozoites were also studied. The merozoites were purified essentially as described above for the preparation of RNA [18]. From these, crude protein extracts were obtained to be used in Western blots. One particular merozoite surface antigen (EAMZ250) was studied by cloning and sequencing [15, 19]. It was also expressed as a galactose binding fusion protein. The recombinant *Escherichia coli* bacteria were directly used to orally immunise chicken [16].

- **Proteins from gametocytes**

For the extraction of proteins from *E.maxima,* [42] incubated 2x10^6 purified gametocytes for 30 min at room temperature with 1 ml of buffer containing 10 mM Tris-HCl, pH 7.4, 50 mM NaCl, 2 mM EDTA, 1 mM PMSF and either 0.5% NP-40 or 0.5% deoxycholate. The suspensions were then spun at 1500 x g for 20 min and the supernatants collected. Sodium deoxycholate extracts were dialysed against 10 mM

phosphate buffer, pH 8.0, and 1 mM PMSF at room temperature for 3 h, and then at 4° C overnight. The extracts were lyophilised and stored dry at -20° C.

4.1.4. Preparation and characterisation of conjugated proteins

Many membrane proteins of many organisms have in recent years been shown to be anchored to membranes via inositol-containing glycolipids [11]. Recently [14] presented indirect evidence for such an anchor on several proteins of *E. tenella.*

- **Procedure**

- Unsporulated oocysts were harvested from chicken caeca 7 days after infection according to [31].

- Oocysts were sporulated at a concentration of $2x10^7$ oocysts per ml in PBS in the presence of penicillin (100 units per ml) and streptomycin (100 µg per ml) at 29° C with continual agitation for a period of 36 h.

- Sporozoites were prepared from sporulated oocysts by the method of [26] and [8].

- Unsporulated and sporulated oocysts ($5x10^7$ per ml) in water were vortexed with glass beads (3 mm diameter) for 5 min at room temperature.

- Sporozoites ($4x10^8$ per ml) were sonicated and all extracts were stored at -70° C.

- The extracts were phase separated [3], treated with phospholipase and glycolipid-anchored proteins were detected on Western blots with an antibody directed against the cross-reacting determinant (or GPI anchor) of *Trypanosoma brucei* variant surface glycoprotein.

4.2. Preparation and characterisation of virus-like particles

RNA molecules resembling viral RNA have been isolated from several *Eimeria* species. Some of these RNAs were shown to be particle-bound, but none of the particles has been demonstrated to be infectious.

Revets et al. [28] showed that *E. stiedai* contained virus-like particles with a non-segmented double stranded RNA of 6.5 kb length.

- **Procedure**

- Oocysts were isolated from livers and bile ducts of experimentally infected rabbits and sporulated in 2.5% potassium dichromate.

- Sporozoites were prepared from $6x10^8$ sporulated oocysts [2].

- Nucleic acids were isolated by a øCIA extraction, followed by a proteinase K treatment and an alcohol precipitation.

- For the preparation of virus-like particles (VLP), sporozoites were sonicated, cleared by centrifugation at 3,000 x g and 12,000 x g, and the final supernatant brought to a density of 1.37 per ml with CsCl.

- The fractions of the gradient containing the VLP were identified by the presence of ds RNA of 6.5 kb length.

- As a probe for blotting filters, 6.5 kb ds RNA was purified in 1% agarose gels [30] and 3' end labeled using [^{32}P]pCp and T4 RNA ligase.

Ellis and Revets [10] found ds RNA of similar sizes (5 to 6 kb) in *E. maxima* and *E. necatrix.*

- **Procedure**

- Sporozoites were lysed with 3% SDS, 50 mM Tris (pH 9.0), 100 mM EDTA and 100 µg/ml proteinase K.

- Following heat treatment at 65° C for 30 min, the supernatants of a 10 min 10,000 x g centrifugation were used for gel electrophoresis.

Evidence was found for a ds RNA of probable viral origin in *E. nieschulzi.* Two RNA species of about 6 kb and 7 kb lengths were observed [29, 33].

Procedure

- Sepp et al. [33] collected oocysts from rat faeces 7 to 10 days after infection.

- The oocysts were isolated and sporulated as described by [7]. Sporulated oocysts were surface sterilized by a 15 min exposure to 5.25% Na hypochlorite and purified by flotation in 1.15 M sucrose.

- They were extensively washed with Puck's saline A [27] and incubated in this solution for 30 min at 37° C.

- They were disrupted by vortexing with glass beads in 50 mM Tris (pH 7.6), 200 mM NaCl and 10 mM EDTA.

- For lysis 10^9 sporozoites per ml were incubated for 2 h at 37° C in 1% SDS and 100 µg per ml proteinase K.

- Nucleic acids were extracted with øCIA and CIA, and then precipitated twice with ethanol.

- The two presumptive viral ds RNAs were found to be quite stable to RNase A at high, but not at low salt concentrations.

Roditi [29] used a similar method to obtain RNase resistant RNA from crude lysates of *E. nieschulzi.*

- **Procedure**

- About 10^8 sporulated oocysts were washed in NTE (100 mM NaCl, 10 mM Tris-HCl, pH 7.5, 5 mMEDTA), resuspended in a minimal volume of the same buffer, mixed with an equal volume of glass beads and vortexed for 2 min.

- The lysate was recovered from the glass beads in 4 ml NTE and to this 200 µl of RNase (1 mg per ml) were added and the suspension incubated at 37° C for 1 h.

- To this, 4 ml polyethylene glycol solution (200 g PEG 6000, 145 g NaCl, 800 ml water) and 8 ml ethanol were added and the mixture kept on ice for at least 30 min.

- After a 10 min centrifugation at 2000 rpm in a Sorvall HB4 rotor the supernatant was discarded and the pellet washed once with 10 ml 75% ethanol.

- Following another centrifugation done under the same conditions as above, the pellet was taken up in 4.5 ml NTE, and 250 µl 10% SDS as well as 250 µl Pronase (20 mg per ml) were added and the mixture incubated at 37° C for 1 h.

- After one extraction each with øCIA and with CIA, the aqueous phase was mixed with 12.5 ml ethanol and nucleic acids were pelleted for 10 min in an SS34 Sorvall rotor run at 10,000 rpm.

- To remove any remaining DNA, the pellet was resuspended in 200 µl RQ buffer (40 mM Tris-HCl, pH 8.0, 10 mM NaCl, 6 mM $MgCl_2$) and 1 µl DNase was added. The incubation was done for 30 min at 37° C.

- Enzyme activity was destroyed by one extraction each with øCIA and with CIA. After adding 10 µl 2 M NaCl to the aqueous phase, the RNA was precipitated with 500 µl ethanol, pelleted and redissolved in 100 µl water. This was stored at -20° C.

The following procedure was used to prepare VLPs [29].

- **Procedure**

- $1x10^8$ sporulated oocysts were washed, broken with glass beads as described above, resuspended in 10 ml NTE and centrifuged for 10 min at 2000 rpm in a Sorvall HB4 rotor.

- To the supernatent Triton X100 was added to a final concentration of 0.1% and to 9 ml of this mixture 6.3 g CsCl were added.

- This was spun in an ultracentrifuge at 18° C and at 40,000 rpm for 4 h in the SW 50.1 rotor.

- From the two centrifuge tubes, corresponding 250 µl fractions were pooled and nucleic acids extracted from 100 µl aliquots from all 23 fractions.

- For RNA extractions the 100 µl aliquots were heated to 80° C to 90° C, supplemented with 400 µl NTE containing 0.5% SDS and were extracted with 500 µl phenol.

- After extraction with chloroform, the nucleic acids were taken up in 10 µl water and analysed in a 1% agarose gel. Those fractions containing the putative viral RNA were combined.

Interestingly, Sepp et al. [33] found in crude extracts of *E. nieschulzi* sporozoites an RNA-dependent RNA polymerase activity. This enzyme activity was able to label putative viral RNA in a similar way to how *Leishmania* viral RNA polymerase labelled the corresponding *Leishmania* RNA [43, 44].

4.3. Establishment and screening of cDNA and genomic libraries

Several laboratories have established gene libraries of different *Eimeria* species using cDNA or, less frequently, genomic DNA as starting materials.
Expression libraries have most frequently been screened with antibodies raised against strongly immunogenic antigens. The establishment, maintenance and expression of gene libraries has met with several difficulties. Some clones seem to be of limited stability only, perhaps because of unusual inverted or direct repeats, which may be eliminated from the bacterial hosts. Sequencing of many different *Eimeria* clones has shown in several different laboratories that this organism has very large amounts of simple repeats of the trinucleotide GCA (or TGC). Such repeat sequences are found both in intergenic regions, but also within regions coding for proteins. These repeats may make up a considerable fraction of the genome. Jenkins et al. [19] prepared and characterised a cDNA library form *Eimeria* and details are given below. Total RNA was isolated from a mixture of sporulated and unsporulated oocysts (for sporozoite mRNA) and from merozoites in CsTFA (Pharmacia) and banded by centrifugation [47].

- **Procedure**

- Poly(A)+ RNA was purified by passage over an oligo(dT) column (Promega) with yields of 12.5 mg of sporozoite mRNA and 11 mg of merozoite mRNA.

- cDNA was prepared, methylated, tailed with EcoRI linkers and ligated to the arms of lambda gt11.

- Phage was packaged in vitro yielding $4.1x10^6$ sporozoite and $2.4x10^6$ merozoite clones.

- Efficiencies of cloning were $3.3x10^5$ sporozoite clones/mg and $2.2x10^5$ merozoite cDNA clones/mg mRNA.

- The cDNA libraries were immunoscreened with a 10^{-2} dilution of sera from rabbits that had been immunised with *E. acervulina* sporozoite and merozoite extracts. The biotin / avidin system was used.

- Positive phage plaques were cloned and used to make lysogens. Insert DNA was cut out, purified by gel electrophoresis and cloned in pUC8 plasmid.

- From lysogens ß-galactosidase fusion proteins were prepared, purified by immunoaffinity chromatography and used to raise antibodies.

- The antibodies were used in Western blots to identify ^{125}I-labeled surface proteins of *E. acervulina* sporozoites and merozoites and to locate proteins by imunofluorescence.

Fried et al. [13] characterised an *E. maxima* gametocyte expression library with vectors pATH1 and pATH2 giving trpE fusion proteins.

4.4. Separation of organelles

In recent years particular attention has been paid in diverse *Eimeria* species to organelles likely to be involved in host-parasite interactions. Of specific interest are the rhoptries and the micronemes, whose contents are thought in all Apicomplexa to be requi-

red for the invasion of the vertebrate host cells and for the formation of parasitophorous vacuoles. Attempts at characterizing membranes have also been undertaken.

Dubremetz and Dissous [7] described a method for the isolation of dense granules and micronemes from *Sarcocystis tenella* that provided the blueprint for [21] for the isolation of micronemes and rhoptries from *E. tenella.*

- **Procedure for dense granules and micronemes from zoites of *Sarcocystis tenella***

All sucrose solutions contain 1 mM EDTA and 5 mM triethanolamine-HCl, pH 7.5.

- Resuspend purified zoites in homogenisation buffer (HM) (250mM sucrose, 1 mM EDTA, 5 mM triethanolamine-HCl, pH 7.5).

- Homogenise zoites in French pressure cell operated at 50 kg/cm^2.

- Centrifuge homogenate at 12,000 x g for 10 min in SW27 rotor. Collect pellet (P1) and supernatant (S1).

- For dense granules transfer pellet (P1) to Dounce homogeniser and resuspend in HM (1 ml for 10^9 cell equivalents) by five up and down strokes of a loose pestle.

- Adjust suspension to 1.6 M sucrose with 2.2 M sucrose, and layer into a discontinuous sucrose gradient (2.2 M: 4 ml; 1.8 M: 10 ml; 1.6 M: 5 ml sample; 1.4 M: 5 ml; 1.0 M: 10 ml, 0.25 M: 2 ml).

- Centrifuge for 1 h at 113,000 x g in SW 27 rotor.

- Collect material at each interphase and dilute with the same sucrose solution as the overlaying cushion.

- Centrifuge each fraction at 113,000 x g for 1 h (SW 40 rotor).

- Recover dense granules from the 1.8 - 2.2 M sucrose interphases.

- For micronemes, layer 8 ml portions of supernatant S1 onto 30 ml linear 1 - 1.4 M sucrose gradients at 72,000 x g for 30 min in SW 27 rotor.

- Remove upper 7.5 ml layer (contains bulk of ribosomes) and replace with HM buffer.

- Centrifuge for additional 3 h at 113,000 x g, then fractionate gradients.

- Dilute micronemes in the 1.4 M sucrose fraction with HM buffer, and sediment for 45 min in SW27 rotor at 72,000 x g.

- **Procedure for rhoptries and micronemes from *E. tenella* sporozoites**

- Resuspend sporozoites at 1 x 10^8/ml in cold HM. All subsequent steps are done at 4° C.

- Sonicate sporozoites and centrifuge at 700 x g for 10 min. Retain supernatant (S1) and discard pellet containing intact parasites and cell debris.

- Centrifuge supernatant S1 at 8,500 x g for 10 min (Sorvall RC-5B, SS34 rotor). Collect supernatant S2 and pellet P1.

- Resuspend pellet (P1) in HM and centrifuge at 5,500 x g for 5 min. Retain supernatant (S3).

- Adjust volume of both supernatants S2 and S3 to 6 ml with HM and layer onto linear 1.0 - 1.6 M sucrose gradients. Centrifuge at 58,000 x g for 30 min in SW40Ti rotor (Sorvall).

- Remove white layer from top of each sample, add more HM and recentrifuge at 70,00 x g for 2 h. Recover 1.4 M fractions from the gradient containing the micronemes.

- Recover pellets from both samples (P2 and P3).

- Combine pellets P2 and P3, resuspend in HM and layer on to a 6 ml pre-formed 1.9 - 2.5 M sucrose gradient and centrifuge at 62,000 x g for 2 h.

- Recover bands from the region around 2.3 M - containing rhoptries - and dilute with 4 volumes of HM. Centrifuge diluted fractions at 130,000 g for 45 min.

- Save pellet and resuspend pellets in 200 - 500 µl of HM.

4.5. PCR identification of species

As mentioned under 2.1.4, species identification of *Eimeria* was successfully performed on the basis of 5S rRNA sequences [38]. Details are described in section 3.2.

4.6. Conclusions

The relative ease with which large numbers of different life cycle stages can be isolated free from host debris, is an attractive feature of studies with eimerian parasites. Clean cultures of oocysts of all species can be recovered from the faeces in large quantities through simple salt flotation techniques, and thereafter they can be treated with strong bleach solutions to remove contaminating bacteria and other debris. Consequently, the isolation of nucleic acids representative of all species is relatively straightforward in the absence of serious technical problems. Zoite life cycle stages (sporozoites and merozoites), and even gametocytes of some species, have also been isolated virtually free from contaminating debris.

Of the avian coccidia, the species most amenable to molecular analysis is *E. tenella.* This parasite has a life cycle characterised by the production of large numbers of both oocysts and second generation merozoites. Merozoites appear relatively synchronously in gut contents and are capable of remaining viable for relatively long periods following their recovery. Up to 3 x 10^9merozoites can be recovered on a single occasion. Moreover, in experimental models, this species will also develop in chorioallantoic membrane of embryonating eggs and, to a limited extent, *in vitro.*

Oocysts and sporozoites of all species of *Eimeria* from many different hosts are also readily accessible as a source of DNA, although access to merozoites may be more restricted than with *E. tenella* as the schizonts may be smaller in size and contain fewer merozoites.

Whilst *E. tenella* continues to provide the principle model, substantial molecular studies have also been done with *E. acervulina* (for technical reasons and because of its

importance economically) and *E.maxima* (a species of high immunogenicity that provides a model for studies on molecules that might have potential value as vaccines).

Whilst the isolation of many eimerian reagents/molecules is straightforward *per se,* some major technical deficiencies are apparent in comparison to studies on other protozoa. For example, no substantial *in vitro* culture system has been developed and no gene(s) that confers the phenotype of drug-resistance has been characterised. If these were to become available it is possible that a transfection system could be devised to enable the full characterisation of some of the increasing number of molecules that are now being isolated.

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5. Techniques for detecting immune responses of avian hosts

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Infection of chickens with *Eimeria* induces the production of specific circulatory and secretory antibodies and a variety of techniques have been used for their detection including radioimmunoassay [16], radial immunodiffusion [3], immunofluorescence [1, 6, 7, 18, 19, 20], a modification of the Sabin-Feldman dye test [9], enzyme-linked immunosorbent assay (ELISA) [4, 8, 10, 11, 12, 13, 15, 17] and western blotting [5, 21, 22, 23]. In this chapter, protocols for the ELISA and western blotting techniques are presented as, in general, these are the most convenient and informative methods for the detection of humoral responses to *Eimeria.*

5.1. Detection of antibodies to antigens of chicken *Eimeria* by ELISA

- **Solutions and materials**

  - Phosphate Buffered Saline pH 7.2 (PBS) = 2.65g/l $NaH_2PO4.H_2O$ + 8.36g/l $Na_2HPO_4.2H_2O$ + 8.5g NaCl

  - ELISA Buffer I (pH 9.6) = 2.5g $NaHCO_3$ + 7.5g Na_2CO_3 + 0.2g NaN_3

  - ELISA Buffer II (pH 7.2) = 0.3% Tween 20 + 0.2g NaN_3 + 0.5g bovine haemoglobin in 1 l PBS.

  - ELISA Buffer III (pH 9.8) = 1.8g $NaHCO_3$ + 3.1g Na_2CO_3 + 0.2g $MgCl_2.6H_2O$ + 500ml H_2O.

  - Wash Buffer = 45g NaCl + 15ml Tween 20 in 5 l H_2O.

  - Substrate Solution (made immediately before use) = 1mg/ml p-nitrophenylphosphate (Sigma Alkaline Phosphatase Substrate 104 : catalogue no. 104.0) dissolved in ELISA Buffer III.

  - Stopping Solution = 3N NaOH.

- 2nd antibody - anti-chicken Ig conjugate to alkaline phosphatase : purchased from Bethyl Laboratories, P.O. Box 850, Montgomery, Texas, 77356, USA; FAX : ++1 409 597 6105. For anti-IgG the catalogue no. is A30-104AP. Working dilution is 1:2000. For anti-IgM and IgA, enquire.

- ELISA plates : Nunc-Immunoplate Maxisorp F96, catalogue no. 439454

- **Preparation of antigens**

- **Oocyst antigen**

- Mix an equal volume of oocysts and PBS and saturate with 1mm glass beads. Vortex at high speed until oocysts, sporocysts and sporozoites are completely ruptured (3-5 min). Check under microscope.

- Separate homogenised oocysts from glass beads and freeze/thaw 3 times with liquid nitrogen and warm water (37°C). After the third thaw, place homogenate on ice and sonicate at 20 watts for 20 seconds.

- Centrifuge homogenate at 12,000 x g for 5 min. Keep supernatant.

- **Merozoite antigen**

- For the preparation of merozoites, chickens are infected at 4 weeks of age (or older) with 200,000 sporulated oocysts and at 96 h post-infection the chickens are killed, their intestines or caeca removed, flushed with cold PBS, and slit open.

- The intestines or caeca are then cut into roughly 1 cm^2 pieces which are incubated for a maximum of 30 min at 40°C in Hank's medium (Gibco, pH 7.6) containing 0.025% trypsin (Difco), 1% taurocholic acid (Fluka) and 10 mM $MgCl_2$.

- The coarse intestinal debris is then removed by filtering through gauze and finer debris is removed by filtration through a 17 µm polymon mesh (Swiss Silk Bolting Cloth Manufacturing Company, Zurich). (This refers to *E. maxima*).

- The merozoites are centrifuged at 1000 x g for 10 min, the supernatant is discarded and the merozoites are resuspended in PBS.

- The merozoites are then filtered through a 10 µm mesh and the centrifugation and washing steps repeated 3 times.

- For *E. tenella,* the merozoites are purified by passage through a DEAE-52 column (Whatman). Merozoite extracts are prepared by suspending merozoites in PBS at 1-2 x 10^8/ml and freeze/thawing and sonication is done as described for the oocysts.

- **Gametocyte antigen**

- For preparation of gametocytes, chickens are infected with 10,000 oocysts (*E. maxima*) at 4 weeks of age (minimum). At 136-138 h p.i., the chickens are killed, the intestines removed and washed with cold SAC (170 mM NaCl, 10 mM Tris-HCl pH 7, 10 mM glucose, 5 mM $CaCl_2$, 1 mM PMSF, 1mg/ml BSA).

- One end of the intestine is then tied with string and the gut filled with 0.5 mg/ml hyaluronidase in SAC. The other end of the intestine is then tied and the filled gut is placed in 37°C PBS in a shaking water bath for 20 min. At the end of the incubation, the intestines are slit open and the contents discarded.

- The intestines are placed on top of a 17 µm mesh and the mucosa is washed through with SAC (room temperature). The material left on the mesh is discarded and the flow through is filtered through a 10 µm mesh.

- The gametocytes accumulate on the filter, are washed off with SAC and centrifuged at 800 x g for 5 min (one 30 x 30 cm mesh is sufficient for 5 intestines). Gametocytes are extracted with 0.5% deoxycholate in DEB (10mM Tris-HCl pH 7.4, 50 mM NaCl, 2 mM EDTA, 1 mM PMSF) for 30 min (room temperature, 2x10^6 gametocytes per ml).

- The parasites are then spun at 1500 x g for 20 min and the supernatant is collected and dialysed against 10mM phosphate buffer (pH 8) plus 1mM PMSF at room temperature for 3 h and then at 4°C overnight. The extract is then lyophilised and stored desiccated at -20°C.

- **Determination of protein concentration of antigen**

- Dilute Biorad protein assay reagent (catalogue no. 500-0006) 1:5 with H_2O.

- Prepare protein standards using bovine serum albumin at concentrations of 0, 0.2, 0.4, 0.6, 0.8 and 1.0 mg/ml in PBS.

- To 2.5 ml of diluted protein assay reagent add 0.05 ml of protein standards or diluted (1:5 and 1:10 in PBS) antigen preparation.

- Mix and leave for between 2 and 30 min. Read absorbance at 595 nm. Plot standard curve on graph paper and determine the protein concentration of the antigen preparation from the graph.

- **Serum and egg yolk samples**

Dilute serum or egg yolk samples using ELISA Buffer II. Normally, serum is diluted 1:100 and yolk 1:500, but this can be varied.

- **ELISA**

  - **Procedure**

  - Dilute antigen preparation with ELISA Buffer I to a concentration of 5 µg/ml.
  - Add 100 µl of diluted antigen to each well (that is, 0.5 µg/well).
  - Leave covered overnight at 4°C.
  - Wash 3 times with Wash Buffer.
  - Fill in protocol sheet to show positioning of samples.
  - Add 100 µls of diluted sera or egg yolk to appropriate wells.
  - Incubate at 40°C for 90-120 min.
  - Wash 3 times with wash buffer.
  - Dilute 2nd antibody in ELISA buffer II (1:2000 for anti-IgG).
  - Add 100 µl of diluted 2nd antibody to each well.
  - Incubate at 40°C for 90-120 min.
  - Wash 3 times with wash buffer.

- Dissolve Sigma alkaline phosphatase substrate 104 with ELISA Buffer III at 1 mg/ml.
- Add 100 µl of substrate solution to each well.
- Incubate at 37°C for 30 min.
- Stop reaction by addition of 50 µls stopping solution (3N NaOH) to each well.
- Pop air bubbles with a sharp point.
- Read absorbance in an automatic ELISA plate reader at 405 nm.

5.2. Detection of antibodies to antigens of chicken *Eimeria* by Western Blotting (WB)

A. Sodium dodecylsulphate (SDS) - polyacrylamide gel electrophoresis (PAGE)

- **Solutions**
- Acrylamide stock solution = 30 g/100 ml acrylamide + 0.8g/100 ml bisacrylamide. (**Note** that non-polymerised acrylamide dust is extremely dangerous. Wear gloves and a mask, handle with extreme care).
- Resolving gel buffer = 182 g/l Tris-HCl (pH 8.8)+ 4 g/l SDS.
- Stacking gel buffer = 60 g/l Tris-HCl (pH 6.8) + 4 g/l SDS.
- Ammonium persulphate : 10 g/100ml (make freshly when required).
- TEMED (commercially available in ready to use form, e.g. Sigma).
- Running buffer = 3 g/l Tris-HCl (pH 6.8) + 14g/l glycine + 0.1 g/l SDS.
- Sample buffer = 1 ml glycerol + 0.5 ml β-mercaptoethanol + 1 g SDS + 0.01 g bromophenol blue; make up to 10 ml with water.

- **Antigen preparation**

  Crude antigens are prepared as described for ELISA but then diluted 1:1 with sample buffer, heated for 3 min in boiling water and briefly centrifuged in a microfuge just prior to loading on the gel.

- **Gel composition**

a. **Resolving gel.** The percentage acrylamide varies with the size of proteins which one wishes to analyse. In general, if good separation of small proteins is desired a higher percentage of acrylamide should be incorporated into the gel.

Tab. 17: Composition of resolving gel

	Volumes in ml:				
% Acrylamide	Resolving gel buffer	Acrylamide stock	water	ammonium persulphate	TEMED
7.5	9.0	9.0	18.0	0.1	0.02
10	9.0	12.0	15.0	0.1	0.02
11	9.0	13.5	13.5	0.1	0.02
15	9.0	18	9.0	0.1	0.02

b. Stacking gel

Tab. 18: Composition of stacking gel

	Volumes in ml:				
% Acrylamide	Stacking gel buffer	Acrylamide stock	water	ammonium persulphate	TEMED
11	2.5	1.5	6.5	0.3	0.01

- **Gel casting and running**
- Thoroughly clean glass plates (20 cm x 20 cm, 6 mm thick) with absolute ethanol.
- Place one glass plate on a level surface, position spacers on 3 sides.
- Cover with a second glass plate and seal edges with boiling agar (2 g/100 ml).
- Allow agar to cool and set then recheck that plates are level.
- Pour in resolving gel of desired acrylamide concentration with a pipette.

- Overlay gel with water or butanol to exclude air and encourage polymerisation and to provide a straight interface between the resolving gel and the stacking gel.
- After polymerisation (about 60 min) pour off overlay solution and blot dry.
- Pour stacking gel on top of the resolving gel.
- Insert sample comb taking care to avoid air bubbles.
- When stacking gel has polymerised remove the bottom spacer.
- Transfer gel (still within glass plates) to electrophoresis apparatus filled with enough running buffer to submerge the bottom.
- Remove any bubbles along the bottom of the gel with a bent needle and syringe.
- Remove comb from the top.
- Rinse sample wells with running buffer and submerge the top end of the gel in running buffer.
- Load samples with a Hamilton syringe or micropipette.
- Run gel at 60-70V overnight.
- Switch off apparatus and remove gel (still in glass plates).
- Remove spacer from one side and use this spacer to carefully separate glass plates.
- Transfer gel (without glass plates) to western blotting solution (see below).

