OXOID

culture

September 1978

Published as a service to microbiology
by Oxoid Limited

Trimethoprim and sulphonamides

Problems associated with their *in vitro* testing

S.G.B. Amyes*

Sulphonamides inhibit the bacterial enzyme, dihydropteroate synthetase, which is the first step in the biosynthesis of tetrahydrofolates. This enzyme does not exist in mammalian cells and thus sulphonamides are selective against bacteria.

Trimethoprim inhibits another enzyme in the same biosynthetic pathway, namely dihydrofolate reductase. Although this enzyme is present in mammalian cells, bacterial dihydrofolate reductase is 100,000 times more susceptible to trimethoprim action.

As sulphonamides and trimethoprim inhibit two steps in the same pathway, they act synergistically with one another. In other words the activity of each drug is greatly enhanced by the presence of the other. It is for this reason that trimethoprim and the sulphonamide, sulphamethoxazole, were marketed in combination with one another as co-trimoxazole.

The Number of Disks

Although both trimethoprim and sulphamethoxazole are effective antibacterial agents *in vivo*, great difficulty has been experienced in producing a reliable *in vitro* sensitivity test. Most laboratories test co-trimoxazole sensitivity using the agar diffusion technique with a single disk of co-trimoxazole, usually containing one part trimethoprim to 20 parts sulphamethoxazole.

The sensitivity of the organism is revealed by a zone of clearing around the disk. However, in this case, this method may give confusing results, for a zone of sensitivity does not give any information about the resistance to the individual components. In Figure 1 the top row of plates shows four organisms all of which are sensitive to the combined disk, whereas on the bottom row are the same organisms tested with individual disks of trimethoprim and sulphamethoxazole. The first organism is sensitive to both components and the synergy between the drugs can be seen by the bridging of the zones. The second organism is sensitive to sulphamethoxazole and resistant to trimethoprim, and the third organism is resistant to sulphamethoxazole and sensitive to trimethoprim. Neither of these two organisms shows any synergy. The last organism is resistant to trimethoprim and sensitive to sulphamethoxazole but synergy does exist this time. Similarly, some trimethoprim-sensitive, sulphamethoxazole-resistant bacteria act in the same way. A final group of sensitive bacteria are resistant to each drug individually and the synergy between them gives a sensitive zone around a combined disk.

Not all clinicians would wish to prescribe co-trimoxazole in all these cases. It seems prudent, therefore, to test the sensitivity of bacteria with one disk of trimethoprim and a separate one of sulphamethoxazole. The results obtained give a more informed view of the bacterial sensitivity on which to prescribe subsequent therapy. If the organism is resistant to one of the components it does not necessarily preclude the use of co-trimoxazole, only that it should be used with more caution. If the organism is resistant to both components the combination *should not be used. The testing* with a separate disk of trimethoprim may have the advantage that if the drug is also released in the future without sulphamethoxazole, as seems likely, no additional test is needed.

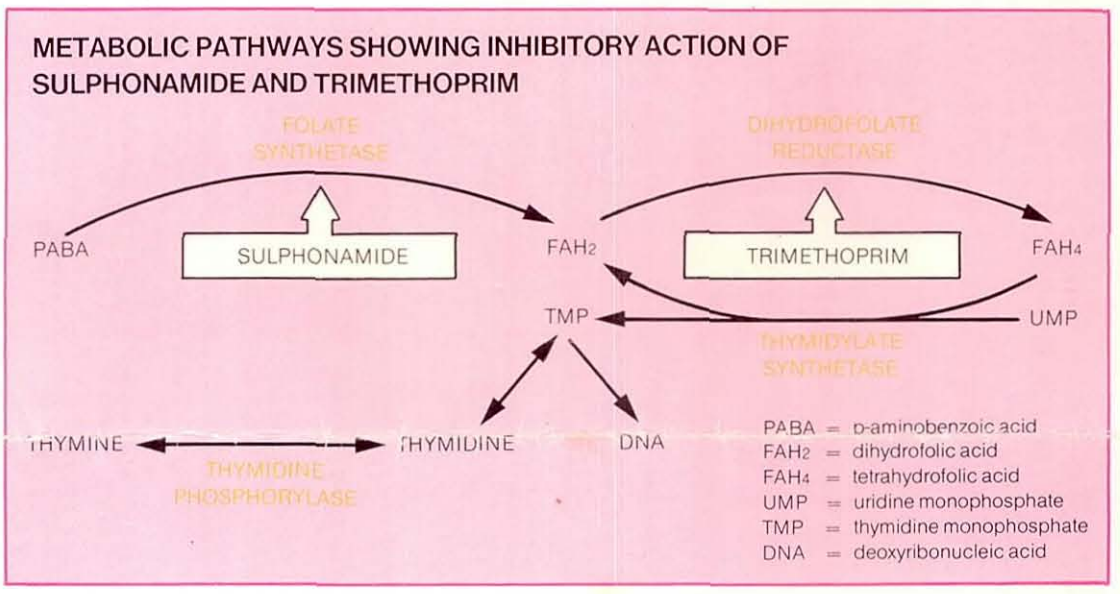


FIGURE 1. Sensitivity tests with four different isolates. The top row of plates contains a single disk of trimethoprim/sulphamethoxazole and on the bottom row the organisms are tested with separate disks of trimethoprim and sulphamethoxazole. The trimethoprim disk is on the left.

