Enzyme-Linked Immunosorbert Assay for Detection of Serum Antibodies to Pasteurella haemolytica Cytotoxin (Leukotoxin) in Cattle†

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An enzyme-linked immunosorbert assay (ELISA) was developed for detection of bovine serum antibodies to the cytotoxin (leukotoxin) of Pasteurella haemolytica. A partially purified, cytotoxic, immunogenic protein obtained from supernatants of logarithmic-phase P. haemolytica was used as the ELISA antigen. Preabsorption of sera with various cytotoxic, somatic, and capsular antigen preparations demonstrated that the assay was specific for anticytotoxin antibodies. ELISA anticytotoxin titers had a strong, significant correlation to cytotoxin-neutralizing-antibody titers. The ELISA, however, was more rapid and allowed for greater numbers of samples to be run than did the neutralization technique. ELISA anticytotoxin titers were high in cattle vaccinated with a live P. haemolytica vaccine, whereas unvaccinated cattle and cattle receiving a P. haemolytica bacterin had low ELISA anticytotoxin titers. A significant positive correlation between ELISA titers and resistance to experimental bovine pneumatic pasteurellosis was present.

Bovine pneumatic pasteurellosis is a severe fibrinous pneumonia of feedlot cattle usually associated with infection by Pasteurella haemolytica biotype A, serotype 1 (5, 16, 17). Various approaches used to prevent the disease have proven largely unsuccessful, and pneumatic pasteurellosis continues to be a major problem for the feedlot industry (16, 19).

Recent studies have concentrated on defining the role played by humoral immunity in resistance to pneumatic pasteurellosis. Clinical use of P. haemolytica bacterins has not proven effective (1, 18, 20). Cattle given bacterins developed an antibody response to somatic antigens, but this response did not consistently result in protection against P. haemolytica challenge (8, 14). Live P. haemolytica vaccines given by aerosol or parenteral routes, however, resulted in both increased antibody titers to somatic antigens and enhanced resistance to experimental challenge (22). Similarly, enhanced resistance to challenge was noted in calves with prior natural exposure to the organism (9). Further studies with live P. haemolytica vaccines suggested a possible protective role for capsular antigens (8). Others have indicated that a neutralizing antibody to a P. haemolytica cytotoxin (leukotoxin) may play a role in increased protection against pneumatic pasteurellosis (4, 14, 24).

Cytotoxin has been incriminated in the pathogenesis of pneumatic pasteurellosis through its toxicity for ruminant alveolar macrophages and peripheral blood leukocytes (2, 23). Antibody to cytotoxin has previously been measured only by labor-intensive cytotoxin neutralization assays. The purposes of this study were to develop an enzyme-linked immunosorbert assay (ELISA) to detect serum antibody to partially purified cytotoxin, to compare antibody responses detected by ELISA to those detected by neutralization assays, and to determine the potential of the ELISA as a predictor of resistance to experimental pneumonic pasteurellosis.

MATERIALS AND METHODS

Serum samples. Serum samples were obtained from 5- to 8-month-old calves which had been used in previous experiments to evaluate the effects of various vaccines on resistance to pneumatic pasteurellosis (9, 10, 22). On days 0 and 7, each calf was vaccinated subcutaneously with phosphate-buffered saline (PBS), a bacterin consisting of 10⁸ CFU of Formalin-killed P. haemolytica in an aluminum hydroxide adjuvant, or 5 × 10⁸ CFU of live P. haemolytica (10, 11, 22). Serum samples were collected on days 0, 7, 14, and 21 and stored at −20°C. On day 21, calves were experimentally challenged by transtracheal injection with 5 ml of a P. haemolytica suspension in PBS containing approximately 10⁸ CFU/ml (21). On day 25, calves were sacrificed, and lung lesions were evaluated to determine the extent of lung resistance to challenge exposure (22). Numerical scores were awarded on the basis of morphologic criteria, which included the size of the lesion and the degree of extension of inflammation from the original lesion site. A maximum score of 20 represented a severe lesion and lack of resistance, whereas lower scores corresponded to increased resistance. A total of 12 calves from each vaccination group were used in the study.

