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Haemolytic and cytotoxic activities of the Tween 80-extracted putative haemolysin of *Pasteurella multocida* B:2

D. Chachra^{*#}, J.G. Coote[#], R. Parton[#] S. K. Jand^{*}

^{*}Department of Veterinary Microbiology, College of Veterinary and Animal Sciences, PAU, Ludhiana, India and [#]Institute of Infection, Immunity and Inflammation, University of Glasgow, Glasgow G12 8TA, UK.

ABSTRACT

The objective of this study was to investigate the haemolytic and cytotoxic activity of *P. multocida* B:2 strains, originally from cases of haemorrhagic septicaemia in cattle. All six *P. multocida* B:2 strains were non-haemolytic on sheep blood agar (SBA) and horse blood agar (HBA) when grown aerobically and on SBA anaerobically but they were haemolytic on HBA when grown anaerobically. No haemolytic activity against horse red blood cells was detected in culture supernates from aerobically or anaerobically grown cultures and only very weak haemolytic activity was obtained in supernates or pellet fractions from sonicated cells. However, after repeated extraction of sonicated cells with Tween 80, haemolytic activity was found in various cell fractions, both Tween-soluble and -insoluble. The Tween-extracted putative haemolysin and other bacterial fractions were also cytotoxic for mouse macrophage-like J774.2 cells. Further characterisation of the putative haemolysin revealed it to be a heat-labile, non-pore-forming protein of molecular weight >10 KDa whose activity was completely destroyed by trypsin and greatly reduced with protease and proteinase K treatment. Congo red also reduced the haemolytic activity. Non-denaturing gel-electrophoresis and RBC agar

overlay revealed clear haemolytic zones but suggested that Tween was bound to some component of the *P. multocida* B:2 fractions and was responsible, to some extent, for the haemolytic activity observed. However, the effect of heat and other reagents on the Tween-extracted fractions and the lack of haemolytic activity in different Tween-extracted cell fractions of organisms other than *P. multocida* suggested that some proteinaceous component of the organism could indeed act as a haemolysin. This putative haemolysin may be one of the virulence attributes of *P. multocida*, but its characterisation and role in pathogenesis require further study.

1. Introduction

Haemolysis is associated with pathogenicity of a number of bacteria but *P. multocida* is not classified as a haemolytic species (Krieg and Holt, 1984). However, browning on blood agar plates as a sign of erythrocyte damage was reported by Carter (1984), and Diallo and Frost (2000) observed a diffuse but obvious zone of haemolysis around avian *P. multocida* (strain VP21) colonies on horse blood agar under anaerobic conditions. Treatment of *P. multocida* strain VP 21 with Tween 80 yielded an extract that lysed red blood cells (RBCs) from the chicken, rabbit, horse, bovine and human, with the highest titres observed on chicken RBCs incubated aerobically (Diallo and Frost, 2000)

The effect that some bacterial haemolysins produce is not always apparent on diagnostic blood agar plates and special methods may be needed to demonstrate their activity. For example, non-ionic detergents and various macromolecules were used to extract streptolysin S from Group A *Streptococcus* (Ginsburg and Harris, 1963; Loridan and Alouf, 1986; Kent *et al.* 1988; Lysons *et al.* 1991). The haemolysin obtained by such methods was assumed to be stabilised by being bound to the carrier-molecule (Tsaihong

and Wennerstrom, 1983). The aim of this study was to further investigate the haemolytic activity of *P. multocida* and to determine its cytotoxic properties.

2. Materials and Methods

2.1 Strains, media and growth conditions

A total of six *Pasteurella multocida* B:2 strains were used in this study. These included strains 90016, 85020, 90025, 8402, 86006 and 85009 originally from cases of haemorrhagic septicaemia in cattle in Sri Lanka. A porcine isolate, *P. multocida* CW5519/93 (serotype D), a bovine strain of *P. multocida* (serotype A3), a bovine strain of *Mannheimia haemolytica* and *Escherichia coli* K12 were included as controls. All strains were stored as lyophilized cultures in the Institute of Infection, Immunity and Inflammation, University of Glasgow.

