The Immunological Responses to Various Cell Wall Fractions of *Pasteurella multocida* in Chicken

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Summary

Various cell wall fractions of *Pasteurella multocida* vaccine strain serotype A:1 including sonicated antigen, heat stable (HS), lipopolysaccharide-protein complex (LPS-P), outermembrane protein (OMP), capsular (CAP) and potassium thiocyanate (KSCN)–extracted proteins were extracted. Their immunogenecity was studied and compared with inactivated whole cell vaccine *in vitro* and *in vivo*. The protein estimation and the polypeptide pattern of these antigens were also compared by Lowery and SDS-PAGE methods. In general the immunological responses to these fractions were varied. The chickens immunized with KSCN–extract antigen and OMP showed the highest protection when they were challenged with homologous strain, while HS and LPS antigens showed the lowest protection. These findings are in correspondence with the end–point titer of antibodies using ELISA. Cross-reaction was also observed by agar gel difussion test between the fractions and raised antisera in chickens especially with KSCN-extract, OMP and CAP antigens. The results suggest that both OMP and KSCN-extract protein as more effective immunogens could be good candidates for development of a subunit vaccine substitute for current whole cell formalized vaccine against fowl cholera infection.

Key word : *Pasteurella multocida*, cell wall immuogens, immunization, vaccine

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Introduction

Pasteurella multocida (*P.multocida*) is the causative agent of many animal diseases including fowl cholera with high rate of morbidity and mortality. The disease happens all over the world with severe losses in wild and domestic fowls, hence prevention of the disease could be a great help for the economic loss (Heddleston & Rhoades 1978). The pathogenesis of the infection is poorly undrestood, however it is known that the severity of the disease may vary considerably depending on the host and bacterial factors. The port of enterance of the organism in birds is mucousmembrane of pharynx, nasal passages, cutaneous and conjuctiva (Rimler & Glisson 1997). Birds that recover naturally from the disease gain solid immunity against reinfection hence various studies on different aspects of this organism including immunogenecity are under progress. Protective immunity can be induced by live, low-virulent strain or inactivated whole cell vaccines but they have some disadvantages like causing systemic or recurrence of infection during outbreaks in vaccinated birds (Bierer & Derioux 1972, Lamont & Wichman 1977). Many attempts have been made to develop a new and effective vaccine. A sensible method for making such an agent is to compare the immunogenecity of various cell wall fractions and choose the fraction which is able to produce better immunity with higher serum titer *in vivo* and *in vitro* (Avakian *et al* 1986).

The use of various cell wall fractions have been investigated. Leu *et al* (1999) showed that native OMP strain X-73 induces homologous protection in chickens and also several synthetic peptides derived from OMP produced practical protection in animals against the bacterial infection. Effects of LPS-P complex and crude capsular antigens (CCA) of *P.multocida* serotype A and delayed type hypersensitivity responses in chickens showed that the antigens have a property enhancing humoral and cell madiated immune responses (Maslog *et al* 1999).

The aim of the present study was to elucidate the immunity of several antigens prepared from the cell wall of *P.multocida* (serotype A:1) and compare the end-point

titer of these fractions *in vitro* by ELISA and *in vivo* by challenge in immunized chickens.

Materials and Methods

Bacterial strains. Five strains of *P.multocida* isolated from north provinces of Iran including PM1030, PM1040, PM1034, PM1033 and PM1036 and, the vaccine serotype A:1 strain (Aerobic Bacterial Vaccines Dept., Razi Institute, Karaj) were cultured on blood agar plates containing 5% sheep blood and incubated at 37°C for 24h. The PM1034 was isolated from duck.