Serologic evaluation. Antibodies to somatic antigens of P. haemolytica were determined by a quantitative fluorometric immunoassay (FIAX; International Diagnostic Technology, Santa Clara, Calif.) with Formalin-killed 22-h cultures of P. haemolytica serotype 1 as the antigens (6).

Serum cytotoxin neutralization titers were determined as previously described (4, 14, 24). In general, these determinations were made by preincubation of serial twofold dilutions of test sera with crude cytotoxin, followed by
cytotoxicity assays that measured cell viability as ⁵¹Cr release from bovine leukocytes.

**Anticytotoxin ELISA.** The antigen used in the ELISA was a partially purified cytotoxic protein obtained from supernatants of logarithmic-phase cultures of *P. haemolytica* (D. A. Mosier, B. A. Lessley, A. W. Confer, S. M. Antone, and M. J. Gentry, submitted for publication). The protein was separated from crude *P. haemolytica* supernatants by anion-exchange chromatography, gel filtration, and chromatofocusing and was designated as partially purified cytotoxin. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting of partially purified cytotoxin revealed two antigenic bands when the cytotoxin was reacted with bovine antisera to live *P. haemolytica* which had been adsorbed with Formalin-killed bacteria to remove antibody to capsular and somatic antigens.

Optimal conditions for the ELISA were determined from preliminary experiments with antigen coating concentrations of 0.01, 0.1, or 1 µg of antigen per well, serum dilutions of 1:50 to 1:1,600, and conjugate dilutions of 1:200 and 1:400.

For routine assays, 100 µl of antigen diluted to 10 µg/ml in carbonate buffer (pH 9.6) was placed in each well of 96-well polystyrene plates (Nunc, Roskilde, Denmark), and the plates were then incubated overnight at room temperature on a rocker platform. The plates were washed three times with PBS containing 0.05% Tween 20 (PBS-Tween). For each sample, 100 µl of a 1:400 serum dilution in PBS-Tween containing 1% bovine serum albumin was added to duplicate wells and incubated for 1 h. Following three washes with PBS-Tween, 100 µl of a 1:200 dilution of horseradish peroxidase-conjugated, affinity-purified, anti-bovine immunoglobulin G in PBS-Tween–1% bovine serum albumin was placed in each well. After 1 h of incubation, the plates were washed six times with PBS-Tween. For color development, 100 µl of substrate containing phenylenediamine and hydrogen peroxide in phosphate-citric acid buffer was added to each well, and the plates were incubated for 45 min in the dark. The reaction was stopped by the addition of 50 µl of 2.5 M H₂SO₄ to each well, and the A₄₉₀ was determined on a manual ELISA reader (Bio-Tek, Burlington, Vt.). The reported A₄₉₀ for each sample was the average of duplicate wells. To compare samples from different plates, we standardized A₄₉₀ readings on the basis of a positive control (serum from a steer hyperimmunized with live *P. haemolytica*) and a negative control (PBS), both included on each plate.

**Adsorption experiments.** Selected sera were used to study the specificity of the ELISA by preadsorption of the sera with several *P. haemolytica* antigens. For each sample, 100 µl of serum was diluted 1:200 in PBS-Tween–1% bovine serum albumin and then incubated with an equal volume of partially purified cytotoxin, cytotoxicity supernatant from *P. haemolytica* (15), Formalin-killed *P. haemolytica*, or PBS. Cytotoxin and capsular extract were used in concentrations of 1,000, 100, 10, and 1 µg/ml, whereas Formalin-killed bacteria were used at 10⁷, 10⁶, and 10⁵ CFU equivalents, as determined spectrophotometrically (6). Mixtures were incubated overnight at 4°C on a rocker platform in 96-well, round-bottom tissue culture plates (Corning Glass Works, Corning, N.Y.). Adsorbed sera were then assayed by the ELISA as described above.

**Statistical evaluation.** Anticytotoxin antibodies were quantitated by the ELISA. These values were then compared with FIAx (somatic antigen) titers, cytotoxin neutralization titers, and lesion scores. Linear correlation among these variables was evaluated by the Pearson product-moment, Spearman, and Kendall tau correlations (Statistical Analysis System, Cary, N.C.). Group means for the different vaccines were compared by multiple t tests. A t test for equal and unequal variances was calculated for the mean titers for each of the comparisons. An F statistic was calculated to determine whether unequal variances were present. If the probability of F was <0.05, unequal variances were used in calculating t test values.

