A new technique for Gram staining paraffin-embedded tissue

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SUMMARY Five techniques for Gram staining bacteria in paraffin sections were compared on serial sections of pulmonary tissue from eight bacteriological necropsies. Brown and Hopps' method was the most satisfactory for distinguishing Gram-positive and Gram-negative bacteria. However, this method cannot be recommended as the preparations were frequently overstained, and the Gram-negative bacteria were stained indistinctly. A modification of Brown and Hopps' method was developed which stains larger numbers of Gram-negative bacteria and differentiates well between different cell types and connective tissue, and there is no risk of overstaining.

The cytochemical reactions of Gram staining are not fully understood. Grouping bacteria as Gram-positive and Gram-negative coincides with a difference in the structure of the cell wall, with the sensitivity to some antibiotics, and with the ability to produce certain toxins and enzymes (Cruickshank *et al.*, 1973).

In the bacteriological laboratory, Gram staining is carried out on flame-fixed smears. Many variations of this 'bacteriological' Gram staining are described. These methods are not suitable for Gram staining bacteria in tissue sections, and special methods are necessary for this purpose. These 'histological' Gram stainings are usually based on a two-step differentiation and counterstaining.

In the present work, five frequently used 'histological' Gram stains are compared with a modification of Brown and Hopps' method developed in this laboratory.

Material and methods

The staining methods were compared on paraffin sections of pulmonary tissue with bronchopneumonia from eight bacteriological necropsies.

Cardiac blood, and pulmonary tissues from several areas in each pulmonary lobe, were aspirated sterily and seeded onto plates containing either 5% blood agar, or bromthymol blue agar, or 10% pre-reduced blood agar for anaerobic cultivation, and in thiogly-

collate medium. Isolation and identification of the cultured bacteria were performed in the Department of Clinical Microbiology, Statens Seruminstitut, Frederiksberg Hospital, Copenhagen.

After evisceration, a frontal section was made through the lungs, and tissue blocks from each lobe were taken for histology. The blocks were fixed for 24 hours in 10% neutral buffered formalin and for 24 hours in 3.6% alcoholic formalin. The blocks were then dehydrated by increasing concentrations of alcohol, cleared in xylene, and embedded in paraplastplus.

Four-micron serial sections of the tissue blocks were stained by the following five methods: Brown and Brenn (1931), MacCallum-Goodpasture (Luna, 1968), Humberstone (1963), Taylor (1966), and Brown and Hopps (1973).

These methods were compared with the following modification of Brown and Hopps' method:

- (1) Deparaffinisation of the sections and hydration with distilled water.
- (2) Crystal violet solution (A) for two minutes.
- (3) Washing in running water to remove excess dye.
- (4) Iodine-potassium iodide solution (B) for five minutes.
- (5) Washing in running water.
- (6) Excess water allowed to run off and then sections placed immediately in absolute acetone.
- (7) Differentiation in four changes of absolute acetone for a total of 100 seconds.
- (8) Brief washing in running water until the acetone is removed.
- (9) Carbolfuchsin solution (C) for 1-1½ minutes.
- (10) Brief washing in running water.

From this stage the preparations must be taken two at a time. (11) Callego's solution (D) for five

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minutes. (12) Brief washing in running water. (13) Excess water allowed to run off and sections then placed immediately in absolute acetone. (14) Three dips in absolute acetone. (15) Three dips in picric acid-acetone (E). (16) Three dips in absolute acetone. (17) Five dips in acetone-xylene (F). (18) Ten dips in xylene. (19) Application of eukitt and cover glass.

SOLUTIONS

(A) Crystal violet: kristallviolett (Merck 1408) 1 gr, distilled water 100 ml. Filter before use. (B) Iodine-potassium iodide: iodine 1 g, potassium iodide 2 g, distilled water 300 ml. (C) Carbol-fuchsin, stock solution: fuchsin (Merck 1358) 1 g, phenol (Merck 206) 5 g, absolute alcohol 10 ml, distilled water 100 ml. (D) Gallego's solution: concentrated formalin 1 ml, glacial acetic acid 0.5 ml, distilled water 50 ml. (E) Picric acid-acetone: pikrinsäure (Merck 623) 0.5 g, absolute acetone (Merck 14) 500 ml. (F) Acetone-xylene: absolute acetone (Merck 14) 50 ml, xylene 50 ml.

