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Microorganisms

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INTRODUCTION

We have all heard the expression, 'The world is getting smaller.' Nowhere is that statement truer than in the world of microorganisms. Microorganisms (also called microbes) are organisms which share the property of being sub-microscopic. Most do not normally cause disease in humans, existing in a state of commensalism, where there is little or no benefit to the person, or mutualism, where there is some benefit to both parties. Pathogens are agents that cause disease. These fall into five main groups (Microbiology at Leicester website):

- Viruses
- Bacteria
- Fungi
- Protozoa
- Helminths.

With the advent of new and more powerful antibiotics, improved environmental hygiene, and advances in microbiological technique, it was widely expected that the need for diagnosis of infectious agents in tissue would diminish in importance. This assumption underestimated the infinite capacity of infectious agents for genomic variation, enabling them to exploit new opportunities to spread infections that are created when host defenses become diminished and inadequate. The following are currently the most important factors influencing the presentation of infectious diseases:

- Increased mobility of the world's population through tourism, immigration, and international commerce has distorted natural geographic boundaries to infection, exposing weaknesses in host defenses, and in knowledge. Some, such as Ebola, have been around for many years but the first human outbreaks were not recorded until 1976. Previous outbreaks would flare up and then burn themselves out, undetected and confined before deforestation and the like altered this state.
- Immunodeficiency states occurring either as part of a natural disease, such as acquired immune deficiency syndrome (AIDS), or as an iatrogenic disease. As treatment becomes more aggressive, depression of the host's immunity often occurs, enabling organisms of low virulence to become life-threatening, and allows latent infections accrued throughout life to reactivate and spread unchecked.
- Emerging, re-emerging, and antibiotic-resistant organisms such as the tubercle bacillus and staphylococcus are a constant concern.
- Adaptive mutation occurring in microorganisms, which allows them to jump barriers of species and explore new physical environments, evading host defenses, and resisting agents of treatment.
- Bioterrorism has become a major concern since September 11, 2001. The world public health systems and primary healthcare providers must be prepared to address varied biological agents, including pathogens that are rarely seen in the developed countries. High-priority agents include organisms that pose a risk to national security because they:
  - Can be easily disseminated or transmitted from person to person
  - Cause high mortality, with potential for a major public health impact
  - Might cause public panic and social disruption, and require special action for public health preparedness.

The following are listed by the Centers for Disease Control and Prevention (CDC) in the United States as high-risk biological agents:

- Anthrax
- Smallpox
- Botulism
- Tularemia
- Viral hemorrhagic fever.

These factors, acting singly or together, provide an ever-changing picture of infectious disease where clinical presentation may involve multiple pathological processes, unfamiliar organisms, and modification of the host response by a diminished immune status.

Size

The term 'microorganism' has been interpreted liberally in this chapter. Space limitation precludes a comprehensive approach to the subject; the reader is referred to additional texts such as that of von Lichtenberg (1991) for greater depth. The organisms in Table 17.1 are discussed, with techniques for their demonstration described.

Safety

Most infectious agents are rendered harmless by direct exposure to formal saline. Standard fixation procedures should be sufficient to kill microorganisms, one exception being material from patients with Creutzfeldt–Jakob disease (CJD). It has been shown that well-fixed tissue, paraffin-processed blocks, and stained slides from CJD remain infectious when introduced into susceptible animals. Treatment of fixed tissue or slides in 96% formic acid for 1 hour followed by copious washing inactivates this infectious agent without adversely affecting section quality (Brown et al 1990). Laboratory safety protocols should cover infection containment in all laboratory areas, and the mortuary, or necropsy area, where handling unfixed material is unavoidable. When available, unfixed tissue samples should be sent for microbiological culture as this offers the best chance for rapid and specific identification of etiological agents, even when heavy bacterial contamination may have occurred.

Table 17.1 Size of organisms

Organisms	Size
Viruses	20–300 nm
Mycoplasms	125–350 nm
Chlamydia	200–1000 nm
Rickettsia	300–1200 nm
Bacteria	1–14 µm
Fungi	2–200 µm
Protozoa	1–50 µm
Metazoans	3–10 mm

DETECTION AND IDENTIFICATION

The diagnosis of illness from infectious disease starts with clinical presentation of the patient, and in most cases a diagnosis is made without a tissue sample being taken. Specimens submitted to the laboratory range from autopsy specimens, where material is plentiful and sampling error presents little problem, to cervical smears where cellular material is often scarce and lesions may easily be missed. A full clinical history is important, especially details of the patient's ethnic origin, immune status, any recent history of foreign travel, and current medication. The macroscopic appearance of tissue, such as abscesses and pus formation, cavitations, hyperkeratosis, demyelination, pseudo-membrane or fibrin formation, focal necrosis, and granulomas can provide evidence of infection. These appearances are often non-specific but occasionally in hydatid cyst disease or some helminth infestations the appearances are diagnostic. The microscopic appearance of routine stains at low-power magnification often reveals indirect evidence of the presence of infection, such as neutrophil or lymphocytic infiltrates, granulomata, micro-abscesses, eosinophilic aggregates, Charcot–Leyden crystals, and caseous necrosis. Some of these appearances may be sufficiently reliable to provide an initial, or provisional, diagnosis and allow treatment to be started even if the precise nature of the suspect organism is never identified, particularly in the case of tuberculosis.

At the cellular level the presence of giant cells, such as Warthin–Finkeldy, or Langhans' giant cells, likely indicates measles and tuberculosis, respectively. Other cellular changes include intracytoplasmic edema of koilocytes, acantholysis, spongiform degeneration of brain, margination of chromatin, syncytial nuclear appearance, 'ground-glass' changes in the nucleus or

cytoplasm, or inclusion bodies, and can indicate infectious etiology. At some stage in these processes, suspect organisms may be visualized. A well-performed hematoxylin and eosin (H&E) method will stain many organisms. Papanicolaou stain and Romanowsky stains, such as Giemsa, will also stain many organisms together with their cellular environment. Other infectious agents are poorly visualized by routine stains and require special techniques to demonstrate their presence. This may be due to the small size of the organism, as in the case of viruses where electron microscopy is needed. Alternatively, the organism may be hydrophobic, or weakly charged, as with mycobacteria, spirochetes, and cryptococci, in which case the use of specific histochemical methods is required for their detection. When organisms are few in number, fluorochromes may be used to increase microscopic sensitivity of a technique. Finally, there are two techniques that offer the possibility of specific identification of microorganisms that extend to the appropriate strain level. There is a growing catalog of *biotinylated antisera* against organism-specific proteins that can be demonstrated immunohistochemically. To date, those developed for protozoan, chlamydial, and viral organisms have been most widely used diagnostically in histopathology; however, this will undoubtedly change in the future.

In situ hybridization has even greater potential for microbial detection. The use of single-stranded nucleic acid probes offers even greater possibilities by identifying latent viral genomic footprints in cells, which may have relevance to extending our knowledge of disease, AIDS and HIV being good examples. The polymerase chain reaction technique, to increase sensitivity and make use of stored blocks and slides to study evolutionary aspects of infectious disease, is being used increasingly in research. Future demonstration methods for infectious diseases may lie with these techniques. While modern advances in technique are important, emphasis is also placed upon the ability of the microscopist to interpret suspicious signs from a good H&E stain. The growing number of patients whose immune status is compromised, and who can mount only a minimal or inappropriate response to infection, further complicates the picture, justifying speculative use of special stains such as those for mycobacteria and fungi on tissue from AIDS patients. It should be remembered that, for a variety of reasons, negative results for the identification of an infectious agent do not exclude its presence. For instance, administration of antibiotics to the patient before a biopsy might be the reason for failure to detect a causal microorganism in tissue.

Detection and identification of bacteria

When bacteria are present in large numbers in an abscess or in vegetation on a heart valve, they appear as blue–gray granular masses with an H&E stain; often organisms are invisible or obscured by cellular debris. The reaction of pyogenic bacteria to the Gram stain, together with their morphological appearance, i.e. cocci or bacilli, provides the basis for a simple classification: see Table 17.2.

Table 17.2 A simplified classification of important bacteria

Gram-positive		Gram-negative		
Cocci	**Bacilli**	**Cocci**	**Bacilli**	**Coccobacilli**
Staphylococcus sp.	*Bacillus*	*Neisseria*	*Escherichia*	*Brucella*
Streptococcus sp. (inc. *Pneumococcus*)	*Clostridium* *Corynebacterium* *Mycobacteria* (weak+) *Lactobacillus* (commensal) *Listeria*		*Klebsiella* *Salmonella* *Shigella* *Proteus* *Pseudomonas* *Vibrio* *Pasteurella*	*Bordetella* *Hemophilus*

Use of control sections

The use of known positive control sections with all special stain methods for demonstrating microorganisms is essential. Results are unsafe in the absence of positive controls, and should not be considered valid. The control section should be appropriate, where possible, for the suspected organism. A pneumocystis-containing control, for instance, should be used for demonstrating *Pneumocystis carinii*. A Gram control should contain both Gram-positive and Gram-negative organisms. Postmortem tissues can often be a good source of control material or, as a last resort, a suspension of Gram-positive and Gram-negative organisms can be injected into the thigh muscle of a rat shortly before it is sacrificed for some other purpose. Gram-positive and Gram-negative organisms can also be harvested from microbiological plates, suspended in 10% neutral buffered formalin (NBF), centrifuged, and small amounts mixed with minced normal kidney, then chemically processed along with other tissue blocks (Swisher & Nicholson 1989).

