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Evaluation of inactivated vaccine against fowl cholera developed from local isolates of *Pasteurella multocida* in Ethiopia

Wubet W.[1], Bitew M.[2], Mamo G.[3], Gelaye E.[1], Tesfaw L.[1*], Sori H.[1], Zewdie T.[1] and Abayneh T.[1]

[1]Research and Development Directorate, National Veterinary Institute, Bishoftu/Debre-zeit, P. O. Box 19, Ethiopia.
[2]Health Biotechnology Directorate, Institute of Biotechnology, Ministry of Technology and Innovation, Addis Ababa, Ethiopia.
[3]Department of Veterinary Microbiology and Immunology, College of Veterinary Medicine and Agriculture, Addis Ababa University, Ethiopia.

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Fowl cholera caused by *Pasteurella multocida* is among the serious infectious diseases of poultry in Ethiopia. This study was conducted to develop a vaccine from local strains of *P. multocida* and evaluate its performance. Inactivated vaccine was prepared following the OIE standards in three adjuvant formulations (oil, alum and gel). The performance of the different formulations was evaluated at different dose rates (0.5 and 1 mL) and routes (subcutaneous, SC and interamuscular, IM) in vaccination-challenge experiment in a total of 160 (six weeks old) chicken. The vaccinated groups showed significantly higher (P<0.05) mean antibody titer at day 21 (1365.49±376.97) and day 35 (1707±193.95) post-vaccination compared to the mean value at day 0 (200.01±4.91) and that of the unvaccinated group (196.72±10.51.147). The highest antibody titer obtained was for group vaccinated with 0.5 mL of alum-adjuvanted vaccine given IM (2472.96±603.47). The differences in antibody titer among vaccinated groups with respect to types of adjuvant and dose rates were insignificant. All vaccine formulations provided significant protection with survival rates ranging from 80 to 100% with alum-adjuvanted vaccine given IM being superior both in protective efficacy (100%) and in the absence of clinical signs post-challenge indicating its potential application in the control of fowl cholera.

Key words: Cholera, *Pasteurella multocida*, vaccines, poultry, Ethiopia.

INTRODUCTION

Poultry production contributes approximately 20% of the protein consumed in developing countries (Jenssen and Dolberg, 2003). Intensive poultry farming has become an important economic activity in Ethiopia particularly in the suburbs of major cities. Besides to its economic and social values, it occupies a unique position in terms of high quality protein source to rural smallholder farming families in Africa including Ethiopia (Dessie and Ogle,

*Corresponding author. E-mail: takeletefera99@gmail.com.

2001). The growing demand for poultry products associated with the rapid increase in urban population in Ethiopia has led to a rapid increase in total chicken population from 50.38 million in 2007 (CSA, 2007) to 56.87 million in 2015 (CSA, 2015).

The poultry industry is growing fast in Ethiopia and is facing many constraints especially infectious diseases causing significant setback to the development of this sector. Most of the infectious diseases are endemic, but some are emerging and re-emerging diseases. Poultry diseases are responsible for a number of adverse economic effects due to mortality and morbidity of chickens, cost of medication, loss in production and ban on international trade and public health significance (Dana et al., 2008).

In Ethiopia, poultry mortalities due to disease are estimated to range from 20 to 50%, but they can rise as high as 80% during epidemics. Fowl cholera has been among the major problems limiting chicken production in Ethiopia (Gebre-Egziabher, 2007). Despite frequent complaints and the reports from public and private poultry farms associated with outbreaks of fowl cholera, no interventions were taken to address the problem (Molalign et al., 2009). Owing to its significant impact on poultry production, control of the disease is essential. Of all the control strategies in areas where the disease is endemic, the use of vaccines is the most efficient from both practical and economical point of view (OIE, 2012). This is due to the fact that the onset of the disease is very rapid, and therefore, high mortality occurs before the disease is diagnosed for subsequent treatment. Antibiotic treatment and prophylaxis are of limited value owing to the emergence of multidrug-resistant strains. Globally, two types of vaccines are being used to immunize birds against fowl cholera namely, live and inactivated vaccines (Rhoades and Rimler, 1991). Live attenuated vaccines provide good protection with long duration of immunity and cross-protection against *P. multocida of* different serotypes or surface lipopolysaccharide (LPS) structures unlike killed vaccines which give protection only against strains with identical or nearly identical surface LPS structures (Harper and Boyce, 2017). However, the use of attenuated (live) vaccine is limited due to the lack of regular maintainable and sustainable attenuation methods and/or vaccine instability problems which may lead to risk of regaining its virulence. In this regard, inactivated vaccines or bacterins have comparative advantages over the attenuated live vaccines and are thus preferred to protect chicken against the disease caused by homologous strains (OIE, 2012).