Three to four corresponding fields on each section were circled with Indian ink and evaluated 'blindly' by the same person. The number of stained bacteria in each area was counted, and the staining of the bacteria was evaluated qualitatively in terms of colour, homogeneity, and contrast with the sur-

rounding tissue.

Colour was defined by the total visual impression of three variables: hue (location in the spectrum), maturation (intensity), and lightness (degree of whiteness or blackness). The colour of the stained bacteria was described with the help of a colour atlas (Kornerup and Wanscher, 1974) using a neutral filter to reduce the light intensity of the microscope.

By homogeneity we mean the ability of the stain to bind to the bacteria, so that the bacteria ideally are stained all through and do not appear moth-eaten or with indistinct outlines.

For evaluation we have used an arbitrary scale from 0 to 4, where 0 = unstained, 1 = poor, 2 = moderately poor, 3 = moderately good, and 4 = good.

Finally, the ability of the staining methods to differentiate between different cell types and connective tissue has been evaluated.

Results

The major features of the most common human pathogenic bacteria, as demonstrated by the six staining methods, are summarised in the Table. Except for Humberstone's method, all methods gave

Table Comparison of six methods for staining Gram-positive and Gram-negative bacteria in paraffin sections

Method*	Staphylococcus	Streptococcus	Corynebacterium	Clostridium	Escherichia	Proteus	Klebsiella	Pseudomonas	Haemophilus
A Number	4	4	4	4	1	1	1	0	0
Colour	Violet blue	Violet blue	Violet blue	Violet blue	Bluish red	Bluish red	Bluish red	—	—
Homogeneity	4	4	4	4	2	2	2	—	—
Contrast	4	4	4	4	2	2	2	—	—
B Number	4	4	4	4	1	1	1	0	0
Colour	Greyish magenta	Greyish magenta	Greyish magenta	Greyish magenta	Pink	Pink	Pink	—	—
Homogeneity	3	3	3	3	1	1	1	—	—
Contrast	3	3	3	3	1	1	1	—	—
C Number	2	2	0	3	3	3	3	2	1
Colour	Pink	Pink	—	Pink	Bluish red	Bluish red	Bluish red	Bluish red	Pink
Homogeneity	1	1	—	1	1	1	1	1	1
Contrast	1	1	—	1	2	2	2	2	2
D Number	4	4	4	4	0	1	1	0	0
Colour	Violet blue	Violet blue	Violet blue	Violet blue	—	Pink	Pink	—	—
Homogeneity	4	4	4	4	—	1	1	—	—
Contrast	3	3	3	3	—	1	1	—	—
E Number	4	4	4	4	3	3	3	3	2
Colour	Greyish magenta	Greyish magenta	Greyish magenta	Greyish magenta	Pink	Pink	Pink	Pink	Pink
Homogeneity	3	3	3	3	3	3	3	3	2
Contrast	3	3	3	3	2	3	3	3	2
F Number	4	4	4	4	4	4	4	4	4
Colour	Deep magenta	Deep magenta	Deep magenta	Deep magenta	Bluish red	Bluish red	Bluish red	Bluish red	Bluish red
Homogeneity	3	3	3	3	4	4	4	4	4
Contrast	3	3	3	3	4	4	4	4	3

*A, Brown and Brenn's method; B, MacCallum-Goodpasture's method; C, Humberstone's method; D, Taylor's method; E, Brown and Hopps' method; F, modification of Brown and Hopps' method developed in this laboratory.

Scale: — unreadable, 0 = unstained, 1 = poor, 2 = moderately poor, 3 = moderately good, 4 = good.

