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Immunopotency of novel oil adjuvant vaccines employing *Pasteurella multocida* biofilm and capsule enhanced organisms in ducklings

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Abstract: *Pasteurella multocida*—a gram-negative bacterium with a broad host range and ubiquitous distribution—is a serious problem in ducks, and the best method of control is vaccination with locally prevalent strains. The present study was conducted to compare the immunopotency of vaccines employing *P. multocida* biofilms and capsule enhanced organisms with ordinary broth grown organisms in 1-month-old ducklings, by measuring the humoral immune response and the protection conferred by each vaccine during homologous challenge with virulent organisms. Formalin inactivated oil adjuvant bacterin vaccines were prepared from *Pasteurella multocida* A: 1. They were grown in conventional broth, capsule enhancement medium, and under biofilm mode. Four different groups of birds were vaccinated with the respective vaccines by intramuscular route, and the immunopotency of the vaccines was assessed by employing passive haemagglutination (PHA) and homologous challenging with virulent organisms. The PHA titres obtained for the biofilm vaccine group on day 14 postvaccination were much higher than those for the other 2 groups. They also provided 10% more protection when challenged with 200 and 100 median "lethal dose" doses. The capsule enhanced vaccine and conventional bacterin revealed similar results.

Key words: *Pasteurella multocida* A: 1, biofilm vaccine, capsule enhanced vaccine, passive haemagglutination, homologous challenging

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Introduction

Among the diseases of ducks, pasteurellosis caused by *Pasteurella multocida* (*P. multocida*) forms a major bottleneck in the burgeoning poultry industry in India. Indiscriminate use of once effective antibiotics has led to drug resistance, making prophylactic immunisation the exclusive alternative to preclude the disease.

In India several commercial vaccines are in vogue for pasteurellosis, but none of them provide a lasting and broad immunity. It was found that organisms grown under conditions mimicking in vivo conferred better immunity than ordinary laboratory grown organisms, plausibly due to the expression of cross protection factors (1). Bacterial biofilms are a structured community of bacterial cells enshrined in a protective covering of exopolysaccharide and are increasingly similar to in vivo growth. They express many novel proteins that could be the immunodominant antigens and, if used as vaccine, may result in a better immune response (2).

Many researchers have insisted on the use of a virulent form of bacteria for the preparation of inactivated vaccines (3). Capsule is an important virulence factor for avian *P. multocida* (4). Serum was found to enhance the capsule formation of bacteria and yeast (5), and such capsule enhanced organisms will be beneficial for vaccine production.

The present study was conducted to compare the immunopotency of vaccines employing *P. multocida* biofilms and capsule enhanced organisms with ordinary broth grown organisms in 1-month-old ducklings by measuring the humoral immune response and the protection conferred by each vaccine during homologous challenge with virulent organisms.

Materials and methods

Vaccine strain: The *Pasteurella multocida* A: 1 strain (DP1) isolated from Niranam Duck Farm (Pathanamthitta District, Kerala, India); serotyped at IVRI, Izatnagar; and maintained in the Department of Veterinary Microbiology, College of Veterinary and Animal Sciences (COVAS), Mannuthy, was used for the entire study.

Pathogenicity in mice and lethal dose in ducklings

Five mice 6 to 8 weeks old were inoculated with 0.3×10^8 organisms/0.1 mL of phosphate buffered saline (PBS, pH 7.4) by intraperitoneal route, keeping appropriate controls.

Median lethal dose (LD_{50}) of DP1 was determined in 1-month-old ducklings. Briefly, *Pasteurella multocida* A: 1 strain was passaged in mice to get a fully encapsulated virulent form, and the virulent organisms isolated from mice were made into serial 10 fold dilutions. The 1-month-old ducklings were randomly assigned to 9 groups of 6 ducklings each. The first 8 groups were separately inoculated with the different dilutions of the bacteria, with a dose of 0.1 mL per bird subcutaneously at the wing web; the ninth group served as control and was sham inoculated with 0.1 mL of sterile PBS (pH 7.4). Mortality was recorded 1 week postinoculation. All the dead ducklings were examined for specific gross lesions caused by *P. multocida*, and reisolation of the organism on blood agar from heart blood, liver, and spleen was attempted. The method described by Reed and Muench (6) was used for LD_{50} calculation.