Poultry enterprises in Ethiopia have experienced problems with the disease due to unavailability of locally produced vaccine. Although these vaccines have been produced in other countries, serotype variations among the pathogen may limit the use of the widely available conventional vaccines, making vaccines developed from locally circulating strains essential and more preferable (et al, 2016). There have been efforts to manufacture the vaccine at the National Veterinary Institute in Ethiopia. The first trial inactivated fowl cholera vaccine (Molalign et al., 2009) developed from local isolates was found to be effective in protecting experimentally challenged layer chickens. This work, however, need to be further validated using different adjuvants and dose rates and moreover there is a need to develop optimized standard operating procedure (SOP) for large scale (industrial) production of the vaccine. This work was aimed at developing inactivated Fowl Cholera vaccine from local *P. multocida* isolates and subsequently evaluate its safety, immunogenicity and protective efficacy in three different adjuvant formulations (Oil, Alum and Gel), at different dose rates and routes of administration.

MATERIALS AND METHODS

Study area and experimental animals

The current experimental study was conducted from November 2016 to May 2017 at the National Veterinary Institute (NVI), Bishoftu/Debrezeit, Ethiopia, located 45 km South East of Addis Ababa. NVI is a center for livestock vaccine research, development and production, and currently produce over 22 vaccines against major bacterial and viral diseases of livestock and poultry.

A total of two hundred nine (four weeks old, Bovans brown, layer chickens) obtained from brood stock farm and four rabbits obtained from NVI's laboratory animal facility were used for the current study. Chicken screened negative for antibody against fowl cholera by Enzyme Linked Immunosorbent Assay (ELISA) were included in the experiment. The chickens were vaccinated against Marek's, Gumboro, Newcastle disease and Fowl pox as per the scheduled time. The chickens were fed with formulated pullet and layer feed and water *ad libitum*. The chickens were kept at the NVI animal experiment facility. All animal experiments were approved by animal ethics committee of NVI and College of Veterinary Medicine and Agriculture (CVMA) of Addis Ababa University.

Experimental design

The safety of vaccines, prepared from three different adjuvants (Alum, Oil and Gel), were tested in three rabbits followed by a safety test in target animal (chicken). A total of 40 chickens randomly assigned into four groups containing 10 chickens each was used for the safety test; three of the groups for each vaccine type while one group was used as control. The immunogenicity and protective efficacy of the trial inactivated fowl cholera vaccine was done in vaccination-challenge experiment using a total of 160 chickens randomly assigned into eight groups of 20 chickens each. Six of the groups were assigned to each vaccine type at two dose rates (0.5 and 1 ml) given subcutaneously while one group was assigned for alum adjuvated vaccine given intramuscularly (0.5 ml) and the remaining as unvaccinated control.

Bacteriological media

Tryptose soya broth, TSB (Oxoid, Hampshire, UK) and Tryptose

soya agar, TSA (Difco, Sparks, USA) supplemented with 10% serum was used for culturing *P. multocida* isolates. *P.multocida* type A inoculum media (PA media), containing peptone (10 g), NaCl (5 g), Na_2HPO_4(3 g), H_2K (2.5 g), $MgSO_4$(1 g), yeast extract (2.5 g), glucose (5 g) and horse serum (5 mL) in 1L of distilled water, was used for growing *P. multocida* vaccine seed strain. Culturing for large scale vaccine preparation was done in *P.multocida* production media containing meat and liver digest broth (1L), yeast extract (2 mL), glucose (5 g) and horse serum(6.25 mL) with PH adjusted to 7.6.

Bacterial strains and growth conditions

Three isolates of *P. multocida* isolated from three different outbreaks (farm A, B and C) and stored at NVI bacterial culture collection were used as vaccine strains for the study. The lyophilized isolates were initially reconstituted with 2ml of TSB, which was then inoculated into the TSB supplemented with 10% horse serum and incubated at 37ºC for 18 h. Bacterial growth was checked by measuring the turbidity and pH of the media.

Identification and characterization of candidate vaccine seed bacterial strains

The three isolates of *P. multocida* strains were cultured on TSA, blood agar and MacConkey agar (Oxoid, Hampshire, UK) after which purity and colony characteristics was observed and recorded. Primary and biochemical (secondary) identifications were done employing Gram's staining and biochemical tests such as oxidase, catalase tests were performed according to standard methods described previously (Quinn et al., 2002).

Identity of the isolates to serotype level was done by *P. multocida* serotype specific multiplex PCR assay using the 5 primer sets specific to each capsular type (A, B, D, E and F) that target capsular biosynthesis gene cluster (*cap)* described previously (Townsend et al., 2001).

