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Phagocytic uptake and killing of *Pasteurella multocida* B: 2 by mouse peritoneal phagocytic cells

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ABSTRACT

The phagocytic uptake and killing of *Pasteurella multocida* B: 2 field isolates (n, 9) and vaccine strain (P52) by mouse peritoneal phagocytic cells, in the absence and presence of opsonization was evaluated using *in vitro* phagocytosis and bactericidal assays. Opsonization either with hyperimmune serum or normal serum caused a nonsignificant increase in the percentage of phagocytosis over non-opsonized bacteria (P<0.01) in all the *P. multocida* B: 2 isolates including P52. It was observed that 99% of the bacteria were killed within 15 min of incubation, irrespective of the serum used. There was a static increase in cfu counts by ~ 5 log at the end of the assay at 240 min. The study revealed that opsonization did not alter the phagocytic uptake and the bacteria were efficiently phagocytosed and killed in *vitro* under different conditions.

Key words: *Pasteurella multocida* B: 2, Phagocytosis

As a first line of defense against invading organisms, both neutrophils and macrophages play an important role in the phagocytosis and killing of *Pasteurella multocida*. The role of phagocytic cells was studied earlier in turkeys (Harmon *et al.* 1991, Priumboom *et al.* 1999), chicken (Poermadjaja and Frost 2000) and rabbits (Anderson *et al.* 1984) using different *P. multocida* strains. However, little is known of the phagocytic uptake and bactericidal effect against *P. multocida* B: 2. The purpose of the present study was to investigate the phagocytic uptake and killing of *P. multocida* B: 2 isolates along with the vaccine strain (P52) by mouse phagocytic cells as well as to study the role of opsonization on these activities.

MATERIALS AND METHODS

Nine isolates of *P. multocida* B: 2 from various outbreaks of HS in buffaloes in Punjab state during July 2007 to July 2008 along with the vaccine strain (P52) were studied. The vaccine strain P52 was obtained from Punjab Veterinary Vaccine Institute, Punjab. All the isolates (n, 9) were virulent and caused mortality in buffaloes. Bacterial cultures were maintained on brain heart infusion (BHI) agar supplemented with 5% defibrinated sheep blood. Hyperimmune serum (HIS) was raised against *P. multocida* vaccine strain P52. The *P. multocida* vaccine strain (P52) was grown on the BHI agar at 37°C. After overnight incubation, (18–20 h) the organisms were harvested in phosphate buffer saline and the turbidity of the cell suspension was adjusted to that of Brown's opacity tube No. 7 (2×10^9 organisms/ml). Formalin was added to the bacterial suspension to a concentration of 0.5% and left at 37°C for 24 h. The formalin killed cell suspension was checked for sterility by inoculating in BHI broth and on to blood agar. The formalin killed suspension was mixed with an equal volume of Freund's incomplete adjuvant to give final concentration of 1×10^9 organisms/ml. Rabbits were injected subcutaneously with 1 ml of the prepared antigen. The presence of antibodies was tested after 3 weeks by slide agglutination test. Two boosters with the same dose and route were given at an interval of 3 and 4 weeks. Heart blood was collected 1 week after the last injection and HIS separated, aliquoted and stored at –20°C, normal rabbit serum was collected from non- immunized clinically healthy adult German Angora rabbits. Sera with active complement were thawed and filtered immediately prior to each experiment.

Mouse peritoneal phagocytic cells were harvested as per Hudson and Hay (1976). Briefly, the phagocytic cells were stimulated 6 days prior to experiment with an intraperitoneal (IP) injection of 1 ml thioglycolate broth which significantly increased the number of macrophages (70–95%) with

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corresponding reduction of polymorphs and lymphocytes. Mouse was sacrificed by cervical dislocation and abdomen was opened aseptically through midline incision without disrupting the peritoneal membrane to ensure that contents remained sterile. Phagocytic cells were collected by IP lavage using 8–10 ml of sterile PBS containing Heparin @ 10 IU/ml. Cell suspension obtained was centrifuged at 250 × g for 10 min at 4°C; supernatant was aspirated and the cell-pellet was washed twice with HBSS-BFS and finally suspended in 1 ml HBSS-BFS. Cell viability was determined using trypan blue exclusion method. Cell number (viable and dead) was determined using haemocytometer and % viable cells were calculated.

