EDITOR
David G. Baker

Flynn's Parasites of Laboratory Animals

SECOND EDITION

American College of Laboratory Animal Medicine

Blackwell Publishing

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David G. Baker

DVM, MS, PHD, DACLAM (EDITOR-IN-CHIEF)

DIRECTOR AND PROFESSOR
DIVISION OF LABORATORY ANIMAL MEDICINE
SCHOOL OF VETERINARY MEDICINE
LOUISIANA STATE UNIVERSITY
BATON ROUGE, LA 70803

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David G. Baker, DVM, MS, PhD, DACLAM, is Director and Professor, Division of Laboratory Animal Medicine, School of Veterinary Medicine at Louisiana State University, Baton Rouge.

Blackwell Publishing Professional
2121 State Avenue, Ames, Iowa 50014, USA

Orders: 1-800-862-6657
Office: 1-515-292-0140
Fax: 1-515-292-3348
Web site: www.blackwellprofessional.com

Blackwell Publishing Ltd
9600 Garsington Road, Oxford OX4 2DQ, UK
Tel.: +44 (0)1865 776868

Blackwell Publishing Asia
550 Swanston Street, Carlton, Victoria 3053, Australia
Tel.: +61 (0)3 8359 1011

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To Dr. Dale L. Brooks, mentor, colleague, and friend,
who envisioned this work fourteen years ago.

PREFACE TO THE FIRST EDITION

ALTHOUGH much is known about the parasites of laboratory animals, information is often lacking and what is available is scattered. It is the purpose of this book to gather what is known in this field so that it is readily accessible to those who need it, and to point out what is not known.

Some of the stated deficiencies in our knowledge are probably incorrect in that the information is available but either has been overlooked or has not been published. It is hoped that these incorrect statements will stimulate persons with contrary information to point out the error or to divulge previously unpublished data.

It is also recognized that in a work of this sort, other errors are likely. It would be appreciated if these are pointed out so that they can be corrected in future editions, should the reception of this book warrant future revisions.

Many people helped write this book. A draft of each chapter was first prepared by the appropriate collaborator and then rewritten by me. The rewriting was done primarily to emphasize laboratory animals and secondarily to provide uniformity of style. The rewritten chapter was then reviewed by the collaborator and, in some cases, by others. Thus, each chapter in the book represents a joint effort of at least two people and, in some cases, of several.

Many people, besides the collaborators, assisted in the preparation of this volume. These include persons who reviewed chapters or parts of chapters, furnished illustrations, made literature searches and helped or advised in various ways.

The parasites described are those that occur spontaneously. Experimentally induced conditions are mentioned only if they are of special significance. No attempt is made to include the parasites of all domestic and wild animals. As a general rule, those of the common laboratory animals (mouse, rat, hamster, guinea pig, rabbit, dog, cat, rhesus monkey, and chicken) are all included, but for the less common species (such as other rodents, other primates, reptiles, amphibians, and fishes), only the commonest parasites of the animal species most likely to be used in the laboratory are described. Agents that occur only in domestic animals of agricultural importance are not described, even though these animals are sometimes used in the laboratory, as this information is readily available elsewhere.

Except for a few rare or uncommon animals, the common name only is used in the text. Although this may appear unscientific, the repeated use, for example, of *Mesocricetus auratus*, when one means the usual laboratory hamster, and *Oryctolagus cuniculus*, when one means the laboratory rabbit, is undesirable. Also, scientific names sometimes change, but common names tend to remain the same. Great care was taken to ensure that the scientific name is given for every common name that appears in the text, and that the common name is specific. Authorities used to determine the appropriate names are cited.

It is my sincere hope that the usefulness of this book will justify the efforts of all who helped prepare it.

Robert J. Flynn

PREFACE TO THE SECOND EDITION

IN the more than 30 years since publication of the first edition of this seminal text, dramatic changes have occurred in the fields of laboratory animal medicine and parasitology. Improvements in laboratory animal production, husbandry, transportation, veterinary care, diagnostics, and treatment, have resulted in dramatic declines in the prevalence of organisms causing parasitic diseases. Nowadays, commercially produced laboratory animals are free of nearly all unwanted organisms, including parasites. Modern facility design and husbandry practices preclude most infections or infestations. This is particularly true for parasites with indirect life cycles.

So, with all of these improvements, why is a new edition of this text warranted? Several reasons may be offered. First, in spite of the improvements in the components of animal care listed above, parasites continue to be found in and on laboratory animals. There are several possible reasons: infections or infestations were never completely eliminated from particular facilities; were inadvertently imported with incoming animals, either as a result of contamination during shipment or because parasitism was enzootic at the original location; entered the facility from feral animals in the local environment; or were carried in or on personnel and transferred to colony animals.

A second justification for revising the first edition is that animals in the wild are occasionally still collected and brought into the animal facility. While quarantine procedures should prevent transmission of parasites from wild to laboratory stock, transmission nevertheless occasionally occurs. Thirdly, the tremendous rise in the use of transgenic animals, some of which are immunologically compromised, provides opportunity for infections and/or infestations to take hold where such would not be the case with immunologically competent animals.

Finally, newer diagnostic and therapeutic approaches to controlling parasitism are available. These may facilitate discovery and elimination of unwanted pathogens. In addition to changes in the field of laboratory animal medicine, the field of parasitology has undergone radical changes. Here, changes have been most profound in the areas of diagnostics and treatment.

The stated purpose of the first edition was to gather into one source, what was known about the parasites of laboratory animals so that it was readily accessible to

those who needed it, and to point out gaps in our knowledge of parasites and the diseases they cause. The purpose of this second edition is essentially the same, with the additional significant task of updating information in a field that has advanced substantially, parasitology of laboratory animals.

As with the first edition, many people contributed to this monumental work. Foremost among them are the chapter authors. Their efforts are greatly appreciated. In addition, all chapters were subjected to peer review. On behalf of the authors, I offer thanks to the reviewers for their many valuable suggestions for improving early drafts. Others contributed illustrations, photographs, or conducted literature searches. These too are greatly appreciated. Lastly, we want to give special thanks to Drs. P. Coan, R. Ermel, S. Feldman, and D. McClure. They constituted an advisory committee charged with assisting the Editor-in-Chief in critically evaluating the first edition, in an effort to identify, if possible, areas in which the second edition could be even more valuable than the first.

The breadth and scope of the original edition has been retained, thereby ensuring continued usefulness to the widest possible readership, including bonafide parasitologists. Introductory chapters have been added, beginning with a chapter on modern diagnostic techniques. The next five chapters present overviews of parasite biology. These should help the reader to better understand information presented in the host-specific chapters. Most significantly, the text has been entirely reformatted, in an attempt to improve utility and readability. The informational content has been reorganized into chapters based on vertebrate host. Parasites are presented phylogenetically within chapters. In addition, information included in comprehensive tables from the first edition has been updated, organized by host body system, and reformatted to coincide with host chapters. Finally, a formulary of drugs, uses, dosages, routes, and mechanisms of action, has been added as an appendix. It is hoped that these changes will increase the usefulness of an already highly valuable reference text.

David G. Baker

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LIST OF CONTRIBUTORS

DAVID G. BAKER, D.V.M., M.S., Ph.D., D.A.C.L.A.M.
Director and Professor
Division of Laboratory Animal Medicine
School of Veterinary Medicine
Louisiana State University
Baton Rouge, LA 70803
Tel: (225) 578-9643
Fax: (225) 578-9649
Email: dbaker@vetmed.lsu.edu

ROBERT A. BAKER, D.V.M.
Clinical Veterinarian
Animal Resources Program
University of Alabama at Birmingham
B10 Volker Hall
1717 7th Ave. South
Birmingham, AL 35294-0019
Tel: (205) 934-5530
Fax: (205) 934-1188
Email: bobbaker@uab.edu

LORA R. BALLWEBER, M.S., D.V.M., D.E.V.P.C.
Associate Professor
Department of Microbiology, Immunology, and Pathology
Colorado State University
1619 Campus Delivery
Ft. Collins, CO 80523-1619
Colorado State University
Tel: (970) 491-5015
Email: lora.ballweber@colostate.edu

DIANA M. PALILA BERGER, D.V.M., M.S.
Clinical Veterinarian and Assistant Director for Large Animal Clinical Medicine
Center for Comparative Medicine
Northwestern University
320 East Superior Street
Searle 13-507
Chicago, IL 60611-3010
Tel: (312) 503-7259
Fax: (312) 908-6428
Email: d-berger@northwestern.edu

DWIGHT D. BOWMAN, M.S., Ph.D.
Professor of Parasitology
Department of Microbiology & Immunology
College of Veterinary Medicine
Cornell University
C4-119 VMC Tower Road
Ithaca NY, 14853-6401
Tel: (607) 253-3406
Fax: (607) 253-4077
Email: ddb3@cornell.edu

RONNIE L. BYFORD, Ph.D.
Professor
Department of Entomology, Plant Pathology, and Weed Science
New Mexico State University
MSC 3BE
Skeen Hall Bldg, Room N141
Las Cruces, NM 88003
Tel: (505) 646-2458
Fax: (505) 646-8085
Email: rbyford@nmsu.edu

SAMUEL C. CARTNER, D.V.M., M.P.H., Ph.D.
Interim Director, Animal Resources Program
Associate Professor, Department of Genetics
University of Alabama at Birmingham
220A Research Support Bldg
1800 9th Ave. South
Birmingham, AL 35294-0019
Tel: (205) 934-8213
Fax: (205) 975-1188
Email: scartner@uab.edu

FRANK COGSWELL, Ph.D.
Director, Parasite Diagnostic Laboratory
Tulane National Primate Research Center
18703 Three Rivers Road

Covington, LA 70433
Tel: (985) 871-6224
Fax: (985) 871-1350
Email: cogswell@tulane.edu

