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Comparison of Three Kinds of Blood and Two Incubation Atmospheres for Cultivation of *Bordetella pertussis* on Charcoal Agar

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We compared the growth of *Bordetella pertussis* strains ($n = 32$) on antibiotic-free and cephalexin (40 μg/ml)-containing charcoal agar supplemented with 10% defibrinated horse blood, defibrinated sheep blood, or anticoagulant-containing human blood. Plates were incubated either in air or in an atmosphere with 5 to 10% CO_2. As assessed by mean colony numbers and rapidity of growth, normal air was preferable to CO_2 enrichment for incubation. Growth on horse blood agar was more abundant and more rapid than on sheep blood agar, but the difference in general was not statistically significant. Human blood was clearly inferior to both horse and sheep blood.

Bordetella pertussis has no CO_2 requirement (4), but some authors have used an atmosphere with 5 to 10% CO_2 for cultivation (1, 3). To our knowledge, no study that compares the two incubation atmospheres with respect to charcoal agar has been published (2).

The manufacturer of charcoal agar base (Oxoid Ltd., Basingstoke, United Kingdom) recommends supplementing the agar with 10% whole defibrinated horse blood. Some authors have used sheep blood instead (1, 3). Comparative studies of horse and sheep blood seem to be lacking. Since animal blood often is not available to bacteriology laboratories in developing countries, the question of whether human blood can be substituted for it is of practical interest. Donated human blood which for some reason cannot be transfused is often the only source of blood available under such circumstances.

We therefore studied the growth of *B. pertussis* on antibiotic-free and cephalexin-containing charcoal agar supplemented with horse, sheep, or human blood and incubated either in normal air or in an atmosphere with 5 to 10% CO_2.

Thirty-two clinical isolates of *B. pertussis* which had been stored at −70°C in glycerol soon after isolation were thawed and grown on antibiotic-free Jones-Kendrick agar (5) in air. For each strain, a suspension in sterile saline was prepared and adjusted to a photometric extinction equivalent to that of a nephelometric McFarland 0.5 standard. This suspension was diluted 1:10,000 with sterile saline. From this dilution, 20 μl (ca. 200 CFU) was spread evenly on each of the test plates.

Oxoid charcoal agar was prepared according to the instructions of the manufacturer. After autoclaving and cooling to 50°C, half of the agar was supplemented with 40 μg of cephalexin per ml. Both the antibiotic-free and the cephalexin-containing agars were further supplemented with 10% horse blood (Oxoid), 10% sheep blood (Gesellschaft für Mikrobiologische Nährmedien, Walldorf, Federal Republic of Germany), or 10% human blood (blood groups A_1 and B) obtained from the blood bank of Tübingen University Hospitals. The two animal blood products had been defibrinated by use of sterile glass beads; the human blood contained the standard anticoagulant citrate-phosphate-dextrose-adenine. When the media were prepared, appropriate sterility control measures were taken.

Each *B. pertussis* strain was seeded on two plates of antibiotic-free charcoal-horse blood agar (HA), charcoal-sheep blood agar (SA), and charcoal-human blood agar (MA). The same procedure was used for the cephalexin-containing media. Altogether, each strain was inoculated onto 12 plates.

Half of all plates were incubated in normal air at 36°C in a humid environment. The remaining plates were incubated in GasPak jars in 5 to 10% CO_2 (CO_2 Systems Envelope; Becton Dickinson and Co., Cockeysville, Md.). Incubation in both atmospheres was for 5 days. On days 3, 4, and 5, colonies were counted and their development stages were assessed semiquantitatively (1+, tiny colonies; 2+, small colonies [ca. 0.5-mm diameter]; 3+, mature colonies [ca. 1-mm diameter]). For statistical evaluation, the tests of Dixon and Mood (6) and of Bowker (7) were used.

All media supported growth of all strains under both incubation conditions, but there were considerable differences. Table 1 shows the mean colony numbers (MCN) of *B. pertussis* counted on the various plates on days 3, 4, and 5. MCN on HA were in general slightly higher than on SA, irrespective of incubation atmosphere and presence of cephalexin. Significantly higher MCN ($P < 0.05$) on HA were observed only on the antibiotic-free plates incubated in 5 to 10% CO_2 on days 3 and 4.

MCN on HA and SA were always significantly higher than on MA ($P < 0.01$), irrespective of atmosphere and antibiotic. MA incubated in 5 to 10% CO_2 yielded the lowest MCN.

Incubation in 5 to 10% CO_2 of plates of all three kinds of blood always yielded significantly lower MCN ($P < 0.01$) than incubation in air of the corresponding plates. This applies both to antibiotic-free and to cephalexin-containing agars.

