ISSN 1682-8356
ansinet.org/ijps

INTERNATIONAL JOURNAL OF

POULTRY SCIENCE

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International Journal of Poultry Science 3 (8): 517-521, 2004

Antigenic Analysis of Outer Membrane Proteins of Biofilm and Free Cells of *Pasteurella multocida* A:1 in Comparison with Local Isolate

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Abstract: Outer Membrane Protein (OMP) - enriched extracts of *Pasteurella multocida* A:1 ("A") and local isolate ("L") obtained from Nutrient rich free cell (FC), Nutrient deficient free cell (DC) and Biofilm cell (BC) growth were analyzed by SDS-PAGE. In general, the OMP profile in all the three growth conditions of "A" was different from that of "L" suggesting that both could be of different serotype. The FC, DC and BC derived OMPs of both "A" and "L" showed a significant variation suggesting that BC are demonstrably and profoundly different from their free cell counterparts (FC and DC). More than four unique proteins were observed in the OMPs of BC when compared to FC and DC. The western blot using Anti-BC hyperimmune serum, elicited maximum number of immunogens that were common to both "A" and "L", including immunogens that were detected by Anti-DC and Anti-FC. Some unique proteins of biofilm cells were also found to be highly immunogenic. Since, fowl cholera is a biofilm associated disease the use of biofilm form of pathogen could be an alternative strategy for evolving effective immunoprophylaxis against fowl cholera.

Key words: *Pasteurella multocida*, fowl cholera, OMP, biofilm, unique proteins

Introduction

Fowl cholera, an acute infectious disease caused by *P. multocida*, is a continuous threat to the poultry industry. So far, research efforts made to evolve safe and effective vaccines have failed to eradicate/eliminate this disease. Killed vaccines are providing protection only against homologous but not heterologous serotype (Heddleston and Rebers, 1972). Live vaccines have been reported to revert to virulent strains (Bierer and Derieux, 1972). Thus both are inadequate for the control of the disease. When *P. multocida* is propagated in an avian host, a protective immune response is induced against heterologous somatic serotypes (Heddleston and Rebers, 1972; Rimler *et al*., 1979a,b; Rimler and Rhoades, 1981). In turkeys, *P. multocida* expresses host specific cross protective antigens *in vivo* during infections but not *in vitro* cultures. The former thus induce a good immunity against heterologous serotype (Rimler and Rhoades, 1989; Rimler, 1994). Evidently, in the search for good immunoprophylaxis against this disease, speculation of fowl cholera as a biofilm associated infection arose. Moreover, the biofilm form of pathogen grown *in vitro* mimics the expression of *in vivo* antigens (Costerton *et al*., 1999). Hence an attempt was made to analyze the immunogens expressed by biofilm mode of growth in comparison with their free cell counterparts.

Materials and Methods

***Pasteurella multocida* isolates:** Standard reference strain of *P. multocida* A:1 obtained from the Indian Veterinary Research Institute, Izatnagar, U.P., India and a local isolate recovered from an outbreak of the disease from a commercial poultry farm located near Bangalore, Karnataka, India abbreviated as "A" and "L" respectively were compared by cultural and biochemical tests and confirmed as *P. multocida* according to Carter (1984).

Preparation of OMP extracts: The OMPs were derived from three day old cultures of both strains "A" and "L" under the following conditions -

1. Nutrient rich free cell (FC) - OMPs - 3.00% TSB without bentonite clay.
2. Nutrient deficient free cell (DC) - OMPs - 0.32% TSB without bentonite clay.
3. Biofilm cell (BC) - OMPs - 0.32% TSB incorporated with 0.3% bentonite clay as inert surface to produce biofilms (Veeragowda, 2003).

Outer membrane proteins of FC, DC, and BC were extracted as per the procedure described by Bolin and Jensen (1987) and subjected to SDS-PAGE analysis according to Laemmli (1970). After the completion of electrophoresis, the gel was subjected to silver staining according to Blum *et al*. (1987).

