Development and evaluation of an enzyme-linked immunosorbent assay for quantification of the humoral response of cattle vaccinated against Campylobacter fetus

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Objective—To develop a reliable ELISA by use of a unique antigen preparation for serum IgG quantification after vaccination against Campylobacter fetus in cattle.

Animals—Twenty-six 24-month-old virgin Hereford heifers and a naturally infected Hereford bull.

Procedures—Five antigens were prepared from a cell suspension of C fetus. Antigen preparations were the same as those reported in the literature, with the exception of antigens that were obtained by detergent solubilization of a C fetus cell suspension. For each antigen preparation, the optimal ELISA conditions for its immobilization were determined. Biotinylated antibodies against bovine immunoglobulins were obtained and used in the ELISA. Two groups of heifers were inoculated with commercial vaccines according to manufacturers’ instructions. A control group was included. The immune response of vaccinated heifers and controls was followed for 6 months.

Results—Detergent solubilized C fetus antigens resulted in better ELISA performance than other antigen preparations. Antigens were optimally immobilized at neutral pH and low ionic strength. All antigen preparations saturated the well with the same amount of protein. The vaccination schedule that advised a booster resulted in higher antibody titers, which were sustained over a longer period than the other schedule.

Conclusions and Clinical Relevance—In the vaccination of cattle against C fetus, the ELISA we have developed may be used to evaluate serum antibody concentrations in response to various vaccines and vaccination schedules. Our results indicate that it is advisable to include a booster in the immunization protocol. (Am J Vet Res 2002;63:586–590)

Bovine genital campylobacteriosis is a venereal disease; the causal agent is Campylobacter fetus, a bacterium with pronounced tropism for the genital system of cattle. In cows it causes infertility, early embryonic mortality, fever, and abortion, resulting in loss of production and breeding inefficiency. The prepuce, glans penis, and urethra of asymptomatic carrier bulls are the natural reservoirs of the bacterium.

The ability of C fetus to produce the disease appears to be associated with surface proteins or S-layer. Strains possessing this S-layer are resistant to the natural bactericidal activity present in serum, phagocytosis, and killing by neutrophils. Failure of C3b to bind to the S-layer explains this resistance. Phagocytosis following opsonization with specific antibodies is the only effective effector mechanism that kills this bacterium.

In countries such as Australia, United States, Argentina, and Uruguay where natural service is the main breeding method, efforts to control the disease are focused on interruption of the transmission cycle. Previous research has revealed the efficiency of vaccination against C fetus, which is a safe and effective procedure for treatment and prevention of the disease.

Various immunoassays have been developed for detection of antibodies against C fetus in vaginal mucus and serum in an attempt to diagnose the infection. Vaccination for campylobacteriosis does not interfere with detection of IgA by ELISA because only IgG is present in the vaginal mucus of vaccinates.

In our experience, the use of the common immunoassays can be problematic. Agglutination assays are of low sensitivity and rely on subjective interpretation of the results. The most commonly used ELISA has a high nonspecific signal or provides results that are not reproducible. Whole crude bacterial extract is used, and this may explain their poor specificity and sensitivity. Currently, there is no reliable technique for determining serum antibody concentrations after vaccination against C fetus or for evaluating the efficiency of the various vaccines and inoculation schedules.

The purpose of the study presented here was to develop a reliable ELISA by using a unique antigen preparation as a tool for serum IgG quantification after vaccination against C fetus in cattle. The ELISA that was developed was also used to evaluate various aspects of C fetus vaccines, including efficiency, formulation, and inoculation schedules.

Materials and Methods

Antigen preparations—Campylobacter fetus ssp. venerealis strain 059 and C fetus ssp. fetus strain 01198 were isolat-
The proteins were fixed with glutaraldehyde and centrifugation at 5,000 g. The supernatant was clarified by centrifugation at 100,000 g for half an hour and lyophilized and studied as antigen 1.

For preparation of antigen 2, the cell pellet obtained in the clarification process was suspended in PBS solution (pH 7.2) and centrifuged at 20,000 g for half an hour. The resulting supernatant was studied as antigen 2.

For preparation of antigen 3, the remaining cell pellet after obtaining antigen 1 and 2 was suspended in a solution containing 0.1 M glycine buffer (pH 9.0), 0.2 M NaCl, and 1% sodium deoxycholate (20 ml of buffer/g of cell pellet) for half an hour at 37 C. After centrifugation at 5,000 g, the cell pellet was discarded, and the supernatant was clarified by centrifugation at 100,000 g and studied as antigen 3.