Bacterial DNA extraction

An overnight pure culture of the bacterial isolate in the log phase was used for DNA extraction. Genomic DNA was extracted using DNeasy Blood and Tissue kit (Qiagen, Germany) following the manufacturer's instructions. The eluted DNA was labeled and stored at -20ºC until analysis.

Serotype specific multiplex PCR assay

Briefly, 50 µL PCR reaction mixture contained 1 U *Taq* DNA polymerase (Qiagen), 3.2 mM of each of the forward and reverse primers (Eurofins Genomics, Austria), 200µM of each dNTP (Qiagen), 1×PCR buffer, and 2 mM $MgCl_2$ (Fermentas, Germany). Amplification protocol used was initial denaturation at 95°C for 5 min, followed by 35 cycles 95°C for 30s, 55°C for 30s, 72°C for 30s and final extension at 72°C for 7 min (Townsend et al., 2001). Each PCR product was detected in electrophoresis after running on a 1.5% agarose gel stained with GelRed for 1h at 100V. PCR products were visualized under UV illuminator and image taken in gel documentation system (UVI TEC, UK).

Sequencing and phylogenetic analysis

The PCR products were purified using the Wizard SV Gel and PCR clean-up system kit (Promega, Germany). The purified PCR products concentration were determined using the NanoDrop 2000c spectrophotometer (Thermo Scientific, USA) and then sequenced through a sequencing service (LGC genomics, Germany) using both primers (forward and reverse).

The sequences were edited using Vector NTI Advances™ 10 software (Invitrogen, Carlsbad, CA, USA) and consensus sequences were generated using BioEdit. For comparative multiple sequence analysis, blastn was used to retrieve *P. multocida HyaD* gene sequences data from the GenBank database. The sequences of the current isolates together with the homologous gene sequences retrieved from GenBank were aligned using BioEdit version 7.1.3.0 (Hall, 1999). Multiple sequence alignment of the nucleotide sequences were performed using the ClustalW and phylogenetic tree was constructed using the Neighbor-Joining method with the maximum composite likelihood nucleotide substitution model and the pairwise deletion. All analysis was done using MEGA version 6 software (Tamura et al., 2013). The accession number and details of the isolates' sequences included in the study is presented in Table 1.

Preparation of experimental vaccine

Of the three identified isolates, the isolate from farm A was considered as a master seed strain for the current trial vaccine due to the highest titer (CFU/mL) it had compared to the other two isolates (Farm B and C) grown under the same conditions. Formalin inactivated fowl cholera vaccine was prepared in three 500 mL flask each containing 300 mL media. One vial of the lyophilized isolate seed was initially reconstituted with 2 ml of TSB, homogenized well and then inoculated into sterile TSA supplemented with10% horse serum and incubated at 37ºC overnight.

The colonies were examined visually and microscopically after gram staining for their purity and cellular morphology, cocco-bacilli organisms and Gram negative. A single colony was transferred to 2 mL tube containing *P. multocida* type A inoculum media and incubated for 7 h at 37°C after which the purity was checked again by Gram staining. Half mL of the broth culture was transferred into 30 mL of *P. multocida* type A (PA) inoculum media and incubated overnight.

The purity of the PA inoculum was checked as described above and inoculated into PA production media at the ratio of 7 mL of *P. multocida* Type A (PA) inoculum, 7 mL of glucose and 3 mL of serum per 300 mL of *P. multocida* production media then incubated for 48 h with slow agitation. After two days, the purity was evaluated by Gram staining while the titer was determined by measuring the pH (5.2 to 5.8) and Optical Density (OD) of the culture medium as well as by titration using streak plate method. At the pH and/or OD value that corresponded to the desired titer (10^8 CFU/mL and above), the culture was inactivated by adding sterile formaldehyde at a proportion of 0.05% followed by incubation at 37°C with slow agitation for 6 days.

In process vaccine quality control tests

Purity and sterility test

The inactivated cultures (the vaccines) were checked for purity using gram stain while sterility was tested by culturing on media such as TSA, TSB, VF broth and Sabouraud agar media. All the test media were incubated at 37ºC except Sabouraud agar which was incubated at room temperature. Un-inoculated media from each type was also incubated as a negative control. All these inoculated media were observed for two weeks for any microbial growth (OIE, 2015).

Table 1. Nucleotide sequences of the local isolates and sequences retrieved from the GenBank included in the present study.

