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Molecular Characterization of Pasteurella Species Isolated from Poultry

[1]Esraa S. Ahmed, [2]Etab. M. AboRemla, [3]Hisham S. Nada and [4]Azza N. Farag

[1]Veterinary Services, Zagazig, Egypt
[2]Department of Bacteriology, Faculty Of Veterinary Medicine,
Kafr El-Sheikh University, Kafr El-Sheikh, Egypt
[3]Department of Bacteriology, Faculty Of Veterinary Medicine,
Kafr El-Sheikh University, Kafr El-Sheikh, Egypt
[4]Department of Bacteriology, Animal Health Institute, Dokki, Egypt

Abstract: *Pasteurella multocida* type A is the etiologic agent of fowl cholera, a highly contagious and fatal disease of chickens. The research work was performed for the isolation, identification and molecular characterization of *P. multocida,* also experimental infection study was conducted to document the clinicopathologic features observed in chickens inoculated with *P. multocida* serotype A, in addition preparation of vaccine from isolated strain and determination of its efficacy, this was performed at Animal Health Institute research for a period of two years. By using standard bacteriological procedures for 440 samples collected from lung, Liver, heart and spleen of suspected chickens that have died of avian pasteuerellosis a total of thirty *P. multocida* isolates were characterized by biochemical profile and analytical Profile Index (API).Capsular typing by using multiplex polymerase chain reaction (PCR), demonstrated that 12 strains were capsular type A. Experimental infection in chickens revealed clinicopathologic features observed by necropsy in chickens inoculated with isolates in the form of hemorrhages on the heart, congestion of the liver with a small number of necrotic and inflammatory foci on its surface, the observable damage on lung included signs of pneumonia and pulmonary hyperemia with fibrinous exudation on its surface, also splenomegaly was recorded. API system and a multiplex capsular PCR could be valuable for the rapid identification of *P. multocida*. The protective efficacy of vaccine was measured by determining the survivability rate of the birds of each vaccinated group by challenge infection. The results of challenge exposure demonstrated that the experimentally prepared fowl cholera vaccine conferred excellent protection.

Key words: Isolation · Identification · Pathogenicity · Vvaccination · *Pasteurella multocida*

INTRODUCTION

We now live in an area where two-thirds of human infectious diseases and three-quarters of emerging or reemerging infectious diseases are zoonotic in origin, i.e., diseases caused by animal-associated pathogens that can be shared with humans [1-4]. *Pasteurella multocida* is an important zoonotic agent that causes wide spectrums of diseases in many species of domestic and wild animals and even humans [5, 6]. Infections with *P. multocida* (otherwise known as pasteurellosis) lead to great economic losses in the farming industry due to their severe morbidity and mortality. *Pasteurella multocida* is a heterogeneous species of Gram-negative bacteria and is a commensal of the upper respiratory tract of many animal species. However, under predisposing circumstances, the organism is the etiology of a wide range of economically important infection in domesticated animals [7]. Clinical findings vary greatly depending on the course of the disease. In acute cases, increased mortality is usually the first indication. Affected birds have swelling of the face or wattles, discharge from the nostrils, mouth and eyes which may become "cheesy", labored breathing and, in some cases, lack of coordination. The face, combs and wattles may become cyanotic (turn a bluish colour) [8- 10]. *Pasteurella multocida* includes capsulated and noncapsulated types, with the former being more virulent than the latter [11, 12]. *P. multocida* can be differentiated

Corresponding Author: Esraa Salem, Veterinary Services, Zagaxig, Egypt. E-mail: Esraasalem135@yahoo.com.

serologically by capsular antigens into serogroups A, B, D, E and F [13]. This assay represented a rapid and reproducible alternative to the serologic and non-serologic methods [14]. Vaccination is the most effective and economic methods to conifer protection to the animals against *Pasteurella* and minimize the symptoms of pasteuerellosis also is widely practiced in endemic areas but significant outbreaks still occur [15]. The aims of this study were the use of standard bacteriological methods for cultivation & identification of *P. multocida and* molecular characterization of isolates. Also study of the pathogenicity of isolates in mice and chicken and applying field trial of chicken vaccination with the isolated strain.