THE GRAM STAIN

In spite of more than a century having passed since Gram described his technique in 1884, its chemical rationale is still obscure. It is probably due to a mixture of factors, the most important being increased thickness, chemical composition, and the functional integrity of cell walls of Gram-positive bacteria. When these bacteria die, they become Gram negative. The following procedure is only suitable for the demonstration of bacteria in smears of pus and sputum. It may be of value to the pathologist in the necropsy room where a quick technique such as this may enable rapid identification of the organism causing a lung abscess, wound infection, septicemic abscesses, or meningitis.

Gram method for bacteria in smears

Method

1. Fix dry film by passing it three times through a flame or placing on a heat block.
2. Stain for 15 seconds in 1% crystal violet or methyl violet, then pour off excess.
3. Flood for 30 seconds with Lugol's iodine, pour off excess.
4. Flood with acetone for not more than 2–5 seconds; wash with water immediately.
5. Alternatively decolorize with alcohol until no more stain comes out. Wash with water.
6. Counterstain for 20 s with dilute carbol fuchsin, or freshly filtered neutral red for 1–2 min.
7. Wash with water and carefully blot section until it is dry.

Results

Gram-positive organisms	blue–black
Gram-negative organisms	red

Modified Brown–Brenn method for Gram-positive and Gram-negative bacteria in paraffin sections (Churukian & Schenk 1982)

Sections

Formalin-fixed, 4–5 micron, paraffin-embedded sections.

Solutions

Crystal violet solution *(commercially available)*

Crystal violet, 10% alcoholic	2 ml
Distilled water	18 ml
Ammonium oxalate, 1%	80 ml

Mix and store; always filter before use.

Modified Gram's iodine *commercially available, or*

Iodine	2 g
Potassium iodide	4 g
Distilled water	400 ml

Dissolve potassium iodide in a small amount of the distilled water, add iodine and dissolve; add remainder of distilled water.

Ethyl alcohol–acetone solution

Ethyl alcohol, absolute	50 ml
Acetone	50 ml

0.5% basic fuchsin solution *(stock) commercially available, or*

Basic fuchsin or pararosaniline	0.5 g
Distilled water	100 ml

Dissolve with aid of heat and a magnetic stirrer.

Basic fuchsin solution *(working)*

Basic fuchsin solution (stock)	10 ml
Distilled water	40 ml

Picric acid–acetone

Picric acid	0.1 g
Acetone	100 ml

Note

With concerns over the explosiveness of dry picric acid in the lab, it is recommended that you purchase the picric acid–acetone solution pre-made. It is available through most histology vendors.

Acetone–xylene solution

Acetone	50 ml
Xylene	50 ml

Staining method

1. Deparaffinize and rehydrate through graded alcohols to distilled water.
2. Stain with filtered crystal violet solution, 1 min.
3. Rinse well in distilled water.
4. Iodine solution, 1 min.
5. Rinse in distilled water, blot slide but NOT the tissue section.
6. Decolorize by dipping in alcohol–acetone solution until the blue color stops running. (One to two dips only!)
7. Counterstain in working basic fuchsin for 1 min. Be sure to agitate the slides well in the basic fuchsin before starting the timer.
8. Rinse in distilled water and blot slide but not section.
9. Dip in acetone, one dip.
10. Dip in picric acid–acetone until the sections have a yellowish-pink color.
11. Dip several times in acetone–xylene solution. At this point, check the control for proper differentiation. (Go back to picric acid–acetone if you need more differentiation.)
12. Clear in xylene and mount.

Results

Gram-positive organisms, fibrin, some fungi, Paneth cells granules, keratohyalin, and keratin	blue
Gram-negative organisms	red
Nuclei	red
Other tissue elements	yellow

Be sure you do not allow the tissue sections to dry at any point in the staining process. If this occurs after treatment with iodine, decolorization will be difficult and uneven.

Gram–Twort stain (Twort 1924; Ollet 1947)

Sections

Formalin fixed, paraffin.

Solutions

Crystal violet solution (see previous method)

Gram's iodine (see previous solution)

Twort's stain

1% neutral red in ethanol	9 ml
0.2% fast green in ethanol	1 ml
Distilled water	30 ml

Mix immediately before use.

Method

1. Deparaffinize and rehydrate through graded alcohols to distilled water.
2. Stain in crystal violet solution, 3 min.
3. Rinse in gently running tap water.
4. Treat with Gram's iodine, 3 min.
5. Rinse in tap water, blot dry, and complete drying in a warm place.
6. Differentiate in preheated acetic alcohol until no more color washes out (2% acetic acid in absolute alcohol, pre-heated to 56°C). This may take 15–20 min; the section should be light brown or straw colored.
7. Rinse briefly in distilled water.
8. Stain in Twort's, 5 min.
9. Wash in distilled water.
10. Rinse in acetic alcohol until no more red runs out of the section; this takes only a few seconds.
11. Rinse in fresh absolute alcohol, clear, and mount.

Results

Gram-positive organisms	blue–black
Gram-negative organisms	pink–red
Nuclei	red
Red blood cells and most cytoplasmic structures	green
Elastic fibers	black

TECHNIQUES FOR MYCOBACTERIA

These organisms are difficult to demonstrate by the Gram technique because they possess a capsule containing a long-chain fatty acid (mycolic acid) that makes them hydrophobic. The fatty capsule influences the penetration and resistance to removal of the stain by acid and alcohol (acid-and alcohol-fastness), and is variably robust between the various species that make up this group. Phenolic acid, and frequently heat, are used to reduce surface tension and increase porosity, thus forcing dyes to penetrate this capsule. The speed with which the primary dye is removed by differentiation with acid alcohol is proportional to the extent of the fatty coat. The avoidance of defatting agents, or solvents, such as alcohol and xylene, in methods for *Mycobacterium leprae*, is an attempt to conserve this fragile fatty capsule.

Mycobacteria are PAS positive due to the carbohydrate content of their cell walls; however, this positivity is evident only when large concentrations of the microorganisms are present. When these organisms die, they lose their fatty capsule and consequently their carbol fuchsin positivity. The carbohydrate can still be demonstrated by Grocott's methenamine silver reaction, which may prove useful when acid-fast procedures fail, particularly if the patient is already receiving therapy for tuberculosis.

A possible source of acid-fast contamination may be found growing in viscous material sometimes lining water taps and any rubber tubing connected to them. These organisms are acid- and alcohol-fast but are usually easily identified as contaminants by their appearance as clumps, or floaters, above the microscopic focal plane of the section.

Ziehl–Neelsen (ZN) stain for *Mycobacterium* bacilli (Kinyoun 1915)

Sections

Formalin or fixative other than Carnoy's, paraffin.

Solutions

Carbol fuchsin *commercially available, or*

Basic fuchsin	0.5 g
Absolute alcohol	5 ml
5% aqueous phenol	100 ml

Mix well and filter before use.

Acid alcohol

Hydrochloric acid	10 ml
70% alcohol	1000 ml

Methylene blue solution (stock) *commercially available, or*

Methylene blue	1.4 g
95% alcohol	100 ml

Methylene blue solution (working)

Methylene blue (stock)	10 ml
Tap water	90 ml

Method

1. Deparaffinize and rehydrate through graded alcohols to distilled water.
2. Carbol fuchsin solution, 30 min.
3. Wash well in tap water.
4. Differentiate in acid alcohol until solutions are pale pink. (This usually only takes 2–5 dips.)
5. Wash in tap water for 8 minutes then dip in distilled water.
6. Counterstain in working methylene blue solution until sections are pale blue.
7. Rinse in tap water then dip in distilled water.
8. Dehydrate, clear, and mount.

Results

Mycobacteria, hair shafts, Russell bodies, Splendore–Hoeppli immunoglobulins around actinomyces, and some fungal organisms	red
Background	pale blue

Notes

a. The blue counterstain may be patchy if extensive caseation is present. Care should be taken to avoid over-counterstaining as scant organisms can easily be obscured.
b. Decalcification using strong acids can destroy acid-fastness; formic acid is recommended.
c. Victoria blue can be substituted for carbol fuchsin and picric acid for the counterstain if color blindness causes a recognition problem.

Fluorescent method for *Mycobacterium* bacilli (Kuper & May 1960)

Sections

Formalin fixed, paraffin.

Solution

Auramine O	1.5 g
Rhodamine B	0.75 g
Glycerol	75 ml
Phenol crystals (liquified at 50°C)	10 ml
Distilled water	50 ml

Method

1. Deparaffinize (1 part groundnut oil and 2 parts xylene for *M. leprae*).
2. Pour on pre-heated (60°C), filtered staining solution, 10 min.
3. Wash in tap water.
4. Differentiate in 0.5% hydrochloric acid in alcohol for *M. tuberculosis*, or 0.5% aqueous hydrochloric acid for *M. leprae*.
5. Wash in tap water, 2 min.
6. Eliminate background fluorescence in 0.5% potassium permanganate, 2 min.
7. Wash in tap water and blot dry.
8. Dehydrate (not for *M. leprae*), clear, and mount in a fluorescence-free mountant.

Results

Mycobacteria	golden yellow (using blue light fluorescence below 530 nm)
Background	dark green

Notes

The advantage of increased sensitivity of this technique is offset by the inconvenience of setting up the fluorescence microscope. Preparations fade over time, as a result of their exposure to UV light.

