

## IDENTIFICATION AND IMMUNOGENICITY OF POLYSACCHARIDE ANTIGENS OF *PASTEURELLA MULTOCIDA* STRAINS INVOLVED IN HAEMORRHAGIC SEPTICAEMIA

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### ABSTRACT

Polysaccharide antigens of *Pasteurella multocida* serotype B:2,5 were identified by immunodiffusion and immunoelectrophoresis. The immunodiffusion tests showed that the first line, near the well containing the whole polysaccharide extract (WPE) represent LPS because this precipitation line occurs by diffusion using antiserum raised against *P. multocida* serotypes B:2,5 and E:2,5 but not with B:3,4. The second precipitation line, near the well containing antiserum raised against *P. multocida* serotype B:2,5, represent the bacterial capsule because this precipitation line also occurs by diffusion using preparation B (capsular antigen) or a whole cell extract of *P. multocida* serotype B:3,4. Immunoelectrophoresis was used to further characterize the polysaccharide antigens. The LPS remained near the antigen well, whereas the less hydrophobic and more acidic bacterial capsule moved further towards the anode. Immune responses against these antigens were measured in sera from a vaccinated buffalo. The analysis of the anti-polysaccharide response after removal of anti-LPS antibodies demonstrated a very low response to capsular material, whereas a 5-times higher response was measured against LPS.

### INTRODUCTION

Haemorrhagic septicaemia (HS) is an acute disease affecting cattle and buffaloes in tropical countries. The infection is caused by *Pasteurella multocida* serotype B:2,5 in Southern and South East Asia and by serotype E:2,5 in most parts of tropical Africa (Carter, 1955 and Heddleston *et al.*, 1972). The type specific antigen (Carter type B and E) refers to the bacterial capsule, whereas the O-group antigen (Heddleston type 2 and 5) refers to the lipopolysaccharide compound. Intravenous injection of animals with crude saline extracts or endotoxin preparations isolated from these strains results in the appearance of clinical symptoms which closely resemble those which appear after the challenge of a susceptible host with a virulent *Pasteurella* strain (Musa *et al.*, 1972 and Rebers *et al.*, 1967).

Protein, polysaccharides and lipopolysaccharides are the important antigens in *P. multocida*. We have conducted various studies on the protein profile of *P. multocida*. A collection of HS-causing strains was analyzed using polyacrylamide gel electrophoresis. It was found that HS-causing strains of *P. multocida* synthesize a number of additional outer membrane proteins when grown under iron limitation. Further analysis of these iron-regulated outer membrane proteins

(IROMPs) by immunoblotting, using sera obtained from healthy carrier buffaloes as well as from vaccinated animals, has shown that these IROMPs are potent immunogens (Veken *et al.*, 1996). An 82-KDa iron-regulated outer membrane protein was found in *P. multocida* B:2,5 which specifically binds bovine transferrin (Veken *et al.*, 1994). In contrast, strains of *P. multocida* serotype B:3,4, isolated from cases of HS in feral ruminants (Rimler, 1993) did not express transferrin-binding proteins.

In search for the presence of protein toxins we investigated the effect of intraperitoneally injected cells of *P. multocida* serotype B:2,5 on mice macrophages. The macrophages showed extensive intracellular vacuolation, whereas macrophages of the control animals did not (Shah *et al.*, 1996). The same effect was observed in in vitro phagocytosis experiments. Vacuolating activity was also observed when cultures of other HS strains of *P. multocida* were used but not with cultures of non-HS control strains. Preincubation of the bacteria with buffalo antiserum prevented vacuolation. These observations indicate that the impairment of innate immunity by *P. multocida* is most likely due to this vacuolating, cytotoxic activity and subsequent macrophage cell death.

The purpose of the present study is to demonstrate the type specific antigen (capsule) and non-type specific antigen (lipopolysaccharide) in *P. multocida* serotype B based on immunodiffusion and immunoelectrophoresis techniques. Antibody titers against the various antigens in sera of vaccinated buffaloes have been analyzed.