Antigen 4 preparation involved centrifugation of a second aliquot of the initially pooled suspension of cells at 10,000 g for half an hour. The cell pellet was suspended in solution containing 0.1 M glycine buffer (pH 9.0), 0.2 M NaCl, and 1% sodium deoxycholate (20 ml of buffer/g of cell pellet) for half an hour at 37 C. After centrifugation at 5,000 g, the cell pellet was discarded, and the supernatant was clarified by centrifugation at 100,000 g and studied as antigen 4.

For preparation of antigen 5, a third aliquot of the initially pooled suspension of cells was disrupted by sonication (3 cycles of 30 seconds each in an ice bath). The suspension of disrupted cells was centrifuged; the supernatant was centrifuged; the supernatant was studied as antigen 5.

**Protein determination in antigen preparations**—The bicinchoninic acid method was used to determine protein concentration, and *bovine serum albumin (BSA)* was used as the standard. The amount of antigen 1 was determined as dry weight after lyophilization.

**Electrophoresis and immunoblotting**—Electrophoretic and immunoblotting techniques were used to monitor reactivity of the serum samples in the ELISA to confirm or check ELISA results. *Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)* was performed according to Laemmli in 8% polyacrylamide gel. The proteins were fixed with glutaraldehyde and silver stained. Another gel was run in identical conditions and blotted onto nitrocellulose sheet by the semi dry method according to Kylshe-Anderson. The sheet was blocked with 1% BSA in PBS solution for 1 hour. During this procedure incubations were done at approximately 20 C. In each step, the washing process was repeated 3 times with 0.05% Tween 20 in PBS solution. After blocking, the sheet was washed. Strips of the nitrocellulose sheet were incubated for 90 minutes with a pooled serum sample from 5 vaccinated heifers (positive pooled serum sample) and a pooled serum sample from 5 healthy unvaccinated heifers (negative pooled serum sample). One strip of each antigen was not incubated with serum samples, thereby serving as control.

The strips were washed and immediately incubated for 60 minutes with rabbit anti-bovine IgG antibodies. After washing, the strips were incubated with alkaline phosphatase and conjugated to streptavidin for 30 minutes. Finally, they were carefully washed and incubated for 20 minutes with the enzyme substrate 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium. The reaction was stopped with distilled water.

**Antiserum**—Bovine IgG was purified by chromatography on a protein G matrix; its purity was controlled by SDS-PAGE. Antiserum to bovine IgG was raised in two 3-kg New Zealand rabbits. An ID injection at day 0 was done as primer (300 mg of protein in Freund complete adjuvant per rabbit). Injections were administered IM on days 30 and 45 (same quantity of protein as primer but in Freund incomplete adjuvant). Rabbits were bled 10 days after the last IM injection. The pooled antiserum was fractionated by ammonium sulfate. The immunoglobulin fraction was applied to a column of immobilized bovine IgG on CNBr-activated Sepharose. Protein was eluted at pH 2.5, dialyzed, and conjugated to biotin (succinimidyl ester) in carbonate-bicarbonate buffer pH 8.3, according to Haugland and You, or to peroxidase by the periodate method.

**Enzyme immunoassay optimization**—The principal reagents used for the ELISA were polystyrene plates, rabbit anti-bovine IgG antibodies conjugated to peroxidase and 3-dimethylaminobenzoic acid, 3-methyl-2-benzothiazolone hydrazone, and hydrogen peroxide as enzyme substrate.

**Determination of the optimal conditions for antigen adsorption**—Adsorption was studied in 3 buffers (acetate, pH 4.7; HEPES, pH 7.0; and glycine, pH 9.2) at low (10 mM) and high (150 mM) ionic strength. High ionic strength was obtained by adding NaCl to the corresponding low ionic strength buffers to reach the desired ionic strength value. Adsorption was performed by dissolving each antigen preparation in the aforementioned buffers at 5 mg/ml, followed by incubation for 2 hours at 37 C.