Hyperimmune sera: Three hyperimmune sera against FC, DC and BC were prepared separately in three groups of birds using respective bacterins of isolate "A". The birds with satisfactory antibody titres as tested by

Indirect Haemagglutination test (Sawada *et al.,* 1982) were bled, sera were separated, pooled group wise and stored in one ml aliquots at -20°C until use.

Western blotting: The OMP extracts of FC, DC and BC of both "A" and "L" isolates were electroblotted onto Nitrocellulose membrane after SDS-PAGE using semi-dry blotting system (3mA/Cm^2, 60 minutes). The membrane was incubated in blocking solution (4% non-fat purified casein in PBS buffer, p^H 7.5) overnight at 4°C. The blotted proteins were subsequently probed with respective immune sera and incubated with 1:100 diluted (secondary antibody) antichicken rabbit IgG conjugated with Horseradish peroxidase and visualized with Ortho dianisidine dihydochloride.

Results and Discussion

SDS-PAGE

OMP profile of *P. multocida* A:1 ("A"): SDS-PAGE profile revealed repression of 89.0, 83.0, 45.0, 33.0 and 17.0 kDa proteins whereas 80.0, 50.0, 46.0, 36.0, 31.0, 27.0, 14.0 and below14.0 kDa proteins were overexpressed in BC when compared to FC. Other proteins viz. 75.0, 68.0, 52.0, 26.0 and 15.0 kDa were expressed in similar fashion in both BC and FC.

Similarly, repression of 87.0, 45.0, 39.0, kDa proteins and overexpression of 75.0, 52.0, 50.0, 46.0, 26.0, 15.0, 14.0 and below14.0 kDa proteins was seen in BC upon comparison with DC, whereas similar expression of 80.0, 68.0 and 31.0 kDa proteins was seen in both BC and DC (Fig. 1). These observations indicate that the biofilm bacteria are demonstrably and profoundly different from their free cell counterparts with respect to their protein profiles. Similar differences also existed between the free cells and biofilm cells of "L" (Vadakel, 2001).

These variations could be attributed to the alteration of gene expression following adhesion of the bacteria to the surface of the bentonite clay particles. These observations are in accordance with the reports of Costerton *et al.* (1995) that the adhesion of bacteria to a surface triggers the expression of a number of genes, making the biofilm cells clearly phenotypically different from their free cell counter parts.

Vadakel (2001) reported the relative molecular weights of biofilm cell OMPs of *P. multocida* serotype A:1 grown in TSB. In the present study also, under identical conditions with the same serotype, about nine similar proteins were observed.

OMP profile of local isolate of *P. multocida* ("L"): SDS-PAGE profile revealed repression of above 97.4, 95.0, 46.0, 42.0, 37.0, 28.0, 20.0 and 15.0 kDa proteins in BC when compared to FC. On the other hand, over expression of 75.0, 63.0, 56.0, 52.0, 45.0, 44.0, 36.0, 33.0, 29.0 and 26.0 kDa proteins occurred in the BC. The 87.0, 68.0 and 47.0 kDa proteins were defined in similar fashion in both FC and BC.

Likely, repression of three proteins of above 97.4, 60.0, 42.0, 31.0, 27.0 and 20.0 kDa proteins, overexpression of 87.0, 75.0, 68.0, 63.0, 56.0, 52.0, 45.0, 44.0, 36.0, 33.0, 29.0 and 26.0 kDa proteins was observed in BC when compared to DC. A 47.0 kDa protein was seen to be expressed in similar fashion in both BC and DC (Fig. 2).

In the present study, *P. multocida* "A" revealed a total of 11, 13 and 15 proteins in FC, DC and BC OMP's respectively. On the other hand, "L" showed a total of 14 proteins both in DC and FC antigens whereas 13 proteins were seen in case of BC.

Comparison of "A" with "L" isolate

A. Nutrient rich free cell (FC) of "A" with "L": "A" expressed one protein of above 97.4, and others are 89.0, 83.0, 75.0, 68.0, 52.0, 45.0, 33.0, 26.0, 17.0, and 15 kDa proteins. Further "L" showed expression of one protein of above 97.4, and others are 95.0, 87.0, 68.0, 52.0, 47.0, 46.0, 42.0, 37.0, 33.0, 28.0, 26.0, 20.0 and 15.0 kDa proteins.