## MATERIAL AND METHODS

### Bacterial strains and cultivation conditions

Strains of *P. multocida* serotype B:2,5 were used in this study which has been described previously (Veken et al. 1994 and 1996). The strain was passaged through mice and subcultured on blood agar plates (5% sheep blood) before being used. For isolation of capsular and lipopolysaccharide antigens the bacteria were grown overnight on Brain Heart Infusion-agar plates (BHI-agar, Gibco) containing 1% yeast extract.

### Isolation of capsular and lipopolysaccharide antigens

Capsular and LPS antigens from serotype B:2,5 were isolated by a modification of the procedure described by (Penn and Nagy in 19974 and 1976). The antigens were extracted from the bacteria by removing the bacterial growth from the BHI-plates with a 2.5% solution of sodium chloride followed by shaking of the bacterial suspension for 5 min at room temperature. Subsequently, the suspension was subjected to centrifugation for 15 min at 10,000 rpm. The resultant supernatant fraction will be referred to as whole cell extract (WCE). To remove the cell surface proteins from this extract the WCE was incubated with 100 µg of proteinase K (Sigma) per ml for one hour at room temperature. Subsequently, the proteinase K was inactivated by heating the mixture for 10 min at 100°C. The proteinase K treated supernatant will be referred to as whole polysaccharide extract (WCE). Subsequently, capsular material and LPS were separated. In the first fraction step, three times the supernatant volume of methanol was added. This mixture was kept overnight at 4°C. The precipitate was collected by centrifugation, resuspended in water and mixed again with three volumes of methanol. The resulting precipitate contained the bacterial LPS (Penn and Nagy 1976). It will be referred to as preparation A. In the second step the combined supernatant of both methanol precipitation steps was supplemented with 0.75 volumes of acetone. The precipitate containing the bacterial capsule was collected by centrifugation (Penn and Nagy 1976). It will be referred to as preparation B. Subsequently, both precipitates (A and B) were resuspended in water and

dialyzed against distilled water for two days. To remove any remaining protein contamination both fractions were incubated with 100 µg of proteinase K per ml in the dark at room temperature. After this treatment the proteinase K was inactivated by heating for 10 min at 100°C. Finally, both fractions were lyophilized.

### Immunodiffusion and immunoelectrophoresis techniques

For immunodiffusion, slides were pre-coated with 0.7% agarose in distilled water and subsequently coated with an overlay of 1% agarose (Sigma type 1, low electroendosmosis) in 50 mM Tris-HCl buffer, pH 7.6. The wells were loaded with 20 µL of undiluted test sera raised against the polysaccharide antigens of *P. multocida* type B and E and with 20 µL of a solution containing 100mg per ml of either WPE or preparation A or B. Immunoprecipitation was read after 48 h of diffusion at room temperature.

Immunoelectrophoresis was carried out in 1% agarose in barbiturate buffer, pH 8.6. The antigen preparations (A and B) for immunoelectrophoresis were the same as used for the immunodiffusion tests. The electrophoresis was carried out at 100 V for 2h.

### Sensitivity to DNase, RNase and periodate degradation

Both DNase and RNase were added to the A and B antigen preparations to a final concentration of 10 µg per ml and incubated for 1 h at 37°C (Van Winkelhoff *et al.*, 1993). To destroy the nucleolytic enzymes proteinase K was added to a final concentration of 100 µg per ml and the mixtures were incubated overnight at 60°C. Finally, the samples were heated for 10 min at 100°C to inactivate the proteinase K.

To investigate the effect of sodium periodate treatment on the heat-stable antigens, samples were subjected to sodium periodate treatment (final concentration of 1mM, 10mM and 20mM) (Van Winkelhoff *et al.*, 1993). Incubations were carried out for 2 h at room temperature in the dark. The reaction was terminated by adding glycerol (Merck, Germany). The results of the various treatments were analyzed by immunodiffusion and immunoelectrophoresis.

