JOURNAL OF CLINICAL MICROBIOLOGY, Jan. 1982, p. 53-57
0095-1137/82/010053-05$02.00/0

Vol. 15, No.1

# Comparison of Atmospheres of Incubation for Primary Isolation of *Campylobacter fetus* subsp. *jejuni* from Animal Specimens: 5% Oxygen Versus Candle Jar

NANCY W. LUECHTEFELD,[1,2]† * L. BARTH RELLER,[3] MARTIN J. BLASER,[1,3] AND WEN-LAN L. WANG[1,2]

*Microbiology Laboratory, Denver Veterans Administration Medical Center, Denver, Colorado 80220,[1] and Department of Pathology[2] and Division of Infectious Diseases, Department of Medicine,[3] University of Colorado School of Medicine, Denver, Colorado 80262*

Received 24 February 1981/Accepted 25 August 1981

An atmosphere with reduced oxygen tension is required for the primary isolation of *Campylobacter fetus* subsp. *jejuni*. Therefore, we compared use of the conventional atmosphere of 5% oxygen and 8% carbon dioxide with use of a candle jar (17% oxygen and 3% carbon dioxide) for primary isolation of *C. fetus* subsp. *jejuni* from 263 positive canine, cattle, and turkey fecal or cecal specimens. At an incubation temperature of 42°C, the atmosphere with 5% oxygen resulted in more *Campylobacter* colonies per plate ($P < 0.005$) and consistently larger *Campylobacter* colonies ($P < 0.005$) than did the candle jar, whereas the growth of interfering flora was similar. Overall, 96% of the 263 specimens were positive for *C. fetus* subsp. *jejuni* with 5% oxygen, and 90% were positive with the candle jar ($P < 0.02$). More striking differences in isolation rates were seen when both the temperature and the atmosphere were varied: 5% oxygen at 42°C enabled recovery of 93% of the isolates from 70 positive specimens, versus 46% recovery with the candle jar at 37°C. Results with 5% oxygen at 37°C were intermediate. The addition of FBP supplement (0.25% each of ferrous sulfate, sodium metabisulfite, and sodium pyruvate) to Campy-BAP selective medium made no improvement over unsupplemented medium at 42°C (whether in 5% oxygen or in the candle jar), but there was significant improvement over unsupplemented medium when both media were incubated at 37°C in the candle jar. We conclude that 5% oxygen is superior to the candle jar for primary isolation of *C. fetus* subsp. *jejuni* from animal specimens and that interaction of the two factors tested (temperature and atmosphere of incubation) greatly decreases isolation rates at 37°C in a candle jar.

Since *Campylobacter fetus* subsp. *jejuni* grows poorly or not at all under either aerobic (21% oxygen) or anaerobic conditions, an atmosphere with reduced oxygen tension is required for the primary isolation of this organism (19). Most methods for achieving the proper atmosphere are relatively expensive or require the use of special equipment such as tank gas and anaerobic jars with manometers. In contrast, the use of a candle extinction jar is inexpensive and requires only an airtight jar and a candle. The purpose of this study was to test whether the atmosphere produced in a candle jar is as effective as an atmosphere of 5% oxygen for the primary isolation of *C. fetus* subsp. *jejuni* from animal specimens.

† Present address: Veterinary Research Laboratory, Montana State University, Bozeman, MT 59717.

## MATERIALS AND METHODS

**Sources of specimens.** Stool samples were obtained from 129 puppies at a local kennel, cecal samples were obtained from 50 cattle at a local abattoir, and cecal samples were obtained from 190 turkeys at a poultry-processing plant. These sources were chosen because puppies (3), cattle (13), and turkeys (12) have high carriage rates of *Campylobacter* and because canines (2), cows (17), and poultry (16, 18) have been implicated as sources of human *Campylobacter* infection.

**Processing of specimens.** One gram of each stool or cecal sample was emulsified in 2 ml of sterile saline. Two drops (0.07 ml) of this suspension from each sample were placed onto duplicate plates of Campy-BAP medium (1), and the plates were streaked in four quadrants with a standard bacteriological loop. One of each pair of selective plates was incubated in a standard microaerophilic atmosphere of 5% oxygen, 8% carbon dioxide, and 87% nitrogen. This was achieved by evacuating 75% of the air from an anaerobic jar and then refilling the jar to atmospheric pressure (627 mm of Hg at a 1,609-m altitude) from tank gas containing

of Hg at a 1,609-m altitude) from tank gas containing 10% carbon dioxide and 90% nitrogen. The other plate in each pair was incubated in a candle jar that contained 15.9 to 18.3% oxygen (100 to 115 mm of Hg at a 1,609-m altitude) at extinction. Oxygen measurements were made at room temperature (25°C) with an oxygen analyzer (model OM-14; Beckman Instruments, Inc., Fullerton, Calif.). All jars were incubated at 42°C for 48 h.