Preparation of bacterial suspensions for vaccine preparation

The freeze-dried stock DP1 was reconstituted with 0.5 mL of Tryptone soya broth (TSB), streaked onto dextrose starch agar (DSA), and following an incubation period of 24 h a typical colony was selected for the preparation of bacterins.

The organisms were grown under the following 3 conditions: (a) 3% TSB for 24 h at 37 °C, conventional vaccine (CV); (b) 3% TSB supplemented with 10% foetal bovine serum (FBS) and 0.5% yeast extract for 12 h at 37 °C, capsule enhanced vaccine (CEV); and (c) TSB (0.32%) supplemented with 0.3% bentonite clay for 3 days at 42 °C, biofilm vaccine (BV).

Organisms grown in TSB (3%) and under capsule enhancement conditions were harvested by centrifugation at 3000 × *g* for 10 min, washed 3 times in the PBS (pH 7.4), and were then resuspended in 0.5% formol saline to a concentration of 3×10^9 cells/mL. The biofilm bacterin was prepared as described by Vadakel (7). Briefly, the bacteria were cultured in Tryptone soya broth (0.32%), supplemented with 0.3% bentonite clay powder (Merck, Mumbai), for 3

days at 42 °C. The attached bacterial mass was then harvested by low speed centrifugation (600 × *g* for 10 min), washed several times, and separated from the bentonite clay by vigorous vortexing.

All bacterial cultures thus prepared were allowed to stand at room temperature for 48 h, with occasional shaking, so that the formalin could act.

The sterility of all the bacterins was tested on blood agar at 37 °C for 72 h under 5% carbon dioxide.

Preparation of vaccines

Oil adjuvanated vaccines were prepared from the above bacterins, and the stability of vaccines was assessed (8). The sterility of the prepared vaccines was tested individually in blood agar and Tryptic soy agar (TSA), modified thioglycolate medium, and Sabouraud's dextrose agar (SDA). The toxicity of the vaccines was assessed by injecting the exact dose (0.5 mL) and a double dose of vaccine intramuscularly into 3 ducklings each. The vaccinated birds were observed for a period of 7 days for any untoward reactions or clinical manifestations.

Potency testing in ducklings

A total of 160 ducklings 4 weeks old purchased from a private breeder were divided into 4 groups with 40 birds each. The vaccination schedule was as follows:

Group I: Conventional vaccine (CV)

Group II: Capsule enhanced vaccine (CEV)

Group III: Biofilm vaccine (BV)

Group IV: Control group

The first 3 groups were inoculated intramuscularly in the thigh region with the respective vaccines (0.5 mL/bird). The control group was sham vaccinated with PBS (pH 7.4).

Serological assay

Collection of serum from vaccinates: Blood was collected from all 160 ducks at weekly intervals up to the 28th day postvaccination (PV) and on day 42 PV by cardiac puncture or by jugular venipuncture; serum was separated from each blood sample.

Passive haemagglutination (PHA)

Passive haemagglutination was performed using sheep red blood cell (SRBC) fixed with glutaraldehyde and sensitised with sonicated antigen of *P. multocida*. Sonicated antigen was prepared as described by Ireland et al. (9), excepting the addition of egg white lysozyme. Passive haemagglutination was performed using the method of Sawada et al. (10).

Homologous challenging of birds postvaccination: From each vaccinated group, 10 birds were subjected to homologous challenge by intramuscular route with 0.1 mL of inoculum containing 200 LD_{50} and 100 LD_{50} of fully encapsulated virulent form of *P. multocida* serotype A: 1 on the 28th and 42nd day PV, respectively. The challenged birds were observed for a period of 2 weeks postchallenge for mortality/clinical signs. All dead birds were examined for specific lesions of pasteurellosis, and reisolation of the organism on blood agar from heart blood, liver, and spleen was attempted.

