Validation of a Monoclonal Antibody-Based Capture Enzyme-Linked Immunosorbent Assay for Detection of *Campylobacter fetus*


Canadian Food Inspection Agency, Ottawa Laboratory Fallowfield, 3851 Fallowfield Rd., P.O. Box 11300 Sn. H, Ottawa, Ontario K2H 8P9, Canada; and Veterinary Laboratory Agency Winchester, Itchen Abbas, Winchester, Hampshire SO21 1BX, England

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A monoclonal antibody (MAb)-based antigen capture enzyme-linked immunosorbent assay (ELISA) was compared with the routine culture methodology for the detection of *Campylobacter fetus* subspecies from bovine and ovine field samples inoculated into Clark’s transport enrichment medium (TEM). The work was a collaboration between two different diagnostic laboratories, one in Canada and the other in England. In both labs, TEM samples were incubated for 4 days at 35°C and then tested by culture and ELISA. The ELISA consisted of initial screening with MAb M1825 against *C. fetus* subspecies core lipopolysaccharide (LPS). All samples positive on ELISA screening were then retested by ELISA with MAb M1825 and MAb M1177, M1183, and M1194, which recognize serotype A- and/or serotype B-specific *C. fetus* subspecies LPS epitopes. The Canadian samples consisted of 1,060 preputial washings from 529 bulls, of which 18 were positive by both culture and ELISA and 1,042 were negative by both methods. The English samples consisted of 321 tissue specimens, mostly stomach contents and placentas, from 190 aborted ovine and bovine fetuses. A total of 262 samples were negative by culture and ELISA, 52 samples were positive by culture and ELISA, and 7 samples were culture negative but ELISA positive. The results for all 70 culture-positive isolates were confirmed by conventional biochemical methods as *C. fetus* subspp. *fetus*, with 39 presumptively identified by the ELISA as serotype A and 30 presumptively identified as serotype B and with one sample containing isolates presumptively identified as serotype A and serotype B. A receiver operating characteristic analysis of the combined ELISA data from both countries resulted in an area under the curve of 0.997, with a sensitivity of 100% and a specificity of 99.5% relative to the results of culture. The data confirm that this ELISA method can be used as an excellent test for the screening of field samples in TEM for the presence of *C. fetus* subspecies.

*Campylobacter fetus* is a fastidious microaerophilic gram-negative curved bacterial rod consisting of two subspecies, *C. fetus* subsp. *fetus* and *C. fetus* subsp. *venerealis* (9, 13). While both subspecies are very closely related at the genomic level (14), they inhabit different ecological niches and have different phenotypic characteristics that are used to differentiate them (2, 13, 29). *C. fetus* subsp. *fetus* normally resides in the intestinal tracts of cattle and sheep, but its ingestion can result in systemic infection and abortion in pregnant cows and ewes. This bacterial subspecies also causes systemic and intestinal infections in humans, usually as a result of a preexisting medical condition (23, 27). In contrast, the only ecological niche for *C. fetus* subsp. *venerealis* is the reproductive tract of cattle, where it produces bovine genital campylobacteriosis (BGC). This disease is characterized by infertility, early embryonic death, and abortion in cows as a result of venereal transfer from carrier bulls. Disease caused by *C. fetus* subsp. *venerealis* is economically important to the cattle industry worldwide. Although Canada is recognized as being free of BGC, it must actively prove pathogen freedom by testing animals, mainly bulls, in artificial insemination (AI) centers for the presence of *C. fetus* subsp. *venerealis* in order to maintain health certification of these animals and their products, especially semen and embryos, for purposes of international trade.

Several testing procedures have been used for the detection of *C. fetus* in animals. Culture and identification have been well described for the diagnosis of this pathogen (7, 8, 18). However, the presence of small numbers of *C. fetus* subspecies in samples, in which other competing commensal bacteria may also be present, and the fastidious nature of this bacterial species mean that samples must be enriched for several days in a transport enrichment medium (TEM). This is followed by culture to selective agar medium for several days under microaerophilic conditions in order to screen for suspect colonies. Once isolates are found, it takes additional time to confirm identification to the species and subspecies levels by traditional biochemical methods. As a result, culture techniques are specific but are laborious, time-consuming, and relatively expensive. To help overcome the problems of culture, other tests have been described. These include fluorescent-antibody assays for the detection of antigen in preputial washes and vaginal mucus samples (7, 19). This test lacks sensitivity for the detection of small numbers of *C. fetus* subspecies, and it lacks specificity because it makes use of a polyclonal antiserum made from killed whole cells of *C. fetus* subspecies which can cross-react with other *Campylobacter* spp. (1, 19). In addition, fluorescent-antibody assays are impractical for use in laboratories doing large numbers of tests. Other tests described for *C. fetus* subspecies have included agglu-
tation and enzyme-linked immunosorbent assay (ELISA) (15, 16) for the detection of antibody in preputial wash and vaginal mucus samples, but they lack sensitivity and specificity and are not recommended for use for the testing of individual animals (7, 16, 29). A PCR test (10) has been developed for the detection of C. fetus subspecies in semen specimens, but it is expensive and has never been evaluated for diagnostic purposes with field samples. As a result of the inadequacies of these tests, routine culture and identification remain the only internationally recognized methods for the diagnosis of infections caused by C. fetus subspecies (29).