MAURICE E. CRAIG, M.S.
Science Specialist
Department of Extension Plant Sciences
New Mexico State University
Las Cruces, NM 88003
Tel: (505) 646-3231
Fax: (505) 646-8085
Email: mcraig@nmsu.edu

THOMAS M. CRAIG, D.V.M., Ph.D.
Professor
Department of Veterinary Pathobiology
College of Veterinary Medicine
Texas A&M University
College Station, TX 77843-4467
Tel: (979) 845-9191
Fax: (979) 862-2344
Email: tcraig@cvm.tamu.edu

JOHN W. FOURNIE, M.S., Ph.D.
Fish Pathologist
U.S. Environmental Protection Agency
National Health and Environmental Effects Research Laboratory
Gulf Ecology Division
1 Sabine Island Drive
Gulf Breeze, FL 32561
Tel: (850) 934-9272
Fax: (850) 934-9201
Email: fournie.john@epa.gov

JAMES G. FOX, D.V.M., M.S., D.A.C.L.A.M.
Professor and Director
Division of Comparative Medicine
Massachusetts Institute of Technology
77 Mass Ave., Bldg 16-8th floor
Cambridge, MA 02139
Tel: (617) 253-9432
Fax: (617) 258-5708
Email: jgfox@mit.edu

LAURETTA W. GERRITY, D.V.M.
Associate Vice President for Research Operations and Compliance
Professor, Department of Genetics
University of Alabama at Birmingham
720 C Administration Bldg
701 20th St. South
Birmingham, AL 35294-0019
Tel: (205) 934-7677
Fax: (205) 975-7886
Email: lwgerrity@.uab.edu

F. CLAIRE HANKENSON, D.V.M., M.S., D.A.C.L.A.M.
Senior Associate Director, University Laboratory Animal Resources
Assistant Professor, Department of Pathobiology
School of Veterinary Medicine
3800 Spruce Street
177E Old Vet Quadrangle
Philadelphia, PA 19104-6009
Tel: (215) 573-3625
Fax: (215) 573-9998
fclaire@pobox.upenn.edu

JOHN E. HARKNESS, D.V.M., M.S., M.Ed., D.A.C.L.A.M.
Professor Emeritus
College of Veterinary Medicine
Mississippi State University
PO Box 6100
Mississippi State, MS 39762
Tel: (601) 325-1131
Fax: (601) 325-1498
Email: harkness@cvm.msstate.edu

AKIRA ITO, M.S., Ph.D., D.Med.Sci.
Director and Professor
Department of Parasitology
Asahikawa Medical College
Midorigaoka-Higashi 2-1-1-1
Asahikawa 078-8510
Hokkaido, Japan
Tel: +81-(0)166-68-2420
Fax: +81-(0)166-68-2429
Email: akiraito@asahikawa-med.ac.jp

MICHAEL L. KENT, M.S., Ph.D.
Director, Center for Fish Disease Research
Department of Microbiology
220 Nash Hall
Oregon State University
Corvallis, OR 97311-3804
Tel: (541) 737-8652
Fax: (541) 737-0496
Email: Michael.Kent@oregonstate.edu

CYNTHIA LANG, D.V.M., M.S.
Resident
Division of Laboratory Animal Medicine
School of Veterinary Medicine
Louisiana State University
Baton Rouge, LA 70803
Tel: (225) 578-9648
Fax: (225) 578-9649
Email: clang@vetmail.lsu.edu

STEPHANIE LEWIS, D.V.M.
Resident
Division of Laboratory Animal Medicine
School of Veterinary Medicine

Louisiana State University
Baton Rouge, LA 70803
Tel: (225) 578-9648
Fax: (225) 578-9649
Email: slewis@vetmed.lsu.edu

DAVID S. LINDSAY, Ph.D.
Distinguished Veterinary Parasitologist
Professor of Parasitology
Center for Molecular Medicine and Infectious Diseases
Department of Biomedical Sciences and Pathobiology
Virginia-Maryland Regional College of Veterinary Medicine
Duckpond Drive, Phase II
Virginia Tech (0442)
Blacksburg, VA 24061
Tel: (540) 231-6302
Fax: (540) 231-3426
Email: lindsayd@vt.edu

JOHN. B. MALONE, JR., D.V.M., Ph.D.
Professor
Department of Pathobiological Sciences
School of Veterinary Medicine
Louisiana State University
Baton Rouge, LA 70803
Tel: (225) 578-9692
Fax: (225) 578-9701
Email: malone@vetmed.lsu.edu

MARK A. MITCHELL, D.V.M., M.S., Ph.D.
Associate Professor
Director, Wildlife Hospital of Louisiana
Department of Veterinary Clinical Sciences
School of Veterinary Medicine
Louisiana State University
Baton Rouge, LA 70803
Tel: (225) 578-9525
Fax: (225) 578-9559
Email: mitchell@vetmed.lsu.edu

CLIFF M. MONAHAN, D.V.M., Ph.D.
Department of Veterinary Preventive Medicine
The Ohio State University
1920 Coffey Road
Columbus, OH 43212
Tel: (614) 292-8335
Fax: (614) 292-4142
Email: monahan.19@osu.edu

TERESA Y. MORISHITA, D.V.M., M.P.V.M., M.S., Ph.D., D.A.C.P.V.
Professor and Poultry Veterinarian
College of Veterinary Medicine
Western University of Health Sciences
309 E. Second Street
Pomona, California 91766
Tel: (909) 469-5512
Fax: (909) 469-5635
email: tmorishita@westernu.edu

MARY PATTERSON, M.S., D.V.M., D.A.C.L.A.M.
Clinical Veterinarian
Division of Comparative Medicine
Massachusetts Institute of Technology
77 Mass Ave., Bldg 16-8th floor
Cambridge, MA 02139
Tel: (617) 324-5403
Fax: (617) 258-5708
Email: mmpatt@mit.edu

JORDAN C. SCHAUL, M.S., Ph.D.
Assistant Director, Laboratory for Wildlife and Environmental Health
College of Veterinary Medicine
Western University of Health Sciences
309 E. Second Street
Pomona, CA 91766
Tel: (909) 469-5512
Fax: (909) 469-5635
Email: jschaul@westernu.edu

TRENTON R. SCHOEB, D.V.M., Ph.D.
Professor, Department of Genetics
Director, Comparative Pathology Laboratory
University of Alabama at Birmingham
724 Kaul Human Genetics Bldg.
720 20th St. South
Birmingham, AL 35294-0024
Tel: (205) 934-2288
Fax: (205) 975-4418
Email: trs@uab.edu

PAT H. SMITH, B.S.
Department of Pathobiological Sciences
School of Veterinary Medicine
Louisiana State University
Baton Rouge, LA 70803
Tel: (225) 578-9710
Fax: (225) 578-9157
Email: psmith@vetmed.lsu.edu

T. BONNER STEWART, Ph.D.
Emeritus Professor
Department of Pathobiological Sciences
School of Veterinary Medicine
Louisiana State University
Baton Rouge, LA 70803
Tel: (225) 578-9684
Fax: (225) 578-9701

CHRISTINE A. SUNDERMANN, M.S., Ph.D.
Professor of Biology
Department of Biological Sciences
131 Cary Hall
Auburn University
Auburn, AL 36849
Tel: (334) 844-3929
Fax: (334) 844-4065
Email: sundeca@auburn.edu

GERALD L. VAN HOOSIER, JR., D.V.M., D.A.C.L.A.M.
Emeritus Professor
Department of Comparative Medicine
Box 357190
University of Washington
Seattle, WA 98195-7190
Tel: (206) 685-3261
Fax: (206) 685-3006
Email: gvanhoo@u.washington.edu

SEKLAU E. WILES, M.Sc.
Research Associate
Department of Pathobiological Sciences
School of Veterinary Medicine
Louisiana State University
Baton Rouge, LA 70803
Tel: (225) 578-9671
Fax: (225) 578-9701
Email: swiles@vetmed.lsu.edu

JAMES D. WILKERSON, J.D., D.V.M., D.A.C.L.A.M.
Associate Director, Laboratory Animal Resource Center
University of California
Box 0564
Medical Science 386D
San Francisco, CA 94143-0564
Tel: (415) 502-2729
Fax: (415) 502-8252
Email: james.wilkerson@ucsf.edu

FLYNN'S PARASITES OF LABORATORY ANIMALS

SECOND EDITION

CHAPTER

1

Collection, Preservation, and Diagnostic Methods

Pat H. Smith, BS; Seklau E. Wiles, MSc; John B. Malone, Jr., DVM, PhD; and Cliff M. Monahan, DVM, PhD

INTRODUCTION

As the scope of this book indicates, the term "laboratory animal" can encompass virtually any animal species used in research. The parasite fauna of such a wide spectrum of hosts seems unlimited. However, within phyla, parasites share many traits. The purpose of this chapter is to describe diagnostic methods useful for parasite phyla likely to be encountered in the research animal environment.

Most laboratory animal facilities should be capable of performing most of the fundamental techniques outlined in this chapter. Performing any of these techniques correctly and reliably requires expertise developed through repetition. For uncommon techniques or obscure parasites, it is often more expedient to send samples to a laboratory with more extensive diagnostic capabilities. Several resources are available for more complete treatment of diagnostic techniques[1–3].

SAMPLE COLLECTION AND PRESERVATION

Feces

Number of Samples to Collect

The number of samples to be collected depends on several factors, including the source and health status of the animals, available financial resources, and the parasite phyla likely to be encountered. For routine screening of an asymptomatic animal, a single sample should suffice. For newly arrived animals with potential parasite exposure or questionable health history, or for symptomatic animals within the colony, sequential fecal examinations are warranted. These are typically performed over three days.