The Choice of Media

A major problem in sensitivity testing has been in the choice of media, because most laboratory media contain antagonists to both drugs and this reduces their efficacy. In the agar diffusion technique this may be seen as a reduction in the zone size or even no zone at all (Fig. 2a). In the 1930's, when sulphonamides were first introduced, all media were as unsuitable as this. Harper and Cawston (1945) found empirically that many laboratory media seemed to be cleared of most of the antagonism if lysed horse blood was added (Fig. 2b) and from then on it was added routinely to media used for sulphonamide sensitivity testing. Later, it was found that lysed horse blood was also effective in clearing the antagonists to trimethoprim though its action was still not known (Waterworth, 1969).

Thymidine

During the last few years the antagonists in laboratory media have been identified. Koch and Burchall (1971) showed that thymidine was a potent antagonist of trimethoprim action and that most laboratory media *contained significant quantities* of this thymine derivative. Amyes and Smith (1974a) showed that thymine was also an antagonist of trimethoprim action which was surprising as it is not usually taken up by prototrophic bacteria.

Workers at the Wellcome Research Laboratories found that lysed horse blood contained significant quantities of the enzyme thymidine phosphorylase (Ferone *et al.*, 1975). This enzyme converts thymidine to thymine. Thymine is a less potent antagonist, about 100 times less active than thymidine and thus providing that the level of thymidine is not too high the addition of lysed horse blood may convert it all to thymine, the concentration of which is not high enough to antagonize trimethoprim.

Lysed horse blood is not available in many countries and a medium that is not dependent on its presence is to be preferred. This must have very low levels of thymine derivatives to produce significant zone sizes (Fig. 2c). At first only Wellcome Nutrient Agar contained low enough levels of thymine derivatives (Koch and Burchall, 1971). More recently other manufacturers have modified the composition of their *media to remove thymine* derivatives. Oxoid Iso-Sensitest Agar and Diagnostic Sensitivity Test Agar, Difco Mueller Hinton Agar and Wellcotest Sensitivity Test Agar are now all suitable in the absence of lysed horse blood for testing both trimethoprim and sulphamethoxazole sensitivity. As the media used can have such a dramatic effect on the results obtained, control organisms that are known to be sensitive should be tested at regular intervals under exactly the same conditions as the test organisms.

One note of caution, the media listed contain, of course, insufficient thymine derivatives to grow thymine auxotrophs. These mutants confer high trimethoprim resistance (Amyes and Smith, 1975) and under certain circumstances can be selected during co-trimoxazole therapy *in vivo* (Okubadejo and Maskell, 1973). They may also be as pathogenic as the prototrophic strain. Therefore, a separate test of the organism for thymine *dependence should be made* (Amyes and Smith, 1974a) to detect the presence of these auxotrophs.

The Action of the Antagonists

In the case of trimethoprim the reversal of the action of the drug by thymine or thymidine in complex media is similar to the requirement of thymine auxotrophs. However, in a minimal

□ *continued on page 2*

☐ continued from page 1

medium containing thymine derivatives, a trimethoprim-treated prototrophic organism does not multiply as it does in complex media (Table 1). Thus other factors are present in complex media that promote the antagonism by thymineless mutants. The metabolites responsible are those in which tetrahydrofolates are involved in the biosynthesis namely methionine, glycine and purines (Table 1) (Amyes and Smith, 1974b).

When these metabolites are present in the absence of thymine derivatives, either in complex media or as supplements to minimal media, the trimethoprim-treated organism will "die" in a similar manner to the classic "thymineless death" of thymine auxotrophs. This is in contrast to the result in minimal media with no supplements when only a bacteriostatic effect is found (Table 1). Thus thymine derivatives are the only "true" antagonists of trimethoprim action although the other metabolites are also required to promote them. These other metabolites have no antagonistic effect by themselves.

The biochemistry of the antagonism to sulphonamides is less clear. The best known antagonist is para-amino benzoic acid (PABA) which inhibits sulphonamide action. PABA is the substrate of dihydropteroate synthetase and sulphonamide is its structural analogue. In normal laboratory media the contribution of PABA in the antagonism of sulphonamides is not great, for it is present in relatively low concentration (Amyes and Smith, 1976). Methionine and derivatives of thymine also have antagonistic effects on sulphonamides and, in addition, the presence of each of these two in a medium potentiates the action of the other antagonist (Amyes and Smith, 1976). Both have a slight effect by themselves, but together they produce a high degree of antagonism. Therefore, if one antagonist can be removed, i.e. a medium containing no thymine derivatives or in the presence of lysed horse blood, the medium may become suitable. The reason why these antagonists behave differently for sulphonamides and trimethoprim is unclear.

	Complex media – no thymine derivatives	Minimal Medium: no supplements	Minimal Medium: + amino acids and purine at 50µg/ml
+ trimethoprim (5µg/ml) and thymidine (5µg/ml)	8.1×10^8	9.6×10^6	1.0×10^8
+ trimethoprim (5µg/ml)	1.0×10^3	9.6×10^6	4.5×10^3
No drug	8.5×10^8	3.0×10^8	3.4×10^8

TABLE I The viable count of the trimethoprim-sensitive prototrophic organism, *Escherichia coli* 114, after 5 hours incubation (organisms/ml). The initial count was 1.0×10^7 organisms/ml.