The heat-stable antigenic epitopes that make up the serotyping scheme for C. fetus subspecies are based on differences in lipopolysaccharide (LPS) O-specific polysaccharide side chain epitopes; and two serotypes, designated A and B, are recognized (3, 24). Brooks et al. (5, 6) developed a number of specific monoclonal antibodies (MAbs) against the heat-stable LPS of C. fetus subspecies. On testing with pure cultures, they were able to select MAbs that were specific for serotype A or B strains of C. fetus subspecies. In addition, MAbs against the core LPS that recognized all serotype A and B strains of C. fetus subspecies but that did not react with other Campylobacter spp. were identified. These MAbs were successfully used in an indirect ELISA procedure to serotype isolates from culture that were confirmed to be C. fetus subspecies (6). Four of these core- and serotype-specific C. fetus subspecies MAbs, identified as M1177, M1183, M1194, and M1825, were selected for further evaluation in a diagnostic capture ELISA procedure (4). The capture ELISA procedure detected as few as 10⁵ to 10⁶ CFU/ml of C. fetus subspecies from TEM vials, and there was a 100% correlation between the ELISA and culture results. The authors concluded that the capture ELISA procedure with specific MAbs had promise as a screening method for the detection of C. fetus subspecies. The purpose of this research was to complete a validation study of the MAAbased antigen capture ELISA procedure as a screening method for the detection of C. fetus subspecies in routine field samples. The validation was a collaborative effort involving two bacteriology laboratories, one in Canada and one in England.

### MATERIALS AND METHODS

#### Preputial wash and tissue samples

In Canada preputial wash samples were routinely collected in the field, by veterinarians, as part of a national health certification program in which animal freedom from C. fetus infection is verified. The samples originated from nine AI and semen distribution centers across the country. At the AI centers, each preputial wash specimen collected was inoculated into duplicate vials of Clark’s TEM and submitted to the CFIA/Ottawa Laboratory Fallowfield (OLF) Germplasm Centre of Expertise diagnostic laboratory for culture of C. fetus subspecies. When they were received in the lab, the inoculated TEM vials were incubated at 35 to 37°C for 4 to 5 days. Following incubation, a sterile swab was inserted into each TEM vial and a portion of the fluid was inoculated onto a selective agar medium for culture of C. fetus subspecies. In addition, 1.0 to 2.0 ml of the TEM fluid was collected for testing by ELISA. For the validation exercise a total of 1,060 preputial wash samples from 529 bulls were submitted between May and November 2003, and these were tested by both culture and ELISA.

In England, on-farm necropsy samples were routinely collected from young animals that had died and from tissues and fetuses associated with abortion in order to determine the etiology. The samples were submitted to the VLA/Winchester Bacteriology laboratory for culture. Upon receipt at the lab, tissues were macerated into 5 ml of sterile saline. One milliliter of stomach contents or macerated tissue fluid was inoculated into a single TEM vial, which was incubated and cultured for C. fetus subspecies according to the Canadian protocol as described below. In addition to culture, 2.0 ml of the incubated TEM fluid was placed into a sterile microcentrifuge tube, heated at 100°C for 15 min, and transported by air back to CFIA/OLF for the completion of ELISA testing. For the validation exercise, a total of 321 samples from 190 animals (105 bovines, 81 ovines, 4 unknown) were submitted between November 2003 and July 2004 and analyzed by culture in England and by ELISA in Canada. They included 193 stomach content samples, 110 placental tissue samples, 10 liver tissue samples, 3 fecal specimens, 2 cotyledon tissue samples, 1 vaginal mucus sample, 1 sheep wash sample, and 1 fetal fluid sample. To ensure objectivity, the results were blinded, such that the culture results for the samples were not known until a final ELISA result was first provided.

