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Pig and Goat Blood as Substitutes for Sheep Blood in Blood-Supplemented Agar Media

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In many developing countries sheep and horse blood, the recommended blood supplements in bacteriological media, are not readily available, whereas pig and goat blood are. Therefore, this study examined the use of pig and goat blood as potential substitutes for sheep blood in blood-supplemented bacteriologic media commonly used in clinical microbiology laboratories. In general, the growth characteristics and colony morphologies of a wide range of aerobic and anaerobic bacteria and *Candida albicans* were similar on media containing pig, goat, and sheep blood, although differences were found. *Enterococcus* sp. uniformly produced alpha-hemolysis when incubated in CO_2, but in anaerobic conditions the hemolysis varied. In contrast, beta-hemolytic streptococci produced identical hemolytic reactions on all three media. Synergistic hemolysis was not observed on pig blood agar in the CAMP test nor on goat blood agar in the reverse CAMP test. The preparation of chocolate agar (heated) with pig blood required heating to a higher temperature than with sheep or goat blood to yield suitable growth of *Haemophilus* species. In general, we conclude that pig and goat blood are suitable alternatives to sheep blood for use in bacteriological media in settings where sheep and horse blood are not readily available.

A variety of animal bloods and banked human blood (BHB) are used to enrich microbiological culture media and to highlight growth characteristics such as hemolysis. In most clinical microbiology laboratories, the selection of colonies from primary cultures for further workup as putative beta-hemolytic streptococci (BHS) is made on the basis of the hemolytic reaction on blood agar (BA) as well as the colonial morphology. On BA, the hemolysis produced by streptococci and enterococci varies depending on the blood type contained in the media. Organisms such as *Haemophilus haemolyticus* also produce hemolytic colonies, which, on the basis of morphology, can be confused with those of BHS (3, 8). Thus, the selection of inappropriate colonies could result in an increase in technical cost to process specimens and also add to the turnaround time. In North American clinical laboratories, defibrinated sheep blood (SB) is accepted as the most efficient blood supplement for routine work because the hemolytic reactions of BHS on BA prepared with SB are deemed "true," and it is used as the standard for defining hemolytic reactions of streptococci (9). Horse blood (HB) is recommended as the second choice and is widely used in European countries (3, 9). In many developing countries, SB and HB are not readily available, probably because of local animal husbandry practices, making these animal bloods relatively expensive in the setting of meager resources. Another blood extensively used in these countries to prepare BA is BHB, which is generally available cost free to laboratories when it is approaching the end of its shelf life and is no longer used as a transfusion product. The anticoagulant used in BHB contains citrate and dextrose, and the adverse effects of these additives in bacteriological media have been well documented (3, 9). In many developing countries there is a high prevalence of blood-borne pathogens, such as hepatitis B virus, human immunodeficiency virus, and hepatitis C virus, and because of the lack of adequate safety precautions in the laboratory, the use of BHB potentially poses a significant risk to the laboratory staff.

In many of these countries, goats, pigs, or both are more readily available than sheep or horses and constitute an alternative source of blood for use in bacteriological media. However, published data on the growth characteristics of pathogenic bacteria on media supplemented with pig blood (PB) and goat blood (GB) are limited (4, 5). This study was undertaken to define the growth characteristics of pathogenic bacteria cultured on PB- and GB-based media compared to those on the SB-based media routinely used in clinical laboratories with a view to offering an alternative in settings where SB and HB are not readily available.

MATERIALS AND METHODS

Blood. Defibrinated blood was collected aseptically by jugular vein puncture from antibiotic-free pigs and goats housed in the animal farm of the University of Calgary, Calgary, Alberta, Canada. Blood was collected from five animals of each species. Defibrinated SB was purchased from Western Biological Products Ltd., Calgary, Alberta, Canada. The blood was stored in plastic containers at 4°C and used within 2 to 7 days of collection.

Media. The media evaluated, which are used in routine microbiology and are enriched with blood, are listed in Table 1. Each medium was prepared with SB, GB, or PB under identical conditions and with identical ingredients according to the instructions in reference 9 or the manufacturers' recommendations.

Chocolatized (heated) blood agar (CHA) was prepared with SB, PB, and GB and Columbia agar base (Oxoid, Ltd., Basingstoke Hampshire, England) as previously described, except the time and temperature of the heating step were varied (9). SB, GB, or PB was added to the prepared molten blood agar base, the batch was divided into nine aliquots, and each aliquot was held at 70, 80, or 100°C for 5, 10, or 15 min. These aliquots were allowed to cool to 50°C, and the plates were poured. Thus, there were nine combinations of time and temperature for each blood type. This step was undertaken in order to determine the optimum temperature and time combinations required to eliminate the thermolabile growth inhibitors for pyridine nucleotide (NAD or NADP)-requiring *Haemophilus* which are naturally present in animal blood (1).

