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Effects of Media, Atmosphere, and Incubation Time on Colonial Morphology of *Arcanobacterium haemolyticum*

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Arcanobacterium haemolyticum **causes pharyngitis as well as skin and other wound infections. Although it is a beta-hemolytic organism, the hemolysis is less well defined than that of beta-hemolytic streptococci and may be overlooked in cultures with heavy growth of commensal throat flora. To determine whether routine throat culture conditions are sufficient to produce recognizable colonies of *A. haemolyticum*, the morphology of six distinct strains was studied after various combinations of incubation time, medium, and atmosphere. The agar media, containing 5% sheep blood, were Trypticase soy agar, Columbia agar, and heart infusion agar. Cultures were incubated in ambient air, 6 to 8% CO_2, or an anaerobic atmosphere. Cultures were compared after 24, 48, and 72 h of incubation for colony size, clarity and size of hemolytic zone, and macroscopic evidence of agar pitting. A minimum of 48 h was needed for expression of beta-hemolysis and pitting. Trypticase soy agar was the superior medium and CO_2 was the superior atmosphere for beta-hemolysis. Agar pitting was not significantly affected by variations in medium or atmosphere. Strains differed in their expression of hemolysis and production of pits at 48 h. After 72 h of incubation, beta-hemolysis and pitting were visible in over 96% of culture observations.**

Arcanobacterium haemolyticum is a beta-hemolytic gram-positive rod that is a well-described etiologic agent of pharyngitis in adolescents and young adults (1, 3, 8, 9, 13, 14, 16, 20–22, 27–29, 32, 33) as well as a cause of skin (21, 23, 29, 32) and other wound infections (4, 6, 11, 29) in all age groups. Because symptoms of *A. haemolyticum* pharyngitis may be confused with those of group A streptococcal scarlet fever, diphtheria, drug allergies, and even toxic shock syndrome (1, 9, 13, 14, 16, 20, 22, 28), *A. haemolyticum* continues to be an organism of clinical interest. Although it is estimated that the frequency of pharyngitis caused by *A. haemolyticum* infection is only about 5 to 13% of that caused by group A streptococci (1, 3, 22), this may be an underestimate.

A. haemolyticum grows more slowly and exhibits less well defined hemolysis than beta-hemolytic streptococci and therefore may be easily masked by commensal throat flora. After 48 h of incubation on blood agar, *A. haemolyticum* characteristically produces colonies of approximately 0.5 mm with a narrow zone of beta-hemolysis. When colonies are scraped away from the agar, a tiny dark pit is visible. Recent studies have used selective media to improve the recovery of *A. haemolyticum* from throat cultures (2, 30). The purpose of the present study was to determine which of the routine throat culture methods produce the most recognizable colonies of *A. haemolyticum*. We examined media, atmospheres, and incubation times that are commonly recommended for throat cultures (7, 15, 18, 19, 24, 25, 31) to determine the best combinations for recognition of the typical *A. haemolyticum* morphology.

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MATERIALS AND METHODS

Test organisms. Strains included the type strain, ATCC 9345, and five clinical isolates selected on the basis of having different *Eco*RI and *Bam*HI restriction fragment patterns of chromosomal DNA in an unpublished analysis of 34 clinical isolates of *A. haemolyticum*. All isolates were previously identified as *A. haemolyticum* on the basis of conventional biochemical tests (negative for catalase, nitrate, urea, gelatin hydrolysis, motility, esculin hydrolysis, mannitol and xylose fermentation; positive for glucose and maltose fermentation) and the Rapid CORYNE strip (Bio-Mérieux, Hazelton, Mo.). Of the five clinical isolates, two were associated with pharyngitis, one was isolated from the throat of an asymptomatic carrier, and one was isolated from a skin lesion. There was no patient history for the fifth clinical isolate. Both reference and clinical isolates had been previously stocked at −70°C. Prior to testing, all strains were subcultured two to three times on heart infusion agar with 5% sheep blood (HIA) and incubated in a CO_2-enriched aerobic atmosphere at 35°C.

Media. Three types of media were studied. Trypticase soy agar with 5% sheep blood (TSA) and HIA were prepared by the University of Washington Medical Center Media Laboratory (Seattle, Wash.). Columbia agar with 5% sheep blood (CLA) was purchased from Prepared Media Laboratory (Tualitin, Oreg.).