Bacteria were grown aerobically at 37°C for 18 h in 500-ml volumes of brain heart infusion (BHI) broth (Difco) in 2-L dimpled flasks in an orbital incubator at 150 rpm. For anaerobic growth, bacteria were inoculated on BHI agar plates and incubated for 48 h at 37°C in an anaerobic jar with Gas Pak. The resulting growth was scraped off in a minimum volume (2-3 ml) of phosphate-buffered saline (PBS). BHI blood agar was prepared by adding 8% v/v horse or sheep blood to BHI agar (Difco). Haemolysin production was evaluated on BHI blood agar after growth aerobically for 24 h or anaerobically, in an anaerobic jar with Gas Pak (Oxoid), for 48 h at 37°C.

2.2 Extraction of haemolysin

2.2. a Sonication

Bacteria from BHI broth cultures grown aerobically, or grown anaerobically on

BHI agar plates and suspended in PBS, were pelleted by centrifugation at 1100g for 15 min. The supernate was removed and the cell pellet resuspended in a minimum volume of PBS, and then sonicated on ice for 10 min, with 10 s on and 10 s off, using a standard ½” diameter probe in a Vibra cell sonicator (Sonics and Materials, Danburg, CT, USA). After sonication, the suspensions were centrifuged at 1800 g for 15 min. The supernates and the pellets, resuspended to the same volume in PBS, were stored at –20°C until used. *P. multocida* B:2 strain Pm 90016 was also grown aerobically for 18 h as described in section 2.1 but in BHI broth containing bovine serum albumin (30 mg/ml) (Sigma). The culture was then processed as described above.

2.2.b Tween 80 extraction

Tween 80 (0.5% v/v) (Sigma) in sterile PBS (Sigma) (pH 7.3) was used to extract the haemolysin from cultures grown both aerobically and anaerobically. Bacterial extracts were prepared as shown in Fig. 1. Briefly, cultures grown for 18h in BHI broth were pelleted by centrifugation and the bacteria suspended in PBS and sonicated as described in section 2.2a. After sonication, the cell fragments were suspended in an equal volume of Tween 80 (0.5 % v/v) and incubated for 2 h at 37°C. After low-speed centrifugation to remove the first Tween-insoluble fraction, the first Tween extract (Fig. 1, fraction A) was centrifuged at 70,000 g for 30 min at 4°C to obtain the cytoplasmic fraction (B) and the second Tween-insoluble fraction (C). This fraction, C, was further treated with Tween 80 for 2 h at 37°C to obtain fraction D then centrifuged at 1100 g for 15 min to obtain the third Tween-insoluble fraction E, and the second Tween extract, F. Fraction F, was centrifuged at 70,000 g for 30 min at 4°C to obtain the fourth Tween-insoluble fraction (G) and the third Tween extract (fraction H). Tween insoluble fractions

E and G were resuspended in Tween to the same volume as the corresponding soluble fractions for haemolysis assay

2.3 Haemolysis assay

Defibrinated horse blood was used except where stated. RBCs were washed thrice with PBS and resuspended at 2% v/v in PBS. Two-fold serial dilutions of the bacterial extracts were made in 500 µl-volumes of PBS in 1.5 ml tubes. To these, equal volumes of the 2% v/v horse RBC suspension were added and, after incubation aerobically for 4 h at 37°C on a shaker, unlysed RBCs were removed by centrifugation at 1100 g for 15 min and the OD_{540nm} of each supernate was determined. The controls for the test included RBCs in the appropriate concentrations of Tween 80 in PBS as negative controls and RBCs in TritonX-100 (0.1% v/v) as a positive control. All assays were performed in duplicate. Percent haemolysis was calculated as:

$$\text{OD of } ((\text{test sample}) - (\text{negative control}) / (\text{positive control}) - (\text{negative control})) \times 100$$

