Differentiation of Toxigenic from Nontoxigenic Isolates of Pasteurella multocida by Enzyme-Linked Immunosorbent Assay

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An enzyme-linked immunosorbent assay (ELISA) was developed for the rapid and simple differentiation of toxigenic from nontoxigenic strains of Pasteurella multocida. The sandwich ELISA is based on two different murine monoclonal antibodies with specificity for the P. multocida toxin. The ELISA, which is now used as a routine test in Denmark, has several advantages compared with previously described biological tests.

The toxin produced by some strains of Pasteurella multocida subsp. multocida is considered the central etiological factor in atrophic rhinitis in pigs (6; M. F. de Jong, H. L. Oie, and G. J. Tettenborn, Proc. 1980 Congr. Int. Pig Vet. Soc., p. 211, 1980). The P. multocida toxin, which is a protein with a molecular weight of approximately 143,000, can induce osteoclastic bone resorption of the nasal turbinates when instilled intranasally in pigs and rats (2).

Differentiation of toxigenic from nontoxigenic strains of P. multocida is essential for the control and diagnosis of atrophic rhinitis in pigs. Previous methods of differentiation have relied on the biological activities of P. multocida toxin, i.e., lethality in mice, dermonecrotic effect in guinea pigs, and cytopathic effect on embryonic bovine lung (EBL) cells (5, 7; de Jong et al., Int. Pig Vet. Soc.). Recently, a less time-consuming technique based on an agar overlay method using a confluent monolayer of EBL cells was reported (1). The present paper describes the use of P. multocida toxin-specific monoclonal antibodies (N. T. Foged, Infect. Immun., in press) in an enzyme-linked immunosorbent assay (ELISA) for the rapid and simple identification of toxigenic strains of P. multocida.

A total of 615 field isolates and 7 reference strains of P. multocida were examined. The field isolates were obtained from nasal swabs (603 isolates) and lungs (12 isolates) of pigs from 156 Danish herds and were identified as P. multocida subsp. multocida (3) by the following criteria: acid produced from glucose, saccharose, mannitol, and sorbitol and not maltose, arabinose, dulcitol, and inositol; and production of indole, ornithine decarboxylase, catalase, and oxidase and not urease.

Extracts for toxin analyses were prepared by harvesting overnight 37°C blood agar (9-cm petri dish) cultures in 2 ml of sterile water. The suspensions were left for extraction at 37°C for approximately 18 h. One part of the extract was examined directly by the ELISA. Another part was centrifuged (30 min at 1,500 × g), and the supernatant was sterilized by filtration and subsequently examined in the EBL cell test as described earlier (4, 7).

The sandwich ELISA was initiated by coating each well of a microtiter plate (96 wells; Immuno Plate II; Nunc, Roskilde, Denmark) with 50 μl of a 2-μg/ml concentration of the anti-P. multocida toxin monoclonal antibody P3F51 in 0.05 M carbonate buffer (pH 9.6) for 16 h at 4°C and 1 h at 20°C. The microtiter plate was emptied, and each well was incubated for 1 h with 200 μl of phosphate-buffered saline containing 0.05% Tween 20 and 1% bovine serum albumin (PBS-T-BSA). After two PBS-T washings, each well was incubated with 50 μl of a dilution of a bacterial extract in PBS-T-BSA for 1 h at 20°C. After three PBS-T washings, each well was incubated with 50 μl of a 0.5-μg/ml concentration of the biotin-conjugated monoclonal antibody P3F37 for 1 h at 20°C, followed by another three PBS-T washings and incubation with 50 μl of a 1:2,500 dilution of horseradish peroxidase-conjugated avidin (Kem-En-Tec, Copenhagen, Denmark) per well for 45 min at 20°C. Finally, 50 μl of an o-phenylenediamine-H2O2 substrate solution per well was added. The reaction was stopped with 2 M H2SO4 after 5 min, and the A492 was determined in a KONTRON SLT-210 photometer (SLT Labinstruments, Zurich, Switzerland) (reference, A620). All absorbances (A) were expressed as percentages of the absorbance obtained by a positive control (A0). The positive control consisted of a 1:1 dilution of an extract of the toxigenic type D reference strain of P. multocida subsp. multocida 4578 (NCT 12178), freshly prepared for each experiment.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Result of</th>
<th>EBL cell test</th>
<th>ELISA*</th>
</tr>
</thead>
<tbody>
<tr>
<td>P. multocida subsp. multocida (250 field isolates)</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>P. multocida subsp. multocida (365 field isolates)</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>P. multocida subsp. septica CCUG 17977T</td>
<td>-</td>
<td>-</td>
<td></td>
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<tr>
<td>P. multocida subsp. gallicida NCT 10204T</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>P. multocida subsp. multocida, type A, NCT 10322T</td>
<td>-</td>
<td>-</td>
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</tr>
<tr>
<td>P. multocida subsp. multocida, type A, reference strain ATCC 12945</td>
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<td>-</td>
<td></td>
</tr>
<tr>
<td>P. multocida subsp. multocida, type A, reference strain NCTC 12177</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>P. multocida subsp. multocida, type D, reference strain ATCC 7707</td>
<td>-</td>
<td>-</td>
<td></td>
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<tr>
<td>P. multocida subsp. multocida, type D, reference strain NCTC 12178</td>
<td>+</td>
<td>+</td>
<td></td>
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</tbody>
</table>

* Corresponding author.

TABLE 1. Differentiation of toxigenic from nontoxigenic strains of P. multocida by the EBL cell test and the P. multocida toxin ELISA

All EBL-positive bacterial extracts had titers above 10^3 (median, 10^4; range, 10^3 to 10^6) in the EBL cell test. EBL-negative extracts were nontoxic.

* All 1:1 diluted ELISA-positive bacterial extracts had relative absorbances above 39% (mean = standard deviation, 94% ± 13%) in the P. multocida toxin ELISA, whereas all ELISA-negative extracts had relative absorbances below 9% (2.1% ± 1.9%).

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The 615 field isolates, all identified biochemically as *P. multocida* subsp. *multocida*, were characterized as toxigenic (*n* = 250) or nontoxigenic (*n* = 365) by the EBL cell test and were of capsular type A (119 toxigenic and 92 nontoxigenic isolates) or D (131 toxigenic and 273 nontoxigenic isolates).

Full agreement between the EBL cell test and the *P. multocida* toxin ELISA was obtained for the 615 field isolates and the 7 reference strains (Table 1).

The cytopathic and nontoxicological extracts of the 615 field isolates were separated into two clearly distinguishable groups by the *P. multocida* toxin ELISA (Fig. 1). Since the mean ± standard deviation of the absorbances obtained from the 1:1 diluted extracts of the 250 toxigenic isolates was 1.72 ± 0.48, visual readings instead of photometric measurements of the ELISA results would be satisfactory for the differentiation of extracts of *P. multocida*. The mean ± standard deviation of the *P. multocida* toxin concentration in the extracts of the toxigenic isolates of *P. multocida* was estimated to be 2.8 ± 1.9 µg/ml, and since the detection limit of the *P. multocida* toxin ELISA is approximately 50 pg of *P. multocida* toxin (1 ng/ml) (Foged, submitted), dilutions of the extracts (Fig. 2) and extracts with low *P. multocida* toxin concentrations can be appropriately tested by using the *P. multocida* toxin ELISA.

The main advantages of the *P. multocida* toxin ELISA compared with existing tests are the independence on cell culture or laboratory animal facilities, the ability of a single laboratory worker to handle several hundred samples per day, and the possibility of obtaining quantitative, objective results from bacterial extracts in 4 h.

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**LITERATURE CITED**