Modified Fite method for *M. leprae* and *Nocardia*

Fixation

10% neutral buffered formalin (NBF).

Sections

Paraffin sections at 4–5 µm.

Solutions

Carbol fuchsin solution *commercially available, or*

0.5 g basic fuchsin dissolved in 5 ml of absolute alcohol; add 100 ml of 5% aqueous phenol. Mix well and filter before use. Filter before each use with #1 filter paper.

5% sulfuric acid in 25% alcohol

25% ethanol	95 ml
Sulfuric acid, concentrated	5 ml

Methylene blue (stock) *commercially available, or*

Methylene blue	1.4 g
95% alcohol	100 ml

Methylene blue, working

Stock methylene blue	5 ml
Tap water	45 ml
Xylene–peanut oil	1 part oil : 2 parts xylene

Method

1. Deparaffinize in two changes of xylene–peanut oil, 6 minutes each.
2. Drain slides vertically on paper towel and wash in warm, running tap water for 3 minutes. (The residual oil preserves the sections and helps accentuate the acid fastness of the bacilli.)
3. Stain in carbol fuchsin at room temperature for 25 minutes. (Solution may be poured back into bottle and reused.)
4. Wash in warm, running tap water for 3 minutes.
5. Drain excess water from slides vertically on paper towel.
6. Decolorize with 5% sulfuric acid in 25 % alcohol, two changes of 1.5 minutes each. (Sections should be pale pink.)
7. Wash in warm, running tap water for 5 minutes.
8. Counterstain in working methylene blue, one quick dip. (Sections should be pale blue.)
9. Wash in warm, running tap water for 5 minutes.
10. Blot sections and dry in 50–55°C oven for 5 minutes.
11. Once dry, one quick dip in xylene.
12. Mount with permanent mountant.

Results *(Fig. 17.1)*

Acid-fast bacilli including *M. leprae*	bright red
Nuclei and other tissue elements	pale blue

Quality control/notes

Be careful not to over-stain with methylene blue and do not allow sections to dry between carbol fuchsin and acid alcohol.

Cresyl violet acetate method for *Helicobacter* sp.

Sections

Formalin fixed, paraffin.

Method

1. Deparaffinize and rehydrate through graded alcohols to distilled water.
2. Filter 0.1% cresyl violet acetate onto slide or into Coplin jar, 5 min.
3. Rinse in distilled water.
4. Blot, dehydrate rapidly in alcohol, clear, and mount.

Results

Helicobacter and nuclei	blue–violet
Background	shades of blue–violet

Notes

This simple method allows for good differentiation of *Helicobacter* sp. from other organisms.

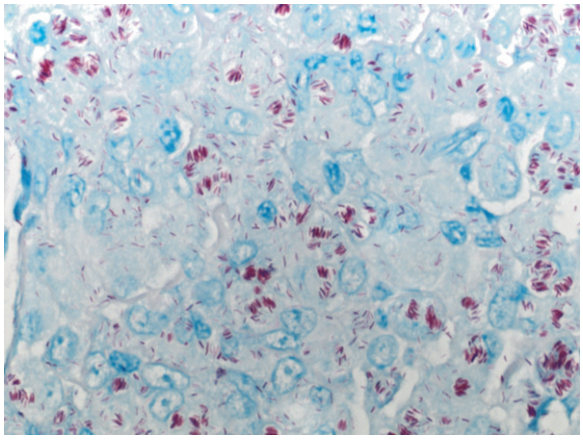

Fig. 17.1 The modified Fite's procedure is necessary to demonstrate *Mycobacterium leprae* due to the organism's fragile, fatty capsule (×63).

Gimenez method for *Helicobacter pylori* (Gimenez 1964; McMullen et al 1987)

Sections

Formalin fixed, paraffin.

Solutions

Buffer solution *(phosphate buffer at pH 7.5, or 0.1 M)*

0.1 M sodium dihydrogen orthophosphate	3.5 ml
0.1 M disodium hydrogen orthophosphate	15.5 ml

Stock carbol fuchsin

Commercial cold acid-fast bacilli stain, or	
Basic fuchsin	1 g
Absolute alcohol	10 ml
5% aqueous phenol	10 ml

Filter before use.

Working carbol fuchsin

Phosphate buffer	10 ml
Stock carbol fuchsin	4 ml

Filter before use.

Malachite green

Malachite green	0.8 g
Distilled water	100 ml

Method

1. Deparaffinize and rehydrate through graded alcohols to distilled water.
2. Stain in working carbol fuchsin solution, 2 min.
3. Wash well in tap water.
4. Stain in malachite green, 15–20 seconds.
5. Wash thoroughly in distilled water.
6. Repeat steps 4 and 5 until section is blue–green to the naked eye.
7. Blot sections dry, and complete drying in air.
8. Clear and mount.

Results

Helicobacter	red–magenta
Background	blue–green

Notes

The greatest problem with this method is over staining, or irregularity of staining, with Malachite green. It is valuable in demonstrating the *Legionella* bacillus in postmortem lung smears.

Toluidine blue in Sorenson's buffer for *Helicobacter*

Sections
Formalin fixed, paraffin.

Solutions
Toluidine blue in pH 6.8 phosphate buffer

Sorenson's phosphate buffer pH 6.8	50 ml
1% aqueous toluidine blue	1 ml

Method
1. Deparaffinize and rehydrate through graded alcohols to distilled water.
2. Stain in buffered toluidine blue, 20 min.
3. Wash well in distilled water.
4. Dehydrate, clear, and mount.

Results
Helicobacter	dark blue against a variably blue background

Warthin–Starry method for spirochetes (Warthin & Starry 1920)

Sections
Formalin fixed, paraffin.

Solutions
Acetate buffer, pH 3.6

Sodium acetate	4.1 g
Acetic acid	6.25 ml
Distilled water	500 ml

1% silver nitrate in pH 3.6 acetate buffer

Developer

Dissolve 3 g of hydroquinone in 10 ml pH 3.6 buffer, and mix 1 ml of this solution and 15 ml of warmed 5% Scotch glue or gelatin; keep at 40°C. Take 3 ml of 2% silver nitrate in pH 3.6 buffer solution and keep at 55°C. Mix these two solutions immediately before use.

Method
1. Deparaffinize and rehydrate through graded alcohols to distilled water.
2. Celloidinize in 0.5% celloidin, drain, and harden in distilled water, 1 min.
3. Impregnate in preheated 55–60°C silver solution (b), 90–105 minutes.
4. Prepare and preheat developer in a water bath.
5. Treat with developer (solution c) for $3^1/_2$ minutes at 55°C. Sections should be golden-brown at this point.
6. Remove from developer and rinse in tap water for several minutes at 55–60°C, then in buffer at room temperature.
7. Tone in 0.2% gold chloride.
8. Dehydrate, clear, and mount.

Results
Spirochetes	black
Background	golden–yellow

Notes
It is wise to take a few slides through at various incubation times to insure optimum impregnation.

Modified Steiner for filamentous and non-filamentous bacteria (Steiner & Steiner 1944; modified Swisher 1987)

Sections
Formalin fixed, paraffin.

Solutions
1.0% uranyl nitrate commercially available, or

Uranyl nitrate	1 g
Distilled water	100 ml

1% silver nitrate

Silver nitrate	1 g
Distilled water	100 ml

Make fresh each time and filter with #1 or #2 filter paper before use.

0.04% silver nitrate

Silver nitrate	0.04 g
Distilled water	100 ml

Refrigerate and use for only 1 month.

2.5% gum mastic commercially available, or

Gum mastic	2.5 g
Absolute alcohol	100 ml

Allow to dissolve for 24 hours then filter until clear yellow before use. Refrigerate unused portion.

2% hydroquinone

Hydroquinone	1 g
Distilled water	25 ml

Make fresh solution for each use.

Reducing solution

Mix 10 ml of 2.5% gum mastic, 25 ml of 2.0% hydroquinone, and 5 ml absolute alcohol. Make just prior to use and filter with #4 filter paper; add 2.5 ml of 0.04% silver nitrate. Do not filter this solution. When gum mastic is added, solution will have a milky appearance.

Method

1. Deparaffinize and rehydrate through graded alcohols to distilled water.
2. Sensitize sections in 1% aqueous uranyl nitrate at room temperature, and place in microwave oven until solution is just at boiling point, approx. 20–30 seconds; do not boil. *Alternatively*, place in preheated 1% uranyl nitrate at 60°C in a water bath for 15 min, or in microwave oven and bring to boiling point—do not boil; 2% zinc sulfate in 3.7% formalin may be substituted.
3. Rinse in distilled water at room temperature until uranyl nitrate residue is eliminated.
4. Place in 1% silver nitrate at room temperature and microwave *until* boiling point is just reached. Do not boil. Remove from oven, loosely cover jar, and allow to stand in hot silver nitrate, 6–7 min; *alternatively*, preheat silver nitrate for 20–30 min in a 60°C water bath, add slides, and allow to impregnate for $1^1/_2$ hours.
5. Rinse in three changes of distilled water.
6. Dehydrate in two changes each of 95% alcohol and absolute alcohol.
7. Treat with 2.5% gum mastic, 5 min.
8. Allow to air dry, 5 min.
9. Rinse in two changes of distilled water. Slides may stand here while reducing solution is being prepared.
10. Reduce in preheated reducing solution at 45°C in a water bath for 10–25 min, or until sections have developed satisfactorily with black microorganisms against a light yellow background. Avoid intensely stained background.
11. Rinse in distilled water to stop reaction.
12. Dehydrate, clear, and mount.