Most nematode infections are easily identified with a single fecal examination because the female worms pass hundreds to thousands of eggs per day. In contrast, low level trematode, cestode, or protozoal infections may not be detected with a single examination because eggs or oocysts may not be passed continuously or daily, or in great number. In these cases, collecting fecal specimens passed on three sequential days will increase diagnostic power. To assess the parasite status of a group of animals, 30 animals or 10% of the group, whichever is greater, should provide adequate sampling coverage.

Sample Collection

Proper collection and preservation methods are critical for finding fecal parasites. A fresh fecal sample, collected rectally or just dropped, is optimal. When feces must be collected from the ground, the specimen should be taken from the middle of the dropping. This will minimize contamination with organisms from the environment. When sampling a group of animals, individual samples should be collected and tested separately. Mixing samples may mask or underestimate the true extent of infection, because parasites are not evenly distributed within host populations. Collected specimens should be placed in clean, wide-mouth plastic containers with screw-top lids, or in sealable plastic bags. Using a permanent marker, specimens should be properly identified with animal identification, date of collection, and species of animal. Specimens should be refrigerated as soon as possible, unless direct smears are to be prepared for the detection of motile protozoa. If collections are made in the field, specimens may be placed among refrigeration packs.

Sample Preservation

Specimens which will not be immediately processed should be immersed in a suitable fixative. The choice of fixative depends on the tests to be performed (Table 1.1). Often, an initial fecal examination is performed on a fresh sample. Positive test results then direct the diagnostician to the appropriate fixation medium for additional testing of the remainder of the sample.

TABLE 1.1 Common fixatives and applications

Fixative	Applications
Formalin	2% in distilled water for modified Knott's recovery of microfilariae 5–10% for concentration techniques (formalin-ethyl acetate; flotations and centrifugations) *Cryptosporidium* and *Giardia* antigen tests Not useful for making permanent mounts of most staining procedures
Schaudin's fluid	Permanent mounts of protozoa stained with trichrome or iron hematoxylin
Polyvinyl alcohol (PVA)	Permanent mounts of protozoa stained with trichrome or iron hematoxylin
Sodium acetate-acetic acid-formalin (SAF)	Concentration techniques (formalin-ethyl acetate; flotations and centrifugations) Permanent mounts of protozoa stained with trichrome or iron hematoxylin
Merthiolate-iodine-formalin	Wet mounts or direct smears Formalin-ethyl acetate sedimentation Limited use for staining of permanent mounts

Adapted from Ash and Orihel (1991) and Garcia (2001).

When sending samples to a commercial diagnostic laboratory, the protocol for preserving and shipping samples should be obtained prior to collection of samples. By adhering to these guidelines, the likelihood of an accurate diagnosis is maximized, and regulatory standards for shipping potential pathogens can be met. Pre-measured fixative vials are available for all of the fixatives described below, and simplify sample processing.

Regardless of the fixation method to be used, sample quality can be improved with centrifugation, or sieving followed by sedimentation. These methods remove water-soluble pigments and debris, and concentrate parasite forms. Diarrheic samples will benefit most by concentration. Ethyl acetate extraction is also useful for removing excess lipid. Once washed or cleaned, droplets of the unfixed sediment can be placed on slides for immediate examination or dried for staining and the remainder of the pellet fixed for shipment to a reference laboratory if necessary. Regardless of the fixative used, samples must be well mixed to ensure complete and uniform fixation of the specimen.

Formalin

Formalin is a readily available fixative that rapidly kills most pathogens, thus decreasing the zoonotic concerns of handling fecal samples. Formalin is not suitable for identifying whole helminths because it makes worms brittle and may interfere with special stains. Formalin fixation also may change the density of parasite structures such that recovery with flotation solutions is decreased. Flotation solutions of higher specific gravity (1.23–1.25) provide optimal recovery of formalin-fixed helminth eggs. Many fecal antigen tests are designed for use with formalin-fixed specimens, but this is not universal and must be verified before use. Also, formalin fixation results in cross-linking of many proteins associated with DNA. This may preclude using formalin-fixed specimens in polymerase chain reaction (PCR)-based assays. For fixation of fecal samples, 5% to 10% neutral buffered formalin solutions (NBF) are most commonly used.

Schaudin's fluid

Schaudin's fluid or fixative is used in-house and for fixing specimens in preparation for shipment. Droplets of a mixture of fresh feces and Schaudin's fluid can be applied directly to microscope slides for drying, then staining. Schaudin-fixed samples are not used in concentration procedures. Specimens can be fixed when passed, or can be prewashed as described below. The latter concentrates parasite forms. Schaudin's fixative provides excellent morphological preservation of trophozoites and amoebic cysts. Schaudin-fixed samples do not adhere well to glass slides, and so must be handled gently. Also, Schaudin's fixative contains mercury and therefore must be handled with caution. Newer preparations are available that employ zinc or copper as a substitute. While there may be a slight decline in the preservation of protozoal morphology, such as the chromatin pattern of amoebic cysts, handling and disposal of reagents with zinc or copper are less problematic than for reagents containing mercury.

Polyvinyl alcohol

Polyvinyl alcohol (PVA) was developed to overcome specimen adherence problems of Schaudin's fixative. While PVA fixation optimizes staining of some parasites, particularly intestinal protozoa, other fixatives are preferred for concentration procedures. Because PVA is carcinogenic, it must be handled with caution.

Merthiolate-iodine-formalin

Merthiolate-iodine-formalin (MIF) is commonly used for fecal specimens to be examined as direct wet mounts or following concentration techniques. It is not useful for preparing permanent mounts or for fixing specimens prior to staining. This fixative will also inactivate most pathogens.

Sodium acetate-acetic acid-formalin

Sodium acetate-acetic acid-formalin (SAF) is a good compromise fixative for shipment of samples destined to be processed either as permanent stains or concentration procedures. There may be a slight decline in protozoal integrity compared to the use of Schaudin's or PVA, but SAF does not contain mercury. Samples fixed with SAF can be stained with iron hematoxylin or trichrome stains.

Blood

Blood-borne parasites include the protozoan hemoparasites and the microfilariae (MF) of filarid nematodes, both of which benefit from collection of blood with an anticoagulant. Blood samples are also collected in tubes lacking anticoagulant, for use for antigen and antibody tests. Protozoan hemoparasites are typically identified by microscopic examination of stained blood smears. Thin films can be prepared immediately or from preserved whole blood. Most staining procedures can be performed on films that have been fixed with methanol. Although MF can often be found on blood films, adequate visualization

is difficult for identification to genus or species. Samples of blood with an anticoagulant are necessary because the MF cannot be removed from a clot for staining.

Collecting adequate blood from small animals can be problematic. Following venipuncture, blood can be drawn into a single hematocrit tube from which a blood smear can immediately be made. The remainder can be centrifuged for determination of packed cell volume. The tube can then be scored and broken at the buffy coat for recovery of MF, and the small quantity of serum or plasma can be harvested for serology.

Urine

Urine samples can be collected and centrifuged to concentrate helminth eggs or microsporidia. These can be stored in saline and refrigerated for days if they cannot be examined immediately. For longer periods, fixation with 10% NBF or 70% ethanol and 5% glycerin are useful preservatives.

Tracheal Lavage Samples

Tracheal lavage samples should be collected from deep within the respiratory tract, using sterile saline. Lavage samples can be viscous in nature, and high viscosity can interfere with sample processing. Viscous samples should be mixed with a solution of 3% sodium hydroxide in saline, then centrifuged to concentrate parasite forms. Very thick mucus plugs can be subjected to ethyl acetate sedimentation as described for fatty fecal samples. Following centrifugation, samples can be preserved in 10% NBF, 70% ethanol (for helminths), or PVA fixative (for protozoa).

PARASITE COLLECTION AND PRESERVATION

Helminths

Helminths collected during necropsy examinations or passed directly by animals should be placed immediately into a container of 70% ethanol heated to 60°C to 63°C. This treatment will cause the helminths to straighten. Also, adult cestodes and acanthocephalans will protrude the rostellum or proboscis, respectively. Worms can then be transferred to 70% ethyl alcohol and 5% glycerin for long-term storage.

Arthropods

Macroscopically visible arthropods should be placed into 70%–90% ethanol. Formalin should not be used because fixation in NBF renders arthropods brittle. Skin scrapings can be collected directly onto microscope slides bearing a drop of mineral oil. However, initial processing with 10% potassium hydroxide (KOH) will facilitate visualization of arthropods by rendering the keratin more transparent. External parasites may frequently be recovered on clear adhesive tape that is brushed across the animal's fur, then adhered to a microscope slide.

FECAL EXAMINATION TECHNIQUES

Direct Smear Method

The direct smear is used only with samples in which motile trophozoites are suspected. The small quantity of sample employed is inadequate for other diagnostic procedures. The fecal sample should be either loose stool or diarrhea. Formed feces are unlikely sources of trophozoites, since under such conditions trophozoites either dehydrate and become distorted or form cysts during normal intestinal transit. Specimens must be examined immediately, before low external temperatures decrease trophozoite motility. Refrigeration of fecal samples renders trophozoites nonmotile and should not be used prior to preparing direct smears.

Materials

- Microscope slide and coverslip
- Saline
- Fecal loop or applicator stick
- Lugol's iodine

Method

1. Place a drop of saline on one end of a microscope slide and a drop of Lugol's iodine on the other.
2. Add a small quantity of fresh fecal specimen first to the saline drop and mix thoroughly, then transfer a small amount of the specimen to the Lugol's iodine drop.
3. Place a coverslip over each mixture.
4. Examine the saline/sample side first, with the light adjusted for ample contrast. Do not mistake Brownian motion for motility. Examine the entire coverslip using the 10 × objective, then 20 fields using the 40 × objective.
5. Examine the drop with Lugol's iodine for comparison.