Strain	Accession number	Source	Reference
P. multocida	AY225345	Iran	Jabbari and Esmaelized (2003a)
P. multocida	AY225346	Iran	Jabbari and Esmaelized (2003b)
P. multocida	AY225347	Iran	Jabbari and Esmaelized (2003c)
P. multocida	AF036004	USA	De Angelis et al. (1998)
P. multocida	AF237926	USA	Fuller et al. (2000)
P. multocida	JF922885	USA	Tahmtan et al. (2011)
P. multocida	KP036621	China	Yang et al. (2014)
*P.multocida (*NVI-01-2017)	MK802880	Ethiopia	This study
P. multocida (NVI-02-2017)	MK802881	Ethiopia	This study

Table 2. Experimental layout of chickens used for immunization and challenge test.

Group	Dose (ml)	Adjuvant	Route of injection
A	0.5	Alum	SC
B	1	Alum	SC
C	0.5	Alum	IM
D	0.5	Oil	SC
E	1	Oil	SC
F	0.5	Gel ($Al(OH)_3$)	SC
G	1	Gel ($Al(OH)_3$)	SC
Control	-	-	-

Safety test of inactivated vaccine

The safety of inactivated vaccine was done in laboratory animals. Three rabbits were injected with 1mL of inactivated culture intramuscularly from each batch (flask) of vaccine, and observed for 14 days for any adverse reaction (OIE, 2015).

Vaccine adjuvant formulation

In this study, sterile Montanide oil, aluminum hydroxide (gel) and Aluminum potassium sulphate (Alum) were used as adjuvants. Oil adjuvant vaccine was used in 1:1 proportion while Aluminum hydroxide gel and Aluminum potassium sulphate (alum) were used at the rate of 1.2 and 1% of the vaccine, respectively. After the addition of each adjuvant, the mixture is continuously agitated using magnetic stirrer and the pH was adjusted to 7.0 before dispensing into 50 mL sterile polypropylene vials.

Quality control of the final prototype vaccine product

Sterility of the final product was evaluated employing same method described for inactivated culture while the safety was done in target animals (Chickens). For safety test, 40 chickens were grouped randomly in to four groups (each with 10 chickens), three of the groups assigned for each vaccine formulation while one was used as control. Chicken in group I, II and III were injected with 1mL each of the vaccine formulations (oil, alum and gel adjuvanted), respectively, through subcutaneous route at the back of the neck and the fourth control group was injected with 1 mL of sterile TSB media. The chickens were observed for 14 days for any adverse effects (OIE, 2015).

Evaluation of vaccine efficacy

Experimental layout

The protective efficacy of the newly prepared vaccine formulations was determined in a vaccination-challenge experiment in chicken. A total of 160 layer pullets (13 weeks old) randomly divided into eight groups each with 20 birds were used for vaccine efficacy trial. Six of the groups were assigned for Oil, gel and Alum adjuvated vaccines and 2 groups per vaccine type for two different doses rates (1 mL and 0.5 mL) subcutaneously. An additional one group was assigned for alum adjuvant vaccine for vaccination using IM route at dose rate of 0.5ml while the remaining group was used as unvaccinated control. The experimental layout is presented in Table 2.

Immunization

The chickens were acclimatized for a week in the experimental facility. At day 21 after the first vaccination, booster vaccination was administered (OIE, 2015). Blood sample was collected from the wing vein from vaccinated and control birds at days 0, 21 and 35 post-vaccination to determine antibody titer. Sera samples were stored at -20°C until analysis.

Determination of immune response and test interpretation

The immune response was evaluated based on the relative level of antibody to *P. multocida* in chicken serum using IDEXX PM ELISA Kit (IDEXX Laboratories, Inc. UK) according to the manufacturer's instruction. Briefly, the test was performed on a 96-well ELISA plate coated with *P. multocida* antigen. Test sera were diluted five hundred fold (1:500) and dispensed in duplicates (100 uL) on coated wells while undiluted negative and positive controls (each 100 uL) were also dispensed on the coated wells. The plate was incubated for 30 min at 20^{o}C so that antibodies specific to *P. multocida* form a complex with the coated antigens. The plate was washed with distilled water to remove any unbound material from the wells followed by addition of a conjugate (100 uL) and incubation at 30 min at 20°C to enable binding to any attached chicken antibody in the wells. Unbound conjugate was washed away as above and enzyme substrate (100 uL) was added. The

Table 3. Phenotypic characteristics the *P. multocida* type A isolates.

