Pasteurellosis in Laboratory Rabbits: Characterization of Lipopolysaccharides of *Pasteurella multocida* by Polyacrylamide Gel Electrophoresis, Immunoblot Techniques, and Enzyme-Linked Immunosorbent Assay

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The lipopolysaccharides (LPSs) of five isolates of *Pasteurella multocida* from rabbits were characterized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, immunoblots, and enzyme-linked immunosorbent assay. Silver-stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis profiles of purified unaggregated LPSs resembled those of semirough strains of gram-negative enterobacteria and consisted of one or two bands that migrated within an interval just ahead or slightly behind the migration of the Ra chemotype of “Salmonella minnesota,” which has a molecular size of 4.3 kilodaltons. Polyclonal rabbit antiserum to *P. multocida* whole cells used in Western blots and enzyme-linked immunosorbent assays of unabsorbed and LPS-absorbed antisera revealed that the LPSs of these isolates of *P. multocida* contained at least two types of antigens: a nerserospecific antigen and a serospecific antigen. The LPSs of four isolates each had a different serospecific antigen. The nersospecific antigen was expressed in two isolates and was the only demonstrable LPS antigen in one isolate.

Mucopurulent rhinitis or “snuffles” in laboratory rabbits is the major clinical manifestation of chronic pasteurellosis caused by *Pasteurella multocida*. The stresses of experimentation on infected rabbits commonly contribute to more serious forms of pasteurellosis, including pneumonia, pulmonary abscesses, and otitis media. These and other manifestations of pasteurellosis in rabbits are refractory to antibiotic therapy. Prolonged clinical infection either kills the animal or prompts euthanasia and thereby limits the usefulness of rabbits as laboratory animals, particularly in long-term studies (9).

Although several capsular and somatic serotypes of *P. multocida* are involved in pasteurellosis of domestic farm animals and birds (3, 10), there appear to be relatively few serotypes that are pathogenic for rabbits (1, 5, 7, 13). In a previous report (15), we evaluated the lipopolysaccharides (LPSs) of 10 rabbit isolates of *P. multocida* by enzyme-linked immunosorbent assay (ELISA) and concluded that there was considerable antigenic homology among their LPSs, even though results of gel diffusion precipitin tests of cell wall extracts suggested much antigenic heterogeneity. We have extended these observations by characterizing the LPSs of five of these isolates of *P. multocida* by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), immunoblots, and ELISA with LPS-absorbed antisera.

**MATERIALS AND METHODS**

**Bacterial isolates.** Several characteristics of the five rabbit isolates of *P. multocida* are shown in Table 1. Purified LPS was prepared by inoculating each isolate into 30 liters of brain heart infusion broth. The broth was incubated at 37°C overnight, and the culture was killed by addition of 0.03 volume of 37% formaldehyde. The organisms were packed by centrifugation and washed three times in phosphate-buffered saline (PBS; 0.02 M Na$_2$PO$_4$ in 0.85% NaCl, pH 7.2). Each batch of organisms was dried by acetone, and LPSs were extracted either as described previously (15) or as described by Darveau and Hancock (6) with the substitution of sodium deoxycholate for SDS. Each method yielded a comparable amount of LPS (1 to 2% of dry cell weight) that contained less than 1.5% protein (15) and had indistinguishable silver-stained SDS-PAGE profiles. Purified LPS and the Ra chemotype of “Salmonella minnesota” were purchased from List Biologics (Campbell, Calif.).

**SDS-PAGE and silver staining.** SDS-PAGE was performed with the Laemmli buffer system (12) on 5-μL samples containing 1 to 3 μg of LPS in Laemmli buffer. The 5% stacking gel and 15% separating gel contained SDS; the separating gel also contained 4 M urea to aid in disaggregation of LPS (16). Electrophoresis (equipment from LKB Sverige AB, Bromma, Sweden) was done at 50 mA constant current for 30 min longer than required for the bromophenol blue dye front to leave the gel (about 5 h) in Tris-glycine (pH 8.3) buffer containing 0.1% SDS. The reservoir was cooled with flowing tap water. In some gels, we also applied a low-molecular-weight protein marker (Bio-Rad Laboratories, Richmond, Calif.) and “S. minnesota” LPS to compare with the banding patterns and mobilities of *P. multocida* LPSs. Also, an estimate of the molecular weights of *P. multocida* LPSs was obtained by including the Ra chemotype of “S. minnesota” in some gels. The gels were fixed and stained for LPS by the silver technique described by Dubray and Bezard (8).