B. Western blotting and immunodetection

- **Solutions**
- Western blotting buffer = 2.4 g/l Tris-HCl (pH 8.5) + 11.3 g/l glycine + 200 ml methanol.
- Blocking buffer = PBS (pH 7.2) + 2.5% foetal calf serum + 2.5% skim milk powder.

Procedure for western blotting

- Soak gel and a piece of nitrocellulose paper cut to the same size as the gel in western blotting buffer.
- Immerse buffer pads from blotting apparatus plus 2-3 pieces of Whatman 3MM paper in western blotting buffer. Remove air bubbles.
- Place gel on 3MM paper and cover with wet nitrocellulose. Remove air bubbles.
- Cover with 2-3 more pieces of Whatman 3MM paper plus more buffer pads.
- Transfer to blotting apparatus.
- Run at 30V overnight.
- Switch off apparatus and remove nitrocellulose.
- Wash briefly in PBS. (If desired, allow nitrocellulose to dry and cut into strips).

- Incubate in blocking buffer at room temperature for 60 min with shaking.
- Remove blocking buffer.
- Dilute serum or egg yolk samples in blocking buffer, add to nitrocellulose, incubate with shaking for 120 min at room temperature.
- Wash 4 times with blocking buffer diluted 1:4 with PBS.
- Dilute anti-chicken Ig conjugated to horseradish peroxidase (Bethyl Laboratories) in diluted (1:4) blocking buffer for 120 min.
- Wash 2 times with diluted (1:4) blocking buffer and 2 times with PBS.
- Add substrate (50 mg/100 ml diaminobenzidine, 0.03 ml/100 ml hydrogen peroxide in PBS)
- Allow colour to develop for at least 1 min and not more than about 30 min (judged by eye for each sample).
- Stop reaction by rinsing nitrocellulose in water.

5.3. Protocol for chicken lymphocyte proliferation assay

Many studies of lymphocyte proliferation have been conducted using chicken lymphocytes yet, until recently, no effort has been made to standardise the conditions required for the reproducible use of the technique. In the last two years, two independent studies [2, 14] have reached remarkably similar conclusions regarding optimum conditions for assessing chicken lymphocyte proliferation *in vitro*. The protocol presented here represents an amalgam of these two studies with minimal discussion of possible variations. A review of alternative conditions can be found in [2] and [14]. Because of wide variations in the responses of individual chickens, including inbred lines, it is recommended that as many chickens as possible be tested simultaneously and certainly not less than 6-8 per experimental group. Cultivation of the cells should be established and maintained under sterile conditions.

- **Lymphocyte isolation**

The best method for isolating chicken lymphocytes appears to be by slow centrifugation of peripheral blood. Isolation of chicken lymphocytes using separation media such as Ficoll does not appear to be a good alternative since cells isolated using such media respond relatively poorly to mitogens *in vitro*. Spleen lymphocytes should be avoided as results with this population are extremely variable and, at present, good methods for isolating intestinal lymphocytes for use in culture have not been published, though ultimately these would be the cells of choice for studying lymphocyte responses to *Eimeria*. A further advantage of peripheral blood cells is that they are easy to collect

(from the wing vein) and the collection procedure may be repeated every 3-4 days so that time course studies may be performed with the same birds.

- **Procedure for isolation of blood lymphocytes**

- Collect blood by puncture of the wing vein with a narrow gauge needle fitted to a 5 ml syringe. (Use birds of at least 6 weeks of age to make blood collection easier).
- Expel blood from syringe into a sterile tube containing 20 units of heparin per ml blood.
- Centrifuge blood at 40 x g for 10 min.
- Collect lymphocytes as the buffy coat layer with plasma.
- To increase yield of lymphocytes, resuspend red cell pellet in 1/5th volume of RPMI-1640 (including 2 mM L-glutamine and 100 units per ml penicillin and 100 µg per ml streptomycin, Gibco) and again centrifuge at 40 x g for 10 min.
- Add second buffy coat to first.
- Sediment lymphocytes by centrifugation at 250 x g for 15 min.
- Discard supernatant.
- Resuspend cells in RPMI-1640.
- Centrifuge at 250 x g for 15 min.
- Discard supernatant.
- Resuspend cells in RPMI-1640.
- Centrifuge at 250 x g for 15 min.
- Discard supernatant.
- Resuspend cells in RPMI-1640.
- Take a 10 µl sample, add to 90 µl of 0.06% trypan blue in 0.15M NaCl plus 2.5% foetal calf serum (FCS), count viable cells in a haemocytometer and calculate total cell number.
- Centrifuge at 250 x g for 15 min.
- Adjust final cell concentration to 10^7 per ml.

- **Lymphocyte cultivation**

- To triplicate wells of 96-well round-bottomed sterile culture plates add :

a) 5 µl of concanavilin A at a variety of concentrations between 10 and 200 µg per ml in RPMI-1640 (a range of concentrations is needed as bird-to-bird responsiveness is extremely variable and the inclusion of the optimum concentration must be

ensured as a positive control for the assay), 5 µl of parasite antigen in RPMI-1640 (again a range of concentrations should be tested) or 5 µl of RPMI-1640;

b) 85 µl of RPMI-1640;

c) 10 µl of FCS;

d) 100 µl of washed buffy coat cells in RPMI-1640, containing 10^7 cells per ml.

- Incubate culture plates at 40°C in 5% CO_2 in humidified air for 24 h.
- Add 1 µCi ^{3}H-thymidine in 5 µl RPMI-1640 to each well.
- Incubate a further 16 h.
- Harvest cells using a commercial cell harvester (eg LKB Wallac, Finland) onto glass fibre filters as per the manufacturers instructions.
- Dry filters.
- Add scintillation fluid as directed by manufacturer.
- Count radioactive emissions in a scintillation counter.
- Express results as counts per minute (cpm) or as the stimulation index (SI = cpm of stimulated cells/cpm of unstimulated cells).

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6. Antigens and immunisation procedures in the development of vaccines against poultry coccidiosis

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6.1. Introduction

Considerable progress has been achieved during the last few years in the development of vaccines against poultry coccidiosis. One important step was the development of live vaccines based on attenuated strains of *Eimeria* species, and their introduction to the market in several European countries [5, 38]. Crucial factors in the development of vaccines include the antigens used and the methods by which they are administered. Some information related to this field is briefly outlined in this chapter.

6.2. Induction of immunity

Immune responses against infection with *Eimeria spp.* can be induced by various types of antigens, namely:

- Viable parasites (sporozoites within oocysts/sporocysts and merozoites);
- Nonviable antigens prepared from different developmental stages;
- Recombinant antigens;
- Cell-culture derived antigens.

Protective immunity can be induced by presentation of live antigens, but non-viable antigens investigated so far produced only very limited or no immunity.

An alternate approach to vaccination of young chickens ist the transfer of maternal antibodies from the laying hen via the egg. This immunisation procedure is discussed in section 6.7. of this chapter.

For the induction of an adequate degree of immunity after antigen application, the age of the host plays an important role. For example, the same inoculum of sporulated oocysts may induce a higher degree of immunity in older animals as compared to young chickens [33, 37]. One-day-old chickens can be infected with oocysts, but a high proportion of them may pass unchanged through the digestive tract. For this reason,

chickens older than one week of age should be used for experimental infections or as the targets of live vaccines.

6.3. Immunisation with viable (live) antigens

At present immunisation of chickens under field conditions can only be achieved by the administration of viable parasites. All vaccines currently used in practice are based on viable oocysts (Tab. 19). Other *Eimeria* stages (isolated sporozoites, merozoites, gametocytes) may infect chickens and induce immune responses but their use for vaccination is still experimental.

6.3.1. Viable oocyst vaccines

Sporulated oocysts derived from wild-type virulent strains or attenuated strains of *Eimeria* are used.

- **Virulent oocysts**

Some vaccines, such as Coccivac® or Immucox® (Tab.19), contain a mixture of sporulated oocysts derived from wild-type virulent *Eimeria* species. The use of such vaccines under field conditions always poses a risk that they may induce outbreaks of clinical coccidiosis which then have to be controlled by chemotherapy. Therefore, the use of attenuated strains is generally preferred as being safer.

- **Irradiated oocysts**

Exposure of sporulated *Eimeria* oocysts to x-ray or Co-ray irradiation (= gamma irradiation) at doses of about 250 Gy reduces pathogenicity, but immunogenicity is maintained [1, 11, 41]. This effect is dose dependent. Doses < 100 Gy do not cause attenuation, and doses > 500 Gy abolish immunogenicity. Results from older studies had suggested that irradiation does not induce attenuation but kills a proportion of the oocysts and thus reduces the number of infective forms which makes the vaccine safer. However, recent studies have shown that sporozoites of *E. acervulina*, *E. maxima* and *E. tenella* released from gamma - irradiated oocysts were not only able to infect host cells but also had a limited asexual multiplication resulting in development of immunity [18-20, 41].

Tab. 19: Commercial live oocyst vaccines against coccidiosis in chickens (modified after 47)

Vaccine	Manufacturer	Antigens	Application	Age of chickens at vaccination (days)	First registration
Coccivac®	Sterwin Lab. USA	Mixture of virulent *Eimeria* species	Drinking water 1 dose	4 - 14	1952/USA
Immucox®	Vetech Lab. Canada	Virulent *E. acervulina*, *E. maxima*, *E. tenella*, *E. necatrix*	Drinking water 2 doses	4 and 7	1985/Canada
VAC M®	Elanco Products Company, USA	Virulent *E. maxima*	Drinking water doses: no data	no data	1989/USA
Paracox®	Mallinckrodt Veterinary Ltd.	Precocious strains of *E. acervulina*, *E. brunetti*, *E. maxima*, *E. mitis*, *E. necatrix* *E. praecox*, *E. tenella*	Drinking water 1 dose	5 - 9	1992/UK
Livacox® D [1)]	Biopharm, Research Institute of Biopharmacy and Veterinary Drugs. Czech Republic	Attenuated strains of *E. acervulina*, *E. tenella*	Drinking water 1 dose	7 - 10	1992/CR
Livacox® T [2)]		*E. acervulina*, *E. maxima*, *E. tenella*	Drinking water 1 dose	7 - 10	1992/CR

[1)] For vaccination of caged chickens; [2)] For vaccination of chickens on litter.

The results of extensive studies have demonstrated that irradiated oocysts of all *Eimeria* species of chickens - when applied in adequate doses - induced solid immunity 14 to 21 days after vaccination of the animals which on challenge tolerated high doses of virulent homologous oocysts [14, 24]. Such vaccines have been used successfully under field conditions. Trials using irradiated oocysts have also been carried out with mammalian *Eimeria* species, for example with *E. bovis* [47] and *E. zuernii* [30].

- **Oocysts derived from biologically attenuated *Eimeria* strains**

The term "biological attenuation" is used for various biological methods by which an avirulent but immunogenic strain can be isolated from a virulent parental *Eimeria* strain. So far, attenuation of *Eimeria* species to low virulence without marked loss of immunogenicity has been achieved either by selection for "precociousness" (see below) or by serial passage in chicken embryos.

Embryo adaptation: Strains of *E. brunetti, E. mitis, E. necatrix, E. tenella* were adapted for development in chicken embryos (see chapter 2.2) [17]. These strains differ from their parental forms in certain features, such as smaller numbers of schizonts and/or merozoites. Following long-term, continuous embryo passages, the virulence of the egg-adapted strains can be substantially diminished.

Precocious *Eimeria* strains: When oocysts excreted at the beginning of the prepatent period are repeatedly used for subsequent infections of chickens, the prepatent period of the parasites is shortened. By sequential selection in this way, strains were obtained with prepatent periods 12 - 40 h shorter than those of the parental lines [16]. These strains were denominated as "precocious" strains (= lines). They are characterised by smaller and/or fewer generations of schizogony and hence lower virulence and reproductive potentials. Attenuation of virulence is stable for at least five passages in absence of further selection pressure. Precocious strains have been selected from parental *Eimeria* species from chickens (*E. acervulina, E. brunetti, E. maxima, E. mitis, E. necatrix, E. praecox and E. tenella*) and several *Eimeria* species from rabbits (see chapter 1.4.).

Laboratory and field trials have clearly demonstrated that monovalent or polyvalent vaccines containing precocious strains of chicken *Eimeria* species are safe and effective in preventing coccidiosis in chickens. Two vaccines using biologically attenuated *Eimeria* strains have recently been marketed (Paracox® [46] and Livacox® [2], Tab. 19).

6.3.2. Application of oocysts

Various possibilities exist for the application of oocysts to infect or vaccinate chickens under experimental or field conditions. The most reliable way of administering oocysts to chickens under field conditions is *via* their drinking water.

- **Application to the crop by tube**

Oocysts may be applied for experimental purposes to the crop by a 1 or 2 ml pipette or syringe in a maximum of 2 ml fluid. When oocysts are given to hungry chickens the subsequent oocyst production is usually lower than in chickens which had been infected when their crops were filled. The immunological response might be influenced by this phenomenon.

- **Application via feed**

Application of oocysts *via* feed gives the most homogenous and reliable infection rates in chickens [25]. Oocysts given as a single dose produce a significantly lower level of immunity than the application of the same number of oocysts given daily in small numbers as a so-called "trickle infection" during several days or weeks [23].
Based on this finding, the administration of small numbers of oocysts *via* the feed was evaluated for vaccination. However, this method of presentation is unsuitable for routine vaccination for several reasons including the relatively quick desiccation and destruction of oocysts in dry poultry feed. In trials, the maximum viability of *E. tenella* oocysts was 2-3 weeks. Prevention of desiccation by encapsulation of oocysts in alginate beads was not sufficient. Neither did coating of these alginate beads with triglycerides prevented destruction of oocysts due to desiccation [2], nor were beads made from a fat/water emulsion any more suitable for practical application [32]. Apart from the above mentioned problem of desiccation of oocysts in dry feed, other practical obstacles also hindered their delivery *via* feed, namely the risk of oocysts killed by low temperatures in silos during the winter or by heat during the summer.

- **Application via drinking water**

This way of delivery of oocysts is recommended for routine vaccination against coccidiosis of poultry by all producers of live vaccines (Coccivac®, Immucox®, Paracox®, Livacox®) (Tab. 19). Oocysts are suspended in a small amount of water which is given to chickens after leaving them for some time without drinking water. Some producers

use preparations which delay the sedimentation of oocysts [28], however, according to our experience [2] the sedimentation of oocysts in drinkers during the first two hours is slow. Application of the vaccine Livacox® through a central watering system did not reveal a special loss of oocysts during water delivery.

- **Application by spraying on litter and feed**

This way of application of oocysts has also been examined [39]. However, protection of chickens achieved by this system was lower than when the same number of oocysts was delivered *via* feed or drinking water primarily due to heavy losses of oocysts [4].

- **Parenteral administration**

Experimentally, oocysts were given by i.v., i.m., i.p. routes [7, 8]. In all of these cases the infection took place in the predilection sites of intestines, but its intensity was lower than when the same number of oocysts was given *per os*. The only advantage of this approach of oocyst delivery for experimental purposes is to avoid the direct stimulation of the mucosal immune system [10].

6.3.3. Viable sporozoite antigens

In vitro excysted sporozoites, when stored at ca. + 4°C, maintain their viability for approximately 2 weeks. When stored in liquid nitrogen, their viability is practically unlimited. To achieve a patent infection with these sporozoites it is necessary to give them to the animals parenterally, usually i.p., i.m., i.v. or per cloacam but not per os [31, 40]. The infection takes place in the usual specific sites of the intestine except in *E. necatrix*. Sporozoites of *E. necatrix*, injected directly into the caecal lumina, complete the entire life cycle in the caecal epithelium without asexual development in the small intestine [15]. One example of cloacal application of sporozoites was described by [40]: One day old chickens were immunised with a dose of 500 sporozoites of *E. tenella* by cloacal application. The challenge after 21 days of vaccination was more or less completely tolerated. The protective effect remained for a long period, i.e. up to 11 weeks.

A phenomenon of immunotolerance has been observed when 10-day old chicken embryos were inoculated with sporozoites of *E. tenella*. When chickens derived from these embryos were later inoculated with oocysts of *E. tenella* a hyperacute form of coccidiosis occurred due to immunotolerance, i.e. the inoculated chickens did not activate defence mechanisms against the antigen they had already seen [3, 19].

However, when 18-days-old embryos were inoculated with a recombinant antigen derived from *E. tenella* and cloned in *Escherichia coli*, the severity of coccidiosis in chickens derived from these embryos was significantly reduced following challenge with *E. tenella* oocysts, but a humoral response did occur [12].

Sporozoites injected to non-specific host animals may survive for about 7 days in their organs [35].

6.3.4. Viable merozoite antigens

Viability of merozoites harvested from the intestines of infected animals is generally very short. They are not able to survive 24 h and must therefore be inoculated parenterally immediately after harvesting. In the case of *E. necatrix* the inoculation of 2nd generation merozoites per cloacam is used for the separation of this species from the mixed infections with *E. tenella* [8]. Merozoites of *E. bovis* obtained from scraping the intestinal mucosa of a calf killed 15 days after oocysts inoculation stimulated the development of immunity when inoculated to the ligated caecum of another calf [13].

6.4. Nonviable *Eimeria* antigens

In recent years the antigen composition of various *Eimeria* species has been studied to some extent using modern techniques of protein chemistry and molecular biology. It is now known that *Eimeria* species contain a broad spectrum of protein antigens which may be specific for certain species, strains or stages, or nonspecific and shared with other *Eimeria* species/stages or even with organisms belonging to other genera [47]. Considerable knowledge has accumulated on surface antigens from sporozoites and merozoites, on microneme and rhoptry antigens, and on antigens derived from amylopectin granules, refractile bodies and the cytoplasma [47]. Among others, a glutamin-rich cytoplasmic antigen of *E. tenella* was detected which has a great degree of homology with an antigen of *E. bovis*. A monoclonal antibody against this antigen de-

rived from *E. tenella* inhibits the development of the parasite *in vitro*. Such shared antigens are of special interest for the development of vaccines against different *Eimeria* species [47].

Results of several studies showed that nonviable antigens prepared from different developmental stages of coccidia (unsporulated or sporulated oocysts, 2nd generation merozoites applied with or without Freund's Complete Adjuvant) did not induce protection against challenge [31]. Administration of such antigens results in production of antibodies, which seem to be of little relevance in protective immunity against *Eimeria* species (see below 6.7.).

6.5. Recombinant antigens

Intensive studies on identification of antigens capable of stimulating protective immunity have been carried out during the last ten years [21, 26, 43, 47]. When promising antigens were found, the corresponding genes were identified by immunoscreening of cDNA libraries prepared from the respective developmental stages of the parasite. However, to be effective in stimulating protective immunity, epitopes of the recombinant proteins need to be expressed in a configuration similar to those found on the native parasite antigens. Therefore, special attention should be paid to the antigen delivery vector. The recombinant antigens may require some other post-translational processing, e.g. glycosylation, or insertion into the host cell membrane to elicit stimulation of host immune cells. For this purpose, antigen delivery vectors have been investigated, such as viruses or bacteria, that are capable of expressing the recombinant coccidian antigens and invading the host intestinal epithelium [22]. The organisms used as vectors include, for example *Escherichia coli, Salmonella gallinarum, Fowlpox virus, Baculovirus*, etc. The corresponding genes are encoded in plasmids which are inserted in different microorganisms [21, 26, 43].

6.6. *Eimeria* cell culture antigens

CLARE et al. [6] tried to immunise chickens by subcutaneous application of culture medium derived from cell cultures inoculated with *E. tenella* sporozoites. This antigen was also given with an immunoadjuvant. From the results presented it seems, however, that rather than a process of strong infection a process of immunisation took place after the infection with 33 x 10^3 oocysts of *E. tenella*.

6.7. Immune mechanisms

It is generally accepted that immunoprotection in chickens against *Eimeria* species is dependent mainly on cell-mediated mechanisms stimulated by asexual stages and that antibodies play only a minor role.

6.7.1. Cell-mediated immunity

Relevant aspects of cell-mediated immunity in chicken coccidiosis have recently been reviewed by Lillehoj [29] and Zahner et al. [47]. It appears that $CD8^+$ and $CD4^+$ cells and other factors are involved, but many details are still unknown [29, 37].

Delayed hypersensitivity (DH) is generally considered as one of the methods appropriate for the detection of cell-mediated immunity (further techniques see part I, 5). In animals infected with coccidia DH can be demonstrated. Antigens prepared from unsporulated *E. stiedai* [27] or sporulated *E. tenella* [36, 42] oocysts or from sporozoites and merozoites were used in some DH experiments.
In such studies antigens are injected intradermally to rabbits on both sides of the body or into the wattle of chickens. PBS is injected in the same way as a control. Readings are made usually 20 min, 4, 24 and 48 h after antigen injection. Erythema, edema and induration are registered and skin or wattle thickness is measured.

6.7.2. Antibodies

Sera from immune animals can be concentrated by lyophylisation and parenterally injected to host animals. Immune serum injected i.v. or s.c. to calves did not induce protection against subsequent peroral infection with oocysts of *E. bovis* [10]. Antibodies against *E. maxima* derived from completely immune chickens provided partial protection in naive animals (reduction of oocyst production) only when injected in large quantities (1 ml) one day before and during the time of oral infection with oocysts [34, 35]. IgA-mediated inhibition of cell penetration of *E. tenella* sporozoites was demonstrated [9]. Recently [44, 45] it was found that 56- and 82-kDa antigens from gametocytes of *E. maxima* can be used for immunisation of laying hens which then transfer large quantities of maternal antibodies via the eggs to chickens which exhibit a high degree of protection against homologous peroral challenge infection.

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7. Guidelines for evaluating the efficacy and safety in chickens of live vaccines against coccidiosis and recommendations for registration

P. Bedrnik, P. Yvoré, Th. Hiepe, D. Mielke, and U. Drössigk

7.1. Introduction

Any live vaccine against coccidiosis in chickens has to fulfil the following basic criteria and should:

- be effective in stimulating protective immunity in vaccinated chickens against all economically important *Eimeria* species;
- be safe for the target-host animal species and for non-target animal or human hosts. It should not produce unacceptable residues in meat, eggs or other food products derived from animals (host-safety);
- not represent an environmental hazard (environmental safety);
- ideally contain parasites of low virulence, either naturally occurring, or derived by attenuation. Attenuation to low virulence must be stable for at least 5 passages through chickens without selection pressure;
- contain parasites of sufficient viability in order to allow storage for reasonable periods of time and distribution to the user.

For evaluating the efficacy and safety of live, attenuated vaccines against coccidia in chickens some specific aspects are discussed here, but definitive recommendations cannot be given. All steps of efficacy and safety evaluation should be carried out according to regulations of „Good Laboratory Practice" (GLP), to national/international legislation for vaccine registration and to Animal Protection Legislation. For countries of the European Union general guidelines for the evaluation of vaccines have been published in 1992 [1].

7.2. Efficacy evaluation

7.2.1. General conditions

- **Animals**

Chickens used in trials with live vaccines should not be younger than 6 days and in no case older than 3 weeks. Attention should be paid to the fact that weight gain and feed consumption of male and female animals are different. Therefore, in battery trials at least 10 birds of the same sex should form an experimental group. In floor-pen trials chickens of broiler type have to be used. When 20 or more birds are allocated to a group in floor-pen trials groups may comprise both males and females in a 1:1. ratio.

- **Vaccination**

Vaccination should be done either individually in battery trials or in such a way which will be recommended for field application. The best mode of application is *via* the drinking water. The oocysts may be mixed in a small amount of water and offered to chickens after they have been deprived of water for 2 h.

- **Challenge**

Challenge infection with laboratory strains should not be performed earlier than 14 days after vaccination. The doses of virulent oocysts should be reasonably high and determined n the basis of the pathogenicity and reproductive capacity of a given *Eimeria* species or strain. They should normally cause a certain degree of disease in control groups. Excessively high doses of oocysts can give misleading results. Graduated challenge doses are only reasonable when the capability of the vaccine is being examined to prevent infections of different degrees of severity.

7. 2.2. Trials

Various types of trials are recommended for efficacy evaluation of a vaccine, namely: battery trials, floor-pen trials and field trials.

7.2.2.1. Battery trials

Battery trials should provide basic information on the efficacy (and target-host safety) of a vaccine.

Procedure for battery trials	
• **Animals:**	Chickens of one sex, at least 6 days old at the beginning of the trial.
• **Maintenance and feeding:**	From day 1 in cages in an isolated room under coccidia-free conditions. Feed supplemented with efficient anticoccidial drug until two days before initiation of the trial. From this day on: non-medicated food.
• **Animal groups:**	Total number of not less than 120 chickens subdivided into the following 4 groups of each 30 (= 3 x 10) animals: • Group. 1: Vaccinated and challenged. • Group 2: Non-vaccinated and challenged. • Group 3: Non-vaccinated on feed medicated with an efficient anticoccidial drug and challenged. • Group 4: Non-vaccinated, non-medicated and non-challenged. **Note:** More groups of type No. 1 have to be added if more than one dose of the vaccine have to be tested.
• **Application of vaccine and challenge oocysts:**	Individually by tube to the crop.
• **Challenge:**	14 days after vaccination (= 21 days of age of the chickens) with virulent sporulated oocysts from all *Eimeria* species which are present in the vaccine (see above: challenge).
• **Duration of trial:**	29 days

Efficacy parameters for battery trials

The following parameters are used for efficacy evaluation in battery trials:

- Mortality rate per group (total mortality and that caused by coccidiosis). Mortality caused by coccidiosis in vaccinated and challenged groups has to be zero.

Necropsy, individual oocyst counts from rectal faeces (day 6 p.i.) and intestinal lesion score (mean 2 or lower) (see chapter 5 and [6]) of each 10 birds per group at the following intervals: (a) 5 days after vaccination , (b) 5 days after challenge, (c) at the end of the trial. After vaccination the output of oocysts of each species used for the vaccination should be observed. After the challenge the output of oocysts in vaccinated groups should be reduced by nearly 90%.

- Individual body weights on days 1, 3, 5 and 8 after vaccination and on days 5 and 8 after challenge.

- Feed conversion rate between days: 1 - 8; 8 - 21; 21 - 26; and 1- 29 (total conversion).

  Calculation of feed conversion:

  $$\frac{\text{total weight (kg) of feed consumed by the birds of a group during a given period}}{\text{total weight gain (kg) of the birds of the same group during a given period (including weight gains of birds which died during the given period).}}$$

7.2.2.2. Floor-pen trials

Floor-pen trials are designed to evaluate the efficacy and safety of the vaccine under simulated field conditions in which circulation of oocysts between the animals and the environment causes a higher infection pressure than in battery trials [3, 7].