#### Culture procedure for the isolation and identification of C. fetus subspecies

The methodology used for the isolation and identification of C. fetus subspecies was as described previously (4, 8). Briefly, after incubation for 4 to 5 days at 35 to 37°C, a portion of the liquid was withdrawn from the sample-containing TEM with a sterile swab and inoculated to cystiene heart agar plates (CH +) supplemented with cycloheximide (20 µg/ml) and novobiocin (2 µg/ml). The inoculum was streaked on the plates to provide well-isolated colonies. The agar plates were placed into anaerobic jars and incubated microaerophilically (4% O₂, 9% CO₂, and the remainder N₂) for 4 days at 35°C. The CH + plates were examined, and typical C. fetus subspecies colonies were examined further. Typical colonies, which were associated with cells which showed a curved rod morphology when they were viewed by wet mount and phase-contrast microscopy, were subcultured for purity onto Mueller-Hinton agar containing 10% sheep blood. All gram-negative curved rods were identified to the Campylobacter genus, species, and

### TABLE 1. Initial screening of TEM samples with C. fetus subspecies core-specific MAbs M1825: ELISA reactivities of C. fetus subsp. fetus-, C. fetus subsp. venerealis-, and C. sputorum biovar sputorum-positive and -negative control antigens

<table>
<thead>
<tr>
<th>Country and control antigen</th>
<th>No. of assays</th>
<th>ELISA OD₅₄₀ with MAb M1825</th>
<th>% Positivity</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Canada</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. fetus subsp. venerealis (serotype A)</td>
<td>24</td>
<td>0.32 ± 0.05 (0.32 ± 0.02)</td>
<td>29 ± 4</td>
</tr>
<tr>
<td>C. fetus subsp. fetus (serotype B)</td>
<td>24</td>
<td>1.10 ± 0.14 (1.10 ± 0.06)</td>
<td>NA</td>
</tr>
<tr>
<td>C. sputorum biovar sputorum</td>
<td>24</td>
<td>0.08 ± 0.01 (0.08 ± 0.00)</td>
<td>8 ± 1</td>
</tr>
<tr>
<td><strong>England</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. fetus subsp. venerealis (serotype A)</td>
<td>9</td>
<td>0.48 ± 0.10 (0.48 ± 0.07)</td>
<td>42 ± 5</td>
</tr>
<tr>
<td>C. fetus subsp. fetus (serotype B)</td>
<td>9</td>
<td>1.15 ± 0.19 (1.15 ± 0.13)</td>
<td>NA</td>
</tr>
<tr>
<td>C. sputorum biovar sputorum</td>
<td>9</td>
<td>0.08 ± 0.00 (0.08 ± 0.00)</td>
<td>7 ± 1</td>
</tr>
</tbody>
</table>

a C. fetus subsp. venerealis (ATCC 19438).
b C. fetus subsp. fetus (ATCC 27374).
c C. sputorum biovar sputorum (ADRI 538; formerly C. sputorum biovar bubulus).
d Mean OD ± SD (mean ± 95% CI).
e Percent positivity (mean OD of the control antigen/mean OD of C. fetus sp. fetus control) × 100. Data represent the mean percent positivity ± SD.

f NA, not applicable (will always be 100%).
subspecies levels by the biochemical tests described by Neill et al. (22), Smibert (28), and Barrett et al. (2) and included the indoxyl acetate test of Mills and Gherna (20). Isolates in Canada that were confirmed to be C. fetus subspecies were serotyped by an indirect ELISA procedure (6). To ensure harmonization of the isolation procedure between the two laboratories, both CH+ agar and TEM vials were produced, quality controlled, and shipped to the VLA laboratory in England from CFIA/OLF in Canada on a monthly basis and were used within the expiration dates.

**ELISA reagents.** The specific reagents used in the ELISA procedure are described elsewhere (4) and included a rabbit polyclonal anti-C. fetus serum, mouse MAbs to C. fetus LPS, and control antigens. The polyclonal anti-C. fetus serum was a hyperimmune serum prepared in rabbits by inoculation of formalin-killed cells made up of a mixture of six C. fetus subsp. fetus and C. fetus subsp. veneralis strains representing both serotype A and B heat-stable antigens. There were four tissue culture-derived murine immunoglobulin G1 MAbs used for ELISA testing: M1825, which recognized a specific core LPS epitope shared by all serotype A and B strains of C. fetus subspecies; M1177 and M1194, which reacted with two different C. fetus subspecies-specific serotype A LPS O-polsaccharide epitopes; and M1183, which reacted with three different C. fetus subspecies-specific serotype B LPS O-polsaccharide epitope. There were three control antigens: (i) C. fetus subsp. fetus ATCC 27374 (American Type Culture Collection), a serotype B-positive control strain which binds to MAbs M1825 and M1183; (ii) C. fetus subsp. veneralis ATCC 19438, a serotype A-positive control which binds to MAbs M1825, M1177, and M1194; and (iii) C. spirochete biovar spirochete ADRI 538, a negative control which does not bind to any of the four MAbs. The concentrated control antigens were aliquoted in 1.0-ml quantities in sterile microcentrifuge tubes and stored at −20°C. Checkboard titration (12) was used to optimize the concentrations and dilutions of these reagents beforehand; and all reagents were brought to room temperature (RT; 22 ± 2°C) prior to use for ELISA testing.