MATERIALS AND METHODS

A total of (110) bird with a history of respiratory distress, cyanosis of comb and wattle freshly died were collected randomly from sporadic different private farms (45 layers, 50 broilers and 15 Baladi breed) from Kotor, Perma, passion, Shopratana. These farms showed different mortality rate during the period from November 2014 to May 2016 from El –Garbia Governorate, Egypt (Table 1).

Samples: The birds were sacrificed, out of (440) tissue pieces from (spleen, liver, heart and lung) were collected. All samples were packed separately in sterile plastic bags tightly closed, labeled and transported immediately to the laboratory at Animal Health Institute research in an ice box. Heart blood smears; tissue impression smears from heart, liver, spleen and lung were prepared and subjected to Geimsa staining for direct bacteriological study, the surface of (pneumonic lung tissue, spleen, liver and heart) was seased by red hot spatula for sterilization, grinded in a sterile mortar with sterile BHI broth [16].

Isolation and Identification of P. Multocida: The isolated pure culture was subjected for Gram staining and Leishman's staining for morphological identification of the bacteria. For biochemical characteristics, different tests such as Methyl red (MR), Voges-Proskauer (VP), Indole production, catalase, oxidase and sugar fermentation tests were done. All the isolates were further confirmed by using analytical Profile Index to generate the API NE using the commercially available kit (API 20-E kit, BioMerieux, France) according to the manufacturer's instructions. Briefly, a single well isolated colony of the bacterium was made into homogenous suspension of 5 ml sterile distilled water and inoculated in the tubes of API-Kit. [17,18]For the maintenance, the *P. multocida strains* were inoculated in NA slant incubated at 37° C overnight Finally, sterile mineral oil was overlaid and kept the slant at room temperature for future use. The colonies of positive samples are of moderate size, round and greyish, viscous, mucoid or rough irregular colonies and non-hemolytic. *P. multocida* has a characteristic sweetish odour. It does not grow on MacConkey's agar and it is a good indole producer and microscopically showing Gram negative coccobacilli. strains were stored at -80ºC in BHI broth containing 40% glycerol [19] twelve single pure non repeated colonies were picked up and inoculated in semisolid nutrient agar for PCR molecular characterization.

M-PCR method

Oligonucleotide Primers Used in cPCR: They have specific sequence and amplify a specific product (Table 2).

Table 1: Poultry and samples tested

Farms	breed	Flock size	age	No of dead Chicken	percentage	No of collected chicken
Bassioun	Layers	10000	2-15/mon.	7/day	0.07%	45
Shopratana	Broilers	7000	1-1.5/mon.	7/day	0.1%	50
Kotor	Baladi	5000	2month	3/month	0.06/m%	15
Total						110

Table 2: Sequence and specificity of primers applied for the detection of capsular-type gene in *Pasteurell:multocida:*strains [20]

Serogroup	Gene name	Sequence	Amplimer
All	KMT1T7	ATCCGCTATTTACCCAGTGG GCTGTAAACGAACTCGCCAC	460bp
A	CAPA-FWD CAPA-REV	TGCCAAAATCGCAGTCAG TTGCCATCATTGTCAGTG	1044bp
B	CAPB-FWD CAPB-REV	CATTTATCCAAGCTCCACC GCCCGAGAGTTTCAATCC	760pb
D	CAPD-FWD CAPD-REV	TTACAAAAGAAAGACTAGGAGCCC CATCTACCCACTCAACCATATCAG	657pb
E	CAPD-EWD CAPE-REV	TCCGCAGAAAATTATTGACTC GCTTGCTGCTTGATTTTGTC	511pb
F	CAPF-FWD CAPF-REV	AATCGGAGAACGCAGAAATCAG TTCCGCCGTCAATTACTCTG	851pb