Organisms. Five different clinical isolates of each of the following organisms and the American Type Culture Collection strains indicated by the numbers in

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TABLE 1. Blood-supplemented media tested, medium base, and concentrations of SB, GB, and PB

Medium evaluated	Base	Blood (% final vol)
BA	Columbia[a]	5
Fastidious anaerobic agar	Fastidious anaerobic agar[b]	5
Phenyl ethyl alcohol agar (PEA)	PEA base[c]	5
CHA	Columbia[a]	5
Regan-Lowe medium	Charcoal agar[a]	10
Skirrow's medium	Brucella agar[d] with supplement (AVPT[e])	10

[a] Oxoid Ltd., Basingstoke, Hampshire, England.
[b] LabM, Toply House, Wash Lane, Bury, England.
[c] Difco Laboratories.
[d] BBL Microbiology Systems.
[e] AVTP, amphotericin, vancomycin, polymyxin B, and trimethoprim (Dylan Laboratory Products, Calgary, Alberta, Canada).

parentheses were tested: group A streptococcus (19615), group B streptococcus (GBS) (13813), group C streptococcus (12388), group F streptococcus, *Streptococcus sanguis*, *Streptococcus pneumoniae* (49619), *Enterococcus faecalis* (29212), *Enterococcus faecium*, *Staphylococcus aureus* (25923), *Staphylococcus epidermidis* (12228), *Corynebacterium diphtheriae* (8028), *Neisseria meningitidis* (13007), *Neisseria gonorrhoeae* (49226), *Haemophilus influenzae* (10211), *Haemophilus parainfluenzae* (7901), *H. haemolyticus* (33390), *Haemophilus aphrophilus* (33389), *Haemophilus ducreyi* (33940), *Escherichia coli* (35218), *Pseudomonas aeruginosa* (27853), *Candida albicans* (14053), *Clostridium difficile*, *Clostridium sporogenes*, *Clostridium perfringens* (13124), and *Bacteroides fragilis* (2528) *Bordetella pertussis* (9340), *Bordetella parapertussis* (15311), and *Campylobacter jejuni* (29428).

The clinical strains were fresh isolates from patient samples or were recovered from lyophilized cultures or isolates preserved at −70°C in brain heart infusion-glycerol broth.

Inoculation of media. A suspension of each isolate in 0.9% saline was prepared from an overnight culture and adjusted to 0.5 McFarland standard density. Ten microliters was inoculated onto separate media containing SB, GB, or PB and streaked for single-colony isolation. A single streak of *S. aureus* was made on blood agar plates inoculated for growth of *Haemophilus* spp. The plates were incubated at 35°C aerobically, in 5% CO_2, or anaerobically as appropriate for the optimum growth of each organism (Table 2) and read at 48 h, except for Regan-Lowe medium, which was incubated for up to 7 days. Plates of Skirrow's medium for the culture of *C. jejuni* were incubated at 42°C under microaerophilic conditions.

CAMP and reverse CAMP tests. The CAMP test for synergistic hemolysis between GBS and *S. aureus* for the presumptive identification of GBS (2) and the reverse CAMP test for synergistic hemolysis between GBS and *C. perfringens* (7) for the presumptive identification of the latter organism were evaluated on SB, GB, and PB. In the CAMP test, a single streak of a beta-hemolytic *S. aureus* was made across the middle of the blood agar plate under evaluation and the GBS was cross-streaked at right angles to within 1 to 2 mm of the first streak. Similarly, for the reverse CAMP test a single central streak of *C. perfringens* and a cross-streak of GBS were made. The plates were incubated in CO_2 for the CAMP test and anaerobically for the reverse CAMP test at 35°C for 24 h. The presence of an arrowhead-shaped area of synergistic hemolysis in the intersecting area indicated a positive result.

RESULTS

The growth characteristics and colony morphologies of organisms were assessed according to medium type and incubation conditions by two of us for comparison. The organisms which displayed similar colony morphologies and growth characteristics on media containing SB, PB, and GB are reported in Table 2.

All 11 strains of *Enterococcus* spp. produced alpha-hemolysis on SBA, GBA, and PBA when incubated in CO_2. Under anaerobic growth conditions, one strain of *E. faecium* was alpha-hemolytic on SBA but nonhemolytic on GBA and PBA. *E. faecalis* ATCC 29212 was beta-hemolytic, five *E. faecalis* strains and two *E. faecium* strains were nonhemolytic, and two strains of *E. faecium* were alpha-hemolytic on SBA, GBA, and PBA.

