Pakistan J. Zool., pp 1-9, 2023. **DOI**: https://dx.doi.org/10.17582/journal.pjz/20221229051239

Comparative Efficacy of Commercial and Autogenous *Avibacterium paragallinarum* Vaccines in Layer Chickens in Pakistan

Aatif Masood Ahmad Khan[1], Masood Rabbani[1]*, Arfan Ahmad[1], Muhammad Wasim[2] and Sohail Raza[1]

[1]*Institute of Microbiology, University of Veterinary and Animal Sciences, Lahore*
[2]*Institute of Biochemistry and Biotechnology, University of Veterinary and Animal Sciences, Lahore*

ABSTRACT

Infectious coryza (IC) is an acute contagious upper respiratory disease of chickens caused by *Avibacterium paragallinarum (A. paragallinarum*), and it causes significant economic losses. Therefore, the current work aimed to assess the efficacy, stability, and safety of autogenous bacterins and two commercial vaccines against *A. paragallinarum* in layers in Pakistan. In the present study, one hundred, six weeks old layer chickens were divided into equally distributed 10 groups. These groups were vaccinated with *A. paragallinarum* autogenous bacterin containing aluminum hydroxide and montanide oil with 10^7, 10^8 and 10^9 CFU/0.5 ml/ dose and two commercial vaccines (A and B; alum-based and mineral oil-based vaccines, respectively). Two groups were the control positive (challenged and non-vaccinated) and the control negative (non-vaccinated or challenged). Booster doses of different vaccines were given at 9 weeks old, and birds were intrasinus challenged at 12 weeks old with *A. paragallinarum* culture. Birds were kept under complete daily observation for 7 days after the challenge. Signs, postmortem lesions, reisolation of the bacteria, protection rate and stability after 3- and 6-months storage were used as criteria for bacterin evaluation. The results showed that montanide oil and alum gel-based vaccines with 10^9 CFU/0.5 ml/ dose and commercial vaccine A gave the highest protection rate (95, 90 and 90%, respectively) and highest stability after storage for 3- and 6-months at 4°C. In conclusion, both autogenous *A. paragallinarum* bacterins with 10^9 CFU/0.5 ml/ dose and commercial vaccine A were safe, stable and more effective in the prevention of *A. paragallinarum* infection in layers in Pakistan when administered at two doses.

Article Information
Received 29 December 2022
Revised 20 April 2023
Accepted 16 May 2023
Available online 20 July 2023
(early access)

Authors' Contribution
Conceptualization: MR, AMAK and AA. Methodology: AMAK, MW and SR. Formal analysis: AMAK and SR. Writing original draft preparation: AMAK, MR and SR. Writing, review and editing: MR, AMAK, AA, MW and SR.

Key words
***Avibacterium paragallinarum*, Infectious coryza, Autogenous bacterins, Aluminum hydroxide vaccine, Montanide oil adjuvant, Layer chickens**

INTRODUCTION

Infectious coryza is an acute and occasionally chronic upper respiratory disease in domestic poultry caused by *Avibacterium paragallinarum*, which was previously known as *Haemophilus paragallinarum* (Gallardo *et al*., 2020; Luna *et al*., 2020; van den Biggelaar *et al*., 2020). This disease is distributed worldwide and characterized by acute inflammation of the upper respiratory tract, conjunctivitis, nasal discharge, sinusitis and facial edema (Liu *et al*., 2016; Paudel *et al*., 2017; Umar *et al*., 2020). The economic significance of IC is associated with a reduction in egg production in layer chicken (10-40%), poor growth performance and an increase in the condemnation rate in broiler chicken because of air sacculitis. Additionally, the economic losses because of IC are more important when other infectious agents like *Mycoplasma gallisepticum* and viruses were involved causing high mortalities (Sun *et al*., 2018; Sayed *et al*., 2019; Xu *et al*., 2019).

In 2011 in Punjab prevalence of Av. Paragallinarum was recorded as 5.3% by Siddique (2012). The prevalence of infectious coryza was recorded as 32.72% in broiler and 29.35 % in layers in Lahore district during the period July, 2011 to June, 2012 by Sultana *et al*. (2012). Hussain *et al*. (2021) observed infectious coryza prevalence rate in Rawalpindi of Punjab as 20% in boilers and 25.63% in layer. He recorded that Prevalence of infectious coryza in broilers was highest in winter 31.58%, followed by autumn 21.4%, summer 11.54% and spring 10.71%. Spring had the highest prevalence of coryza infections in layer 32%, followed by winter 30.77% and autumn 26.32%, while summer had the lowest prevalence 9.38%. Infectious coryza was predominantly seen in broilers aged 3-6 weeks. Poor management techniques, such as temperature fluctuations during the winter and increased ammonia gas

* Corresponding author: deanfvs@uvas.edu.pk
0030-9923/2023/0001-0001 $ 9.00/0

concentrations due to poor ventilation, appear to be the cause of such a high prevalence of the disease.

