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# Coagglutination Test for Identification of *Pasteurella multocida* Associated with Hemorrhagic Septicemia

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Two serogroups (B and E) of *Pasteurella multocida* associated with hemorrhagic septicemia in cattle were differentiated by a coagglutination test with antibody-coated staphylococci. The group antigen was soluble and heat stable. It was not detectable in unencapsulated cells or their heated extracts. Adsorbed onto human type O erythrocytes, it could be demonstrated by an indirect hemagglutination test with specific antiserum or by coagglutination with specific antibody-coated staphylococci. Antibody-coated staphylococci could detect the soluble group antigen in the plasma and liver extract of mice experimentally infected with *P. multocida*. The group antigen did not affix to mouse erythrocytes in vivo.

*Pasteurella multocida* which produce hemorrhagic septicemia (HS) of cattle and buffalo belong to serogroups B and E (2). The group antigens are specific to encapsulated strains, and only encapsulated strains are pathogenic. Group B HS strains have been isolated in Asia, Africa, Europe, and, rarely, North America; group E strains have been reported only from outbreaks of HS in central Africa.

The mouse passive immunization test (13) and the indirect hemagglutination (IHA) test (1) are used in the serological identification of HS *P. multocida*. Neither test is performed routinely in diagnostic laboratories.

Recent studies have described a method for the rapid serological identification of capsular antigens from both gram-positive and gram-negative bacteria with antibody-coated *Staphylococcus aureus* (3, 6, 7, 14). Protein A, a component of some staphylococci, binds specifically to the Fc segment of immunoglobulin G (8). This leaves the Fab segment of the globulin free to participate in antigen-antibody reactions. The addition of homologous antigen to the antibody-coated staphylococci is visualized by an agglutination reaction (coagglutination). The test can be used with both soluble and particulate antigen.

This report describes the use of a slide coagglutination test for the identification of the group-specific antigen of HS *P. multocida* from bacteria grown in vitro and from tissue extracts.

## MATERIALS AND METHODS

**Bacterial strains.** Ten strains of *P. multocida* from the National Animal Disease Center collection representing various geographical origins and three distinct serogroups were used (Table 1). Strains X-73 and P-1059 were used as controls. Colonies of strains P-1248 and P-1237 were not iridescent in oblique transmitted light on dextrose starch agar plates (DSA; Baltimore Biological Laboratory). The presence of a capsule and group-specific antigen correlates with the presence of iridescence.

The Cowan 1 strain of *S. aureus* (ATCC 12598) was used for antibody coating.

**Preparation of antiserum.** Strain M-1404 (group B) or P-1235 (group E), grown for 18 h on DSA, was harvested in 0.3% Formol-treated saline and adjusted to a turbidity equivalent to a no. 10 MacFarland nephelometer standard. A portion of this aqueous suspension was mixed 1:1 with Freund incomplete adjuvant (Difco Laboratories) and stored at 4°C until it was used.

Young adult male New Zealand white rabbits were inoculated subcutaneously at four sites along the back with a total of 1.0 ml of adjuvant bacterin. After 2 weeks they were inoculated subcutaneously with 1.0 ml and intramuscularly with 0.5 ml of the same adjuvant bacterin. Sera tested 1 week later had little or no titer by the IHA test. Therefore, a second course of injections was begun. Four intravenous injections (0.1, 0.2, 0.3, and 0.4 ml) of the original aqueous suspension were given on alternate days, and the rabbits were exsanguinated at 1 week after the last injection. Sera were preserved with 0.01% thimerosal and 0.06% phenol.

**Adsorption of antisera.** Group B and group E antisera were adsorbed with washed cells of strains P-1235 and M-1404, respectively. Cells for adsorption were harvested in Formol-treated saline from 18- to 24-h growth on DSA and washed twice by centrifugation in 0.01 M phosphate-buffered saline (PBS; pH 7.2) containing 0.01% thimerosal and 0.06% phenol. Antiserum was diluted 1:5 with PBS, and 9 volumes was added to 1 volume of packed washed cells. The

TABLE 1. *Origins, colonial iridescence in oblique transmitted light, and serogroups of P. multocida strains*

| *P. multocida* strain | Continent of origin | Colony iridescence | Serogroup (previously reported) |
|---|---|---|---|
| M-1404 | North America | + | B |
| P-1248 | Africa | − | B |
| P-1256 | Asia | + | B |
| P-1458 | North America | + | B |
| P-1487 | Asia | + | B |
| P-1234 | Africa | + | E |
| P-1235 | Africa | + | E |
| P-1237 | Africa | − | E |
| X-73 | North America | + | A |
| P-1059 | North America | + | A |

cells were suspended, and the suspension was incubated at 37°C for 3 h and then at 4°C overnight. Cells were removed by centrifugation. The antiserum was adsorbed twice. Antiserum was concentrated to its original volume with a Minicon S125 concentrator (Amicon Corp.).