The phagocytosis and killing assays were performed as per Anderson *et al*. (1984) with minor modifications. To determine the cfu/ml, *Pasteurella multocida* vaccine strain (P52) was grown in BHI broth at 37°C and OD was recorded spectrophotometrically at 540 nm wavelength after regular intervals of time and corresponding cfu were counted on agar plates. A standard absorbance curve was drawn by taking OD and cfu on×and y axis respectively. Two colonies of each *Pasteurella multocida* B: 2 isolate were inoculated in BHI broth and incubated for 6 h at 37°C and the optical density of the bacterial culture was recorded by the spectrophotometer. The cfu/ml for the corresponding bacterial culture was interpolated by using the standard absorbance curve. The bacterial cultures were centrifuged, washed twice with phosphate buffered saline (PBS) and then finally suspended in hanks' balanced salt solution (HBSS) supplemented with 10% heat inactivated bovine fetal serum (HBSS-BFS) to give final concentration of 1×10^7 cfu/ml. Bacterial suspension (0.4 ml) at 1×10^7 cfu/ml was opsonized with 0.1 ml of HIS and normal serum by incubating at 37° C for 15 min, with rotation. Phagocytic cell suspension (0.4 ml) at a concentration of 1×10^6 cells/ml was added to give 10: 1 ratio of bacteria to phagocytic cells. The final volume was made 1.0 ml by adding HBSS-BFS. The reaction mixture was incubated for 15 min at 37° C with rotation to permit phagocytosis to occur. After 15 min, tubes were set in ice to stop phagocytosis. The phagocytic cell – bacterial suspensions were centrifuged (115 g × 10 min), and the number of unphagocytozed (non-cell associated) bacterial colony forming units (cfu) was determined from the aliquots of the supernatant, using standard dilution and plate counting techniques. Supernatant samples (0.1 ml) were serially diluted in PBS and inoculated onto BHI agar plates in duplicate. Controls consisted of (i) bacteria in HBSS-BFS and (ii) serum and bacteria without phagocytic cells. Per cent of phagocytosis at 15 min was calculated according to the following equation:

$$[(N_o-N_{15})/N_0] \times 100$$

where N_o, number of cfu in the initial inoculum and $N_{15,}$ number of cfu in supernatant at 15 min.

The cell pellets containing phagocytic cells and cell-associated bacteria were washed in HBSS-BFS and then suspended in 1 ml HBSS-BFS. This suspension was incubated at 37° C with rotation, and aliquots were removed at 15, 30, 60, 120, 180 and 240 min to determine the number of remaining viable cell associated bacteria. 0.1 ml aliquot of the cell suspension was added to 1 ml of distilled water and vortexed for 60 s with incubation for 15 min at room temperature to lyse the phagocytic cells for release of the bacteria. Serial dilutions were made and inoculated onto duplicate BHI plates. The % of bacterial killing or growth at 15, 30, 60, 120, 180 and 240 min was calculated according to the following equation:

$$[P_{15}-P_t)/ P_{15}] \times 100$$

where P_{15}, number of phagocytic cell associated cfu at 15 min and P_t, number of phagocytic cell associated cfu at 30, 60,120, 180 or 240 min.

Data from phagocytosis and killing assays was analyzed statistically using one way analysis of variance (ANOVA) and 2- way analysis of variance (ANOVA) respectively at 1% level of significance ($P\geq0.01$).

RESULTS AND DISCUSSION

There are various factors which affect the susceptibility of bacteria to phagocytosis and subsequent killing by phagocytes. Though there are many reports regarding *in vitro* phagocytosis and killing assays using various combinations of *Pasteurella multocida* serotypes and phagocytic cells as mentioned earlier but no specific roles for innate defense mechanisms have been demonstrated in *P. multocida* B: 2 isolates. The phagocytic uptake of *P. multocida* B: 2 isolates (n,9) and the vaccine strain (P52) by mouse peritoneal phagocytic cells was measured for both opsonized and non-opsonized bacteria *in vitro*. The phagocytic uptake in the absence of serum ranged from 85% to 99.66%, while 93.5% to 99.8% and 96.8% to 99.99% bacteria were phagocytozed when normal serum and HIS were used as the opsonic source respectively (Table 1, Fig.1). The susceptibilities to killing by mouse peritoneal phagocytic cells for all the *P. multocida* B: 2 isolates alongwith the vaccine strain (P52) were examined in continuation of the phagocytosis assay. There