Incubation conditions. Each organism was inoculated onto duplicate plates of the three media and incubated at 35°C in three different atmospheres: ambient air, 6 to 8% CO_2, and an anaerobic atmosphere (GasPak generator kit; Becton Dickinson Microbiology Systems, Cockeysville, Md.).

Measuring results. One hundred and eight cultures were examined, representing duplicates of all combinations of organisms, media, and atmospheres. Each culture plate was examined in random order by three independent technologists at 24, 48, and 72 h, yielding a total of 972 observations. In addition, pitting under the colony was read with the

TABLE 1. Effect of incubation time on colonial morphology

Duration (h)	Avg colony size (mm)	Avg zone of hemolysis (mm)	Hemolysis visible (% of cultures)	Pit visible to eye (% of cultures)[a]
24	—[b]	—[c]	12.7	1.9
48	0.48	0.97	89.8	87.0
72	0.68	1.45	96.4	99.1

[a] Pit was visible in all cases with the use of a hand lens or dissecting microscope.
[b] Colonies were too small for accurate measurement in 321 of 324 culture readings.
[c] There was no zone of measurable hemolysis in 317 of 324 cases.

unaided eye, a hand lens, and a dissecting microscope. The diameter of a typical colony on each plate was measured with the unaided eye to the nearest 0.1 mm with calipers. The presence or absence of hemolysis was recorded. Hemolysis was further characterized as either an obvious clearing of the blood agar or a brightening of the medium around the colony when viewed with transmitted light. The diameter of the zone of hemolysis, or brightening, was measured, if possible, to the nearest 0.1 mm with calipers. When hemolysis or brightening occurred without a measurable zone, it was recorded as 0 mm in diameter. The presence or absence of a pit beneath the colony was also recorded.

Statistical analysis. The chi-square test was used for statistical analysis for both the presence of hemolysis and a visible pit. For the analysis of the effects of medium and atmosphere on quantitative parameters (sizes of colonies and zones of hemolysis), the initial data management was done with Microsoft EXCEL (version 3.0, for the Macintosh; Microsoft Corporation). Sorted data were imported to SYSTAT 5 (version 5.1, for the Macintosh; SYSTAT, Inc.) for statistical analysis. Numerical measurements were analyzed with analyses of variance (ANOVAs) in the multivariate procedure called General Linear Model (ANOVA tables). The differences between different categories of a factor were estimated and compared with the procedure called CONTRAST after every ANOVA (34).

RESULTS

The effect of incubation time on colonial morphology is shown in Table 1. After 24 h of incubation, 321 of 324 culture observations found colonies that were too small for accurate measurement. In addition, pitting and measurable hemolysis were observed in only six and seven readings, respectively. After incubation for 48 h, the mean colony diameter was 0.48 mm, and the mean zone of hemolysis was 0.97 mm, with no hemolysis detected in 33 culture readings. After 48 h, a pit was visible to the unaided eye in 282 of 324 culture readings. Pitting was observed in an additional 21 readings with the aid of a hand lens (total, 293). In all cases, pitting was observed with the dissecting microscope. After 72 h, the mean colony size was 0.68 mm, the mean zone of hemolysis was 1.45 mm, hemolysis was evident in over 96% of culture readings, and a pit was visible to the unaided eye in almost all cases.

There were some major differences between strains. The mean colony size of cultures at 48 h ranged from 0.32 mm (strain 4) to 0.57 mm (ATCC 9345). Strain 1 was less hemolytic than the others. Because strain 4 produced such small colonies at 48 h, only one-third of cultures had pits that were visible to the naked eye. After 48 h, the type strain produced the most recognizable morphology with the largest colonies (mean, 0.57 mm), the widest zones of hemolysis (mean, 1.1 mm), and consistently visible pits.