Test	Isolates		
	1	2	3
Hemolysis on blood agar	No-hemolytic	No-hemolytic	No-hemolytic
Growth on MacConkey's agar	No growth	No growth	No growth
Gram's reaction	-	-	-
Cellular morphology	Coccobacilli	Coccobacilli	Coccobacilli
Indole production	+	+	+
Catalase test	+	+	+
Oxidase test	+	+	+
MP test	-	-	-
VP test	-	-	-
TSI test	Y/Y	Y/Y	Y/Y
Citrate test	+	+	+
Urease test	-	-	-
Interpretation	*P. multocida*	*P. multocida*	*P. multocida*

+, Positive; -, negative; Y/Y=yellow slant and yellow butt

absorbance or optical density (OD) of the subsequent color development was measured by spectrophotometer at 650nm and the corresponding OD value was directly related to the amount of antibody to *P. multocida* present in the test sample. The corresponding antibody titer was determined from the OD values using the method stated in the test kit instruction which was given as:

$$\text{Titre} = \text{antilog}\,(1.09(\log_{10} S/P) + 3.36$$

Where:

$$S/P = \frac{\text{Mean OD of test sample} - \text{Mean OD of negative control}}{\text{Mean OD of positive control} - \text{Mean OD of negative control}}$$

The value 3.36 relates S/P at a 1:500 dilution as endpoint titre. The test result was interpreted as S/P ≤ 0.20 as negative and S/P ≥ 0.20 (titer greater than 396) as positive.

Challenge experiment

P. multocida Kombolcha strain used as seed for vaccine preparation was also used as a challenge strain. Before the actual challenge test, a pilot challenge experiment was carried out to determine LD_{50} of the challenge strain and to optimize the pathogenicity and the challenge dose, which was found to be $5x10^8$ CFU/mL. At day 35, chicken in all groups (vaccinated and controls) were challenged with a suspension of 6 hr culture of *P. multocida* containing $5x10^8$ CFU/mL IM on breast muscle.

The chickens were then followed-up for two weeks for any development of clinical signs and mortality and the results were recorded. All dead birds were necropsied and specimen from organs was cultured for re-isolation of *P. multocida* by streaking on TSA with 10% serum. Surviving chickens were slaughtered at the end of experiment and specimen collected from internal organs for re-isolation of *P. multocida*. Isolation was done by culturing on blood agar followed by identification through morphology, staining, cultural, biochemical test, and finally by species-specific PCR.

Ethical declaration

All animal experiment has been approved by the Animal Research Ethics Committee of the National Veterinary Institute regarding its conformity to the ethical standards set in international guiding principles for animal experiment research.

Data analysis

Raw data were entered in to Microsoft Excel spreadsheet and transferred to SPSS 23.0 for analysis. Descriptive statistics such as proportions, averages and frequencies were used in summarizing quantitative data as required. The Analysis of Variance (ANOVA) was used to find out the differences in the mean antibody titers among immunized groups vaccinated with the different vaccine formulations and dose rates. The desired level of precision and confidence level used in the analysis was 5 and 95%, respectively (Thrusfield, 2005).

RESULTS

Identification of isolates

The three isolates obtained from three different outbreaks suspected of fowl cholera showed similar phenotypic characteristics consistent with *P. multocida*. The results of phenotypic characteristics of the isolates are presented in Table 3. Molecular identification of the isolates confirmed that all belong to *P. multocida* with the same capsular type, that is, capsular type A.

Molecular identification

Molecular identification in capsular typing multiplex PCR assay showed that all the three isolates were positive for capsular type A with PCR product of 1044 bp similar to the positive control (Figure 1).

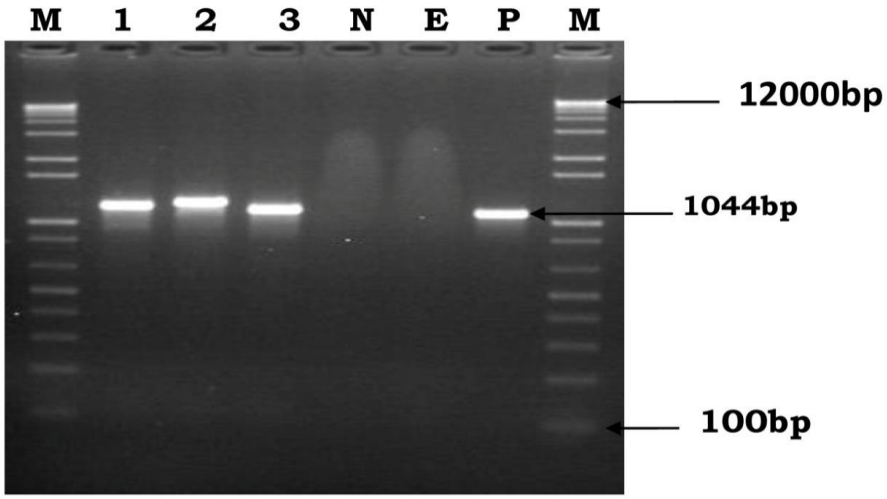

Figure 1. Multiplex PCR using four sets of specific primers targeting capsular biosynthesis genes (*capA, capB, capD* and *capF*) of *P. multocida*. Lanes: M, Molecular marker (started 100bp 1kb plus, Invitrogen); 1, *P. multocida* Kombolcha isolate; 2, *P. multocida* Genesis isolate; 3, *P. multocida* Tadesse farm isolate; N, Negative control; E, extraction internal control; P, positive control (*P. multocida* capsular