**Immunoblotting.** After SDS-PAGE, LPSs were electrophoretically transferred (Western blot) from the gel onto nitrocellulose sheets (Transphor Unit; Hoefer Scientific Instruments, San Francisco, Calif.) in the presence of 150 mM glycine-20 mM Tris (pH 8.3) in 20% methanol at 0.6 A for 18 h in a reservoir cooled with flowing tap water. After electrotransfer, the nitrocellulose sheets were immersed in a
TABLE 1. Capsular and somatic types of P. multocida isolates from rabbits

<table>
<thead>
<tr>
<th>Isolate no.</th>
<th>Source</th>
<th>Capsular type</th>
<th>Somatic type</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Skin abscess</td>
<td>A</td>
<td>NADC 4, Lab 12, LPS 12, 4, 3, 7</td>
</tr>
<tr>
<td>2</td>
<td>Scrotal abscess</td>
<td>D</td>
<td>3, 4, 12, 4, 3</td>
</tr>
<tr>
<td>6</td>
<td>Mesenteric abscess</td>
<td>D</td>
<td>4, 12, 4, 5, 7</td>
</tr>
<tr>
<td>10</td>
<td>Pennsylvania cottontail</td>
<td>D</td>
<td>1, 3, 4, 5</td>
</tr>
<tr>
<td>11</td>
<td>Rabbit from Brazil</td>
<td>A</td>
<td>Untypeable (15)</td>
</tr>
</tbody>
</table>

* Type A isolates produce a hyaluronic acid capsule, the production of which is inhibited by hyaluronidase; type D isolates aggregate in 0.1% solution of acriflavine (15).

Minor (faint) or delayed (faint and occurring after 24 h) precipitin lines are included within parentheses. Somatic typing was done in a gel diffusion precipitin test in which type-specific chicken antiserum (kindly provided by the National Animal Disease Center, Ames, Iowa) was reacted against a Formalin-saline heat-stable extract of the isolate either at the Ames Laboratory (NADC) or in our laboratory (Lab) with chicken antiserum kindly provided by Billy Blackburn of the NADC (10). In the column labeled LPS, purified LPS was used as antigen, and the tests were done in our laboratory.

Kindly provided by the Microbiology Laboratory, National Animal Disease Center, Ames, Iowa.

4. LPS of this isolate was present mainly in the phenol layer when cells were extracted by the hot phenol-H2O method of Westphal and Jann (21). LPSs from other isolates were within the aqueous phase.

blocking buffer (1% bovine serum albumin, 0.85% NaCl, 20 mM NaHPO4, pH 7.2, 0.01% thimerosal) for 3 h and then in immune serum diluted 1:100 in blocking buffer for 1 h at room temperature. The sheets were washed three times for 10 min in PBS and immerser for 1 h at room temperature in a solution of protein-A-conjugated horseradish peroxidase (Sigma Chemical Co., St. Louis, Mo.) diluted to 2 μg/ml in blocking buffer. After three 10-min washes in PBS, substrate solution consisting of 0.05% (wt/vol) 3,3',5-diaminobenzidine, 0.01% (vol/vol) hydrogen peroxide, 0.05 M citric acid, and 0.05 M sodium citrate in distilled water was added. The brown reaction product was allowed to form for 10 to 40 s. The sheets were rinsed in distilled water and air dried.

Immune sera. Immune sera to killed whole cells were obtained from pasteurella-free New Zealand White rabbits maintained and immunized as described previously (15).