**RESULTS**

Serial twofold dilutions of four bovine serum samples from 1:50 to 1:1,600 gave absorbance readings typical of an antibody response curve (Fig. 1). Preadsorption of one of the serum samples with PBS or various concentrations of a saline extract of *P. haemolytica* or Formalin-killed *P. haemolytica* had no effect on the cytotoxin antibody response. Preadsorption of the same serum sample with cytotoxin, however, resulted in dramatic decreases in the cytotoxin antibody response at the two highest concentrations of cytotoxin (Fig. 2).

In calves vaccinated with live *P. haemolytica*, there was a marked increase in ELISA-detected antibody to cytotoxin (ELISA cytotoxin antibody) (Fig. 3). From days 0 to 21, the bacterin group had a small, insignificant (P > 0.05) increase in the ELISA cytotoxin antibody response as compared with the PBS group. During the same period, the ELISA cytotoxin antibody response for the PBS group remained relatively constant. On days 7, 14, and 21, a significant difference (P < 0.05) was seen between the responses of the live-vaccine group and both the bacterin and PBS groups. No significant difference (P > 0.05) was present between the mean antibody responses for the bacterin and PBS groups on any day.

The means and standard deviations for day-21 samples for the ELISA cytotoxin antibody response, cytotoxin neutralization titer, FIAx titer, and lesion score for each vaccine group are given in Table 1. t tests between these three groups showed a significant difference (P < 0.0001) between the means regardless of which of the four parameters was used as the dependent variable. The results of t tests between groups for each parameter indicated that ELISA cytotoxin antibody responses and cytotoxin neutralization titers for the live-vaccine group were significantly different (P < 0.05) from those for both the bacterin and PBS groups. A significant difference was not detected between bacterin and PBS group mean responses. For FIAx titers and lesion scores, a
significant difference ($P < 0.05$) was present between the means of all three groups.

Correlation coefficients ($r$) and probabilities ($P$) for all samples organized by parameter are given in Table 2. Correlations of ELISA cytotoxin antibody responses with cytotoxin neutralization titers, FIAX titers, and lesion scores were all significant at $P < 0.01$. The strongest correlation was between the ELISA cytotoxin antibody response and cytotoxin neutralization titers, whereas the lowest was between the ELISA cytotoxin antibody response and FIAX titers. As an indicator of resistance to pneumonic pasteurellosis, the ELISA correlated better with lesion scores than did the FIAX. Cytotoxin neutralization titers had the best correlation with lesion scores of all the parameters examined. Significant correlations were not obtained between any parameters when samples were evaluated within individual vaccine groups.

**DISCUSSION**

The result of this study was the development of a primary binding immunoassay (ELISA) which is suitable for screening large numbers of bovine serum samples for antibody to *P. haemolytica* cytotoxin. Previously reported ELISAs for *P. haemolytica* have used sodium salicylate extracts (3, 12), KSCN extracts (26), or saline extracts (7) of *P. haemolytica* as antigens. The assay in the current report is the first direct binding assay that uses a partially purified cytotoxic *P. haemolytica* antigen. The ELISA results provide information similar to that obtained by cytotoxin neutralization while eliminating the need for serial dilutions of sera, preparation of living cells, and use of radioisotopes.

Preadsorption studies with various *P. haemolytica* antigens demonstrated the specificity of the ELISA reaction for cytotoxin. When preincubated with high concentrations of cytotoxin, antigen-binding sites on immunoglobulins which were specific for cytotoxin apparently became saturated, resulting in a marked decrease in the ELISA absorbance. In contrast, high concentrations of Formalin-killed organisms or saline extracts of *P. haemolytica* had only minimal effects on the ELISA absorbance. These results suggest that cytotoxin is not associated with structural components of the bacteria and further supports its classification as a true exotoxin (25).