Statistical analysis: A one-way analysis of variance (ANOVA) of the logarithm of IHA titres of sera collected from the ducks on different days was done to compare the immunopotency of the vaccines. The significance of mean differences between vaccine groups was tested using Bartlett's test.

Results

Pathogenicity and lethal dose testing in mice and ducklings

Pasteurella multocida serotype A: 1 killed the mice within 8 h when injected by intraperitoneal route. The median lethal dose was $10^{-7.4}$/0.1 mL subcutaneous dose (Table 1).

Biofilm formation of DP1

The organism adhered over the inert material, bentonite clay, under nutrient restricted conditions; the biofilm formation peaked on day 3, with a mean plate count of 1.54×10^6 CFU/g of bentonite clay.

Preparation of vaccines

All the prepared vaccines were homogeneous suspensions, which facilitated intramuscular administration. The prepared vaccines were sterile and did not cause any untoward effects except for a transient lameness in ducks, which subsided after 1 day postinoculation.

Passive haemagglutination (PHA)

Antibodies were detected in the postvaccination sera

Table 1. LD_{50} of *Pasteurella multocida* (DP1) in 1-month-old ducklings.

Dilution	Organisms present in 0.1 mL of inoculum	No. of birds inoculated	No. dead	No. live	Accumulative value*		Ratio of dead birds	% of dead birds
					Dead	Live		
10^{-1}	3×10^7	6	6	0	41	0	41/41	100
10^{-2}	3×10^6	6	6	0	35	0	35/35	100
10^{-3}	3×10^5	6	6	0	29	0	29/29	100
10^{-4}	3×10^4	6	6	0	23	0	23/23	100
10^{-5}	3×10^3	6	6	0	17	0	17/17	100
10^{-6}	3×10^2	6	6	0	11	0	11/11	100
10^{-7}	3×10^1	6	4	2	5	2	5/7	71.4
10^{-8}	3×10^0	6	1	5	1	7	1/8	12.5

*Accumulative values for the total number of animals that died or survived were obtained by adding in the direction of lowest to highest values. Median lethal dose was calculated from the proportionate distance, as per Reed and Muench (6).

as early as 7 days in the first 3 vaccinated groups, and the titre increased during subsequent days, although the pattern differed on different days. The control group, which was sham vaccinated with PBS, did not reveal many antibody titres irrespective of the day of collection. There was no significant difference in mean titres among the groups during the seventh day. At day 14 the PV biofilm group was significantly different from the other 2 vaccine groups and the control. By days 21 and 28 all the vaccine groups were similar. At day 42 the biofilm vaccine group was found to maintain the titres while the titres of groups I and II decreased, making them significantly different. All the vaccine groups showed a significant difference from the control group at all stages of the study. Groups I and II had no significant difference in the mean titre during the entire study. Notably, only the biofilm group was able to maintain its titre up to the 42nd day. The mean logarithmic PHA titres of individual sera collected at days 7, 14, 21, 28, and 42 PV are shown in Table 2.

Direct homologous challenge

When challenged with a 200 LD_{50} dose, more ducklings were dead in all groups; however, at both 100 and 200 LD_{50} doses, the biofilm group showed 10% more protection. CV and CEV revealed similar protection rates (Table 3). The postmortem examination of dead birds from all vaccine groups revealed gross lesions that were less severe than those in the control group. The culture of heart blood and liver of dead birds onto blood agar revealed typical colonies of *P. multocida* even though the lesions were less severe.

Table 2. Mean logarithmic PHA titres of serum samples of birds, postvaccination.