Because growth at 24 h is insufficient for recognition of *A. haemolyticum* and 72 h is unrealistic for routine throat cultures in most clinical laboratories, only the results at 48 h were subjected to statistical analysis. The effect of medium on colonial morphology at 48 h is shown in Table 2. The average colony size was significantly greater on TSA than on either CLA or HIA ($P < 0.001$). The average zone of hemolysis was also significantly larger on TSA ($P < 0.001$ versus CLA, $P < 0.05$ versus HIA). Not all visibly hemolytic colonies had a measurable zone; in some cases ($n = 75$), the clearing or brightening was due to hemolysis directly beneath the colony, without a measurable zone beyond the edge of the colony. Of the 33 completely nonhemolytic cultures, 10 were incubated on TSA, 9 were incubated on HIA, and 14 were incubated on CLA. Strain 1 accounted for 22 of the 33 nonhemolytic cultures. Although a pit could be detected in all cases with the use of a hand lens or dissecting microscope at 48 h, slightly more pits were visible to the unaided eye when colonies were grown on TSA (90.7%) than were visible on either CLA (88.9%) or HIA (81.5%). Strain 4 accounted for 36 of the 42 cultures in which a pit was not visible to the unaided eye.

The effect of atmosphere on colonial morphology at 48 h is shown in Table 3. The average colony size was smaller when cultures were incubated anaerobically; there was no significant difference in colony size between either aerobic or anaerobic incubation and CO_2 incubation. The mean zone of hemolysis was significantly larger with CO_2 incubation than in either aerobic ($P < 0.05$) or anaerobic ($P < 0.001$) incubation. Of the 33 nonhemolytic cases, 13 were incubated in air, 14 were incubated anaerobically, and only 6 were incubated in CO_2. Although a pit could be detected in all cases with the use of a hand lens or dissecting microscope at

TABLE 2. Effect of medium on colonial morphology at 48 h

Blood agar medium	Avg colony size (mm)	Avg zone of hemolysis (mm)[a]	Hemolysis visible (% of cultures)	Pit visible to eye (% of cultures)[b]
TSA	0.52	0.79	90.7	90.7
CLA	0.45	0.54	87.0	88.9
HIA	0.44	0.62	91.7	81.5
P value	<0.001, TSA vs CLA; <0.001, TSA vs HIA; NS, CLA vs HIA[c]	<0.001, TSA vs CLA; <0.05, TSA vs HIA; NS, CLA vs HIA	NS	NS

[a] Zones of hemolysis or brightening that were too narrow or indistinct to measure were recorded as zero.
[b] Pit was visible in all cases with the use of a hand lens or dissecting microscope.
[c] NS, not significant.

TABLE 3. Effect of atmosphere on colonial morphology at 48 h

Atmosphere	Avg colony size (mm)	Avg zone of hemolysis (mm)[a]	Hemolysis visible (% of culture)	Pit visible to eye (% of cultures)[b]
CO_2-enriched (CO_2)	0.47	0.77	94.4	91.7
Aerobic (Air)	0.48	0.61	88.0	83.3
Anaerobic (Ana)	0.45	0.57	87.0	86.1
P value	<0.05, Air vs Ana; NS, CO_2 vs Ana[c]; NS, CO_2 vs Air	<0.001, CO_2 vs Ana; <0.05, CO_2 vs Air; NS, Air vs Ana	NS	NS

[a] Zones of hemolysis or brightening that were too narrow or indistinct to measure were recorded as zero.
[b] Pit was visible in all cases with the use of a hand lens or dissecting microscope.
[c] NS, not significant.

48 h, slightly more pits could be seen with the unaided eye when cultures were incubated in CO_2 (91.7%) than with either anaerobic (86.1%) or aerobic (83.3%) incubation. Although both medium and atmosphere had significant effects on the diameter of hemolytic zones, there was no significant effect on the percentage of cultures with hemolysis.

DISCUSSION

This study compared growth conditions and media commonly used for recovery of group A beta-hemolytic streptococci (GABHS) from throat cultures and wounds to determine their effects on the colonial morphology of *A. haemolyticum*, a beta-hemolytic organism that can cause pharyngitis and wound infections. We found significant differences in colonial morphology of *A. haemolyticum* colonies with changes in incubation duration, atmosphere, and medium. However, it was reassuring to note that four of the six strains yielded the characteristic hemolytic pitting colonies of *A. haemolyticum* in 96.3% of the observations at 48 h for all combinations of medium and atmosphere.

Our unpublished DNA analysis of the type strain and 34 clinical isolates of *A. haemolyticum* revealed only seven different restriction fragment length polymorphisms in digests with *Bam*HI and *Eco*RI. For the present study, we selected one strain from each of six DNA types which represented 79% of all isolates available in our *A. haemolyticum* collection. Therefore, it seems likely that the relatively small number of strains in this study represent a good cross section of *A. haemolyticum* diversity in this region.