ELISA. Lyophilized LPS was dissolved in carbonate buffer (0.05 M carbonate, pH 9.6) to 100 μg/ml and sonicated for 1 min at a 35% setting (Fisher Scientific Co., Pittsburgh, Pa.). Sonicated LPS solution (0.1 ml; 10 μg of LPS) was put into wells of polystyrene plates (Falcon 3070; Becton Dickinson Labware, Oxnard, Calif.), which were then covered and kept at 37°C overnight. The wells were washed three times with PBS. Blocking buffer (PBS with 10% fetal bovine serum, 1% bovine serum albumin, and 0.3% gelatin) was added to fill each well, and the plates were kept at room temperature for 3 h. The wells were washed three times with PBS. Antiserum (0.1 ml) diluted 1:1,000 in diluting buffer (PBS containing 1% bovine serum albumin and 0.3% gelatin) was added; the plates were kept at room temperature for 20 min and were washed five times with PBS. Protein-A-conjugated horseradish peroxidase (0.1 ml, original concentration, 1 mg/ml; Sigma), diluted 1:500 in diluting buffer was added; the plates were kept at room temperature for 20 min and were again washed five times with PBS. Substrate, consisting of 0.05% O-phenylenediamine (Sigma) in a buffer composed of 0.05 M citric acid, 0.05 M sodium citrate, and 0.01% hydrogen peroxide, was prepared just before use, and 0.1 ml was added to each well. After 1 h at room temperature, the reaction was stopped by the addition of 0.1 ml of 1.6 N sulfuric acid to each well. Optical densities were read at 492 nm (Titer tek Multiscan; Flow Laboratories, Inc., McLean, Va.). Control wells lacked only LPS, and all determinations were done in duplicate.

Serum absorption. Antiserum, diluted 1:500 in diluting buffer, was added to an equal volume of diluting buffer containing 20 μg of LPS per ml. The sera were absorbed for 1 h at room temperature with constant rocking. Unabsorbed antiserum was diluted at 1:1,000 served as a control.

RESULTS

SDS-PAGE profiles of silver-stained LPSs are shown in Fig. 1. None of the LPS profiles of the five isolates demonstrated the ladderlike profile characteristic of seminor fus Escherichia or Salmonella strains including "S. minnesota" (11). Rather, the LPS profiles of P. multocida contained one or two (isolate 6 occasionally had a third trailing band) argentophilic bands which migrated somewhat faster than a protein standard of 14 kilodaltons. All the bands were within an interval slightly ahead to slightly behind the Ra chemotype of "S. minnesota." The Ra chemotype has an estimated molecular weight of 4,311 (16) and consists of lipid A and core polysaccharide but lacks repeating oligosaccharide units (11).

Immunoblots and ELISA of absorbed antisera. The possible antigenic relatedness among the LPSs of these five isolates of P. multocida was evaluated by use of immunoblots and ELISAs. The results of several Western blots and ELISAs are shown in Fig. 2 and 3. Antiserum to isolate 1 reacted strongly to moderately with LPSs of isolates 1, 2, and 6 and weakly with isolate 11 LPS (Fig. 2a). The strong cross-reactivity of isolate 1 antiserum to LPSs of isolates 2 and 6 was confirmed by ELISAs on immune sera and LPS-absorbed immune sera (Fig. 3A). Nearly all the antibody to LPS in isolate 1 antiserum was removed by absorption with LPS of isolates 2 and 6. Conversely, LPSs of isolates 10 and 11 had negligible absorptive potency on isolate 1 antiserum.

The reactivity of antiserum to isolates 2 and 6 (Fig. 2b and c) was very similar to that of isolate 1 antiserum in immunoblots, except that isolate 6 antiserum reacted very weakly with isolate 2 LPS (Fig. 2c). Based upon the activity of isolate 6 antiserum to isolate 2 LPS in the ELISA (Fig. 3C), a stronger reaction was expected in the immunoblot. The faint band shown in Fig. 2c, lane 2, is a minor band of isolate 2 that was also seen occasionally in silver-stained SDS-polyacrylamide gels. This band, though not seen in Fig. 1, migrated just ahead of the band shown in Fig. 1, lane 2, and coincided with the band of isolate 1 (Fig. 1, lane 1). It would appear from these observations that the LPS of isolate 2 includes traces of incomplete LPS molecules composed mainly of common (nonserospecific) antigen and that the minor disparity between the ELISAs and immunoblots is influenced by the amount of nonserospecific LPS antigen within a given preparation of LPS. In this instance, the batch of LPS from isolate 2 used for the ELISA contained more nonserospecific LPS than the batch of LPS used in the immunoblot. ELISAs on LPS-absorbed antiserum to isolate 2 and isolate 6 revealed that each isolate possessed a distinct serospecific LPS antigen not possessed by the other isolates (Fig. 3B and C), in addition to the nonserospecific LPS antigen shared by isolates 1, 2, and 6.