B. Nutrient deficient free cell (DC) of "A" with "L": Isolate "A" expressed one protein of above 97.4, and others are 87.0, 80.0, 68.0, 52.0, 45.0, 39.0, 36.0, 33.0, 31.0, 27.0, 26.0, and 15.0 kDa proteins. On the other hand, "L" showed expression of 3 proteins of above 97.4, and others are 87.0, 68.0, 60.0, 52.0, 47.0, 45.0, 42.0, 33.0, 31.0, 27.0 and 20.0 kDa proteins.

C. Biofilm cell (BC) of "A" with "L": Isolate "A" expressed one protein of above 97.4 and others are 80.0, 75.0, 68.0, 52.0, 50.0, 46.0, 36.0, 33.0, 31.0, 27.0, 26.0, 15.0, 14.0, and below 14.0 kDa proteins. Further, isolate "L" showed expression of 87.0, 75.0, 68.0, 63.0, 56.0, 52.0, 47.0, 45.0, 44.0, 36.0, 33.0, 29.0 and 26.0 kDa proteins.

Thus analysis and comparison of OMP profile of "A" and "L" isolates showed wide variation between the two isolates (Fig. 1 and 2). This reflects that both the isolates may not belong to the same serotype.

Unique proteins identified: Unique proteins of "A" seen only in BC were 50.0, 46.0, 14.0 and below 14.0 kDa. On the otherhand, nutrient deficient conditions (both in DC and BC) showed 80.0, 36.0, 31.0 and 27.0 kDa proteins. Similarly, BC of "L" showed unique proteins of 75.0, 63.0, 56.0, 44.0, 36.0, and 29.0 kDa. Whereas DC and BC combined had 45 kDa band as unique protein.

Western blotting: Western blot is a useful technique extensively used to detect antibody targeted to individual antigenic determinants in a crude antigen mixture. In the present study three different hyperimmune sera (Anti-FC, Anti-DC and Anti-BC) raised against "A" were used to

Table 1: Comparative analysis of immunogens detected by western blot with three different hyperimmune sera

Blot Antigens	Hyperimmune sera against "A" of		
	DC	FC	BC
"A" DC	>97.4, 87.0, 80.0, 68.0, 45.0, 33.0, 26.0 **(7)**	>97.4, 68.0, 33.0 **(3)**	Nil **(0)**
"A" FC	>97.4, 68.0, 33.0, 26.0 **(4)**	>97.4, 68.0, 33.0, 26.0, 15.0 **(5)**	>97.4, 68.0, 52.0, 33.0 **(4)**
"A" BC	80.0, 68.0 **(2)**	68.0, 33.0 **(2)**	>97.4, 75.0, 68.0, 52.0 50.0, 33.0, 31.0, 26.0 **(8)**
"L" DC	87.0, 68.0 **(2)**	68.0 **(1)**	68.0, 52.0, 33.0 **(3)**
"L" FC	>97.4, 68.0 **(2)**	68.0, 33.0, 26.0 **(3)**	68.0, 52.0 **(2)**
"L" BC	68.0, 45.0 **(2)**	68.0, 33.0 **(2)**	75.0, 68.0, 52.0, 33.0, 26.0 **(5)**

Note - 1. FC-Nutrient rich free cell DC-Nutrient deficient free cell. BC-Biofilm cell. 2. Three hyperimmune sera X 6 antigens = 18 combinations.

identify the immunogenic proteins resolved in the SDS-PAGE.

DC hyperimmune serum: The DC hyperimmune serum recognized seven, four and two proteins of A-DC, FC and BC respectively. This serum indicated good antibody response to proteins of above 97.4, 87.0, 80.0, 68.0, 45.0, 33.0, and 26.0 kDa of "A"-DC as indicated by thick prominent band. Likewise, above 97.4, 68.0, 33.0 and 26.0 kDa of "A"-FC had good antibody response. Out of "A"-BC only 80.0 and 68.0 kDa proteins reacted with the DC hyperimmune serum as indicated by thin light band. Upon blotting with the same hyperimmune serum, two proteins of "L"-DC viz. 87.0 and 68.0 kDa were detected. Similarly, two proteins of "L"-FC viz. 97.4 and 68.0 kDa reacted in a strong manner. Likewise, strong reaction was exhibited to two proteins of "L"-BC 68.0 and 45.0 kDa.