Results *(Fig. 17.2)*

Spirochetes, cat-scratch organisms, Donovan bodies, non-filamentous bacteria of *L. pneumophila*	dark brown-black
Background	bright yellow to golden yellow

Notes

Bring all solutions to room temperature before using. All glassware making contact with silver nitrate should be chemically cleaned. Avoid the use of metal forceps in silver solutions. When doing a bacterial screen, Gram controls should be run along with diagnostic slides. As spirochetes take longer to develop, Gram controls should be used in addition to spirochete controls. When Gram controls have a yellow appearance, remove them to distilled water, and check on microscope for microorganisms. Return to silver solution if they are not ready, and repeat, realizing that spirochetes will take longer. Most solutions can be made in large quantities and kept in the refrigerator.

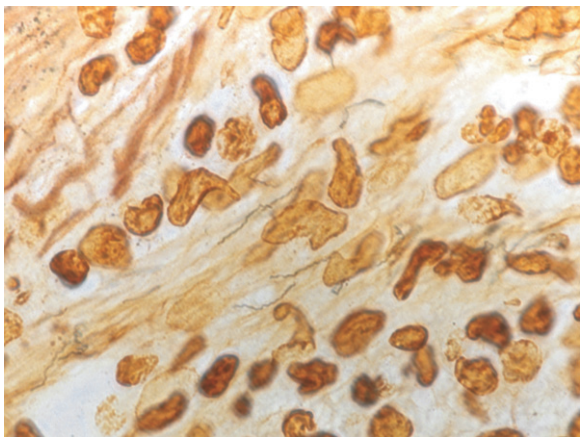

Fig. 17.2 Syphilis bacilli, *Treponema pallidum*, demonstrated here with the modified Steiner technique. The resistance to coloration is shared by *Helicobacter*, spirochetes, and *Legionella* (×100).

SOME IMPORTANT BACTERIA

Staphylococcus aureus is perhaps the most important pathogen of this group. It causes boils, wound and burn infections, and a form of cavitating pneumonia in children and adults. Septicemic states and the formation of multiple scattered abscesses sometimes occur. Staphylococci tend to form clusters (cf. streptococci). Multiresistance to antibiotics is sometimes encountered.

Neisseria meningitidis (meningococcus) is a common cause of meningitis, and may produce a fulminating septicemia. Organisms can be seen in histological sections of meningococcal meningitis, but are difficult to see because they are usually within neutrophil cytoplasm.

Neisseria gonorrheae (gonococcus) is the cause of gonorrhea. Organisms may be seen within polymorphs in sections of cervix, endometrium, or fallopian tubes in cases of gonorrhea, but, again, are difficult to find. Members of the *Neisseria* family are generally difficult to see in histological sections, although easily detectable in smears of fresh pus or cerebrospinal fluid (CSF), characteristically in pairs. They are easier to detect using the Gram–Twort method.

Lactobacillus acidophilus (Doderlein's bacillus) is a normal inhabitant of the human vagina and is seen in cervical smears taken in the secretory phase of the cycle.

Corynebacterium vaginale is a short Gram-negative bacillus which may cause cervicitis, and is present in about 6% of women of childbearing age. It may be seen in cervical smears where it accumulates as blue-stained masses on the surface of squamous cells stained by Papanicoloau's method; these cells are known as 'clue cells'.

Helicobacter pylori is frequently seen in endoscopic biopsies. A spiral vibrio organism is heavily implicated as the organism causing many cases of chronic gastritis. It is seen as small, weakly hematoxyphilic organisms (usually in clumps) in the lumina of gastric glands, often adherent to the luminal surface of the epithelial cells. With practice, these can be identified from an H&E stain, but Warthin–Starry, Steiner, Gimenez, toluidine blue, or cresyl violet acetate methods demonstrate them more clearly. A commercial specific antiserum has recently become available for their demonstration.

Clostridium difficile causes pseudomembranous colitis, an inflammation of the large bowel. This arises following the administration of broad-spectrum antibiotics; the balance of the normal anerobic gut microflora is disturbed, allowing the organism to proliferate unchecked. *C. difficile* is difficult to stain but the 'explosive lesions' of purulent necrosis of the epithelium and lamina propria of the gut, giving rise to a 'mini volcano' effect, are a good indicator.

Listeria monocytogenes is the cause of a rare form of meningitis and may cause septicemia in humans. Focal necrosis with macrophages that contain tiny intracellular rods arranged in a 'Chinese letter' formation, and staining variably with the Gram stain, are the hallmark of this disease.

Mycobacterium tuberculosis remains a significant pathogen in developed countries where the familiar caseating granulomatous lesion and its associated 1–2-μm, blunt-ended, acid and alcohol-fast bacilli can still be seen. In Africa and other countries, this organism has developed an opportunistic relationship with AIDS, where it is a major cause of death.

Mycobacterium avium/intracellulare are representatives of a group of intracellular opportunistic mycobacteria that are frequently present in the later stages of immunosuppression, particularly that associated with AIDS. They frequently persist in spite of treatment, and are often lethal. The lesions produced are non-caseating and consist of collections of vacuolated macrophages that often contain vast numbers of organisms. On occasion, there is little evidence of a cellular reaction on an H&E-stained section, and the organism is detected only by routinely performing an acid-fast stain, such as the ZN, on all tissue from AIDS patients. This group also includes *M. kansasii*.

Mycobacterium leprae is an obligate intracellular, neurotrophic mycobacterium that attacks and destroys nerves, especially in the skin. Tissue reaction to leprosy depends on the immune status of the host; it can be minimal with a few macrophages packed with crescentic, pointed, intracytoplasmic bacilli (lepromatous leprosy), or may contain scanty organisms and show florid granulomatous response (tuberculoid leprosy). *M. leprae* is only acid-fast and can often be demonstrated with a standard Ziehl–Neelsen technique.

Legionella pneumophila was first identified in 1977 as the cause of a sporadic type of pneumonia of high mortality. The small Gram-positive coccobacillus is generally spread in aerosols from stagnant water reservoirs, usually in air-conditioning units. The bacterium may be difficult to stain except with the Dieterle and modified Steiner silver stains, and specific antiserum.

Treponema pallidum is the organism causing syphilis, and is infrequently seen in biopsy specimens as the primary lesion or 'chancre' is diagnosed clinically. The spirochete is quite obvious using dark-ground microscopy, as an 8–13-μm corkscrew-shaped microorganism that often kinks in the center. Dieterle, Warthin–Starry, or modified Steiner methods demonstrate the organism; specific antiserum is also available.

Leptospira interrogans is the organism causing leptospirosis or Weil's disease. It is a disease characterized by

spirochetes, and is spread in the urine of rats and dogs, causing fever, profound jaundice, and sometimes death. Spirochetes can be seen in the acute stages of the disease where they appear in Warthin–Starry and modified Steiner techniques as tightly wound 13-µm microorganisms with curled ends resembling a shepherd's crook.

Intestinal spirochetosis appears as a massive infestation on the luminal border of the colon by spirochete *Brachyspira aalborgi* (Tomkins et al 1986). It measures 2–6 µm long, is tightly coiled, and arranged perpendicularly to the luminal surface of the gut, giving it a fuzzy hematoxyphilic coat in an H&E stain. There is no cellular response to the presence of this spirochete. It is seen well with the Warthin–Starry and the modified Steiner techniques.

Cat-scratch disease presents as a self-limiting, local, single lymphadenopathy appearing about 2 weeks after a cat scratch or bite. Histologically the node shows focal necrosis or micro-abscesses. Two Gram-negative bacteria (*Afipia felis* and *Bartonella henselae*) have been implicated. Because of the timing or maturation factor of the bacterium, it is difficult to demonstrate on paraffin sections, but the modified Steiner and the Warthin–Starry methods are valuable techniques for demonstrating this organism.

FUNGAL INFECTIONS

Fungi are widespread in nature, and humans are regularly exposed to the spores from many species, yet the most commonly encountered diseases are the superficial mycoses that affect the subcutaneous or horny layers of the skin or hair shafts, and cause conditions such as athlete's foot or ringworm. These dermatophytic fungi belong to the *Microsporum* and *Trichophyton* groups and may appear as yeasts or mycelial forms within the keratin. They are seen fairly well in the H&E stain, but are demonstrated well with the Grocott and PAS stains. As with other infections, the increase in the number of patients with diminished or compromised immune systems has increased the incidence of *systemic mycoses*, allowing opportunistic attacks by fungi, often of low virulence, but sometimes resulting in death.

When fungi grow in tissue they may display primitive asexual (imperfect) forms that appear as either spherical *yeast* or *spore* forms. Some may produce vegetative growth that appears as tubular *hyphae* that may be septate and branching; these features are important morphologically for identifying different types of fungi. A mass of interwoven hyphae is called a fungal *mycelium*. Only rarely, when the fungus reaches an open cavity, the body surface, or a luminal surface such as the bronchus, are the spore-forming fruiting bodies called *sporangia*, or *conidia*, produced.