Prevalence of *A. paragallinarum* was recorded by (Muhammad *et al*., 2021) in Balochistan; 19.2% for layers, 18.4% for broilers, 16.0% for quails, 15.8% for chukars and 11.0% for pigeons. Among positive cases, serotype A was 24%, serotype B was 29% and serotype C was 27.40%. Prevalence of IC need to be studied in other parts of Pakistan

Prevention and control of IC can be achieved by strict biosecurity, antimicrobial therapy and vaccination (Charoenvisal *et al*., 2017). Effective biosecurity should prevent chicken's exposure to *A. paragallinarum* infection, but vaccination should be utilized to reduce the effect of IC in the endemic area. Treatment of IC infected birds could be of value, but the recovered birds become reservoirs and sources of infection. Therefore, vaccination is one of the best procedures to prevent IC (Blackall and Soriano-Vargas, 2019).

Vaccination against *A. paragallinarum* is the best preventive measure under the present management types where depopulation is impossible. Vaccination against IC cannot prevent infection with *A. paragallinarum,* but it reduces the disease symptoms and minimizes bacterial shedding and spreading (Blackall and Soriano-Vargas, 2019). Commercial IC vaccines are widely utilized worldwide and are prepared from standard inactivated strains of *A. paragallinarum*. Still, in some cases, these vaccines are not protective against the local variants of *A. paragallinarum*. Thus, there is a need to prepare vaccines from local strains (Abd El-Ghany, 2011).

Several adjuvants have been reported to be effective for IC bacterins, such as mineral oil, aluminum hydroxide gel and saponin, but mineral oil adjuvants may cause an adverse reaction at the injection site (Blackall and Soriano-Vargas, 2019).

There are no available studies on the efficacy of *A. paragallinarum* autogenous bacterins and commercial vaccines in layer chickens in Pakistan. Therefore, the current study aimed to (i) prepare *A. paragallinarum* autogenous bacterins containing either aluminum hydroxide or montanide oil adjuvant with 10^7 and 10^8 and 10^9 CFU/0.5 ml/dose of the bacteria, (ii) assess the efficacy of the prepared autogenous bacterins and two commercial vaccines (one is aluminum-based and the other one is oil-based vaccine) in layer chickens in Pakistan and (iii) assess the stability of different vaccine after storage for 3- and 6-months at 4°C.

MATERIALS AND METHODS

Preparation of the autogenous vaccine

The preparation method was performed as mentioned previously (Blackall, 1991; Abd El-Ghany, 2011). Briefly, Brain Heart Infusion broth (Sigma Aldrich, Germany) supplemented with 0.0025% NADH (Sigma Aldrich, Germany) and 1% chicken serum (Sigma Aldrich, Germany) (BHI/SN) was inoculated with the fully characterized field strain of *A. paragallinarum* serogroup B obtained in previous study (Masood *et al*., 2023) and incubated at 37 °C for 12 h in a shaking incubator in air. After incubation, an aliquot was taken out, and the bacterial count was estimated using the standard plate count technique and colony forming unit (CFU) was estimated for each dilution. The bacterial culture was inactivated by adding thimerosal sodium 0.01 and 0.005%. After 48 h incubation, the samples were taken from each kind of inactivated vaccine and cultured on BHI/SN, and checked for bacterial growth. Results were expressed as averages of duplicate trials in a single experiment. The lowest concentration of thimerosal sodium, which inactivates the bacterial culture, was selected for the final vaccine production.

After confirmation of inactivation, vaccine broth cultures were prepared with different concentrations of local isolate of *A. paragallinarum* serogroup B obtained in previous study (Masood *et al*., 2023) (10^7, 10^8 and 10^9 CFU/0.5 ml/dose of the bacteria) and with 2 different adjuvants. Aluminum hydroxide gel precipitated adjuvant (alum gel-based vaccine) (Fisher Scientific, USA) was added at a concentration of 25% of the final volume, and another adjuvant was made by adding montanide oil-based vaccine (ISA 71 R VG , SEPPIC) at a concentration of 60% of the final volume (Blackall, 1988). Sterility and safety testing of the prepared vaccines were done as mentioned previously (Gong *et al*., 2014).

Experimental design

One hundred, 6 weeks old layer chickens were obtained from a commercial farm known to be free of IC and non-vaccinated. One-hundred-layer chickens were divided into equally distributed 10 groups, each comprising 10 birds. The bacterial inoculation vaccine dose was estimated using method adopted by (Chukiatsiri *et al*., 2009). All types of vaccines were administered I/M at the dose of 0.5 ml/bird. Groups 1, 2 and 3 were vaccinated with alum gel-based vaccine with 107, 108 and 109 CFU/0.5 ml/dose, respectively while groups 4, 5 and 6 were given montanide oil-based vaccine with 107, 108 and 109 CFU/0.5 ml/dose, respectively. Additionally, group 7 and 8 received two different commercially available vaccines in Pakistan; vaccines A and B (trivalent inactivated vaccine in aluminum hydroxide gel and mineral oil emulsion, respectively). Finally, group 9 was the unvaccinated positive control group (challenged), and

group 10 was the control negative group (not vaccinated or challenged). All types of vaccines were administered intra muscular (I/M) in a dose of 0.5 ml/bird. Booster doses of vaccines were given 21 days after the primer dose in groups 1-8. Birds in groups 1-9 were challenged 3 weeks post-vaccination with Intra sinus inoculation of 0.2 ml (2 x10^8 CFU) of a local live isolate of *A. paragallinarum* serogroup B. Birds after the challenge were kept for 7 days under complete daily observation (García *et al*., 2008). Results were expressed as averages of duplicate trials in a single experiment.