### Enzyme-linked-immunosorbent-assay (ELISA)

ELISA tests were carried out as described previously (Shah *et al.*, 1997) to measure the antibody response to capsular and LPS antigens in vaccinated and non-vaccinated buffalo calves. Whole cell extracts and whole polysaccharide extracts described in 2.2 were used as antigen to coat an ELISA tray at a concentration of

1 $\mu$ g per mL in carbonate buffer pH 9.6 and incubated overnight at room temperature. The plates were washed three times with washing solution consisting of 0.5% Tween 80 in distilled water. Serial dilutions of test sera were added to the wells and the trays were incubated for 1 h at 37°C. Subsequently, the plates were washed again three times with the 0.5% Tween 80 solution. Rabbit anti-bovine IgG peroxidase conjugate was then added (1:2000) and the plates were incubated at 37°C for 1 h. After washing three times, the substrate (O-phenylenediamine in 0.01% hydrogen peroxidase) was added and the plates were kept in the dark during 40 min. The reaction was stopped by adding sulphuric acid (1:10 ratio) and the plates were read at 492nm. The ELISA titer was assigned as a reciprocal of the highest dilution of serum that gave an maximum average absorbance.

A serum concentration just high enough to give maximum colour development was chosen to be adsorbed twice with 25  $\mu$ g of control LPS (obtained from Dr. Richard Rimler who isolated from *P. multocida* serotype B:2,5) by incubation at 37°C during 1 h. The precipitated LPS was removed by centrifugation at 14,000 rpm for 15 min. The supernatant containing the anti-capsular antibodies was used in ELISA with WPE-coated plates to measure the anti-capsular response. The response to LPS was measured by coating the plates with purified LPS.

## RESULTS AND DISCUSSION

### Identification of capsular and lipopolysaccharide antigen

Immunodiffusion and immunoelectrophoresis were used to identify the capsular and LPS antigens *P. multocida* serotype B:2,5. Three antigen preparations were used (i) whole polysaccharide extract (WPE), (ii) preparation A containing the bacterial LPS, (iii) preparation B containing the bacterial capsule. The results of immunodiffusion tests showed that WPE contained two antigens (Fig.1). The first precipitation line, near the well containing the WPE, represents the LPS because this precipitation line also occurs by diffusion using antiserum raised against *P. multocida* serotype E:2,5 (Fig.1) but not with antiserum against *P. multocida* serotype B:3,4 (not shown). The second precipitation line, near the well containing antiserum raised against *P. multocida* serotype B:2,5 represents the bacterial capsule because this precipitation line also occurs by diffusion using preparation B (Fig.1) or a

whole cell extract of *P. multocida* B:3,4 (not shown). No precipitation line was observed in immunodiffusion using preparation B and E:2,5 antiserum (Fig.1) suggesting that preparation B did not had detectable levels of LPS.

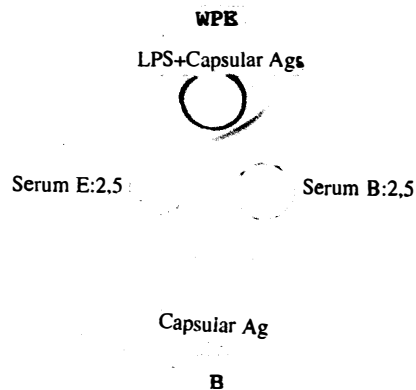


Fig.1. Double immunodiffusion test showing comparison of lipopolysaccharide and capsular antigen from *P. multocida* serotype B:2,5 reacting with E:2,5 and B:2,5 anti-sera.

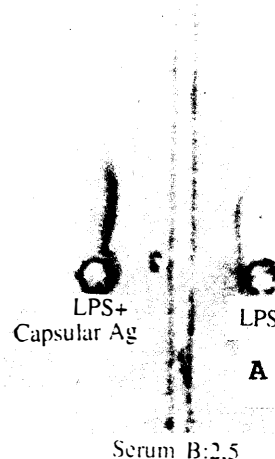


Fig.2(a). Immunoelectrophoresis showing the presence of lipopolysaccharide in preparation A by using B:2,5 anti-serum.