The Level of Drug in the Disk

In a complex medium containing insignificant levels of thymine derivatives the minimum inhibitory concentration (MIC) of trimethoprim is the same for most organisms as it is in minimal medium. The MIC of sulphonamides, however, is always higher in this type of complex media than it is in minimal media. This is because methionine and PABA are always present and have some antagonistic effect.

Thus sulphonamide sensitivity testing must still remain a compromise. In normal circumstances this may not matter for a zone of sensitivity is usually found. However, in order to be more precise, perhaps higher content disks of sulphamethoxazole should be used to take this into account and maintain the normal 1:20 trimethoprim: sulphamethoxazole ratio. This gives a clearer result and so, as complex medium usually gives an MIC about 2½ times that on minimal medium, a disk of 50 µg of sulphamethoxazole, instead of 20 µg, would take this into account.

The Inoculum of Bacteria

The inoculum of bacteria used is critical in testing the components of co-trimoxazole. If an overnight culture of a sensitive organism is flooded on the surface of a plate and disks of trimethoprim and sulphamethoxazole are applied very small zone sizes are found after incubation (Fig. 3a). This is not due to "carry-over" from the antagonists in the original growth medium because cultures that have been thoroughly washed with antagonist-free medium give the same result.

When the culture is diluted 1 in 100, a small zone around the sulphamethoxazole disk and a slight enlargement of the trimethoprim zone is seen (Fig. 3b). However, when the culture is diluted 1 in 10,000, significant zone sizes are found (Fig. 3c). At dilutions greater than this, no improvement is found and indeed, the inoculum becomes so low that it becomes difficult to see the zones properly.

Conclusions

The agar diffusion technique is by no means perfect and with the drugs trimethoprim and sulphamethoxazole there are more problems than most. However, there are no suitable alternatives to this method which can give a result as quickly or as easily. If the agar diffusion technique is used carefully it can provide a valuable indication to therapy. In general, an overnight culture of the test organism should be diluted 1 in 10,000 in an antagonist-free medium. This dilution is flooded on an agar plate of suitable composition. When the excess fluid is removed, one disk of 1 or 1.25 µg trimethoprim and one of 50 µg sulphamethoxazole should be placed on the surface. The zone sizes, obtained after suitable incubation, should be related to those found with a sensitive control.

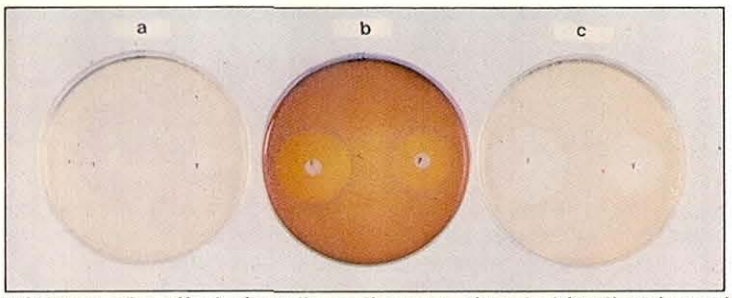


FIGURE 2. The effect of media on the zone sizes to trimethoprim and sulphamethoxazole disk. The trimethoprim disk is on the left. (a) An unsuitable medium (b) The same medium contained 5% lysed horse blood (c) Medium containing no thymine derivatives.

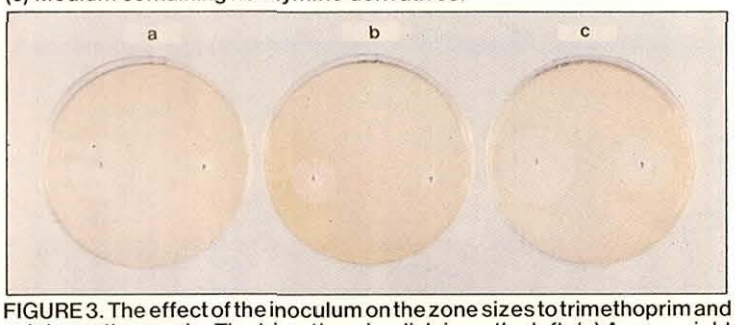


FIGURE 3. The effect of the inoculum on the zone sizes to trimethoprim and sulphamethoxazole. The trimethoprim disk is on the left. (a) An overnight culture (b) 1 in 100 dilution (c) 1 in 10,000 dilution.