The handling of birds was in accordance with the approved Guidelines provided by University of Veterinary and Animal Sciences, Lahore ethical institutional review board. All birds groups were housed in separate isolation facilities, with feed and water provided ad libitum at the University Animal House facility.

Protective efficacy of the prepared vaccines

For protective efficacy three criteria were selected; clinical signs at 2 days post-challenge to 7 days, mucus in sinus at 7 days post-challenge (post mortem) and presence of *A. paragallinarum* in sinus at 7 days post-challenge (post mortem) by streaking sinus swabs onto Blood Agar (Fisher Scientific, USA) supplemented with 1% chicken serum and 0.0025% NADH (reduced nicotinamide adenine dinucleotide) (Sigma Aldrich, Germany) (BA/SN agar). Birds were considered protected if they showed no IC clinical signs or gross lesions at post mortem and didn't yield any growth on BA/SN agar (Rimler *et al*., 1976; Abd El-Ghany, 2011).

Evaluation of the shelf life of vaccines

The shelf life of the prepared vaccines was evaluated in the same way as described before, but with the best vaccine obtained as a result of the challenge trial. The shelf life was evaluated at 3- and 6-months storage of vaccines at 4°C.

Statistical analysis

A one-way ANOVA test was used to compare the efficacy of different vaccines. We also applied independent samples t-test to compare the protection rate of freshly prepared, 3- and 6-months stored vaccines. Both tests were done using the SPSS Inc. version 26 (IBM Corp., NY, USA). P> 0.05 was considered as statistically significant.

RESULTS

Sterility and safety tests of the prepared vaccines

The obtained field strain of *A. paragallinarum* was tested for purity and identified through the general criteria of *A. paragallinarum* bacteria as follows; it didn't grow on MacConkey agar (Fisher Scientific, USA)., but it was NAD-dependent with satellites growth on Blood Agar (BA) (Fisher Scientific, USA). Moreover, it didn't produce catalase enzyme or acid from lactose and arabinose, but it was positive for sugar fermentation.

Thimerosal 0.005% (Fisher Scientific, USA) was the least concentration that inactivated *A. paragallinarum* in 24 h. Thus, it was used for the preparation of different autogenous vaccines.

Interestingly, the final products of the prepared vaccines were proven to be sterile as there were no growth or contamination after inoculation of 0.2 ml of any of the prepared vaccines into brain heart infusion broth (Sigma Aldrich, Germany) and sabouraud agar (Sigma Aldrich, Germany) (supplemented with NADH) under different condition.

At the end of the safety study, no side effects were observed in alum gel-based vaccines, but chickens were off feed on the first day of oil-based vaccines, and some granuloma was formed.

Protection efficacy of different vaccines against A. paragallinarum *serogroup B challenge*

After single-dose administration of different vaccines, three weeks later each bird except control layer chickens were challenged with infra-orbital sinus inoculation of 0.2 ml of 12 h incubated culture in BHI/SN (approximately 2×10^8 CFU as challenge dose). Clinical signs of IC such as nasal discharge, sneezing and swelling of the infraorbital sinuses 2 days post challenge were recorded in the control positive group (9; non-vaccinated and challenged) and all vaccinated groups (1-8) except group 2 (alum gel-based vaccine with 10^8 CFU/0.5 ml/dose). No clinical signs were detected in the control negative group (10; non-vaccinated or challenged) (Table I). There was a significant difference among all groups after single-dose administration of different vaccines (P> 0.05) (Table I).

The efficacy of different types of vaccines after booster dose administration is shown in Table II. Three weeks later after the administration of booster dose of various vaccines, challenge was given. Two days post challenge clinical signs of IC were recorded in the control positive group (9) and all vaccinated groups (1-8) except group 7 (commercial vaccine A) (Table II). No clinical signs were detected in the control negative group (10) (Table II). There was a significant difference among all groups after booster dose administration of different vaccines (P> 0.05) (Table II).

The post mortem lesions (7 days post challenge) were mucus in the sinus, catarrhal rhinitis, laryngitis and

subcutaneous edema in the tissue around the infraorbital sinuses. The presence of mucus in the sinus and reisolation of *A. paragallinarum* from sinus swabs after single and booster dose administration of different vaccines were detected in all groups except the control negative group (10) (Tables I and II). Clinical signs, presence of mucus in sinus and reisolation of *A. paragallinarum* from sinus swabs after single and booster dose administration of different vaccines were highly observed among groups 9, 4 and 1 (control positive, vaccinated with montanide oil and alum gel-based vaccines with 10^7 CFU/0.5 ml/dose, respectively) (Tables I and II).

Table I. The efficacy of different types of autogenous *A. paragallinarum* bacterins and two commercially available vaccines in layer chickens three weeks after single-dose administration.