Remaining free sites on the polystyrene plates were blocked with 1% BSA in PBS solution for 1 hour at approximately 20 C. After washing, the plates were incubated with positive and negative pooled serum samples (diluted 1 to 1,000 in 0.05% Tween 20 in PBS solution) for 90 minutes at approximately 20 C and then washed with 0.05% Tween 20 in PBS solution. To each plate a predetermined working dilution of a rabbit anti-bovine IgG antibodies conjugate was added. After incubation for 60 minutes at approximately 20 C, the plates were washed. A streptavidin-peroxidase conjugate antiantibody was added and incubated for 90 minutes at approximately 20 C and washed again. Finally, the plate was incubated with the enzyme substrate for 20 minutes, and the optical density (OD) at 600 nm was measured. The assay was repeated 3 times on 3 plates for antigens 1, 3, 4, and 5. In each plate, pooled serum samples for each condition of adsorption were studied in triplicate. Positive and negative pooled serum samples were thus assayed 9 times with each antigen.

**Determination of the maximum amount of antigen per well**—The peroxidase saturation technique was used to determine the maximum amount of antigen adsorbed per well at the pH and ionic strength selected in the previously described assay. Briefly, 3 plates were coated with antigens 3, 4, and 5, respectively, for 2 hours at 37 C. Concentrations of each antigen from 0.3 to 40.0 mg/ml were applied to each plate in triplicate. Then a peroxidase solution (100 mg/ml of PBS solution) was added to all the wells and incubated for 60 minutes at 37 C and washed with 0.05% Tween 20 in PBS solution. The enzyme substrate was added for 30 minutes at approximately 20 C; the OD was measured at 600 nm. A graph of the amount of antigen added versus mean OD (at 600 nm) was plotted for each antigen (n = 3 for each data point). For each branch of the curve linear regression was applied, and the maximum amount per well was derived from the intersection of both regression lines.

**Data treatment**—To determine the optimum antigen, pH, and ionic strength conditions for adsorption and to max-
Imimize the signal-to-noise ratio to minimize the effect of non-specific interactions, the variable \( I \) was used as follows:

\[
I = \frac{ODS_+ - ODS_-}{ODS_+}
\]

where \( ODS_+ \) is the mean value (\( n = 9 \)) of OD obtained for the positive pooled serum sample (pooled serum sample from 5 vaccinated heifers), and \( ODS_- \) is the mean value of OD (\( n = 9 \)) obtained for the negative pooled serum sample (pooled serum sample from 5 healthy unvaccinated heifers). \( I \) is a measure of nonspecific interactions. The smaller the value of \( I \), the greater the nonspecific interactions.

Vaccination protocol—Two groups of 10 virgin Hereford heifers were vaccinated with 2 (vaccine A and B) oil-based commercial vaccines according to manufacturers’ specifications and schedules. Group-1 heifers were inoculated with a single dose of vaccine A (5 ml) on day 0. Group-2 heifers were primed with a dose of vaccine B (5 ml) on day 0, and a booster was administered on day 22. A control group of 6 heifers that were not vaccinated was included. The heifers were bled at day 0 and then twice a month for 6 months. The same bleeding schedule was applied to the control group. The humoral immune response of vaccinated heifers was followed by use of the optimized ELISA. Each serum sample was assayed in triplicate and on 2 plates (\( n = 6 \)). A standard curve was used in each plate by use of a pooled serum sample obtained from vaccinated cows at various stages in the immune response (4 blood collections per cow). Concentration of the standard was assayed in quadruplicate. One arbitrary unit (AU) was defined as the concentration of antibodies in the 1 to 1000 dilution of the standard. For each serum sample, the mean OD of 6 assay results was interpolated in the standard curve of each plate, and the titer for each sample was expressed in AU. Serum assay results with a coefficient of variation of \( \geq 10\% \) in OD were not included.

Additional studies—Additional ELISA were done to compare reproducibility of results by use of antigen 4 with that by use of antigen 5. A positive pooled serum sample (pooled serum sample from 5 vaccinated heifers) and a negative pooled serum sample (pooled serum sample from 5 healthy unvaccinated heifers), both diluted 1 to 8,000, were used in 5 plates in triplicate for each antigen. Serum samples of 4 vaccinated heifers and a healthy heifer that was not vaccinated were also used and diluted 1 to 1,500 in 3 plates in triplicate. A standard curve was used in each plate by use of the same serum samples described for the vaccination protocol.

Studies on a naturally infected bull—The serologic immune status of a naturally infected bull (confirmed by pathogen isolation from prepuce) was also studied. Immunoblotting and ELISA were performed as described. Samples were collected by prepuce scraping and placed in a transport enrichment medium containing equine serum and antibiotics. The samples were maintained in this medium for 3 days at 37 C. Then they were cultured in Columbia blood agar in 5% oxygen, 10% carbon dioxide, and 85% nitrogen atmosphere at 37 C for 5 days. Campylobacter colonies were observed under optical microscopy.