Interpretation

The direct smear is a method for finding motile trophozoites. The quantity of sample used is so small that this method is not likely to accurately reflect the range of parasites which may be discovered using a concentration technique. Even when a direct smear is found to be positive, a concentration technique is still warranted to detect additional parasite forms. Not all protozoa observed in direct smears are parasitic, and therefore responsible for the clinical signs observed. During bouts of loose stool or diarrhea, intestinal or cecal protozoa can be expelled that are not normally seen during fecal examinations of asymptomatic animals. This is particularly true with herbivores, including reptiles and amphibians, because several ciliates and flagellates participate in digestion. Unwarranted treatment of these protozoa may alter the normal intestinal flora and prolong the symptoms.

Fecal Concentration Methods

The recovery of fecal parasites is enhanced by concentration procedures. These include flotation and sedimentation techniques, both of which depend on differences in specific gravity (sg) between the parasite form and the surrounding solution. Flotation techniques concentrate parasites by employing hypertonic solutions so that parasite forms rise to the surface of the flotation solution, while most debris fall (Table 1.2).

Sedimentation techniques employ solutions less dense than the parasites, so that parasite forms concentrate at the bottom of the collection vessel. Sedimentation methods generally allow for the recovery of more parasites than do flotation methods. With sedimentation, everything can be recovered, whereas with flotation techniques only those items of lower specific gravity than the flotation medium are recovered. Sedimentation techniques also are more easily performed in the field. In contrast, sedimentation has the disadvantage of greater debris, which can complicate examination. Furthermore, when examining sediment, one must focus through multiple focal planes because parasite forms will drift at different levels within the solution between the slide and the coverslip. This results in longer examination time, versus flotations.

TABLE 1.2 Common flotation solutions.

Solution	Specific Gravity	Ingredients/1 L H_2O
Sodium chloride	1.20	311 g sodium chloride
Sodium nitrate	1.20	338 g sodium nitrate
Sodium nitrate	1.30	616 g sodium nitrate
Sugar	1.20	1170 g sugar*
Sheather's sugar	1.27–1.30	1563 g sugar*
Zinc sulfate	1.20	493 g zinc sulfate

*Requires refrigeration of stock solution or addition of 9 ml phenol as preservative

Passive Flotation

Passive flotation relies solely on gravity to separate parasites and debris, and is therefore much less sensitive than centrifugal flotation (discussed below). The densities of many parasite forms are too similar to those of the common flotation media to be recovered without the added force provided by centrifugation.

Although both zinc sulfate or sodium nitrate solutions can be used, zinc sulfate is preferable to sodium nitrate because the latter is more caustic and will degrade many helminth eggs, as well as protozoan cysts. Additionally, sodium nitrate solutions crystallize more quickly than zinc sulfate, and crystallization can distort parasitic structures.

Common mistakes in performing passive flotation include setting up multiple samples at one time and reading each sample as time permits. This results in nonuniformity in flotation time, and greater potential for crystallization to render slides unreadable. To minimize crystallization, slides may remain in place on top of the flotation apparatus until they are ready to be read. However, exceeding the recommended 15-minute flotation may result in salt solutions equilibrating with the internal milieu of the egg or oocyst, either by passive diffusion or by extraction of water into the hypertonic float solution through osmotic forces. As a result, eggs or oocysts will become distorted and no longer buoyant, and may fall away from the microscope slide. False negative results are more often obtained with the last slides to be read.

If zinc sulfate solution is used, all of the slides could be removed and coverslips applied at the 15 minute time point. Slides should then be placed on a rack in a simple humidified chamber to decrease the rate of crystal formation (Figure 1.1). These slides can be removed from the chamber and read as soon as possible, or the chamber placed in a refrigerator to be read later in the day. All salt solutions will crystallize, thus the timing of microscopy is very important.

Materials

- Pill vial or sputum jar
- Small petri dish or watch glass

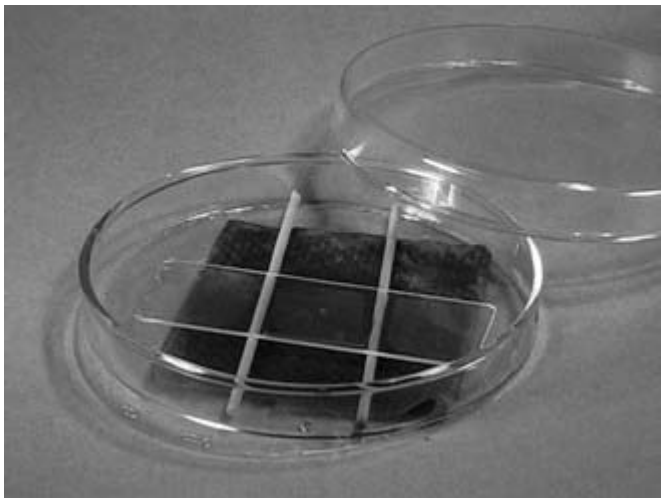

Fig. 1.1 A simple humidified chamber can be assembled to decrease the rate of crystal formation of flotation solutions.

- Disposable cup
- Applicator sticks
- Flotation medium (1.20 sg)
- Microscope slides and coverslips
- Tea strainer

Method

1. Place 2 to 3 g of fecal sample in the disposable plastic cup using the applicator sticks.
2. Add a small quantity of flotation medium and mix into a slurry.
3. Continue adding flotation medium, stirring to mix thoroughly.
4. Place the pill vial in the small petri dish as a guard against overflow.
5. Pour the mixture through the tea strainer into the pill vial, stirring with the applicator sticks to facilitate flow through the strainer.
6. Add drops of the float medium until a slight, bulging meniscus forms above the rim of the vial.
7. Place the microscope slide on top of the meniscus.
8. Allow 15 minutes for parasite forms to rise to the surface.
9. Gently lift the slide from the pill vial, invert the slide, and place a coverslip on the droplets of sample adhering to the slide.
10. Examine the entire coverslip using the 10 × objective, followed by 20 fields using the 40 × objective.

Interpretation

Passive flotation can be used effectively when the technician understands the limitations of the technique. Only a small subset of parasite forms will be recovered even when the technique is performed optimally. Strongyle-type eggs and coccidian oocysts are often passed in sufficient numbers that the poor sensitivity of passive flotation is overcome during routine fecal screening. Other parasite forms may not be sufficiently recovered. For this reason, passive flotation is not the diagnostic method of choice where accuracy is required.

Centrifugal Flotation

Centrifugal flotation is more sensitive than passive flotation because it magnifies gravitational forces, thereby accelerating the downward movement of more dense debris and the upward movement of less dense parasite forms.

The basic process of preparing a fecal sample for centrifugation is identical regardless of the flotation medium to be used. The sample should first be centrifuged with water to remove water-soluble pigments, free lipids, and other small debris.

Flotation solutions range from 1.20–1.30 sg (Table 1.2). The preferred salt solution for examination of fecal samples from carnivores is zinc sulfate at 1.20 sg. Zinc sulfate is sufficiently gentle to protozoal cysts that it enhances their recovery without distortion. Zinc sulfate at 1.20 is less effective at recovery of very dense parasite forms, such as *Physaloptera* eggs. For improved visualization of *Giardia* cysts, drops of Lugol's iodine can be added to the fecal pellet and mixed thoroughly for 30 seconds prior to addition of the zinc sulfate.

In general, sugar solutions are less sensitive than zinc sulfate. Sugar solutions are more viscous than salt solutions, and therefore are not very useful for passive flotation. Sugar solutions should be prepared with a preservative (e.g. formalin) to retard bacterial or yeast growth, since digestion of the sugar molecules will lower the specific gravity. Sheather's sugar is a more concentrated or super-saturated solution (1.30 sg) that is particularly suited for recovery of *Cryptosporidium* sp. oocysts.

Sugar solutions are superior to salt solutions in many ways. Sugar solutions are less expensive to make, do not distort eggs or oocysts to the same degree as salt solutions, and will not crystallize rapidly. The latter advantages mean that prepared slides may be refrigerated for days prior to

examination, without loss of parasite structural integrity. Sugar solutions are particularly useful for processing herbivore fecal samples. Flotation solutions should be compared through side-by-side preparations using known positive samples.

Centrifuges with swinging bucket rotors are preferred because they allow each tube to be filled more than is possible with fixed-head rotors. Many diagnosticians prefer to place the coverslip on the sample tube during the centrifugation steps. This is not possible with fixed-head rotors. Because small vibrations can cause a coverslip to be lost during centrifugation, many laboratories perform the centrifugations with the fluid level in the tube at the maximum possible, then transfer the tube into a stationary rack before placing the coverslip on the sample to allow parasite stages to adhere to the coverslip. Sensitivities are equivalent for the two variations, and the difference in time required is negligible.

Materials

- Disposable plastic cups
- Applicator sticks
- Water or saline for washing
- Plastic centrifuge tubes and screens
- Centrifuge; swinging-bucket preferred, but fixed-head is also possible
- Test tube rack
- Flotation solution

Method

1. Place 2 to 3 g of feces in a disposable plastic cup. Mix very well with a small quantity of water and when mixed thoroughly, increase quantity of water to create a loose slurry. The quantity of water used should be approximately the volume of the centrifuge tube being used (approximately 15 ml).
2. Pour this mixture through a screen into a centrifuge tube and assist the passage through the screen by agitating with the applicator sticks.
3. Bring the volume of water in the sample tube to the top of the centrifuge tube, and equal to the volume in a second (balance) tube.
4. Centrifuge at 400 g for 3–5 minutes.
5. Remove sample tube from centrifuge and decant supernatant. If it is difficult to visualize the pellet apart from the supernatant, repeat this washing step by mixing the pellet thoroughly with water or saline a second or third time until the supernatant is clear.
6. Place a small drop of the washed pellet onto a microscope slide and examine as a sediment, or dry for staining.
7. Mix the remainder of the pellet thoroughly with a small volume of the flotation solution of choice, until a loose paste is achieved.
8. Bring the volume of the flotation solution to within millimeters of the rim of the centrifuge tube. Return the tube to the centrifuge. Place a balance tube opposite the sample tube. The specific gravity of water is only 1.00, thus a separate balance tube for flotation solutions is necessary.
9. Centrifuge for 5 minutes; 10 minutes if anticipating *Cryptosporidium* oocysts.
10. Transfer the tubes from the centrifuge to a test tube rack.
11. Add drops of the flotation solution to the top of the tube until a slightly bulging meniscus is formed. Do not overfill the tube because the floating parasite stages will be lost.
12. Place a coverslip on the slightly bulging meniscus and allow to stand 10 additional minutes.
13. Remove the coverslip to a microscope slide for examination.