**Capture ELISA procedure.** Coated antibody plates were prepared ahead of time. Briefly, 100 μl of a 1:5,000 dilution of a rabbit polyclonal anti-C. fetus subspecies serum (4) in 0.06 M carbonate buffer (pH 9.6) was added to each well of Nunc MaxiSorp 96-well polystyrene plates (Nalge Nunc International, Rochester, NY), and the plates were incubated for 1 h at RT. The ELISA plates were sealed and kept frozen at −20°C, and any unused frozen coated antibody plates were discarded after 30 days. On the day of testing coated plates were removed from the freezer, brought to RT, and washed with 0.01 M phosphate-buffered saline containing 0.15 M NaCl and 0.05% Tween 20 (PBST). After inoculation of the TEM samples to CH+ agar plates, 1.0 to 2.0 ml of the TEM fluid and antigen controls was heated at 100°C for 15 min in a block heater and allowed to cool to RT prior to ELISA testing. One hundred microliters of each heated test fluid sample was added in duplicate, followed by incubation at RT for 1 h. For each antigen control, 100 μl of the heated antigen solution was added in duplicate wells to each microtiter plate except if a single ELISA plate was being set up, in which case quadruple wells were used. The plates were washed as described above; and 100 μl of MAB M1825, diluted 1:100 in PBST, was added to each well and the plates were incubated for 1 h at RT. The plates were washed, and 100 μl of a horseradish-peroxidase goat anti-mouse immunoglobulin G (heavy and light chains) conjugate (Jackson ImmunoResearch Laboratories Inc., West Grove, PA), diluted 1:5,000 in PBST, was added, followed by incubation for 1 h at RT. The plates were washed, and 100 μl of 3,3',5,5'-tetramethylbenzidine–hydrogen peroxide substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD) was added to each well. The plates were placed on a shaker for 10 min at RT and the optical densities (ODs) at 620 nm (OD620) were determined for each well by using a Titertek microtiter MCC/340 microplate reader (Labsystems, Needham Heights, MA). A test solution which was positive on initial screening with M1825 was retested by using all four C. fetus subspecies core LPS- and serotype-specific MAbs as described above.

**Analysis of data and interpretation of results.** From each ELISA plate, after the initial screening with MAB M1825, a mean OD620 value was determined for each heated TEM fluid and control antigen suspension tested. In order to account for day-to-day variability and provide more accurate results, the mean OD values were converted to percent positivity by using the following formula: (mean OD620 of the test sample with MAB M1825/mean OD620 of the C. fetus subsp. fetus ATCC 27374 positive control with MAB M1825) × 100.

A test result giving a percent positivity of ≥14% was tentatively designated positive, and all test TEM samples yielding a result of ≥14% were retested against all four C. fetus subspecies-specific MAbs. A repeat percent positivity ≥14% with M1825 and an OD620 ≥0.2 with M1177, M1183, or M1194 confirmed the positive ELISA result for a sample. Samples reacting with (i) M1825 and M1177 and/or M1194 or (ii) M1825 and M1183 were presumptively positive for serotype A and serotype B C. fetus subspecies isolation.
TABLE 3. Initial screening: culture results and ELISA reactivities of diagnostic test samples in TEM with C. fetus subsp. LPS core-specific MAb M1825

<table>
<thead>
<tr>
<th>Country and culture result</th>
<th>ELISA result</th>
<th>No. of samples (no. of animals)</th>
<th>ELISA OD(_{620}) with MAB M1825 (b)</th>
<th>Positivity (d)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Canada</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>Negative</td>
<td>1042 (521)</td>
<td>0.09 ± 0.01 (0.09 ± 0.00)</td>
<td>8 ± 1</td>
</tr>
<tr>
<td>Negative</td>
<td>Positive</td>
<td>0</td>
<td>NA (a)</td>
<td>NA</td>
</tr>
<tr>
<td>Positive</td>
<td>Negative</td>
<td>0</td>
<td>NA (a)</td>
<td>NA</td>
</tr>
<tr>
<td>Positive</td>
<td>Positive</td>
<td>18 (8)</td>
<td>0.88 ± 0.26 (0.88 ± 0.12)</td>
<td>75 ± 18</td>
</tr>
<tr>
<td><strong>England</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>Negative</td>
<td>262 (161)</td>
<td>0.08 ± 0.01 (0.08 ± 0.00)</td>
<td>7 ± 1</td>
</tr>
<tr>
<td>Negative</td>
<td>Positive</td>
<td>7 (6)</td>
<td>0.85 ± 0.35 (0.85 ± 0.26)</td>
<td>80 ± 20</td>
</tr>
<tr>
<td>Positive</td>
<td>Negative</td>
<td>0</td>
<td>NA (a)</td>
<td>NA</td>
</tr>
<tr>
<td>Positive</td>
<td>Positive</td>
<td>52 (22)</td>
<td>1.00 ± 0.25 (1.00 ± 0.07)</td>
<td>84 ± 18</td>
</tr>
</tbody>
</table>