Procedure for floor-pen trials	
• **Animals:**	One-day-old broiler type chickens of both sexes (ratio 1:1).
• **Maintenance and feeding:**	Floor-pens in the same environmentally controlled room. Each group should consist of 2 to 6 floor-pens, each with at least 20-30 chickens. Maximum of 18 - 20 chickens per m^2. Feeding of chickens in vaccinated group: diet free of anticoccidial drug since 1st day of life; chickens in the medicated group: diet supplemented with efficient anticoccidial drug.
• **Animal groups:**	Floor-pen groups with a total number of up to 120 chickens subdivided into 2-4 pens of each 20-30 animals: • Group 1: Vaccinated and challenged. • Group 2: Non-vaccinated and challenged. Group 3: Non-vaccinated on medicated feed with a highly efficient anticoccidial drug and challenged. • Group 4: Non-vaccinated, unmedicated and non-challenged. **Note**: More groups of type 1 have to be added if more than one dose of the vaccine have to be tested.
• **Application of vaccine:**	Vaccine application according to recommendation of manufacturer, challenge: via feed or drinking water.
• **Challenge:**	14 days after vaccine application: mixture of virulent *Eimeria* species which are also present in the vaccine. (Doses of each species: see 7.2.1., challenge).
• **Duration of trial:**	42-45 days

Efficacy parameters for floor-pen trials

- Mortality rates in all groups (total mortality and that caused by coccidiosis).
- Lesion score at necropsy on day 5 post challenge in 10 chickens per group.
- Oocyst counts on days 6-8 after challenge from faecal samples collected from each pen.
- Individual body weights on day 1 of the trial and on days 3, 5 and 7 after challenge and at the end of the trial.
- Feed conversion at the same intervals as body weights.
- Titres of circulating antibodies [2, 9] against *E. tenella*-antigens 14 days after the date of vaccination in all groups 1-3 by IFAT or ELISA (see: chapter 5).

7.2.2.3. Field trials

Field trials should be performed either in broilers or in pullets depending on the type of birds in which the respective vaccine is intended for use. In contrast to the battery and floor-pen trials the birds are not challenged after vaccination but are exposed to the natural infection pressure. Therefore, at least 3 trials of the type described below should be carried out in three different farms of a given area [3, 4].

A. Field trials with broilers

A.1. Procedure for field trials with broilers	
• **Animals:**	Broiler chickens from the same hatchery and the same batch.
• **Maintenance and feeding:**	Two groups in separated pens of the same poultry house; maximum of 18-20 birds per m^2. Feed for both groups has to contain same batches of raw materials and has to be produced in the same feed mill. Both animal groups should be attended by the same personnel.
• **Animal groups:**	At least 10,000 broiler chickens subdivided into the following 2 groups each of at least 5,000 animals: • Group 1: Vaccinated and non-medicated. • Group 2: Medicated and non-vaccinated. Medication: with highly efficient anticoccidial drug.
• **Vaccination:**	According to manufacturer's recommendation.

A. 2. Efficacy parameters for field trials with broilers

- Mortality rates in both groups (total mortality and that caused by coccidiosis).
- Lesion score of each 5 animals per group 7, 14 and 21 days after date of vaccination.
- Average live body weights of the groups at the end of the fattening period.
- Feed conversion rate between days: 1-8; 8-21; 21-26 and 1-29 (total conversion).
- It is advisable to follow antibody titres in 10 animals per group on days 0 and 14 after challenge and at the end of the trial.

B. Field trials with pullets

The trials with pullets are performed in the same way as in broilers, i.e. on litter, not in cages and the medicated group should be treated with an efficient anticoccidial drug. The trials should not be terminated sooner than 2 months after beginning of the laying period.

B.1. Efficacy parameters for field trials with pullets

- Mortality rate (total mortality and that caused by coccidiosis).
- Egg production during the laying period.
- Mean live weight during the breeding period.
- Additionally, titres of circulating antibodies against *E. tenella*-antigens can be followed 14 days or one month after vaccination.

7.3. Host-safety evaluation

7.3.1. Contaminants of the vaccine

Samples of the vaccine have to be tested for potential contaminants by suitable and sensitive methods for the detection of pathogenic viruses, bacteria, mycoplasms or parasites. The vaccine has to be free of organisms pathogenic for animals or humans.

7.3.2. Target-host safety

Samples for host-safety evaluation should be taken from a batch of the vaccine produced according to the manufacturing process described in the application for registration.

The vaccine should be safe for the target animal species (chickens) in doses up to 10 - fold of the dose recommended for vaccination (= recommended dose: RD). Trials for host-safety evaluation can be carried out in combination with trials for stability of attenuation according to procedures described under 7.4.(see below).

Substances added to the vaccine for oocyst preservation or for other reasons should not be toxic at the highest concentrations possibly taken up by the target-host and should not produce unacceptable residues in meat, organs or eggs.

7.3.3. Non-target host safety

As *Eimeria* species are highly host-specific, a risk of infection of non-target host species, including humans, does not exist. Therefore, there is no need for safety evaluation of the vaccine in non-target hosts. (Regarding substances added to the vaccine see 7.3.2. and 7.5.).

7.4. Stability of attenuation

Biologically attenuated strains of *Eimeria* (see chapter 6) must be genetically stable for at least five generations without selection pressure. The five passages should not give any indications for reversal of the *Eimeria* strains to virulence [5, 8].

Data on stability of attenuation have to be worked out by the manufacturer for each single *Eimeria* species included in the vaccine and provided to the registration authority. For stability testing the following procedures are recommended:

7.4.1. Procedures for testing stability of attenuation

7.4.1.1. Passages of *Eimeria* strains through chickens

- Groups of 5-10 chickens must be kept under coccidia-free conditions until the age of 14 days (see 7.2.2.1.).

- Two days after transfer to a feed free from anticoccidial drugs, each of the chickens is inoculated with 10-20 x 10^3 oocysts of the respective strain of *Eimeria* to be examined.

- Oocysts collected from faeces during the entire patent period are isolated and sporulated (see chapter 1.1.).

- Groups of 5-10 new chickens are infected with 10-20 x 10^3 oocysts per animal. During patency oocysts are collected and sporulated. This procedure is carried out at least 5 times.

- Oocysts from the last passage without any selection pressure should be used for testing the stability of the vaccine according to the procedure described below.

7.4.1.2. Combined procedure for testing target-host safety and stability of attenuation	
• Number of animals:	50 (= 5 groups of each 10 animals)
• Age of animals:	8 days
• Application of oocysts and doses:	to the crop by tube
• Animal groups:	• Group 1: Recommended dose (RD) of the last (at least 5th) passage (see 7.4.1.1.). • Group 2: 10-fold of RD of the same oocysts as in group 1. • Group 3: RD of oocysts of original inoculum (see initial infection under 7.4.1.1.). • Group 4: 10-fold of RD of oocysts as in group 3. • Group 5: no dose (uninfected control).
• Housing of chickens:	Floor-pen conditions.
• Food and water:	Food free of anticoccidial drugs, water ad libitum.
• Duration of trial:	7 days (lesion score 5 days after inoculation).
• Follow-up parameters:	Daily clinical observation, lesion score at necropsy.

7.4.1.3. Criteria for target-host safety and for stability of attenuation

The vaccine is regarded as safe for the target-host species and stable for attenuation if the following criteria are fulfilled:

- Mortality: zero.
- Average intestinal lesion score of 10 animals lower than 2.
- Clinical symptoms lacking or only very mild, transient decrease in weight gain and minor impairment of feed conversion.
- Substances added to the vaccine for oocyst preservation or for other reasons should not be toxic at the highest concentrations possibly taken up by the target-host and should not produce unacceptable residues in meat, organs or eggs.

7.5. Environmental safety

Special care should be taken that in vaccines only *Eimeria* species are used which also occur in natural cycles in a given area. There is an urgent need for the development of rapid and simple techniques for the identification of *Eimeria* species and strains on the one hand and attenuated vaccine species and strains on the other hand.

Substances used in the vaccine for oocyst preservation or for other reasons with unknown toxicity and degradability have to be tested according to national regulations for environmental safety testing.

7.6. Recommendations for registration

In the development of anticoccidial vaccines or drugs efficacy is evaluated first, followed by safety studies. In registration procedures safety studies might be of primary interest [1].

7.6.1. Declaration of antigens and other components

Basic information on antigens (*Eimeria* species, strains) and other components used in the vaccine has to be submitted to the registration authorities by the producer.

7.6.2. Trials for registration

Trials for registration should be carried out according to the guidelines described above. For registration, battery trials for efficacy evaluation are unnecessary if adequate data from such trials are provided by the manufacturer. The following trials for efficacy evaluation should be carried out by institutions *independent* from the manufacturer:

- One floor-pen trial.
- At least 3 field trials in different farms of a given area.

The data on host-safety and environmental safety evaluation provided by the manufacturer have to be evaluated by the registration authority. In unclear cases complementary trials have to be performed by independent institutions.

Results of trials which have been carried out according to these guidelines and under the conditions of GLP in one country should be accepted for registration in other countries.

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Part II. *Cryptosporidium*

J. Peeters and I. Villacorta

1. Introduction

Cryptosporidium was first described by Tyzzer in 1907 in the gastric glands of laboratory mice (*Cryptosporidium muris*). In 1912 he identified a new species, *Cryptosporidium parvum*, in the small intestine of the same host. He also reported first on avian cryptosporidiosis [26]. Since 1971 *Cryptosporidium* has been found to be associated with bovine diarrhoea, which stimulated veterinary interest. Now *Cryptosporidium* is recognized as an important parasite with worldwide distribution. There exist at least 6 valid species: *Cryptosporidium parvum* and *C.muris* in mammals, *C. meleagridis* and *C. baileyi* in birds, *C. serpentis* in reptiles and *C. nasorum* in fish [26]. Probably also *C. wrairi* in guinea pigs has to be considered as a valid species [76].

C. parvum is considered as the economically most important species. It appears to be infectious for all mammals including humans and can easily be transmitted from one mammalian species to another. In calves *C. parvum* is considered as the second most common cause of diarrhoea [3]. Unlike other coccidial oocysts, *C. parvum* oocysts are fully sporulated and ready to initiate infection upon excretion. These oocysts may contaminate drinking water resources and cause human cryptosporidiosis [24]. *C. parvum* is of special significance in patients with AIDS as it causes severe forms of diarrhoea [26]. Unfortunately, only ozone is effective in disinfecting drinking water [41, 54]. Moreover, only few of 94 anticoccidial and antimicrobial drugs [26] such as halofuginone lactate [72], a chemical variant of the chicken anticoccidial halofuginone bromohydrate, and lasalocid sodium show clear activity against experimental cryptosporidiosis but they are not commercially available. Few desinfectants are effective within a short time of exposure. Most effective are OO-Cide [10], ozone [34, 41, 54] and chlordioxide [54].

In chickens disease associated with *C. baileyi* manifests itself most often as a respiratory disease in immunosuppressed birds. Normally the parasite develops in the bursa Fabricii. Only occasionaly the parasite causes intestinal or renal disease although combinations with tracheitis may be observed [26].

The prevention of cryptosporidiosis is difficult. Whereas most other species of enteric coccidida have a genetically programmed series of developmental stages in the life cycle and are incapable of recycling within the host, *Cryptosporidium* spp. have two stages that initiate auto-infectivity: type I merozoites and sporozoites derived from thin-walled oocysts. These characteristics allow *Cryptosporidium* spp. of developing severe infections in hosts exposed to only a small number of thick-walled oocysts [6]. Only the development of sufficient immunity is able to stop the cycle.

2. Maintenance of *Cryptosporidium* in animal hosts

2.1. Life cycle of *Cryptosporidium* species

Studies of different isolates (calf and human) of *C. parvum* in suckling mice [21] revealed that the life cycle of this parasite is similar to that of *Eimeria* and *Isospora* spp. The life cycle differs somewhat from other monoxenous coccidia as each intracellular stage of *C. parvum* resides within a parasitophorous vacuole confined to the microvillous region of the host cell (Fig. 17), whereas comparable stages of *Eimeria* and *Isospora* spp. occupy parasitophorous vacuoles deep within the host cells. Oocysts of *C. parvum* undergo sporogony while they are within the host cells and are infective when released in the faeces, whereas oocysts of *Eimeria* and *Isospora* spp. do not sporulate before they are passed into the faeces. Approximately 20% of the oocysts of *C. parvum* within host enterocytes do not form a thick, two-layered environmentally resistant oocyst wall. Soon after being released from the host cell, the membrane surrounding the four sporozoites ruptures and the sporozoites reinitiate a new life cycle. Also type I meronts are able to recycle. Studies of *C. baileyi* in experimentally infected chickens [22] have revealed that this species has a life cycle similar to that described for *C. parvum* in suckling mice. The major difference in the life cycle of these two species is that *C. baileyi* has three distinct types of meronts rather than the two types found in *C. parvum*. Little is known on the development of *C. meleagridis*.

Apart from morphological differences of the oocyst, *C. parvum* also may be differentiated from *C. muris* by ultrastructural differences [21, 70].

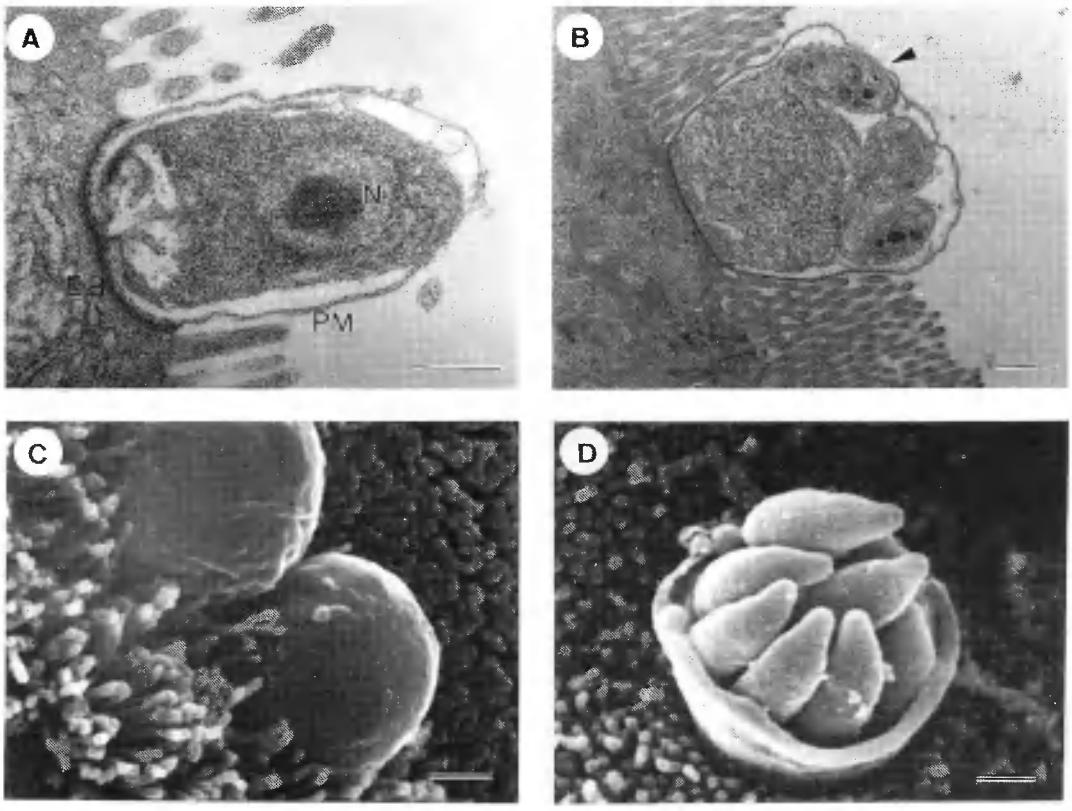

Fig. 17 (part 1): Life cycle of *Cryptosporidium parvum*: electron microscopy (bar = 0.5 µm). Courtesy of G. Charlier, National Institute of Veterinary Research, Brussels, Belgium.

A. TEM: A trophozoite in a parasitophorous membrane (PM), connected with the cell membrane. An electron dense band (EB) is formed on the fixation site. N = nucleus.

B. TEM: Meront showing 3 developing merozoites (arrow).

C. SEM: Micrograph of two meronts situated on an enterocyte between the microvillous border.

D. SEM: Opened mature meront containing 8 merozoites.

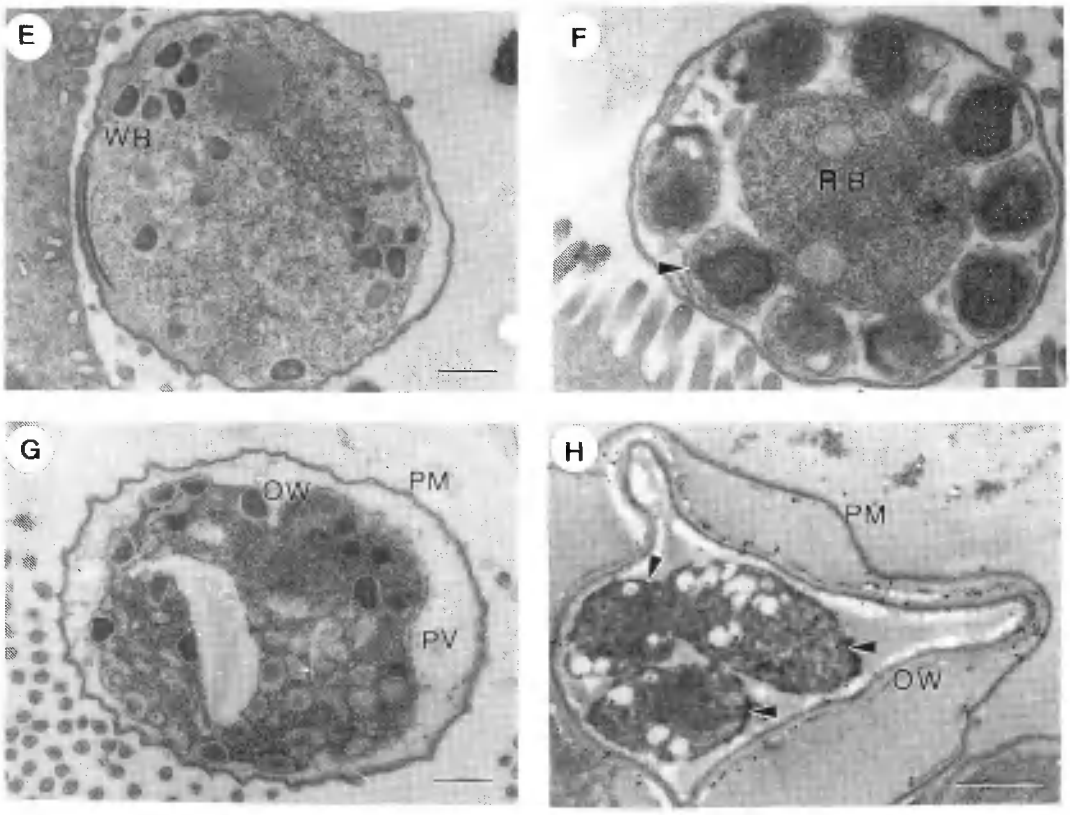

Fig. 17 (part 2): Life cycle of *Cryptosporidium parvum*: electron microscopy

E. TEM: Developing macrogametocyte. WB = wall forming bodies.

F. TEM: Developing microgametocyte containing microgametes (arrow) and a residual body (RB).

G. TEM: Developing oocyst: PM = parasitophorous membrane, PV = parasitophorous vacuole, OW = oocyst wall.

H. TEM: Sporulating oocyst showing 3 developing sporozoites (arrows). PM = parasitophorous membrane, OW = oocyst wall.

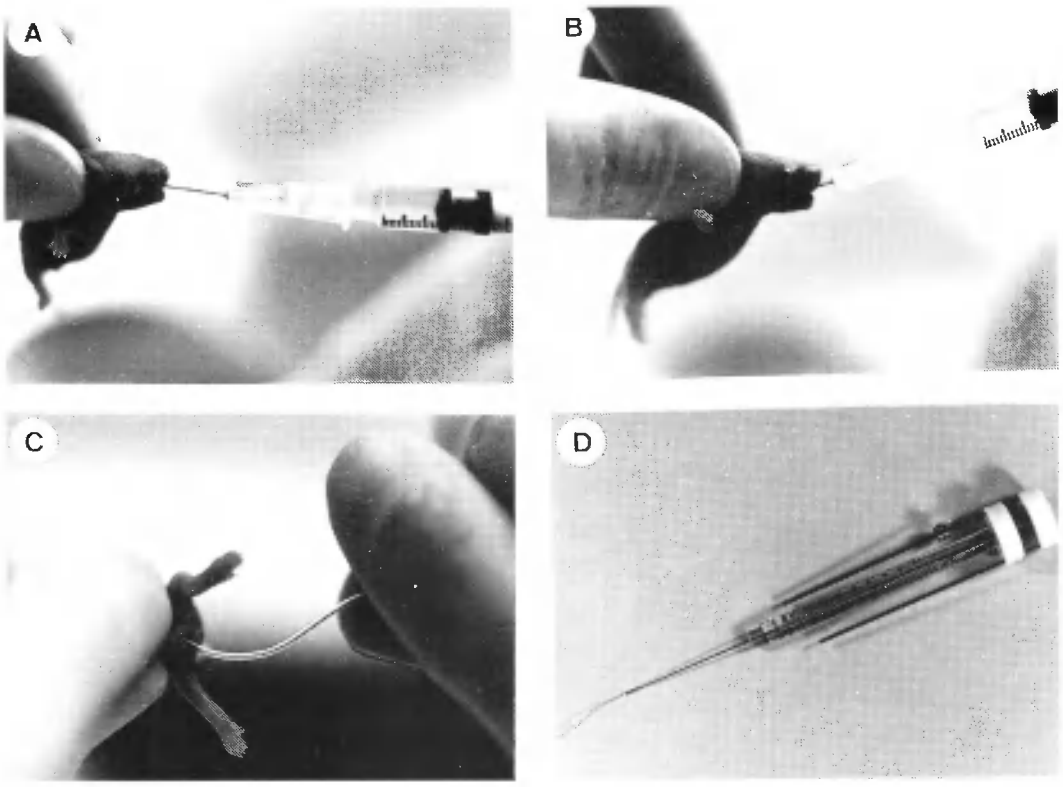

Fig. 18: Intragastric and intra-intestinal administration of *Cryptosporidium parvum* oocysts into neonatal mice: electron microscopy.

A. Intragastric administration: a tuberculin syringe with a 16 mm long 26-G needle fitted with plastic tubing is used to infect a 3-day-old mouse. The protected needle is inserted into the back of the animal's throat. In a first movement the needle is held horizontally and parallel with the animal's head.

B. Intragastric administration: once the mouse has swallowed the needle, it is inclined about 30° in order to penetrate into the oesophage of the mouse. The needle is inserted further at its full length, which corresponds with the mean distance between mouth and gastric entrance.

C. Intra-intestinal administration; a Hamilton syringe provided with a needle fitted with plastic tubing is inserted 1 cm into the colon through the anus.

D. A Hamilton syringe with a 25 mm long 23-G needle is fitted with 0.61 mm outer diameter plastic tubing that extens about 1-1.5 cm beyond the tip of the needle. The tubing is lubricated with oil before being inserted into the anus.

2.2. Maintenance of *Cryptosporidium parvum* in various hosts

- **Suckling mice or rats**

•• Intragastric administration of oocysts (Fig. 18)

Mice and rats are quite suitable [27] for the production of small numbers of *C. parvum* oocysts ($0.5\text{-}10 \times 10^6$/animal) as they are supplied as *Cryptosporidium*-free animals and can be maintained easily in strict isolation. Extensive infections occur in neonates of 1 to 4 days of age. Oocyst shedding starts 5-7 days p.i. and persists for 13 to 16 days. Usually the animals do not exhibit detectable signs of disease. Neonatal mice or rats are inoculated intra-gastrically with a 100 µl suspension containing 1 to 5 x 10^5 oocysts (ID_{50} = 500 oocysts). Inoculation is possible by using a 16 mm long 26-gauge needle fitted with plastic tubing. It is also possible to infect the animals by placing a disposable tip fitted to a micropipet into the back of the animal's throat and slowly delivering 100 µl of oocyst suspension. The animals should be maintained with their dams until oocysts are harvested 7 days p.i. Then, the animals are sacrificed as collection of faeces from suckling rats and mice is nearly impossible. The lower half of the small intestine, the caecum and the entire colon of each animal in the litter is pooled in +/- 10 ml of cold PBS containing 0.02% Tween-20 (PBS-T).

Oocysts may be recovered by various techniques: either the intestines are minced with a pair of scissors, followed by repeated passage of the mixture through 18-G and 19-G needles (insufficient recovery of oocysts), or they may be subjected to ultrasonication (causes indentation of the oocyst wall) or to homogenizing with an ultra-turrax (3 x 10 sec). Both latter techniques cause a 40% destruction of oocysts [54]. The homogenate should be washed three times in PBS-T at 1000 x g for 10 min and then resuspended in 2.5% potassium dichromate and stored at 4°C.

•• Intra-intestinal administration of sporozoites and merozoites [57, 60] (Fig. 18)

After sero-neutralisation of sporozoites or merozoites, infectivity should be checked in a live model. As sporozoites and merozoites are not infective when administered orally, an elegant technique has been developed for intra-intestinal administration.

After excystation and purification (see section 10) 2 x 10^5 sporozoites (ID_{50} = 8000 oocysts) are suspended in 25 µl HBSS and administered to 4- to 6-day-old SPF mice using a Hamilton syringe with a 2.5 cm long 23-gauge needle fitted with 0.61 mm outer diameter polyethylene tubing that extends about 1-1.5 cm beyond the tip of the needle. The tubing is lubricated with petrolatum and inserted 1 cm into the colon

through the anus. Injection of dye showed that the substance spreads to the entire colon, caecum and terminal 2 cm of small intestine. After 25 µl of parasite suspension is injected and the tubing withdrawn, a small rubber patch consisting of rubber cement (plasticine) is applied immediately to the perianal area to prevent defaecation. Mice are then maintained in paper drinking cups in a 37°C incubator for 30 min. (oocysts lose infectivity at 37°C in dry environment), then the patch is removed and the pups are returned to their dams. Animals are housed in large cages provided with feed, water and a filtered air supply.

- **T-cell deficient nude mice [36]**

Athymic nude (nu/nu) mice represent an elegant model for the maintenance of laboratory *Cryptosporidium* strains. They can be maintained without any problem in filter-capped cages at 27°C ambient temperature. Nu/nu mice can be distinguished from their nu/+ littermates by their crisped whiskers. Female mice of 6 days old are inoculated orally via a 22-gauge feeding needle with 10^5 *C. parvum* oocysts in 100 µl of PBS-pH 7.2. The mice start eliminating oocysts from 3 to 5 days p.i. and the intensity of shedding peaks on days 11 to 13. Where nu/+ littermates cease oocyst output between 15 and 24 days p.i., nu/nu mice continue oocyst output for at least 50 days p.i. At days 24 p.i. some nu/nu mice may develop diarrhoea, associated with increased oocyst output and weight loss. Mortality starts from day 36 p.i.