Identification of fungi

Some fungi may elicit a range of host reactions from exudative, necrotizing, to granulomatous; other fungi produce little cellular response to indicate their presence. Fortunately, most fungi are relatively large and their cell walls are rich in polysaccharides, which can be converted by oxidation to dialdehydes and thus detected with Schiff's reagent or hexamine–silver solutions. Fungi are often weakly hematoxyphilic. Some fungi, such as sporothrix, may be surrounded by a stellate, strongly eosinophilic, refractile Splendore–Hoeppli precipitates of host immunoglobulin and degraded eosinophils.

Fluorochrome-labeled specific antibodies to many fungi are available, and are in use in mycology laboratories for the identification of fungi on fresh and paraffin sections. These antibodies have not found widespread use, however, on fixed tissue where identification still relies primarily on traditional staining methods.

An H&E stain, a Grocott methenamine (hexamine)–silver (GMS), a mounted unstained section to look for pigmentation, and a good color atlas (Chandler et al 1980) when experience fails, permit most fungal infections to be identified to levels sufficient for diagnoses. However, there is no substitute for microbiological culture.

Grocott methenamine (hexamine)–silver for fungi and *Pneumocystis* spp. organisms (Gomori 1946; Grocott 1955; Swisher & Chandler 1982)

Sections

Formalin fixed, paraffin.

Solutions

4% chromic acid *commercially available, or*

Chromic acid	4.0 g
Distilled water	100 ml

1% sodium bisulfite

Sodium bisulfite	1 g
Distilled water	100 ml

5% sodium thiosulfate

Sodium thiosulfate	5.0 g
Distilled water	100 ml

0.21% silver nitrate *(stock)*

Silver nitrate	2.1 g
Distilled water	1000 ml

Refrigerate for up to 3 months

(A) Methenamine–silver borate solution *(stock)*

Methenamine	27 g
Sodium borate decahydrate (borax)	3.8 g
Distilled water	1000 ml

Refrigerate for up to 3 months.

(B) Methenamine–silver sodium borate solution *(working)*

Equal parts of solutions A and B. Make fresh each time and filter before use.

0.2% light green *(stock)*

Light green	0.2 g
Distilled water	100 ml
Glacial acetic acid	0.2 ml

Light green *(working)*

Stock light green	10 ml
Distilled water	50 ml

Prepare working solution fresh before each use.

Method

1. Deparaffinize and rehydrate through graded alcohols to distilled water.
2. Oxidize in 4% aqueous chromic acid (chromium trioxide), 30 minutes.
3. Wash briefly in distilled water.
4. Dip briefly in 1% sodium bisulfite.
5. Wash well in distilled water
6. Place in preheated (56–60°C water bath) working silver solution for 15–20 minutes. Check control after 15 minutes. If section is 'paper bag brown' then rinse in distilled water and check under microscope. If it is not ready, dip again in distilled water and return to silver. Elastin should not be black. Check every 2 minutes from that point onwards. (See Note a.)
7. Rinse well in distilled water.
8. Tone in 0.1% gold chloride, 5 seconds. Rinse in distilled water.
9. Place in 5% sodium thiosulfate, 5 seconds.
10. Rinse well in running tap water.
11. Counterstain in working light green solution until a medium green (usually 5–15 seconds).
12. Dehydrate, clear, and mount.

Results

Fungi, pneumocystis, melanin	black
Hyphae and yeast-form cells of fungi	sharply delineated in black
Mucins and glycogen	taupe to dark gray
Background	pale green

Notes

a. Incubation time is variable and depends on the type and duration of fixation, and organism being demonstrated. Impregnation is controlled microscopically until fungi are dark brown. Background is colorless at this point. Over-incubation produces intense staining of elastin and fungi that may obscure fine internal detail of the hyphal septa. This detail is essential for critical identification, and is best seen on under-impregnated sections. To avoid excess glycogen impregnation in liver sections, section may be digested prior to incubation. A water bath may be used effectively to insure an even incubation temperature.
b. Borax insures an alkaline pH.
c. Sodium bisulfite removes excess chromic acid.
d. Some workers prefer a light H&E counterstain. This is especially useful when a consulting case is sent with only one slide, providing morphological detail for the pathologist.
e. Solutions A and B need to be made and stored in chemically clean glassware (20% nitric acid), as does the working solution. This includes graduates and Coplin jars. Do not use metal forceps.
f. Allow all refrigerated solutions to reach room temperature before using.

McManus' PAS method for glycogen and fungal cell walls

Fixation

10% NBF.

Sections

3–5-µm paraffin sections.

Solutions

Schiff's reagent *(see p. 171), also commercially available*

0.5% periodic acid solution

Periodic acid	0.5 g
Distilled water	100 ml

0.2% light green *(stock)*

Light green	0.2 g
Distilled water	100 ml
Glacial acetic acid	0.2 ml

This is the same stock solution used in the GMS.

Light green *(working)*

Stock light green	10 ml
Distilled water	50 ml

Make fresh before each use.

Method

1. Deparaffinize and hydrate slides to distilled water.
2. Oxidize in periodic acid solution for 5 minutes.
3. Rinse in distilled water.
4. Place in Schiff's reagent for 15 minutes.
5. Wash in running tap water for 10 minutes to allow pink color to develop.
6. Counterstain for a few seconds in working light green solution.
7. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene.
8. Mount in resin-based mountant.

Results

Fungal cell walls and glycogen	magenta to red
Background	pale green

Quality control/notes

A solution of 5% aqueous sodium hypochlorite reduces over-staining by Schiff's.

A SELECTION OF THE MORE IMPORTANT FUNGI AND ACTINOMYCETES

Actinomyces israelii is a colonial bacterium which can be found as a commensal in the mouth and tonsillar crypts. It can cause a chronic suppurative infection, actinomycosis, which is characterized by multiple abscesses drained by sinus tracts. Actinomycotic abscesses can be found in liver, appendix, lung, and neck. The individual organisms are Gram-positive, hematoxyphilic, non-acid-fast, branching filaments 1 micron in diameter. They become coated in 'clubs' of Splendore–Hoeppli protein when the organism is invasive. These clubs are eosinophilic, acid-fast, 1–15 µm wide, and up to 100 µm long, and stain polyclonally for immunoglobulins. This arrangement of a clump of actinomyces or fungal hyphae, which measures 30–3000 µm, surrounded by eosinophilic protein, is called a 'sulfur' granule and is an important identification marker for certain fungal groups. These granules may be macroscopically visible and their yellow color is an important diagnostic aid.

Nocardia asteroides is another actinomycete. It is filamentous and may be visible in an H&E stain, but is Grocott positive and variably acid-fast using the modified Ziehl–Neelsen for leprosy; however, it is difficult to demonstrate even with the acid-fast bacillus. Its pathology is similar to that of actinomycosis, but its organisms are generally more disseminated than those of actinomycosis.

Candida albicans is a common fungus, but with immunosuppression can become systemic. It infects the mouth as thrush, the esophagus, the vagina as vaginal moniliasis, the skin and nails, and is in heart-valve vegetations. It is seen as both ovoid budding yeast-form cells of 3–4 µm, and more commonly as slender 3–5-µm, sparsely septate, non-branching hyphae and pseudohyphae. While difficult to see on H&E, this organism is strongly Gram positive, and is obvious with the Grocott and PAS techniques.

Aspergillus fumigatus is a soil saprophyte and a commensal in the bronchial tree. It may infect old lung cavities (Fig. 17.3) or become systemic in immunosuppressed patients. The fungus has broad, 3–6-µm, parallel-sided, septate hyphae showing dichotomous (45°C) branching. It may be associated with Splendore–Hoeppli protein and sometimes forms fungal balls within tissue. This fungus may be seen in an H&E stain and is demonstrated well with a PAS or Grocott. When it grows exposed to air, the conidophoric fruiting body may be seen as *Aspergillus niger*, a black species that can cause infection of the ear.

Zygomycosis is an infrequently seen disease caused by a group of hyphated fungi belonging mainly to the genera *Mucor* and *Rhysopus*. They have thin-walled hyphae (infrequently septate) with non-parallel sides, ranging from 3 to 25 µm in diameter, branch irregularly, and often show empty bulbous hyphal swelling.

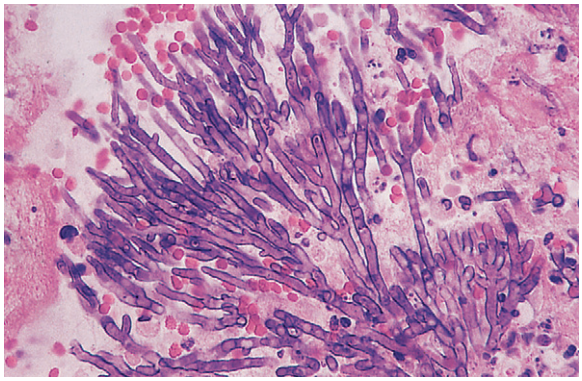

Fig. 17.3 A strong hematoxylin (Ehrlich's and eosin stain) will show the fine detail of many infectious agents. The hyphal structure identifies this as *Aspergillus* which was colonizing an old tuberculosis cavity in the lung (×100).