\(a\) All culture-positive isolates were C. fetus subsp. fetus.
\(b\) Mean OD ± SD (mean ± 95% CI).
\(c\) NA, not applicable.
\(d\) Percent positivity = (mean OD of the test sample/mean OD of C. fetus subsp. fetus antigen control [ATCC 27374]) × 100. Data represent the mean percent positivity ± SD.

Kappa and chi-squared statistics and receiver operating characteristic (ROC) analysis of the ELISA results from both countries were performed using the Statistics (for ROC analysis) and Tests (for chi-square and interrater agreement, i.e., kappa statistic) tools in MedCalc, version 7.3, software (MedCalc Software, Mariakerke, Belgium), according to the manufacturer’s instructions.

RESULTS

ELISA reactivities of control antigens. The mean OD\(_{620}\) results for the three control antigens on ELISA testing after initial screening of all TEM test samples from both Canada and England with C. fetus subsp. core LPS-specific MAb M1825 are found in Table 1. C. fetus subsp. fetus ATCC 27374 was a positive control for serotype B LPS which produced a strong mean OD\(_{620}\), and its percent positivity was 100%, since it was the standard for comparison of all control and test samples. C. fetus subsp. venerealis ATCC 19438 was a positive control for serotype A LPS and produced a weaker mean OD\(_{620}\) result when it was used to screen samples from Canada and England. When C. fetus subsp. venerealis ATCC 19438 was compared to the C. fetus subsp. fetus ATCC 27374 positive control, the mean percent positivities were 29% ± 4% (range, 22 to 41%) for the samples from Canada and 42% ± 5% (range, 32 to 47%) for the samples from England. C. sputorum biovar sputorum ADRI 538 was a negative control which produced a nonreactive ELISA OD\(_{620}\) result, with mean percent positivities of 8% ± 1% (range, 6.5 to 9.4%) for the samples from Canada and 7% ± 1% (range, 5.5 to 8.0%) for the samples from England when the results were compared to those for the C. fetus subsp. fetus ATCC 27374 positive control. For the initial screen, the OD values observed for each of the three controls with M1825 were all within 2 standard deviations (SDs) of the mean with the exception of that for a C. fetus subsp. fetus control in one of the assays, which failed at 2 SDs but passed at 3 SDs, and that for a C. sputorum biovar sputorum control in another assay, which failed at 3 SDs. Similarly, in each of nine assays in England (number of wells = 26), the result for all three controls were within 2 SDs of the mean with the exception of that for a C. fetus subsp. fetus control in one of the assays, which failed at 2 SDs but passed at 3 SDs. For all controls in ELISA testing of samples from both Canada and England, the coefficients of variation (CVs) were less than 20% with the exception of that for the C. fetus subsp. venerealis ATCC 19438 control in the England data, the CV for which was 21%.

Table 2 presents the mean OD\(_{620}\) results for the three control antigens against all four core LPS- and serotype-specific C. fetus subsp. MAb M1825, MAb M1177, M1183, M1194, and M1825, upon retesting with ELISA-positive TEM samples. In addition to reacting more weakly with the core LPS-specific MAb M1825, C. fetus subsp. venerealis ATCC 19438 reacted with MAb M1177 and M1194 but not serotype B-specific MAb M1183. In addition to reacting strongly with the core LPS-specific MAb M1825, C. fetus subsp. fetus ATCC 27374 reacted with serotype B-specific MAb M1183 but not the serotype A-specific MAb Mab M1177 and M1194. C. sputorum biovar sputorum ADRI 538 did not react with any of the four MAb in Canada or England. In eight assays from Canada (number of wells = 26) and six assays from England (number of wells = 30), the OD values observed for all three control organisms on repeat testing with MAb M1825 were all within 2 SDs of the mean with the exception of that for a C. fetus subsp. venerealis control in one of the assays in the England data, which failed at 2 SDs but passed at 3 SDs.