FC hyperimmune serum: This serum showed strong antibody response to five proteins of "A"-FC proteins viz. above 97.4, 68.0, 33.0, 26.0 and 15 kDa. Similarly, strong reaction with only two proteins of "A"-BC viz. 68.0 and 33.0 kDa. On the other hand antibody response was comparatively less to three proteins of "A"-DC viz. above 97.4, 68.0 and 33.0 kDa.

With, the same hyperimmune serum, three, two and one proteins were recognized from "L"-FC, BC and DC respectively. Strong antibody response was seen only to 26.0 kDa protein and next in the order of expression was 33.0 and 68.0 kDa proteins of "L"-FC. From "L"-BC, only 68.0 and 33.0 was expressed whereas for "L"-DC, only 68.0 kDa was expressed.

BC hyperimmune serum: BC hyperimmune serum showed antibody response to eight and four proteins of "A"-BC and FC respectively whereas no response was seen to "A"-DC. This serum showed strong antibody response to above 97.4, 68.0, 52.0, 50.0 and 31.0 kDa proteins and expressed to 75.0, 33.0, and 26.0 kDa proteins of "A"-BC. Likewise, "A"-FC proteins viz. above 97.4, 68.0, 52.0 and 33.0 kDa exhibited strong reaction. There was no evidence of serum antibody response to the proteins of "A"-DC.

Five proteins of "L"-BC viz. 75.0, 68.0, 52.0, 33.0 and 26.0 kDa were showing strong antibody response with the homologous hyperimmune serum. Similarly antibody response was noticed for two proteins of "L"-FC viz. 68.0 and 52.0 kDa, and to three proteins of "L"-DC viz. 68.0, 52.0 and 33.0 kDa.

But biofilm hyperimmune serum did not react even with one protein of DC antigen. Although, it is very difficult to assign single reason for this, it may be due to any one or a combination of several factors such as:

1. Variation in the antigenic epitope (Sequence and Conformational).
2. Alterations in the proteins expressed under nutrient deficient conditions.
3. DC proteins may be expressed in low concentration and denatured rapidly.

Common immunogens detected: Blotting experiment using three hyperimmune sera with six different antigens of the two isolates "A" and "L" resulted in 18 combinations.

The analysis of these results revealed that 68 kDa protein was found in maximum of 17 combinations, followed by 33 kDa and above 97.4 kDa proteins which were identified at a frequency of 11 and 7 combinations respectively (Table 1). This indicates that these three

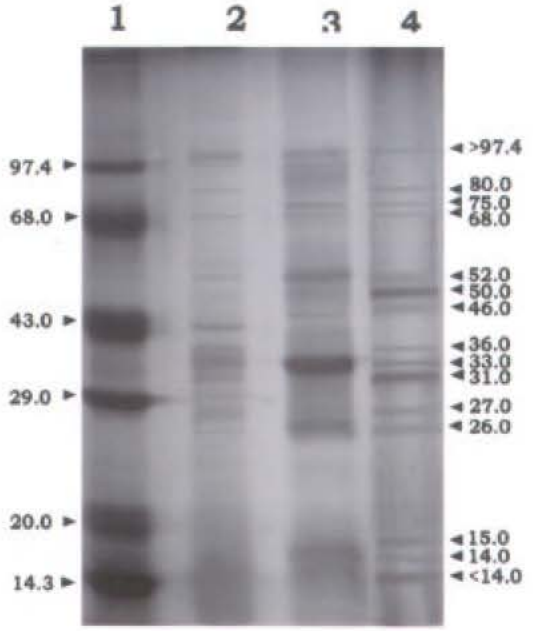

Fig. 1: SDS-PAGE resolved OMP's of free (DC & FC) and biofilm (BC) cells of *Pasteurella multocida* A:1 strain ("A")