**Preparation of antibody-coated staphylococci.** A stabilized suspension of the Cowan 1 strain of *S. aureus* was prepared by the method of Kronvall (7). *S. aureus* was grown in Trypticase soy broth for 18 h. The cells were harvested and washed three times by centrifugation in 0.12 M NaCl–0.03 M $Na_2HPO_4$ at pH 7.3 (PBSS), suspended in 0.5% Formol-treated PBSS, and kept at 25°C for 3 h. The cells were again washed three times, adjusted to 10% (vol/vol) in PBSS, and then heated at 80°C for 1 h. After cooling, 0.1 ml of antiserum was added to 1.0 ml of this cell suspension, and the mixture was held at 25°C for 1 h with occasional shaking. The cells were washed once and suspended in 3 ml of PBSS.

Stabilized uncoated staphylococci or normal rabbit serum-coated staphylococci were used as controls.

**Bacterial antigens.** Growth from 18- to 24-h DSA cultures was harvested in 3 ml of PBS per plate. Cell suspensions were heated at 56°C for 30 min or 100°C for 1 h. The cells were pelleted by centrifugation, and the supernatant fluid was designated 56 or 100°C extract, respectively. The cells were washed once and suspended in 2 ml of PBS. Westphal lipopolysaccharide of strain M-1404 was provided by P. A. Rebers (National Animal Disease Center).

**Preparation of tissue extracts.** Groups of 5 or 10 female CF-1 mice (Charles River Breeding Laboratories, Inc.) weighing 20 to 22 g were inoculated intraperitoneally with 500 to 1,000 live cells of *P. multocida* strain M-1404, P-1256, P-1234, P-1235, X-73, or P-1059 in 0.1 ml of brain heart infusion broth (Difco Laboratories). Livers from 1, 2, or 3 just-dead or moribund sacrificed mice were pooled and then ground with sand by using a mortar and pestle. A 2-ml quantity of Formol-treated saline was added for each liver, and the suspension was mixed with a Vortex mixer (Scientific Products Inc.) and then centrifuged at 10,000 × *g* for 20 min. The supernatant fluid was removed and centrifuged at the same rate. The supernatant fluid from the final centrifugation was designated mouse liver extract.

When possible, mouse blood was collected into tubes containing 20 U of heparin. This blood was added to 1.5 ml of PBS, suspended, and centrifuged at 10,000 × *g* for 20 min. The supernatant fluid was removed, filtered through 0.45-μm Swinney filters (Millipore Corp.), and then concentrated 10-fold in a Minicon S125 concentrator. The erythrocytes were washed twice in PBS before they were suspended to 1.0% (vol/vol).

**IHA test.** The IHA test for identification of group-specific antigen was done by the method of Carter (1), except that a microtiter system (Cooke Engineering Co. [Dynatech Corp.]) was used.

Human type O erythrocytes were washed by centrifugation three times in PBS, and 0.1 ml of the packed cells was added to 3 ml of 56 or 100°C *P. multocida* extract. The suspension was incubated for 2 to 3 h at 37°C; the cells were then washed twice and suspended to 1.0% (vol/vol) in PBS. Serial twofold dilutions of unadsorbed antiserum were made in PBS, and 0.05 ml of the sensitized erythrocytes was added to 0.05 ml of antiserum dilution in U-bottom microtiter plates (Cooke Engineering Co. [Dynatech Corp.]). The IHA titer was determined from the patterns of the sedimented erythrocytes after 2 h at 25°C. Unsensitized erythrocytes were used as controls.

**Agglutination and coagglutination tests.** For slide agglutination or coagglutination tests, 0.05 ml of antiserum or antibody-coated staphylococci was added to 0.05 ml of test material on a glass slide. Clumping within 2 min was considered to be a positive test.