ELISA reactivity and culture results of TEM test samples. Table 3 summarizes the culture results and ELISA reactivities of diagnostic test samples in TEM with C. fetus subsp. LPS core-specific MAb M1825. In Canada there were 1,042 TEM samples from 521 animals that were negative by culture and initial ELISA screening, while 18 samples from 8 animals were both culture and ELISA positive. There were no culture-positive and ELISA-negative or culture-negative and ELISA-positive samples observed. Compared to the results for the C. fetus subsp. fetus ATCC 27374 positive control, the corresponding mean percent positivities were 8% ± 1% (range, 5.5 to 13%) and 75% ± 18% (range, 40 to 103%) for the negative and the positive samples, respectively. All 18 culture-positive isolations were confirmed to be C. fetus subsp. fetus by conventional methods. Serotyping of the isolates by an indirect ELISA method (6) found that 14 and 4 of the isolates were serotype A...
and serotype B, respectively. In England there were 262 TEM samples from 161 animals that were culture negative and ELISA negative, with a mean percent positivity of 7% ± 1% (range, 4.6 to 10%); 52 samples from 22 animals that were culture positive and ELISA positive, with a mean percent positivity of 84% ± 18% (range, 4.5 to 122%); and 7 samples from 6 animals that were culture negative and ELISA positive, with a mean percent positivity of 80% ± 20% (range, 48 to 113%). These seven samples included four placenta tissue samples, two stomach contents, and one liver tissue sample. There were no culture-positive and ELISA-negative samples observed. All 52 culture-positive isolates were confirmed to be C. fetus subsp. fetus by conventional methods. Cultures were not available from England, and thus, it was not possible to serotype the cultured isolates in this part of the validation exercise.

Table 4 summarizes the culture and ELISA reactivities of all positive TEM samples upon retesting with the four C. fetus subspecies-specific MAbs. In Canada, 14 TEM fluid samples reacted with M1177 and M1194, in addition to M1825, but not with M1183, indicating a serotype A specificity. There were four TEM samples that reacted with M1825 and M1183 but not with M1177 or M1194, indicating a serotype B specificity. In Canada, there was a 100% correlation between the serotyping results obtained for isolates cultured from TEM by the indirect ELISA and the serotype specificity observed by the capture ELISA procedure used directly with the TEM samples. In England, three patterns of reactions were observed for the 52 culture-positive and ELISA-positive test samples from the initial screening. The first pattern included 25 samples which showed C. fetus subspecies serotype A specificity, reacting to MAbs M1825 and M1194 but not M1183. As expected, 22 of these samples reacted to M1177, but 3 did not (mean OD, 0.12 ± 0.04). The second pattern of results observed was for 25 samples which showed serotype B specificity, reacting with M1825 and M1183 but not M1177 or M1194. The third pattern, which was detected for a single sample, showed both serotype A and serotype B specificity, reacting to all four MAbs. In addition, there was a culture-positive and ELISA-positive sample that had an insufficient fluid quantity and could not be retested against all four MAbs. The same patterns of ELISA reactivity were observed for the seven culture-negative and ELISA-positive samples observed from England. Two samples showed serotype A specificity, reacting to M1825 and M1194 but not M1183. Only one of these samples reacted with M1177, while the other did not (OD, 0.18). Three samples showed serotype B specificity, reacting with M1825 and M1183 but not M1177 or M1194; and two samples showed both serotype A and serotype B specificity, reacting to all four MAbs.

**Statistical analysis of data.** The results of the ROC analysis of the culture versus capture ELISA results are found in Fig. 1. The analysis is for a combination of the results for all samples tested in both Canada and England, and the ELISA results represent those obtained from initial screening against C. fetus subspecies core MAb M1825 and are expressed as percent positivity. The ROC curve (Fig. 1A) yielded an area under the curve of 0.997, with a 95% confidence interval (CI) from 0.993 to 0.999. Figure 1B shows the scatter plot of the ROC analysis of the culture-positive and -negative results versus their corresponding ELISA percent positivities. This analysis showed an optimum cutoff value of 13.1% positive at the highest sensitiv-

<table>
<thead>
<tr>
<th>Country</th>
<th>Culture and ELISA reactivities of all positive TEM samples with the C. fetus subspecies LPS core-specific MAb (M1825) and serotype-specific MAbs (M1177, M1183, M1194)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ELISA OD&lt;sub&gt;620&lt;/sub&gt; with MAb&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Mean (min–max)</td>
</tr>
<tr>
<td>Canada</td>
<td>M1177</td>
</tr>
<tr>
<td></td>
<td>M1183</td>
</tr>
<tr>
<td></td>
<td>M1194</td>
</tr>
<tr>
<td></td>
<td>M1825</td>
</tr>
</tbody>
</table>

<sup>a</sup>Mean OD ± SD (min–max) of all culture-positive and ELISA-positive test samples from the TEM samples. No. of samples: 25. RESULTS OF ELISA-positive TEM samples with the C. fetus subspecies LPS core-specific MAb (M1825) and serotype-specific MAbs (M1177, M1183, M1194). Data represent the mean percent positivity ± SD.
ity and specificity of 100% (95% CI, 94.8 to 100) and 99.5% (95% CI, 98.9 to 99.8), respectively. The chi-square statistic was 0.259 ($P = 0.611$), and the kappa statistic was 0.950 (95% CI, 0.913 to 0.987).