Antigens preparation. For extraction of KSCN-extract of protein, capsular (CAP) and LPS-P complex, from strain A:1, bacteria were grown in 2 liters of BHI broth (OXOID) at 37°C for 18 h. Cells were harvested, washed with PBS (pH 7.2) and centrifuged at 12000*g* at 4°C for 25min. Finally 10gr (wet weight=ww) of bacteria was obtained. The KSCN-extract protein was prepared using 4gr ww of the bacteria in 200ml of 0.5M potassium thiocyanate buffer with 0.08M NaCl (pH6.3) and slowly stirring for 5h at 37°C. The suspension was centrifuged at 19000*g* for 35min at 4°C and the supernatant was dialysed against four changes of 0.1M Tris–HCl buffer with 0.32M NaCl (pH8) for 34h. CAP antigen was prepared from 2gr ww of the bacteria in 0.0425% NaCl buffer and stirring at 56°C for 1h (Kodama *et al* 1981). The suspension was cetrifuged at 19000*g* for 35min at 4°C and the supernatnt was dialysed against one change of 0.15% NaCl for 24h at 4°C. LPS-P was extracted from 4gr ww of the bacteria in 0.85% NaCl and 0.3% formalin, stirred for 30h at fridge temperature (Ganfield *et al* 1976, Kodama *et al* 1983). The suspension were centrifuged at 19000*g* at 4°C for 1h and the supernatant was collected and centrifuged at 105000*g* for 2h. The pellet and the buttom one ml was used as LPS–P complex. The HS antigen was prepared according to the method of Heddleston *et al* (1972).

Sonicated antigen. For sonication 4gr (ww) of the bacteria washed from blood

agar plates. The suspension was adjusted to an absorbance of 1 at 540nm (equivalent to 2×10^{10}CFU/ml. The bactreia was sonicated for 20min at 4°C.

Preparation of OMP by SDS method. The OMP was perpared with a few modification as described by Van Gelder *et al* (1994). The bacteria were grown in tripticase soy agar (BBL) overnight, washed in 10mM of Hepes buffer and finally 2gr ww of bacterial suspention was transferred into sonicating tube. The cells were sonicated for 10min at 4°C. The suspension was centrifuged at 10000*g* for 30min at 4°C and the supernatant was collected and centrifuged again for 1h. The clear pellet was suspended in 4ml of 10mM Hepes buffer containing 2% Triton X-100. 2ml of 2% SDS was added and left at 56°C for 15min. and the suspension was centrifuged at the same manner for 1h. The pellet (mixture of protein complex and peptidoglycan) was suspended in one ml of distilled water and dialysed against 2ml of 5M NaCl contain 2% SDS and left overnight at 37°C. The dialysate was centrifuged at 5000*g* at 4°C for 10min. and the supernatant was dialysed three times against distilled water for 48h at room temperature, which separates SDS and NaCl from protein complex.

Preparation of OMP by sarcosyl method. 4gr ww of bacteria was suspended in PBS equivalent to 2×10^{10}CFU/ml and OMP was prepared according to the method described by Ojanen et al (1993).

Protein estimation. Protein concentrations of various preparations were estimated by the method of Lowery *et al* (1951) with bovine serum albumin as the standard and folin ciocalteus phenol reagent.

Electrophoresis. Sodiumdodecyle sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) was carried out by the method of Laemmli (1970) to assay the purity and determine the molecular weight of protein components by using high and low molecular weight markers (Sigma).

Immunization of chickens. Eight groups of twelve chickens were injected with 1ml solution containing 500µl antigen in ten days intervals and were challenged

with of 75CFU/ml of homologous strain. The injections were carried out with complete Freund's adjuvant for the first and incomplete adjuvant for the rest of injections. Control chickens were injected only with PBS and adjuvant. The amount of protein injection for the fractions in 50µl were as follow, KSCN-extract 0.05mg, CAP 0.03mg, HS 0.025mg, OMP (sarcosyl method) 0.02mg, OMP (SDS method) 0.03mg, LPS 0.02 and sonicated antigen 0.5mg.

ELISA. The optimum concentration of antigens and antisera was determined by checker-board method. Antigens and antisera used at concentration of 1/50 to 1/1600 and 1/50 to 1/2566 repectively and further diluted serialy twofold.

Immunodiffusion tests. Antigens and antisera were tested in Ouchterlony gel diffusion and were checked after 24, 48 and 72h for immunoprecipitin reactions.

Results

Comparison between polypeptide pattern of 5 isolates of *P.multocida* as well as the vaccine strain is shown in figure 1. As it is shown there were very similarities between the polypeptide pattern of the local and the vaccine strain .The main polypeptide bands were common between the strains although the density of some of the bands (tracks J and I) was less than the other strains.