2.4 Effect of heat, enzymes and Congo red on the haemolytic activity of *P. multocida* B:2

Putative haemolysin (fraction H, Fig.1) was subjected to various degrees of heat (60, 80, 100°C) treatment for 30 min and then tested for haemolytic activity. Unheated sample was included as control. Various enzymes were tested for their effect on haemolytic activity. Trypsin (0.1 mg/ml), protease (0.5 mg/ml) from *Streptomyces griseus* and proteinase K (0.1 mg/ml) (all from Sigma) dissolved in PBS were mixed 1:1 with test sample, incubated for 30 min at 37°C, and the suspension was then measured for haemolytic activity, as described. Controls included Tween 80 and enzymes at appropriate concentrations, RBCs in PBS and RBCs in Triton X-100 (0.1% v/v). Congo

red dissolved in PBS was added to the Tween 80 extracted-soluble fraction (fraction H) at a final concentration of 0.25% w/v, incubated at 37°C for 30 min and then the samples were tested for haemolytic activity as described.

2.5 Cytotoxicity test

2.5.1 Preparation of cells

Mouse macrophage-like cells (J774.2 cells) were grown in RPMI-1640 medium (Cambrex Bioscience) containing L-glutamine (2 mM, Gibco), penicillin-streptomycin (100 IU/ml penicillin, 100 µg/ml streptomycin (Gibco)) and fungizone (amphotericin B 2.5 µg/ml) (Gibco) and heat-inactivated foetal bovine serum (Biowest) 10% v/v. After 2-3 days, the spent medium was decanted and cells were washed twice with fresh, non-phenol red RPMI-1640 medium (Cambrex Bioscience). The cells were scraped off into 5 ml of fresh, non-phenol red medium and centrifuged for 5 min at 1100 g. The cells were then resuspended in fresh non-phenol red medium, counted by Trypan Blue exclusion and the cell concentration was adjusted to 5×10^5 cells/ml. A 50 µl aliquot of this suspension was added to each well of 96-well flat-bottomed cell culture microtitre plates and incubated for 1 h at 37°C, in CO₂ (5% v/v).

2.5.2 MTT assay

Samples of bacterial extracts were diluted in non-phenol red RPMI-1640 medium and 50 µl of each dilution were added to wells containing 50 µl of cells, mixed gently and plates were incubated for 2 h at 37°C in CO₂ (5% v/v). After this time, 15 µl of MTT dye (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (CellTiter 96 Non-Radioactive Cell Proliferation Assay, Promega) were added to each well and plates were incubated for 4 h at 37°C in CO₂ (5% v/v). A 100 µl volume of stop solution was then

added and plates were incubated overnight at 37°C in CO₂ (5% v/v). Controls included cells in RPMI medium alone and cells in the appropriate concentration of Tween 80 (negative control). Cells treated with Triton X-100 (0.55% v/v concentration) was used as a positive control. The OD_{540nm} of the samples was measured and the percentage cell death was obtained from the following equation:

$$1 - \frac{(\text{OD of test sample} - \text{OD of negative control})}{(\text{OD of positive control} - \text{OD of negative control})} \times 100.$$

2.6 Polyacrylamide gel electrophoresis and RBC agar overlay

Tween 80 extracts (fraction H) were separated by non-denaturing gel electrophoresis with 12% acrylamide in the separating gel, according to the method of Bollag and Edelstein (1991). The gel was run at 70 volts overnight at 4°C. After electrophoresis, the gel was removed from the cast and washed three times with Tween 80 (0.5% v/v) in PBS at 37°C on a shaker, changing the Tween every 15-20 min. The gel was then washed three times with PBS at 37°C on a shaker changing the PBS every 15-20 min. The washed gel was placed in a square Petri dish and overlaid with 30 ml of molten 0.7% w/v microagar (Oxoid) in PBS which had been allowed to cool to 45°C before adding 10 ml of washed horse RBCs (2% v/v). The agar was allowed to set at room temperature for half an hour and then the Petri dish was incubated at 37°C. The RBC overlay was observed every hour for development of haemolytic zones. If no haemolytic zone appeared after 6 h, incubation was continued overnight. A sample containing Tween 80 (0.5% v/v) alone was run on the gel alongside the test samples, as a control.