Groups (10 birds each)	Clinical signs	Mucus in the sinus (post mortem)	Reisolation of *A. paragallinarum* from sinus swab[s]	Protection rate (%)
Alum gel-based vaccine (CFU/0.5 ml/dose)				
1. 10^7 CFU	3±0.5 ^{b,c}	3±0 ^{b,c}	4±0.5 ^{b,c}	65±5 ^{b,c}
2. 10^8 CFU	0 ^{d}	1±0.5 ^{c,d}	2±0.5 ^{c,d}	85±5 ^{c,d}
3. 10^9 CFU	1±0.5 ^{c,d}	1±1 ^{c,d}	2±0.5 ^{c,d}	85±5 ^{c,d}
Montanide oil-based (CFU/0.5 ml/dose)				
4. 10^7 CFU	4±0.5 ^{a,b}	5±1 ^{a,b}	6±1 ^{b}	40±10 ^{b}
5. 10^8 CFU	2±0.5 ^{b,c,d}	3±0 ^{b,c}	3±0.5 ^{c,d}	75±5 ^{c,d}
6. 10^9 CFU	1±0 ^{c,d}	2±0.5 ^{c,d}	2±0.5 ^{c,d}	85±5 ^{c,d}
7. Commercial vaccine A	1±0.5 ^{c,d}	1±0 ^{c,d}	1±0 ^{c,d}	90±0 ^{c,d}
8. Commercial vaccine B	2±0 ^{b,c,d}	2±0 ^{c,d}	3±0.5 ^{c,d}	75±5 ^{c,d}
9. Control positive	6±0.5 ^{a}	8±0.5 ^{a}	10±0 ^{a}	0 ^{a}
10. Control negative	0 ^{d}	0 ^{d}	0 ^{d}	100±0 ^{d}

[s]Results were the average of duplicate trials in a single experiment (mean ± SEM). Superscripts with different letters in the same column represent a significant difference between groups (P< 0.05).

Table II. The efficacy of different types of autogenous *A. paragallinarum* bacterins and two commercially available vaccines in layer chickens after booster dose administration.

Groups (10 birds each)	Clinical signs	Mucus in the sinus (post mortem)	Reisolation of *A. paragallinarum* from sinus swabs	Protection rate (%)
Alum gel-based vaccine (CFU/0.5 ml/dose)				
1. 10^7 CFU	3±0.5 ^{b,}c	4±0.5 ^{b,c}	3±0 ^{b,c}	70±0 ^{b,c}
2. 10^8 CFU	1±0.5 ^{c}	2±0 ^{c,d}	2±0.5 ^{c,d}	85±5 ^{c,d}
3. 10^9 CFU	1±1 ^{b,c}	2±1 ^{c,d}	1±1 ^{c,d}	90±10 ^{c,d}
Montanide oil-based (CFU/0.5ml/dose)				
4. 10^7 CFU	4±0 ^{a,b}	6±0.5 ^{a,b}	6±0.5 ^{b}	45±5 ^{b}
5. 10^8 CFU	2±0.5 ^{b,c}	4±0.5 ^{b,c}	2±0 ^{c,d}	80±0 ^{c,d}
6. 10^9 CFU	1±0.5 ^{c}	2±0.5 ^{c,d}	1±0.5 ^{c,d}	95±5 ^{c,d}
7. Commercial vaccine A	0 ^{c}	2±0.5 ^{c,d}	1±0 ^{c,d}	90±0 ^{c,d}
8. Commercial vaccine B	1±0 ^{b,c}	3±0.5 ^{c,d}	2±0.5 ^{c,d}	85±5 ^{c,d}
9. Control positive	6±1 ^{a}	8±0 ^{a}	10±0 ^{a}	0 ^{a}
10. Control negative	0 ^{c}	0 ^{d}	0 ^{d}	100±0 ^{d}

[s]Results were the average of duplicate trials in a single experiment (mean ± SEM). Superscripts with different letters in the same column represent a significant difference between groups (P< 0.05).

Table III. The efficacy of different types of autogenous *A. paragallinarum* bacterins and two commercially available vaccines in layer chickens three weeks after single-dose administration of various vaccines stored for 3 months at 4°C.

Groups (10 birds each)	Clinical signs	Mucus in the sinus (post mortem)	Reisolation of *A. paragallinarum* from sinus swabs	Protection rate (%)
Alum gel-based vaccine (CFU/0.5 ml/dose)				
1. 10^8 CFU	2±0.5 ^{b}	4±0.5 ^{b}	3±0.5 ^{b}	75±5 ^{b}
2. 10^9 CFU	1±0.5 ^{b}	2±0.5 ^{b,c}	1±0 ^{b,c}	90±0 ^{b,c}
Montanide oil-based vaccine (CFU/0.5 ml/dose)				
3. 10^8 CFU	2±1 ^{b}	4±1 ^{b}	3±0.5 ^{b}	75±5 ^{b}
4. 10^9 CFU	1±0 ^{b}	2±0.5 ^{b,c}	2±0.5 ^{b,c}	85±5 ^{b,c}
5. Commercial vaccine A	2±0.5 ^{b}	3±0 ^{b,c}	2±0.5 ^{b,c}	85±5 ^{b,c}
6. Commercial vaccine B	2±1 ^{b}	4±1 ^{b}	3±0.5 ^{b}	75±5^{b}
7. Control positive	9±0.5 ^{a}	9±0 ^{a}	10±0 ^{a}	0 ^{a}
8. Control negative	0 ^{b}	0 ^{c}	0 ^{c}	100±0 ^{c}

*Results were the average of duplicate trials in a single experiment (mean ± SEM). Superscripts with different letters in the same column represent a significant difference between groups (P< 0.05).