Since some laboratories use 37°C incubators for the isolation of *Campylobacter*, a third set and a fourth set of plates from 70 of the turkey specimens were inoculated, streaked, and incubated at 37°C in candle jars or in 5% oxygen for 48 h. Additionally, modified FBP medium was prepared by adding 0.25% each of ferrous sulfate, sodium metabisulfite, and sodium pyruvate (7) to the Campy-BAP medium; specimens from 189 of the puppies and turkeys were inoculated onto several sets of this medium for comparison with unsupplemented Campy-BAP.

**Grading of plates.** *Campylobacter* colonies were visually identified on the plates by their characteristic smooth, flat, tan or gray, sometimes spreading appearance and were generally easily distinguishable from interfering flora. This visual impression was confirmed by Gram staining one to five *Campylobacter* colonies per plate to demonstrate the vibrioid morphology of the organisms. Gram stains were also made of many of the small colonies of interfering flora to be certain that they were not unusual *Campylobacter* colonies. Each plate was given numerical grades for each of three parameters: the number of *Campylobacter* colonies, the size of *Campylobacter* colonies, and the amount of growth of interfering flora (Table 1). All plates for all experiments were graded by the same individual. The sign test for paired data was used for the statistical analysis of the numerical grades, and the rates of positivity for given atmosphere-temperature conditions were compared with the McNemar chi-square test for correlated proportions (15). The Bradley modification of the Friedman test was used to test for the main effects and interaction of the two factors: atmosphere and temperature (5). For each stool or cecal sample tested, one *Campylobacter* colony was picked for isolation and identification as *C. fetus* subsp. *jejuni* by methods described previously (1).

TABLE 1. Criteria for grading plates in comparisons of incubation conditions

| *C. fetus* subsp. *jejuni* | | | Interfering flora |
|---|---|---|---|
| Grade | No. of colonies | Size of largest colony (diam [mm]) | No. of colonies |
| 0 | No Cfj[a] present | No Cfj present | No interfering flora present |
| 0.5 | ≤3 colonies on plate | <0.5 | ≤3 colonies on plate |
| 1 | >3 colonies, 1st quadrant | 0.5–1 | >3 colonies, 1st quadrant |
| 2 | >3 colonies, 2nd quadrant | >1–2 | >3 colonies, 2nd quadrant |
| 3 | >3 colonies, 3rd quadrant | >2–3 | >3 colonies, 3rd quadrant |
| 4 | >3 colonies, 4th quadrant | >3–7 | >3 colonies, 4th quadrant |
| 5 | | >7 | |

[a] Cfj, *C. fetus* subsp. *jejuni*.

## RESULTS

Overall, 71% of 369 specimens were positive for *Campylobacter* by at least one of the incubation conditions, including 40% of 129 puppy, 42% of 50 cattle, and 100% of 190 turkey specimens.

Table 2 shows comparisons between plates incubated at 42°C in either 5% oxygen or in the candle jar. The amount of growth of *C. fetus* subsp. *jejuni* (as measured by the quadrant in which the organism was present) was greater in the 5% oxygen than in the candle jar for specimens from both puppies and turkeys, but no significant difference was noted for the cattle specimens. The size of the *Campylobacter* colonies was consistently larger on plates incubated in 5% oxygen compared with the candle jar for both puppies and turkeys. Growth of interfering flora was not significantly different between the two test atmospheres for any of the three sources of specimens. Isolation rates for *C. fetus* subsp. *jejuni* were similar in both test atmospheres for specimens from turkeys and cattle; however, 5% oxygen yielded more isolates from puppies.

Table 3 shows comparisons among plates incubated under different combinations of temperatures and atmospheres. Use of 5% oxygen at 42°C yielded a much higher isolation rate and consistently more and larger *Campylobacter* colonies than did use of the candle jar at 37°C. Use of 5% oxygen at 37°C yielded intermediate results for all parameters tested.