Interpretation

Common mistakes in the performance of centrifugal flotation, which result in false negative results include:

1. Failure to thoroughly mix the sample with water prior to passage through the screen into the centrifuge tube, resulting in failure of parasite forms to pass through the screen. Often, too much water is added initially, so that the fecal sample drifts about without breaking apart.
2. Failure to stir or agitate the fecal slurry as it passes through the screen, rather than allowing it to simply drip through the tube, resulting in the buildup of debris on the screen that traps the suspended eggs or oocysts. This mat must be disrupted by stirring with the applicator sticks.
3. Failure to mix the pellet formed after centrifugation with a small quantity of flotation medium before filling the tube. The pellet is difficult to mix when the tube is too full with solution. Failure to mix adequately will trap any eggs or oocysts within the pellet, reducing sensitivity.
4. Overfilling the tube so that instead of forming a meniscus, parasite forms spill out of the tube and are lost.

Baermann Sedimentation

The Baermann technique uses simple gravity sedimentation to recover nematode larvae, either from a fecal culture or from tissue digests that liberate any larvae that may be present. The sample is placed into a funnel with warm water to facilitate nematode motility. Pulmonary tissues may be homogenized in a blender to recover lungworms, and diaphragm or other muscle tissues may be homogenized and placed in a Baermann apparatus for recovery of *Trichinella spiralis* larvae.

Materials

- Fine screen mesh or sieve, nylon coffee filter, or cheesecloth
- Funnel with latex tubing attached, with clamp
- Ring stand to hold funnel
- Collection tube
- Dish to collect spillage
- Petri plate for microscopic examination of the collected sediment
- Warm water to fill the Baermann apparatus

Method

1. Place clamp on latex tubing in open position and attach one end of the tubing to the funnel.
2. Insert collecting tube into the other end.
3. Place funnel assembly into a ring stand.
4. Add warm water to fill latex tubing and collecting tube until the funnel is half full.
5. Loosely wrap fecal or tissue sample in cheesecloth or place into sieve or coffee filter.
6. Place the sample into the funnel and gently fill with warm water until the sample is covered.
7. Leave the sample in the funnel for 12 to 18 hours.
8. Clamp the latex tube to prevent excess water from draining when the collecting tube is removed from the latex.
9. Decant the collected volume into a petri plate and examine this sediment for larvae.

Interpretation

The Baermann sedimentation is a technique often requested inappropriately due to a misunderstanding of its strengths and weaknesses. Historically, the Baermann has been used to recover cattle lungworm and strongylid larvae from feces. These larvae are very active and will swim free of the fecal sample. With parasitic infections that pass eggs or oocysts, or less active larvae, the Baermann sedimentation is far less sensitive than centrifugal flotation techniques. The first-stage larvae of most Metastrongyloidea are not active enough to free themselves from the feces in which they were passed, since these nematodes use gastropods as intermediate hosts. Gastropods are drawn to feces for the nitrogenous meal that feces can provide, thus active larvae that leave the feces are less likely to be consumed by gastropods. This feature favors larvae that remain with the feces. In contrast, cattle lungworms and larvae of strongylid nematodes develop directly on pasture without an intermediate host. Larvae of these nematodes more actively extricate themselves from the fecal sample.

Simple Gravity Sedimentation

Simple gravity sedimentation can be performed without a centrifuge and is intended to collect parasite eggs too dense to recover with common flotation media, such as eggs of *Fasciola hepatica.* It also cleans some debris and water-soluble pigments in the process of decanting. The process involves a two-step sedimentation and decanting method whereby the first step follows a brief sedimentation that removes the densest debris while the parasite forms remain in the water column that is decanted into a second vessel for the second, longer sedimentation step. A pilsner glass or funnel-shaped vessel provides an advantage over a flat-bottom beaker in that the sediment is concentrated into the narrow bottom of the pilsner glass.

Materials

- Fecal sample and mixing container
- Water or saline
- Pilsner glasses or conical, round-bottomed vessels, approximately 250 ml capacity
- Petri dish for microscopic examination
- Methylene blue as an optional stain

Method

1. Mix the fecal sample in a container using water or saline of the approximate volume of the pilsner glass or other vessel.
2. Suspend the sample well and pour into the pilsner glass.
3. Allow the heaviest debris to sediment for about 2 minutes.
4. Decant the suspended sample into the second pilsner glass and allow this to sediment for at least 2 hours.
5. Decant the supernatant carefully so as to leave the sediment undisturbed.
6. Pour aliquots of the sediment into a petri dish and examine with a dissecting microscope. Several drops of methylene blue can add contrast to aid in visualization.

Interpretation

This sedimentation technique is applicable for heavy eggs, such as those of *Fasciola hepatica* or *Schistosoma* sp. It is readily applicable to field work.

Formalin-ethyl Acetate Sedimentation

Formalin-ethyl acetate sedimentation uses ethyl acetate as a non-miscible solvent to extract lipid from feces. Ethyl acetate forms a layer above the water or saline. The extracted portion of lipid is drawn into the ethyl acetate plug and can be discarded with the supernatant (Figure 1.2).

Formalin-ethyl acetate sedimentation is useful for cleaning steatorrhea or other diarrheic feces, thereby facilitating microscopic examination. The pellet can be examined directly, to prepare smears for staining, or for flotation procedures. Formalin-ethyl acetate sedimentation is highly efficient in recovering all parasite forms, including trophozoites, cysts, oocysts, eggs, and larvae. It is commonly used in human diagnostic laboratories. Formalin is used to kill any pathogens present, but water or saline can be employed to retain motility of protozoan trophozoites as an aid in identification. The use of formalin is most desirable where the concern of zoonotic disease is high, such as with non-human primates.

Most common intestinal parasites can be recovered using flotation techniques; however, sedimentation procedures are warranted where test sensitivity must be maximized. Viewing sediments can be tedious due to the range of focal planes that must be traversed. Formalin-ethyl acetate sedimentation, followed by centrifugal flotation, results in high sensitivity and economy of effort.

Materials

- Phosphate buffered saline (PBS) with or without 10% formalin (water can be used when trophozoites are not anticipated)
- Disposable plastic cup for mixing the fecal sample
- Applicator or stir sticks
- Polypropylene centrifuge tubes with caps
- Filter funnel
- Ethyl acetate
- Cotton swabs

Method

1. Place sample in a sealable container; add PBS with 10% NBF. Allow approximately 20 minutes for fixation.
2. Pour the sample through the filter screen into a polypropylene centrifuge tube to a volume of 10 ml. Polypropylene tubes are necessary due to the solvent activity of the ethyl acetate.
3. Add 3 ml of ethyl acetate to the sample; cap and shake vigorously to mix.
4. Centrifuge at 1,000 rpm for 5 to 10 minutes (10 minutes if *Cryptosporidium* is suspected).
5. Remove the tube from the centrifuge, remove the cap, and, using a stir stick, ring the plug of ethyl acetate and any trapped debris, freeing the plug from the wall of the centrifuge tube.
6. Decant the supernatant and ethyl acetate plug into a disposal container that will not be degraded by the ethyl acetate.

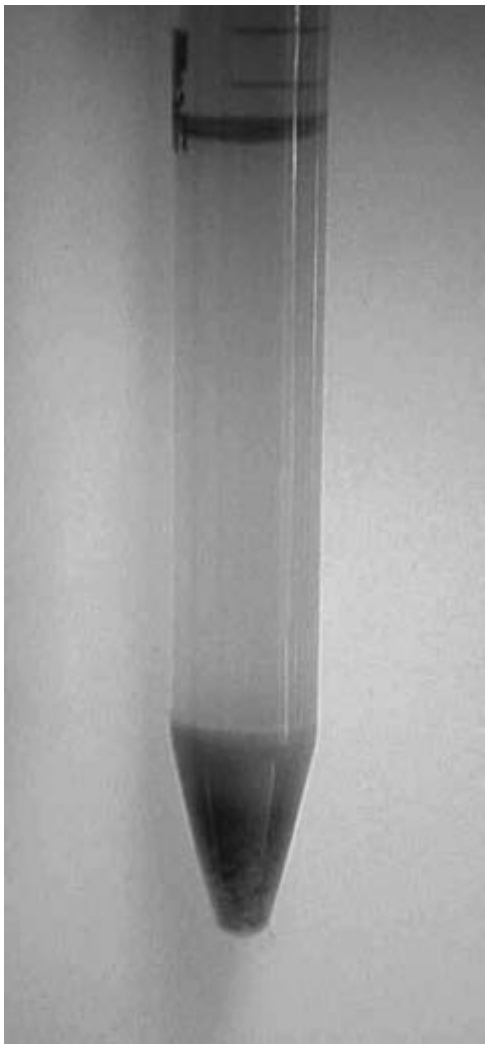

Fig. 1.2 Formalin–ethyl acetate sedimentation. Ethyl acetate forms a layer above the water or saline. The extracted portion of lipid is drawn into the ethyl acetate plug and can be discarded with the supernatant.