Considering the protection rate against *A. paragallinarum* after single-dose administration of different vaccines, Table I showed that commercial vaccine A gave the highest protection (90%), followed by alum gel-based vaccine with 10^8 and 10^9 CFU/0.5 ml/dose and montanide oil-based vaccine with 10^9 CFU/0.5 ml/dose (85% each). While, montanide oil-based vaccine with 10^7 CFU/0.5ml/dose showed the lowest protection rate (40%), followed by the alum gel-based vaccine with 10^7 CFU/0.5ml/dose (65%), montanide oil-based vaccine with 10^8 CFU/0.5ml/dose and commercial B vaccine (75% each). There was a significant difference among all groups after single-dose administration of different vaccines (P> 0.05) (Table I).

Considering the protection rate against *A. paragallinarum* after booster dose administration of different vaccines, Table II showed that montanide oil-based vaccine with 10^9 CFU/0.5 ml/dose gave the highest protection (95%), followed by alum gel-based vaccine with 10^9 CFU/0.5 ml/dose and commercial vaccine A (90% each). While, montanide oil-based vaccine with 10^7 CFU/0.5 ml/dose showed the lowest protection rate (45%), followed by the alum gel-based vaccine with 10^7 CFU/0.5 ml/dose (70%) and montanide oil-based vaccine with 10^8 CFU/0.5 ml/dose (80%). There was a significant difference among all groups after booster dose administration of different vaccines (P> 0.05) (Table I).

The protection rate of different vaccines against *A. paragallinarum* after single and booster dose administration are shown in Figure 1. There were no significant differences in the protection rate between the single and booster dose administration among the tested groups (P< 0.05) (Fig. 1).

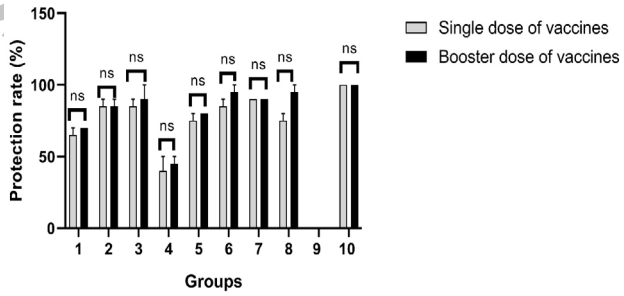

Fig. 1. Protection rate of different vaccines against *A. paragallinarum* after single and booster dose administration. Results were the average of duplicate trials in a single experiment (mean ± SEM). * Represents the significant differences between single and booster dose administration, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns: no significance. 1,2,3: Alum gel-based vaccine with 10^7, 10^8 and 10^9 CFU/0.5 ml/dose, respectively, 4,5,6: Montanide oil-based vaccine with 10^7, 10^8 and 10^9 CFU/0.5 ml/dose, respectively, 7, 8: commercial vaccine A and B, respectively, 9: control positive and 10: control negative.

Protection efficacy of different vaccines against A. paragallinarum *after 3- and 6-months storage at 4°C*

The efficacy of different vaccines three weeks after single-dose administration of various vaccines stored for 3- and 6-months at 4°C are shown in Tables III and IV, respectively. Two days post challenge, clinical signs, presence of mucus in sinus, and 7 days pot challenge re-

isolation of *A. paragallinarum* from sinus swabs, were recorded in all 8 groups except the control negative group (8). Clinical signs, presence of mucus in sinus and re-isolation of *A. paragallinarum* from sinus swabs (challenged three weeks after the single-dose administration of different vaccines stored for 3- and 6-months) were highly observed among groups 7, 6 and 3 (control positive, commercial vaccine B and montanide oil-based vaccine with 10^8 CFU/0.5 ml/dose, respectively) (Tables III and IV). There was a significant difference among all groups after single-dose administration of different vaccines stored for 3- and 6-months at 4° C (P> 0.05) (Tables III and IV).

Considering the protection rate against *A. paragallinarum* serogroup B, after single-dose administration of different vaccines stored for 3 months at 4° C, Table III showed that alum gel-based vaccine with 10^9 CFU/0.5 ml/dose gave the highest protection (90%), followed by montanide oil-based vaccine with 10^9 CFU/0.5 ml/dose and commercial vaccine A (85% each). While alum gel and montanide oil-based vaccines with 10^8 CFU/0.5 ml/dose and commercial vaccine B showed the lowest protection rate (75% each). There was a significant difference among all groups after single-dose administration of different vaccines stored for 3 months at 4° C (P> 0.05) (Table III).

Considering the protection rate against *A. paragallinarum* serogroup B after single-dose administration of different vaccines stored for 6 months at 4° C, Table IV showed that montanide oil-based vaccine with 10^9 CFU/0.5 ml/dose gave the highest protection (90%), followed by alum gel-based vaccine with 10^9 CFU/0.5 ml/dose and commercial vaccine A (80% each). While, commercial vaccine B showed the lowest protection rate (65%), followed by montanide oil and alum gel-based vaccines with 10^8 CFU (75% each). There was a significant difference among all groups after single-dose administration of different vaccines stored for 6 months at 4° C (P> 0.05) (Table IV).

A protection rate of freshly prepared, 3- and 6-months stored different vaccines after single-dose administration are shown in Figure 2. There were no significant differences in the protection rate between freshly prepared, 3- and 6-months stored different vaccines after single-dose administration among the tested groups (P< 0.05) (Fig. 2).