Table 4 shows the effect of adding FBP supplement to the Campy-BAP medium. At 42°C, there was no significant difference in *Campylobacter* isolation rates, colony size, or colony numbers between FBP-supplemented and unsupplemented Campy-BAP, whether in 5% oxygen or in the candle jar. In contrast, FBP supplement increased the isolation rate as well as the size and number of *Campylobacter* colonies when the colonies were incubated at 37°C in a candle jar; however, this improvement was still significantly less effective than incubating at 42°C.

## DISCUSSION

There are few reports regarding the exact atmospheric requirements for the growth of *C. fetus*. Several earlier studies (6, 10, 14) were performed with pure cultures of *Vibrio fetus* (now *C. fetus*); however, it is not known which of these strains were actually *C. fetus* subsp. *jejuni*, and none of these studies compared at-

TABLE 2. 5% Oxygen vs candle jar for the primary isolation of *C. fetus* subsp. *jejuni* from animal specimens incubated at 42°C

| Specimen source and incubation condition | Rate of isolation from positive specimens (%) | Growth (mean numerical grades)[a] | | |
|---|---|---|---|---|
| | | No. of colonies (Cfj)[b] | Colony size (Cfj) | No. of colonies (interfering flora) |
| Puppies ($n^c$ = 52) | | | | |
| 5% Oxygen | 100 | 2.5 | 2.0 | 1.6 |
| Candle jar | 88 | 2.2 | 0.8 | 1.5 |
| | $P < 0.02$ | $P < 0.005$ | $P < 0.005$ | NS[d] |
| Cattle ($n$ = 21) | | | | |
| 5% Oxygen | 71 | 1.4 | 3.3 | 2.3 |
| Candle jar | 62 | 1.2 | 3.0 | 2.7 |
| | NS | NS | NS | NS |
| Turkeys ($n$ = 190) | | | | |
| 5% Oxygen | 97 | 2.9 | 3.9 | 3.2 |
| Candle jar | 94 | 2.3 | 3.6 | 3.2 |
| | NS | $P < 0.005$ | $P < 0.005$ | NS |
| Total ($n$ = 263) | | | | |
| 5% Oxygen | 96 | 2.8 | 3.6 | 2.8 |
| Candle jar | 90 | 2.4 | 3.3 | 2.8 |
| | $P < 0.02$ | $P < 0.005$ | $P < 0.005$ | NS |

[a] Grading criteria from Table 1.
[b] Cfj, *C. fetus* subsp. *jejuni*.
[c] *n*, Number.
[d] NS, Not significant.

mospheres for primary isolation of the organism from fecal specimens. These early investigations showed that, for laboratory isolates of *V. fetus* (subspecies not determined), 5 to 6% oxygen was optimal (6, 10, 14), and carbon dioxide was required for growth (6), but not in quantities greater than those found in air (10, 14). In one study, none of 34 isolates grew on solid medium anaerobically or in air (10), whereas others report that some laboratory strains become "adapted" to growth in air (14).

The focus of our study was *C. fetus* subsp. *jejuni*, and we compared atmospheres for the primary isolation of the organism rather than for the growth of laboratory strains. Despite these differences, our results are consistent with the earlier studies and with our preliminary report (20): an atmosphere of 5% oxygen and 8% carbon dioxide was superior to an atmosphere generated in the candle jar (ca. 17% oxygen and 3% carbon dioxide). The results of our studies were consistent among the three animal species tested although the trends in specimens from cattle often did not achieve statistical significance (perhaps owing to the smaller number of cattle specimens tested).

In the experiment testing different combinations of incubation temperatures and atmospheres (Table 3), there was a statistically significant interaction or synergism occurring between the two variables ($P < 0.005$). That is, the effect of the incubation temperature on the growth of *Campylobacter* was influenced by the particular atmosphere used, and vice versa. Thus, if only one of the two factors is suboptimal (i.e., use of 37°C with 5% oxygen or use of the candle jar with 42°C), then the incubation system is only slightly less effective than when optimal conditions are used (42°C with 5% oxygen). But if both factors are suboptimal (i.e., use of 37°C with a candle jar), then their interaction produces markedly inferior incubation conditions which yield strikingly lower *Campylobacter* isolation rates and growth parameters.