References

1. Amyes, S.G.B. and Smith, J.T. (1974a). "Trimethoprim Sensitivity Testing and Thymineless Mutants" *Journal of Medical Microbiology* **7** 143–153.
2. Amyes S.G.B. and Smith J.T. (1974b). "Trimethoprim Action and its Analogy with Thymine Starvation". *Antimicrobial Agents and Chemotherapy* **5** 169–178.
3. Amyes S.G.B. and Smith J.T. (1975). "Thymineless Mutants and their Resistance to Trimethoprim". *Journal of Antimicrobial Chemotherapy* **1** 85–89.
4. Amyes S.G.B. and Smith J.T. (1976). "Antagonism in Sulphonamide Sensitivity Testing". *Journal of Pharmacy and Pharmacology* December Supplement p52.
5. Ferone R., Bushby S.R.M., Burchall J.J., Moore W.D. and Smith D. (1975). "Identification of Harper-Cawston Factor as Thymidine Phosphorylase and Removal from Media of Substances interfering with Susceptibility Testing to Sulfonamide and Diaminopyrimidines". *Antimicrobial Agents and Chemotherapy* **7** 91–98.
6. Harper G.J. and Cawston W.C. (1945). *"In vitro* Determination of Sulphonamide Sensitivity in Bacteria". *Journal of Pathology and Bacteriology* **57** 59–66.
7. Koch A.E. and Burchall J.J. (1971). "Reversal of the Antimicrobial Activity of Trimethoprim by Thymidine in Commercially Prepared Media". *Applied Microbiology* **22** 812–817.
8. Okubadejo O.A. and Maskell R.M. (1973). "Thymine-requiring Mutants of *Proteus mirabilis* selected by Co-trimoxazole *in vivo*". *Journal of General Microbiology* **77** 533–535.
9. Waterworth P.M. (1969). "Practical Aspects of Testing Sensitivity to Trimethoprim and Sulphonamide". *Postgraduate Medical Journal* **45** (Suppl.), 21–28.

*S.G.B. Amyes is from the Department of Bacteriology, The Medical School, University of Edinburgh, Teviot Place, EH8 9AG.

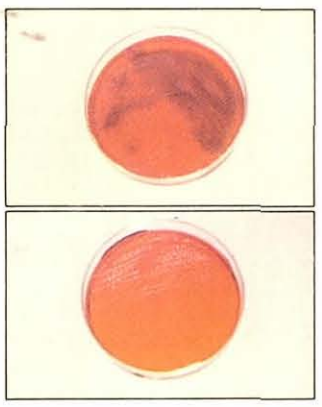
OXOID NEWSLINES

Campylobacter supplement

CODE SR69

The development of a selective culture medium has now made the isolation of campylobacters from faeces a simple matter. All that is required is a blood agar medium, the Oxoid Antibiotic Supplement (SR69), an Oxoid Gas Generating Kit, a vacuum jar and an incubator set at 43°C.

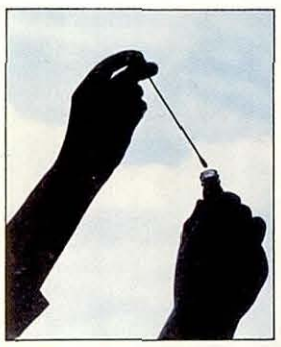
The illustrations of growth on the culture plates show the remarkable selective effect of the supplement. Subculture and identification of *C. jejuni – C. coli* from the medium containing the supplement is made very simple.



Without Supplement
Enteric isolate containing Campylobacter species will normally be overgrown by commensurates such as *E. coli, Strep. faecalis* and Proteus spp. when grown on lysed blood agar.

With Supplement
By adding Oxoid Campylobacter Supplement (SR69) a pure culture of campylobacter can be obtained from the same isolate.

Amies transport medium



The charcoal transport medium of Amies combines the virtues of Stuart's medium with the up-dated modifications learned during the last twenty years, such as added charcoal to protect neisseria and glycerol-free phosphate buffer. It is now the best transport medium for *Neisseria gonorrhoeae.*

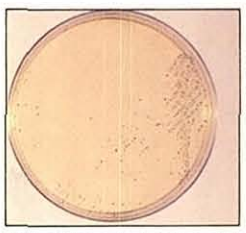
Neisseria gonorrhoea subcultured from Amies Transport Medium, growing on Oxoid G. C. Medium

Tinsdale's medium CODE CM487 & SR65

As the incidence of diphtheria becomes smaller, the risk of not recognizing the disease increases. Laboratory staff, who have never seen colonies of *Corynebacterium diphtheriae* outside a classroom, could overlook a positive culture from a carrier of the disease.

The dark halo around colonies of *C. diphtheriae* on Tinsdale's medium immediately alerts the microbiologist to the organism. Identification can then follow and may save a life or stop an epidemic.

C. diphtheriae showing characteristic dark halo around colonies



Typical throat diphtheroids

BRILLIANT GREEN IN SELECTIVE CULTURE MEDIA

Brilliant green is the most important dye used in culture media for the selection of salmonellae. It follows, therefore, that attention should be paid to the quality and stability of the dyestuff.

Before Edel and Kampelmacher[1,2,3] embarked on their large-scale international testing survey for salmonella isolation, they had first carried out tests in their own laboratories with various selective media. One of the reasons for the international test was an apparent conflict of results between the authors' laboratories and other workers, using the same test samples. The results suggested that the methods and media used in Utrecht were superior to those used elsewhere.

In particular, much work was carried out on the R.I.V. Brilliant Green Agar that had been used in the authors' laboratories for many years. Some of this work was described by Edel[4], in which the co-operative investigation carried out by the Rijks Instituut voor de Volksgenzondheid (R.I.V.) and Oxoid Limited resulted in Brilliant Green Agar (Modified) Oxoid CM329.