- **Hamsters [64]**

Hamsters weighing 80-100 g are immunosuppressed by subcutaneous injection of 8-10 mg of hydrocortisone acetate (100 mg/kg body weight) administered on days 4 and 1 before infection and 3 days afterwards. A dose of X rays at the day on infection as outlined in the original publication may be skipped. Animals are infected with 1 x 10^5 oocysts by administering a 100 µl suspension in the cheek-pouch or by sticking a pasta containig the oocysts directly onto the tongue. Oocyst shedding starts 3 to 5 days p.i. with a peak on day 9 p.i., when a mean production of 3-5 x 10^7 oocysts per gramme of faeces may be obtained. Hamsters produce 3 to 4 g of faeces per day. Total oocyst output between 3 and 15 days p.i. may reach 1.6-2.4 x 10^8. Faeces may be homogenized in a ground-glass homogenizer, filtered through a 300 µm-pore-size sieve to exclude larger particles and suspended in phosphate-buffered saline after washing. This model is suitable to propagate strains of *C. parvum* isolated from different animal species.

- **Guinea pigs [4, 16]**

Spontaneous infection of guinea pigs causes watery caecal contents, diarrhoea and low mortality. This species represents an interesting animal model as in contrast with other rodents both juvenile and adult guinea pigs up to 16 weeks of age are susceptible. Unfortunately, not all strains of *C. parvum* are able to infect guinea pigs. Angus [4] for instance failed to infect young guinea-pigs with a bovine *C. parvum* isolate. This was confirmed in our laboratory. A dose study showed that 6-week-old guinea pigs may be experimentally infected with dosages as low as 325 oocysts of a guinea-pig isolate of *Cryptosporidium* [16]. After experimental infection by oral gavage of 2 x 10^4 oocysts in 200 µl of PBS, oocyst output starts after 3-4 days and continues for up to 2 weeks. For the maintenance of laboratory strains of *Cryptosporidium*, guinea pigs are less suitable as it is rather difficult to purify oocysts from the very dry and concentrated faeces.

- **Rabbits [53]**

Cryptosporidium strains can easily be processed in suckling rabbits. When the isolates originate from weaned rabbits, they are often contaminated by *Eimeria* oocysts. As the latter are not sporulated in fresh faecal material, isolates free of *Eimeria* may be obtained by passing directly fresh faeces in suckling rabbits. Fresh faeces may also be maintained at 4°C until use. Suckling rabbits are inoculated orally with 2 x 10^4 oocysts in 200 µl of PBS. They show diarrhoea from 5 to 7 days p.i., a stop of weight gain between 6 - 13 days p.i. and mortality between 10 - 14 days p.i. High numbers of oocysts are present in the gut between days 7-9. Infection of weaned rabbits results in weak oocyst output between days 2 to 12 p.i. (peak values days 7 - 8) and a 32% reduction of weight gain during the second week p.i.

- **Ruminants**

Suckling calves, goats and lambs are the best species to produce large numbers of *C. parvum* oocysts [2, 3, 37]. Important is to obtain *Cryptosporidium*-free animals before infection. This requires special isolation facilities. Animals that are less than 3 days of age are fed 10^6 - 10^7 oocysts suspended in milk or milk-replacer by a bottle. In most cases diarrhoea will start 3 to 4 days p.i. Then, it is advisable to replace the milk or milk-replacer by an oral electrolyte solution. The use of this solution reduces the

amount of fat in the faeces, which may interfere with oocyst recovery. The faeces should be collected daily in 2.5% (w/v) potassium dichromate and stored at 4°C until processing. It is best to store each daily collection separately, as samples with the highest oocyst numbers are most suitable for purification of the parasite. Oocyst output starts after 2 days with peak values between day 5 and 9. Calves may eliminate between 10^6 and 10^8 oocysts per gramme of faeces and the total number may reach 10^{10} oocysts per animal [1, 9].

2.3. Maintenance of *Cryptosporidium baileyi* and *C. meleagridis* in animal hosts

Chickens are susceptible to both *C. baileyi* and *C. meleagridis*. Isolates issued from young birds are often contaminated with *Eimeria* oocysts. As the latter are not sporulated in fresh faecal material, isolates free of *Eimeria* may be obtained by passing directly fresh faeces into recipient chickens. Fresh faeces may also be maintained at 4°C until use. Three-week-old chickens are infected in the crop using a tuberculination syringe with a 1 ml suspension containing $2x10^5$ oocysts. This is generally not followed by clinical signs. Some birds may appear depressed and weight gain may be reduced after *C. baileyi* infection. Parasite-induced lesions are generally confined to the bursa Fabricii and the cloaca. Faecal excretion starts at day 6 for two weeks with a peak on day 13. Purification of oocysts from faeces is difficult. Pure suspensions may be obtained by scraping the mucosa of the bursa Fabricii or by homogenizing the bursa with an ultraturrax. Usually a bursa contains approximately 10^7 oocysts.

2.4. Maintenance of *Cryptosporidium muris* in animal hosts [77]

Cryptosporidium muris is infective to various laboratory animals, such as mice, guinea pigs, rabbits, dogs, and cats. Among these host species, mice and cats are highly susceptible to the parasite. In 3-week-old SPF mice oocyst output starts 5-16 days p.i. and lasts about 4-6 weeks. Between days 16-28 p.i. animals may excrete 1-5 x 10^6 oocysts. The total number of oocysts discharged per mouse during the patent period may reach 2-6 x 10^8. Also cats infected at 1-2 months of age discharge a large number of oocysts for a long period. Guinea pigs, rabbits and dogs show low susceptibility: the number of excreted oocysts is extremely small and the patent period lasts less than 3 weeks. The entire endogenous development of *C. muris* occurs in the stomach (only pars glandularis affected) and not in the small and large intestine.

3. Collection, preservation and storage of oocysts

Oocysts are mainly collected from faecal samples. For diagnostic purposes faeces should be submitted as fresh material or in 10% buffered formalin. As the number of oocysts shed may fluctuate, it is advisable to collect a minimum of three samples. If parasites are not going to be used for subsequent experimental purposes, fixed samples are recommended to avoid contamination of people handling the specimen. For purification purposes, it is important to start with fresh faecal samples that are as rich as possible in oocysts and that are preferentially collected by rectal sampling. In anticipation of further purification (see section 9.3. of this chapter on oocyst purification) or after purification and washing by centrifugation, oocysts are suspended in 2.5% $K_2Cr_2O_7$ and stored at 4°C. Most oocysts will remain viable for at least 3 months, although viability may remain for up to 15 months [53]. Since oocysts begin to loose viability after 3 months of storage, it is advisable to make passages every 3-4 months. As chromium is an environmental contaminant requiring special disposal and handling procedures, it is advisable to store oocysts in Hank's balanced salt solution (HBSS) containing penicillin 10,000 i.u., streptomycin 10 mg, amphotericin B 0.05 mg and nystatin 500 i.u./ml.

4. Morphological identification of oocysts

C. parvum may be differentiated from *C. muris* based on oocyst dimensions: *C. parvum* measures 4.8 x 5.0 µm [71], whereas *C. muris* measures 7.4 x 5.6 µm [15]. Also avian species may be differentiated by morphological differences: *C. meleagridis* measures 4.5 x 4.0 µm [65] against 6.2 x 4.6 µm for *C. baileyi* [22].

5. Counting and concentration of oocysts

Oocysts can be detected in faeces, in sputum [42] or in scrapings from the bursa Fabricii, whereas endogenous stages may be diagnosed by histological examination of intestinal, respiratory or bursa epithelium. Until 1980, the latter technique was the main diagnostic method [18]. Both fresh and fixed samples can be examined as wet mounts or can be concentrated or stained [31, 33]. If the faecal sample is not fluid, it may be necessary to dilute it with water and pass it through cheesecloth or a sieve to remove coarse material.

5.1. Counting of oocysts

Because of their small size, oocyst numbers of *Cryptosporidium* spp. are usually counted in a Neubauer haemocytometer (see Fig. 5). Therefore 0.2 ml of oocyst suspension is mixed with 0.8 ml of malachite green (malachite green 0.16 g, sodium dodecyl sulphate 0.1 g, 100 ml aq. dest.). The total number of oocysts present in 10 large squares of 1 mm^2 is counted. As the counting chamber has a depth of 0.1 mm, this number is equivalent to the number of oocysts present in 1 mm^3. To obtain the number of oocysts per ml this figure has to be multiplied by a factor of 5000 (x 1000: conversion to 1 ml of diluted suspension; x 5: conversion from 0.2 ml of original oocyst suspension of 1 ml). Oocysts appear as bright, birefringent 4.5 to 5.5 µm spherical bodies (*C. muris* 6-8 µm) containing one to four dark granules. Yeast cells usually are larger, stain greenish blue and sometimes show budding.

5.2. Semiquantitative examination of samples for oocysts

For experimental purposes requiring the evaluation of a large number of samples, oocyst numbers may also be assessed semiquantitatively in negatively stained faecal preparations (Heine's technique). The infection rate is estimated at a magnification of 500 x by bright-field microscopy as follows:

0: no oocysts
1: fewer than 5 oocysts per microscopic field
2: between 5 and 25 oocysts per field
3: more than 25 oocysts per field.

A score of 1 corresponds with 10^4-10^5 oocysts per g of faeces, a 2-score with 10^5-10^6 oocysts and a 3-score with more than 10^6 oocysts per g.

5.3. Concentration techniques for oocysts

Usefull concentration techniques [14, 31, 46, 48, 52, 75] include flotation of oocysts in Sheater's sugar solution (d=1.18), in zinc sulfate solution (d=1.20) or in saturated sodium chloride solution (d=1.20). Physico-chemical concentration techniques using sedimentation include formalin-ether and formalin-ethyl acetate. There exist no signifi-

cant differences between the different concentration techniques. Sheater's sugar flotation technique is most widely used.

- **Sheater's sugar flotation technique [1, 18, 52]**

- Approximately 1 ml of faecal (sputum) suspension is thoroughly mixed with 10 ml of Sheater's sugar solution (500 g sugar, 320 ml tap water and 6.5 g phenol) in a 15-ml tube.
- After centrifugation for 10 min at 500 x g the meniscal fluid is examined microscopically. Phase-contrast microscopy will allow to distinguish oocysts from contaminating yeast cells [18].

C. parvum oocysts appear as bright, birefringent 4.5 to 5.5 µm spherical bodies containing one to four dark granules. With good-quality phase-contrast oil immersion objectives the four sporozoites surrounding the centrally located oocyst residuum can often be seen. Oocysts of other species of *Cryptosporidium* will appear similar to *C. parvum* but with a different size and shape (see morphological identification of oocysts, section 4 of this chapter). Contaminating yeasts do not appear as bright, birefringent bodies, do not contain dark granules and sometimes show budding. When viewed with bright-field microscopy oocysts are often difficult to distinguish from yeast cells [1, 31]. After +/- 15 min in the hyperosmotic solution, oocysts begin to collapse.

- **Formalin-ethyl acetate sedimentation technique [14, 31, 52]**

This is a modification [74] of the original Ritchie's formalin-ether-sedimentation method [61].

- Samples are suspended in an equal amount of tap water and strained through a sieve. Then they are centrifuged for 2 min at 500 x g.
- The sediment is thoroughly mixed with 9 ml of neutral buffered 10% formalin and subsequently 4 ml ethyl acetate is mixed to each tube. After shaking the tubes in an inverted position for 30 sec, they are centrifuged for 2 min at 500 x g. This results in 4 layers: solvent, a plug of debris, formalin and sediment.
- The top 3 layers are decanted after loosening the plug of debris.

- The sediment is resuspended in a saturated salt solution and after centrifugation for 10 min at 500 x g, the meniscal fluid is examined microscopically. Cryptosporidial oocysts lay immediately against the coverglass and show the same characteristics as outlined above.

6. Staining of oocysts

Any preparation can be stained with the following techniques. Only stools preserved in polyvinyl alcohol (PVA) fixative are not suitable. It is important that faecal smears are not too thick to ensure adequate penetration of the reagents. One should take in mind that direct staining techniques of faeces or sputum only allow the detection of 1 x 10^3-10^4 oocysts/ml or more.

6.1. May-Grünwald Giemsa method [44]

After making a 1/1 suspension of faeces in tap water, a thin smear is made on a defatted glass slide, airdried, fixed in methyl alcohol and stained. *C. parvum* oocysts appear as round to slightly oval structures of 4-4.5 µm, frequently containing 2-5 dense red granules, contrasting with the blue cytoplasm. Confusion is possible with yeast cells. As faster and reliable staining techniques are available now, this technique is less used.

6.2. Acid-fast staining

This technique is based on the acid-fast staining properties of the oocyst wall. Several techniques have been used without marked differences in sensitivity and specificity [11, 31, 33, 45, 46, 58]. The most commonly used is the modified cold Kinyoun technique [45].

- A sample is spread on a microscope slide and allowed to air-dry.
- Then the sample is fixed in absolute methanol for 5-10 min and air-dried.
- Subsequently the smear is covered with Kinyoun acid-fast stain (carbol-fuchsin [Merck No. 9215] for 2 min. The stain may also be prepared by dissolving 4.0 g of basic fuchsin in 100 ml distiled water plus 20 ml 95% ethanol. After the stain is dissolved, slowly ad 8 ml of liquified phenol.

- Then rinse briefly in tap water and decolorize the smear with 10% H_2SO_4 for 1 min and rinse briefly again in tap water.
- Subsequently the smear is counterstained with light green SF yellowish stain (Harleco) or with methylene blue (0.3 g in 100 ml distilled water) for 1 min rinsed again in tap water, air-dried and examined microscopically.

C. parvum oocysts appear as 4.5 to 5.5 µm red spherical bodies against a green or blue background. Most faecal debris and yeast cells will take up the color of the counterstain.

6.3. Negative staining (Heine's carbol-fuchsin method)

This technique [17, 35, 52] requires less preparation time and is suitable for examining large series of samples. Yet, there does not remain a permanent slide. Best results are obtained with fresh faecal samples, but also formalin fixed samples will do.

- One drop of a stool-suspension is mixed thoroughly with one drop of carbol-fuchsin (Merck No. 9215) on a microscope slide and a thin smear is made.

- As soon as the smear has air dried, a drop of immersion oil is added directly to the smear, a coverslip is placed on top of the immersion oil and the preparation is examined with a bright-field microscope.

- *C. parvum* oocysts appear as bright, birefringent round bodies of 4.5-5.5 µm against a red-staining background. A prominent black dot is regularly visible. Faecal debris, yeast and bacteria all stain dark-red. After approximately 15 min the oocysts begin to collapse.

7. Detection of oocysts by immunological and molecular techniques

7.1. Immunofluorescent antibody test (IFAT) [32, 47, 51]

The detection limit of this technique is restricted to 10^3 oocysts/ml or g [5]. However, most faecal samples from diseased animals contain sufficient oocysts to allow diagnosis. The necessary hyperimmune polyclonal anti-*Cryptosporidium* spp. serum can easily be prepared in rabbits. Otherwise immunofluorescent diagnostic kits based on mo-

noclonal antibodies specific to the oocyst wall of *C. parvum* are available commercially from different companies (for example: Monofuo® Cryptosporidium Kit, DiagnosticsPasteur).

- **Preparation of antisera.** Suspend oocysts (10^8/ml) in 2 mM TRIS buffer (pH 8.0) containing 10 mM phenylmethyl-sulfonfluoride (PMSF) and disrupt by three freeze-thaw cycles. Thereafter put the sample in liquid nitrogen for 10 min and incubate them at 37°C until the suspension is thawed. Disruption of oocysts and sporozoites can be monitored microscopically. Oocysts may also be disrupted by ultrasonic treatment. Immunise rabbits s.c. with approximatley 10^7 disrupted oocysts mixed in complete Freund's adjuvant, followed 2 weeks later by a first i.m. boosting injection with 10^7 disrupted oocysts in incomplete Freund's adjuvant and a similar injection 4 weeks later. Also other adjuvants and/or routes of administration may be used.

- **Test procedure**

- Dilute the faecal samples in water and add 50 µl of this suspension to each well of a 12-well multispot microscope slide.
- Air-dry the preparations for 1 h at 37°C.
- Fix the smear in cold 95% ethanol or aceton for 10 min, wash in PBS (pH 7.2) and air-dry.
- Cover the smear with rabbit anti-*Cryptosporidium* serum (diluted 1:100 to 1:1000 in PBS) and incubate for 30 min at 37°C in a moist chamber.
- Then wash five times by incubating in PBS for 3 min and air-dry. An appropriate blocking agent such as 1% bovine serum albumin or 1% gelatin may be used at this step to avoid background fluorescence.
- Cover the smear with 50 µl of appropriately in PBS diluted FITC-conjugated goat anti-rabbit IgG to each well, incubate for 30 min at 37 °C in a moist chamber, wash five times by incubating in PBS for 3 min and air-dry.
- Add one drop of buffered glycerin (9:1) to each well and read at a magnification of 400 x. Addition of 0.01% (w/v) Evans blue to the diluted FITC-conjugate helps distinguish bright green fluorescing oocysts from red fluorescing contaminating debris and yeast cells.

7.2. Capture enzyme-linked immunosorbent assay (C-ELISA) [5, 62]

The detection limit of this technique is restricted to 3 x 10^5 oocysts/ml, which makes the test only suitable for the screening of calves in the acute stage of the disease. The test requires monoclonal antibodies (MAB) directed against the oocyst wall of the parasite and polyclonal goat anti-*Cryptosporidium* antibodies.

- Microtitration plates are coated with 5 µg of MAB per ml in 0.05% sodium carbonate-bicarbonate buffer (pH 9.6) by adding 50 µl per well and incubating them overnight at 4°C. Wells are washed five times with 200 µl of blocking buffer (PBS containing 2% bovine serum albumin [PBS-2 % BSA]) per ml at room temperature and 2 min per wash.
- The plates are then incubated for 2 h at 4°C with 200 µl of blocking buffer per well. Plates are used on the day of preparation or stored at -20°C for up to 7 weeks before use. Immediately before use, the plates are blocked a second time with 100 µl of PBS-10% BSA per well for 30 min at 37°C.
- Faecal oocysts are concentrated by centrifugation through formalin-ethyl acetate solution (see physico-technical concentration technique, section 9.2. of this chapter) and the pellet is suspended in 0.5 ml of PBS-2 % BSA.
- Then 50 µl samples of concentrated faecal pellet are added to appropriate wells and incubated for 30 min at 37°C.
- Plates are washed five times at 2 min per wash with PBS-2% BSA.
- Then samples (50 µl) of immune or nonimmune anti-*Cryptosporidium* goat serum are added.
- Following 30 min of incubation at 37°C, the plates are washed five times at 2 min per wash with PBS-2% BSA.
- Peroxidase conjugate of rabbit anti-goat IgG (50 µl) is added to the wells and incubated for 30 min.
- The plates are washed five times at 2 min per wash with PBS containing 0.05 % Tween 20.
- Finally add 100 µl of appropriate substrate such as TMB (3,3', 5, 5'-tetramethylbenzidine, Kirkegaard Perry Laboratories, Gaithersburg, MD, USA, No. 50-76-00) and allow to react at room temperature for 15 min. Stop the reaction by adding 1 M o-phosphoric acid and read the optical densities (OD) at 450 nm in a spectrophotometer. OD-values larger than twice the mean OD of negative control samples are considered as positive.

7.3. Detection of *Cryptosporidium parvum* by PCR

Recently, the design and use of polymerase chain reaction (PCR) primers and probes for the detection of *Cryptosporidium parvum* have been described [78]. Sensitive and specific amplification of a 329 base pair product was demonstrated by ethidium bromide staining and hybridisation of radiolabelled probes. Under optimal conditions, this PCR was able to reproducibly detect as little as 20 oocysts. No amplification products were obtained with *C. baileyi*, *C. muris*, *Giardia lamblia*, *Trichomonas foetus*, mixed ovine *Eimeria* cultures, *Escherichia coli* and bovine DNA. Environmental monitoring and epidemiological surveys should confirm the reliability of this PCR in practical conditions in the near future.

8. Identification of endogenous stages

Routine histological methods can be used to detect endogenous stages of *Cryptosporidium* spp. in tissue samples. Haematoxylin and eosin stain will visualize the parasites as basophylic spherical bodies of 2-5 µm on the surface of the epithelium. Yet, identification of the different life cycle stages will be difficult. Transmission electron microscopy (Fig. 17) is better suited to identify developmental stages [18, 21]. For the identification of *Cryptosporidium* stages in fresh mucosal scrapings [21], cultured cells [20] or the chorioallantoic membrane of chicken embryos [19] phase contrast or better interference contrast light microscopy can be used.

9. Purification of oocysts

9.1. General aspects

Most purification procedures involve a considerable loss of oocysts up to 90%. Therefore it is important to start with faecal samples that are as rich as possible in oocysts. Diarrhoeic faeces from heavily infected calves (up to 10^7 oocysts/g) are the best source. Fresh faecal material should be collected by rectal sampling, suspended immediately 1/1 in cold 5% (w/v) aqueous $K_2Cr_20_7$ solution and stored at 4°C until purification within 48 h. Faecal materials stored at 4°C without dichromate for more than 7 days are not suitable for purification. Efficiency of oocyst recovery varies from sample to sample. Presence of black yeasts makes it virtually impossible to obtain pure oocysts.

Prior to initiate the concentration procedure it is important to minimize the amount of faecal debris in the sample. Therefore, faeces are diluted 1:20 in PBS and washed through sieves (Endocotts Ltd., England) with meshes of 150 and 45 µm, respectively. The resulting fluid is centrifuged at 500 x g for 10 min and the sediment washed two more times.

The procedure of oocyst purification includes two main steps: (a) concentration and (b) purification.

9.2. Procedures for oocyst concentration

Oocysts may be concentrated by the Sheater's sugar flotation procedure, but as faeces often contain considerable amounts of fat we prefer a physico-chemical concentration technique, followed by percoll gradient centrifugation.

- **Physico-chemical concentration**

The sediment is resuspended in 20 ml of PBS (pH 7.2) and 20 ml of diethyl ether. The tube is shaken vigourously in an inverted position for 30 sec and then spun down at 500 x g for 10 min. Lipid material will concentrate into the upper ethylacetate layer, whereas oocysts will remain in the aqueous phase. The top 3 layers are decanted. This step is repeated until the sediment is free from lipids. After 3 further washings at 1500 x g for 10 min the sediment is resuspended in PBS. It is advisable to remove the supernatant with a pipette.

- **Sheater's sugar flotation**

If the sample does not contain an excessive amount of fat, a modification of the Sheater's sugar flotation procedure may be used after the large faecal debris is removed.

Add 0.2% w/v Tween-20 in water to the faecal suspension and mix thoroughly. Then mix thoroughly 5 ml of this suspension with 30 ml of Sheater's sugar solution (see concentration techniques, section 5.3.) and centrifuge at 100 x g for 10 min. Then layer carefully 5 ml of water containing 0.2% Tween-20 on the top of the flotation medium. Stir the top layer of water with the tip of a pipette. One can see a wisp of oocysts being washed off of the surface of the flotation medium into the uppermost water layer. Aspirate carefully the water layer. The 5 ml of water containing oocysts from each

harvest can be pooled into the same tube. Thoroughly remix the remaining flotation medium and repeat these steps 2 to 4 times.

Add an equal volume of water containing 0.2% Tween-20 to the tube(s) containing the harvested oocysts and centrifuge at 1000 x g for 15 min. The pellet often has two layers, the whitish oocysts on the bottom and a darker upper layer of fine faecal debris. The upper layer can be removed by careful aspiration and discarded with the supernatant resulting in suspensions containing highly purified oocysts.

9.3. Procedures for purification of oocysts

- **Discontinuous percoll gradient centrifugation (Fig. 19)**

The oocysts obtained after one of the concentration procedures may further be purified by percoll gradient centrifugation.

- Layer one ml samples of the resulting suspension on a discontinued Percoll (Pharmacia Fine Chemicals, Uppsala, Sweden) gradient. This gradient is constituted of four 2.5 ml layers with a density of 1.13 (undiluted percoll), 1.09 (1.5 ml percoll + 0.5 ml H_20), 1.05 (0.7 ml percoll + 1.3 ml H_20) and 1.01 g/ml (0.1 ml percoll + 1.9 ml H_20) which are carefully superposed with a pipette in a transparent conic plastic or better glass tube of 15 ml (critical ∅ of 17 mm) and centrifuged at 650 x g for 15 min.
- Then, the band containing purified oocysts between the 1.09 and 1.05 layer is removed, washed three times in PBS at 1500 x g for 10 min and then resuspended in PBS.

- **Isopycnic percoll gradient centrifugation [6]**

This is a variant of the above described procedure.

- Prepare 9 ml of a Percoll (Pharmacia, Uppsala, Sweden) solution of 1.09 g/ml (320 mOsm, pH 7.4) in a 15-ml high-speed centrifuge tube. The solution is prepared by mixing thoroughly nine parts Percoll, one part 10 x Alsever's (preparation see page 223) solution and nine parts 1x Alsever (can be substituted for HBSS pH 7.2 in this and next steps). The percoll solution should be prepared fresh and used immediately.

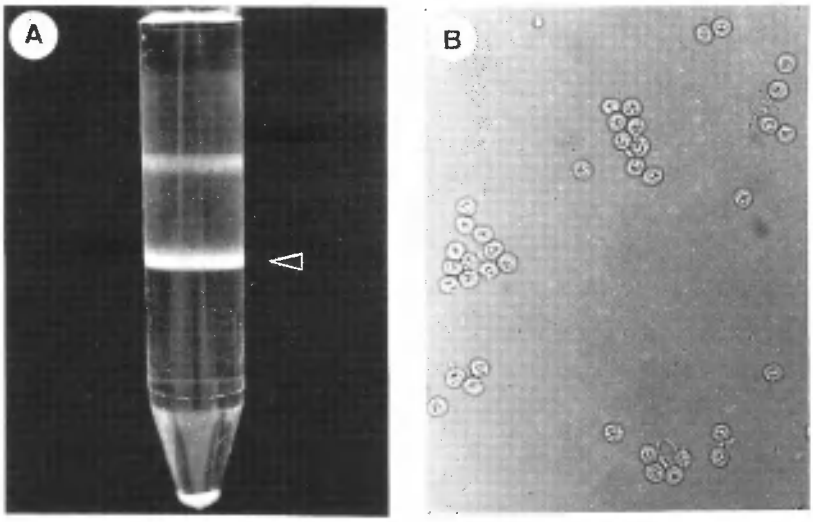

Fig. 19: Discontinuous percoll gradient centrifugation

A. Percoll gradient showing different bands: the band between the 1.09 d and 1.05 d layer (arrow) contains purified oocysts.

B. Direct view of purified oocysts recovered after percoll gradient centrifugation (bright field, 400 X). The oocysts are only slightly contaminated with bacteria.

- Layer on the top of the Percoll solution 1 ml of an oocyst suspension (2-4 x 10^8 oocysts per ml in Alsever's) obtained from the concentration technique and centrifuge at 22,000 x g for 30 min at room temperature.

Oocysts walls form a dense band approximately one third the distance from the bottom, below an upper layer of bacteria. Remove the band of oocysts and wash three times in PBS-pH 7.2 at 1000 x g for 10 min. If the oocysts are not pure enough, the process may be repeated using the oocysts recovered from the first attempt.