The most significant variable in the present study was incubation time, which is consistent with analogous studies for the recovery of GABHS from throat cultures. Kellogg recommended an incubation duration of 48 h for optimal recovery of GABHS (15). In all but one of the studies reviewed, incubation of cultures for 48 h rather than for 24 h significantly increased the recovery of GABHS regardless of incubation atmosphere or medium (15). Our study also suggests enhanced recognition of *A. haemolyticum* with increased duration of incubation. At 24 h, most colonies of *A. haemolyticum* are too small to measure and hemolysis is not produced. Therefore, routine throat cultures must be held longer than one day if recovery of *A. haemolyticum* is desired. At 72 h, all colonies are fairly large and most are obviously hemolytic; unfortunately, this is an unrealistic length of time to hold routine throat cultures in the clinical laboratory. Incubation for at least 48 h is therefore recommended for recovery of both *A. haemolyticum* and GABHS. While doing various projects with many cultures of *A. haemolyticum*, we have not noticed that hemolysis changes during the period between 40 and 48 h of incubation, but this has not been tested in a formal manner.

MacLean's discovery of *A. haemolyticum* was made from throat cultures on human blood agar, on which, at 24 h, colonies were 0.75 mm in diameter and partially beta-hemolytic (20). After 48 h of incubation, colonies were about 1.5 mm in diameter and zones of beta-hemolysis were 7 to 8 mm. Hermann found that rabbit and human blood agar yielded the same colonial morphology of *A. haemolyticum* but that sheep blood yielded much smaller colonies that were not hemolytic until 48 h of incubation (10). Most descriptions of *A. haemolyticum* colonies on sheep and horse blood agar indicate that hemolysis is not evident in cultures at 24 h (8, 10, 26, 29), but others have found beta-hemolytic colonies after 24 h on sheep blood (5) and horse blood (5, 12). These differences may be the result of different medium bases that were not described in most reports. We confirmed the superior performance of rabbit blood for providing good growth and obvious zones of hemolysis with all *A. haemolyticum* strains in this study after 24 h on all media in the CO_2 atmosphere. Rabbit blood agar was not included in the present report because this medium is not used for routine throat cultures in the vast majority of clinical laboratories, and it is known to be far superior to sheep blood agar for hemolysis by *A. haemolyticum* colonies.

After 48 h of incubation, both medium and atmosphere had measurable effects on the colonial morphology of *A. haemolyticum*. Both colony size and zone of hemolysis were significantly greater on TSA medium. The CO_2 atmosphere was clearly superior for production of larger hemolytic zones. Not only was the average zone of hemolysis larger in CO_2, but also fewer cultures were described as nonhemolytic. Anaerobic incubation produced the smallest colonies and zones of hemolysis. Since production of noticeable hemolysis is critical for the recognition of *A. haemolyticum* colonies in the presence of other flora, our results suggest that routine throat culture specimens should be cultured on TSA and incubated in CO_2 to improve recovery of this organism. However, authors of throat culture studies that compared all three atmospheres for recovery of GABHS did not recommend incubation in CO_2 (17, 19, 24). It is postulated that recovery of GABHS in CO_2 is lower than that in either aerobic or anaerobic incubation owing to enhanced growth of normal aerobic and facultative throat flora (15). In spite of the significantly larger colonies and zones of hemolysis on TSA medium in CO_2, it is notable that neither medium nor atmosphere had a statistically significant effect on the number of nonhemolytic cultures of *A. haemolyticum*; this suggests that any conventional throat culture

procedure that includes a 48-h incubation should be an adequate method for recovery of most strains of this species.

It was interesting that the six strains in this study exhibited diverse colonial morphology, including one strain that produced beta-hemolytic colonies in fewer than 60% of the 48-h cultures and another strain that produced pits in only 33% of 48-h cultures. This variability suggests that some strains of *A. haemolyticum* could be overlooked in cases of heavy growth of commensal throat flora, even under optimal medium and incubation conditions. For cases in which *A. haemolyticum* is suspected, but not recovered after a 48-h incubation on routine throat culture media, extended incubation for 72 h or use of selective media as described by Brenwald et al. (2) may be appropriate.

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