Serotype A;1 strain was choosen for antigen preparation because of the polypeptide patern simillarities with the other isolates. Protein analysis of *P.multocida* antigens was as follow, HS 0.59, KSCN-extract protein 1.01, CAP 0.68, LPS 0.45, OMP (sarcosyl method) 0.4 and OMP (SDS method) 0.6mg/ml. The comparison of polypeptide pattern of the antigens showed that OMP prepared by both sarcosyl and SDS methods, CAP and KSCN-extract proteins had very similar pattern (Figure 2). The antigens showed a major peptide band with 75KD MW approximately, also OMP extract showed two faint bands with MW's of 220 and 200KD, which only the later band was seen in KSCN-extract. Another major band with 30 to 35KD was also seen in OMP extract. HS antigen showed few fussy bands

and so many bands were seen in sonicated antigen as it was expected.

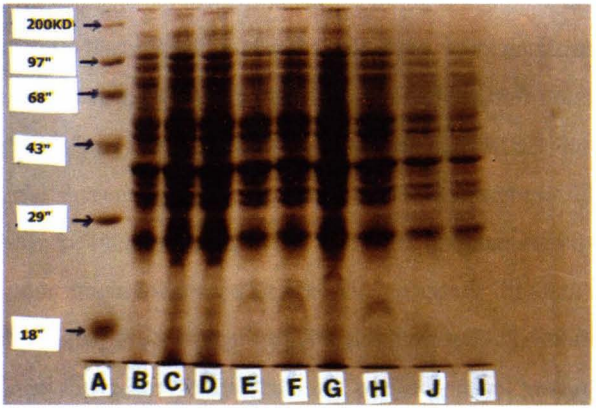

Figure 1. *Comparison between different isolates of P.multocida and the vaccine strain. Protein marker, vaccine strain A:1 (tracks A, B), strains PM 1030 (tracks C, D), 1040 (tracks E, F), 1034 (track G), 1033 (track H) and PM 1036 (tracks J, I).*

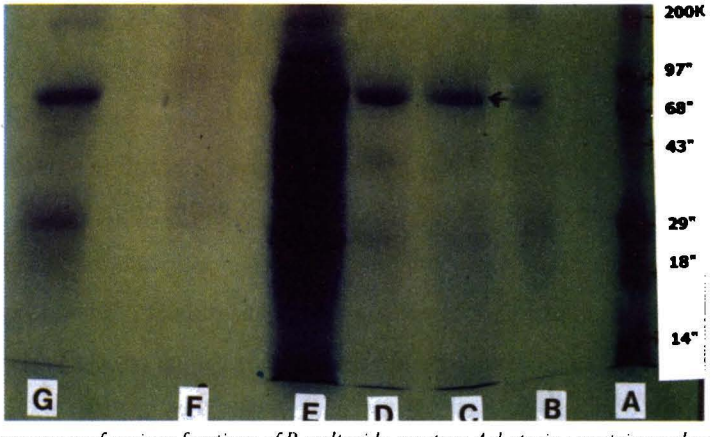

Figure 2. *Comparison of various fractions of P.multocida serotype A:1 strain ; protein marker (track A), CAP (track B), KSCN-extract (track C), OMP SDS method (track D), sonicated antigen (track E), HS (track F) and OMP sarcosyl method (track G)*

Immunization of chickens. As it is shown in table 1 OMP and KSCN-extract gave the highest protection while LPS and HS showed the least. The current inactivated whole cell vaccine showed less protection than OMP and KSCN extract.

Table 1. *Immunization of chickens and challenged with homologous strain*

Antigen	No. Chickens	No.Injections	Dose of Injection (µl)	Days of Intervals	Amount of Challenge (ml)	Challenge Dose CFU/ml	No. Death
KSCN-extract	12	3	500	10	1	75	1/12
Capsular	12	3	500	10	1	75	3/12
Heat-stable	12	3	500	10	1	75	10/12
LPS	12	3	500	10	1	75	11/12
Sonicate	12	3	500	10	1	75	4/12
OMP (SDS)	12	3	500	10	1	75	2/12
OMP (Sarcosyl)	12	3	500	10	1	75	1/12
Control	12	3	500	10	1	75	12/12

ELISA. Serum antibodies formed in response to some *P.multocida* fractions were detected by ELISA. The results are shown in table 2.