Slide agglutination tests were done with live 18-h DSA-grown bacterial cells. Slide coagglutination tests with antibody-coated staphylococci were done with live bacterial cells, 56°C extracts and bacterial cells, 100°C extracts and bacterial cells, 56°C extract-sensitized human erythrocytes, 100°C extract-sensitized human erythrocytes, and tissue extracts, plasma, and washed erythrocytes from infected mice.

**Gel diffusion tests.** For gel diffusion precipitin tests (GDP), 0.9% Noble agar with 8.5% NaCl was used. Agar was poured to a depth of 5 mm in standard petri dishes, and 4-mm wells were cut and spaced 3 mm apart. Center wells were filled with antigen, and opposing wells were filled with antiserum. The filled agar plates were incubated under moist conditions at 37°C, and results were read at 48 h.

## RESULTS

The results of agglutination tests with live bacterial cells and coagglutination tests with live cells, 56 and 100°C extracts, and bacterial cells are shown in Table 2. Live cells, heated extracts, and heated cells of group E organisms cross-reacted with unadsorbed group B antibody-coated staphylococci. These cross-reactions were eliminated by the use of adsorbed antiserum. Coagglutination with cells and extracts from noniridescent strains was also eliminated by the use of adsorbed antiserum. Stabilized uncoated or normal rabbit serum-coated staphylococci controls did not react. Group A *P. multocida* cells or extracts, which were also used as controls, also did not react.

The results of IHA tests and coagglutination tests with erythrocytes sensitized with 56 or 100°C extracts are shown in Table 3. Little or no difference in IHA titer was observed between 56 and 100°C extract-sensitized erythrocytes. Correlation was complete between reactions to group-specific material when the IHA and coagglutination tests were used. The fact that adsorbed antisera did not have to be used suggests that additional heat-stable antigen(s) observed in extracts of both iridescent and noniridescent cells was not adsorbed to erythrocytes.

Adsorbed antibody-coated staphylococci and heated extract coagglutinated after removal of sensitized erythrocytes from the sensitizing reaction mixture. This coagglutination indicated that not all of the group-specific antigen was adsorbed to erythrocytes.

Table 4 shows the coagglutination reactions between unadsorbed or adsorbed antibody-coated staphylococci and mouse liver extracts. Some cross-reactions occurred with unadsorbed antiserum, but these were eliminated by the use of adsorbed antiserum. When the expected coagglutination reaction did not take place, mouse liver extracts were concentrated 10-fold with a Minicon S125 concentrator. After concentration, the expected reactions occurred.

Filtered plasma prepared from the heparinized blood of mice inoculated with strain P-1256 or P-1234 reacted specifically with adsorbed antibody-coated staphylococci. Washed mouse erythrocytes did not react with unadsorbed or adsorbed antibody-coated staphylococci, which suggests that, although group-specific antigen is soluble in plasma, it may not absorb to eryth-

TABLE 2. *Comparisons of agglutination or coagglutination when unadsorbed or adsorbed antiserum was used with bacterial cells or extracts*

| *P. multocida* strain | Agglutination or coagglutination reactions with live bacterial cells when unadsorbed antiserum was used | | Coagglutination reactions with 56 or 100°C extracts and cells when unadsorbed antiserum was used[a] | | Coagglutination reactions with 56 or 100°C extracts and cells when adsorbed antiserum was used[a] | |
|---|---|---|---|---|---|---|
| | Group B antiserum | Group E antiserum | Group B antiserum | Group E antiserum | Group B antiserum | Group E antiserum |
| M-1404 | + | − | + | − | + | − |
| P-1248 | + | − | + | − | − | − |
| P-1256 | + | − | + | − | + | − |
| P-1458 | + | − | + | − | + | − |
| P-1487 | + | − | + | − | + | − |
| P-1234 | − | + | − | + | − | + |
| P-1235 | − | + | + | + | − | + |
| P-1237 | + | + | + | + | − | − |
| X-73 | − | − | − | − | ND[b] | ND |
| P-1059 | − | − | − | − | ND | ND |

[a] Reactions were the same with 56°C extracts, 56°C cells, 100°C extracts, and 100°C cells.
[b] ND, Not done.