Bowdre et al. reported that the aerotolerance of a strain of *C. fetus* subsp. *jejuni* was greatly increased by adding iron salts (ferrous or ferric) to Brucella agar medium (4). George et al. (7) subsequently tested 18 laboratory strains of *C. fetus* subsp. *jejuni* grown either on Brucella agar (no blood added) or on FBP agar (Brucella agar with added ferrous sulfate, sodium metabisulfite, and sodium pyruvate). They found that all

TABLE 3. 5% Oxygen (42° and 37°C) vs candle jar (37°C) for the primary isolation of *C. fetus* subsp. *jejuni* from 70 turkey cecal specimens

| Incubation condition | Rate of isolation from positive specimens (%) | Growth (mean numerical grades)[a] | | |
|---|---|---|---|---|
| | | No. of colonies (Cfj)[b] | Colony size (Cfj) | No. of colonies (interfering flora) |
| 42°C, 5% Oxygen | 93 | 2.4 | 3.2 | 3.3 |
| 42°C, Candle jar | 93 | 2.2 | 3.0 | 3.2 |
| 37°C, 5% Oxygen | 88 | 1.9 | 2.0 | 3.2 |
| 37°C, Candle jar | 46 | 0.8 | 0.8 | 2.8 |

[a] Grading criteria from Table 1.
[b] Cfj, *C. fetus* subsp. *jejuni*.

TABLE 4. Effect of adding FBP supplement to Campy-BAP medium for isolation of *C. fetus* subsp. *jejuni* from animal specimens under various incubation conditions

| Specimen source and incubation condition | Plate medium | % Positive for Cfj[a] | Growth of Cfj (mean numerical grades)[b] | |
|---|---|---|---|---|
| | | | No. of colonies | Colony size |
| Puppies ($n^c$ = 52) | | | | |
| 42°C, 5% Oxygen | FBP[d] | 100 } NS[e] | 2.8 } NS | 2.0 } NS |
| | CBAP[f] | 100 } | 2.5 } | 1.9 } |
| 42°C, Candle jar | FBP | 90 } NS | 2.1 } NS | 0.8 } NS |
| | CBAP | 88 } | 2.2 } | 0.7 } |
| Turkeys ($n$ = 60) | | | | |
| 42°C, 5% Oxygen | CBAP | 100 | 3.1 | 3.1 |
| 42°C, Candle jar | FBP | 95 } NS | 2.6 } NS | 3.3 } NS |
| | CBAP | 92 } | 2.6 } | 3.0 } |
| 37°C, Candle jar | FBP | 60 } $P < 0.001$ | 1.5 } $P < 0.001$ | 0.8 } $P < 0.005$ |
| | CBAP | 25 } | 0.6 } | 0.2 } |

[a] Cfj, *Campylobacter fetus* subsp. *jejuni*.
[b] Grading criteria from Table 1.
[c] *n*, Number.
[d] FBP, Campy-BAP selective plate medium plus 0.25% each of $FeSO_4 \cdot 7H_2O$, sodium metabisulfate, and sodium pyruvate.
[e] NS, not significant.
[f] CBAP, Campy-BAP selective plate medium without FBP supplement.

18 strains grew on both media when the strains were incubated in an atmosphere of 6% oxygen; at 17% oxygen, all 18 strains grew on FBP agar, but only 13 strains (72%) grew on Brucella agar. Also, growth appeared on more quadrants, and the size of *Campylobacter* colonies was larger compared with those on Brucella agar incubated under the same conditions. In our study, 5% sheep blood as well as antimicrobial agents were added to Brucella agar base (Campy-BAP medium). The addition of FBP to this medium did not significantly improve the isolation rates or increase the size or number of *Campylobacter* colonies from animal specimens incubated at 42°C, whether in 5% oxygen or in the candle jar. The presence of catalase and superoxide dismutase, as well as other reducing agents in sheep blood, probably enhanced the aerotolerance of *Campylobacter* and decreased the need for FBP supplement in this medium (8, 11). The addition of FBP supplement did, however, improve all three growth parameters at 37°C in the candle jar when compared with unsupplemented Campy-BAP incubated under the same conditions.

It is possible that primary isolation using the candle jar could be achieved because interfering flora growing on the plates consume oxygen and may thereby decrease the oxygen tension in the candle jar to levels more suitable for the growth of *Campylobacter* (9). The extent of this effect would depend upon the number of plates in each jar and the number and type of interfering organisms growing on each plate, which varies from species to species. Puppies, cattle, and poultry have all been implicated as sources of human *Campylobacter* infections (1, 3, 16–18). In epidemiological studies with these animals, 5% oxygen with 8% carbon dioxide is clearly superior to the candle jar for primary isolation. The applicability of our work to the primary isolation of *C. fetus* subsp. *jejuni* from humans requires further investigation.