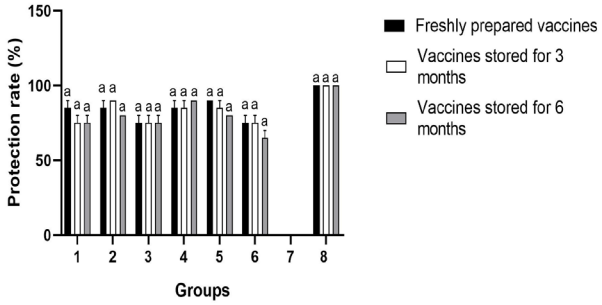

Fig. 2. Protection rate of freshly prepared, 3- and 6-months stored different vaccines after single-dose administration. Results were the average of duplicate trials in a single experiment (mean ± SEM). Bars with different letters in the same group represent a significant difference (p< 0.05). 1,2: Alum gel-based vaccine with 10^8 and 10^9 CFU/0.5 ml/dose, respectively, 3,4: Montanide oil-based vaccine with 10^8 and 10^9 CFU/0.5 ml/dose, respectively, 5,6: commercial vaccine A and B, respectively, 7: control positive and 8: control negative.

Table IV. The efficacy of different types of autogenous *A. paragallinarum* bacterins and two commercially available vaccines in layer chickens three weeks after single-dose administration of various vaccines stored for 6 months at 4°C.

Groups (10 birds each)	Clinical signs	Mucus in the sinus (post mortem)	Reisolation of *A. paragallinarum* from sinus swabs	Protection rate (%)
Alum gel-based vaccine (CFU/0.5 ml/dose)				
1. 10^8 CFU	3±0.5 ^{b}	4±1 ^{b}	3±0.5 ^{b,c}	75±5 ^{b,c}
2. 10^9 CFU	2±0.5 ^{b}	3±0.5 ^{b,c}	2±0 ^{b,c}	80±0 ^{b,c}
Montanide oil-based vaccine (CFU/0.5 ml/dose)				
3. 10^8 CFU/0.5	2±1.5 ^{b}	5±0.5 ^{b}	3±0.5 ^{b,c}	75±5 ^{b,c}
4. 10^9 CFU	1±0 ^{b}	3±0.5 ^{b,c}	1±0 ^{c,d}	90±0 ^{c,d}
5. Commercial vaccine A	2±0 ^{b}	3±0.5 ^{b,c}	2±0 ^{b,c}	80±0 ^{b,c}
6. Commercial vaccine B	3±1 ^{b}	6±0.5 ^{a,b}	4±0.5 ^{b}	65±5 ^{b}
7. Control positive	8±1 ^{a}	9±0.5 ^{a}	10±0 ^{a}	0 ^{a}
8. Control negative	0 ^{b}	0 ^{c}	0 ^{d}	100±0 ^{d}

* Results were the average of duplicate trials in a single experiment (mean ± SEM). Superscripts with different letters in the same column represent a significant difference between groups (P< 0.05).

DISCUSSION

IC is an acute contagious upper respiratory disease of chickens caused by *A. paragallinarum* and it considers a serious problem in the chicken industry despite the widespread utilization of IC vaccines because there is only partial cross-protection between serogroups (Wahyuni *et al*., 2019). Therefore, the utilization of commercial vaccines containing multiple serovar B strains or the autogenous bacterins that contains field (local) serovars would be more effective in controlling IC and these vaccines should be utilized in birds at 10-20 weeks of age (before going into productions) (Patil *et al*., 2018; Blackall and Soriano-Vargas, 2019; Xu *et al*., 2019). Thus, the present study aimed to assess the efficacy and stability of commercial and autogenous killed bacterins against *A. paragallinarum* in layers in Pakistan.

In the present work, at the end of the safety study, no side effects were observed in alum gel-based vaccines, but chickens were off feed on the first day of oil-based vaccines and some granuloma was formed. These results are in complete agreement with previous studies conducted in China (Gong *et al*., 2014; Xu *et al*., 2019).

In the current study, the protection rates against *A. paragallinarum* serovar B after booster dose administration of commercial vaccines A and B (alum and oil-based vaccines, respectively) were 90 and 85%, respectively. These results are lower than those reported in a previous study in Thailand, which reported 100% protection against *A. paragallinarum* serogroup B after using commercial vaccines A and B (Charoenvisal *et al*., 2017).

Herein, the protection rate against *A. paragallinarum* after booster dose administration of different vaccines, showed that montanide oil-based vaccine with 10^9 CFU/0.5 ml/dose gave the highest protection (95%), followed by alum gel-based vaccine with 10^9 CFU/0.5 ml/dose (90%). These results were similar to those reported in a previous study conducted in Egypt, which reported high protection rate using mineral oil and alum gel-based bacterins (88 and 92%, respectively) (Abd El-Ghany, 2011).

In the present study, there were no significant differences in the protection rate between the single and booster dose administration of the different examined vaccines. These results were in contrast with previous reports, which stated that for longer-term protection against IC, different vaccines should be administrated at 2 doses, at least 3 weeks apart (Abd El-Ghany, 2011; Blackall and Soriano-Vargas, 2019).

In the current study, autogenous bacterins gave good protection rate against *A. paragallinarum* field strain. These results are in complete agreement with previous studies conducted in India (Patil *et al*., 2018) and China (Sun *et al*., 2018; Xu *et al*., 2019).