Finally, immunoblots of these LPSs reacted with antiserum to isolates 10 and 11 are shown in Fig. 2d and e. Each
antiserum reacted strongly with homologous LPS and weakly or not at all with LPSs of the other isolates. ELISA of absorbed and unabsorbed antisera to isolates 10 and 11 demonstrated clearly that the major antigenic characteristics of LPSs from these isolates were serospecific (Fig. 3D and E).

DISCUSSION

The semicrude characteristics of P. multocida LPSs resemble the SDS-PAGE profiles of LPSs from several other bacterial genera, including Neisseria gonorrhoeae (16), Campylobacter jejuni (18), Neisseria meningitidis (19), and Bacteroides fragilis (20). Moreover, LPS profiles of these five isolates of P. multocida are very similar to those of P. multocida isolated from the nasal cavities of swine (14), except that LPS PAGE profiles of our isolates had fewer bands. In our initial studies on P. multocida, we also noticed up to seven variably spaced LPS bands within an estimated molecular-size range of 15 to 26 kilodaltons in silver-stained gels. These LPS bands appear to be the result of clustering of LPS molecules to form aggregates with various molecular weights as has been described for the LPSs of N. gonorrhoeae (16). Aggregation is inhibited or diminished either by incorporation of 4 M urea into the separating gel or by mild alkaline hydrolysis of LPS before SDS-PAGE (16). Either procedure proved satisfactory for our studies and resulted in very similar SDS-PAGE LPS profile banding patterns (Fig. 1).

Although the LPSs of these P. multocida isolates lack the several repeating oligosaccharide units responsible for serospecificity among several genera of the Enterobacteriaceae family, these LPSs contain, within a relatively small molecule that is similar in size to "S. minnesota" chemotype.
Ra (4.3 kilodaltons), both serospecific and nonserospecific antigens. Isolates 2, 6, 10, and 11 each had a different serospecific LPS antigen. The serospecificity of LPSs of isolates 10 and 11 confirms an earlier report by Cary et al. (4), who evaluated the LPSs of these two isolates by ELISA inhibition tests.

The nature of the nonserospecific antigen(s) is not yet clear. However, the occurrence of common antigens in the LPSs of Pasteurella multocida has also been suggested by other investigators (4, 17). If this antigen is common to the LPSs of all five isolates, it appears to be sterically shielded in isolates 10 and 11. Alternatively, there may be different core antigens among these LPSs of Pasteurella multocida, or there may be a variable LPS antigen, as has been demonstrated for the LPSs of Neisseria gonorrhoeae (16). The nonserospecific antigen on our isolates appears to be common to many rabbit isolates of Pasteurella multocida because Western blots of LPSs of five other isolates (15) reacted strongly with antisera of isolates 1 and 6 (unpublished observations). Moreover, this nonserospecific antigen is probably the LPS antigen responsible for broad cross-reactivity demonstrated by extracts of isolates 1, 2, and 6 with several typing sera in the gel diffusion precipitin tests (2, 4, 10) shown in Table 1 (column 5).

In summary, the LPSs of five rabbit isolates of Pasteurella multocida were purified and evaluated by SDS-PAGE, ELISA, and Western blots. The SDS-PAGE profiles were similar to semirough LPSs of enterobacteriaceae. The LPSs contained a nonserospecific antigen, a serospecific antigen, or both. Four of the isolates each had a different serospecific LPS antigen. The nonserospecific antigen was expressed on the LPS preparation of two isolates (strongly in isolate 6 and weakly in isolate 2) and was the only demonstrable LPS antigen in one isolate other.

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LITERATURE CITED