Strains of BHS groups A, B, C, F, and G grew equally well and gave identical hemolytic reactions on SBA, PBA, and GBA, although individual isolates displayed variation in the size of the hemolytic zone and/or sharpness of the zone edge on the different BAs. Colonies of *S. pneumoniae* were dome shaped and more mucoid on PBA compared to colonies on SBA and GBA, which were flat with a central depression.

Table 3 illustrates the differences observed on the various media. The maximum growth of *H. influenzae* was observed on SBCHA prepared at 80°C for 5 min, with an average colony size of 5 mm; on GBCHA prepared at 80°C for 15 min, with an average colony size of 4 mm; and on PBCHA prepared at 100°C for 15 min, with a colony size of 3 mm. For *H. parainfluenzae*, maximum growth was observed on SBCHA and GBCHA prepared at 100°C for 15 min, with colony sizes of 3 and 2 mm, respectively, compared to 1.5 and 0.5 mm on SBCHA and GBCHA, respectively, prepared at 80°C for 15 min, whereas on PBCHA prepared at 100°C for 15 min, a colony size of 0.5 mm was observed. On media produced at 70°C for 15 min, *H. influenzae* produced colonies 0.5 mm in size on SBCHA and GBCHA and only a hazy growth on PBCHA, whereas *H. parainfluenzae* failed to grow.

TABLE 2. Organisms with similar colonial morphologies and growth characteristics on media containing SB, PB, and GB

Medium	Incubation conditions[a]	Organism(s)
BA	Anaerobic	BHS groups A, B, C, F, and G; *Enterococcus* spp., *S. pneumoniae*
BA	CO_2	BHS groups A, B, C, F, and G; *Enterococcus* spp., *S. pneumoniae*; *S. sanguis*; *S. aureus*; *S. epidermidis*; *E. coli*; *P. aeruginosa*; *H. aphrophilus*; *C. diphtheriae*; *C. albicans*; *H. influenzae*; *H. parainfluenzae*; *H. haemolyticus* (with *Staphylococcus* streak)
Fastidious anaerobic agar	Anaerobic	*C. difficile*, *C. sporogenes*, *B. fragilis*
Phenyl ethyl alcohol agar	Aerobic	
Inhibition		*E. coli* and *P. aeruginosa*
Growth		*Streptococcus* group A, *S. aureus*, and *E. faecalis*
CHA	CO_2	*N. meningitidis* and *N. gonorrhoeae*
Regan-Lowe medium	Aerobic	*B. pertussis* and *B. parapertussis*
Skirrow's medium	Microaerophilic	*C. jejuni*

[a] Temperature of incubation was 35°C, except for Skirrow's medium, which was 42°C.

H. haemolyticus failed to grow on PBA and GBA except as satellite growth around the *Staphylococcus* streak, and on PBCHA and GBCHA the growth characteristics were similar to those of *H. influenzae*.

The results of the CAMP and reverse CAMP tests are presented in Table 3. Notably, synergistic hemolysis was not observed in the CAMP test on PBA or in the reverse CAMP test on GBA.

DISCUSSION

Published data on the growth characteristics of pathogenic bacteria on media supplemented with GB are limited to two studies (4, 5), and as far as we could ascertain, there are no studies of PB. Feinsod and Kim (4) examined GB with a limited range of bacteria and yeasts and did not examine specialized media supplemented with blood required for the growth of organisms such as *Bordetella* spp., *Campylobacter* spp., and anaerobic bacteria or procedures such as the CAMP test, which require blood-based media. Furthermore, the base media which they used to prepare BA and CHA with SB were different from those used to prepare BA and CHA with GB; therefore, the effect of blood per se could not be assessed. Gratten et al. (5) evaluated selective and nonselective BA and CHA prepared with GB and compared them to similar media prepared with HB, but their study was limited to growth of *H. influenzae* and *S. pneumoniae*.

In this study we compared most media commonly used in a clinical laboratory for which blood supplement is required prepared with PB or GB, using identically prepared SB as a reference. We are confident that PB and GB can be substituted for almost all media for which SB is used within certain limitations discussed below.