Notation of the colour area (Kornerup and Wanscher, 1974): violet blue 19B7, greyish magenta 14C6, deep magenta 14E8, bluish red 12B7, pink 12A5.

acceptable staining of Gram-positive bacteria. Because their method showed more stained bacteria and greater homogeneity and contrast, Brown and Brenn achieved the highest score. Humberstone's method did not stain the corynebacteria and stained other Gram-positive bacteria pink. The original Brown and Hopps' method decolorised the central area of corynebacteria, so that they became pale pink encircled by a blue rim.

With Gram-negative bacteria, the six methods showed much greater variability. Acceptable staining was achieved only with the original and the modified Brown and Hopps' methods, but the modified method achieved the highest score due to larger numbers of stained bacteria and greater homogeneity and contrast of the stained bacteria.

Both the original and the modified Brown and Hopps' methods stained the tissue well, so that the localisation of the bacteria could easily be evaluated in relation to the alveolar wall, inflammatory cells, metastases, etc. The other methods were less satisfactory. Brown and Brenn's and Taylor's methods stained the tissues so faintly that it was impossible to identify the tissue, and Brown and Brenn's, MacCallum-Goodpasture's, and Humberstone's methods stained the erythrocytes so strongly that it was difficult to localise the bacteria in tissues with a high blood content.

Discussion

In the initial stages of our experiments, all staining methods greatly overstained the preparations. Microscopical examination after each step in the procedures showed that the preparations were not being decolorised with acetone.

In the staining procedures, it is stated that, before differentiation, excess water should be pressed off with blotting paper without the preparations becoming dry. This step is difficult to control. If the preparations become a little too dry, an irreversible hardening of the dyes occurs, and the preparations are not decolorised with acetone. The results in the Table are the best that we could achieve with these methods.

Brown and Hopps' method proved the most satisfactory of the original methods for differentiating between Gram-positive and Gram-negative bacteria. However, for three reasons it was necessary to improve it, so that it could be used in routine work: (i) the preparations often became overstained because they dried during staining, (ii) the Gram-negative bacteria were stained indistinctly, and (iii) not all Gram-negative bacteria in the tissue were stained.

To prevent the preparations drying during staining,

we omitted pressing with blotting paper at step 6 and 13 and transferred the preparations directly to acetone. This modification was possible if four rinses with acetone were used instead of one, and it had the advantage that only from step 10 was it necessary to proceed with two slides at a time.

The original Brown and Hopps' method used a basic fuchsin solution as counterstain. This counterstain has the disadvantage that it washes off crystal violet from the Gram-positive bacteria (Adams, 1975). With the aim of improving the staining of Gram-negative bacteria, we tried to increase the concentration of the dye and the staining time, but this caused further decolorisation of Gram-positive bacteria. We therefore tested a number of other cationic dyes—neutral red, safranin, eosin, pyronine Y, Bismarck brown, and carbol-fuchsin. Carbol-fuchsin solution stained the Gram-negative bacteria most satisfactorily and apparently did not wash the crystal violet off Gram-positive bacteria. Carbol-fuchsin is a solution of basic fuchsin and phenol and is more soluble in lipoids than is pure basic fuchsin. The greater affinity of carbol-fuchsin to Gram-negative bacteria could be due to the higher content of lipoids in the cell wall of Gram-negative bacteria, enabling carbol-fuchsin to penetrate more easily into these bacteria than basic fuchsin.

Because carbol-fuchsin does not remove the crystal violet from Gram-positive bacteria, the staining time could be varied, and the differentiation time at step 7 could be increased 10-fold in comparison with the original method without any decolorisation of Gram-positive bacteria taking place.

In summary, the present modification stains larger numbers of Gram-negative bacteria. Gram-negative bacteria are stained with greater homogeneity, the contrast between Gram-positive and Gram-negative bacteria is increased, and the staining of the tissue is such that the tissue can be identified with confidence, even intracellular bacteria standing out clearly.

The modified Brown and Hopps' method has been used for routine bacteriological necropsies at Frederiksberg Hospital for more than three years with very satisfactory results.

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Letters to the Editor

Book reviews

Another well-written chapter is by W. D. and J. E. Edwards on pulmonary vasculature, particularly the description of plexogenic pulmonary arteriopathy. L. Reid has brought us up to date with research on mucus secretion in the lung.