Herein, montanide oil-based vaccine with 10^9 CFU/0.5 ml/dose gave the highest protection rate (95%), followed by alum gel-based vaccine with 10^9 CFU/0.5 ml dose (90%). These results are in contrast with previous studies, which reported that oil-based vaccines were more effective because of the minimal local reaction around the injection site, which minimizes the stress and leads to proper carcass development (Abd El-Ghany, 2011; Charoenvisal *et al*., 2017).

Herein, the protection rate against *A. paragallinarum* after single-dose administration of montanide oil-based vaccine with 10^9 CFU/0.5 ml/dose stored for 3- and 6-months at 4° C were 85 and 90%, respectively. These results are in contrast with the results of a previous study conducted in China, which reported that the protection rate of oil-based vaccine after 3- and 6-months storage were 93 and 80%, respectively (Gong *et al*., 2014).

The difference in the protection rate of different vaccines against *A. paragallinarum* among various studies attributed to the geographical location, type and severity of the local serovar and type of adjuvant and bacterin used.

CONCLUSION

In conclusion, both autogenous *A. paragallinarum* bacterins (montanide oil and aluminum hydroxide adjuvants with 10^9 CFU and commercial vaccine A (alum-based vaccine) were found to be safe and more effective when administrated as two I/M shots at 6 and 9 weeks of age in layers and they were still providing significant protection after 3- and 6-months storage at 4°C.

It was the first IC autogenous killed bacterins against *A. paragallinarum* in layers in Pakistan that contains field (local) serovar B. It proved to be as efficacious and stable as imported commercial vaccines. Therefore, efforts should be made to produce IC indigenous vaccines from local isolates belonging to different serogroups (A, B and C) of *A. paragallinarum* and use them in poultry of Pakistan to reduce import bill and earn foreign exchange by exporting surplus of vaccine.

ACKNOWLEDGEMENT

Being an indigenous PhD scholar I highly acknowledge financial support provided by the Higher Education Commission, Islamabad towards my PhD studies. I am thankful to Dr. Patrick Blackall, Principal Research Fellow, Centre for Animal Science (CAS), Queensland Alliance for Agriculture and Food innovation (QAAFI) and his Microbiology Research group. He gave me Infectious Coryza (IC) specific research training and

equipped me to handle with difficulties in my Infectious Coryza PhD research work.

Funding

This study was funded by Higher Education Commission, Islamabad PIN No. 213-60434-2BM2-186.

Internal review board approval

The study was approved by Advanced Studies and Research Board (ASRB), UVAS (DAS/4917-230216).

Ethical statement

During the samples collection, animals were handled according to the approved guidelines provided by University of Veterinary and Animal Sciences, Lahore ethical institutional review board.

Statement of conflict of interest

The authors have declared no conflict of interest.

REFERENCES

Abd El-Ghany, W.A., 2011. Evaluation of autogenous *Avibacterium paragallinarum* bacterins in chickens. *Int. J. Poult. Sci.* **10**: 56–61. https://doi.org/10.3923/ijps.2011.56.61

van den Biggelaar, R.H.G.A., Eden, W. van, Rutten, V.P.M.G. and Jansen, C.A., 2020. Macrophage activation assays to evaluate the immunostimulatory capacity of *Avibacterium paragallinarum* in a multivalent poultry vaccine. *Vaccines*, **8**: 1–16. https://doi.org/10.3390/vaccines8040671

Blackall, P.J., 1988. Further comparison of adjuvants for an inactivated infectious coryza vaccine. *Avian Dis.,* **32**: 831–835. https://doi.org/10.2307/1591007

Blackall, P.J., 1991. An evaluation of the cross-protection afforded by inactivated infectious coryza vaccines. *Aust. Vet. J.,* **68**: 266-267. https://doi.org/10.1111/j.1751-0813.1991.tb03237.x

Blackall, P.J. and Soriano-Vargas, E., 2019. Infectious coryza and related bacterial infections. *Dis. Poult.*, pp. 890–906. https://doi.org/10.1002/9781119371199.ch20

Charoenvisal, N., Chansiripornchai, P. and Chansiripornchai, N., 2017. Efficacy of four commercial infectious coryza vaccines on prevention of *Avibacterium paragallinarum* serovar A , B and C infection in Thailand. *Pak. Vet. J.*, **37**: 287-292

Chukiatsiri, K., Sasipreeyajan, J., Neramitmansuk, W. and Chansiripornchai, N., 2009. Efficacy of autogenous killed vaccine of *Avibacterium paragallinarum*. *Avian Dis.*, **53**: 382–386. https://doi.org/10.1637/8563-121908-Reg.1

Gallardo, R.A., Da Silva, A.P., Egaña-Labrin, S., Stoute, S., Kern, C., Zhou, H., Cutler, G. and Corsiglia, C., 2020. Infectious coryza: Persistence, genotyping, and vaccine testing. *Avian Dis.*, **64**: 157–165. https://doi.org/10.1637/0005-2086-64.2.157

García, A., Romo, F., Ortiz, A.M. and Blackall, P.J., 2008. The vaccination-challenge trial: the gold standard test to evaluate the protective efficacy of infectious coryza vaccines. *Avian Pathol.*, **37**: 183–186. https://doi.org/10.1080/03079450801929941

Gong, Y., Zhang, P., Wang, H., Zhu, W., Sun, H., He, Y., Shao, Q. and Blackall, P.J., 2014. Safety and efficacy studies on trivalent inactivated vaccines against infectious coryza. *Vet. Immunol. Immunopathol.*, **158**: 3–7. https://doi.org/10.1016/j.vetimm.2013.01.015

Hussain, D., Yousaf, A., Wakeel, A., Noori, B., Aijaz, H., Kiran, T.S., Naazir, S., Gul Soomro, A., Khalil, R. and Sharif, A., 2021. Prevalence of respiratory dieases in different broiler and layer poultry farms in Rawalpindi of Punjab, Pakistan. **5**: 85–91.