Days postvaccination	(Mean ± SE)			
	CV	CEV	BV	Control
7*	0.386 ± 0.13	0.718 ± 0.14	0.631 ± 0.18	0.266 ± 0.1
14	0.84 ± 0.14^b	0.77 ± 0.16^b	1.397 ± 0.1^a	0.250 ± 0.08^c
21	0.863 ± 0.06^a	0.889 ± 0.06^a	1.008 ± 0.08^a	0.302 ± 0.05^b
28	0.946 ± 0.05^a	0.989 ± 0.06^a	1.057 ± 0.05^a	0.34 ± 0.05^b
42	0.801 ± 0.11^b	0.795 ± 0.09^b	1.108 ± 0.05^a	0.363 ± 0.07^c

* Values are not significant ($P > 0.05$).
**Values with same superscripts in a row have no significant difference ($P > 0.05$).

Table 3. Homogeneous challenge with 200 and 100 LD_{50} doses of virulent DP1 in ducklings.

Type of vaccine	Dose of inoculum	Days postinoculation	Survived/total tested	% protection
CV	200 LD_{50}	28	6/10	60
	100 LD_{50}	42	8/10	80
CEV	200 LD_{50}	28	6/10	60
	100 LD_{50}	42	8/10	80
BV	200 LD_{50}	28	7/10	70
	100 LD_{50}	42	9/10	90
Control	200 LD_{50}	28	2/10	20
	100 LD_{50}	42	1/10	10

Discussion

The *P. multocida* A: 1 strain used in this study was found to be an in vitro biofilm former. However, it was very difficult to obtain enough biomass for vaccine preparation. As a result, the biofilm mode of growth was first induced, and then it was subcultured onto nutrient agar. Bacteria were found to retain the biofilm mode of growth for a few subcultures in agar media, which was consistent with the findings of Kaplan and Mulks (11) in the case of *Actinobacillus pleuropneumoniae* field isolates. This modification is useful, as it makes the process of vaccine preparation more amenable to commercial production of the vaccine.

It is interesting to note that the pattern of antibody response elicited by BV simulates subunit vaccines employing outer membrane proteins (12) and live vaccines (13). The antibody response was unusually rapid; it peaked during the 14th day PV and also maintained a reasonable titre during the entire period of study (45 days). This may be attributable to the uncanny semblance of biofilms to conditions in vivo and the better presentation of antigens. Organisms grown in vivo express the cross protective factors that make them capable of providing a broader immunity in terms of heterologous protection (14). Biofilms exhibit many novel proteins that are conserved, and out of these many could be immunodominant (15). Most of the bacterial diseases involve biofilm formation inside the host body, and the presentation and familiarisation of such epitopes to the host body may be a reason for the better immune response of biofilm vaccine. To exploit the expression of novel heat shock proteins and simulate the natural body condition of the birds, biofilm cultures in the present study were subjected to nutrient limitation and a higher temperature (42 °C) (16).

Sometimes even if antibodies against a particular organism are present in the host body, they may not be capable of providing the host with sufficient protection against biofilm mediated infections (17). This highlights the importance of familiarising the host body with antigens specific to biofilm, which may not be present in planktonic bacteria. Shivaraj (18) reported an exceptional 83.33% protection for a biofilm based oral vaccine for *E. coli*; the conventional vaccine could induce only 33.33% protection. Although the BV in the present study did not produce such an enormous improvement over the conventional vaccine, it did ensure 10% more protection than both CV and CEV.

CV and CEV revealed similar titres and protection rates, in accordance with the findings of Ramanatha (19) and Akand et al. (20). With capsule enhancement no added advantage was obtained, which was consistent with the findings of Somarajana et al. (21).

Conclusion

In conclusion, the biofilm vaccine was the best among the 3 vaccines examined in the present study, as it provided stronger and lasting immunity. The protection rates of capsule enhanced bacterin

and the conventional bacterin were the same. The present study is only a preliminary investigation and provided some information on the biofilm formation of *P. multocida* and the utility of its biofilm as vaccine. Outer membrane subunit vaccines were found to be effective by various researchers, and they would be more effective if the outer membrane proteins of biofilm cells were used.

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