Table 1. Percentage phagocytosis of different isolates under different conditions at 15 min

Isolate	No serum	Normal serum	HIS
P52	99	99	99.5
LDH 67	85	96.3	98.96
LDH 68	99.5	99.7	99.8
LDH 69	95.3	99.2	99.7
LDH70	88.7	93.5	96.8
LDH71	93.2	97.5	98.3
LDH72	91.76	93.5	99.87
LDH73	85.9	97.5	98
LDH74	89.39	95.69	98.33
LDH75	99.66	99.8	99.99

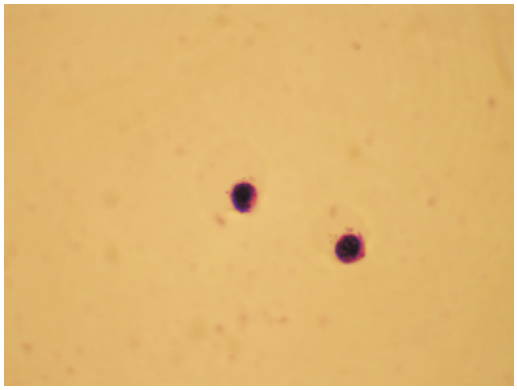

Fig 1. Uptake of bacteria by phagocytic cells in the presence of hyperimmune serum.

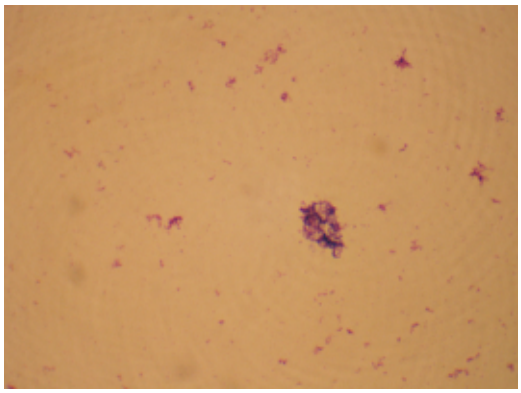

Fig 2. Release of engulfed bacteria after lysis of phagocytic cell.

was no change in killing % at 15, 30 or 60 min of incubation. Also, killing % did not change in the absence or presence of normal serum and HIS at different time intervals. So, incubation period for phagocytic cells' lysis to release the engulfed bacteria was chosen to be 15 min. During the initial 15 min incubation, more than 99% killing by phagocytic cells was observed for all the *P. multocida* B: 2 isolates (n,9) and the vaccine strain (P52); both in the absence or presence of normal serum and HIS (Figs 2,3). *In vitro* killing of bacteria by phagocytic cells within 15 min has been reported earlier by Guckian *et al*. (1978). After further incubation for 30, 60 or 120 min, no additional killing was observed both in the

Table 2. Percentage killing of different isolates under different conditions at different time intervals