Results

The SDS-PAGE—For all antigen preparations, most of the bands on SDS-PAGE appeared in the 40 to 130 kd range. Antigen 1 had the lowest number of bands of proteins, whereas antigens 3, 4, and 5 had the maximum number, mainly between 50 and 100 kd. Antigen 2 had a mixed pattern of bands (Fig 1). Proteins that are usually described\(^{20,21}\) for \( C \) fetus were observed from all antigen preparations at 98 and 127 kd. The study of antigen 2 was discontinued because it appeared to be a mixture of surface proteins (shed antigens) and the total extract.

Immunoblotting—In this assay, the positive pooled serum sample contained antibodies that specifically recognized the antigens preparations, particularly bands at 98 and 127 kd (Fig 2). Neither the negative pooled serum sample nor the serum of the naturally infected bull showed any bands.
infected bull (data not shown) contained antibodies capable of specific recognition.

The ELISA optimization—The variable \( I \) was used to determine the best antigen preparation and the optimal pH and ionic strength for its adsorption. Antigen 3, 4, and 5 had higher \( I \) values than antigen 1. Mean \((\pm SD)\) values for antigens 3, 4, and 5 were similar and overlapped (Table 1). In accordance with these results, the maximum amount of protein per well was determined only for antigens 3, 4, and 5. The maximum amount of protein immobilized for all 3 antigen preparations was achieved with a concentration of \(11.0 \pm 0.5\) mg/ml by using low ionic strength HEPES buffer (pH 7.0) and the coating solution.

Immune response of vaccinated animals—Antigen 4 was used in all ELISA at 11 mg/ml of low ionic strength HEPES buffer (pH 7.0) for the study of the immune response of all the cattle (vaccinated heifers, nonvaccinated heifers, and infected bull). The time course after vaccination of mean antibody titer in AU for group-1, group-2, and control-group heifers by use of antigen 4 was determined (Fig 3-5). The ELISA results provided excellent within- and between-run precision reproducibility. In addition, the slope and the regression constant (linear equation) of the standard curve were reproducible for every plate. Assays results by using antigen 5 did not have the reproducibility of those using antigen 4 (Table 2). The ELISA cut off value for antibody titers against \(C\) fetus (antigen 4) was 25 AU. Adding 2 SD to the mean titer of serum samples from 36 seronegative cattle determined the cut off value for seropositivity.

Immune status of naturally infected bull—Results of immunoblot studies and ELISA on serum samples from the infected bull indicated that the bull was seronegative (ie, contained no specific anti-\(C\) fetus antibodies). The value obtained by ELISA was 6 AU (data not shown).

<table>
<thead>
<tr>
<th>Antigen No.</th>
<th>Buffer type</th>
<th>(150 \text{ mM} )</th>
<th>(10 \text{ mM} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Glycine (pH 9.2)</td>
<td>0.77 ± 0.069</td>
<td>0.70 ± 0.073</td>
</tr>
<tr>
<td></td>
<td>HEPES (pH 7.0)</td>
<td>0.90 ± 0.066</td>
<td>0.78 ± 0.077</td>
</tr>
<tr>
<td></td>
<td>Acetate (pH 4.7)</td>
<td>0.79 ± 0.079</td>
<td>0.62 ± 0.091</td>
</tr>
<tr>
<td>3</td>
<td>Glycine (pH 9.2)</td>
<td>0.91 ± 0.09</td>
<td>0.92 ± 0.091</td>
</tr>
<tr>
<td></td>
<td>HEPES (pH 7.0)</td>
<td>0.93 ± 0.096</td>
<td>0.92 ± 0.092</td>
</tr>
<tr>
<td></td>
<td>Acetate (pH 4.7)</td>
<td>0.92 ± 0.091</td>
<td>0.93 ± 0.093</td>
</tr>
<tr>
<td>4</td>
<td>Glycine (pH 9.2)</td>
<td>0.95 ± 0.095</td>
<td>0.93 ± 0.084</td>
</tr>
<tr>
<td></td>
<td>HEPES (pH 7.0)</td>
<td>0.96 ± 0.075</td>
<td>0.96 ± 0.086</td>
</tr>
<tr>
<td></td>
<td>Acetate (pH 4.7)</td>
<td>0.96 ± 0.075</td>
<td>0.95 ± 0.085</td>
</tr>
<tr>
<td>5</td>
<td>Glycine (pH 9.2)</td>
<td>0.91 ± 0.100</td>
<td>0.91 ± 0.109</td>
</tr>
<tr>
<td></td>
<td>HEPES (pH 7.0)</td>
<td>0.80 ± 0.099</td>
<td>0.91 ± 0.090</td>
</tr>
<tr>
<td></td>
<td>Acetate (pH 4.7)</td>
<td>0.91 ± 0.100</td>
<td>0.90 ± 0.108</td>
</tr>
</tbody>
</table>