**DISCUSSION**

In a previous evaluation study (4), 725 TEM preputial wash and vaginal mucus samples were cultured for *C. fetus* subspecies, and the fluids were frozen for up to 12 months before being tested by the capture ELISA procedure with all four *C. fetus* subspecies-specific MAbs described here. That study was conducted in two different laboratories at CFIA/OLF, with the ELISA testing being completed by three technologists. There was a 100% correlation between the culture and the ELISA results, and the data strongly suggested that screening for the presence of *C. fetus* subspecies could be accomplished with the use of the LPS core-specific MAb M1825 alone rather than all four MAbs at the same time. This strategy would allow testing of up to 45 samples rather than 9 samples on a microtiter plate and economize the use of valuable reagents. The validation work completed here was done to verify this approach and to expand the scope of the testing to two different laboratories on different continents in order to increase the numbers and types of samples tested.

It has been recognized that the analysis of control data is a useful means of tracking and determining the performance of tests (11). The inclusion of the SD data for the three controls in Tables 1 and 2 is useful, since they measure precision, or repeatability, by measuring the dispersion of data around a mean. With the exception of a couple of assay points in both countries, the OD values of the controls remained within 2 SDs of the mean; and all values except that for a *C. sputorum* biovar *sputorum* control in Canada were within 3 SDs. In addition, the CVs for control reagents should not exceed 15 to 20% (11). This was true for all screening test data in this work with the exception of that for the *C. fetus* subsp. *venerealis* control in England, where the CV was 21%. Thus, the ELISA showed good precision.

There was a 100% correlation between the culture and the ELISA results for samples tested in Canada, but there were seven culture-negative and ELISA-positive samples from England which affected the overall specificity. Whether the type of sample inoculated into the TEM vials, i.e., tissue samples versus preputial washing samples, affects culture results is not known with certainty at this time. One of the seven samples was overgrown with fungal contamination on culture, and this type of overgrowth by the commensal microflora may be a reason for the difference observed between the culture and the ELISA results for these seven samples. However, all seven samples reacted against at least one of the other *C. fetus* subspecies LPS serotype-specific MAbs, two with serotype A-specific MAbs, three with serotype B-specific MAbs, and two with both serotype A- and B-specific MAbs (Table 4). In addition, these seven samples were from six animals; and other samples taken from three of the six animals were positive by culture and ELISA, confirming that these animals were infected with *C. fetus*. We have experienced similar discrepant results when comparing culture with the capture ELISA procedure. As part of the evaluation study (4), multiple preputial wash samples in TEM were taken from three bulls known to be naturally infected with *C. fetus* subsp. *fetus*, two with serotype A and one with serotype B. Of the 66 samples analyzed, 65 were positive by ELISA but only 49 were positive by culture. Although the method used for the culture of *C. fetus* subspecies is reliable, the inoculum size, the presence of competing microflora, and the environmental conditions that affect the TEM vials during transport are known to influence the success of culture (9, 21). As such, two TEM samples are routinely inoculated for every sample in Canada. However, for this validation exercise, culture was the “gold standard,” and these seven culture-negative and ELISA-positive samples from England were considered to have false-positive results. As a

![Figure 1](http://cvi.asm.org/)

**FIG. 1.** ROC analysis of culture versus ELISA results, based on percent positivity, for all 1,381 TEM samples from Canada and England tested on initial screening with MAb M1825. (A) ROC curve; (B) scatter plot.
result, the capture ELISA procedure used as a screening method had a sensitivity of 100% and a specificity of 99.5% at a cutoff value of 13.1% positivity.