Our observations regarding the hemolytic reactions of the BHS groups A, B, C, F, and G are similar to those reported by Updyke, who also noted that variations in hemolytic activity were restricted to *Enterococcus* spp. (8). In her report, 88% of group D strains were alpha-hemolytic on SBA but beta-hemolytic on rabbit, horse, and human blood agars. Our observations differed in that the hemolytic reactions of all enterococci strains that we tested were alpha-hemolytic on SBA, PBA, and GBA when incubated in CO_2 but under anaerobic incubation were either alpha- or nonhemolytic, except for *E. faecalis* ATCC 29212, which was beta-hemolytic. Although there was interstrain variability in the type of hemolysis, each individual strain gave identical hemolysis on all three blood types. Therefore the majority of *Enterococcus* isolates would not be selected for further testing as potential BHS on the basis of their hemolysis on GBA and PBA, although the occasional isolate, as exemplified by *E. faecalis* ATCC 29212, may be confused. The literature shows that animal blood naturally contains the thermolabile growth inhibitors for pyridine nucleotide (NAD or NADP)-requiring *Haemophilus* spp. and that the heat susceptibilities of these vary with the animal species (1, 6). Furthermore, there is a critical time and temperature threshold that must be achieved during medium production to eliminate them (1, 6). The quality of CHA prepared with heated animal blood can therefore vary depending on the animal blood can therefore vary depending on the animal blood and the time and temperature used in its preparation. In developed countries, chocolate agar prepared with a defined base, such as Mueller-Hinton agar or GC medium base, and supplemented with a mixture of hemoglobin and a synthetic cocktail of chemically defined supplements, such as Isovitelex (BBL, Cockeysville, Md.) has largely replaced CHA (heated). In developing countries these supplements are often cost prohibitive, and

TABLE 3. Organisms showing variations in colonial morphology and growth characteristics on media containing SB, PB, and GB

Organism or test	Medium/incubation conditions	Variation in test or growth characteristics		
		SB	GB	PB
H. influenzae	CHA[a]/CO_2	5 mm	4 mm	3 mm
H. parainfluenzae		3 mm	2 mm	0.5 mm
H. haemolyticus		5 mm	4 mm	3 mm
CAMP test	BA/CO_2	Arrowhead hemolysis	Smaller arrowhead hemolysis	No synergistic hemolysis
Reverse CAMP test	BA/anaerobic	Arrowhead hemolysis	Small, oval hemolysis; no arrowhead	Arrowhead narrower than on SB
E. faecium (one isolate)	BA/anaerobic	Alpha-hemolytic	Nonhemolytic	Nonhemolytic
C. perfringens	Fastidious anaerobic agar/anaerobic	Double zone of hemolysis (second zone, 6 mm)	No double zone	Double zone of hemolysis (second zone, 3 mm)

[a] SB, PB, GB media heated to 100°C for 15 min.

CHA (heated), which is much cheaper and simpler to prepare, still has utility for the isolation of fastidious organisms such as *Neisseria* spp. and *Haemophilus* spp. However, its preparation needs to be carefully controlled to ensure adequate removal of these inhibitors. We found the time-temperature combination of 80°C for 15 min, as recommended by Vera and Powers (9) for SBCHA, to be adequate for both SBCHA and GBCHA but not for PBCHA, which required a higher temperature of 100°C for 15 min for better growth. However, we did not evaluate longer times at this temperature for this organism. Our finding for the optimum time-temperature combination for GB is at variance with that of Gratten et al. (5), who recommended 100°C for 15 min. However, we support their recommendation, since heating the medium to this temperature removes inhibitors of *H. influenzae* and *H. parainfluenzae* from all blood types which we tested without adversely affecting the medium for the isolation of *Neisseria* spp. Thermolabile inhibitors of *Haemophilus* spp. are known to be qualitatively variable in blood of different animals, and both SB and GB are known to possess high NADase activity compared to HB and rabbit blood (1). Our findings suggest that NADase activity in PB is likely to be even greater. We recommend greater care in the preparation of heated PBCHA and GBCHA and subsequent quality control to ensure that an adequate time-temperature combination has been achieved.

A drawback of PB is that it cannot be used for the presumptive identification of GBS with the CAMP test, which would be useful in developing countries, where there is often a limited availability of antisera for serogrouping streptococci. Similarly, the reverse CAMP test, which is a simple test for the presumptive identification of *C. perfringens*, works satisfactorily on PBA. On GBA, the lack of synergistic hemolysis in the form of an arrowhead makes this reaction unreliable, and the results need to be interpreted with caution. This phenomenon may be related to the observation that the double zone of hemolysis produced by *C. perfringens* on SBA and PBA was absent on GBA.

A point that we noted in the phlebotomy of the animals is that it is easier to obtain blood from goats than from pigs. Therefore, where access to both animals is equal, GB would be the preferred choice.

In conclusion, PB and GB can almost always be substituted for SB for bacterial isolation and in identification steps for organisms commonly encountered in a clinical laboratory.

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