- **Cesium chloride step gradient centrifugation [39]**

In comparison with the Percoll centrifugation method this technique results in more highly purified oocysts that are easily separated from the purification medium.

- Prepare a stock solution of CsCl with a specific gravity of 1.8 g/ml distilled water. If a refractometer is used, first dissolve 10.0 g CsCl in 5 ml distilled water, check

then the density and add water to obtain a density of 1.8 g/ml (refraction index of 1.409 at 25°C).

- Also prepare a stock TRIS buffer pH 7.2 solution of 50 mM TRIS (6.055 g/l) and 10 mM EDTA (2.923 g/l). From these stock solutions prepare the three following solutions.
  - solution 1 (1.4 g/ml: mix 1 part stock CsCl solution with 1 part stock TRIS buffer)
  - solution 2 (1.1 g/ml: mix 1 part stock CsCl solution with 7 part stock TRIS buffer)
  - solution 3 (1.05 g/ml: mix 1 part stock CsCl solution with 15 part stock TRIS buffer)
- Carefully layer into a 15 ml high-speed centrifuge tube 3 ml of each solution, starting with the most dense solution.
- In the mean time wash concentrated oocysts twice in distilled water (1500 x g, 10 min) and once in stock TRIS buffer. On the top layer 1 ml of oocysts (approximately 10^8/ml) in the stock TRIS buffer and centrifuge at 16,000 x g for 60 min at 4°C. Three bands form within the step gradient.
- The top band formed at the interface between solution 2 and 3 contains the purified oocysts. The other two bands contain debris and a few oocysts. If the oocysts are not pure enough the process may be repeated using the oocysts recovered from the first attempt.
- The recovered oocysts should then be dialysed overnight against 10 mM TRIS/1.0 mM EDTA buffer to remove the CsCl.

10. Isolation and purification of sporozoites

10.1. Isolation [10, 18, 59, 66, 73]

Sporozoites may be obtained in vitro by simulating the gastrointestinal environment of the host in which the parasite stages are liberated from oocysts (= excystation). The following procedure results in 60-90 % of excystation.

- Purified oocysts are washed and suspended in cold PBS (pH 7.2) at a concentration of 10^8 oocysts/3 ml.
- Add 3 ml of sodium hypochlorite solution of 10.5 % in order to obtain a final concentration of 5.25% (commercial laundry bleach contains 12 % sodium hypo-

chlorite). Sodium hypochlorite treatment increases excystation rate, dissolves residual microscopic debris and inactivates microbial contamination.

- Stir the mixture on ice for 10 min. It may be useful to stop the reaction with 1.05 % thiosulphate.
- Then centrifuge the suspension at 1500 x g for 10 min at 4°C.
- Resuspend the pellet of oocysts in cold PBS and recentrifuge. This procedure should be repeated 4 times until all hypochlorite is removed. The percentage of sporozoites excysting can be increased by preincubating oocysts in PBS for 1 h at 37°C.
- After the last washing step, mix the sediment with 6 ml of prewarmed excystation fluid consisting of 0.75 % (w/v in PBS) sodium taurocholate (Aldrich No. 86, 196-0) with 0.25 % (w/v in PBS) bovine trypsin (Difco No. 840-7250) and incubate for 30 to 45 min at 37°C in a warm water shaking bath.
- The excystation process can be monitored microscopically: first sporozoites are released after 15-20 min. Depending on the age of the oocysts and the excystation solution 60 to 90 % of excystation may be expected. Sporozoites are then washed free of excystation fluid in Alsever solution at 1500 x g for 10 min at 4°C (Alsever: NaCl 4.2 g/l, glucose 20.5 g/l, sodium citrate 8.8 g/l, aqua dest. 1000 ml - adjust to pH 7.2 with a few drops of 10 % citric acid - osmolarity 320 mOsm). The procedure should be repeated twice. Alsever may be substituted for Hank's balanced salt solution (HBSS, pH 7.2).

10.2. Purification

Three purification techniques may be used depending on the volume of sporozoites and the purity required. The first technique is a simple filtration method that results in suspensions of sporozoites that are usually free from oocysts and oocyst walls. The other two methods result in 1-4 % contamination with intact oocysts. However, the percentage of excysted sporozoites obtained with the latter techniques will be higher. Purified sporozoites may be kept alive for 12 h at 4 °C in Alsever's solution or in Hank's balanced salt solution.

- **Filtration technique [26]**

At maximal excystation, place the warm excystation fluid containing the mixture of free sporozoites, oocyst walls and intact oocysts into a syringe and gently force it through a polycarbonate membrane with a pore size of 2 µm (Millipore). Then force warm Alsever or HBSS through the membrane (twice the volume of the excystation

fluid used). Because sporozoites are thin (1-2 μm in diameter) and motile, they will pass through the pores of the membrane, whereas the 4.5 to 5.5 μm oocysts and oocyst walls are retained. The percentage of sporozoites that will pass depends on the amount of debris that plug the pores of the membranes. Therefore the best yields are obtained when the number of oocysts forced onto the filter is below $10^7/cm^2$ of membane surface. Sporozoites which pass through the membrane can be washed free of excystation fluid as described above.

- **Isopycnic Percoll Centrifugation [6]**

Following excystation wash the sporozoites free of excystation fluid as described above. Resuspend the pellet in Alsever (can be substituted for HBSS pH 7.2 in this and next steps) up to an equivalent of 5 x 10^8 oocysts per ml and carefully layer 1 ml on top of 9 ml of a Percoll (Pharmacia, Uppsala, Sweden) solution of 1.09 g/ml (320 mOsm, pH 7.4) in a 15-ml high-speed centrifuge tube. The solution is prepared by mixing thoroughly nine parts Percoll, one part 10x Alsever and nine parts 1x Alsever. The percoll solution should be prepared fresh and used immediately. After layering the sporozoite suspension, centrifuge at 22,000 x g for 30 min at room temperature. A control tube containing density marker beads (Sigma) may be used to calibrate the density gradient. Oocyst walls form a band at the top of the gradient, whereas the sporozoites are found approximately one third the distance from the bottom. As intact oocysts sediment immediately below the band of sporozoites, the band of sporozoites recovered from the gradient may be contaminated with 2-4 % intact oocysts. Sporozoites can be washed free of Percoll solution in Alsever's solution or HBSS as described above.

- **Anion exchange [26, 60]**

Add 5 g of DEAE-52 preswollen matrix (Pharmacia No. 45059) to 50 ml of column buffer. Column buffer is prepared by adding 100.78 g Na_2HPO_4 (anhydrous), 0.62 g $NaH_2PO_4.H_20$, 3.4 g NaCI and 10.0 g glucose to 1000 ml of distilled water. Adjust to pH 8.0. Ionic strength should be 290 mOsm.

Wash the matrix in column buffer three times by stirring gently during 10 min followed by letting to settle the matrix to the bottom and carefully aspirating the supernatant with a pipette. Do not use a stir because it can crush the matrix into fine particles. Store overnight in 50 ml of column buffer at 4°C. Check the matrix buffer slurry the following day, readjust to pH 8.0 if necessary and degas. Use 10 ml of this slurry to

prepare the column. Columns are prepared by placing flexible tubing equipped with clamps on the end of a 3-ml syringe barrel containing a plug of nylon wool and 1 cm of DE-52 matrix. Let the DE-52 matrix settle to the bottom of the column, allow excess column buffer flow away and close the column at the moment that the matrix is still lightly covered with buffer. Adjust to 1 cm of matrix with more slurry if necessary.

After excystation, wash the mixture of sporozoites and oocyst walls twice in 10 ml of column buffer by centrifugation. Add another 10 ml of matrix buffer slurry (or an amount equal to the one to form 1 cm of DE-52 matrix in the column) to the 10 ml of column buffer containing the sporozoites, oocyst walls and oocysts recovered from the excystation of approximately 10^8 oocysts. Swirl gently for 1-2 min and then add to the column. Elute with 30 to 50 ml of column buffer (flow rate 2 to 3 ml/min), collect sporozoites by centrifugation into a pellet and resuspend sporozoites to desired concentrations in Alsever's solution or HBSS.

11. Isolation and purification of merozoites [7, 38]

11.1. Isolation from neonatal calves

Merozoites may be obtained from neonatal calves as follows [7].

- After transporting calves to a facility to minimise environmental exposure, they are given colostrum within 3 h of birth and hand-reared on bovine milk or milk replacer.
- The calves are inoculated orally with 10^8 oocysts and euthanised 65 h p.i. before oocyst production and shedding. Calves shedding oocysts should be considered as infected from another source.
- At necropsy the most heavily infected region, i.e. the last 12 m of small intestine measured proximally from the ileocaecal valve is sectioned into 10-cm portions and split lenghtwise, then vigorously stirred for 20 min in 2 liters of calcium- and magnesium-deficient HBSS containing 5 mM dithiothreitol and 10 mM EDTA (pH 7.4) at 4°C.
- The resulting suspension is centrifuged in 500 ml bottles for 10 min at 1800 x g to remove cellular and faecal debris. The supernatant is centrifuged for 30 min at 4100 x g to pellet the merozoites, which are resuspended in 1000 ml of RPMI 1640 medium (Sigma), centrifuged for 30 min at 4100 x g, then resuspended in 150 ml of RPMI and transferred to 15-ml conical centrifuge tubes.

- The suspension is centrifuged for 5 min at 1000 x g to remove residual cellular and faecal debris. The supernatant is centrifuged for 20 min at 4100 x g, and the resulting merozoite pellet, washed an additional three times with RPMI, is resuspended in 15 ml of RPMI.
- Then, a Percoll density gradient is used to purify 1-ml fractions of this merozoite suspension (see isopycnic Percoll centrifugation, section 9.3.).
- The fraction of Percoll containing merozoites is washed in 12 ml of RPMI and centrifuged for 20 min at 4100 x g. They are resuspended in RPMI and counted with a haemocytometer using interference-contrast microscopy. Merozoite viability may be determined with fluorescein diacetate and confirmation that the isolated organisms are merozoites and not contaminating sporozoites may be obtained by transmission electron microscopy.

11.2. Isolation from suckling mice

Smaller numbers of merozoites may be obtained from suckling mice as follows [38]. SPF five-day-old mice are orally inoculated by stomach tube (see suckling mice or rats, section 2.2.) with 10^6 oocysts and euthanized 65 h p.i. before oocyst production and shedding. At necropsy, the small intestine is severed at the ileocaecal junction, dissected free of mesentery, laid out and divided into four parts, each approximately 30 mm in length. Each segment is flushed vigorously with 1 ml of calcium- and magnesium-deficient HBSS containing 5 mM dithiothreitol and 10 mM EDTA (pH 7.4) at 4°C. About 10^6 merozoites per mouse are obtained.

12. Cultivation of *Cryptosporidium*

12.1. Cultivation in embryonating eggs [19, 43]

Both *C. parvum* [19] and *C. baileyi* [43] can complete their entire life cycle in chicken embryo's. *C. baileyi* grows in endoderm cells of the chorio-allantoic membrane (CAM) of chicken embryos from virtually any flock and large numbers of oocysts can be harvested from the chorio-allantoic fluid, making it possible to pass the parasite from embryo to embryo. *C. baileyi* has been transferred from embryo to embryo every week for more than 3 years without noticeable changes in infectivity for chicken embryos or chickens nor in pathogenicity [23]. In contrast to *C. baileyi* the mammalian parasite (*C. parvum*) will grow only in embryos obtained from certain flocks [23]. Trial and er-

ror is the only way to determine if the embryos from a certain origin are suitable. Unlike *C. baileyi*, oocysts of *C. parvum* are often not released from endoderm cells of the CAM into the allantoic fluid and are therefore difficult to recover in large numbers.

- **Procedure**

The following cultivation technique gives good results.

- Candle 10-days-old embryos and mark an inoculation site approximately 0.5 cm below the air sac avoiding blood vessels in the CAM.
- Sterilise the inoculation site and make a small hole in the egg shell with a 23-G needle. To prevent bacterial contamination it may be advisable to inject 0.1 ml containing 1000 i.u. of penicillin and 1 mg of streptomycin or other suitable antibiotics with a 26-G needle.
- Resuspend washed sporozoites in HBSS at 10^7/ml and inoculate 0.1 ml of the sporozoite suspension into each embryo using a 1 ml syringe with a 26-G needle.
- Seal the inoculation site with melted wax and further incubate the embryos for 7 days.
- Cut away the egg shell covering the air sac at the top of the embryo using scissors and moisten the CAM surface with sterile HBSS in order to visualize the blood vessels with a bright light.
- Puncture the CAM avoiding large blood vessels in an area near the shell with a sterile Pasteur pipette and aspirate the allantoic fluid. Approximately 10-15 ml of fluid can be obtained from each embryo.
- Pool the allantoic fluids and centrifuge at 1000 x g for 15 min. Resuspend the pellet containing oocysts in distilled water with 0.02 % Tween-20 (H_20-T), wash twice in H_20-T by centrifugation and store the oocysts overnight at 4°C.

Oocysts may be purified from debris by incubating in HBSS with cold 20 % commercial bleach for 10 min followed by 4 times washing with HBSS by centrifugation. The pellets that are formed may have two layers: a darker upper layer with cellular debris from CAM and a bottom white layer of oocysts. Aspiration of the top layer results in highly purified oocysts.

12.2. Maintenance in cell cultures [20, 25]

C. parvum can complete its life cycle in a variety of cultured cells. Yet, the multiplication ratio and the number of oocysts produced is much lower than in experimentally infected animals or chicken embryos. This may be attributed to the absence of the auto-infective oocysts that develop in the natural host and in the CAM of chicken embryos. Attempts to grow other species of *Cryptosporidium* in cultured cells have been disappointing: several attempts to grow *C. baileyi* have been unsuccessful [43] and at least one attempt to grow a bovine strain of *C. muris* [26] has also failed. The procedures outlined below are similar to those used for growing the parasite in chicken embryos.

- **Procedure**

- Select an appropriate cell line [e.g. human fetal lung cells (HFL) or human colon carcinoma cells (CACO2 cells)] or a primary cell culture (porcine kidney or chikken kidney) and grow to approximately 75 % confluency on round coverslips in the bottom of 24-well plates.
- These cultures may be inoculated with unpurified or purified sporozoites. Yet it is easier to monitor the different developmental stages of the parasite in the cell layer when oocyst walls and intact oocysts are removed from the inoculum.
- Resuspend the excysted sporozoites in tissue culture medium supplemented with 5-10 % fetal bovine serum, 2 mM l-glutamine, 50 mg/ml streptomycin and 50 i.u./ml penicillin G.
- Inoculate 2-5 x $10^4/cm^2$ of cell monolayer and incubate at 37°C in 5% CO_2 and 95% air.
- After 4-12 h the monolayer should be washed by aspiration with a pipette and the culture medium should be changed in order to remove the sporozoites that did not invade the cells.
- Culture medium should be changed every 2 to 4 days. Once the cell layer becomes confluent, it may be necessary to reduce the fetal bovine serum to 1-2 % to prevent overgrowth.

13. Cryopreservation of oocysts and sporozoites [30, 63]

Failure to infect mice with **sporozoites** following a variety of protocols for type and concentration of cryoprotectant, as well as variations in rate of cooling, indicates that

Cryptosporidium differs from several species of the related genus *Eimeria* that can be deep-frozen in this manner.

Oocysts frozen at - 0.3°C per min from 4°C to -70°C in HBSS with 5% DMSO were infectious for hamsters immunosuppressed one week before and 2 and 3 days after challenge with hydrocortisone acetate and x-irradiated at 500 rad on the day of challenge [63], whereas similarly treated oocysts failed to induce infection in neonatal mice [30].

14. Detection of immune responses of hosts

14.1. Indirect immuno-fluorescent antibody test (IFAT) [12, 13, 23, 24, 40, 67]

The first publications mentioned infected mouse intestine as antigen [12, 67]. This technique allows to detect antibodies directed against all life cycle stages. However, as oocysts are more readily available, most authors prefer an IFA procedure based on purified oocysts as antigens.

- **Procedure**

- *Cryptosporidium* sp. oocysts are purified as described above and resuspended in distilled water at a concentration of approximately 10^6 oocysts per ml.
- A volume of 40 µl of the oocyst suspension (40,000 oocysts) is added to each well of a 10-well multispot microscope slide. The suspension should be shaken regularly before adding in order to obtain an even distribution. After allowing the oocysts to sediment for 30 min at room temperature, the preparations are air-dried for 1 h at 37°C. If multispot slides are not available, wells can also be made with a wax pencil. Air dried antigen preparations may be kept for up to 12 months if the slides are frozen (-70°C) in a desiccator.
- Before use, the oocysts are fixed in cold 95% ethanol or aceton for 10 min and air-dried.
- Three-fold dilutions of test-serum are made in PBS and 50 µl are added to each well. Then the preparations are incubated for 30 min at 37°C in a moist chamber. An appropriate initial dilution of test serum is 1:10 in order to avoid unspecific reactions. After incubation, the slides are washed three times in PBS for 5 min and air-dried.

- Subsequently 50 µl of appropriately in PBS diluted FITC-conjugated antibody that will recognise the host species and the class of desired antibody is added to each well, incubated for 30 min at 37°C in a moist chamber, washed three times by incubating in PBS for 5 min and air-dried. To avoid background fluorescence, an appropriate blocking agent such as 1% bovine serum albumin or 1% gelatin may be added to the PBS. Addition of 0.01% (w/v) Evans blue counterstain to the diluted FITC-conjugate results in a red fluorescence of contaminating debris and yeast cells.
- Then one drop of buffered glycerin (9:1) is added to each well and the slides are read at a magnification of 400 x. Oocysts fluoresce bright green against a dark background. Take as end-titer the last dilution in which at least 50% of oocysts show a clear fluorescence.

For experimental purposes the same technique may be used with purified sporozoites. Infected mouse intestine can be used to detect antibodies to all life cycle stages of the parasite.

14.2. Enzyme linked immuno-sorbent assay (ELISA) [23, 55, 68, 69]

Purified *Cryptosporidium* sp. oocysts are suspended in coating buffer (0.1% M sodium carbonate bicarbonate buffer, pH 9.6) at a concentration of approximately 10^8 oocysts per ml and disrupted by sonication. The disruption process may be monitored microscopically. Antigen may also be prepared by repeated freeze-thaw cycles as outlined for the preparation of polyclonal antisera in section 7.1. After the last thawing step add 1% (w/v) sodium dodecyl sulfate to the ruptured oocyst-sporozoite suspension, centrifuge at 10,000 x g for 20 min at 4°C and recover the supernatant. The stock antigen is prepared by diluting the supernatant 1:10 in coating buffer. This antigen may be stored at -70°C as 1 ml aliquots.

- **Procedure**

- Dilute the stock antigen in carbonate buffer (pH 9.6) to a concentration of 7 µg of protein per ml as determined by the Lowry method (1 µg of protein = +/- 150,000-170,000 oocysts/ml). A total of 100 µl of the diluted stock-antigen is added to the wells.
- After incubation for 1 h at 37°C and then at 4°C overnight, the plates are washed with PBS-pH 7.2 containing 0.05% Tween 20 (PBS-T). Wells are blocked with 1%

gelatin, 1% horse serum or other appropriate blocking agents in carbonate buffer at 37°C for 1 h to limit non specific reactivity. The plates may be processed immediately or stored up to 14 days at 4°C.

- The plates are washed three times with PBS-T and test serum of faecal extracts are diluted in PBS-T containing 1% of the blocking agent (BA) used above (PBS-T-BA). A volume of 100 µl of diluted faecal extract or serum is added to the wells and incubated for 1 h at 37°C.
- After washing three times with PBS-T, the wells are incubated for 1 h at 37°C with 100 µl of appropriately diluted (in PBS-T-BA) peroxidase-conjugated antibody that will recognise the host species and class of antibody of interest.
- When class specific conjugates are not available for the host species of the test serum, a sandwich technique may be used. In this case the wells are incubated for 1 h at 37°C with host and class specific antibody raised for instance in goats after reaction of the test serum or faecal extract with the antigen. After further washing, the wells are subsequently incubated with peroxidase-conjugated anti-(goat) IgG for 1 h at 37°C.
- After a further wash 100 µl of appropriate substrate such as TMB (3,3', 5.5'-tetramethylbenzidine, Kirkegaard Perry Laboratories, Gaithersburg, MD, USA, No. 50-76-00) is allowed to react at room temperature for 15 min. The reaction is stopped by adding 1 M o-phosphoric acid and the optical densities (OD) are read at 450 nm in a spectrophotometer.
- The absence of non-specific binding of the various anti-Ig may be tested by direct incubation on the plates with antigen on them. Moreover, each test should be run with negative and positive faecal or serum samples. The last dilution of each sample giving an OD value larger than twice the mean OD of 10 negative control samples is considered as end-titer.

14.3. Sporozoite neutralization assay [60]

After obtaining pure sporozoites (see section 9) 15 µl of HBSS suspension containing 2 x 10^5 sporozoites are added to each well of a 96-well round-bottom tissue culture plate and mixed with an equal volume of HBSS or heat-inactivated serum diluted 1:5 with HBSS (final serum dilution 1:10). Then the plates are incubated at 37°C for 30 min in a 5% CO_2 atmosphere. The viability of the isolated sporozoites before and after incubation may be determined with fluorescein diacetate [50] or tested "in vivo" in 4 to 6-day-old mice by rectal administration. Therefore sporozoites are suspended by drawing up and then gently expulsing the well contents 5 times with a 25-µl Hamilton

syringe. Then 7-day-old SPF mice are injected through the rectum (see section 2.2.) and scored for intensity of *Cryptosporidium* infection (see section 15.1.).

14.4. Oocyst neutralization assay [57]

Oocyst neutralisation properties may be tested by a similar approach. Mice, 3 to 5 days old, are separated from their dams for 1 h to minimise gastric content and then dosed with 65 µl of the appropriate immune or control solution by gastric intubation as outlined in section 2.2. on suckling mice or rats. Two hours later they receive intragastrically a suspension containing 2 x 10^4 purified oocysts (treated with 3% peracetic acid at room temperature for 10 min to kill non-oocyst microbial agents) in 10 µl of medium that were mixed with 55 µl of the appropriate immune or control solution and incubated at 37°C for 30 min. Then individual mice are placed in paper cups and kept at 37°C for 2 h before beeing returned to their dams. Each mouse is treated again by gastric intubation with 65 µl of relevant solution at 20 h post oocysts injection. They are scored for intensity of *Cryptosporidium* infection as outlined in section 15.1.

15. Measurement of disease impact on host animals

15.1. Lesions score [8, 60]

The infectivity of *Cryptosporidium*-strains or the neutralising activity of specific monoclonal or polyclonal antibodies on sporozoites may be evaluated histologically. Mice are inoculated intra-gastrically or into the colon through the anus and euthanized according to animal protection regulations 96 h post challenge. The entire intestinal tract is collected immediately after euthanasia, cut into 2.5-cm sections, and fixed longitudinaly in buffered neutral formalin. Hematoxylin-eosin-stained longitudinal sections representing the entire length of the intestinal tract are prepared and examined histologically. Infection scores reflecting the density of organisms in the intestinal mucosa are assigned for duodenum, jejunum, ileum, caecum and colon as follows:

0 : absence of infection
1 : 1-33% of mucosa parasitised
2 : 34-66% of mucosa parasitised.
3 : more than 66% of mucosa parasitised.

The sum of individual scores for the 5 different intestinal regions are calculated and may vary between 0 (absence of infection) and 15 (maximal infection). Also the biliary and pancreatic-duct epithelium may be infected and should be scored in order to determine if therapeutic agents directed against the autoinfective sporozoite and merozoite stage have access to the biliary and pancreatic ductal system [8].

15.2. Weight gain

When performing trials in chickens and rabbits changes in weight gain due to the parasite may be taken into account. When working with large animals (calves, sheep) disease impact is difficult to asses: the first week after birth most calves lose weight and recover during the second week, which makes evaluation of *Cryptosporidium* associated weight loss difficult.

15.3. Diarrhoea

In most animal species cryptosporidiosis causes diarrhoea. Whereas in laboratory animals diarrhoea may be scored on a 0-3 scale (1: pastose faeces, 2: semi-liquid faeces and 3: liquid faeces) assessment in diarrhoeic calves is difficult: 60-70% of animals show diarrhoea and faeces is liquid in only half of diarrhoeic calves. Fayer et al. [29] consider bovine faeces as diarrheal when it is liquid, watery or immediately formless upon collection, as opposed to normally soft faeces that require a few min or longer to become formless. During our trials evaluating efficacy of anticryptosporidial drugs in calves [72], diarrhoea was scored as in laboratory animals. Often faeces contain strands of mucus or take a pure mucinous aspect. This occurs mostly simultaneously or shortly after *Cryptosporidium* oocyst output starts.

16. Production of hyperimmune colostrum in cows [29]

Hyperimmune colostrum may be obtained by inoculating cows parentally with 2×10^7 purified *C. parvum* oocysts in 2 ml HBSS mixed with 2 ml of complete Freund's adjuvant 10 weeks before parturition. Half of the inoculum is given i.m. at 2 sites on the neck. At 8 weeks before parturition 2×10^7 oocysts in 1 ml HBSS mixed with 1 ml incomplete Freund's adjuvant is infused through the orifice of 2 teats into the udder; at 6 weeks and 4 weeks before parturition 5×10^6 oocysts mixed with incomplete Freund's

adjuvant are similarly infused into the same two quarters of the udder. Following the infusions, each of the 2 quarters of the udder is massaged to distribute the oocyst mixture throughout the cysternae of the gland. Within 24 h after parturition colostrum is collected twice by hand milking. The colostrum is centrifuged at 1300 x g for 25 min and the center whey fraction is collected and stored at -70°C. Hyperimmune colostrum contains high levels of specific IgG1, IgM and IgA antibodies. IgG2 is normally not present.

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Part III. *Sarcocystis* species

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In this chapter methods are described that have been used in research or diagnosis of *Sarcocystis* spp. Methods that are used regularly and with success in at least one of our laboratories are outlined in detail. In addition, we have given references for those methods that have been used by other researchers, but with which we have no own experience.

To date about 130 *Sarcocystis* spp. have been described which differ in life-cycle, biology, morphology and pathogenicity. Therefore, it may be necessary to optimise conditions if a method described in this manual is used for a *Sarcocystis* species different from that for which it was originally developed, and not all methods described here may be useful for each *Sarcocystis* species.

1. Host animals and maintenance

The various species of *Sarcocystis* are highly specific for their intermediate hosts, and most of them are family-specific for their final hosts. It is generally not possible to passage *Sarcocystis* spp. from intermediate host to intermediate host, or from final host to final host.

Intermediate hosts used for the production and isolation of parasites have to be reared coccidia-free. Mice purchased from specific-pathogen-free (SPF) breeding stations are usually free of coccidia. The safest way to obtain coccidia-free domestic animals is to buy pregnant animals and to use their offsprings for the experiments. All animals must be kept under special conditions as described below (see 1.1.).