The high sensitivity of the ELISA procedure for the detection of *C. fetus* subspecies is enhanced by the use of Clark’s TEM (8, 13), which, when incubated, allows the growth and selection of small numbers of this bacterium to levels detectable by ELISA. The high specificity of the ELISA procedure is due to the development of very specific LPS core- and serotype-specific *C. fetus* subspecies-specific MAbs (6). While initial screening with LPS core-specific MAb M1825 alone provided an excellent correlation between the culture and the ELISA results, it is known that at least one strain of *Arcobacter butzleri* will bind to this antibody reagent. In addition, while *Campylobacter hyointestinalis* will not grow in the Clark’s TEM, we have found that two of eight strains of this bacterial species will also bind to M1825 and react in the capture ELISA (unpublished data). However, neither of these bacterial species binds to the other three *C. fetus* subspecies serotype-specific MAbs. Thus, it is important that all samples that react with M1825 on initial ELISA screening be tested further by using all four MAbs. Further ELISA testing also allows the presumptive serotype identification of the *C. fetus* subspecies isolate in the original sample. In Canada in both this and the previous evaluation study (4), there was a 100% correlation between the capture ELISA result with all four LPS core- and serotype-specific MAbs and the results of an indirect ELISA with the same MAbs conducted with the cultured isolate obtained from the same TEM vial. Although it was not possible to obtain the *C. fetus* subsp. *fetus* isolates recovered from TEM in England and, hence, to determine the serotype of these strains, a presumptive serotype could be stated based on the capture ELISA results obtained with the original sample fluid.

The O-specific polysaccharide chain in LPS forms the basis for the heat-stable serotyping scheme in *C. fetus* (3, 24). There are two heat-stable serotypes described, serotypes A and B, and their chemical structures have been determined. *C. fetus* subspecies serotype A LPS is due to a partially O-acetylated 1,3-linked α-D-mannan (26), while serotype B LPS is due to the presence of a β-D-rhamnan terminated with 3-O-methyl-D-rhamnose (25). *C. fetus* serotype AB strains have also been reported infrequently in the literature. All *C. fetus* subsp. *fetus* strains possess the serotype A, B, or AB LPS, while strains of *C. fetus* subsp. *venerealis* contain the serotype A LPS only (24). All isolates from culture-positive samples from both Canada and England were identified as *C. fetus* subsp. *fetus*. By using the results of the capture ELISA procedure, LPS serotypes A and B were presumptively detected from culture-positive samples from Canada; and serotype A, serotype B, and both serotype A and serotype B were presumptively detected from the culture-positive samples from England. In addition, the ELISA procedure used two MAbs, M1177 and M1194, in order to recognize different O-side-chain serotype A-specific epitopes originally described by Brooks et al. (5). As such, reactivity against either or both of these MAbs is considered a positive reaction for serotype A. From England, there were four samples that reacted with M1825 and M1194 but not M1177. Thus, in addition to recognizing the three main serotypes, the ELISA was able to discern different serotype A epitopes, which is important for accurate detection and possibly for epidemiological considerations as well.

There was a lack of *C. fetus* subsp. *venerealis*-positive samples in this study. However, the MAbs used in the ELISA procedure were developed for the detection of shared *C. fetus* subspecies LPS core- and serotype-specific epitopes, which are well characterized (24, 25, 26). In addition, *C. fetus* subsp. *venerealis* grows well in Clark’s TEM (4, 8, 13); and on testing with numerous different strains of this subspecies, we have never failed to detect it in previous studies using the ELISA and the MAbs described here (4, 5, 6).

The capture ELISA procedure cannot be used to identify accurately the subspecies of *C. fetus* serotype A strains that may be present in a sample. Obviously, if a serotype B ELISA result was obtained, then it would be strong evidence that the positive reaction was due to the presence of *C. fetus* subsp. *fetus*. However, a serotype A reactivity could mean the presence of either *C. fetus* subsp. *fetus* or *C. fetus* subsp. *venerealis*. In addition, while it is uncommon, reactivity with both serotype A- and serotype B-specific MAbs may be due to *C. fetus* subsp. *fetus* or to the presence of two different strains, a serotype A strain and a serotype B strain, in the same sample. Thus, culture is still necessary to confirm the ELISA reaction and the identity of the *C. fetus* subspecies present.

In summary, the capture ELISA procedure for detection of *C. fetus* subspecies was performed with a large number of samples and a broad range of sample types by five different technologists using different ELISA equipment in both a research and diagnostic laboratory and in collaboration between international partners. In comparison to the results of culture, it had a sensitivity of 100% and a specificity of 99.5%, was repeatable, and met the standards for the validation of tests described by others (11, 17). Any TEM sample showing a positivity of ≥14% on initial ELISA screening with LPS core-specific MAb M1825 would be retested by using all four *C. fetus* subspecies LPS core- and serotype-specific MAbs. Reactivity with M1825 and at least one of the other serotype-specific MAbs would confirm that the ELISA reaction was positive. The validation results in this work indicate that the capture ELISA could replace routine culture as a reliable screening test for the presumptive detection of *C. fetus* subspecies from inoculated TEM vials. However, since (i) the capture ELISA does not differentiate *C. fetus* subsp. *fetus* from *C. fetus* subsp. *venerealis* in the case of serotype A results and (ii) culture is the OIE reference method, culture would be required to confirm all positive results by ELISA.

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**REFERENCES**


102.