7. Using a cotton swab, remove residual ethyl acetate around the tube or perform another washing step to clean the pellet of any additional ethyl acetate droplets. These droplets can resemble amoebic cysts and confuse the microscopic interpretation.
8. Add a small amount of saline to the pellet and resuspend.
9. Place drops of this suspension on a microscope slide, place a coverslip on top, and examine.

Interpretation

The ethyl acetate plug can trap some eggs and cysts. Specific examples include *Alaria canis* and *Giardia intestinalis.* Ethyl acetate must not be discarded into municipal sewage systems but must be collected for disposal through an appropriate chemical processing system. For this reason alone, many laboratories may find it preferable to do several washing steps with saline or tap water to remove free lipid droplets and water-soluble pigments, instead of performing formalin-ethyl acetate sedimentation.

Fecal Stains

Preparation of slides for staining should be part of a routine diagnostic workup for symptomatic patients, but it serves little purpose for asymptomatic animals. Once prepared, the slides can be stained or held pending results of flotation or sedimentation procedures. Slides can also be prepared from the cleaned fecal pellet prior to the final centrifugation with the flotation medium.

Basic staining procedures adaptable to most laboratories include the Gram stain and the modified acid-fast stain. More specialized stains, such as Gomori's trichrome or Masson's trichrome stains, and the iron hematoxylin stain, can be included in the repertoire if consistent need dictates. Otherwise, it is generally more expedient and reliable to send fixed slides or samples to specialized laboratories that perform these techniques regularly. The shelf-life of reagents affects the decision whether to perform specialized stains in-house or to outsource them. Older reagents give unsatisfactory results. The Gram stain can be used for differentiating *Giardia* sp. from *Candida*-like, elliptical yeast, which stain Gram positive, whereas *Giardia* contain both positive and negative structures within the cyst. The method for performing the Gram stain is presented in microbiology laboratory manuals and will not be described here.

Modified Acid-fast Stain

The modified acid-fast stain can be used to detect *Cryptosporidium* sp. and fecal microsporidia. This procedure does not require heating of the slide or stain and uses a brilliant green counterstain, which facilitates visualization.

Materials

- Microscope slides, coverslip optional
- Absolute methanol
- Carbol fuchsin stain
- Acid alcohol decolorizer
- Brilliant green stain
- Immersion oil

Method

1. Air dry droplets of the fecal sample or smear on a clean glass slide.
2. Fix the dried slide in methanol for 2 minutes.
3. Cover the fecal smear with carbol fuchsin for 2 minutes.
4. Rinse gently with tap water.
5. Apply drops of the acid alcohol decolorizer for 1 to 6 seconds.
6. Rinse gently with tap water.
7. Cover the smear with brilliant green counterstain for 2 minutes.
8. Rinse gently with tap water, then blot or air dry until ready to examine microscopically.

Interpretation

The best results will be obtained when the fecal sample has been washed by centrifugations with water, or subjected to the ethyl acetate extraction process prior to placing droplets on slides. Smears must be uniformly thin and translucent for even decolorization, otherwise over-decolorization can result in false negatives.

The staining procedure is easy to perform but does require perfecting several steps in the process. Thin, homogeneous smears are very important because small particles of debris will leach carbol fuchsin during the decolorizing step and this may lead to excessive decolorizing time. A positive sample should be obtained and used to perfect the technique.

When the slide is dry it can be examined with oil immersion, but the red-stained oocysts are readily visible with lower power objectives. Place a drop of immersion oil on the stained sample before adding a coverslip. This

compensates for light refraction from an otherwise irregular surface, and facilitates identification of stained oocysts.

Antigen and Fluorescent Antibody Diagnostics

Parasite antigens pass in feces and in some cases, are more readily identified using antigen tests than are parasite forms using microscopic examination. Test procedures include direct and indirect immunofluorescent antibodies directed at parasite antigens, enzyme immunoassays, and membrane chromatographic assays. Fluorescent antibodies directed against parasite antigens facilitate detection, but require use of a microscope with a UV light source. Enzyme immunoassays are labor intensive and are therefore most susceptible to operator error. Rapid chromatographic membrane assays are the most applicable to a typical diagnostic laboratory. They are very simple to perform but are more expensive per individual than enzyme immunoassay.

Antigen tests are not intended to replace microscopic examination, but to serve as useful adjuncts to microscopy. Eggs, oocysts, and larvae that do not react with the primary antibodies used in the assay will go undetected if an antigen test is used as the sole diagnostic procedure. However, some protozoan cysts or oocysts are not passed consistently, resulting in the need to perform multiple fecal examinations to ensure accurate diagnostic outcome. This is particularly true for the genera *Giardia, Cryptosporidium,* and *Entamoeba.* In these cases, antigen detection tests may facilitate diagnosis.

Most of the commercially available tests for fecal antigen detection are marketed for human diagnostics. The range of commercially available antigen detection kits applicable to laboratory animals is limited. Lists of currently available commercial human parasite antigen detection kits are available online through the Centers for Disease Control and Prevention.

DETECTION OF MICROFILARIA

Filarid nematodes produce MF that are ingested by biting arthropod intermediate hosts during a blood meal. Depending upon parasite species, blood or tissue samples may be collected and processed for the recovery and identification of MF. For select filarid infections of animals, antigen detection is possible using commercially available antigen detection kits such as for *Dirofilaria immitis.* These may be used regardless of host species. In contrast, detection kits that recognize host antibodies generated to parasite-specific antigens rarely have cross-species applications.

Blood

For large animals, a 1 ml venous blood sample provides sufficient sample for testing. For animals too small to provide this volume, a microhematocrit tube can be filled, and, following centrifugation, the tube placed on a microscope stage and the area of the buffy coat examined for MF. The interface between the buffy coat and the plasma fraction is most productive. The microhematocrit tube can be scored and broken at this junction to harvest any MF that may be present, saving the red cells for blood films and the serum or plasma for other tests.

Direct Smear or Wet Mount

The volume used in preparing a wet mount is so small that this is not intended to serve as a definitive diagnostic test, but merely as a quick screening tool for the presence of MF.

Materials

- Saline
- Applicator stick
- Microscope slides and coverslips

Method

1. Place a drop of saline on a microscope slide.
2. Mix a drop of the blood sample into the saline droplet.
3. Apply a coverslip and examine for MF.

Interpretation

A wet mount typically will not provide sufficient visualization to make identification of genus or species possible. Saline is important to dilute the red cells without rupturing them, as might a hypotonic solution. Samples that have been refrigerated may require brief warming for MF to resume activity.

Modified Knott's Test

The modified Knott's test uses a hypotonic solution to lyse red blood cells (RBC), leaving the white blood cells (WBC) and MF intact. A solution of 2% formalin in distilled water is often used to straighten the MF, facilitating identification. If visualization alone is desired, using distilled water without formalin allows the MF to continue

moving, which can aid in detection. A centrifuge is most desirable for pelleting the lysate, but recovery of MF can be achieved by passive sedimentation.

Materials

- Centrifuge tubes, 12 to 15 ml
- Lysing and fixative solution (2% formalin/distilled water)
- 0.1% new methylene blue
- Microscope slides and coverslips

Method

1. Add 1 ml of blood to a centrifuge tube.
2. Add 9 ml of hypotonic lysing buffer (2% formalin/distilled water).
3. Invert the tube several times, causing the RBCs to rupture.
4. Centrifuge for 3–5 minutes at low speed.
5. Decant the supernatant carefully to retain the pellet in the bottom of the tube.
6. Add a drop of 0.1% new methylene blue and mix with the pellet.
7. Place a drop of this mixture on a microscope slide and coverslip for examination.

Polycarbonate Filter Technique

The polycarbonate filter technique uses a filter and filter housing unit as components of a commercially available kit. Therefore, this test is more expensive to perform than the Knott's test. However, the polycarbonate filter technique does not require a centrifuge. MF can be recovered, but it is not as easily visualized and identified to species as with the Knott's test. The polycarbonate filter technique is intended to detect only the presence of MF in animals known or suspected to be infected with adult filarid nematodes, such as dogs already determined to be antigen positive for *Dirofilaria immitis* infection.

Materials

- Syringe, 12 to 15 ml
- Filter housing and filters
- 0.1% methylene blue
- Lysing buffer (distilled water will suffice)
- Microscope slides and coverslips

Method

1. Draw 1 ml of blood into a 12 to 15 ml syringe.
2. Draw at least 9 ml lysing buffer into the syringe.
3. Invert for 2 minutes.
4. Place polycarbonate filter into filter holder, verifying that the gasket seal is in place.
5. Affix the filter apparatus to the syringe.
6. Slowly expel the lysed blood solution through the filter.
7. Remove the filter apparatus and refill the syringe with water.
8. Replace the filter apparatus onto the syringe.
9. Slowly expel the wash water through the filter to remove excess debris.
10. Remove the filter apparatus and refill the syringe with air.
11. Reattach the filter apparatus and flush the filter with air.
12. Remove the filter from the holder and place on a microscope slide with the side containing MF facing up.
13. Add a drop or two of methylene blue solution.
14. Place a coverslip on the liquid and examine for MF.

Interpretation

The polycarbonate filter technique is used when the genus or species of the MF, if present, is known. Clear visualization of MF, for identification of genus or species, requires the Knott's test.

Skin Samples

Some arthropods which transmit filarid nematodes feed on serum or plasma rather than whole blood. In these cases, MF are found within tissues such as the skin, rather than circulating in the blood stream. Skin biopsies must be processed to free the MF for visualization.