Some mouse strains are more susceptible to *Sarcocystis muris* than others. For example, NMRI-nu/nu and AKR/N Han mice are highly susceptible, i. e. infection rates can be as high as 100 %. By contrast, C3H/He mice are less susceptible, i. e. infection rates are less than 25 % [6]. Nothing is known about the susceptibilities of different breeds of domestic animals.

Final hosts should not excrete sporocysts upon consecutive examinations during a period of at least 14 days before the beginning of an experiment. Since final hosts develop at least partial immunity, it is advisable to rear them free of *Sarcocystis*, especially if immunological studies are to be carried out.

1.1. Maintenance of host animals

- **Stables**

  Intermediate hosts that are going to be used for propagation of pure *Sarcocystis* strains must be reared and kept in an isolation unit for the whole period of the experiment. Animal attendants must wash their hands thoroughly and change their clothes when entering the unit. Animals have to be bedded on decontaminated material only. Infectious stages (sporocysts) are killed at 60 °C after 30 min [27]. It is extremely difficult to keep a control group of mice free of *Sarcocystis* when other groups have to be infected simultaneoulsy and when there is only one person available to look after the animals.

  No special care has to be paid to final hosts. However, care needs to be taken that they are not fed with raw meat and that they cannot hunt rodents.

- **Cages**

  Mice should be kept in Makrolon cages. Small ruminants and pigs are best kept on plastic slatted floors that may be covered with rubber mats. Cattle may be kept on concrete floors covered with decontaminated straw. All housing equipment has to be decontaminated before animals are brought into the isolation unit.

- **Food and water**

  No special diet is necessary for intermediate hosts but it has to be made sure that all food and drinking water is free of infective sporocysts and free of coccidiostats. This is best achieved by feeding the animals on heat-treated food pellets. Hay and straw for small ruminants or cattle must be autoclaved.

  Final hosts should receive only thoroughly cooked or otherwise sterilised food. It is advisable to feed carnivores on commercial pellets with low fat content during

the experimental period, because a high fat content in the faeces interferes with the flotation of sporocysts.

1.2. Infection of animals

- **Mode of infection**

Intermediate hosts are infected orally by stomach tube, by bringing the infective material into the buccal cavity with a syringe (risk of contaminating the environment), by application of the sporocyst suspension in a gelatine capsule (small ruminants) or by mixing the sporocyst suspension into a small amount of mashed food (pigs). The infection dose should not exceed 50-100 infective sporocysts of *S. muris* and 25,000 infective sporocysts of *Sarcocystis* spp. infecting livestock. Higher doses may result in no cyst formation at all (e. g. *S. muris*) or in an acute and sometimes lethal disease (e. g. *S. cruzi*, *S. tenella*, *S. arieticanis*, *S. miescheriana*, *S. suihominis*).

The safest way to avoid accidental infections of control animals is to infect the principals by injecting them excysted sporozoites intrapertioneally.

Final hosts are infected by feeding them meat containing cysts of the respective *Sarcocystis* sp. Cats and dogs must be accustomed to the type of meat before the infection. For this conditioning meat must be derived from animals that are free of coccidia (e. g. SPF mice). Whereas cats and dogs infrequently respond to high doses of some *Sarcocystis* spp. (e. g. *S. capracanis*) showing mild clinical symptoms such as transient diarrhoea, human volunteers may develop serious clinical symptoms (e. g. diarrhoea, vomiting, dizziness, tachycardia) after ingesting cysts of *S. suihominis*.

- **Preparation of infective doses**

Sporocysts are counted using a McMaster counting chamber, and their numbers are adjusted to the required infective dose by adding water. According to McKenna and Charleston [38] counting of sporocysts using a haemocytometer is more accurate than using a McMaster counting chamber.

Meat containing cysts for the infection of final hosts can be stored at 4 °C for about one week, but for highest infectivity it should be used as fresh as possible. It is not possible to count cysts in meat.

2. Isolation of parasites from host animals

2.1. Sporocysts and sporozoites

2.1.1. Recovery of sporocysts

Sporocysts are either collected from the faeces or from mucosal scrapings of the small intestine of the final host. The latter method is much less time-consuming, yields greater numbers of sporocysts and prevents accidental infection of other susceptible intermediate hosts.

Faecal material is flotated with $NaCl/ZnCl_2$ solution or $ZnSO_4$ solution (specific gravity 1.3). The sporocysts are aspirated from the surface with a Pasteur pipette the tip of which is broken off to give an oval opening. When working with larger volumes, the sporocysts may be aspirated using a 10-20 ml glass pipette with pipette bulb. The opening of the pipette is immersed in the flotation fluid in a way that only half of it is submerged to make sure that exactly the surface is aspirated. If the mouth of the pipette is totally immersed only flotation fluid may be aspirated, leaving the sporocysts on the surface of the fluid. Aspirated sporocysts are washed at least 3 times by centrifugation and resuspension in distilled water. It is important to know that only low numbers of sporocysts are excreted in the faeces of final hosts. Therefore, large amounts of faeces have to be processed to obtain a reasonable number of sporocysts. Other authors have described methods that employ NaCl solution (specific gravity 1.2) for the isolation of sporocysts from faeces [39].

For isolation of large numbers of sporocysts from the mucosa of the small intestine, the final host should be euthanatised in the early phase of the patency period, i. e. 3-5 days after the appearance of the first sporocyst in the faeces. The animals are fasted for 1 day to have as few intestinal content as possible. The mucosa of the entire small intestine is rinsed with water and scraped off with a microscope slide or scalpel blade. To obtain optimal sporocyst yields only those parts of the mucosa that contain sporocysts upon microscopic examination of squash preparations should be used. Portions of the mucosa scrapings are mixed with 300 ml of digestion fluid (0.25 % trypsin in phosphate-buffered saline, pH 7.2-7.4; Trypsin 1:250; Difco Laboratories, Detroit, Mich., USA) at 22 °C and are minced in a blender at high speed for 3 min. For the

preparation of the whole intestine of a dog, up to 3 litres of digestion fluid may be required. To check whether the intestinal villi have been destroyed and whether sporocysts have been released, a drop of the suspension is examined microscopically. If necessary, mincing has to be repeated. Immediately afterwards, each portion is passed consecutively through sieves with 250 µm and 30-40 µm mesh wire into water. The digest is washed at least 3 times by centrifugation (700 *g*, 10 min) and resuspension in water. The sporocysts are then counted (see 1.2.) and stored in water at 4 °C (see 2.1.2.).

2.1.2. Storage and preservation of sporocysts

Sporocysts are stored at 4 °C in water containing penicillin (200 i.u./ml), streptomycin (200 µg/ml) and amphotericin B (1 µg/ml). They remain viable for at least 6 months. The addition of potassium dichromate, which is recommended as a preservative for oocysts of other genera of coccidia, may kill *Sarcocystis* sporocysts [35].

Sporocysts of *S. cruzi* were still infective for calves after storage in water at -22 °C for 10 days, and *S. tenella* sporocysts were infective for lambs after storage in HBSS-PMSF medium at -15 °C to -20 °C for 19 months [34].

2.1.3. Excystation of sporozoites

There are several descriptions of methods for the excystation of sporozoites of different *Sarcocystis* spp. Some of these methods yield sterile sporozoites. Bile appears to be an essential supplement of the excystation fluid. The following method is used in the Berlin laboratory to obtain sterile sporozoites of *Sarcocystis* spp. of goats and sheep [28] or of *S. miescheriana* or *S. muris* [52].

Procedure

- Wash the sporocysts twice in distilled water (1600 x g, 10 min). Resuspend the pellet in 6-8 % sodium hypochlorite, mix well and allow to stand for 10-20 min at 22 °C. Then wash the sporocysts 5 times in distilled water.

- For sonication, a Branson sonifier cell disruptor (W 250, Branson, Conn., USA) can be used. Resuspend sporocysts in 1.5 ml RPMI 1640 medium and sonicate (30 % duty cycle, output control 3) for 2 min on ice.

- Add 10 % fetal calf serum and 15 % bovine bile to the suspension and incubate at 39 °C for 30-60 min. Then wash the sporozoites twice with RPMI 1640 medium (250 x g, 10 min) and count them using a haemocytometer:

$$\% \text{ Excystation} = \frac{\text{Free sporozoites}}{\text{Sporozoites within sporocysts} + \text{Free sporozoites}} \times 100$$

Because sporocysts of different *Sarcocystis* spp. respond differently to pretreatment with sodium hypochlorite, the sodium hypochlorite concentration should be optimised for each *Sarcocystis* sp. Moreover, sonication should be applied according to the prospective use of the sporozoites: If large numbers of sporozoites are needed for use in cell cultures or for parasite antigen preparations, sonication leads to better and faster excystation. However, as sonication is known to cause alteration of surface membranes, it should not be applied if sporozoites are needed for immunological studies or for investigations of sporozoite surface membranes. For a more detailed description see Horn et al. [28].

Other excystation methods have been described for *S. cruzi* [9,19], *S. capreolicanis* [5], *S. suihominis* [5], *S. miescheriana* [5, 56], *S. muris* [16, 71], *S. capracanis* [9, 28, 47], *S. tenella* [9, 28, 47], *S. gigantea* [40], *S. arieticanis* [28, 47] and *S. hircicanis* [28].

2.1.4. Purification of sporozoites

Several methods have been described for purification of excysted sporozoites. The most suitable of these methods are column purification and discontinuous density gradient centrifugation.

S. cruzi sporozoites may be passed through a nylon wool column prepared as described by Larsen et al. [33] [see 54]. Passage of excysted sporozoites through a DE-52 anion exchange column has yielded high recovery rates (70-99 %) for sporozoites of *Sarcocystis* spp. of small ruminants [45] and of *S. muris* [52]. Sporozoites of *S. miescheriana* have been purified by discontinuous density gradient centrifugation as described below for cystozoites (see 2.2.3.), but using a gradient consisting of 90 %, 75 %, 50 % and 30 % isotonic Percoll® stock solution. The sporozoites can be harvested from the 75 % Percoll layer [52].

Here a more detailed description of the method used in the Berlin laboratory is presented: The DE-52 cellulose column is prepared according to Schmatz et al. [49], with some modifications: DE-52 cellulose (Chromedia, Whatman, Maidstone, UK) is prepared with phosphate-buffered saline supplemented with 1 % glucose, pH 8.0 [32]. The barrel of a 1 ml disposable plastic syringe (diameter 5 mm) is plugged at the outlet with a wisp of nylon wool (Fenwal Laboratories, Deerfield, Ill., USA) and loaded with DE-52 cellulose up to a column height of 6 mm. Following in vitro excystation, parasites are washed twice in RPMI 1640 medium and are then resuspended in 2 ml RPMI 1640 medium that has been prewarmed to 39 °C (2×10^6 to 7×10^7 sporozoites per sample). The column is washed with 1-2 ml RPMI 1640 medium, and the parasite suspension is layered on top of the cellulose. Sporozoites are eluted with 10 ml RPMI 1640 medium that has been prewarmed to 39 °C. The eluate is centrifuged at 260 x g, and the pellet is resuspended in 1 ml RPMI 1640 medium. The suspension is examined microscopically for appearance, purity and motility of sporozoites using a haemocytometer [45].

2.1.4.1. Sporozoite viability and infectivity test

The viability of in vitro excysted and purified sporozoites can be assessed by microscopic examintaion of the sporozoite motility or by the trypan blue dye exclusion test.

Procedure

- Prepare a 0.4 % trypan blue solution from a 40 % trypan blue stock solution (T6146; Sigma Chemical Company, St. Louis, Mo., USA). Mix 500 µl of 0.4 % trypan blue solution with 300 µl phosphate-buffered saline in a reaction tube and add 200 µl parasite suspension (about 1×10^6 sporozoites). Incubate for 10-15 min at 22 °C. Viable sporozoites do not incorporate the dye and can be counted in a haemocytometer.

- For assessment of infectivity, sporozoites may be inoculated into monolayer cultures of the appropriate cell type. Infective sporozoites will penetrate the cells and then develop to schizonts (see 3.1.).

2.1.5. Cryopreservation of sporozoites

Excysted sporozoites of *S. capracanis*, *S. tenella* or *S. arieticanis* are washed twice in RPMI 1640 medium and resuspended in cryo-medium (RPMI 1640 containing 10 % fetal calf serum and 7.5 % dimethylsulfoxide) at a concentration of 5×10^4 to 5×10^5 viable sporozoites per ml. Sporozoites are frozen using an automatic deep freezer

(-1 °C/min; Cryoson, Schöllkrippen, Germany) and stored in liquid nitrogen until used. Sporozoites frozen in this way are still viable after 28 days; longer periods of time have not yet been tested. Prior to animal inoculation, parasites are washed in RPMI 1640 medium. Sporozoites are then inoculated intraperitoneally into the respective intermediate host.

Note: Animals infected via the intraperitoneal route show fewer tissue cysts than animals infected orally with a comparable number of sporocysts.

2.2. Cysts and cystozoites

2.2.1. Isolation of cysts

The isolation of intact cysts from host muscles is difficult, and although several methods have been described [2, 37, 68] their success depends greatly on the size of the cysts formed by the *Sarcocystis* sp. under study and on the infection grade of the host. If infection grades are high, cysts $\geq$ 500 µm may be isolated by dissecting muscle samples using a stereo-microscope and recovering the cysts with dissecting needles. To isolate smaller cysts, about 10 g of muscle samples are minced for 30 sec in 80 ml saline using a blender. The suspension is passed consecutively through 1.5 mm and 1.0 mm aperture sieves, and then centrifuged (3000 rpm, 2 min). The supernatant fluid is carefully removed from the top of the tube up to the bottom 3 ml of fluid. This is then mixed thoroughly with the sediment, and drops of this suspension are checked microscopically (magnification 15-50×) for intact cysts.

2.2.2. Isolation of cystozoites

To obtain optimal yields of cystozoites, it is important to know the time needed for maturation of the *Sarcocystis* cysts. Cyst maturation times vary among different *Sarcocystis* spp., and the cysts of some *Sarcocystis* spp. degenerate over time. Immature cysts and degenerated cysts do not contain cystozoites. Therefore, it will not be possible to isolate cystozoites from animals that are slaughtered too early or too late after infection. For *S. muris* and most *Sarcocystis* spp. forming microscopic cysts in livestock, e. g. *S. tenella*, *S. arieticanis*, *S. capracanis*, *S. hircicanis* or *S. miescheriana*, 85-100 days after infection is an optimal time for isolation of cystozoites. By contrast,

the cysts of some *Sarcocystis* spp. forming macroscopic cysts, e. g. *S. gigantea* or *S. moulei*, need 1-4 years to mature.

The method used for the isolation of cystozoites depends greatly on the size of the cysts from which cystozoites have to be liberated, on the infection grade of the host and on the fat content of the host muscles. Because some of the methods that involve enzymatic digestion of large amounts of muscle samples from livestock are very time-consuming, it is advisable to keep the muscle samples from which cystozoites are going to be isolated in phosphate-buffered saline (PBS, pH 7.2-7.4) at 4 °C until they can be processed. This will keep the cysts intact for at least 48 h. Mechanical isolation (see 2.2.2.2.) of cystozoites can be achieved for cysts that are macroscopically visible, such as those of *S. gigantea* or *S. moulei*, or for cysts of *S. muris* in those cases in which susceptible mice (see 1.) have been treated immunosuppressively to increase infection grades [59, 64]. For the isolation of cystozoites from microscopically small cysts, such as those of *S. tenella* or *S. capracanis*, enzymatic digestion of host tissue will yield much greater numbers of cystozoites than mechanical isolation methods.

2.2.2.1. Mechanical isolation using scissors

This is a quick and convenient method for isolation of cystozoites of *S. gigantea* or *S. moulei*. To avoid contamination of the isolated cystozoites with bacteria, which are always present when oesophageal muscles are being dissected, all steps should be carried out using aseptical or sterile conditions. Equipment and buffers should be autoclaved.

Procedure

- Excise intact *Sarcocystis* cysts from host tissue using scissors and transfer the cysts into a dish containing phosphate-buffered saline (PBS, pH 7.2-7.4).

- Wash the cysts repeatedly in the dish with fresh PBS until the buffer stays clear. The following steps should ideally be carried out in a tissue-culture cabinet.

- Finely dice the cysts into pieces with scissors in PBS.

- Transfer the PBS containing the diced cysts to a glass homogeniser. Use 10-20 gentle strokes with the homogeniser to liberate the cystozoites from the cyst walls.

(Alternatively, cystozoites can be liberated from the cyst walls using glass beads as described below for *S. muris*.)

- Transfer the homogenate to a centrifuge tube, and wash the cystozoites 3-5 times by centrifugation and resuspension in PBS until the supernatant buffer is clear.

- Purify cystozoites from cyst wall and host tissue debris as described below (see 2.2.3.).

2.2.2.2. Mechanical isolation using glass beads

This method can be used for small amounts of muscle samples containing large numbers of cysts, such as mouse muscles infected with *S. muris*. Infection grades with *S. muris* in mice can be increased by immunosuppressive treatment with cyclophosphamide (Endoxan®, Asta-Werke, D-Bielefeld; intraperitoneal injection of 5 mg per mouse, 4 times at intervals of 1 week, starting 1 week before infection) or dexamethasone (Voren®-Depot, Boehringer D-Ingelheim; subcutaneous injection of 9 mg/kg body weight, 3 times at intervals of 3 days, starting 2 days before infection). All of the procedures described below should be carried out using aseptical or sterile conditions. Equipment and buffers should be autoclaved.

Procedure

- Anaesthetise the mice according to local animal welfare regulations (for example with ether) and collect as much blood as possible by punctuation of the *plexus retroorbitalis* or by heart puncture. This will reduce the amount of red blood cells, which may interfere with some methods used for the purification of cystozoites (see 2.2.3.).

- Excise pieces of muscles with visible cysts from thorax and extremities of the mice and transfer them to a dish containing phosphate-buffered saline (PBS, pH 7.2-7.4).

- Finely dice the pieces of excised muscles with scissors in PBS.

- Vigorously stir the diced muscle pieces in 200 ml PBS with an equal volume of glass beads (∅ 3-4 mm) using a magnetic stirrer at high speed (45 min, 22 °C).

- Allow glass beads and all material that has not been ground to settle (1-2 min). Then decant the supernatant suspension and filter it through three layers of gauze. Concentrate the filtrate by centrifugation (2500 x g, 10 min).

- Discard the supernatant fluid and wash the sediment 3-5 times by resuspension in PBS and centrifugation as above until the supernatant fluid is clear.

- Resuspend the sediment in PBS and purify cystozoites from host tissue as described below (see 2.2.3.).

2.2.2.3. Tryptic digestion of host tissue

This method is the method of choice in the Hannover laboratory for isolation of cystozoites from skeletal muscles of sheep, goats and cattle, e. g. for cystozoites of *S. tenella*, *S. arieticanis*, *S. capracanis*, *S. hircicanis* or *S. cruzi*. The method has been optimised so that most of the host tissue is digested, but most of the isolated cystozoites will stay intact. Cystozoites isolated by this method can be used for extraction of proteins (see 4.) or nucleic acids (see 6.). A comparison of proteins and RNA of *S. muris* cystozoites isolated by three different methods showed that short-time digestion of host tissue with trypsin does not adversely affect the biological activity of total protein or RNA extracts derived from the isolated cystozoites [64]. By using this method, 10^{10}-10^{11} cystozoites of *S. tenella* and 10^{8}-10^{9} cystozoites of *S. arieticanis* can be isolated from one experimentally infected sheep.

Procedure

- Excise skeletal muscles from the carcasses of slaughtered animals. Remove all fat, ligaments and connective tissue, because these may interfere with the digestion.

- Finely mince 30-50 g of muscle samples with 150 ml of digestion fluid* (see below) for 20 sec at high speed in a 1 l laboratory blender (Waring®, Dynamics Corporation of America, New Hartford, Conn., USA). The ratio of muscles to digestion fluid depends on the type of muscles and the amount of fat and connective tissue present. A ratio of 1/4 (w/v) should not be exceeded. For some types of muscles it may be necessary to decrease this ratio to 1/5 (w/v) or 1/6 (w/v). The volume of

muscle samples and digestion fluid filled into the blender should not exceed 1/5th of the blender's capacity, because otherwise muscle samples will not be mixed properly with the digestion fluid.

*2.5 g trypsin (Trypsin 1:250; Difco Laboratories, Detroit, Mich., USA) per litre 0.01 M phosphate-buffered 0.15 M saline (PBS). The pH value of this digestion fluid is 7.2-7.4.

- Stir the suspension at 37° C for 10-15 min in a magnetic water bath. This step should not be carried out longer, because otherwise cystozoites will be released from the cysts and may be affected by the digestion.

- Filter the digest through three layers of gauze. Add 0.5-1 volume of cold PBS and centrifuge (2500 x g, 15 min, 10° C) this suspension immediately to stop the digestion.

- Discard the top 3/4 of the supernatant fluid. Resuspend the bottom 1/4 contained in the centrifuge tube in fresh PBS and centrifuge as above.

- Discard the supernatant fluid and wash the sediment 3-5 times (i. e. until the supernatant fluid is clear) by resuspension in PBS and centrifugation as above to remove all of the digestion fluid.

- The isolate containing *Sarcocystis* cysts and cystozoites can now be stored at 4° C in PBS for up to one day. It can then be combined with isolates obtained from other muscle samples for purification of the cystozoites (see 2.2.3.).

Note: If the infection grade of the animal host is low, i. e. if a large amount of undigested host tissue is still present in the isolate obtained at this stage, the isolate may be digested a second time to reduce the amount of material that has to be purified using the method described below (see 2.2.3.). For this proceed as follows:

- Suspend the isolate in PBS and mix with an equal volume of 2x digestion fluid (5 g trypsin/l PBS). Stir the suspension at 37° C for 10-20 min in a magnetic water bath. Check the result of the digestion every 5 min by microscopical examination.

- Filter the digest through three layers of gauze, add 0.5-1 volume of fresh PBS and centrifuge (2500 x g, 15 min, 10° C) immediately to stop the digestion.

- Discard the supernatant fluid. Wash the sediment 3 times by resuspension in PBS and centrifugation as above to remove all of the digestion fluid.

- Purify cystozoites from tissue debris as described below (see 2.2.3.).

2.2.2.4. Peptic digestion of host tissue

This method may be more feasible than digestion with trypsin if *Sarcocystis* cystozoites have to be isolated from host muscles containing a high amount of fat (e. g. cystozoites of *S. miescheriana* or *S. suihominis*). Tryptic digestion is usually less efficient when fat is present in the digestion fluid. In these cases, digestion of contaminating host tissue with pepsin may give higher yields of cystozoites. Short-time digestion of host tissue with pepsin does not adversely affect the biological activity of RNA extracts derived from the isolated cystozoites [64]. Therefore, cystozoites isolated by this method can be used for extraction of nucleic acids (see 6.). However, some quantitative differences were found between the protein profile of *S. muris* cystozoites isolated by peptic digestion and that of *S. muris* cystozoites isolated mechanically [64]. Therefore, this method may have some disadvantages if proteins are to be extracted from the isolated cystozoites.

Procedure

- Excise skeletal muscles from the carcasses of slaughtered animals. Remove as much fat, ligaments and connective tissue as possible.

- Finely mince 50 g of muscle samples with 150 ml of digestion fluid* for 20 sec at high speed in a 1 l laboratory blender (Waring®, Dynamics Corporation of America, New Hartford, Conn., USA).

  * 1,500 FIP-U pepsin (E. Merck, D-Darmstadt) per litre PBS containing 0.25 % v/v HCl. The pH value of this digestion fluid is 1.9-2.1.

- Stir the suspension at 37 °C for 20 min in a magnetic water bath.

- Filter the digest through three layers of gauze, add an equal volume of cold PBS (pH 7.2-7.4) and immediately centrifuge (2500 x g, 15 min, 10° C) this suspension to stop the digestion and restore physiological pH conditions.

- Discard the top 3/4 of the supernatant fluid. Resuspend the bottom 1/4 contained in the centrifuge tube in fresh PBS and centrifuge as above.

- Discard the supernatant fluid and wash the sediment 3-5 times (i. e. until the supernatant fluid is clear) by resuspension in PBS and centrifugation as above to remove all of the digestion fluid.

- The isolate containing *Sarcocystis* cystozoites can now be stored at 4 °C in PBS for up to one day. It can then be combined with isolates obtained from other muscle samples to purify the cystozoites from tissue debris as described below (see 2.2.3.).

2.2.3. Purification of cystozoites

The method of choice in the Hannover laboratory for the purification of *Sarcocystis* cystozoites from cyst wall and host tissue debris is a discontinuous density gradient centrifugation. This method yields preparations containing >95 % cystozoites [59]. If the cystozoites have been isolated mechanically (see 2.2.2.), some contamination may occur with host red blood cells which sometimes have a density similar to that of *Sarcocystis* cystozoites. If the cystozoites have been isolated by digestion of host tissue (see 2.2.2.), it is possible to obtain preparations containing >99.9 % cystozoites. No host cell nuclei were detected in Giemsa stained smears of *S. tenella* and *S. arieticanis* cystozoites that were obtained by digestion of sheep muscles with trypsin and subsequent purification of the isolated cystozoites by density gradient centrifugation.

It is important to know that the density of *Sarcocystis* cystozoites varies with the age of the cysts and among different *Sarcocystis* spp. Therefore, the composition of discontinuous density gradients for purification of cystozoites has to be optimised accordingly. However, the following method can be regarded as a general guideline. All steps should be carried out using sterile conditions.

Procedure

- Prepare an isotonic stock solution of Percoll® (density 1.13 g/ml, osmolarity 13-16 mosmol/kg H_2O; Pharmacia LKB Biotechnology, Uppsala, Sweden) by adding 1 volume of 10× PBS (pH 7.2-7.4) to 9 volumes of Percoll. For the bottom layer of the gradient, dilute the isotonic Percoll stock solution with 1× PBS to give a solu-

tion of 80-90 % Percoll. For the top layer of the gradient, dilute the isotonic Percoll stock solution with 1× PBS to give a solution of 40-50 % Percoll.

- Pipette 4 ml of high-density Percoll solution (80-90 %) into 15 ml conical centrifuge tubes and overlay them with 4 ml of low-density Percoll solution (40-50 %) to prepare discontinuous density gradients. The number of tubes needed to purify about 10^9 cystozoites depends greatly on the *Sarcocystis* sp., on the method used for isolation of the cystozoites (see 2.2.2.) and on the degree of contamination with host tissue. For example, 2 tubes may be sufficient for the purification of *S. gigantea* cystozoites whereas 200 tubes or more may be necessary for the purification of *S. arieticanis* cystozoites.

- Homogenise preparations of *Sarcocystis* cystozoites isolated by one of the methods described above (see 2.2.2.) by 10-20 gentle strokes in a glass homogeniser. This should remove all lumps in the preparation to prevent clotting at the interphases of the gradient. Dilute the homogenate with PBS to give a transparent suspension. If the suspension from which cystozoites are to be purified is too concentrated, clotting may occur during centrifugation.