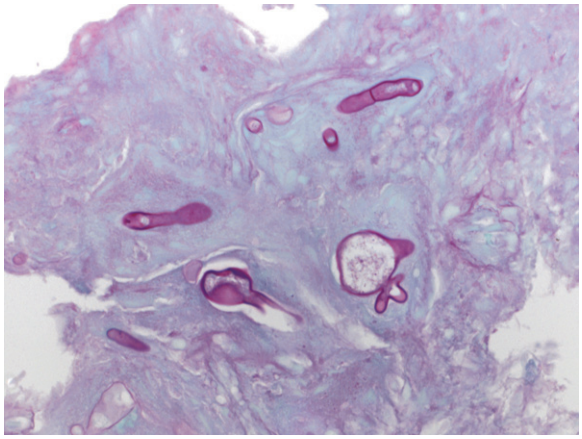

Fig. 17.4 *Rhizomucor* spp. (a cosmopolitan, filamentous fungus) is well demonstrated by this PAS stain with light green counterstain (×40).

Grocott and PAS are the staining methods of choice (Figs 17.4 and 17.5).

Cryptococcus neoformans exists solely in yeast-form cells, variable in diameter (2–20 μm) with ovoid, elliptical, and crescentic forms frequently seen. There is an extensive mucopolysaccharide coat around the yeasts that is mostly dissolved during processing, but, when present, appears as a halo around the organism and is visible with special stains such as Mayer's or Southgate's mucicarmine procedures. Yeasts may be free form or within the cytoplasm of giant cells, staining faintly with

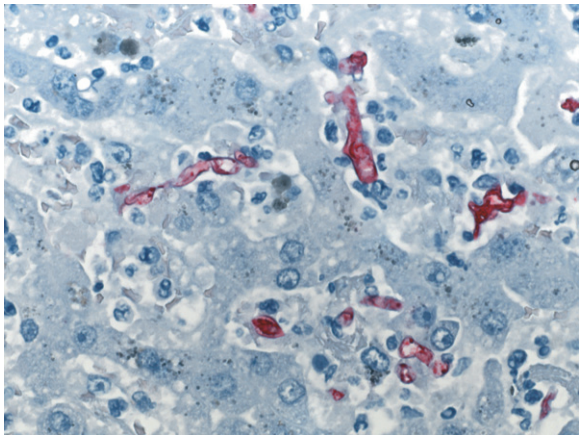

Fig. 17.5 Immunohistochemistry is being increasing applied to the demonstration of microorganisms using labeled specific antibody. This figure demonstrates *Zygomycetes*, a fast-growing fungus, with fast red chromogen (×20).

an H&E stain. The PAS and Grocott procedures demonstrate these cells well. Infection is found in the lungs and in the brain within the parenchyma or in the leptomeninges. Usually these patients are immunosuppressed.

Histoplasma capsulatum is another soil-dwelling yeast that can cause a systemic infection in humans called histoplasmosis. It is especially common along the southern border of the United States, and where there are large bird populations. The organism is usually seen within the cytoplasm of macrophages that appear stuffed with small, regular, 2–5-μm yeast-form cells that have a thin halo around them in H&E and Giemsa stains. Langhans' giant cells forming non-caseating granulomas may be present. PAS and Grocott stains demonstrate this fungus well (Fig. 17.6).

Pneumocystis carinii. There is still some debate over the taxonomy of this organism, although recent analysis of its ribosomal RNA has placed it nearer to a fungal than a protozoan classification (Edman et al 1988). It came to prominence as a pathogen following immunosuppressive therapies associated with renal transplants in the 1960s, and has become a life-threatening complication of AIDS. It most frequently causes pneumonia, where the lung alveoli are progressively filled with amphophilic, foamy plugs of parasites and cellular debris. It is found rarely in other sites such as intestines and lymph nodes. The cysts are invisible in an H&E stain, and can barely be seen in a Papanicolaou stain, as they

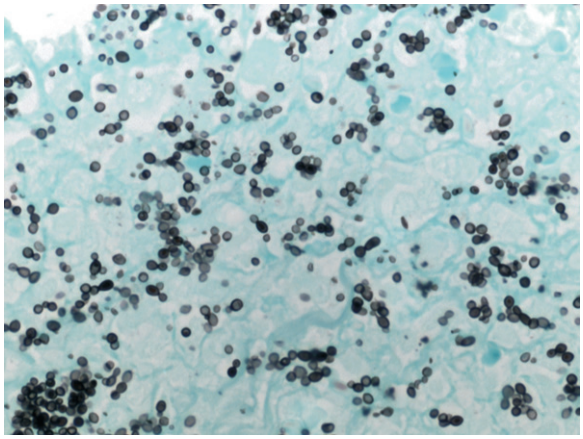

Fig. 17.6 Grocott's methenamine–silver stains a wide variety of infectious agents. Here seen with light green counterstain is the method of choice for *Histoplasma capsulatum*, a dimorphic endemic fungus (×63).

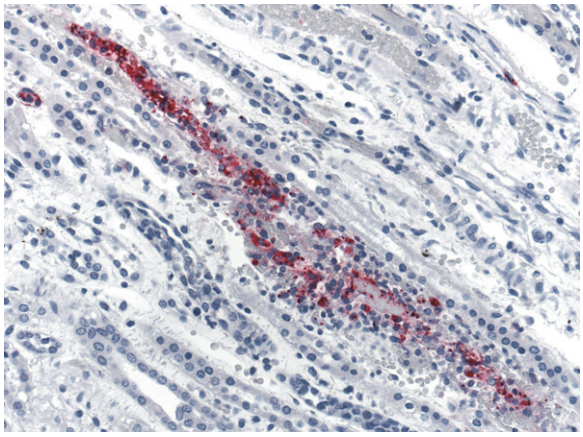

Fig. 17.7 Immunohistochemical method demonstrating Rocky Mountain spotted fever in kidney. It is caused by the bacterium *Rickettsia rickettsii*, which is carried by ticks (×20).

appear refractile when the microscope condenser is racked down. Specific antiserum is available to use; otherwise Grocott methenamine–silver is recommended.

Only electron microscopy or an H&E stain on a resin-embedded thin section will show their internal structure. The cysts are 4–6 μm in diameter and contain 5–8 dot-like intracystic bodies. The cysts rupture and collapse, liberating the trophozoites which can be seen as small hematoxyphilic dots in a good H&E and Giemsa stain; these attach to the alveolar epithelium by surface philopodia.

THE DEMONSTRATION OF RICKETTSIA

Rickettsial organisms, such as those causing Q fever, Rocky Mountain spotted fever, or typhus, rarely need to be demonstrated in tissue sections. They can sometimes be seen with a Giemsa stain, or by using the Macchiavello technique which also demonstrates some viral inclusion bodies (Fig. 17.7).

Macchiavello's stain for rickettsia and viral inclusions, modified (Culling 1974)

Sections

Formalin fixed, paraffin.

Method

1. Deparaffinize and rehydrate through graded alcohols to distilled water.
2. Stain in 0.25% basic fuchsin, 30 min.
3. Differentiate in 0.5% citric acid, 3 seconds.
4. Wash in tap water, 2 min.
5. Counterstain in 1% methylene blue, 15–30 seconds.
6. Rinse in tap water.
7. Dehydrate, clear, and mount.

Results

Rickettsia and some viral inclusions	red
Background	blue

THE DETECTION AND IDENTIFICATION OF VIRUSES

While the cytopathic effects of viruses can often be seen in a good H&E stain, and may be peculiar to a single viral group, the individual viral particles are too small to be seen with the light microscope, thus requiring the electron microscope to reveal their structure. This allows a rapid and accurate diagnosis in viral infections; an outline of the value of electron microscopy in the diagnosis of viral lesions is given in Chapter 30. Some viruses aggregate within cells to produce *viral inclusion bodies*, that may be intranuclear, intracytoplasmic, or both. These inclusion bodies may be acidophilic and usually intranuclear, or can be basophilic and cytoplasmic. Most special staining methods are modified trichromes, using

contrasting acid and basic dyes to exploit these differences in charges on the inclusion body and the host cell. These methods include Mann's methyl blue–eosin stain for the Negri bodies of rabies, Machiavello's method, and more recently the elegant Lendrum's phloxine–tartrazine stain. Unfortunately, the need for optical differentiation in these methods increases the chance of technical error.

The introduction of commercially available monoclonal antibodies to viruses, which are either class- or species specific, has revolutionized the tissue detection of viruses. Hepatitis B virus is a good example of the diagnostic value of this technique where the surface antigen (HBs or Australia antigen) and the core antigen (HBc) can be specifically detected immunohistochemically, providing clinically important information about the stage of this disease. More recently, nucleic acid hybridization probes have become available and can be used to detect genomically inserted viral nucleic acid in situ, in cells and tissues that are frozen or formalin fixed. It should be remembered, however, that the detection of microorganisms using nucleic acid probes, unlike specific biotinylated antiserum, does not necessarily mean active disease.

Phloxine–tartrazine technique for viral inclusions (Lendrum 1947)

Sections

Formalin fixed, paraffin.

Solutions

Phloxine

Phloxine	0.5 g
Calcium chloride	0.5 g
Distilled water	100 ml

Tartrazine

A saturated solution of tartrazine in 2-ethoxyethanol, or cellosolve.

Method

1. Deparaffinize and rehydrate through graded alcohols to distilled water.
2. Stain nuclei in alum hematoxylin (Carazzi's or Harris's), 10 min.
3. Wash in running tap water, 5 min.
4. Stain in phloxine solution, 20 min.
5. Rinse in tap water and blot dry.
6. Controlling with the microscope, stain in tartrazine until only the viral inclusions remain strongly red, 5–10 min on average.
7. Rinse in 95% alcohol.
8. Dehydrate, clear, and mount.