Materials

- Skin biopsy instruments
- Scalpel blades
- Pipettes
- Saline without preservative
- Microscope slides and coverslips
- Centrifuge tubes, 15 to 50 ml depending on sample size

Method

1. Macerate biopsy sample using a scalpel blade.
2. Place macerated material into a centrifuge tube with saline.
3. Incubate overnight at 37°C.
4. Remove tissue, then centrifuge for 3 to 5 minutes at low speed.
5. Carefully decant supernatant.

6. Using a pipette, place drops of the sediment on a microscope slide.
7. Place a coverslip on the drops and examine at low power (10 × objective).

Interpretation

The tissue sample does not need to be finely homogenized because the MF have motility and can extricate themselves from the macerated sample. The tube does need incubation near body temperature to facilitate motility.

MICROSCOPY TECHNIQUES

Standard Practice for Reading Microscope Slides

A standard approach to microscopic examinations of prepared specimens is essential for effective and efficient diagnoses of parasitic infections.

For fecal examinations, the 10 × objective is most commonly used, and the entire coverslip is examined systematically. Beginning at one corner of the coverslip, the slide is moved either vertically or horizontally in a direct line to the other corner of the coverslip. A mental notation is made of a small object just at the edge of the optical field, and the slide is moved just far enough to bring the object to the boundary of the new optical field. The objective is moved in a direct line back across the coverslip. The process is repeated until the entire coverslip has been examined with the 10 × objective. During these direct horizontal or vertical sweeps, the operator must learn to use the fine focus adjustment to change focal planes continuously because the smallest objects will be visible in different focal planes than those of larger diameter. Once the entire coverslip has been examined with the 10 × objective, 20 random fields should be examined with the 40 × objective, again, using the fine adjustment to compensate for changes in focal plane.

For blood films, or permanent mounts to be examined with the oil immersion lens (typically the 100 × objective), each laboratory must establish a standard number of fields to be examined, or a standard number of sweeps across the coverslip. The size of coverslip used should also be standardized. Coverslip dimensions of 22 × 22 mm are most commonly used. Larger sizes may have special application, but are too large for standard use.

Use of the Ocular Micrometer

An ocular micrometer is essential for accurate diagnoses. Measurements are often important for differentiating parasites from pseudoparasites, such as differentiating a grain mite egg from a parasitic strongyle-type egg. Differentiating larvae in a fecal sample or MF recovered from a Knott's test relies on measurements. A micrometer is placed in one of the ocular pieces of the microscope. The micrometer must be calibrated using a standardized, commercially available, etched microscope slide. Parasite eggs and oocysts are described in reference texts within a range of measurements because the area under the coverslip is three-dimensional, and eggs or oocysts can rotate within that space, so that objects may be viewed and measured while lying at different angles.

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CHAPTER

2

Biology of the Protozoa

David S. Lindsay, PhD, and Christine A. Sundermann, MS, PhD

INTRODUCTION

Protozoa are single-celled eukaryotes. They are a structurally and genetically diverse multiphyletic group of organisms (Figures 2.1, 2.2, 2.3, and 2.4)[1,2,3]. Many protozoa have features in common with fungi or algae, and the term "protist" is often used instead of "protozoa" to emphasize this fact.

FLAGELLATES: PHYLA EUGLENOZOA, PARABASALIA, RETORTAMONADA, AXOSTYLATA, CHROMISTA

Flagellated protozoans, sometimes referred to as "mastigophorans," can be either free-living or parasitic. The major groups which contain parasitic species are the kinetoplastid flagellates, parabasalians, and retortamonads; these group names are sometimes elevated to class or phylum by various taxonomists[4].

Special Organelles

Whereas free-living flagellates typically possess a full complement of organelles, many parasitic flagellates lack one or more common organelles, and instead possess unique structures. For example, the trophozoite stage of most flagellates contains one nucleus, whereas *Giardia* spp. are binucleated (Figure 2.5)[5].

The kinetoplastid flagellates (e.g., *Leishmania* spp., *Trypanosoma* spp.) contain a unique structure, the kinetoplast. The kinetoplast is a large mass of mitochondrial DNA positioned near the flagellar basal body. Kinetoplast DNA exists as mini- and maxicircles and is located at one end of the cell's single mitochondrion[6], which is sometimes

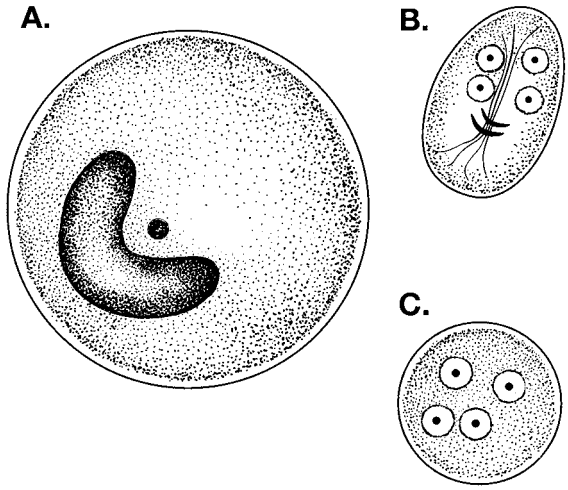

Fig. 2.1 Examples of diversity among protozoal cysts. (A) Cyst of a ciliate. (B) Cyst of a flagellate. (C) Cyst of an amoeba.

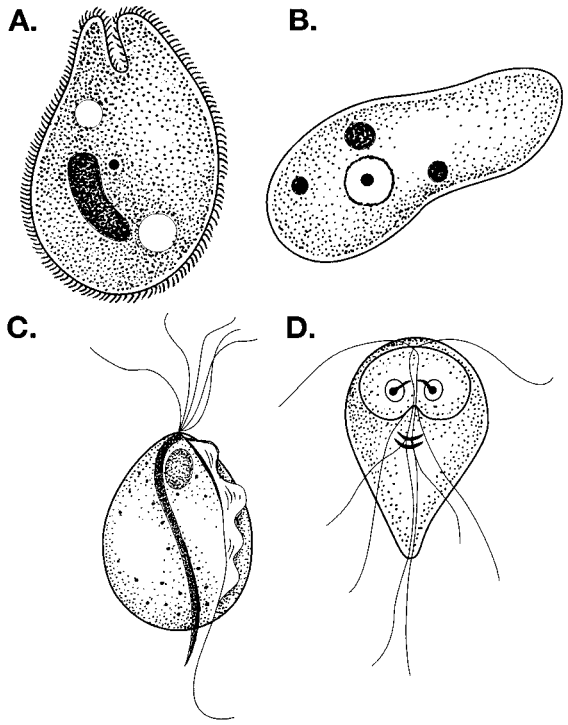

Fig. 2.2 Examples of diversity among protozoal trophozoites. (A) Trophozoite of a ciliate. (B) Trophozoite of an amoeba. (C) and (D) Trophozoites of flagellates.

referred to as the chondriome. Kinetoplast DNA readily stains with traditional DNA stains, and is easily observable under light microscopy. The glycosome is another organelle unique to some developmental stages of kinetoplastid flagellates. This membrane-bound organelle contains enzymes

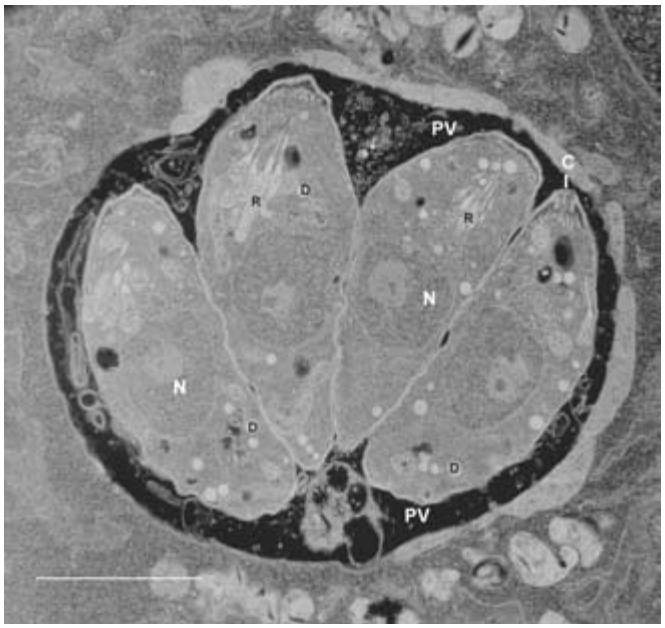

Fig. 2.3 A group of *Toxoplasma gondii* tachyzoites in a parasitophorous vacuole (PV) in a human fibroblast cell. The apical end (C) is clearly visible in 1 tachyzoite. The nucleus (N), rhoptries (R), and dense granules (D) are also readily visible in several tachyzoites. Transmission electron micrograph. Bar = 2 micrometers.

that function in the glycolytic pathway, and thus it is responsible for efficient, compartmentalized oxidation of glucose, resulting in high rates of glycolysis[7].

The parabasalians (e.g., *Tritrichomonas* spp., *Histomonas* spp.) possess an array of cellular features (e.g., nucleus, flagella, basal bodies, axostyle, costa, parabasal body, parabasal fiber) organized into a "karyomastigont system"[8] (Figure 2.6). The axostyle is a bundle of parallel, cross-linked microtubules extending from the anterior basal bodies of the flagella to the posterior end of the cell. The costa is a flexible rod-like structure with a striated appearance. It also originates near the anterior end of the cell and extends posteriorly. Functionally, the parabasal body is a large Golgi complex visible under light microscopy. It is associated with a parabasal fiber which originates near the anterior basal bodies. Trichomonad parabasalians lack mitochondria and instead possess hydrogenosomes. These are membrane-bound organelles that metabolize sugars and produce H_2 as an end product[9]. Ingestion of nutrients by many flagellates is typically by pinocytosis, although some use a cytostome (e.g., *Chilomastix* spp.).