3. Results

3.1 Effect of media and growth conditions on haemolysin production

All six *P. multocida* B:2 strains were non-haemolytic on sheep blood agar (SBA) and horse blood agar (HBA) when grown aerobically and on SBA anaerobically but they were haemolytic on HBA when grown anaerobically. The approximate size of zones was 1-2 mm, with no discolouration. The *P. multocida* A:3 and D strains, *M. haemolytica* and *E. coli* K12 were non-haemolytic on HBA and SBA grown either aerobically or anaerobically.

3.2.a Effect of cell sonication on recovery of haemolysin

No haemolytic activity was detected against horse RBCs in supernates from cultures grown aerobically or anaerobically and only very weak haemolytic activity was obtained from supernates of sonicated cells of *P. multocida* B: 2 strains grown in BHI broth under either aerobic or anaerobic conditions. The sonicated supernates were concentrated 25x and 4x by ultrafiltration through 30 kDa and 10 kDa molecular weight cut-off Amicon filters (Microcon), respectively, but no increase in haemolysis was detected. Pellets from cells grown aerobically or anaerobically, then sonicated, did not show any haemolytic activity (data not shown). None of the above cell fractions were cytotoxic for the J774.2 cells in the MTT assay (data not shown). No haemolytic activity was observed in culture supernates or supernates from sonicated cells of *P. multocida* B:2 grown in BHI broth containing BSA.

3.2.b Effect of Tween 80 on extraction of haemolysin

In preliminary experiments, both aerobically- and anaerobically-grown *P. multocida* B: 2 strains 90016 and 85020 whole bacterial cells, and supernates and pellets from sonicated

cells were incubated with Tween 80 (0.5% v/v) for 4h at 37°C and tested for haemolytic activity. Most of the haemolytic activity was found in the pellet fraction of the sonicated cells irrespective of whether the bacteria had been grown aerobically or anaerobically (data not shown). For convenience, the subsequent extractions were done on cells grown aerobically. After Tween extraction of cells grown aerobically (as in Fig. 1), haemolytic activity was found in various cell fractions, both Tween soluble and insoluble. Fig. 2 shows the percent haemolysis of different cell fractions from *P. multocida* B:2 strain 90016. The highest activity was found in fractions D, H and A, with 50% haemolytic titres of >16, 8-16 and 8-16, respectively. There was lower activity in fractions B and G, with titres of 2-4 and 4-8, respectively. Fraction F had activity similar to fraction D whereas fraction E had little haemolytic activity (data not shown). Similar haemolytic activity was present in fractions A, B, D, G, H (Fig.1) derived from *P. multocida* serotypes A3 (bovine strain) and D (pig strain). *P. multocida* A3 showed highest haemolytic activity in fractions A, D and G, all with 50% haemolytic titres of 8-16. There was lower activity in fractions H and B, with titres of 4-8 and 2-4, respectively. In *P. multocida* serotype D, maximum haemolytic activity with 50% haemolytic titres of >16 were observed in fraction G. Titres of 8-16, 4-8 and 2-4 were observed in fractions A, H and D, and fraction B, respectively. However, no haemolytic activity was seen in any fraction of *E. coli* K12 or *M. haemolytica*, using the same method of extraction, or with Tween alone at concentrations corresponding to those in the bacterial extract samples. No haemolytic activity could be detected when BSA was used in place of Tween 80 for the extraction. The Tween extractions described above (as in Fig.1) were repeated twice, with similar results.

3.3 Cytotoxic activity of the Tween-extracted putative haemolysin

In order to test whether the factor responsible for the haemolytic activity was also cytotoxic to cells other than RBCs, the same fractions were tested for cytotoxicity on a J774.2 mouse macrophage cell line (Fig. 3). Fractions A, D, G and H showed similar cytotoxicity with 50% cytotoxicity titres of around 16. The cytotoxicity data, therefore, correlated to some extent with the haemolytic activity (Fig. 2). Fractions D, H and A were highly active in both assays but fraction G was more active against J774.2 cells than against RBCs. Fraction B had low activity in both assays.

3.4 Effect of heat, enzymes and Congo red on the haemolytic activity of *P. multocida*

After heating a 1 in 2 dilution of the third Tween extract (fraction H, in Fig.1) of *P. multocida* B:2 strain 90016 to different temperatures for 30 min, there was a decline in haemolytic activity from 100% haemolysis with the unheated sample to 60% haemolysis at 60°C, 40% at 80°C and no haemolysis after heating to 100°C.