Results

Viral inclusions	bright red
Red blood cells	variably orange–red
Nuclei	blue–gray
Background	yellow

Notes

All tissue is stained red with phloxine which is then differentiated by displacement with the counterstain, tartrazine. The red color is first removed from muscle, then other connective tissues. Paneth cells, Russell bodies, and keratin can be almost as dye retentive as viral inclusions, and can occasionally be a source of confusion.

Shikata's orcein method for hepatitis B surface antigen (modified Shikata et al 1974)

Sections

Formalin fixed, paraffin.

Solutions

Acid permanganate

0.25% potassium permanganate	95 ml
3% aqueous sulfuric acid	5 ml

Orcein

Orcein (synthetic)	1 g
70% alcohol	100 ml
Concentrated hydrochloric acid (gives a pH of 1–2)	1 ml

Saturated tartrazine in cellosolve (2-ethoxyethanol).

Method

1. Deparaffinize and rehydrate through graded alcohols to distilled water.
2. Treat with acid permanganate solution, 5 min.
3. Bleach until colorless with 1.5% aqueous oxalic acid, 30 seconds.
4. Wash in distilled water, 5 min, then in 70% alcohol.

5. Stain in orcein solution at room temperature, 4 hours, or in a Coplin jar of 37°C pre-heated orcein, 90 min.
6. Rinse in distilled alcohol and examine microscopically to determine desired staining intensity.
7. Rinse in cellosolve, stain in tartrazine, 2 min.
8. Rinse in cellosolve, clear, and mount.

Results

Hepatitis B-affected cells, elastic and some mucins	brown–black
Background	yellow

Notes

The success of this method largely depends on the particular batch of orcein used, and on freshly prepared solutions. This method relies on permanganate oxidizing of sulfur-containing proteins to sulfonate residues that react with orcein. Results compare well with those obtained using labeled antibodies, but the selectivity is inferior.

IMMUNOHISTOCHEMISTRY

Immunohistochemistry is now a routine and invaluable procedure in the histopathology lab for the detection of many microorganisms. There are many commercially available antibodies for viral, bacterial, and parasitic organisms. Most methods today utilize (strept)avidin–biotin technologies. These are based on the high affinity that (strept)avidin (*Streptomyces avidinii)* and avidin (chicken egg) have for biotin. Both possess four binding sites for biotin, but due to the molecular orientation of the binding sites fewer than four molecules of biotin will actually bind. The basic sequence of reagent application consists of primary antibody, biotinylated secondary antibody, followed by either the preformed (strept)avidin–biotin enzyme complex of the avidin-biotin complex (ABC) technique or by the enzyme-labeled streptavidin. Both conclude with the substrate solution. Horseradish peroxidase and alkaline phosphatases are the most commonly used enzyme labels. (Handbook of Immunohistochemical Staining Methods, 3rd edn. DAKO Corporation.)

SOME IMPORTANT VIRAL INFECTIONS

This summary is presented because of the viruses that are likely to be encountered in surgical and post-mortem histopathology and cytopathology (Table 17.3).

Viral hepatitis. To date, five hepatitis viruses have been reported, hepatitis viruses (HV) A, B, C, D, and E, that show great biological diversity, and three of which are incompletely characterized. The liver is the target organ and damage varies with the viral strain, ranging

Table 17.3 Viral infections seen in histopathology

Virus	Family	Genome	Disease
Measles	Paramyxo	SS RNA	Measles
Varicella-zoster	Herpes	DS DNA	Chickenpox, shingles
Herpes simplex	Herpes	DS DNA	Cold sores
Herpes genitalis	Herpes	DS DNA	Genital herpes
Cytomegalovirus (CMV)	Herpes	DS DNA	Cytomegalic inclusion disease
Epstein–Barr virus	Herpes	DS DNA	Glandular fever, African Burkitt's lymphoma
Human T-cell leukemia virus (HTLV-1)	Retro	SS RNA	Adult T-cell leukemia
Human immunodeficiency virus (HIV)	Retro	SS RNA	AIDS
Human papilloma virus (HPV)	Papova	DS DNA	Human wart viruses
JC virus	Papova	DS DNA	Progressive, multifocal leucoencephalopathy
Poliovirus	Picorna	SS DNA	Poliomyelitis
Molluscum virus	Pox	DS DNA	Molluscum contagiosum
Lyssavirus	Rhabdo	SS RNA	Rabies

DS = double-stranded; SS = single-stranded

from massive acute necrosis to chronic 'piecemeal necrosis' of liver cells, leading to cirrhosis. An eosinophilic 'ground glass' appearance is seen in the cytoplasm of some hepatocytes, due to dilated smooth endoplasmic reticulum that contains tubular HB surface antigen. It is this component that can be demonstrated using Shikata's orcein method, or by specific antiserum.

Herpes viruses are usually acquired subclinically during early life and enter a latent phase, to be reactivated during times of immunological stress. These viruses cause blistering or ulceration of the skin and mucous membranes, but can cause systemic diseases, including encephalitis, in immunosuppressed or malnourished individuals. The cytopathic effects of the herpes virus are well seen in Tzanck smears of blister fluid, and include the margination of chromatin along nuclear membranes, Cowdry type A ('owl's eye') inclusion bodies, and syncytial or 'grape-like' nuclei within giant cells. *Cytomegalovirus* (CMV) is sometimes seen as a systemic opportunistic infection in AIDS patients. It is seen in the endothelial cells, forming prominent intranuclear inclusions that spill into the cytoplasm where they form granular hematoxyphilic clusters. The CMV virus causes obvious cytomegaly in the cells it infects. All herpes viruses have an identical electron microscopic appearance of spherical, 120-nm, membrane-coated particles.

Papilloma viruses are a family of about 50 wart viruses that cause raised verrucous or papillomatous skin warts, or flat condylomatous genital warts. Cytologically, evidence of hyperkeratosis may be present together with koilocytosis (irregular nuclear enlargement and cytoplasmic vacuolation forming perinuclear halos). Skin verrucas are associated with HPV 1–4 strains, genital condylomas with HPV 6, 11, 16, and 18, and cervical cancer with HPV 16 and 18. These uncoated viruses measure 55 nm, are mainly intranuclear, and can be detected using electron microscopy, or immunoperoxidase and gene probes on paraffin sections.

JC virus is a papova virus that causes progressive multifocal leucoencephalopathy, a demyelinating disease, in AIDS and other immunosuppressed patients. Intranuclear hematoxyphilic inclusions may be seen within swollen oligodendrocytes.

Molluscum virus produces a contagious wart in children and young adults called molluscum contagiosum. Large eosinophilic, intracytoplasmic inclusion bodies can be seen in maturing keratinocytes, and are seen well with phloxine–tartrazine. The large 1-μm viral particles have a typical pox virus structure: brick-shaped with a superimposed figure-of-eight nucleic acid sequence.

Rabies virus. This neurotrophic rhabdovirus forms intracytoplasmic eosinophilic inclusions best seen in the axonal hillocks of hippocampal neurons of the brain. Machiavello, phloxine–tartrazine, Mann's methyl blue–eosin, or PAS stains are recommended.

Human immunodeficiency virus (HIV) consists of at least two retrovirus strains. The virus is best seen in cultured lymphocytes and is rarely seen in tissues from AIDS patients. It produces a distinctive neuropathological lesion in AIDS encephalitis consisting of microglial nodules, or stars, containing collections of giant cells, microglia, and astrocytes. Synthetic nucleic acid probes have been prepared to HIV genomes.

Influenza virus (flu) is a contagious respiratory illness caused by influenza viruses (Fig. 17.8). It can cause mild to severe illness, and at times can lead to death. According to the Centers for Disease Control (CDC), every year in the United States, on average:

- 5–20% of the population suffers from the flu
- more than 200,000 people are hospitalized from flu complications
- about 36,000 people die from flu.

Some people, such as older people, young children, and people with certain health conditions, are at high risk for serious flu complications.

SARS (severe acute respiratory syndrome) is a viral respiratory illness caused by a coronavirus called SARS-associated coronavirus (SARS-CoV) (Fig. 17.9). SARS

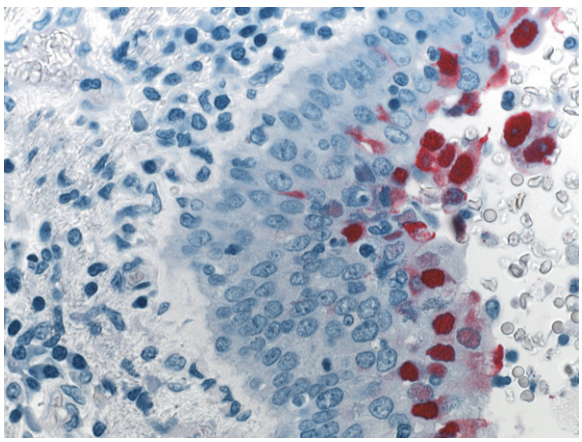

Fig. 17.8 Immunohistochemical method demonstrating Flu A (which is caused by influenza viruses) in bronchus (×40).