- Overlay the Percoll gradients with 7 ml of the homogenised suspension and centrifuge the tubes for 30-40 min at 2500 x g and 18° C. After centrifugation, a white layer containing the purified cystozoites should be found in the conical part of the centrifuge tube. Host cell debris is usually found at the interphase between the top, low-density Percoll layer and the supernatant PBS, while some cyst wall debris forms a pellet underneath the bottom, high-density Percoll layer. If cystozoites and debris have not separated properly, the density of the two Percoll layers has to be optimised.

- Remove and discard the top 12 ml of fluid from the centrifuge tubes.

- Collect the layer containing the cystozoites and combine it with those collected from other density gradient tubes. Add at least 1/3 volume of PBS and centrifuge (2500 x g, 10 min, 15° C) to remove the Percoll.

- After centrifugation, the purified cystozoites should be pelleted at the bottom of the tube. Resuspend the pellet in PBS and wash the cystozoites 3 times by centrifugation (2500 x g, 10 min, 4 °C) and resuspension in PBS.

2.2.4. Storage of cystozoites

Cysts in host muscles or isolated cystozoites do not survive freezing. Cryopreservation of viable cystozoites in cryo-medium in liquid nitrogen has not yet been attempted. To preserve the molecular composition of purified cystozoites (see 2.2.2.) for immunological or molecular biological studies cystozoites should be stored as follows.

- **Lyophilisation**

Pellet the cystozoites by centrifugation and remove the supernatant buffer. Suspend the cystozoites in a small volume of ultrapure or double-distilled water and transfer them to a vial that is suitable for lyophilisation. Snap-freeze the cystozoites in liquid nitrogen or freeze them overnight at -80° C. Lyophilise the frozen preparation and store this at -20° C. Cystozoite preparations stored by this method can be used for extraction of proteins (see 4.) or for extraction of genomic DNA (see 6.1.).

- **Storage at -80 °C**

For extraction of proteins (see 4.): Pellet the cystozoites by centrifugation. Suspend the cystozoites in a small volume of 0.01 M phosphate-buffered 0.15 M saline (pH 7.2-7.4). Freeze and store this preparation at -80 °C.

For extraction of genomic DNA (see 6.1.): Pellet the cystozoites by centrifugation. Remove and discard the supernatant buffer. Add 0.5-1 ml of 10 mM Tris·HCl / 10 mM EDTA (pH 8.0) to the pellet and gently mix by finger-tapping. Freeze and store this preparation at -80° C.

For extraction of cellular RNA (see 6.2.): Pellet the cystozoites by centrifugation. Remove and discard the supernatant buffer. Add 1 ml of 6 M guanidine hydrochloride / 0.2 M sodium acetate (pH 5.2) / 1 mM 2-mercaptoethanol to the pellet and gently mix by finger-tapping. Snap-freeze this preparation in liquid nitrogen and store it at -80° C.

- **Storage in liquid nitrogen**

This is the method of choice for storage of cystozoites in the Hannover laboratory. Pellet the cystozoites by centrifugation in a cryopreservation tube. Completely remove and discard the supernatant buffer. Snap-freeze and store the pellet in liquid nitrogen. This method allows the use of the cystozoites for extraction of proteins (see 4.) as well

as nucleic acids (see 6.). The respective extraction buffer is added to the pellet immediately after removal from storage. Cystozoites stored by this method can also be used for extraction and purification of messenger RNA (see 6.3.); however, in these cases it may be preferable to add 1 ml of 6 M guanidine hydrochloride / 0.2 M sodium acetate (pH 5.2) / 1 mM 2-mercaptoethanol to the cystozoite pellet before storage in liquid nitrogen.

3. In vitro cultivation

3.1. Stages of the vascular phase

Methods have been developed for the cultivation of the vascular phase of *S. cruzi* [53, 54], *S. capracanis* [55], *S. tenella* [55] and *S. hirsuta* [10]. All cultures are started with sporozoites. *S. capracanis* can be grown in bovine monocytes, *S. hirsuta* in bovine pulmonary artery endothelial cells, and *S. cruzi* and *S. tenella* in both types of culture systems. *S. cruzi* has now been maintained in culture for several years by continuous subculturing of merozoites [1]. Recently, *S. neurona* has been cultured from the homogenate of spinal cord tissue derived from an infected horse and inoculated into bovine monocyte cultures [13].

3.2. Gamogonic stages

Gamogonic stages can be grown right down to the stage of oocysts in tissue cultures inoculated with cystozoites. The first species cultivated was a *Sarcocystis* sp. from grackles (*Quiscalus quiscula*) in bovine kidney cells [70] or in bovine tracheal cells [20]. Since then, *S. suihominis* has been cultured in human skin fibroblasts [4, 41], *S. cuniculi* in feline embryonic fibroblasts [58], and *S. tenella* in ovine embryonic kidney cells [14] or in canine kidney cells [4]. *S. muris* can be grown in feline lung cells [4], in canine kidney cells [15] or in feline embryonic cells [17].

4. Extraction of proteins

4.1. Extraction of sporozoite proteins

Proteins have been extracted from sporozoites and subjected to various electrophoretic procedures using standard techniques such as those of Laemmli [31] or O'Farrell [44]. They have also been extracted for isoenzyme analysis and studies on surface proteins using biotinylation (see below). For all these applications, it has to be taken into account that the protein content of *Sarcocystis* sporozoites is only about half of the protein content of cystozoites.

4.1.1. Extraction of sporozoite proteins for isoenzyme analysis

Whereas isoenzyme patterns of *Sarcocystis* cystozoites have been studied using cellulose acetate gel electrophoresis [21, 43], Greiner et al. [24] analysed isoenzyme patterns of *Sarcocystis* sporozoites by polyacrylamide gel electrophoresis using the modified method of Harris and Hopkinson [26] described below. This method gives better resolution of bands than cellulose acetate gel electrophoresis.

Procedure

- In vitro excyst and purify sporozoites as described above (see 2.1.3. and 2.1.4.).
- Resuspend sporozoites in 0.05 M Tris·HCl, pH 8.0.
- Lyse sporozoites by freezing (-80°C) and thawing the suspension 3 times followed by sonication (see 2.1.3.).
- Centrifuge the suspension at 50,000 x g (30 min, 4° C) and collect the supernatant fluid containing water-soluble proteins. Membrane proteins can be extracted from the pellet with 1 % Triton X-100.
- Electrophorese the extracted proteins on polyacrylamide gels for study of isoenzyme patterns [24].

4.2. Extraction of cystozoite proteins

Proteins have been extracted from *Sarcocystis* cystozoites for analyses using various electrophoresis or chromatography methods [61, 63], or for application in diagnostic assays [see 10.1.). Cystozoites used for extraction of proteins can either be isolated mechanically or by tryptic digestion of host tissue (see 2.2.2.). The latter method does not adversely affect the biological activity of the extracted proteins [64]. *Sarcocystis* cystozoites contain a large amount of protein. Therefore, it is usually not a problem to prepare protein extracts of *Sarcocystis* cystozoites using standard techniques, such as those of Laemmli [31] or O'Farrell [44].

4.2.1. Extraction of cystozoite proteins for isoelectric focusing and two-dimensional electrophoresis

In the Hannover laboratory, a modification of the lysis buffer described by O'Farrell [44] is used preferably to extract proteins from *S. tenella* and *S. gigantea* cystozoites for analysis by two-dimensional electrophoresis, using immobilised pH gradient gels as the first dimension and SDS pore gradient gels as the second dimension [61].

Procedure

- Isolate cystozoites mechanically or by tryptic digestion of host tissue (see 2.2.2.).

- Purify cystozoites by discontinuous density gradient centrifugation (see 2.2.3.). Store pellets of purified cystozoites in liquid nitrogen until required (see 2.2.4.).

- Suspend a pellet containing 10^9-10^{10} cystozoites in 600 µl lysis buffer* (see below). Allow the cystozoite proteins to dissolve for 10 min at 22° C. Mix the solution by finger-tapping every few min. Then centrifuge the solution at 15,000 x g for 5 min to remove non-dissolved components. Usually only a very small pellet is observed after centrifugation. If the pellet is large, the cystozoite proteins are not dissolved properly. In this case more lysis buffer needs to be added to the preparation.

  * 9 M urea / 2 % v/v Triton X-100 / 2 % v/v 2-mercaptoethanol / 2 % v/v Pharmalyte 3-10 (Pharmacia LKB Biotechnology, Uppsala, Sweden).

- If the pellet obtained by centrifugation of the extract is small, take off the supernatant fluid containing cystozoite proteins and transfer this to a new tube. Estimate the protein concentration of this preparation and adjust it to 2 mg/ml with lysis buffer. About 12 mg protein can be extracted from 10^9 *Sarcocystis* cystozoites.

- Distribute the protein extracts into portions of 100 µl and use them immediately or store them at -80° C for up to 3 months.

4.2.2. Extraction of cystozoite proteins for chromatofocusing

The following method proved to be useful to obtain protein extracts of *S. tenella*, *S. arieticanis*, *S. gigantea* and *S. muris* cystozoites for analysis and fractionation by chromatofocusing [65].

Procedure

- Isolate cystozoites mechanically or by tryptic digestion of host tissue (see 2.2.2.).

- Purify cystozoites by discontinuous density gradient centrifugation (see 2.2.3.).

- Suspend the cystozoites in phosphate-buffered saline (pH 7.2) and disintegrate them using a Willems Polytron® (Brinkmann Instruments, Westbury, N.Y., USA) for 30-60 sec at medium speed.

- Add an equal volume of 8 M urea and allow cystozoite proteins to dissolve for 15 min at 22° C. Then filter the suspension through Whatman No. 1 filter paper.

- Desalt the filtrate by gel filtration using a Sephadex G-25 M column (Column PD-10; Pharmacia LKB Biotechnology, Uppsala, Sweden). Equilibrate and elute the column with deionised water. Lyophilise the protein extracts to remove the water and store them at -80° C.

- For chromatofocusing, reconstitute the protein extracts and adjust the protein concentration to 10 mg/ml with start buffer* (see below). Remove non-dissolved components by centrifugation at 135,000 x g for 30 min.

* The start buffer used depends on the pH interval used for chromatofocusing, e.g. 75 mM Tris·acetic acid (pH 9.3) for a pH interval 9-6 or 25 mM bis-Tris·iminodiacetic acid (pH 7.1) for a pH interval 7-4 on a Mono P HR 5/20 column (Pharmacia LKB Biotechnology, Uppsala, Sweden).

- Take off the supernatant fluid containing cystozoite proteins, and use it immediately or store it at 4° C for up to 2 days.

5. Labelling of proteins

5.1. Biotinylation and analysis of surface proteins

Intact or disintegrated *Sarcocystis* sporozoites and cystozoites can be labelled with biotin using the method of Lima and Villalta [36]. Biotinylated polypeptides can then be resolved by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), Western blotted and stained as described by Sommer et al. [51].

Procedure

- Resuspend 2×10^7 parasites in 50 µl phosphate-buffered saline (PBS pH 7.2), add 1.4 µl NHS-Biotin (N-hydroxysuccinimide biotin; Bio-Rad Laboratories, Richmond, Calif., USA) and incubate at 22° C for 15 min.

- Wash the parasites 3 times by centrifugation (2000 x g, 10 min) and resuspension in cold PBS.

- Solubilise the parasite polypeptides in sample buffer as described by Laemmli [31], electrophorese and blot the resolved polypeptides onto a nitrocellulose membrane.

- Saturate the membrane with 3 % bovine serum albumin in PBS for 1 h.

- Wash the membrane 3 times for 5 min with PBS-Tween (PBS containing 0.1 % v/v Tween 20), then once with PBS.

- Incubate the membrane with avidin-horseradish peroxidase (Bio-Rad Laboratories, Richmond, Calif., USA) diluted 1:1000 in PBS containing 1 % bovine serum albumin for 1 h. Then wash the membrane 4 times with PBS-Tween and once with PBS.

- Develop the reaction with substrate solution (about 5 min): 50 ml of 20 mM Tris, 500 mM NaCl (pH 7.5) / 30 µl of 30 % w/v H_2O_2 / 10 ml of 30 mg 4-chloro-1-naphtol in methanol.

- Stop the reaction by washing the membrane with distilled water.

5.2. Pulse-chase labelling of cystozoites with [^{35}S]-methionine

Proteins can be labelled by several techniques, such as by treatment with radioactive sodium borohydride or by iodination [51, 52]. Most commonly, proteins are labelled by growing cells in the presence of a radioactive amino acid or the radioactive precursor of an amino acid. The most commonly used radioactive precursor for labelling proteins is [^{35}S]-methionine. The ^{35}S decay is easier to detect than either that of ^{3}H or ^{14}C, and the ^{35}S signal is easily enhanced using fluorography. In addition, the intracellular concentration of methionine is lower than that of many other amino acids and, hence, exogenous methionine can easily be incorporated into proteins. The rates of turnover of a protein can be determined by using short labelling times (for example 30 min) followed by the removal of the label and addition of saturating amounts of cold amino acids. These so-called pulse-chase experiments can be used to analyse many time-dependent processes, such as post-translational modification, transport, secretion or proteolytic cleavage of newly synthesized proteins.

A modification of the method described by Coligan et al. [12] has been used to biosynthetically label proteins of *S. muris* cystozoites with [^{35}S]-methionine. *S. muris* cystozoites were isolated from the muscles of experimentally infected mice. After tryptic digestion of host tissue, the parasite suspension was passed through metal sieves. The suspension was centrifuged (2000 x g, 10 min, 4° C), and the supernatant fluid was discarded. The sediment was washed 3-5 times by resuspension in phosphate-buffered saline and centrifugation. Proteins were labelled as follows.

Procedure

- Resuspend 2×10^7 cystozoites per chase in 10 ml short-term labelling medium* (see below) and incubate the suspension 15 min at 37°C to deplete intracellular pools of methionine. Shake gently.

  * Short-term labelling medium: RPMI 1640 medium without methionine supplemented with 5 % heat-inactivated fetal calf serum, 30 µg/ml gentamycin and 25 mM HEPES (pH 7.5).

- Centrifuge (300 g, 5 min, 22° C) the cystozoites. Discard the supernatant fluid.

- Thaw [^{35}S]-methionine stock solution and prepare a working solution of short-term labelling medium containing 0.2 mCi [^{35}S]-methionine per ml. Pre-warm the working solution to 37° C.

- Resuspend the cystozoites in 4 ml [^{35}S]-methionine working solution. Incubate the cystozoites 30 min at 37° C. Shake gently.

- After pulse-labelling, remove the [^{35}S]-methionine medium, wash the cystozoites once with 10 ml chase medium (pre-warmed to 37° C),* (see below) and then re-suspend the cystozoites in 10 ml chase medium.

  * Chase medium: short-term labelling medium supplemented with 15 mg/ml of non-radioactive methionine.

- Incubate the suspension for the desired time at 37° C. Shake gently.

- Collect the cystozoites by centrifugation (300 x g, 5 min, 4° C). Discard the supernatant fluid. The cystozoites are now ready for lysis.

- Incubate the labelled cystozoites in lysis buffer* (see below) (2×10^7 cells/ml) for 1 h at 4° C.

  * Lysis buffer: TSA buffer (0.01 M Tris·HCl, pH 8.0 / 0.14 M NaCl / 0.025 % NaN_3) supplemented with 1 % Triton X-100, 1 % bovine haemoglobin, 0.2 U/ml aprotinin, 1 mM iodoacetamide and 1 mM phenylmethylsulfonyl fluoride.

- Centrifuge (3000 x g, 10 min) the lysate to remove the cell nuclei and collect the supernatant fluid.

- Centrifuge (10,000 x g, 30 min) the preparation containing labelled proteins and again collect the supernatant fluid.

Antigenic proteins can be isolated from the radio-labelled mixture by specific immunoprecipitation using monoclonal or polyclonal antibodies and can then be analysed by SDS-PAGE followed by autoradiography. This permits the estimation of their molecular sizes and the identification of subunits of associated proteins as described in detail by Harlow and Lane [25].

6. Extraction of nucleic acids

Nucleic acids can be extracted from *Sarcocystis* cystozoites isolated mechanically or by digestion of host tissue with trypsin or pepsin (see 2.2.2.). Enzymatic digestion of host tissue does not adversely affect the biological activity of nucleic acids extracted from the isolated cystozoites [64].

6.1. Extraction of genomic DNA

The following method is used routinely in the Hannover laboratory for extraction of genomic DNA from *Sarcocystis* cystozoites, e. g. those of *S. tenella*, *S. arieticanis*, *S. gigantea*, *S. capracanis*, *S. moulei*, *S. cruzi*, *S. miescheriana* or *S. muris*. Genomic DNA extracted by this method has been used for RAPD-PCR, for PCR amplification of the small subunit ribosomal RNA gene [67] and for the construction of genomic DNA libraries (see 8.).

Procedure

- Isolate cystozoites by one of the methods described above (see 2.2.2.).

- Purify cystozoites by discontinuous density gradient centrifugation (see 2.2.3.). Check the degree of purity by microscopic examination of Giemsa stained smears. Preparations used for the extraction of genomic *Sarcocystis* DNA should contain more than 99.98 % cystozoites, i. e. no host cell nucleus per 5000 cystozoites. Store pellets of purified cystozoites in liquid nitrogen until required (see 2.2.4.).

- Suspend a pellet containing 10^8-10^9 cystozoites in 7.5 ml Tris buffer* (see below) using a 50 ml screw cap centrifuge tube. Add SDS to a final concentration of 1 % (i. e. 2.5 ml SDS buffer**). Add Proteinase K to a final concentration of 200 µg/ml (i. e. 100 µl of a stock solution containing 20 mg/ml). Incubate the suspension in a water bath at 56° C for 2 h. Swirl the suspension periodically.

  * 10 mM Tris·HCl / 10 mM EDTA, pH 8.0.
  ** 10 mM EDTA (pH 8.0) containing 4 % SDS.

- Add fresh Proteinase K to a final concentration of 400 µg/ml (i. e. 100 µl of a stock solution containing 20 mg/ml). Incubate the suspension at 56° C for another 2 h with periodical swirling.

  Note: *Sarcocystis* cystozoites contain a large amount of protein. Therefore, it may not be possible to remove all of the protein by digestion with Proteinase K, and further purification of the extracted *Sarcocystis* DNA may be necessary to obtain highly pure DNA preparations (see below).

- Add 2.0 ml of 6 M sodium perchlorate.

- Add an equal volume (ca. 12 ml) of phenol / chloroform / isoamylalcohol (50/48/2), pH 8.0. Gently mix the two phases by turning the tube end over several times. Then, separate the two phases by centrifugation (10,000 x g, 10 min, 10° C).

- Slowly take off the aqueous phase and repeat step 6 until the interphase is clear. Then, extract once with chloroform.

- Add 200 µl of 10 mg/ml RNAase A and incubate on ice for 15 min.

- Precipitate DNA with ethanol using one of the following methods (step a or b):

  (a) Mix the aqueous phase with 1/10 volume (ca. 1 ml) of 3 M sodium acetate (pH 5.5) and 2.5 volumes (ca. 25 ml) of ice-cold ethanol. Precipitate *Sarcocystis* DNA at -20° C overnight.

  (b) Mix the aqueous phase with 1/2 volume (ca. 5 ml) of 7.5 M ammonium acetate (pH about 7.5) and 3 volumes (ca. 30 ml) of ice-cold ethanol. *Sarcocystis* DNA will precipitate at -70° C for 30 min, at -20° C for 2 h, or at 4° C overnight.

- Centrifuge (10,000 x g, 10 min, 0° C) the precipitate to pellet DNA. Carefully decant and discard the supernatant fluid. Wash the pellet twice with ice-cold 70 % ethanol. Then wash the pellet once with ice-cold 100 % ethanol. Allow the pellet to dry at 22° C.

- Dissolve the pellet in 0.5-1 ml TE buffer (10 mM Tris·HCl / 1 mM EDTA, pH 8.0) overnight at 4° C before use.

- Store the DNA at 4° C.

  Note: We find that the purity of *Sarcocystis* DNA preparations obtained by this method is usually sufficient for most molecular biological applications. However, for some applications such as the construction of DNA libraries (see 8.) or restriction enzyme analysis it may be necessary to further purify the DNA preparation by cesium chloride density gradient centrifugation using standard techniques [46].

Genomic DNA of *S. muris* has also been extracted using a modification of the methods described by Blin and Stafford [7] and Sambrook et al. [46], and this is the method of choice in the Bochum laboratory. If this method is used, the extracted DNA preparation needs to be dialysed several times against a large volume of TE buffer (10 mM Tris·HCl / 1 mM EDTA, pH 8.0) before it can be used for restriction enzyme analysis or DNA cloning experiments (see 8).

6.2. Extraction of cellular RNA from cystozoites

A modification of the method described by Brooker et al. [8] is used routinely in the Hannover laboratory for extraction of RNA from *Sarcocystis* cystozoites, e. g. those of *S. tenella*, *S. arieticanis*, *S. gigantea*, *S. capracanis*, *S. cruzi* or *S. muris*. The method will yield 1.3-3.4 mg cellular RNA per 10^9 *Sarcocystis* cystozoites [62, 64]. RNA preparations obtained by this method have been used for Northern blot analysis [62, 64] and for reverse transcription [66] of ribosomal RNA.

All steps should be carried out on ice. Wear gloves when handling samples containing RNA to avoid contamination with RNAases. All reagents and utensils should be autoclaved or treated with diethyl pyrocarbonate (DEP).

Procedure

- Isolate cystozoites by one of the methods described above (see 2.2.2.).

- Purify cystozoites by discontinuous density gradient centrifugation (see 2.2.3.). Check the degree of purity by microscopic examination of Giemsa stained smears. Preparations used for the extraction of RNA should contain $\geq$ 99.98 % cystozoites, i. e. no host cell nucleus per 5000 cystozoites. Store the cystozoites in extraction buffer I at -80° C or in liquid nitrogen (see 2.2.4.).

  Extraction buffer I:
  6 M guanidine hydrochloride / 0.2 M sodium acetate (pH 5.2) / 1 mM 2-mercaptoethanol:
  - add 57.32 g guanidine hydrochloride to about 30 ml H_2O
  - add 6.7 ml of 3 M sodium acetate (pH 5.2)
  - make up to 99.0 ml with H_2O
  - take out 50 ml and add 3.5 µl of 2-mercaptoethanol
  - add one drop DEP and leave overnight before use.

  Use the remaining 49.0 ml to prepare extraction buffer II (see below).

- Suspend 10^9 -10^{10} cystozoites in 10 ml ice-cold extraction buffer I. Transfer this suspension to a glass homogeniser and homogenise by 10-15 gentle strokes with a teflon pestle.

- Transfer the homogenate to a 50 ml screw cap polypropylene tube. Place the tube into an ethanol-ice bath and disrupt the cystozoites by ultrasonication using a microtip (2 bursts of 30 sec).

  Note: Sterilise the ultrasonic tip with 0.1 M NaOH and subsequently wash 3 times with sterile distilled water (1 burst of 30 sec each) before use.

- Add 15 ml of ice-cold ethanol to the sonicate and allow to precipitate for 2 h at -20° C.

- Centrifuge (12,000 x g, 20 min, 4° C) the precipitate. Decant and discard the supernatant fluid. Resuspend the pellet in 15 ml ice-cold extraction buffer II. Transfer this suspension to a glass homogeniser and homogenise by 10-15 gentle strokes with a teflon pestle.

Extraction buffer II:

6 M guanidine hydrochloride / 0.2 M sodium acetate (pH 5.2) / 10 mM EDTA:

- take the remaining 49.0 ml of 6 M guanidine hydrochloride / 0.2 M sodium acetate (pH 5.2) left over from the preparation of extraction buffer I (see above)
- add 1.0 ml of 0.5 M EDTA
- add one drop DEP and leave overnight before use.

- Transfer the homogenate to a 50 ml polypropylene tube. Add 15 ml of ice-cold ethanol and allow to precipitate for 2 h at -20° C.

- Centrifuge (12,000 x g, 20 min, 4° C) the precipitate. Decant and discard the supernatant fluid. Resuspend the pellet in 7.5 ml ice-cold extraction buffer III. Transfer this suspension to a glass homogeniser and homogenise by 10-15 gentle strokes with a teflon pestle.

Extraction buffer III:

7 M urea / 100 mM Tris (pH 8.5) / 0.1 mM EDTA / 0.1 % SDS:

- add 21.02 g urea to about 30 ml H_2O
- add 0.44 g Tris-HCl
- add 0.87 g Tris-base
- add 500 µl of 10 % SDS
- add 10 µl of 0.5 M EDTA (pH 8.0)
- make up to 50.0 ml with H_2O
- add one drop DEP and leave overnight before use.

- Transfer the homogenate to a 50 ml polypropylene tube. Add 15 ml of phenol / chloroform / isoamylalcohol (50/48/2), pH 8.0. Cover the lid of the tube with parafilm and vortex the tube for 1 min to mix the two phases. Then, separate the two phases by centrifugation (12,000 x g, 5 min, 4° C).

- Slowly take off the upper aqueous phase containing RNA and transfer this to another 50 ml polypropylene tube.

Note: Do not disturb the interphase!

- Extract the bottom organic phase a second time with 15 ml ice-cold extraction buffer III (see above). Vortex the tube for 1 min to mix the two phases. Then, separate the two phases by centrifugation (12,000 x g, 5 min, 4° C).

- Slowly take off the upper aqueous phase and combine it with the first one derived from step 10. Then, extract once with chloroform.

- Divide the aqueous phase into two equal portions in two 50 ml screw cap polycarbonate tubes. To each tube, add 1/10 volume (ca. 1 ml) of 3 M sodium acetate (pH 5.2) and 2.5 volumes (ca. 25 ml) of ice-cold ethanol. Allow precipitation of *Sarcocystis* RNA at -20° C overnight.

The following day, purify the *Sarcocystis* RNA with lithium chloride as follows:

- Centrifuge (12,000 x g, 15 min, 4° C) the precipitate to pellet RNA. Carefully decant and discard the supernatant fluids of each tube. Resuspend each pellet in 5 ml of 2 M lithium chloride. Combine the suspensions, then vortex the tube for 1 min.

- Centrifuge (12,000 x g, 15 min, 4° C) the suspensions to pellet RNA. Carefully decant and discard the supernatant fluid and resuspend the pellet in 3.0 ml of 100 mM potassium acetate (pH 5.0) and 7.0 ml ice-cold ethanol.

Note: The pellet does not dissolve well at this stage.

- Centrifuge (12,000 x g, 15 min, 4° C) the suspensions to pellet RNA. Carefully decant and discard the supernatant fluid. Cover the top of the tube with parafilm, punch a few holes into the parafilm. Dry the RNA pellet in a vacuum desiccator for 10-15 min at 22° C.

- Dissolve the dried RNA pellet in 0.5-1 ml of ultrapure or double-distilled water and store the *Sarcocystis* RNA at -20° C.

In the Bochum laboratory the methods of choice for extraction of RNA from *S. muris* cystozoites are those described by Chomczynski and Sacchi [11] or Ausubel et al. [3]. RNA preparations obtained by these methods have been used to select polyadenylated RNA for Northern blot analysis and for the construction of a cDNA library (see 8.).