Isolates		Percentage killing					
		15 min	30 min	60 min	120 min	180 min	240 min
P52	No serum	99.96	99.96	99.97	99.95	99.92	69.2
	Normal serum	99.97	99.97	99.96	99.94	99	70
	HIS	99.99	99.99	99.99	99.49	99.35	69.5
LDH 67	No serum	99.9	99.97	99.59	99.94	99.3	70
	Normal serum	99.81	99.97	99.73	99.98	93.5	69
	HIS	99.99	99.99	99.85	99.93	99.2	68.5
LDH 68	No serum	99.56	99.94	99.82	99.96	97.85	72.3
	Normal serum	99.72	99.98	99.87	99.97	92.39	71.5
	HIS	99.81	99.9	99.93	99.99	95.4	73.5
LDH 69	No serum	99.98	99.83	99.73	99.84	93.1	71
	Normal serum	99.95	99.89	99.83	99.89	91.08	69.8
	HIS	99.99	99.99	99.96	99.96	96.3	68.3
LDH 70	No serum	99.81	99.73	99.94	99.99	98.45	74.5
	Normal serum	99.97	99.88	99.97	99.99	93.18	76
	HIS	99.99	99.95	99.49	99.99	97.42	69.3
LDH 71	No serum	99.98	99.99	99.93	99.84	99.02	68.5
	Normal serum	99.98	99.99	99.81	99.94	97.08	70.8
	HIS	99.99	100	100	100	100	100
LDH 72	No serum	99.98	99.98	99.98	99.94	99.3	71.3
	Normal serum	99.91	99.95	99.95	99.9	99.5	68.3
	HIS	99.96	99.99	99.9	99.8	93	70
LDH 73	No serum	99.94	99.98	99.96	99.96	99.35	72.9
	Normal serum	99.81	99.83	99.97	99.95	99.43	70.5
	HIS	99.97	99.99	99.98	99.93	99.5	67.8
LDH 74	No serum	100	100	100	100	100	100
	Normal serum	99.98	99.88	99.9	99.95	99	80
	HIS	99.99	99.99	99.9	99.81	98.9	75
LDH 75	No serum	99.99	99.99	99.98	99.93	99.2	70
	Normal serum	99.99	99.98	99.94	99.89	99.15	69.8
	HIS	99.99	99.99	99.98	99.97	99.29	69.5

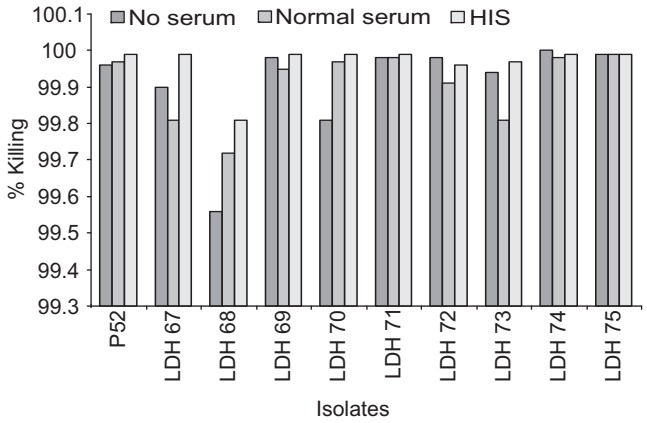

Fig 3. Percentage killing of different isolates under different conditions at 15 min.

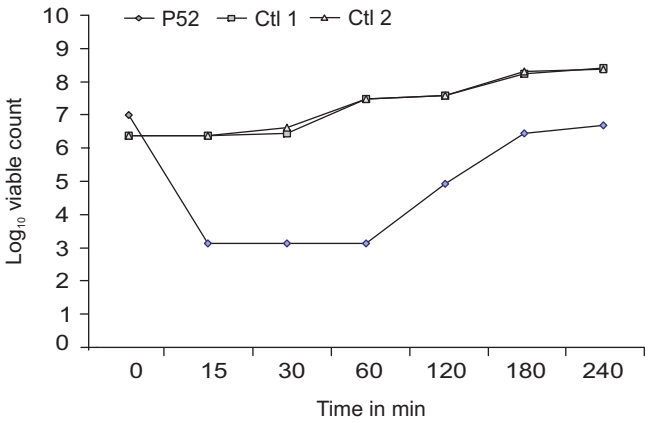

Fig 5. Growth pattern of vaccine strain (P52) in the presence of HIS and controls.

absence or presence of normal serum and HIS. Similar results were obtained for all other isolates (Table 2). Both opsonized and non-opsonized bacteria increased during the course of the assay after 120 min of incubation in the presence of phagocytic cells. CFU counts increased ~ 5 log at the end of the assay at 240 min in the presence of phagocytic cells. Bacterial count increased from $\log_{10}$ 6.47 to $\log_{10}$ 8.95 in the presence of normal serum and from $\log_{10}$ 6.6 to $\log_{10}$ 8.90 in HIS for vaccine strain P52 after 240 min of incubation (Figs 4, 5). Similar results were obtained for all other isolates. For all the nine *P. multocida* isolates and the vaccine strain (P52), opsonization either with HIS or normal serum did not increase the% of phagocytosis significantly over non-opsonized bacteria ($P \leq 0.01$). It indicates that serum opsonins (complement and immunoglobulins) are not mandatory for phagocytosis of bacteria by phagocytic cells. Surface characteristics; either physical or physicochemical, favour the bacterial interaction with the phagocytic cells in the absence of serum (Rest *et al.* 1982). Almost complete phagoctyosis in the presence of normal serum suggests that serum from clinically normal animals contained antibodies against the components of *P. multocida*. The organism may