Liu, C.C., Ou, S.C., Tan, D.H., Hsieh, M.K., Shien, J.H. and Chang, P.C., 2016. The fimbrial protein is a virulence factor and potential vaccine antigen of *Avibacterium paragallinarum*. *Avian Dis.*, **60**: 649–655. https://doi.org/10.1637/11410-031316-Reg.1

Luna, L.P., Buter, R., Pantoja-Nuñez, G.I., Acuña-Yanes, M.A.Y., Ceballos-Valenzuela, K., Talavera-Rojas, M., Salgado-Miranda, C., Heuvelink, A., de Wit, S., Soriano-Vargas, E. and Feberwee, A., 2020. Research note: Identification, HPG2 sequence analysis and antimicrobial susceptibility of *Avibacterium paragallinarum* isolates obtained from outbreaks of infectious coryza in commercial chickens in Sonora State, Mexico. *Avian Dis.*, **65**: 95–101. https://doi.org/10.1637/aviandiseases-D-20-00103

Masood, A., Khan, A., Rabbani, M., Ahmad, A., Wasim, M. and Raza, S., 2023. Molecular characterization of indigenous isolates of *Avibacterium paragallinarum* and media optimization of its growth for vaccinal seed production. *Pakistan J. Zool.,* pp. 1-8. https://dx.doi.org/10.17582/journal.pjz/20221013061003

Muhammad, G., Kamran TAJ, M., Taj, I., Panezai, I. and Abbas, F., 2021. Incidence and antimicrobial susceptibility profile of *Avibacterium paragallinarum* isolated from commercial birds. *Taiwania*, **66**: 160–164. https://doi.org/10.21203/rs.3.rs-36937/v1

Patil, V., Mishra, D., Mane, D. and Surwase, S., 2018. Development of autogenous vaccine for effective control of Infectious coryza in chicken. *Int. J. Infect. Dis.*, **73**: 355–356. https://doi.org/10.1016/j.ijid.2018.04.4220

Paudel, S., Hess, M. and Hess, C., 2017. Coinfection of *Avibacterium paragallinarum* and *Gallibacterium anatis* in specific pathogen free chickens complicates clinical signs of infectious Coryza, which can be prevented by vaccination. *Avian Dis.*, **61**: 55–63. https://doi.org/10.1637/11481-081016-Reg

Rimler, R.B., Shotts, E.B., Brown, J. and Davis, R.B., 1976. The effect of atmospheric conditions on the growth of *Haemophilus gallinarum* in a defined medium. *J. Gen. Microbiol.*, **92**: 405–409. https://doi.org/10.1099/00221287-92-2-405

Sayed, R.H., Shasha, F.A., Khalaf, N.M., Saber, S.M. and El-Tantawy, L.A., 2019. Efficacy of preliminary prepared mucosal vaccine of Infectious Coryza in chicken. *Int. J. Vet. Sci.*, **8**: 182–186.

Siddique, A.B., 2012. *Molecular characterization of opportunistic pathogens from avian* Mycoplasma *cases in Punjab*. PhD thesis, University of Agriculture, Faisalabad, Pakistan

Sultana, R., Siddique, B., Ali, R. and Chaudhary, S., 2012. A study on the prevalence of respiratory diseases in broiler and layer flocks in and around Lahore district. *J. Zool.,* **27**: 13–17.

Sun, H., Xie, S., Li, X., Xu, F., Li, Y., Boucher, C.E. and Chen, X., 2018. Selection of *Avibacterium paragallinarum* Page serovar B strains for an infectious coryza vaccine. *Vet. Immunol. Immunopathol.*, **199**: 77–80. https://doi.org/10.1016/j.vetimm.2018.04.001

Umar, S., Ongor, H., Bayraktar, E., Gurgen, H.O., Sigirci, B.D., Esra, S., Ozkan, K., Turan, N., Yilmaz, A., Gurel, A., Ak, S., Cetinkaya, B. and Yilmaz, H., 2020. First report on the molecular detection, phylogeny, virological and pathological investigations of *Avibacterium paragallinarum* in chickens in Turkey. *Med. Weter,* **76**: 226–231. https://doi.org/10.21521/mw.6378

Wahyuni, A.E.T.H., Ramandani, D., Prakasita, V.C. and Widyarini, S., 2019. Efficacy of tetravalent coryza vaccine against the challenge of *Avibacterium paragallinarum* serovars A and B isolates from Indonesia in chickens. *Vet. World,* **12**: 972–977. https://doi.org/10.14202/vetworld.2019.972-977

Xu, Y., Cheng, J., Huang, X., Xu, M., Feng, J., Liu, C. and Zhang, G., 2019. Characterization of emergent *Avibacterium paragallinarum* strains and the protection conferred by infectious coryza vaccines against them in China. *Poult. Sci.,* **98**: 6463–6471. https://doi.org/10.3382/ps/pez531