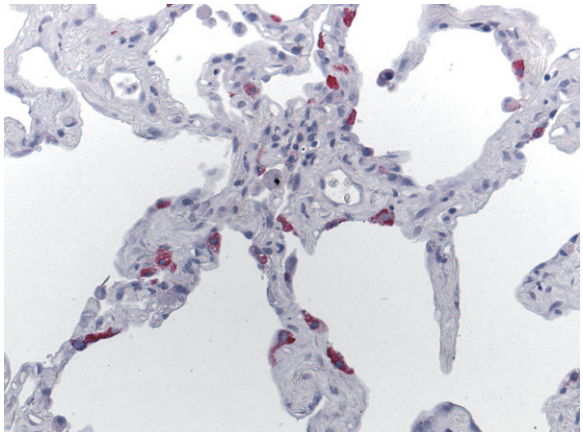

Fig. 17.9 Immunohistochemical method demonstrating the previously unrecognized SARS-associated coronavirus which is responsible for severe acute respiratory syndrome (SARS) (×20).

was first reported in Asia in February 2003. Over the next few months, the illness spread to more than two dozen countries in North America, South America, Europe, and Asia before the SARS global outbreak of 2003 was contained.

PRION DISEASE

To date, more than eight transmissible neurodegenerative diseases have been described affecting the central nervous system (CNS). The diseases caused by prions include Creutzfeldt–Jakob disease (CJD) and variant CJD (vCJD), Germann–Straussler–Shienker disease, fatal familial insomnia, and kuru in humans, bovine spongiform encephalopathy (BSE, also known as 'mad cow disease'), scrapie (in goats and sheep), and chronic wasting disease (CWD) (in mule deer and elk). In addition, prions are not microbes in the usual sense because they are not alive, but the illness they cause can be transmitted from one animal to another. All usually produce a characteristic spongiform change, neuronal death, and astrocytosis in affected brains. The infectious agent is a prion, a small peptide, free of nucleic acid and part of a normal transmembrane glycoprotein which is not, strictly speaking, a virus. Antibodies have been prepared from prion protein that strongly mark accumulated abnormal protein in these diseases (Lantos 1992).

The CJD Surveillance Center in the USA is an invaluable source for monitoring and testing of human prion disease in the United States. The Center is supported by the CDC and by the American Association of Neuropathologists. Visit their website (http://www.cjdsurveillance.com) for details on how to submit specimens for testing; they perform these tests at no charge for laboratories in the USA. In addition, both CDC and the World Health Organization (WHO) also offer guidelines regarding the handling of suspected and known cases of prion disease. Visit http://www.cdc.gov and search for CJD for a fact sheet and other relevant information. WHO offers a manual in pdf form for downloading. It gives information about what to do should you find yourself with a suspected or known positive case in your lab: http://who.int/bloodproducts/TSE-manual2003.pdf. Remember that these types of cases should never knowingly be handled in a routine histology lab. Contact your local health department for additional guidelines.

THE DEMONSTRATION OF PROTOZOA AND OTHER ORGANISMS

The identification of protozoa is most often made on morphological appearance using H&E and, particularly, Giemsa stains. The availability of antisera against organisms such as entamoeba, toxoplasma, and leishmania has made diagnosis much easier in difficult cases (Fig. 17.10).

Giemsa stain for parasites

Sections

Fixative is not critical, but B5 or Zenker's is preferred; thin (3 µm) paraffin sections. (If Zenker's is not used, post-mordant in Zenker's in a 60°C oven for 1 hour before staining.)

Solutions

Giemsa stock (commercially available) or

Giemsa stain powder	4 g
Glycerol	250 ml
Methanol	250 ml

Dissolve powder in glycerol at 60°C with regular shaking. Add methanol, shake the mixture, and allow to stand for 7 days. Filter before use.

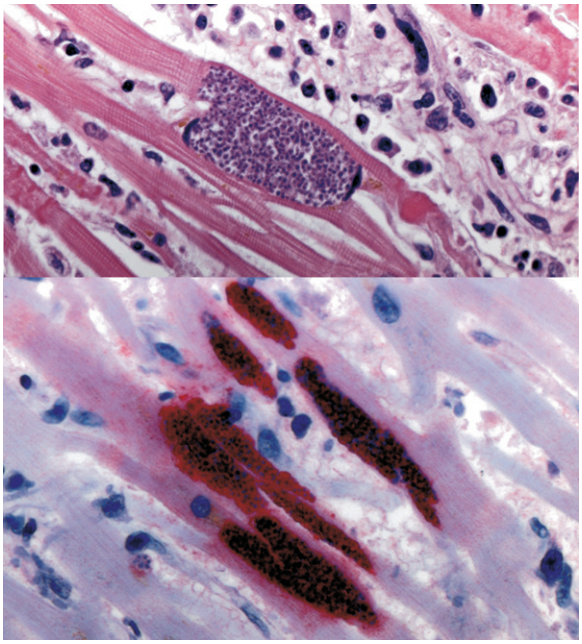

Fig. 17.10 H&E and immunohistochemical methods demonstrating the single-celled parasite *Toxoplasma gondii* in heart (×40).

Working Giemsa for parasites

Giemsa stock	4 ml
Acetate buffered distilled water, pH 6.8	96 ml

Method

1. Deparaffinize and rehydrate through graded alcohols to water.
2. Rinse in pH 6.8 buffered distilled water.
3. Stain in working Giemsa, overnight.
4. Rinse in distilled water.
5. Rinse in 0.5% aqueous acetic acid until section is pink.
6. Wash in tap water.
7. Blot until almost dry.
8. Dehydrate rapidly through alcohols, clear, and mount.

Results

Protozoa and some other microorganisms	dark blue
Background	pink–pale blue
Nuclei	blue

Protozoa

Entamoeba histolytica, the organism causing amebic colitis or dysentery, can be found in ulcers that occur in infected colon and in amebic liver abscesses. The trophozoite (adult form) measures 15–50 μm, contains a small nucleus, and has a foamy cytoplasm containing ingested red cells and white cell debris. They may be seen in granulation tissue within ulcers, or in the luminal mucus overlying normal appearing mucosa, and are PAS positive; brief counterstaining in 1% aqueous metanil yellow emphasizes the ingested red cells.

Toxoplasma gondii, a commonly encountered organism that is spread in cat litter, causes an acute lymphadenopathy which is often subclinical. Affected nodes show non-specific changes and no organisms. In AIDS and other immunosuppressed patients this protozoon causes systemic diseases, including meningoencephalitis where encysted bradyzoites and free tachyzoites can be seen in necrotic brain tissue. Cysts also occur in other tissues such as cardiac muscle, and measure up to 40 μm with tachyzoites 4–6 μm, which can be seen on H&E. A Giemsa stain can also be used, but the use of labeled specific antiserum is recommended.

Leishmania tropica is transmitted by sandfly bite and causes a chronic inflammatory disease of the skin sometimes called cutaneous leishmaniasis. The injected parasite forms (2 μm), or amastigotes, are found in large numbers within the cytoplasm of multiple swollen histiocytes that congregate in early lesions in the dermis. A related organism, *L. donovani*, causes a systemic visceral infection, kala azar, in which the organisms are seen within histiocytes in spleen, lymph nodes, liver, and bone marrow. The organisms are hematoxyphilic and can be emphasized with a Giemsa stain.

Giardia duodenalis (lamblia) is a flagellate protozoon that is ingested in cyst form from drinking water with fecal contamination; the trophozoites migrate to the duodenum where they may cause severe diarrhea and malabsorption. These organisms can easily be missed on an H&E stain, where they appear as eosinophilic, sickle-shaped flakes with indistinct nuclei resting on intestinal mucosa that may show little evidence of inflammation. When seen in a fresh Giemsa-stained duodenal aspirate, they appear kite-shaped, 11–18 μm in size, binucleate, and have faint terminal flagella.

Trichomonas vaginalis is a similar flagellate protozoon most frequently seen in a Papanicolaou stain. Inflammatory cells and mildly dysplastic squamous cells often

accompany this parasite as it causes cervicitis in the female, and urethritis in both sexes.

Cryptosporidium is one of a group of protozoa (including *Isospora* and *Microsporidium*) that causes severe and relentless outbreaks of diarrhea among AIDS patients. Cryptosporidial gametes are seen on H&E stain as blue dots arranged along the mucosal surface. Mature cysts are shed into feces and are acid fast in a ZN stain of fecal smears.

WORMS

Schistosoma species cause the disease schistosomiasis or 'bilharzia'. Various manifestations of the disease differ according to the particular *Schistosoma* species involved, but granulomata containing schistosome ova are found in the liver, bowel, and bladder mucosa, and sometimes in the lungs. The ova have thick, refractile, eosinophilic walls and are easily detected in H&E-stained sections. The PAS, Grocott, and ZN techniques are positive for these ova. Where the plane of section allows, the presence of a terminal spine to the ovum indicates *S. haematobium* whereas *S. mansoni* and *japonicum* have lateral spines. Any good trichrome procedure will demonstrate worm development.

Echinococcosis. Echinococcus granulosus is a tapeworm found in dogs; humans and sheep may become intermediate hosts and develop hydatid cyst disease. These cysts form in many organs, particularly liver and lung. The walls of the daughter cysts are faintly eosinophilic, characteristically laminated, and produced by the worm, not by its host. The walls are PAS positive and Congo red positive, showing green birefringence. The scolicial hooklets survive inside old, burnt-out cysts, are of diagnostic shape, and stain brilliant yellow with picric acid.

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