Blocking Enzyme-Linked Immunosorbent Assay for Detection of Antibodies to *Actinobacillus pleuropneumoniae* Serotype 2

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Received 9 November 1990/Accepted 21 January 1991

A blocking enzyme-linked immunosorbent assay (ELISA), based upon a polyclonal rabbit antiserum specific to *Actinobacillus pleuropneumoniae* serotype 2, was developed for the detection of antibodies to *A. pleuropneumoniae* serotype 2 in pigs. By testing sera from pigs experimentally infected with the 11 recognized serotypes of *A. pleuropneumoniae*, the assay was proven to be specific for *A. pleuropneumoniae* serotype 2. With field sera from herds infected with *A. pleuropneumoniae* serotype 2, the assay was found to be more sensitive than the complement fixation test. Positive results were not observed with field sera from herds known to be free from *Actinobacillus* infection or with sera from two herds infected with either *A. pleuropneumoniae* serotype 6 or 8. The high diagnostic sensitivity and specificity of the blocking ELISA will make it useful in field diagnostic work.

*Actinobacillus pleuropneumoniae* is the cause of a contagious pleuropneumonia in swine which has become a major problem in the swine industry throughout the world. The economic losses due to *A. pleuropneumoniae* infection are results of deaths caused by acute illness and of lowered production in chronically infected herds (11). Until now, 12 serotypes have been recognized. The prevalence of the different types varies in different countries. Thus, in continental Europe and Japan, serotype 2 is the dominating serotype (2, 5, 8), and in Canada the incidence of this type has increased in recent years (10).

Serology has proven its value in the diagnosis of *A. pleuropneumoniae* infection in pigs and in the control of the disease. The complement fixation (CF) test has been a valuable tool in this context in various countries, but because of its technical complexity, the test is not appropriate for use in all diagnostic laboratories.

Work done in Switzerland and Canada (6, 12) has indicated a potential use for different indirect enzyme-linked immunosorbent assays (ELISAs) as alternatives to the CF test for detection of *A. pleuropneumoniae* infection. Thus, comparative analyses, based on sera from pigs infected with *A. pleuropneumoniae*, have shown good correlations between the results obtained with one of the indirect ELISAs and those obtained with the CF test (6).

Several soluble antigen preparations of *A. pleuropneumoniae* have been used in a previously described indirect ELISA with variable results (6). In the present study, the supernatant of heat-treated *A. pleuropneumoniae* serotype 2 cells was used as a coating material for the wells of the microtiter plates used for the blocking ELISA. The detection of antibodies to *A. pleuropneumoniae* serotype 2 was based upon a competitive reaction between the serum to be tested and a polyclonal rabbit antiserum specific for *A. pleuropneumoniae* serotype 2.

**MATERIALS AND METHODS**

**Immunization of rabbits.** A 6-h-old culture of the serotype 2 strain 4226 of *A. pleuropneumoniae* grown on PPLO agar (5) was harvested in phosphate-buffered saline (PBS) containing 0.3% Formalin. Two milliliters of a suspension containing 10⁷ organisms per ml was mixed with Freund incomplete adjuvant (1:1 [vol/vol]) and injected intramuscularly in 6-month-old White Landrace rabbits (mean weight, approximately 3 kg). Starting 2 weeks later, a series of immunizations were performed twice a week by intravenously injecting live *A. pleuropneumoniae* 4226 without adjuvant. The first injection was 0.5 ml of a bacterial suspension containing 10⁷ organisms per ml. The following injections were 0.5 ml of a suspension containing 10⁶ organisms per ml. In the indirect hemagglutination test and gel diffusion assay (9), this antiserum was specific for serotype 2.

**Blocking ELISA.** Antigen for the ELISA was prepared as a supernatant of heat-treated cells (3). A 6-h culture of strain 4226 grown on PPLO agar was washed off the plates with 0.85% saline containing 0.5% Formalin. After overnight storage at 4°C, the concentration of cells was adjusted to 10⁸ cells per ml in Formol saline. The suspension was heated at 100°C for 1 h. The cells were removed by centrifugation, and the supernatant, filtered through 0.22-μm-pore-size membrane filters (sterile Millex 65; Millipore), was used as the antigen.

Checkerboard titrations were performed in ELISA plates to determine the optimal concentrations of antigen, serum, enzyme conjugate, and substrate.