On antibiotic-free agar plates of all three kinds of blood, MCN were always higher than on the corresponding plates

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TABLE 1. MCN of 32 *B. pertussis* strains[a]

Type of charcoal agar	Day	MCN (range) in:					
		Air			5–10% CO_2		
		Horse blood	Sheep blood	Human blood	Horse blood	Sheep blood	Human blood
Antibiotic free	3	60 (5–120)	56 (1–116)	28 (1–72)	32 (3–80)	17 (2–62)	2 (1–7)
	4	72 (5–144)	72 (2–139)	51 (2–129)	59 (3–155)	48 (3–116)	9 (1–28)
	5	79 (6–150)	76 (3–140)	59 (2–135)	64 (3–140)	61 (2–116)	24 (1–73)
Cephalexin containing	3	47 (19–84)	45 (20–90)	12 (1–32)	15 (5–34)	16 (6–37)	1 (1–4)
	4	58 (20–124)	57 (24–93)	30 (2–62)	46 (17–119)	44 (14–79)	3 (1–6)
	5	72 (29–153)	70 (38–128)	36 (5–68)	60 (25–125)	59 (20–100)	6 (2–14)

[a] Strains cultured in air or in 5 to 10% CO_2 on antibiotic-free or cephalexin-containing charcoal agar supplemented with 10% horse, sheep, or human blood.

containing cephalexin, but the difference was not statistically significant for HA and SA, with the exception of SA incubated in air on days 3 ($P < 0.02$) and 4 ($P < 0.05$).

Table 2 shows the rapidity of growth of *B. pertussis* as assessed by three stages of colony development. During incubation in air, colony development on HA was in general more rapid than on SA, but statistical significance ($P < 0.05$) was reached only on day 3 on the antibiotic-free plates.

During incubation in air, colony development on HA and SA was always significantly more rapid than on MA, irrespective of antibiotic. On MA incubated in air, *B. pertussis* never developed mature colonies (3+ by day 5), but on MA incubated in 5 to 10% CO_2, colony development was even poorer.

On the plates incubated in 5 to 10% CO_2, development of mature colonies was never observed (with one exception), irrespective of antibiotic and the kind of blood used.

On cephalexin-containing HA and SA, growth of *B. pertussis* on days 3 and 5 (but not on day 4) was more advanced than on the corresponding antibiotic-free plates. This observation is not easily explained.

In conclusion, incubation in normal air is clearly preferable to incubation in an atmosphere with enriched CO_2 for cultivation of *B. pertussis*. Growth of the organisms on HA was more abundant and more rapid than on SA, but the difference in general was not statistically significant.

In our study, human blood was clearly inferior to both horse and sheep blood. The possibility that the anticoagulant contained in human blood (but not in horse and sheep blood) introduced a bias cannot be excluded. However, it was one intention of this study to evaluate donated human blood as an agar supplement for *B. pertussis*. MA supported growth of all test strains, but incubation in air for at least 7 days is advisable if human blood is used. In a pilot study in Nigeria, MA was evaluated under field conditions. Nasopharyngeal swab samples taken from 210 children with suspected early whooping cough yielded 33 *Bordetella* isolates, including two strains of *Bordetella parapertussis* (total isolation rate, 15.7%) (J. E. Hoppe and A. Rockenstiehl, manuscript in preparation). Thus, culturing for *B. pertussis* is possible even if animal blood is not available.

TABLE 2. Rapidity of growth as shown by stage of colony development of *B. pertussis*[a]

Type of charcoal agar	Day	Stage[b]	Growth rate (%) in:					
			Air			5–10% CO_2		
			Horse blood	Sheep blood	Human blood	Horse blood	Sheep blood	Human blood
Antibiotic free	3	1+	34	66	97	100	97	100
		2+	66	34	3	0	3	0
		3+	0	0	0	0	0	0
	4	1+	16	16	63	47	56	100
		2+	47	63	38	53	44	0
		3+	38	22	0	0	0	0
	5	1+	9	13	25	25	16	97
		2+	3	13	75	75	84	3
		3+	88	75	0	0	0	0
Cephalexin containing	3	1+	0	9	100	97	100	100
		2+	100	91	0	3	0	0
		3+	0	0	0	0	0	0
	4	1+	0	0	69	31	69	100
		2+	94	91	31	69	31	0
		3+	6	9	0	0	0	0
	5	1+	0	0	34	0	0	100
		2+	3	6	66	100	97	0
		3+	97	94	0	0	3	0

[a] Strains cultured in air or in 5 to 10% CO_2 on antibiotic-free or cephalexin-containing charcoal agar supplemented with 10% horse, sheep, or human blood. Data expressed as percentage of 32 strains.

[b] For explanation, see text.

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