Lane 1: Molecular weight Markers
Lane 2: Nutrient deficient free cell (DC) OMPs
Lane 3: Nutrient rich free cell (FC) OMPs
Lane 4: Biofilm cell (BC) OMPs

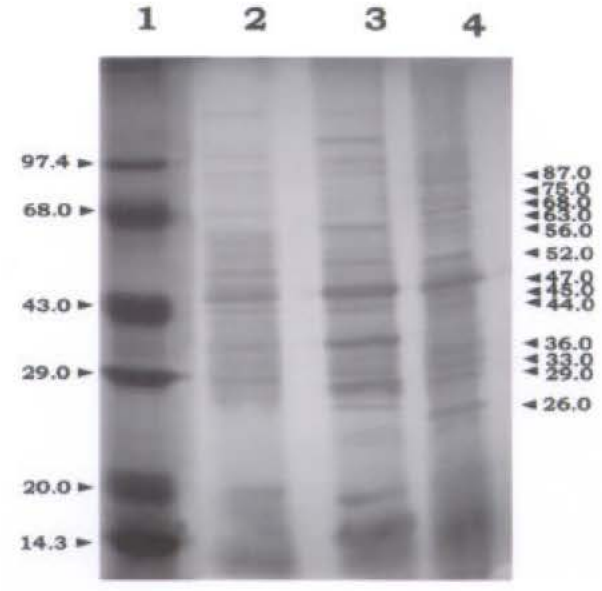

Fig. 2: SDS-PAGE resolved OMP's of free (DC & FC) and biofilm (BC) cells of Local isolate of *Pasteurella multocida* ("L")

Lane 1: Molecular weight Markers
Lane 2: Nutrient deficient free cell (DC) OMPs
Lane 3: Nutrient rich free cell (FC) OMPs
Lane 4: Biofilm cell (BC) OMPs

proteins could be the potent immunogens for prophylaxis and diagnosis.

In general, FC hyperimmune serum reacted with five proteins of homologous antigen whereas reacted with only three proteins of heterologous antigen. On the otherhand, DC reacted with seven proteins of its homologous antigen and reacted with only two proteins of heterologous antigen. The BC hyperimmune serum reacted with eight and five proteins of homologous and heterologous antigens respectively. Out of these, 75.0, 68.0, 52.0, 33.0 and 26.0 kDa proteins were common to both, reflecting maximum cross reaction among the three hyperimmune sera. Also, it included four and three proteins that were recognized by the DC and FC hyperimmune serum respectively with its homologous antigens (Table 1).

Further, biofilm hyperimmune serum reacted with the unique proteins of biofilm which were 50.0, 31 kDa of *P. multocida* "A" and 75 kDa protein of *P. multocida* local isolate ("L").

Earlier researchers, performed western blot with *P. multocida* A:3 and other serotypes to identify the immunogenic proteins. Lu *et al.* (1988) identified 27.0, 37.5, 49.5, 58.7 and 64.4 kDa proteins as protective immunogens. Pande *et al.* (2000) detected 78.0, 59.0 and 37.0 kDa as predominant protein in western blot.

These workers have used different serotypes of *P. multocida* and hyperimmune serum raised against free cell antigens. Further they have raised the hyperimmune serum in other species of animal like rabbit, whereas in this study it was raised in chicken to simulate natural host. Hence, it is reasonable, to extrapolate the result of the biofilm hyperimmune serum with that of convalescent serum from the recovered bird. However, further study in this direction is desirable.

To conclude, the differences in protein profiles of the three types of antigens of the two isolates and in general between the antigens of these isolates were evident. Further, western blot experiment with their respective homologous and heterologous hyperimmune serum raised against three types of antigens of "A" clearly identified a maximum of eight immunogenic proteins in BC and also covered wide range of antigenic determinants shared by the two isolates and among their three types of antigens of both the isolates analyzed. Hence biofilm form of pathogens could be an alternative strategy for evolving effective immunoprophylaxis against fowl cholera.

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