TABLE 3. *Comparisons of IHA titer and coagglutination of 56 and 100°C bacterial extract-sensitized erythrocytes when unadsorbed or adsorbed antiserum was used*

| *P. multocida* strain | IHA titer of unadsorbed antiserum when 56°C extract-sensitized erythrocytes were used | | IHA titer of unadsorbed antiserum when 100°C extract-sensitized erythrocytes were used | | Coagglutination with 56 or 100°C extract-sensitized erythrocytes when unadsorbed or adsorbed antiserum was used[a] | |
|---|---|---|---|---|---|---|
| | Group B antiserum | Group E antiserum | Group B antiserum | Group E antiserum | Group B antiserum | Group E antiserum |
| M-1404 | 128[b] | −[c] | 128 | − | + | − |
| P-1248 | − | − | − | − | − | − |
| P-1256 | 256 | − | 512 | − | + | − |
| P-1458 | 1,024 | − | 1,024 | − | + | − |
| P-1487 | 1,024 | − | 512 | − | + | − |
| P-1234 | − | 512 | − | 1,024 | − | + |
| P-1235 | − | 128 | − | 128 | − | + |
| P-1237 | − | − | − | − | − | − |

[a] Reactions were the same for 56 and 100°C extract-sensitized erythrocytes. No differences occurred between adsorbed and unadsorbed antisera.
[b] Expressed as reciprocal of titer.
[c] −, No reaction at ≥ 1:2 dilution.

rocytes in vivo.

Table 4 and Figure 1 show the GDP reactions between unadsorbed antisera and mouse liver extracts. Reactions were group specific when they occurred. Figure 2 compares the GDP reactions of unadsorbed rabbit antisera versus mouse tissue extracts, 100°C extracts of strains M-1404 and P-1235, and Westphal lipopolysaccharide of strain M-1404. The fact that no reaction occurred with the latter indicated that little or no antibody was formed by rabbits against this cell wall component.

## DISCUSSION

The coagglutination test with adsorbed antibody was specific for the heat-stable group-specific B or E antigen of HS *P. multocida*. This antigen corresponds to the group-specific antigen present in iridescent organisms described by Carter (1).

Prince and Smith (12) described a heat-stable antigen ($\beta$ antigen) with GDP, which was probably responsible for the specific reaction observed with the IHA test of Carter. This antigen was present in cell-free pleural fluid of cattle

TABLE 4. *Coagglutination and GDP reactions with mouse liver extracts from P. multocida-inoculated mice when unadsorbed or adsorbed antiserum was used*

| Inoculated strain | No. of livers per extract | Coagglutination with liver extract when unadsorbed antiserum was used | | Coagglutination with liver extract when adsorbed antiserum was used | | GDP reactions when unadsorbed antisera were used | |
|---|---|---|---|---|---|---|---|
| | | Group B antiserum | Group E antiserum | Group B antiserum | Group E antiserum | Group B antiserum | Group E antiserum |
| M-1404 | 2, 3[a] | + | − | + | − | + | − |
| M-1404 | 2 | + | − | (+)[b] | − | + | − |
| M-1404 | 2 | + | − | + | − | − | − |
| M-1404 | 1 | −[c] | − | −[c] | − | − | − |
| P-1235 | 2, 3[a] | − | + | − | + | − | + |
| P-1235 | 2 | (+) | + | − | + | − | + |
| P-1235 | 1 | − | + | − | (+) | − | − |
| P-1235 | 1, 1[a] | − | −[c] | − | −[c] | − | − |
| P-1256 | 1, 2[a] | + | − | + | − | + | − |
| P-1256 | 2 | + | − | + | − | − | − |
| P-1234 | 2, 2[a] | − | + | − | + | − | + |
| P-1234 | 1 | (+) | −[c] | − | −[c] | − | − |
| X-73 | 2, 3[a] | − | − | − | − | − | − |
| P-1059 | 2, 3[a] | − | − | − | − | − | − |
| Normal mouse tissue | 4 | − | − | − | − | ND[d] | ND |

[a] Different liver extracts that gave the same reactions.
[b] (+), Weak reaction.
[c] Positive when liver extract was concentrated 10-fold.
[d] ND, not done.