6.3. Purification of polyadenylated RNA from cystozoites

Polyadenylated RNA can be purified from preparations of cellular RNA of *Sarcocystis* cystozoites (see 6.2.) by affinity chromatography on oligo(dT)-cellulose using a modification of the method described by Sambrook et al. [46]. Poly(A)$^+$ RNA of *Sarcocystis* derived by this method has been used for in vitro translation studies [62, 64] and for the construction of cDNA libraries (see 8.)

Take care that all steps are carried out using sterile conditions. All reagents and utensils should be autoclaved or treated with diethyl pyrocarbonate (DEP). Wear gloves to avoid contamination with RNAases.

Procedure

- Allow 100 mg of oligo(dT)-cellulose type 7 to swell in excess (3-5 ml) loading buffer* (see below) for about 1 h at 22° C (swells to about 400 µl).

  * Loading buffer: 20 mM Tris·HCl (pH 7.6) / 0.5 M LiCl / 1 mM EDTA (pH 8.0) / 0.1 % SDS.

- Plug a 1 ml insulin syringe with siliconised glass wool, attach a 3-way stopcock to it, and clamp it to a retort stand. Pour the swollen oligo(dT)-cellulose into the syringe using a Pasteur pipette. Check the flow rate with loading buffer (see above).

- Sterilise the oligo(dT)-cellulose column by sequential washing with:
  - 5 ml H_2O
  - 5 ml of 0.1 M NaOH / 5 mM EDTA
  - 20 ml H_2O.

  Check that the pH of the effluent is <8.0. Then wash the column with 5 ml loading buffer (see above).

- Remove *Sarcocystis* RNA preparation (see 6.2.) from storage at -20° C and heat the solution to 65° C for 5 min. Cool the solution quickly on ice to 22° C, and add an equal volume of 2× loading buffer. Apply the solution to the column and collect the effluent in a sterile tube. When all of the RNA solution has entered the column, add 1 column volume of loading buffer (~ 400 µl) and again collect the effluent. Then heat the effluent to 65° C for 5 min and reapply this to the column.

  2x loading buffer: 40 mM Tris·HCl (pH 7.6) / 1.0 M LiCl / 2 mM EDTA (pH 8.0) / 0.2 % SDS.

- Collect the effluent containing non-polyadenylated RNA. Wash the column with 4 ml loading buffer and collect 400 µl fractions of the effluent.

- Read the absorbance at 260 nm of each fraction collected from the column. Combine the fractions with high OD_{260} values, i. e. the fractions containing poly(A)$^-$ RNA, and store them on ice. Wash the column until the OD_{260} value of the effluent is about 0.

- Elute the poly(A)$^+$ RNA from the oligo(dT)-cellulose column with 1.5 ml elution buffer and collect 300 µl fractions of the eluate.

  Elution buffer: 10 mM Tris·HCl (pH 7.5) / 1 mM EDTA (pH 8.0) / 0.05 % SDS.

- Read the absorbance at 260 nm of each eluted fraction and combine the fractions containing poly(A)$^+$ RNA.

  Note: The eluted poly(A)$^+$ RNA fraction can be purified further by adjusting the LiCl concentration of the eluted fraction to 0.5 M and repeating the last five steps.

- To the collected poly(A)$^+$ RNA fraction (ca. 900 µl), add 1/10 volume (ca. 90 µl) of 3 M sodium acetate (pH 5.2) and 2.5 volumes (ca. 2.25 ml) of ice-cold ethanol. Allow precipitation of *Sarcocystis* poly(A)$^+$ RNA at -20° C overnight.

  Likewise, precipitate the collected poly(A)$^-$ RNA fraction (ca. 4 ml) by adding 1/10 volume (ca. 400 µl) of 3 M sodium acetate (pH 5.2) and 2.5 volumes (ca. 10 ml) of ice-cold ethanol. The fraction of *Sarcocystis* poly(A)$^-$ RNA can be used for studies on ribosomal RNA or other non-polyadenylated RNA species.

- For storage, wash the oligo(dT)-cellulose column sequentially with 5 ml H_2O and 5 ml of 0.1 M NaOH / 5 mM EDTA. Store the column at 4° C. For further use, regenerate the column as described above.

  The following day recover the *Sarcocystis* poly(A)$^+$ RNA and poly(A)$^-$ RNA as follows:

- Centrifuge (10,000 x g, 20 min, 4° C) the precipitate to pellet the poly(A)$^+$ RNA and poly(A)$^-$ RNA. Carefully decant and discard the supernatant fluids. Wash the pellets twice with ice-cold 70 % ethanol and recentrifuge briefly. Allow the pellets to dry at 22° C.

- Dissolve the dried pellet of *Sarcocystis* poly(A)$^+$ RNA in 30 µl of ultrapure or double-distilled water. Dissolve the dried pellet of *Sarcocystis* poly(A)$^-$ RNA in 0.5-1 ml of water. Store the RNA preparations at -20° C.

About 1.3-2.3 % of cellular RNA derived from *Sarcocystis* cystozoites is polyadenylated (62, 64). Therefore, about 30 µg of poly(A)$^+$ RNA should be isolated and purified from about 10^9 *Sarcocystis* cystozoites.

7. In Vitro translation of mRNA from cystozoites

Preparations of polyadenylated RNA of *Sarcocystis* cystozoites derived by the method described above (see 6.3.) can be used for in vitro translation of mRNA using cell-free wheat germ or rabbit reticulocyte translation systems [62, 64]. It is important to know that not all commercially available translation systems give equally good results with *Sarcocystis* mRNA. In addition, in vitro translation results may vary with different batches of the in vitro translation system. The following translation systems and conditions were found to be optimal for in vitro translation of cystozoite-derived mRNA of *S. tenella* and *S. gigantea* [62].

Wheat germ translation system

- 50 % wheat germ extract (Promega Corporation, Rozelle, Wis., USA; Lot No. 01910)
- 98 mM potassium acetate
- 2.5 mM magnesium acetate
- 18.5 MBq/ml L-[^{35}S]methionine

Rabbit reticulocyte translation system

- 75 % rabbit reticulocyte lysate (Amersham International, Amersham, UK; N. 90, Lot No. 81)
- 129 mM potassium
- 2.3 mM magnesium
- 111 MBq/ml L-[^{35}S]methionine.

For use in the in vitro translation systems, the concentration of the poly(A)$^+$ RNA preparations of *S. tenella* or *S. gigantea* was adjusted to 1 µg/µl with water. The RNA

preparations were heated at 67° C for 10 min to destroy local regions of secondary structure, cooled on ice, and 2 µg of each preparation were added to a total volume of 50 µl of each translation reaction. Wheat germ translation reactions were incubated at 25° C and rabbit reticulocyte translation reactions at 30° C.

Co-translational and initial post-translational processing of *Sarcocystis* polypeptides can be studied using canine pancreatic microsomal membranes that are added to the rabbit reticulocyte translation system. The following conditions were found to be optimal for processing of *S. tenella* or *S. gigantea* polypeptides [62].

- 75 % rabbit reticulocyte lysate (Amersham International, Amersham, UK; N. 90, Lot No. 81)
- 129 mM potassium
- 2.3 mM magnesium
- 3.6 equivalents of microsomal membranes/25 µl reaction (Promega Corporation; Lot No. T408)
- 111 MBq/ml L-[^{35}S]methionine.

The *S. tenella* or *S. gigantea* poly(A)$^+$ RNA preparations were heated at 67° C for 10 min, cooled on ice, and 1 µg of each preparation (concentration of 1 µg/µl) was added to a total volume of 25 µl of each translation reaction. Translation reactions were incubated at 30° C.

8. Construction of DNA libraries

Genomic DNA libraries have been constructed for *S. cruzi*, *S. tenella* and *S. gigantea* by restricting genomic DNA preparations (see 6.1.) with *Eco*RI and subsequent cloning of the DNA fragments into bacteriophage λgt10 or λgt11 (Promega Corporation, Rozelle, Wis., USA) using standard techniques [46]. Details of the cloning procedure used for *S. cruzi* are given by Kibenge et al. [30].

Cystozoite-derived cDNA libraries have been constructed for *S. muris* in bacteriophage λZAP (Stratagene, La Jolla, Calif., USA) and for *S. tenella*, *S. arieticanis* and *S. gigantea* in λgt11 (Promega Corporation, Rozelle, Wis., USA). Details of the cloning procedures are given by Eschenbacher et al. [18] and Mertens [42]. *Sarcocystis* gene fragments expressing polypeptides have subsequently been subcloned into the plasmid vectors pGEX-3X or pGEX-2T, and recombinant *Sarcocystis* polypeptides have been expressed in *Escherichia coli* as glutathione *S*-transferase fusion polypeptides [18, 42].

9. Preparation of chromosomal DNA

A modification of the method described by Van der Ploeg et al. [69] has been used to embed chromosomal DNA of *S. gigantea* and *S. muris* in agarose for analysis by pulsed field gel electrophoresis [57]. The chromosomal DNA of *S. gigantea* or *S. muris* used for the preparation of agarose plugs was derived from mechanically isolated cystozoites (see 2.2.2.).

Procedure

- Isolate cystozoites mechanically by one of the methods described above (see 2.2.2.).

- Purify cystozoites by discontinuous density gradient centrifugation (see 2.2.3.). Check the degree of purity by microscopic examination of Giemsa stained smears. Cystozoite preparations used for the preparation of chromosomal *Sarcocystis* DNA should contain $\geq$ 99.98 % cystozoites, i. e. no host cell nucleus per 5000 cystozoites.

- Immediately after purification, suspend 2.5×10^9 cystozoites in 500 µl phosphate-buffered saline (PBS, pH 7.2) in a microfuge tube and place the tube into a water bath at 37°C. Add 500 µl of 2 % low-melting-point agarose (Incert Agarose, FMC Bioproducts, Rockland, Me., USA) kept at 37°C. Gently mix the suspension by finger-tapping and immediately place the tube back into the water bath at 37°C.

- Quickly dispense 250 µl of the agarose/cell suspension into each of four wells (2×5×20 mm) of a slot holder. Allow the agarose to set by transferring the slot holder to 4° C for at least 20 min.
- Remove the agarose plugs from the slot holder and place them in lysis buffer. Incubate the plugs at 42° C for 48 h.

  Lysis buffer: 10 mM Tris·HCl (pH 9.5) / 0.5 M EDTA / 1 % N-lauroyl-sarcosine, supplemented with 2 mg/ml Proteinase K (Boehringer Mannheim Biochemicals, Mannheim, Germany).

- Store the agarose plugs in lysis buffer at 4° C.

Recently, chromosomal DNA of *S. tenella*, *S. arieticanis* and *S. gigantea* has been embedded in agarose using a modification of the method described for a commercially available IMBED™ KIT (New England Biolabs, Beverly, Mass., USA).

Procedure

- Isolate cystozoites mechanically (*S. gigantea*) or by digestion of host tissue with trypsin (*S. tenella*, *S. arieticanis*) as described above (see 2.2.2.).

- Purify cystozoites by discontinuous density gradient centrifugation (see 2.2.3.). Check the degree of purity by microscopic examination of Giemsa stained smears. Cystozoite preparations used for the preparation of chromosomal *Sarcocystis* DNA should contain $\geq$ 99.98 % cystozoites, i. e. no host cell nucleus per 5000 cystozoites.

- Immediately after purification, transfer 1×10^9 to 5×10^9 cystozoites to a microfuge tube and pellet the cells by centrifugation. Completely remove and discard the supernatant buffer. Resuspend the cystozoites in 500 µl cell suspension buffer and place the suspension in a water bath at 42° C.

  Cell suspension buffer: 10 mM Tris·HCl (pH 7.2) / 20 mM NaCl / 100 mM EDTA.

- Add 500 µl of agarose solution (1 % low-melting-point agarose in H_2O heated to 42° C) to the cell suspension. Gently mix the suspension by finger-tapping and immediately place the suspension back into the water bath at 42° C.

- Pour the agarose/cell suspension into a GelSyringe™ (supplied with the IMBED™ KIT) and allow to set for at least 15 min at 22° C. Then place the syringe at 4° C for 30 min.

- Extrude the agarose rod onto a sterile dish and cut it in half. Place the two 0.5 ml rods into a 15 ml conical screw cap tube and add 3 ml Proteinase K buffer. Incubate the rods for at least 24 h (up to 48 h) at 42° C with gentle shaking.

  Proteinase K buffer: 1 mg/ml Proteinase K / 100 mM EDTA (pH 8.0) / 1 % N-lauryl-sarcosine / 0.2 % sodium deoxycholate.

- Remove the Proteinase K solution and wash the agarose rods in 5 ml wash buffer (20 mM Tris·HCl (pH 8.0) / 50 mM EDTA) for 15 min at 22° C with gentle shaking.

- Remove the wash buffer and wash the agarose rods in 5 ml fresh wash buffer (see above) for 15 min at 22° C with gentle shaking. Repeat this step 20 times.

- Remove the wash buffer, and wash the agarose rods in 5 ml storage buffer (2 mM Tris·HCl, pH 8.0 / 5 mM EDTA) for 15 min at 22° C with gentle shaking. Repeat this step twice.

- Load the agarose rods into a GelSyringe™. Fill both ends of the syringe with storage buffer (see above). Store the agarose plugs at 4° C.

10. Diagnostic methods

10.1. Examination of muscle digests for cystozoites

To determine the number of *S. miescheriana* cystozoites per g of muscle, host tissue is digested with trypsin [50]. Five g of minced muscle samples are added to 200 ml of digestion fluid (0.25 % trypsin 1:250, Difco no. 0152-15, in PBS) and incubated for 2 h at 37° C by gentle stirring. The digest is passed through a 1 mm aperture sieve and centrifuged for 10 min at 3000 rpm. The supernatant fluid is decanted and three drops of the sediment are examined microscopically for the presence of cystozoites. The liberated cystozoites are counted in a Fuchs-Rosenthal chamber.

10.2. Immunohistochemistry

Methods for the detection and identification of life cycle stages of the vascular phase of *Sarcocystis* spp. (schizonts and merozoites) in histological sections have been described by Jeffrey et al. [29] and Granstrom et al. [22].

10.3. Serology

Various serological tests have been developed for the diagnosis of *Sarcocystis* infections in intermediate hosts. The most reliable and widely used tests are the indirect fluorescent antibody test (IFAT), the enzyme-linked immunosorbent assay (ELISA) and the dot-ELISA. The methodology of these tests using cystozoite-derived antigen preparations have been described in detail by Tenter [59, 60]. The IFAT and ELISA have also been carried out with antigen preparations derived from in vitro cultures of schizonts and merozoites [23, 48]. A disadvantage of all these tests is that they are

only genus-specific. Thus far, no species-specific serological test is available for *Sarcocystis* spp.

10.4. DNA probes

Recently, specific PCR primers have been developed that allow the species-specific amplification of small subunit ribosomal RNA gene fragments of *S. tenella*, *S. arieticanis* and *S. gigantea* [6]. The development of diagnostic PCR assays based on these primers that allow species-specific diagnosis of infections with pathogenic and non-pathogenic *Sarcocystis* spp. in sheep is now underway.

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Part IV. Annexes

1. International coding system for coccidia species and strains

In order to facilitate the exact identification and registration of species and strains of coccidia a uniform system should be internationally adopted. It should contain the following information:

- Name of species
- Host species from which the isolate was obtained
- Location, country, month and year of isolation
- Normal (N), modified (M) or drug resistant (R) strain (for example, a modified strain would be a precocious strain). If a strain is modified or drug resistant some details should be given.

- Reference number should be given composed of the abbreviated name of the laboratory and the number (for example CVL 100 = Central Veterinary Laboratory No. 100).

Example

Eimeria tenella (Chicken IHA, Compton/UK, 12/1989, R [Monensin]) IHA 105.

2. Coccidia species and strains held in various laboratories

Note: List without claim for completeness
N.= No known drug resistance; R.= Resistance to drugs listed; D.= Drug dependent. Maintenance: in h
passages or cryopreserved.

2.1. *Eimeria* species and strains

2.1.1. Central Veterinary Laboratory (CVL), Weybridge, New Haw, Addlestone, Surrey KT 15 3 NB, U.K.

Species	Host	Where isolated	Date	Drug resistance	Ref. N
Eimeria acervulina	Chicken	Weybridge, UK	before 1961	N.	PS28a
Eimeria acervulina	Chicken	Houghton, UK	1956	N.	PS28b
Eimeria acervulina	Chicken	Weybridge, UK	1963	N. (M) strain	PC28c
Eimeria acervulina	Chicken	Lasswade, UK	1977	N.	PS28d
Eimeria acervulina	Chicken	Weybridge, UK	1974	R. Pancoxin	PS28e
Eimeria acervulina	Chicken	Weybridge, UK	1979	R. Robenidine	PS28f
Eimeria adenoeides	Turkey	Weybridge, UK	1952	N.	PS20
Eimeria ahsata	Sheep	Weybridge, UK	early 1970's	N.	PS11
Eimeria bakuensis	Sheep	Weybridge, UK	early 1970's	N.	PS9
Eimeria bateri	Quail	Weybridge, UK	1970	N.	PS33
Eimeria brunetti	Chicken	Weybridge, UK	1961	N.	PS31a
Eimeria brunetti	Chicken	Houghton, UK	1961	N.	PS31b
Eimeria caprina	Goat	Weybridge, UK	early 1970's	N.	PS16
Eimeria christenseni	Goat	Weybridge, UK	early 1970's	N.	PS17
Eimeria coecicola	Rabbit	Weybridge, UK	1969	N.	PS8
Eimeria colchici	Pheasant	Weybridge, UK	1965	N.	PS22
Eimeria crandallis	Sheep	Weybridge, UK	early 1970's	N.	PS12
Eimeria dispersa	Turkey	Weybridge, UK	1978	N.	PS21
Eimeria duodenalis	Pheasant	Weybridge, UK	1965	N.	PS23
Eimeria faurei	Sheep	Weybridge, UK	early 1970's	N.	PS13
Eimeria flavescens	Rabbit	Weybridge, UK	1979	N.	PS5
Eimeria intestinalis	Rabbit	Weybridge, UK	1969	N.	PS4
Eimeria irresidua	Rabbit	Weybridge, UK	1969	N.	PS6
Eimeria legionensis	Partridge	Weybridge, UK	1981	N.	PS25
Eimeria magna	Rabbit	Weybridge, UK	1968	N.	PS3
Eimeria maxima	Chicken	Weybridge, UK	1958	N.	PS26a
Eimeria maxima	Chicken	Weybridge, UK	1956	N.	PS26b
Eimeria maxima	Chicken	Weybridge, UK	1971	R. Buquinolate	PS26d
Eimeria maxima	Chicken	Weybridge, UK	1974	R. Coyden	PS26e
Eimeria maxima	Chicken	Weybridge, UK	1975	R. Robenidine	PS26f
Eimeria maxima	Chicken	Weybridge, UK	1973	D. Robenidine	PS26g
Eimeria maxima	Chicken	Weybridge, UK	1973	R. Sulphaquinoxaline	PS26h
Eimeria maxima	Chicken	Weybridge, UK	1977	R. Sulphaquinoxaline	PS26i
Eimeria maxima	Chicken	Weybridge, UK	1974	R. Sulphaquinoxaline	PS26j
Eimeria maxima	Chicken	Weybridge, UK	1975	R. Robenidine/Methyl benzoquate	PD26k
Eimeria maxima	Chicken	Weybridge, UK	1975	R. Robenidine/Coyden	PS261
Eimeria maxima	Chicken	Weybridge, UK	1978	R. Bisporocystic	PS26m

Species	Host	Where isolated	Date	Drug resistance	Ref. No.
Eimeria maxima var indentata (Jungle fowl)	Chicken	Weybridge, UK	1975	N.	PS26c
Eimeria media	Rabbit	Weybridge, UK	1969	N.	PS7
Eimeria meleagridis	Turkey	Weybridge, UK	1965	N.	PS19
Eimeria meleagrimitis	Turkey	Weybridge, UK	1965	N.	PS18
Eimeria mitis	Chicken	Weybridge, UK	1977	N.	PS29a
Eimeria mitis	Chicken	Wreay, UK	1980	N.	PS29b
Eimeria mitis	Chicken	Saron, UK	1981	N.	PS29c
Eimeria mitis	Chicken	Cumbernauld, UK	1981	N.	PS29d
Eimeria mitis	Chicken	Houghton, UK	1969	N.	PS29e
Eimeria mitis	Chicken	Weybridge, UK	1978	R. Methyl benzoquate	PS29f
Eimeria mitis	Chicken	Weybridge, UK	1979	R. Robenidine	PS29g
Eimeria mitis	Chicken	Weybridge, UK	1979	R. Sulphaquinoxaline	PS29h
Eimeria necatrix	Chicken	Weybridge, UK	1961	N.	PS32a
Eimeria necatrix	Chicken	Weybridge, UK	1979	R. Lerbek	PS32b
Eimeria ninakohl-yakimovae	Goat	Weybridge, UK	early 1970's	N.	PS15
Eimeria ovinoidalis	Sheep	Weybridge, UK	early 1970's	N.	PS14
Eimeria phasiani	Pheasant	Weybridge, UK	1965	N.	PS24
Eimeria piriformis	Rabbit	Weybridge, UK	1967	N.	PS2
Eimeria praecox	Chicken	Houghton, UK	1966	N.	PS30
Eimeria stiedai	Rabbit	Weybridge, UK	1975	N.	PS1
Eimeria tenella	Chicken	Weybridge, UK	before 1947	N.	PS27a
Eimeria tenella	Chicken	Houghton, UK	1949	N.	PS27b
Eimeria tenella	Chicken	Weybridge, UK	1969	R. Buquinolate	PS27c
Eimeria tenella	Chicken	Weybridge, UK	1978	R. Pancoxin	PS27d
Eimeria weybridgensis	Sheep	Weybridge, UK	1973	N.	PS10

2.1.2. Institute of Parasitology and Tropical Veterinary Medicine, Königsweg 65 D-14163 Berlin, Germany

Eimeria tenella	Chicken	Wusterhausen, D	9/1992	N.	-

2.1.3. Institute of Parasitology, School of Veterinary Medicine, Bünteweg 17, D-30559 Hannover, Germany

Eimeria acervulina	Chicken	unknown/NL	5/1993	not characterised, 3 isolates	-
Eimeria acervulina	Chicken	Hannover/D	8/1993	not characterised	-
Eimeria acervulina	Chicken	Möckern/D	10/1993	not characterised	-
Eimeria acervulina	Chicken	Hannover/D	1/1993	not characterised	-
Eimeria mitis/ acervulina	Chicken	unknown/D	4/1993	R. Maduramicin, Monensin, Salinomycin, Nicarbazin, Diclazuril, Halofuginone	-
Eimeria mitis/ acervulina	Chicken	unknown/D	4/1993	not characterised	-
Eimeria mitis/ acervulina	Chicken	Cuxhaven/D	6/1993	not characterised	-

Species	Host	Where isolated	Date	Drug resistance	Ref. No.
Eimeria tenella	Chicken	Möckern/D	5/1993	R. Maduramicin, Monensin, Salinomycin, Nicarbazin, Halofuginone	-
Eimeria tenella	Chicken	Hannover/D	5/1993	R. Maduramicin, Monensin, Salinomycin, Nicarbazin, Halofuginone	-
Eimeria tenella + *E. mitis/acervulina*	Chicken	Rechterfeld/D	6/1993	not characterised	-
Eimeria tenella	Chicken	Hannover/D	6/1993	not characterised	-
Eimeria tenella	Chicken	Möckern/D	8/1993	not characterised	-
Eimeria tenella	Chicken	Cuxhaven/D	8/1993	not characterised	-

2.1.4. Institute of Parasitology, University of Zürich, Winterthurerstr. 266a, CH-8057 Züri Switzerland

Eimeria acervulina	Chicken	Netherlands	since 1990 in Zürich	not characterised	E.a. ZH 0
Eimeria maxima	Chicken	Houghton/UK	since 1990 in Zürich	not characterised	E.m. ZH (
Eimeria nieschulzi	Rat	Bonn/D	1966, since 1989 in Zürich	not characterised	E.n. ZH 0
Eimeria tenella	Chicken	Houghton/UK	since 1990 in Zürich	not characterised	E.t. ZH 0
Eimeria vermiformis	Mouse	Australia	since 1989 in Zürich	not characterised	E.v. ZH 0

2.2. *Isospora suis*

2.2.1. National Veterinary Laboratory, 27 Bülowsvej, DK-1790 Copenhagen V, Denmark

Isospora suis	Pig	Denmark	-	not characterised	-

2.3. *Cryptosporidium* species and strains

2.3.1. National Institute of Veterinary Research, Groeselenberg 99, B-1180 Brussels, Belgium

Cryptosporidium parvum	Calf	Kasterlee/B	1994	not characterised	-
Cryptosporidium baileyi	Chicken	Netherlands	1990	not characterised	-

2.3.2. National Veterinary Laboratory, 27 Bülowsvej, DK-1790 Copenhagen V, Denmark

Cryptosporidium parvum	Calf	Denmark	-	not characterised	-

2.4. *Sarcocystis* species and strains

2.4.1. Institute of Parasitology and Veterinary Tropical Medicine, Königsweg 65, D-14163 Berlin, Germany

Sarcocystis arieticanis	Sheep/ Dog	Berlin/D	12/1972	N.	-
Sarcocystis capracanis	Goat/ Dog	Thessaloniki/GR	9/1977	N.	-
Sarcocystis hircicanis	Goat/ Dog	Thessaloniki/GR	9/1977	N.	-

Species	Host	Where isolated	Date	Drug resistance	Ref. No.
Sarcocystis ovicanis (tenella)	Sheep/ Dog	Berlin/D	12/1972	N.	-
Sarcocystis suicanis (miescheriana)	Pig/Dog	Berlin/D	7/1993	N.	-

2.4.2. Institute of Special Zoology and Parasitology, Ruhr-University, Universitätsstrasse 150, Gebäude ND/05/755, D-44780 Bochum, Germany

Sarcocystis miescheriana	Pig/Dog	Berlin/D	7/1973, since 1980 in Bochum	N.	-
Sarcocystis muris	Mice	Bochum/D	12/1993	N.	-
Sarcocystis muris	Cat	Bochum/D	5/1993	N.	-
Sarcocystis muris	Mouse/ Cat	San José/Costa Rica	1976, since 1977 in Bochum	N.	-
Sarcocystis suicanis	Pig	Bochum	8/1991	N.	-
Sarcocystis suicanis	Dog	Bochum	5/1991	N.	-

2.4.3. Institute of Parasitology, School of Veterinary Medicine, Bünteweg 17, D-30559 Hannover, Germany

Species	Host	Where isolated	Date	Drug resistance	Ref. No.
Sarcocystis muris	Mouse/Cat	San José/Costa Rica	1976, since 1978 in Hannover	N.	
Sarcocystis miescheriana	Pig/Dog	Berlin/D	7/1973, since 1978 in Hannover	N.	

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