Haemolytic activity in fraction H was completely destroyed by trypsin treatment and greatly reduced with the other two proteases. The haemolytic activity of a 1 in 2 dilution of fraction H was reduced from 100% to 32% and 67%, when treated with protease (0.5mg/ml w/v) and proteinase K (0.1 mg/ml w/v), respectively.

Treatment of fraction H (1 in 4 dilution) with Congo red (0.25% w/v) also reduced the haemolytic activity, from 85% haemolysis to 20%.

3.5 Detection of haemolysin by non-denaturing electrophoresis and RBC agar overlay

A number of samples were subjected to non-denaturing polyacrylamide gel electrophoresis. These included fraction H from *P. multocida* strain 90016 that was either unconcentrated or concentrated 30-fold by ultrafiltration (Centiplus YM-30, Amicon). The RBC agar overlay showed clear haemolytic zones after 18 h (Fig. 4) particularly with the *P. multocida* B:2 concentrated samples but also with the unconcentrated samples. The *M. haemolytica* Tween-extract and the solution of Tween 80 also showed zones of haemolysis but they had not migrated as far as the *P. multocida* B: 2 fractions. No distinct bands coinciding with the zones of clearing were seen in a Coomassie blue-stained duplicate gel before overlay.

4. Discussion

The aim of this study was to determine and characterise the haemolytic and cytotoxic activity of *P. multocida* B:2 strains. The presence of haemolysis on HBA plates incubated under anaerobic but not aerobic conditions indicated either that the active component is inactivated in the presence of oxygen or that it is released from the bacteria only under anaerobic conditions. However, the latter explanation is most likely as haemolytic activity could be extracted from both aerobically- and anaerobically-grown bacteria, as described below. No haemolytic activity or cytotoxic activity for mouse macrophage J774.2 cells was detected in culture supernates of *P. multocida* B:2 strains even after concentration with 10 KDa and 30 KDa cut-off membranes. Shah *et al.* (1996) and Diallo and Frost (2000) had also reported little vacuolating and cytotoxic activity in the culture supernates of *P. multocida* B:2 strains. Moreover, in the present study, little haemolytic activity was detected in supernate or pellet fractions from sonicated cells of *P. multocida* B:2.

A non-ionic detergent Tween-80 was used to extract the putative haemolysin, as reported previously by Diallo and Frost (2000) with an avian strain of *P. multocida*. Repeated extractions with Tween 80 to extract the haemolysin into the soluble fraction led to the idea that the component is either in an inactive form before extraction or is cell-bound. However, other possibilities, as assumed for the haemolysin of Group B streptococci, were that the haemolysin was continually synthesized and released by the bacteria but was rapidly inactivated unless it was associated with other molecules, like Tween 80, that act as carriers for this haemolysin (Marchlewicz and Duncan, 1980).

The Tween-extracted haemolytic activity from both aerobically and anaerobically-grown bacteria was found to be genus-specific since extracts from *P. multocida* A:3 and D strains showed haemolytic activity whereas those from *E. coli* and *M. haemolytica* treated in the same way did not. The haemolytic extract from *P. multocida* B:2 was heat-labile and was inactivated by various proteases which suggested it was a protein. These contrasts with the findings of Diallo and Frost (2000), working with a Tween extract from an avian *P. multocida*, who found it to be heat-stable. The extraction procedure adopted by Diallo and Frost (2000) was different in that whole cells were treated with Tween 80, rather than the sonicated cells as used in the present study. The apparent molecular weight of the putative haemolysin was >10 KDa as the haemolytic activity was seen in the concentrate, rather than the filtrate of haemolytic extract, when passed through a 10 KDa cut-off filter (data not shown).