\(*I\) is a measure of nonspecific interactions. The smaller the value of \(I\), the greater the nonspecific interactions. \(I\) values are reported as \(\leq 1.00\).
Discussion

The ELISA developed in our study may be used as a tool to quantify serum IgG concentrations as a means of monitoring response to vaccination against \textit{C. fetus} in cattle. Of the antigens we prepared, we determined the best immobilized antigen to be used in the ELISA. In our study, commonly used methods to obtain antigen preparations (ie, use of shed antigens or whole cell sonication) were associated with serious disadvantages. Shed antigens are of interest, because they originate from the external cell layer, toward which the immune response will be preferentially directed. Preparation of shed antigens is also easy and inexpensive. But in our study, ELISA using shed antigens had the highest nonspecific signal, and this dramatically affected the sensitivity of the assay. The ELISA using a total extract of cells obtained by sonication yielded results that were not reproducible (Table 2); such extracts contain products of degradation of nucleic acids and other cellular components that are the source of random variations in nonspecific signals.

We found that using antigen preparations obtained from detergent extraction (ie, antigens 3 and 4) provided similar reliable ELISA results (antigen 3 does not include shed antigens). On the basis of this finding, we recommend working with detergent (deoxycholate) extracts of whole cells as an easy way to obtain reproducible assay results. For a given antigen, we found no variation in nonspecific interactions associated with pH or ionic strength at adsorption; therefore, we chose to use HEPES buffer (pH 7) at low ionic strength.

On the basis of findings on electrophoresis and immunoblotting, antigen 1 had a clearly unique protein and immune recognition pattern, which corresponded with a weak ELISA result for the positive pooled serum sample. Antigen 2 was eliminated from our study because of its SDS-PAGE results and immunoblot pattern that suggested it was a mixture of shed and somatic antigens.

On immunoblot testing the main IgG specific activity was observed at 97 and 128 kd for our antigen preparations. Previous reports\(^3\)\(^4\) suggest that 97 and 128 kd proteins share biochemical characteristics and that they elicit strong humoral antibody responses. If this is so, the differences in ELISA results may be caused by other components (nucleic acids, lipids, carbohydrates) included in each antigen preparation, the nature of which is dependent on the methods used to prepare them. Most of the nonspecific signals and nonreproducible results may be attributed to these other components.

We tested the effectiveness of 2 commercial oil-based vaccines, using our optimized ELISA. Oil-based vaccines generally produce a stronger humoral response than those that contain adjuvants such as aluminium hydroxide. According to the manufacturers’ recommendations, vaccine A was administered to cattle once, and vaccine B was administered twice at a 20-day interval. Our ELISA results indicate that the use of vaccine B provided higher serum antibody concentrations for a longer period (between 33 and 80 days after first vaccination), compared with vaccine A. The use of vaccine A did not result in a clearly safe concentration of serum antibodies over a long period. On the basis of our findings, it is advisable to include a booster in the immunization protocol in cattle for \textit{C. fetus}.

\(^{1}\)ICN, Lisle, Ill.
\(^{2}\)Sigma-Aldrich, St Louis, Mo.
\(^{3}\)Pierce Chemical Co, Rockford, Ill.
\(^{4}\)Merck, Darmstadt, Germany.
\(^{5}\)HiTrap Protein G Column, Amersham Pharmacia Biotech, Piscataway, NJ.
\(^{6}\)CNBr-activated Sepharose 4 Fast Flow column, Amersham Pharmacia Biotech, Piscataway, NJ.
\(^{7}\)Maxisorb, Nunc, Naestrup, Denmark.
\(^{8}\)Multispec Plus MCC340, Titertek, Huntsville, Ala.

References