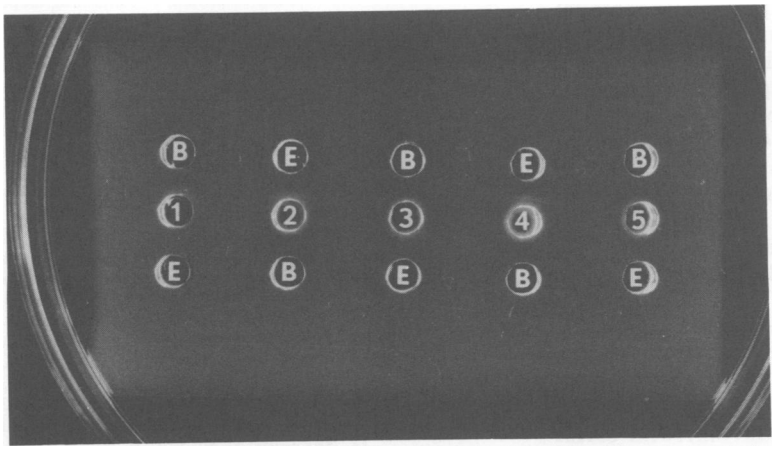


FIG. 1. *GDP reactions of unadsorbed antisera B and E with mouse liver extracts. B, Group B antiserum; E, group E antiserum; 1 and 5, liver extracts from mice inoculated with strain M-1404; 2 and 4, liver extracts from mice inoculated with strain P-1235; 3, liver extract from mice inoculated with strain X-73.*

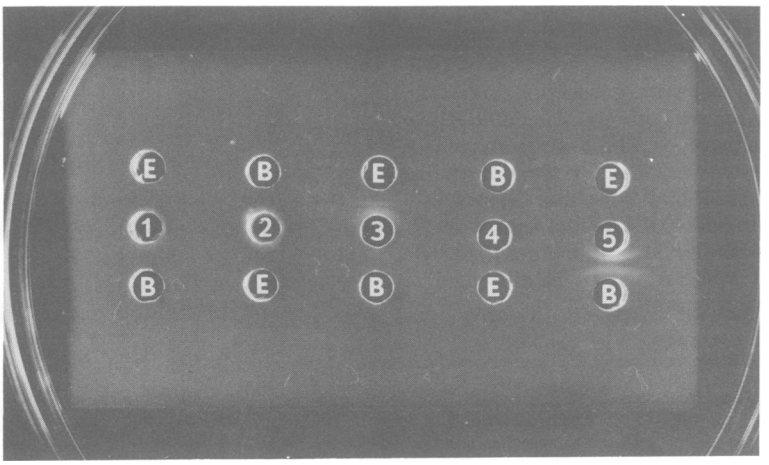


FIG. 2. *GDP reactions of unadsorbed antisera B and E with mouse liver extracts. B, Group B antiserum; E, group E antiserum; 1 and 2, liver extracts from mice inoculated with strain M-1404; 3, 100°C extract from P-1235 bacterial cells; 4, Westphal lipopolysaccharide from strain M-1404; 5, 100°C extract from strain M-1404.*

dying from experimental HS and could be detected with GDP (11). A soluble antigen was also demonstrated in cattle tissue fluids by Dhanda (4).

Soluble antigens of other bacterial species in tissue fluids have been described (5, 9, 10, 14). Detection of soluble antigens of *Haemophilus influenzae* in cerebrospinal fluid, urine, and serum with a coagglutination test has been described recently by Suksanong and Dajani (14). The test was more sensitive than countercurrent immunoelectrophoresis (14).

In this study, soluble antigen (probably $\beta$ antigen) was identified in plasma and liver extracts of infected mice. Other data (not presented) have shown that the coagglutination test reacts equally well, if not better, with crude liver homogenates. The only requirement was removal of the larger particulate material.

Although mice were tested in this study, the results, together with documentation of the presence of group-specific antigen in pleural fluids and tissues of infected cattle, suggest that this test would be useful for the presumptive diagnosis of HS using animal fluids or tissues as well as the isolated bacteria. Precaution would have to be taken that antibodies do not react with normal tissue.

The coagglutination test is simple, rapid, and inexpensive. The quantities of reagents required for the test are much less than those required for the mouse passive protection test or the IHA test. These qualities would make the coagglutination test applicable in diagnostic laboratories.

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