Edel[4] showed that the selective action of brilliant green depended on its concentration in the medium.

An optimum concentration must be chosen which prevents the swarming of Proteus species but still gives adequate colony size and recovery of salmonellae.

Prior to the development of the dehydrated medium (Oxoid CM329) instructions for the preparation of the medium[1] included the preparation of a stock aqueous solution of brilliant green (0.5% w/v) which had to be stored in the *dark* without any heating or sterilization.

As this dyestuff is so important to the medium, Oxoid were asked by the Dutch I.S.O. Group to investigate the quality of batches of brilliant green dye. Edel (personal communication) had previously reported that "Chroma" (Gesellschaft Schmid & Co. Stuttgart) and B.D.H. (B.D.H. Chemicals Limited, Poole, Dorset) were the dyes of choice. Enquiries were therefore made to B.D.H. about quality tests of dye batches. During this enquiry it became evident that *aqueous* solutions of brilliant green were not stable, either in the light or in the dark. Measurement of optical density at E' 630 showed a continuous fall over a short time span. This result, discovered at B.D.H. and confirmed in Oxoid, was independent of pH. It has never been observed in Oxoid because there the standard practice was to prepare brilliant green in 50% v/v ethanol and then dilute in aqueous buffer. The alcohol had a stabilizing effect on brilliant green. Fig I shows these results to be independent of storage in the *light* or *dark*.

There appears to be no adequate explanation for this effect, although dimerization may be more likely than chemical instability. If ethanol favoured the monomer this would explain why absorbences are higher in alcohol than in water.

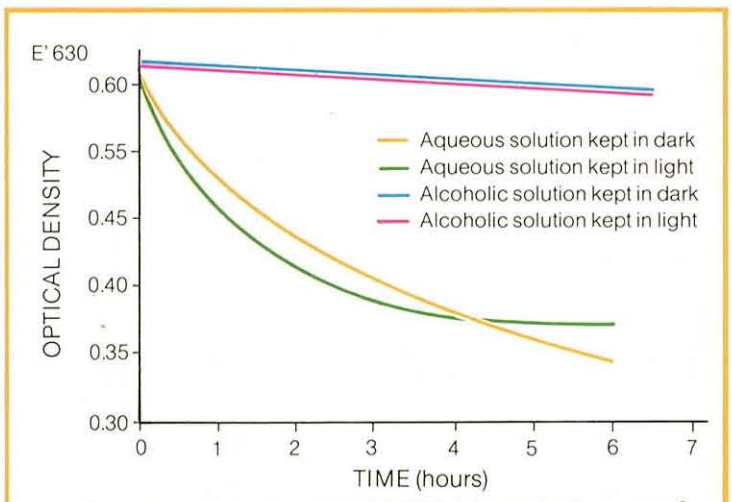
However, the seventy years of experience with brilliant green in culture media did not suggest a basic deterioration of the dye in aqueous solutions. The E' 630 densities were, therefore, measured in Lauryl Tryptose Broth (Oxoid CM451) as an example of a buffered aqueous broth. Fig 2 shows that the dye in aqueous media is as stable as in ethanol. It is evident that other molecules than ethanol can stabilize brilliant green in aqueous solutions.

The full significance of this curious phenomenon has yet to be measured, but it would seem to be a wise precaution to prepare stock solutions of brilliant green, using ethanol as the primary solvent. After this, storage in the light or dark is immaterial, although all dyestuff solutions must be protected from direct sunlight.

Brilliant Green in ml/l	Diameter of Salmonella colonies in mm	Proteus Spreading
5.50	3.5 – 4.5	++++
5.75	3.0 – 4.5	++
6.00	2.5 – 4.0	+
6.25	2.5 – 3.5	–
6.50	1.5 – 3.0	–
6.75	1.0 – 1.5	–

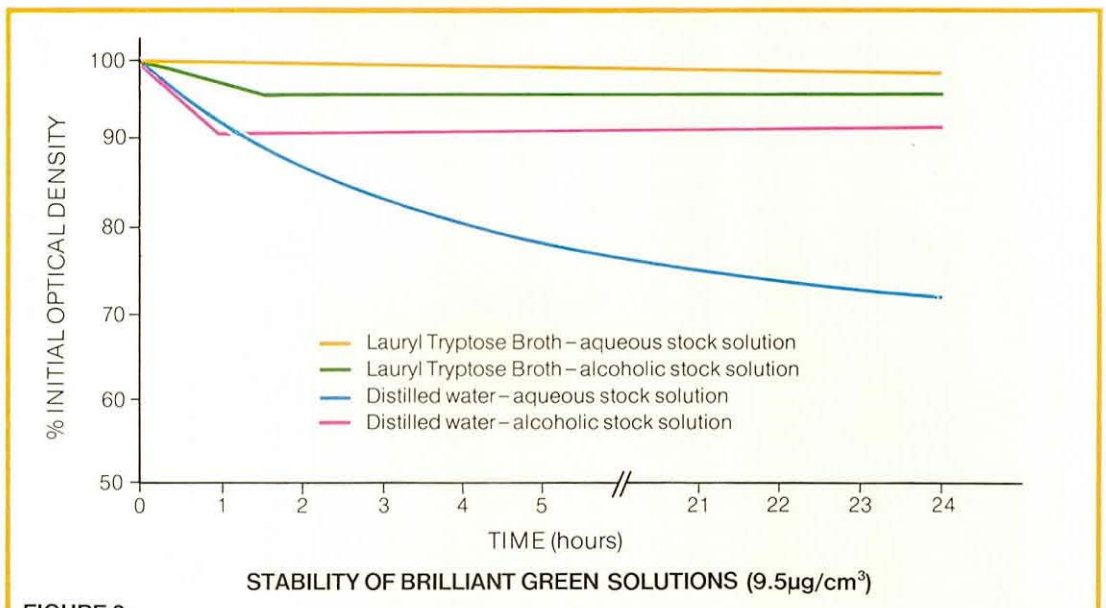
++++ = spreading all over the plate
++ = spreading over a part of the plate
\+ = spreading only around the colonies (c. 1 cm)
– = no spreading