Table 2. *The end point titer of the pooled sera from the last injections against the OMP (sarcosyl method), KSCN -extract, CAP and HS antigens*

Antigen	Protein conc.(mg/well)	Antigen conc.	Antibody conc.	Antigen cut-off	End-point (reciprocal)
OMP	0.02	1/1600	1/50	0.407	2.869
KSCN-extract	0.05	1/800	1/100	0.164	1.798
CAP	0.03	1/400	1/50	0.225	0.683
HS	0.025	1/50	1/50	0.182	0.406

Immunodiffusion test. Double gel diffusion of the extracted antigens, vaccine strain and pooled antisera obtained from each group of chickens after antigen injection was studied in agar gel diffusion test. The precipitin bands were observed after second injection and maximum two bands were observed after the third injection. Anti KSCN-extract antibody showed at least three precipitation bands against KSCN–extract antigen after the third injection and also showed a band against CAP, indicating the presence of CAP in protein extract. Anti OMP antibody (prepared by

sarcosyl method) showed one precipitin band against OMP after the first injection and at least three to four bands after the second and the third injections. Also there was a precipitation band between the first anti OMP antibody and KSCN-extract, which shows some similarities between the two antigens (Figure 3).

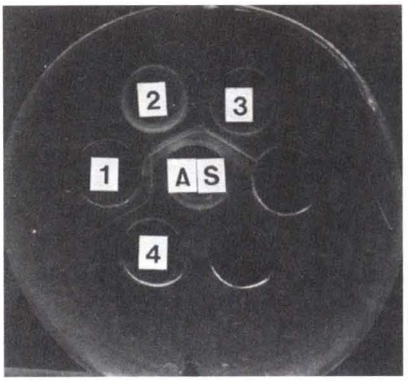

Figure 3. *Immunodiffusion gel showing the reaction between antibody to OMP antigen (centre well) and the first, second and the third antisera to OMP (wells 1, 2, 3). KSCN extract antigen is in well 4*

Discussion

Despite the use of hygiene and biosecurity, in conjunction with currently available bacterins, live vaccines or use of auxotrophic mutants, control of fowl cholera remains a problem. Many scientists have shown that various, cell wall antigens can play an important role in the development of immunity against *P.multocida* infoction in chickens (Maslog *et al* 1999, Avakian *et al* 1986, Leu *et al* 1999).

In this work we extracted several antigens from *P.multocida* serotype A:1 strain and compared and highlihted their immunogenecities with the current whole cell vaccine in chickens. The SDS-PAGE pattern of two immunogenic antigens, OMP and KSCN-extract showed very similarities with a major band of 75KD and few minor bands ranging from 18 to 90KD. In challenge studies, the same fractions revealed a good protection in immunized chickens compared to other fractions. The

same result also reported by Mukkur and Pyliotis (1981). Leu *et al* (1999) could also induce homologous protection using OMP from strain X-73. Ibrahim *et al* (2000) showed that X-73 and 1059 serotypes had similar peptide bands between 58-100KD when they were grown *in vivo*.

ELISA as a mean of determining the serological responses of poultry to *P.multocida* is a rapid and more sensitive assay (Hofacre *et al* 1986). The end-point titer of collected sera from chickens was measured by ELISA test and was shown that OMP had the highest titer followed by KCSN and CAP extracts. These results are in correlation with the protection study indicating the role of antibody titer in our challenge studies. In double gel diffusion test, anti OMP antibody showed at least 3 precipitation lines against OMP antigen and one common line against KCSN-extract indicating the antigen similarities between OMP and KSCN-extract.

In our study other fractions including LPS and HS hardly showed any protection and there was no precipitation reaction in agar gel diffusion test between the antigens and antisera raised in treated chickens. The immunogenecity of CAP antigen was also showed that, following OMP and KSCN-extract, there was a highest survival rate in CAP immunized chickens. The important role of CAP and somatic antigens of *P.multocida* have been reported previously (Boriasenkevas 1978, Kodama *et al* 1980). It is reported previously that the use of whole cell inactivated vaccines have lack of a complete protection. Whatever the reason, it somewhat justifies the recurrence of fowl cholera in vaccinated chicken during the outbreaks (Avakiain *et al* 1986). The work on use of auxtrophic mutants as a vaccine of *P.multocida* by creating nonreverting mutation in the aro-A gene (Roberts *et al* 1990) that rendered them growth dependent on the absence of particular amino acids in host tissues were safe and induced protection in mouse model. Two auxotrophic aro-A mutants of *P.multocida* designated PMP1 (serotype 1) and PMP3 (serotype 3) were tested as vaccine candidates to protect chickens against fowl cholera (Scott *et al* 1999). The results of our experiments showed that OMP and KSCN-extract

antigens, which had shown very similar polypeptide pattern are very good candidates to develop a sub-unit vaccine against fowl cholera infection.

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