Fifty microliters of the antigen solution diluted 500 times in PBS (pH 7.2) was added to each well of flat-bottom microtiter plates (Immuo Plate II; Roskilde, Denmark). The plates were incubated overnight at 4°C and after that at room temperature for 0.5 h. The plates were blocked with 200 μl (per well) of a blocking buffer consisting of PBS with 0.05% Tween 20 and 1% bovine serum albumin (PBS+T+BSA). Plates were incubated for 1 h at room temperature and washed three times with PBS containing 0.05% Tween 20 (PBS+T). Sera were serially diluted (start dilution, 1:4) in PBS+T+BSA, and 50 μl of diluted serum was added to the wells, which were then incubated at room temperature for 1 h. Fifty microliters of rabbit antiserum to strain 4226 (RA 4226) was then added to each well in an optimal dilution of 1:2,000 in PBS+T+BSA. After 0.5 h at room temperature, the plates were washed three times with PBS+T. After the wash, 50 μl of peroxidase-conjugated swine anti-rabbit immunoglobulin (Dakopatt, Copenhagen, Denmark) diluted
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A. pleuropneumoniae serotype

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enriched
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CF test. Antibodies to A. pleuropneumoniae
erosotypes 2, 6, and 8 in swine sera were
measured by the modified CF test
(1, 7). Test sera were heat treated at 60°C for 30 min.
Normal, unheated serum from 8-week-old pigs free from A.
pleuropneumoniae infection was used in a dilution of 1:150
in Veronal-buffered diluent as the reconstituting fluid for
lyophilized guinea pig complement. Apart from this
modification, the general procedure described in the
Laboratory Branch Complement Fixation Method adapted to a
microtechnique (1) was followed. Five 50% hemolytic units
were used in the test, and the endpoint of serum titration
was the highest dilution showing 30% hemolysis or less as
compared with hemoglobin color standards (0 to 100% hemolysis).

Sera. The sera tested by the blocking ELISA originated
from the following sources.

(i) Pigs experimentally infected with reference strains of A.
pleuropneumoniae. The 11 reference strains representing
serotypes 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, and 12 of A.
pleuropneumoniae used for intranasal inoculation of pigs to
provide reference immune sera were Shope 4074, S1536,
S1421, M62, K17, Femb, WF83, 405, 13261, D13039, and
8329, respectively. Eight-week-old specific pathogen-free
(SPF) pigs were inoculated intranasally with 10^8 live
organisms of an 18-h culture grown on enriched PPD agar. Three
weeks later the pigs were sacrificed and bled. Sera were
stored at -20°C.

(ii) Pigs experimentally infected with the Danish serotype 2
strain 4226 of A. pleuropneumoniae. The serotype 2 strain
4226 of A. pleuropneumoniae originated from a Danish
feeder pig with acute pleuropneumonia. Thirty-two 8-week-
old SPF pigs were inoculated intranasally with 10^8 live
organisms from an 18-h culture of strain 4226. Sera were
collected at sacrifice 3 weeks later. All pigs had lung
necroses at slaughter, and A. pleuropneumoniae serotype 2 was
isolated from the necroses.

(iii) Pigs from a conventional herd without a history of
pleuropneumonia. Forty-eight serum specimens were
collected from 6-month-old pigs from a conventional breeding
herd without a history of pleuropneumonia. At necropsy,
pathological lesions were not observed and A. pleuropneu-
moniae was not isolated. The sera were negative in the CF
test to all serotypes.

(iv) Pigs from two SPF herds. Eighty-three serum speci-
mens were collected from breeding animals in two SPF
herds. Both herds have been monitored for several years for
freedom from A. pleuropneumoniae infection by monthly

blood testing of breeding animals by the CF test. The 83
serum specimens used in this study were negative in the CF
test for all serotypes of A. pleuropneumoniae.

(v) Pigs from herds naturally infected with A. pleuropneu-
moniae serotype 2. Ninety-six field serum specimens were
collected from breeding animals in 11 herds infected with A.
pleuropneumoniae serotype 2 as diagnosed by a herd history
of pleuropneumonia. The clinical diagnosis was confirmed
by the finding of positive reactors to serotype 2 by the CF
test.

(vi) Pigs from herds infected with A. pleuropneumoniae
serotype 6 or 8. Eighteen field serum specimens were
collected from breeding animals in a herd naturally infected
with serotype 6. This serotype was isolated from lung lesions
of pigs submitted for necropsy. The sera were positive for
serotype 6 in the CF test. Eighteen field serum specimens
were collected from breeding animals in a herd naturally
infected with serotype 8. Serotype 8 was isolated from the
lungs of pigs submitted for necropsy. The sera were positive
in the CF test for serotype 8.

RESULTS

Serum specimens from two pigs infected with the serotype
2 reference strain S1536 of A. pleuropneumoniae had a mean
relative absorbance of 10%, whereas the reference sera from
pigs inoculated with serotypes 1, 3, 4, 5, 6, 7, 8, 9, 10, and 12
had a mean absorbance (± standard deviation) of 105% ±
5% (Fig. 1).