TABLE I



STABILITY OF BRILLIANT GREEN SOLUTIONS (3µg/cm³)

FIGURE 1



STABILITY OF BRILLIANT GREEN SOLUTIONS (9.5µg/cm³)

FIGURE 2

References

1. Edel W. & Kampelmacher E.H. (1968) *Bull. Wld. Hlth. Org.* **39** 487.
2. Edel W. & Kampelmacher E.H. (1969) *Bull. Wld. Hlth. Org.* **41** 297.
3. Edel W. & Kampelmacher E.H. (1973) *Bull. Wld. Hlth. Org.* **48** 167.
4. Edel W. (1974) *Thesis for Doctorate.* Rijks universiteit Utrecht, Holland.

A BLOOD CULTURE REVIEW

The clinical importance of bacteraemia makes the earliest possible isolation, identification and antibiotic susceptibility testing of positive blood cultures a priority for any hospital microbiology laboratory[1].

In the absence of a clear history, with symptoms and signs pointing to a specific infecting organism, the blood culture system should be capable of *isolating a range of organisms* from obligate aerobes (Candida spp and Pseudomonas spp) through capnoeic organisms (micro-aerophiles or CO_2-dependent) to obligate anaerobes (both Gram negative and Gram positive). Almost all known pathogenic organisms have at some time been isolated from blood cultures.

The demand for blood culture investigations has increased[2] and the number of positive findings has increased even more[3]. The pattern of organisms isolated has also changed over the years. The predominantly isolated Gram positive organisms being displaced by Gram negative infections[4,5], and more recently an increasing number of anaerobic Gram negative organisms[6,7] and fungi[8].

The reasons for the increase in bacteraemia and the greater variety of organisms isolated are various and include the following.

i The selective effect of indiscriminate antibiotic treatment.
ii The widespread use of immuno-suppressive drugs for organ transplants, cancer treatment.
iii Increased use of implanted prostheses, pace-makers, plastic valves, even the much abused intravenous drip.
iv More adventurous surgery, especially on the aged whose resistance to infection is poor.

Stokes[9] describes five kinds of clinical condition where blood culture is demanded.

a Acute spontaneous generalized infection which may proceed to endocarditis, e.g. staphylococcal septicaemia.
b Specific fevers, often insidious in onset, e.g. typhoid fever, brucellosis.
c Subacute bacterial endocarditis.
d Septicaemia, including Gram-negative shock or pyaemia, secondary to some other pathological condition or to surgical manipulation.
e Invasion by low-grade pathogens in abnormally susceptible patients.

The opportunity for diagnosing bacteraemia may be fleeting as the urgency to commence antimicrobial treatment increases. Once such treatment has started there is little chance of obtaining viable cultures. Therefore, the culture system should cope with all eventualities as there is little opportunity for going back.

Blood Samples

The technique of drawing blood aseptically from the patient has been fully described[9].

The number of samples taken has been discussed by Crowley[10]. The use of more than one bottle of medium to identify possible contaminant organisms arising during venepuncture or subculture is a wise precaution.

The volume of blood used is compromise between two opposing factors.

i The greater the volume of *blood, the larger the* probability of isolating a low-level infection.
ii The blood sample has bactericidal properties and must be diluted in broth. Ratios of blood to medium of 1 : 10 to 1 : 20 have been recommended[11]. Dilutions of blood up to 1 : 60 have been recommended in the absence of any neutralizing substance[12]. Such dilutions call for large volumes of broth if more than 3 – 5 ml of blood is used.

Culture Media Used

The list of media used in blood culture techniques is long, with many formula variations. They vary from simple glucose-broth to more complex media containing growth factors and reducing *agents for anaerobic organisms.* Organisms such as *Brucella species* often have specific media designed for them and blood is added to the special bottles if the clinical indications suggest the disease. However, it is better to have media capable of growing most of the known pathogens as the clinical symptoms of diseases such as brucellosis are often misleading. Complex broths such as Brain-Heart Infusion are often used for this purpose.

The addition of a small quantity of agar (0.1 % w/v) will produce a stable range of eH values in the cooled broth, which will support the growth of organisms at their optimum eH value.