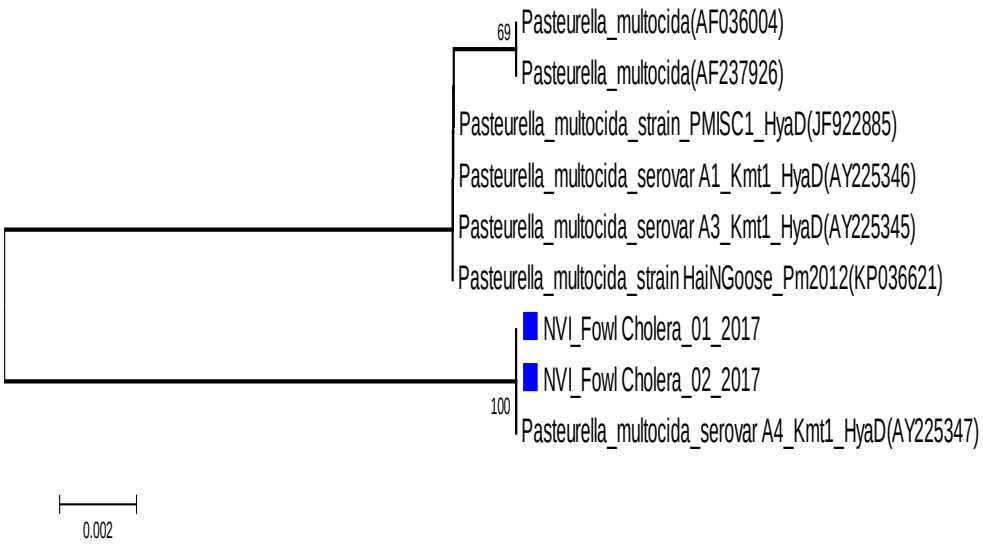

Figure 2. Phylogenetic analysis of the *HyaD* gene coding sequence of *Pasteurella multocida* Isolates.

Phylogenetic analysis

Phylogenetic analysis based on the sequence data of *HyaD* gene indicated that the local *P. multocida* isolates were closely genetically related to one of a previously characterized reference isolate *P. multocida* serovar A4 of Iran origin while divergent from the remaining strains included in the analysis as shown in Figure 2.

The tree was constructed using neighbor-Joining method with the pairwise deletion option in MEGA6.

Vaccine titre and safety

The titre of the current vaccine was $5x10^8$ CFU/mL and with pH and OD (optical density) value of 5.62 and 0.738, respectively. Follow-up of rabbits and chicken injected with formalin inactivated ana-culture and final vaccine

Table 4. Mean serum antibody titer of chickens in different groups at day 21 after primary vaccination with inactivated Fowl Cholera vaccine.

Group	Dose (ml)	Adjuvant	Route	Mean±SE of Ab titer	S/P ratio	F value	Significance level (P value)
A	0.5	Alum	SC	599.35±114.9689	0.285		
B	1	Alum	SC	648.54±233.88	0.298		
C	0.5	Alum	IM	1205.28±296.59	0.535		
D	0.5	Oil	SC	761±193.30	0.350	1.18	0.321*
E	1	Oil	SC	2035.43±763.24	0.836		
F	0.5	$Al(OH)_3$	SC	775.12±255.39	0.349		
G	1	$Al(OH)_3$	SC	3533.33±2463.47	1.304		

* P>0.05, no significant difference among the means of antibody titers.

Table 5. Mean serum antibody titer of chickens in different groups 2 weeks after booster vaccination (day 35) with inactivated fowl Cholera vaccine.

Group	Dose (ml)	Adjuvant	Route	Mean±SE of Ab titer	S/P ratio	F-value	Significance level (P value)
A	0.5	Alum	SC	1852.24±431.06	0.797		
B	1	Alum	SC	1500.76±772.43	0.611		
C	0.5	Alum	IM	2472.97±603.47	1.034		
D	0.5	Oil	SC	1177.59±276.07	0.52	0.71	0.642*
E	1	Oil	SC	1471.37±331.67	0.642		
F	0.5	$Al(OH)_3$	SC	1466.23±345.20	0.640		
G	1	$Al(OH)_3$	SC	2009.93±643.84	0.85		

* P>0.05, no significant difference among the means of antibody titers.

with adjuvant, respectively, did not show any adverse effect or clinical signs of infection during the 14 days of period indicating that the product was safe.