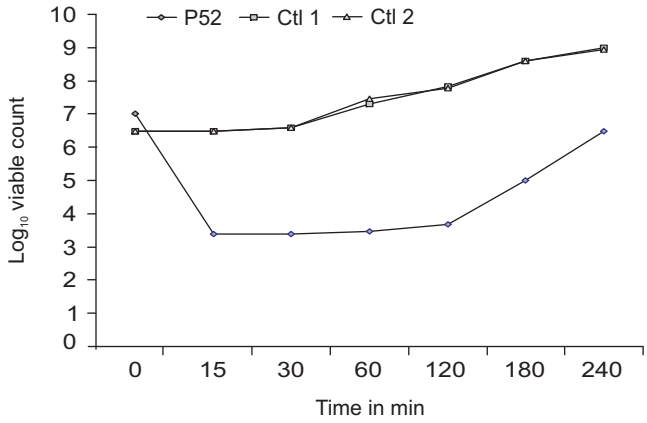

Fig 4. Growth pattern of vaccine strain (P52) in the presence of normal serum and controls.

be carried in the nasal passages of clinically normal animals (Lu *et al.* 1978). Further explanation for increased opsonization could be that the non-immune serum contained sufficient cross-reactive antibodies to be opsonic in the presence of active complement. Rush *et al.* (1981) made similar observations for different *P. multocida* serotypes. Inspite of high titer of HIS used (1: 640), no significant difference was apparent in opsonizing capability with phagocytosis as an indicator. It is quite conceivable that the biological property of the specific antibody may only be in serological reactions and not in the opsonizing reactions. Similar findings were reported by Maheswaran *et al.* (1980) regarding the phagocytic uptake of the opsonized bacteria. HIS had little effect in promoting the killing of any of the isolates when compared with the corresponding non-opsonized bacteria. It clearly indicates that they became cell-associated in the absence of serum opsonins. Hence killing could be attributed to the loss of bacterial virulence due to prolonged cultivation *in vitro*. Moreover, certain bacteria produce virulence factors only during growth *in vivo* (Collins 1977, Frost *et al.* 1972 and Petkus *et al.* 1979). Antigenic changes in *P. multocida* during growth *in vivo* were reported by Petkus *et al.* (1979). All the 9 *P. multocida* isolates and the vaccine strain P52 when compared among themselves, for killing by mouse peritoneal phagoytic cells under different conditions and at different time intervals differed insignificantly at 1% level of significance ($P \leq 0.01$). The viability of the bacteria was checked in sera as control. The normal serum or HIS was neither growth inhibiting nor bactericidal. Antibacterial immunoglobulin did not appreciably affect the ability of bacteria to grow and divide (Young and Armstrong 1972), since bacterial numbers increased in immune serum at the same rate as in normal serum. The viability of phagocytic cells was 95 to 99% at the start of the assay and remained unaltered till the end of the experiment. Similar results were obtained by other workers using different serotypes of *P. multocida* (Anderson *et al.* 1984, Poermadjaja and Frost 2000). During the course

of the assay after 120 min of incubation, both opsonized and non-opsonized bacteria were increasing. CFU counts were increased ~ 5 log at the end of the assay at 240 min. Similar findings were reported for *P. haemolytica* (Czuprynski *et al.* 1987), *P.multocida* serotype D strains (Anderson *et al.* 1984) and *P. multocida* serotype A (Poermadjaja and Frost 2000).

Thus phagocytic cells efficiently killed the bacteria *in vitro* under different conditions used in the study. But, further study on phagocytic uptake and killing of *P.multocida* B: 2 need to be carried out *in vivo* to know the exact role of phagocytic cells to determine the pathogenesis of the disease.

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