ACKNOWLEDGMENTS

These studies were supported in part by a grant from BBL Microbiological Systems, Cockeysville, Md.

LITERATURE CITED

1. **Blaser, M. J., I. D. Berkowitz, F. M. LaForce, J. Cravens, L. B. Reller, and W.-L. L. Wang.** 1979. Campylobacter enteritis: clinical and epidemiologic features. Ann. Intern. Med. **91**:179–185.
2. **Blaser, M., J. Cravens, B. W. Powers, and W.-L. L. Wang.** 1978. Campylobacter enteritis associated with canine infection. Lancet **ii**:979–981.
3. **Blaser, M. J., F. M. LaForce, N. A. Wilson, and W.-L. L. Wang.** 1980. Reservoirs for human campylobacteriosis. J. Infect. Dis. **141**:665–669.
4. **Bowdre, J. H., N. R. Krieg, P. S. Hoffman, and R. M. Smibert.** 1976. Stimulatory effect of dihydroxyphenyl compounds on the aerotolerance of *Spirillum volutans* and *Campylobacter fetus* subsp. *jejuni*. Appl. Environ. Microbiol. **31**:127–133.
5. **Bradley, J. V.** 1968. Distribution-free statistical tests, p. 87–145. Prentice-Hall, Inc., Englewood Cliffs, N.J.
6. **Fletcher, R. D., and W. N. Plastridge.** 1964. Gaseous environment and growth of microaerophilic vibrios. J. Bacteriol. **87**:352–355.
7. **George, H. A., P. S. Hoffman, R. M. Smibert, and N. R. Krieg.** 1978. Improved media for growth and aerotolerance of *Campylobacter fetus*. J. Clin. Microbiol. **8**:36–41.
8. **Hoffman, P. S., H. A. George, N. R. Krieg, and R. M. Smibert.** 1979. Studies of the microaerophilic nature of

*Campylobacter fetus* subsp. *jejuni*. II. Role of exogenous superoxide anions and hydrogen peroxide. Can. J. Microbiol. **25**:8–16.

9. **Karmali, M. A., and P. C. Fleming.** 1979. Application of the Fortner principle to isolation of *Campylobacter* from stools. J. Clin. Microbiol. **10**:245–247.
10. **Kiggins, E. M., and W. N. Plastridge.** 1956. Effect of gaseous environment on growth and catalase content of *Vibrio fetus* cultures of bovine origin. J. Bacteriol. **72**:397–400.
11. **Kingscote, B.** 1961. Use of catalase in the culture of *Vibrio fetus*. Can. J. Microbiol. **7**:951–952.
12. **Luechtefeld, N. W., and Wang, W.-L. L.** 1981. *Campylobacter fetus* subsp. *jejuni* in a turkey processing plant. J. Clin. Microbiol. **13**:226–268.
13. **Prescott, J. F., and C. W. Bruin-Mosch.** 1981. Carriage of *Campylobacter jejuni* in healthy and diarrheic animals. Am. J. Vet. Res. **42**:164–165.
14. **Reich, C. V., E. V. Morse, and J. B. Wilson.** 1956. Gaseous requirements for growth of *Vibrio fetus*. Am. J. Vet. Res. **17**:140–143.
15. **Remington, R. D., and M. A. Schork.** 1970. Statistics with applications to the biological and health sciences, p. 310–325. Prentice-Hall, Inc., Englewood Cliffs, N.J.
16. **Richardson, N. J., and H. J. Koornhof.** 1979. Campylobacter infections in Soweto. S. Afr. Med. J. **55**:73–74.
17. **Robinson, D. A., W. J. Edgar, G. L. Gibson, A. A. Matchett, and L. Robertson.** 1979. Campylobacter enteritis associated with consumption of unpasteurized milk. Br. Med. J. **1**:1171–1173.
18. **Skirrow, M. B.** 1977. Campylobacter enteritis: a "new" disease. Br. Med. J. **2**:9–11.
19. **Smibert, R. M.** 1978. The genus *Campylobacter*. Annu. Rev. Microbiol. **32**:673–709.
20. **Wang, W.-L. L., N. A. Wilson, L. B. Reller, and M. J. Blaser.** 1980. Selective methods for isolation of *Campylobacter fetus* subsp. *jejuni*, p. 940–942. *In* J. D. Nelson and C. Grassi (ed.), Current chemotherapy and infectious disease, Vol. 2. American Society for Microbiology, Washington, D.C.