The monograph should interest anyone concerned with teaching pulmonary pathology or wanting to learn about it.

BRIAN E. HEARD

Clinical Gastrointestinal Immunology. By H. C. Thomas and D. P. Jewell. (Pp. viii + 264; illustrated; £12.00.) Oxford: Blackwell Scientific Publications. 1979.

In the past 10-15 years both basic and applied immunology have produced an 'explosion' (as the authors state) not only in gastroenterology but in most of the clinical specialties; with the probable exception of nephrology, however, gastroenterology represents the specialty in which both basic and applied immunology have contributed most to our understanding. This is borne out by the number of texts which have now appeared and continue to appear dealing with various aspects of the immunology of the gastrointestinal tract and liver. The need for a further monograph such as the present one is in no doubt; it provides a most lucid and concise 'up-date' of current knowledge and with a judicious and careful critical approach in those areas where the evidence as yet remains equivocal.

The first three chapters provide a basic immunological background against which the subsequent chapters are set. One wonders whether the authors have not been a little patronising in assuming that their colleague gastroenterologists have not as yet become sufficiently familiar with the language and concepts of immunology; if they have not, then it is surely an indictment of our undergraduate and postgraduate medical education. If there is need to include a glossary in which, for example, gluten and reticuloendothelial system appears, are they justified in assuming a sufficient virological knowledge among their readers to be able to understand nucleocapsid, DNA polymerase, and others? Having said this, however, these three chapters represent an excellent precis of current knowledge, and for any gastroenterologist who has missed out on his immunology the book is worth purchase for this alone. The subsequent chapters deal with particular diseases, including intestinal and hepatic infections

(perhaps the least satisfying in its rather sketchy and brief handling of the topic), coeliac disease, chronic inflammatory bowel diseases, acute and chronic hepatitis, and others. There are some minor inconsistencies here and there, for example, in a balanced review of Crohn's disease and ulcerative colitis immune responsiveness in the former becomes immunological competence in the latter. The chapter on acute and chronic viral hepatitis is a masterpiece, in which the complexities of the host/viral interrelationships are clearly and skilfully outlined. The authors are to be congratulated on this excellent text, and it is a pleasure to commend it.

R. N. M. MACSWEEN

Post Mortem Procedures. (An Illustrated Textbook). By G. A. Gresham and A. F. Turner. (Pp. 160; illustrated; £8.00.) London: Wolfe Medical Publications Ltd. 1979.

Sex and violence pervade the media and intrude into this book. The first page advises us that sexual intercourse is a cause of sudden death, and the later chapters are full of high-quality gruesome photographs of the mutilated victims of violence. This type of presentation, with a tabloid-type format of numerous photographs (thankfully in monochrome) and a concise text in short, easily read sentences, may attract a mortuary technician with limited literary horizons.

To a hospital pathologist trying to raise the standards of his postmortem room the 60 pages on the technique of the postmortem examination are outstanding. The step-by-step illustrations of how to remove the organs are excellent, and most of them speak for themselves. Specialised techniques and mortuary administration are also described, and pathologists in training will benefit from a quick perusal of this book. It is well-printed on good paper, does not take long to read, and is not expensive. Recommended for the mortuary staff to read and to imitate. The pathologist who works with technicians who can reproduce these procedures is fortunate.

R. A. B. DRURY

Corrections

A new technique for Gram staining paraffin-embedded tissue by K. ENGBAEK, K. S. JOHANSEN, and M. E. JENSEN (*Journal of Clinical Pathology*, 1979, 32, 187-190.)

On page 188, column 1, paragraph 2, line 7, under solution (C) should be added: 'Working Solution: stock solution 10 ml, distilled water 50 ml.'

Winter vomiting disease caused by calicivirus by W. D. CUBITT, D. A. MCSWIGGAN, and W. MOORE (*Journal of Clinical Pathology*, 1979, 32, 786-793).

Results:

Buoyant density in CsCl

Feline calicivirus 1.36-1.42 g/cm⁻³

'Human' calicivirus 1.36-1.38 g/cm⁻³