Evaluation of the cytotoxin-neutralizing ability of sera from feedlot cattle demonstrated significantly lower cytotoxin-neutralizing activity in the sera of cattle that died of fibrinous pneumonia than in the sera of cattle that died for other reasons (24). Similar associations between pneumonia and cytotoxin neutralization were found in studies of experimentally induced disease (4, 14). A direct positive correlation was demonstrated between resistance to experimental challenge with *P. haemolytica* and serum cytotoxin neutralization titers (14). As determined by a modified indirect complement fixation test, however, antibodies to somatic antigens of *P. haemolytica* appeared to be unrelated to the development of pneumonia (4).

The results of this study support these findings and suggest that exposure to live organisms or vaccines containing cytotoxic antigens may be necessary to produce an anticytotoxic immune response and that this response is a better predictor of resistance to pneumonia than the immune response to somatic antigens. ELISA cytotoxin antibody responses and cytotoxin neutralization titers for the live-vaccine group were both significantly higher than those for the bacterin group. In association with this, mean lesion scores for the live-vaccine group were significantly lower

![Antigen/Bacteria Concentration](antigen_bacteria_concentration.png)  
**FIG. 2.** $A_{490}$ of anticytotoxic bovine serum samples following adsorption with four concentrations of various *P. haemolytica* antigens. A. Nonadsorbed; B. Formalin-killed *P. haemolytica*; C. saline extract of *P. haemolytica*; D. cytotoxin.

![Absorbance at 490 nm](absorbance_490nm.png)  
**FIG. 3.** Mean $A_{490}$ and standard deviations of serum samples from calves receiving live vaccine (A), bacterin (B), or PBS controls (C).
TABLE 1. Antibody responses and lesion scores for cattle challenged with P. haemolyticaa

<table>
<thead>
<tr>
<th>Group</th>
<th>ELISA cytotoxin antibody response (A490)b</th>
<th>Cytotoxin neutralization titerc</th>
<th>FIAX titerd</th>
<th>Lesion scored</th>
</tr>
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<tbody>
<tr>
<td>PBS</td>
<td>0.39 ± 0.18</td>
<td>6.4 ± 5.1</td>
<td>28.5 ± 15.1</td>
<td>16.9 ± 4.5</td>
</tr>
<tr>
<td>Bacterin</td>
<td>0.30 ± 0.10</td>
<td>8.5 ± 5.2</td>
<td>102.7 ± 88.1</td>
<td>10.3 ± 5.4</td>
</tr>
<tr>
<td>Live vaccine</td>
<td>0.70 ± 0.22</td>
<td>88.0 ± 36.4</td>
<td>173.2 ± 46.7</td>
<td>3.7 ± 2.5</td>
</tr>
</tbody>
</table>

a All antibody titer were detected on day 21 after the initial vaccination.
b Arithmetic mean.
c Geometric mean.
d Higher scores indicate a greater susceptibility to challenge.

(indicating greater resistance) than those for controls or the bacterin group. The most significant correlation with lesion scores was obtained with cytotoxin neutralization titer, suggesting this to be the test of choice in experimental trials with small sample sizes. For rapid analysis of large numbers of serum samples, however, the ELISA is an excellent alternative.

The relationship between the antigens of P. haemolytica and resistance to disease is becoming more clearly defined. Bacterins stimulate a somatic antibody response which does not consistently provide protection from disease and in some cases has been incriminated in enhancing disease (13, 27). In the current study, there was a significant reduction in the lesion scores of bacterin-vaccinated animals as compared with controls, however, indicating that some degree of protection may have been afforded by antibodies to somatic antigens. Alternatively, this reduction may have been caused by the presence of low levels of anticytotoxin antibodies or antibodies to somatic antigens which cross-react with cytotoxotics. Animals which received live vaccine and possessed high levels of cytotoxin antibodies determined by the ELISA and neutralization assays had lesion scores which were significantly lower (indicating greater resistance) than those for the bacterin group. Although additional protection was associated with increased levels of anticytotoxin antibodies, animals with good neutralization titers or ELISA cytotoxin antibody responses occasionally still developed severe pneumonia lesions. The reasons or factors involved in these cases are unknown.

The pathogenesis of pneumonia pasteurellosis is complex. A successful product for immunologic prevention of the disease will most likely have to take into account multiple antigenic features of Pasteurella spp. The ELISA described in this report provides a helpful tool for more clearly defining the role played by cytotoxin in pneumonia pasteurellosis.

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LITERATURE CITED