Immunoelectrophoresis was used to further characterize the various antigens (Fig.2). Using the WPE preparation and B:2,5 antiserum two antigens moving towards the anode were observed, indicating an acidic (polysaccharide) nature for these antigens. Using the preparation A and B the two precipitation lines could be identified as LPS and capsule, respectively (Fig.2a and b). The LPS remained near the antigen well, whereas the less hydrophobic and more acidic bacterial

capsule moved further towards the anode. Protein antigens were not detected in this experiment because the antigen preparation used were treated with proteinase K. Also nucleic acids did not play a role because previous treatment of the antigen preparations with RNase and DNase did not affect the results described above (not shown). However, treatment of preparation A and B with 1.0 or 10.0 mM of sodium periodate appeared to result in partial degradation since the precipitation lines became hardly visible. Complete degradation of both antigens was observed with 20mM sodium periodate. Which suggests that preparation A and B are purely polysaccharides.

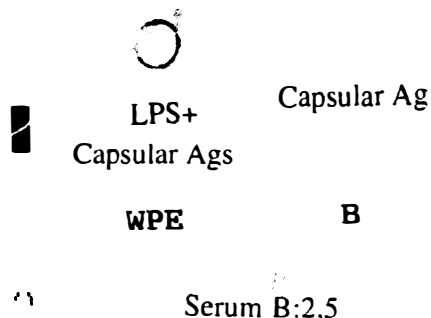


Fig.2(b). Immunoelectrophoresis showing the presence of capsule in preparation B by using B:2,5 antiserum.

#### Antibody titers against polysaccharide antigens

Antibody titers against the polysaccharide antigens described above were analyzed in sera obtained from a buffalo immunized with an experimental oil adjuvant vaccine (Shah *et al.*, 1997). The antisera used were collected just before vaccination and at 78 and 230 days after vaccination, respectively. The highest antibody titers were observed against the whole cell extract (Fig.3). Considerably lower titers were measured after treatment of this antigen preparation with proteinase K. These 5-10 times lower titers most likely results from the degradation of protein antigens present in the whole cell extract. Further analysis of the anti-polysaccharide response after removal of anti-LPS antibodies demonstrated a very low response to capsular material, whereas a 5-times higher response was measured against LPS (Fig.3). Polysaccharide capsules are known to be

poor immunogen which may explain the low anti-capsule response observed. On the other hand anti-capsular antibodies are supposed to play a major role in protection against haemorrhagic septicaemia (Nagy and Penn 1976). We observed a considerable response against protein antigens and like to suggest that the vacuolating cytotoxic activity observed in HS-causing strains of *P. multocida* (Shah *et al.*, 1996) is another possible protective antigen.

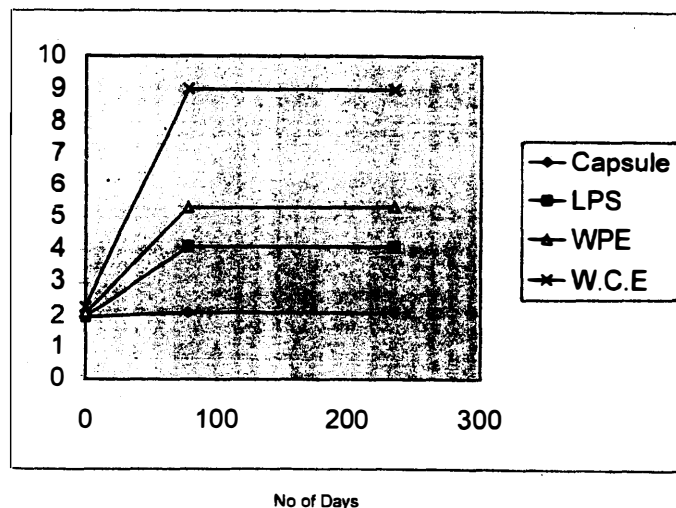


Fig.3. Antibody titers against four different antigen preparations of *P. multocida* serotype B:2,5 in buffaloes immunized with an experimental oil adjuvant vaccine.

WCE = Whole cell extract,  
WPE = Whole polysaccharide extract,  
LPS = Lipopolysaccharides

#### ACKNOWLEDGMENT

The research described in this study was granted by the Netherlands foundation for the advancement of Tropical Research (WOTRO) with financial support from the Directorate-General for Development Cooperation (DGIS).

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