Evaluation of immunogenicity

In immunization test, the pre-vaccination mean serum antibody (Ab) titer in chickens of all groups was 200.01±4.91(S/P ratio, 0.11) while that of unvaccinated control group was 196.72±10.51.147 (S/P ratio, 0.13). The Mean serum Ab titers at day 21 and 35 were 1365.49±376.97 and 1707±193.95, respectively which were both significantly higher (P<0.05) than the control group and background titer at day 0 of the respective groups. The highest serum Ab titer was obtained for chicken groups vaccinated with 0.5 mL of alum adjuvanted vaccine given IM (2472.96±603.47). However, no significant differences were observed in antibody titers between the respective groups of birds vaccinated with vaccine formulations of different adjuvants and dose rates. The mean serum antibody titer of chicken's in the different groups at day 21 and 35 after primary vaccination is summarized in Tables 4 and 5.

Evaluation of protective efficacy

Protective efficacy test showed that some chicken in all vaccinated groups, except 0.5 mL Alum IM group, showed signs of depression, ruffled feathers, loss of appetite and drop in egg production a day after challenge which lasted from 2 to 5 days. None of the chicken vaccinated with 0.5 mL alum adjuvant vaccine given through IM route showed any signs and all were active after challenge. Chicken in non-vaccinated control group showed clinical signs including depression, loss of appetite, greenish diarrhea, conjunctivitis, cloudiness of the eye with unilateral or bilateral blindness, labor breathing, lameness with swollen joint in some cases with death starting from the second day of challenge. Number of Chickens showing clinical signs indicative of fowl cholera is summarized in Table 6.

The protective efficacy evaluation showed that alum ($AlK(SO_4)_2$) adjuvant vaccine given intramuscularly provided 100% protection with none of the birds showing clinical signs after challenge. Although 1 mL of Gel ($Al(OH)_3$) adjuvated inactivated vaccine given SC provided 100% protection, two of the birds showed clinical signs consistent to Fowl cholera from which later *P. multocida* was re-isolated (Table 7). In all chicken that

Table 6. Number of chicken showing different clinical signs indicative of fowl cholera starting a day after the challenge experiment.

Group	Adjuvant and route of injection	Labor breathing	Depression	Greenish diarrhea	Swelling of joints and lameness	Conjunctivitis	Ruffled feathers	**
A	0.5 mL Alum, SC	3	3	2	3	2	3	3
B	1 mL Alum, SC	3	3	2	2		2	3
C	0.5 mL Alum IM	-	-	-	-	-	-	0
D	0.5 mL Oil, SC	2	2	3	2	2	4	4
E	1mL Oil, SC	3	3	1	3	-	3	3
F	0.5 mL Gel, SC	3	3	2	3	1	3	3
G	1 mL Gel, SC	2	2	1	2	1	2	2
H	Control, 1mL SC	20	20	16	20	20	20	20

** = Total No of Chickens which showed clinical sign.

Table 7. Protective efficacy of inactivated fowl cholera vaccine formulated with three types of adjuvants.

Group	Birds (no)	Dose (ml)	Adjuvant	Route	No of chicken Survived (%)	No of chicken died (%)
A	20	0.5	Alum	SC	19 (95)	1 (5)
B	20	1	Alum	SC	20 (100)	0 (0)
C	20	0.5	Alum	IM	20 (100)	0 (0)
D	20	0.5	Oil	SC	19 (95)	1 (5)
E	20	1	Oil	SC	19 (95)	1 (5)
F	20	0.5	$Al(OH)_3$	SC	19 (95)	1 (5)
G	20	1	$Al(OH)_3$	SC	18 (90)	2 (10)
H	20	NA	NA	NA	4 (20)	16 (80)

died during the course of the experiment, *P. multocida* was re-isolated from blood, joint lesion and internal organs (liver, spleen and heart) and subsequently identified using both phenotypic and molecular methods.

P. multocida was isolated from the liver, spleen, heart, blood and lung of the control group in 4 randomly selected birds from each group at the end of the experiment (14 days after challenge), whereas none from vaccinated groups.