Oxygen-stable haemolysins of streptococci have been shown to be induced by certain agents like serum and serum albumin (Ginsburg and Harris, 1963). The

haemolysins induced by these agents were all non-antigenic and their activity was inhibited by trypan blue and Congo red (Ginsburg and Harris, 1963). In our study, haemolysin was extracted by Tween 80 but not by bovine serum albumin. Duncan and Mason (1976) suggested that, in the case of streptolysin S, certain agents removed the active peptide from the surface of the bacteria and served as carrier molecules for the haemolysin. The carrier molecule Tween 80 is thought to bind to streptolysin S by substituted oxyethylene polymers via hydrogen bonding (Ginsburg and Harris, 1963). There is also a report of a requirement for a carrier of the extracellular haemolysin released by *S. agalactiae* during growth (Tsaihong and Wennerstrom, 1983). In this report, there was enhanced production of extracellular haemolysin in the presence of Tween or starch added to the culture medium and there was evidence of an association of the haemolysin with the molecules eliciting its release and determining some of its properties. With *P. multocida*, Tween might be sequestering some haemolytic component of the organism and may be acting as a carrier molecule, as with streptolysin S, but the mechanisms by which this is achieved are unknown.

The Tween-extracted putative haemolysin and other bacterial fractions as prepared in Fig. 1 were cytotoxic for J774.2 cells. The cytotoxicity results correlated to some extent with the haemolytic activity, suggesting the cytotoxic effect of the putative haemolysin against J774.2 cells. Haemolysins and cytolysins are membrane-damaging agents. They play a multifunctional role in disease due to their ability to lyse erythrocytes, mast cells, polymorphs and other cells (Braun and Focarte, 1991). Membrane-damaging toxins form a large, heterogenous group comprising substances which are characterized functionally by their ability to act either as surfactants,

solubilizing the membrane through a detergent-like action, or through enzymatic or pore-forming activity (Braun *et al.* 1993, Titball, 1993, Rowe and Welch, 1994). The pore-forming cytotoxins from Gram-negative bacteria are considered to be virulence determinants (Welch, 1991), although their precise role in the pathogenesis of any disease has been difficult to define. In the present study, the putative haemolysin of *P. multocida* B:2 did not seem to be a pore-forming toxin as no osmotic protection was afforded by suspending the RBCs in solutions of polyethylene glycols of different sizes (Kuga, 1981) (data not shown).

The results of non-denaturing gel electrophoresis and RBC agar overlay showed clear haemolytic zones in lanes containing samples of *P. multocida* B:2 haemolysin, especially when concentrated by ultrafiltration. However, there were haemolytic zones in lanes containing *M. haemolytica* extract and Tween alone although the mobility of the putative haemolysin was greater in the *P. multocida* B:2 samples. These results suggest that PAGE concentrates the Tween and it stays within the gel, despite the washing step. They could also suggest that, in the Tween-extracted samples, the Tween remains bound to some component of the *P. multocida* B:2 fractions and it is this concentrated detergent that is responsible, to some extent, for the haemolytic activity observed. However, the production of haemolysis on HBA under anaerobic conditions, the effect of heat and other reagents on the Tween-extracted fractions and the lack of activity in different Tween-extracted cell fractions of organisms other than *P. multocida* suggests that some, presumably proteinaceous, component of the organism can indeed act as a haemolysin. This putative haemolysin may be one of the virulence attributes of *P. multocida*, but its characterization and role in pathogenesis require further study.

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366

CAPTIONS OF FIGURES

367

368 Fig. 1. Flow diagram of the method used for the extraction of putative haemolysin of *P.*
369 *multocida*

370

371 Fig. 2. Haemolytic activity in different bacterial fractions of *P. multocida* B: 2 strain
372 90016, prepared as described in Fig. 1, by Tween extraction.

373

374 Fig. 3. Cytotoxicity for J774.2 cells in different bacterial fractions, prepared as described
375 in Fig. 1, by Tween extraction of *P. multocida* B: 2 strain 90016

376

377 Fig. 4. PAGE and RBC agar overlay to demonstrate haemolytic activity in Tween-
378 extracted fractions. Lanes 1, 2: *P. multocida* B: 2 strain 90016, fraction H concentrated
379 fractions, from different extracts prepared on different occasions; Lane 3: *P. multocida*
380 B:2 strain 90016, fraction H unconcentrated; Lane 4: *M. haemolytica* fraction H
381 unconcentrated; Lane 5: Tween 80 (0.5 % v/v)

382