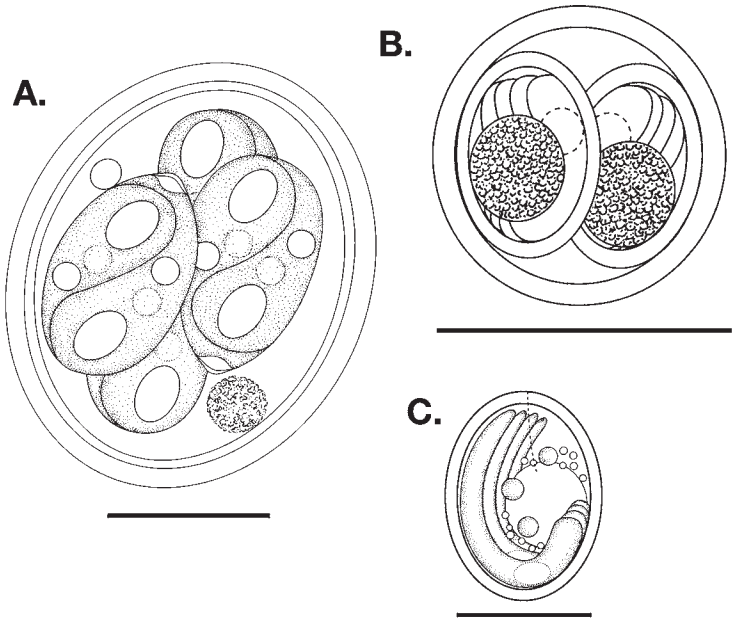

Fig. 2.4 Sporulated oocyst of coccidia. (A) *Eimeria* type. Bar = 10 µ. (B) *Cystoisospora* type. Bar = 10 µ. (C) *Cryptosporidium* type. Bar = 5 µ. (A) Reproduced from Lindsay, D.S., Upton, S.J., and Hildreth, M.B. (1999) with permission. (B) Reproduced from Lindsay, D.S., Upton, S.J., and Dubey, J.P. (1999) with permission. (C) Reproduced from Lindsay, D.S., Upton, S.J., Owens, D.S., Morgan, U.M., Mead, J.R., and Blagburn, B.L. (2000) with permission.

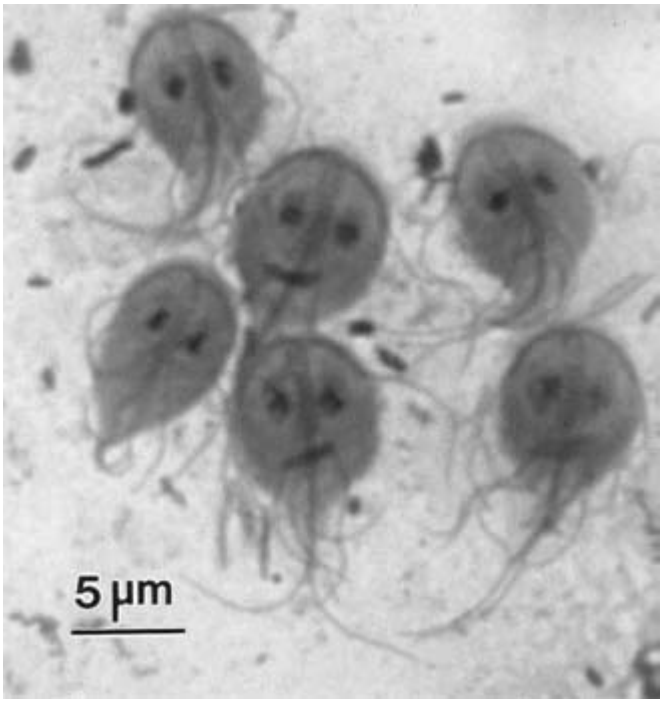

Fig. 2.5 Trophozoites of *Giardia* sp. Giemsa-stained intestinal smear from a hamster.

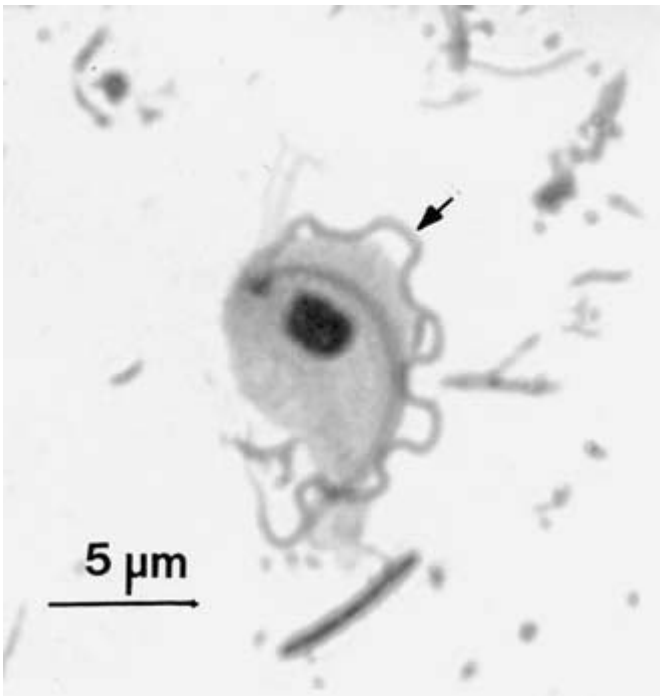

Fig. 2.6 Trichomonad. Note the undulating membrane (arrow).

Locomotion

Most flagellates possess one or more flagella; however, a few parasitic species have no visible external flagellum (e.g., *Dientamoeba* spp. and amastigotes of kinetoplastid flagellates)[10]. Flagella, although usually longer, are structurally similar to cilia in that they contain a 9 + 2 arrangement of microtubules. Most flagellates move by beating flagella in an undulating, whip-like, ATP-dependent manner. In some species, the flagellum is modified to form an "undulating membrane" such that an expanded membrane connects the flagellum to the cell surface. The result is a cell that appears to have a ruffled or curled area on one edge. A few flagellates have no external flagellum, although they retain basal bodies. Some species (e.g., *Dientamoeba fragilis* and *Histomonas meleagridis*) move in an amoeboid manner.

Life Cycles

The majority of flagellates reproduce by longitudinal binary fission. Sexual reproduction is practiced by very few species[11]. Intestinal dwellers are usually extracellular and are transmitted by the fecal-oral route. Most enteric flagellates encyst before exiting the host; and nuclear multiplication in the cyst may (e.g., *Giardia* spp.), or may not (e.g., *Chilomastix* spp.), occur. In one group, encystment is linked to production of hormones by the host so that the life cycles of protozoan (e.g., *Opalina* spp.) and host are linked. A few species (e.g., *Dientamoeba fragilis, Histomonas meleagridis*) do not encyst, and transfer to the next host relies on rapid transmission of trophozoites or other means, such as transfer via nematode eggs.

Kinetoplastid flagellates live in the extra- or intracellular environment, depending on the parasite species. Some (e.g., *Trypanosoma cruzi, Leishmania* spp.) have both intracellular (amastigote) and extracellular (trypomastigote, promastigote) stages in their life cycles. Most of these blood and tissue parasites rely on a hematophagous vector (arthropods, leeches) for transmission to a new host. In some kinetoplastid species, distinct developmental stages occur in the vector, while other species undergo no development but merely use the vector for mechanical transmission to the next host. Antigenic variation during developmental stages is a well known phenomenon[12]. A few important flagellated parasites, including *Tritrichomonas foetus* and *Trypanosoma equiperdum,* are venereally transmitted.

AMOEBA: PHYLUM HETEROLOBOSA

This phylum contains free-living, parasitic, and opportunistically parasitic amoebae, also commonly called "sarcodines." Phyla that contain species that are not parasitic in laboratory animals are not included here. Genera with the greatest medical and veterinary importance include *Acanthamoeba* spp., *Entamoeba* spp., and *Naegleria* spp. Many enteric amoebae are obligate commensals and not associated with disease.

Special Organelles

Amoebae are noted for possessing pseudopods for locomotion and food capture and ingestion, a single type of nucleus, and a lack of centrioles and cytoplasmic microtubules, though they do possess mitotic spindle microtubules. Pseudopods possessed by amoebae include (a) lobose pseudopods, which are transitory, cytoplasmic extensions with blunt or pointed ends, (b) actinopods, which are pointed and contain extrusomes and an inner core of microtubules, (c) filopods, which are very slender and filiform, and (d) reticulopods, which are web-like networks.

Many amoebae have two distinct regions of the cell. The ectoplasm is an outer, clearer, more viscous region surrounding the second distinct region, the endoplasm. The endoplasm is less viscous cytoplasm, and therefore appears denser. Some species have no identifiable anterior region of the cell unless the cell is moving; the anterior region then appears at the leading edge. The posterior region of the cell, the "uroid," is where myosin filaments are often concentrated. Actin microfilaments are found in the ectoplasmic regions. Most amoebae contain Golgi bodies, contractile vacuoles, food vacuoles, and mitochondria.

Amoebae obtain dissolved nutrients by pinocytosis, and larger particulate nutrients by phagocytosis. In both processes, invagination of the cell membrane occurs so that food enters the cell in a food vacuole that is completed when the membrane pinches off. Invagination of the membrane is accomplished through an actin-myosin system[13]. Several species of amoebae form a more prominent ingestion area, the amoebostome, which is large, rounded, and cup-shaped. Digestion in the food vacuole is initiated by hydrolytic enzymes, and waste products are released from the cell by exocytosis. Ingestion of prey is more complex for amoebae with axopods. Many species of parasitic amoebae contain a glycogen vacuole, which is a storage depot for carbohydrates.