The use of bile salt broth for the isolation of salmonella from the blood is still in use, especially when typhoid fever is suspected[13]

The lethal effect of glucose in blood culture media, caused by acid pH shift, has been reported[14]. It was also reported by other workers when isolating *Bacteroides species*[15,16]. Hypertonic, as well as isotonic media, are used in blood cultures. The addition of 10% – 30% w/v sucrose to conventional media is used to raise the osmotic value. Blood culture studies comparing hypertonic and isotonic media have indicated that the use of hypertonic medium permits higher isolation rates of micro-organisms and often permits more rapid detection[17,18]. The results suggest that antibiotic – cell – wall – damaged organisms can repair the damage in hypertonic media but lyse in isotonic media. The relevance of this hypothesis to the *in vivo* isotonic situation is not yet established, but the possibility that blood samples taken from patients may contain antibiotics still acting on the organisms in the sample suggests that hypertonic media could play a useful role.

When shaken isotonic media were compared with hypertonic media there were no advantages when isolating aerobic organisms[19], but the authors concluded that optimal recovery of bacteria from blood is accomplished with an aerobic isotonic medium that is agitated and an anaerobic hypertonic medium that is incubated under stationary conditions.

Neutralization of Blood Bactericidal Activity

To overcome the bactericidal activity of blood a considerable dilution with broth is essential, as reported above. The discovery that the synthetic anticoagulant, sodium polyanethole sulphonate (S.P.S. or "Liquoid") neutralized the complement-mediated bactericidal activity in human blood samples was a great step forward[20]. It can also inhibit complement-dependent phagocytosis[21] as well as the classical and alternative pathways of human complement activation[22]. However, the inhibition of S.P.S. (Liquoid) on the growth of anaerobic streptococci[23,24] and neisseria[25] meant that it had to be used with caution. The concentration of S.P.S. (Liquoid) used in blood culture systems is 0.025 – 0.05%[22]. Another synthetic anticoagulant, sodium amylosulphate (SAS) has been introduced for use in blood cultures[26]. It is claimed that it has no deleterious effects against peptostreptococci[27]. A comparative trial of S.P.S. and S.A.S. showed no significant difference between them. However, it was noted that certain Gram-negative bacilli were less frequently recovered from blood culture media containing S.A.S. at concentrations of 0.025 to 0.05%. The failure of S.A.S. to completely neutralize the bactericidal activity of blood was confirmed[28] and a recommendation was made that blood samples should be diluted at least five-fold ($\geqslant$ 20% v/v) with broth to ensure inactivation of complement.

Gaseous Environments

Most blood cultures are carried out using the slightly reduced pH present in culture media after heat sterilization.

The addition of 5 – 10% CO_2 is an advantage when isolating *Brucella species*[9] and *Actinobacillus species*[29].

Pre-reduced media, gas flushed with hydrogen-carbon dioxide mixture ensure that strict anaerobes will be recovered.

It is essential that blood culture bottles containing special gas atmospheres are adequately sealed and preferably sampled by syringe. Once the cap has been removed the gaseous atmosphere is lost or irreversibly changed.

Glass containers are necessary as plastic bottles are permeable to gases, particularly carbon dioxide.

Methods of Use

The addition of blood to sterile broth and subsequent incubation still leaves the problems of detection of microbial growth and isolation of the organism.

The inoculated bottle is incubated at 35–37°C and visually inspected for 7 to 21 days. Blood from patients suspected of brucellosis may have to be kept up to eight weeks[9]. Routine or blind subculture is carried out every 2 or 3 days, with the attendant risk of contamination[30]. The difficulty of recognizing bacterial growth by visual examination is well known. In an attempt to improve the process, a *method of blood culture* examination using blue tetrazolium dye has been proposed[31].

A di-phasic blood culture system was devised for the isolation of brucellae, in which an agar slope of nutrient medium is washed with the blood-broth. Sampling into the bottle is made only when visible colonies are seen[32,9]. This technique is far less laborious and costly than numerous blind samples. To improve detection of bacterial colonies on the agar slope, triphenyl tetrazolium (2–3–5 TTC) indicator has been added to the agar slope[33].

Blood culture systems where the organisms in the blood are separated by membrane filtration have been described[34,35]. The technique involves lysing the blood and aseptically filtering the lysate. The difficulties associated with the method are not small.

Impedance method

A technique which has attracted a lot of attention is the bacterial impedance method. A monitoring apparatus permits detection of alteration of impedance ratios in culture media due to microbial metabolism and growth[36]. This is still a new technique and reports of results are very scanty. A full report of a "mock" blood culture system suggested that aerobic organisms could be detected in 6 to 18 hours of incubation. Anaerobic organisms varied from 3 hours for *Clostridium perfringens* to 48 hours for *Bacteroides melaninogenicus*[37]. A similar technique involves adding blood to broth prepared with a ^{14}C radioisotope of glucose and other substrates. An automated radioisotopic detection system[38] measures $^{14}CO_2$ formation by organisms growing in the medium[39]. There have been conflicting reports in the evaluation of the system and a review of the reports and variable factors has been published[40]. A publication, drawing attention to the need to make a terminal subculture before discarding radiometrically-negative bottles after 7 days incubation, suggests that the technique is not yet reliable[41].

Group D streptococci accounted for most of the organisms undetected by the radiometric procedure.

Conclusions

In spite of the great importance of accurate detection of bacteraemia the current techniques are still cumbersome, expensive in terms of time and labour, and not reliable. Whether the pathway forwards points towards greater automated equipment has yet to be determined.