DISCUSSION

Fowl cholera caused by *P. multocida* is a highly contagious disease of poultry presenting one of the major challenges of the poultry industry worldwide (Singh et al., 2014). The fast growing poultry sector in Ethiopia has been confronted by a number of infectious diseases among which one is fowl cholera. However, there is no information on the genotypes of *P. multocida* causing fowl cholera and neither locally available vaccine. The current studies is aimed at characterizing P. multocida strains, from cases of fowl cholera and develop vaccine from local strains, which is pertinent strategy to address the problem associated with the disease.

The identification of capsular biotype A *P. multocida* isolates from an outbreak in the current study was supported by the results of phylogenetic characterization where both isolates

were found to be of same genotype. However, this may not reflect nationwide distributions of genotypes that may be involved in causing fowl cholera thus requiring further studies to elucidate its molecular epidemiology. The phylogenetic relationships of the current isolates from Ethiopia with the Iranian isolate (Jabbari and Esmaelized, 2003a) suggest the strains may have global distribution and are common causes of fowl cholera elsewhere.

The significantly higher immune response as seen from antibody titre between vaccinated and non-vaccinated category, despite the high variation within a group, shows that the vaccine is highly immunogenic. The variation within a group observed may be due inherent individual differences since precautions were taken during the study design to minimize 'within group effects' where same breed and age group of birds were included. The significantly higher immune response was further substantiated by the results of the protective efficacy where all the three adjuvant formulations of the current vaccine conferred significant protection of chicken. The findings of significantly higher immune response in groups vaccinated through IM route than the SC groups unlike the absence of significant differences among the vaccines with three adjuvant formulations (alum, oil and gel) may suggest that the route of administration is more important than the type of adjuvant used. This is in agreement with previous reports where IM delivery of inactivated fowl cholera vaccine provided better antibody response and protective efficacy than the SC route of administration (Rahman et al., 2004). Previous study by Molalign et al. (2009) also found that alum ($AlK(SO_4)_2$) adjuvant fowl cholera vaccine given IM provided better HAI titer at bacterial dose of 10^{12} CFU/mL than the Oil based (Montanide ISA 50) and $Al(OH)_3$ adjuvants.

The absence of significant differences in immune response with respect to vaccine formulations prepared with three adjuvants, however, contradicts the fact that oil adjuvant evokes an enhanced and better immune response due to delayed absorption from site of injection providing prolonged source of antigen. It has been demonstrated that the antibody response to a protein antigen in oil adjuvant vaccine remained constant for over 300 days in contrast to the antibody response to the same antigen in aqueous solution or alum precipitation, which declined after 10 days (Talmage and Maurer, 1953). On the other hand, the current finding is in agreement with a previous similar study where the three adjuvants showed no significant difference in stimulating the immune response against *P. multocida* (Molalign et al., 2009). This may suggest that the differences in immune response among vaccine formulations with respect to the three adjuvants may not be significant for the short term requiring long term evaluation to have conclusive information.

In evaluation of protective efficacy, the clinical pictures observed in un-vaccinated controls in the challenge experiment agree with previous studies where similar clinical signs were also reported (Akhtar et al., 2016). The absence of clinical signs and death in groups vaccinated with 0.5 mL of Alum adjuvated Fowl Cholera vaccine given through IM route substantiates the finding of significantly higher antibody titer of this group than the remaining vaccinated groups. Although all the chicken survived in group vaccinated with 1mL Alum adjuvant vaccine given through SC route, few of them showed the characteristic clinical signs of fowl cholera. The lack of 100% protection (Table 7) and the clinical signs observed in some of the chicken in four of the vaccinated groups all administered subcutaneously (Table 6) may indicate that the route of administration had more effect on the immune response than the type of adjuvant used. The higher protective efficacy of IM administered Fowl cholera vaccine was also documented in previous works (Rahman et al., 2004). The lower mortality (80%) recorded in the unvaccinated group compared to previous similar study in Ethiopia where 86% mortality was recorded (Molalign et al., 2009) may be attributed to the older age of the chickens used in the current study and other intrinsic factors such as breed or differences in individual immune status. Thus, further optimization study of the IM route is required using the different adjuvants not only due to its better immune response but also from practical point of view of its suitability during vaccine administration.

In conclusion, isolates of *P. multocida* obtained from outbreak cases characterized in this study including the isolate used for vaccine preparation fall into the same genotype, which may suggest the important role of these strains in causing fowl cholera in Ethiopia. However, further study on outbreak investigation covering wider areas of the country is necessary to demonstrate the most dominant strains causing fowl cholera which will be important to develop multivalent vaccine. The formalin inactivated alum adjuvant fowl cholera vaccine induced better antibody titer and conferred better protection in a challenge experiment indicating its potential use as a vaccine against Fowl cholera but requires further evaluation of its field performance.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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