Serum specimens from 32 pigs experimentally infected
with A. pleuropneumoniae serotype 2 strain 4226 had a mean
relative absorbance of 10% ± 10%, whereas a group of 48
serum specimens collected from a conventional herd known
to be free from pleuropneumonia and a group of 83 serum

FIG. 1. Analysis by blocking ELISA of 1:4-diluted sera from
pigs intranasally infected with one of the 11 reference strains of A.
pleuropneumoniae. The average A490 obtained for the negative
control sera from noninfected pigs was 2.679. The A490 was signifi-
cantly lowered in sera from pigs infected with A. pleuropneumoniae
serotype 2, whereas none of the sera from pigs infected with the
other serotypes of A. pleuropneumoniae showed any blocking
effect.
DISCUSSION

The blocking ELISA described here was developed for the detection of antibodies to A. pleuropneumoniae serotype 2, which is the dominating serotype in Danish swine herds, causing approximately 70% of diagnosed outbreaks of pleuropneumonia.

Only a few reports on the ELISA as a tool for diagnosis and control of A. pleuropneumoniae infections are available (4, 6, 12), and none of them have used a blocking ELISA.

With the 50% cutoff level, the blocking ELISA with polyclonal rabbit antiserum as a detector allowed for a very specific test while maintaining a sensitivity which was higher than that of the CF test. No cross-reactions to other serotypes of A. pleuropneumoniae were seen.

The specificity and sensitivity of an ELISA using bacterial antigens depend on the quality of the antigen preparation. In two reports (6, 12) EDTA-treated antigen from A. pleuropneumoniae was used for coating wells of microtiter plates in indirect ELISAs. The sensitivity and specificity of these tests was claimed to be sufficient for use in commercial herds as an aid for determining the exposure of pigs to A. pleuropneumoniae. In comparative analyses of the CF test, the indirect ELISA using different antigen preparations, and a modified tube agglutination test performed in the presence of 2-mercaptoethanol, it was found that none of the tests was

![Graph](https://example.com/graph.png)

**FIG. 2.** Comparison by the serotype 2 blocking ELISA of 1:4-diluted sera collected from pigs (n = 32) in an experimental (Expm.) herd infected with A. pleuropneumoniae serotype 2, from pigs (n = 48) in a conventional (Conv.) herd free from infection with A. pleuropneumoniae, and from pigs (n = 83) in two SPF herds in which A. pleuropneumoniae could not be isolated. The mean ± standard deviation of the absorbances of these sera collected from SPF herds free from infection with A. pleuropneumoniae both had mean relative absorbances of 100% ± 10% (Fig. 2).

The specific detection of antibodies to A. pleuropneumoniae serotype 2 by the blocking ELISA was further sustained with field sera collected from pigs infected with either A. pleuropneumoniae serotype 6 or 8, since the relative absorbances of these sera were 100% ± 10%.

The 32 serum specimens collected from pigs experimentally infected with A. pleuropneumoniae serotype 2 were titrated and analyzed by both the CF test for detection of serotype 2 antibodies and the blocking ELISA. A good correlation was observed, but higher titers were observed in the ELISA for the weakly positive sera (Fig. 3).

Of 96 field samples from herds infected with A. pleuropneumoniae serotype 2, 86 (90%) were positive by the blocking ELISA, with a mean relative absorbance of 10% ± 10%. The remaining 10 serum specimens were negative, with a relative absorbance of 96% ± 14%. When the same 96 serum specimens were analyzed by the CF test for detection of serotype 2 antibodies, 67 samples (70%) were seropositive (Table 1).

**TABLE 1.** Detection of antibodies to A. pleuropneumoniae serotype 2 by the CF test and the blocking ELISA

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<thead>
<tr>
<th>ELISA result</th>
<th>No. of CF test results</th>
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<tr>
<td>+</td>
<td>67</td>
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<td>-</td>
<td>19</td>
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<td>Total</td>
<td>86</td>
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<tr>
<th>ELISA result</th>
<th>No. of CF test results</th>
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<tr>
<td>+</td>
<td>0</td>
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<tr>
<td>-</td>
<td>10</td>
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<tr>
<td>Total</td>
<td>10</td>
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The 86 serum specimens resulted in a mean relative absorbance ± standard deviation of 10% ± 10% by the blocking ELISA.

The 10 serum specimens resulted in a mean relative absorbance ± standard deviation of 96% ± 14% by the blocking ELISA.

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completely satisfactory and that a combination of at least two of these tests was necessary to interpret results (4).

The favorable results obtained with the blocking ELISA described here may be due to the use of a heat-extracted antigen preparation which mainly contains important polysaccharide components in combination with a rabbit antiserum showing a high titer to these polysaccharides (9). The blocking ELISA is a realistic alternative to existing tests for monitoring herds for A. pleuropneumoniae as well as for eradication programs. It is therefore important that the high sensitivity and specificity found with the relatively low number of serum specimens in this study be documented with a large number of field samples.

ACKNOWLEDGMENT

This work was supported by grant 5.23.06 from the Danish Agricultural and Veterinary Research Council.

REFERENCES

3. Lariviere, S. Personal communication.