The diphasic method or simple poured plate prepared at the bedside may still give the fastest results, as the more sophisticated detection systems require further subculture to isolate, identify and determine the antibiotic sensitivity of the organism.

REFERENCES

1 Sonnenwirth A.C., 1973, *Bacteraemia, Clinical and Laboratory Aspects.* Springfield, Ill., Chas. C. Thomas.
2 Brown P.P. & Blair J.N., 1977, *Lancet,* **i,** 808.
3 Williams G.T., Houang E., Shaw E. & Tabaqchali S., 1976, *Lancet,* **ii,** 1291.
4 Finland M., Jones W.F. Jr. & Barnes M.W., 1959, *J.A.M.A.* **170,** 188.
5 McGowan J.E., Barnes M.W. & Finland M., 1975, *J. Infect. Dis.*, **132,** 316.
6 Wilson W.R., Martin W.J., Wilkowske C.J. & Washington J.A., 1972, *Mayo Clin. Proc.*, **47,** 639.
7 Chow A.W. & Guze L.B., 1974, *Medicine Baltimore,* **53,** 93.
8 Gantz N.M., Swain J.L., Medeiros A.A. & O'Brien T.F., 1974, *Lancet,* **ii,** 1174.
9 Assoc. Clin. Pathologists, Broadsheet 81, April 1974, Blood Culture Technique.
10 Crowley N., 1970, *J. Clin. Path.*, **23,** 166.
11 Bartlett R.C., Ellner P.D. & Washington J.A., 1974, Cumitech. 1, Amer. Soc. Microbiol., Washington D.C.
12 Roome A.P.C.H. & Tozer R.A., 1968, *J. Clin. Path.,* **21,** 719.
13 Foster W.D., 1975, *Lancet,* **ii,** 80.
14 Waterworth P.M., 1972, *J. Clin. Path.*, **25,** 227.
15 Shanson D.C., 1974, *J. Clin. Path.*, **27,** 273.
16 Forgan-Smith W.R. & Darrell J.H., 1974, *J. Clin. Path.*, **27,** 280.
17 Rosner R., 1975, *Amer. J. Clin. Path.*, **63,** 149.
18 Gross P.A., Fryda R. & Reilly K., 1977, *J. Clin. Microbiol.*, **6,** 362.
19 Ellner P.D., Kiehn T.E., Beebe J.L. & McCarthy L.R., 1976, *J. Clin. Microbiol.*, **4,** 216.
20 von Haebler T. & Miles A.A., 1938, *J. Path. Bact.*, **46,** 245.
21 Belding M.E. & Klebanoff S.J., 1972, *Appl. Microbiol.*, **24,** 691.
22 Traub W.H. & Kleber I., 1977, *J. Clin. Microbiol.*, **5,** 278.
23 Graves M.H., Morello J.A. & Kocka F.E., 1974, *Appl. Microbiol.*, **27,** 1131.
24 Wilkins T.D. & West S.E.H., 1976, *J. Clin Microbiol.*, **3,** 393.
25 Eng. J. & Ireland H., 1975, *J. Clin. Microbiol.*, **1,** 444.
26 Kocka F.E., Magoc T. & Searcy R.L., 1972, *Proc. Soc. Exp. Biol. Med.*, **140,** 1231.
27 Kocka F.E., Arthur E.J. & Searcy R.L., 1974, *Amer. J. Clin. Pathol.*, **61,** 25.
28 Traub W.H., 1977, *J. Clin. Microbiol.*, **6,** 128.
29 Macklon A.F., Ingham H.R., Selkon J.B. & Grinley Evans J., 1975, *B.M.J.*, **1,** 609.
30 Blazavic D.J., Stemper J.E. & Matsen J.M., 1974, Appl. *Microbiol.*, **27,** 537.
31 Shih C.N. & Balish E., 1977, *J. Clin. Microbiol.*, **6,** 249.
32 Castaneda M.R., 1947, *Proc. Soc. Exp. Biol and Med.*, **64,** 114.
33 Coetzee E.F.C. & Johnson R.S.A., 1974, *Med. Lab. Techn.* **31,** 299.
34 Sullivan N.M., Sutter V.L. & Finegold S.M., 1975, *J. Clin. Microbiol.*, **1,** 37.
35 Ziert C.H., Kagan R.L. & MacLowry J.D., 1977, *J. Clin. Microbiol.*, **5,** 46.
36 Bactometer 32, Bactomatic Inc. Palo Alto Calif.
37 Specter S., Throm R., Straus R. & Friedman H., 1977, *J. Clin. Microbiol.*, **6,** 489.
38 Bactec, Johnson Laboratories, Cockeysville, Md.
39 Deland F.H. & Wagner H.N., 1975, *J. Lab. Clin. Med.*, 1970–75, 529.
40 Brooks K. & Sodeman T., 1974, *Amer. J. Clin. Path.*, **61,** 859.
41 Strauss R.R., Throm R. & Friedman H., 1977, *J. Clin. Microbiol.*, **5,** 145.

Culture is published as a service to microbiology by Oxoid Limited, Wade Road, Basingstoke, Hampshire RG24 0PW, England.