Source: Metabion (Germany), Catalogue no.51304

Extraction of DNA According to QIAamp DNA Mini Kit Instructions: Into the bottom of a 1.5 ml microcentrifuge tube (20 µl QIAamp protease),then added 200 µl of the sample followed by addition of 200 µl buffer, then mixed by pulse vortexing for 15 seconds. The mixture was incubated at 56°C for 10 min and then centrifuged to remove drops from the inside of the lid. 200 µl ethanol (96%) was added and then mixed again by pulse vortexing for 15 seconds. After mixing the tube was briefly centrifuged to remove drops from the inside of the lid. Applied the mixture carefully in a 2ml collecting tube to the QIAamp mini spin column. Closed the cap and centrifuged at 8000 rpm for 1 min. The QIAamp mini spin column was carefully opened and 500 ml bufferAW1 were added without wetting the rim then the cap was closed and centrifuged at 8000 rpm for 1 min. After that placed in a clean 2 ml collection tube, while the tube containing the filtrate was discarded. The QIAamp mini spin column was carefully opened and 500 ml buffer AW2 was added without wetting the rim. The cap was closed and centrifuged at full speed for 3 min. The QIAamp mini spin column was placed in a new 2 ml collection tube and discarded the old collection tube with the filtrate, then centrifugation at full speed for 1 min. The QIAamp mini spin column was placed in a clean 1.5 ml micro centrifuge tube and discarded the collection tube containing the filtrate. After that the QIAamp mini spin column was opened carefully and 100 µl buffer AE were added and incubated at room temperature (15-25°C) for 1 min and Closed the cap and centrifuged at 8000 rpm for 1 min.

Preparation of PCR Master Mix according to Emerald Amp GT PCR master mix (Takara) Code No RR310A kit as shown in table (3):

Table 3: Preparation of either kmt gene uniplex:PCR Master Mix

Component	Volume/reaction
Emerald amp GT PCR mastermix (2x premix)	12.5µl
PCR grade water	4.5 µl
Forward primer (20 pmol)	1 µl
Reverse primer (20 pmol)	1 µl
Template DNA	6 µl
Total	25 µl

Table 4: Preparation of serotyping multiplex PCR Master Mix

Component	Volume/reaction
Emerald amp GT PCR mastermix (2x premix)	25µl
PCR grade water	12 µl
Forward primer (20 pmol)	1 µl each
Reverse primer (20 pmol)	1 µl each
Template DNA	10 µl
Total	50 µl

Cycling Conditions of the Primers During cPCR: Temperature and time conditions of the primers during PCR are shown in (Table 5) according to specific author and Emerald Amp GT PCR master mix (Takara) ki

Table 5: Cycling conditions of the different primer during cPCR

Target gene	Primary den	Sec. den.	Ann.	Ext.	Final ext.
Detection	94°C	94°C	55°C	72°C	72°C
and typing	5 min.	30 sec.	1min	1min.	10 min.

DNA Molecular Weight Marker: The ladder was mixed gently by pipetting up and down 6 µl of the required ladder then loaded.

Agarose Gel Electrophoreses [21] with Modification: Twenty µl of each uniplex PCR product and 30 µl of each multiplex PCR product, negative control and positive control were loaded with ethidium bromide to the gel. The power supply was 1-5 volts/cm of the tank length. The run was stopped after about 30 min and the gel was transferred to UV cabinet. The gel was photographed by a gel documentation system and the data was analyzed through computer software.

Pathogenicity Test in Mice: Thirty three white mice weighting about 18-22 g were used for purification and pathogenicity of *P. multocida.* All strains of *P. multocida* were grown for 18 h in a shaker/incubator at 37ºC in BH) broth. Approximately 0.2 ml of each culture, containing approximately 2.4×10^8 colony forming units (CFU)/ml was inoculated into each of thirty mice by the intraperitoneal route and observed for 72 h, to study the mortality pattern. Control mice (n=3) were inoculated with 0.1 ml of phosphate buffered saline (PBS PH7.4) Keeping appropriate then observed for 72 hrs to study the mortality pattern. Organisms were re-isolated on a blood agar plate using heart blood collected from dead mice and an impression smear from the liver was prepared on microscopic slides for bacterial observation, using the Giemsa staining method [22]

Vaccine Trial:

Preparation of Oil Adjuvant Killed Vaccine: The bacterium P. multocida serotype (A) was recovered from chickens with clinical cases of fowl cholera in this study and confirmed by biochemical test, API and PCR at Animal Health Institute Research.The minimum lethal dose (MLD) as a virulence indicator used was described previously [23- 25].After reconstitution with 0.5ml tryptose phosphate broth (TPB), each strain was streaked

on sheep blood agar plate and incubated for 24hrs at 37°C. Isolated P. multocida was cultured in BA media and kept in Bacteriological shaker incubator at 37°C for 24 hours. The purity of culture was examined and subsequently sub-cultured in the same media for 24 hours. The isolated colonies were then inoculated in 200 ml nutrient broth containing yeast extract and beef extract and kept in shaker incubator at 37°C for 24 hours for massive growth. Bacterial count was determined and calculated as 3.2 x 10^8CFU/ ml. Later on formalin was added in the broth culture and incubated at room temperature for 24 hours. Liquid paraffin and arlacil-80 was mixed slowly for 30 minutes with the help of magnetic stirrer. Then formalin killed culture was slowly dispensed in vials and stored at 40C as vaccine. A single typical colony was cultured in 300ml TPB and incubated for 18hrs at 37°C with shaking. Cell suspensions was inactivated by adding 0.3% formaldehyde and was left to stand for 24h. The cells were separated by centrifugation at (pH7.2) [26]

Chickens Used for Experimental Infection and Vaccination: A total of 31 chickens obtained at one day age from a commercial hatchery in El-Garbiya prevalence, were placed in concrete block pens until reach nine week old, before the experiment, birds were wing marked with a number, then the rooms were cleaned, disinfected and 8 cm of clean shavings was placed on the floor. All chickens were fed with commercial pelleted. Feed and drinking water were available all the time (out of them 12 chickens for experimental infection, 4 for toxicity of the vaccine and 15 chicken were divided into 4 groups (three for each) for vaccination.

Experimental Infection: A total of (12) 9 week-old healthy chickens were divided into 3 challenge groups (Group 1 to 3) containing 3 chickens each and one negative control (NC) group of 3 chickens. Chickens in the challenge groups were given intramuscularly challenge of *P. multocida* bacterial suspension at different CFUs as Group one was administered 0.1 ml intramuscularly of 10^6 CFU contained in tryptose soya broth, the second group received 10^7 CFU of the same serotypes and the last group received 10^8 CFU of the same serotypes. Each chicken in the NC group was challenged with 0.5 mL of sterile normal saline (NS) using the same routine. Each group of chickens was housed in a separate room. The chickens were monitored for a signs of infection throughout the study at 12, 24, 36 and 48 hrs post challenge and death occurred within the period was recorded. Findings from clinical and postmortem examination of carcasses were recorded, the chickens were sacrificed and swabs or tissue materials were taken from liver, lungs and spleen. Immediately after collection, the samples were streaked onto BA and NA. Plates and were incubated at 37oC overnight. The positive cases were reconfirmed by re-isolation of *P. multocida* type A by following the standard procedures.The collected samples (heart, liver and spleen) from the artificially infected chickens were collected and examined.

Immunization and Challenge Procedure: Total of 15 nine - weeks old chickens were used. The chickens were divided into 2 groups vaccinated (n=10) and control groups (n=5). Chickens of each, one week before the onset of the study were screened for the presence of *P. multocida* by taking oropharyngeal swabs. All birds were inoculated intramuscularly at thigh region with the prepared vaccine 1 ml / bird except the control group was inoculated with PBS (PH7.4). Vaccination was repeated 3 weeks later. The chickens were challenged 2 weeks after booster injection. To challenge a typical colony of each strain was transferred to 10ml TPB and incubated for 6h at 37°C. The optical density of these cultures was then adjusted spectrophotometrically to 0.440 at 540nm. The concentration of these cultures was approximately 2×10^9CFU/ml. The cultures were diluted serially ten-fold in PBS and selected dilutions were used for challenging. For estimating the concentration of viable organisms, 0.5ml of selected suspensions was spread on BA plates. The colonies were counted after 24h incubation at 37°C.

RESULTS AND DISCUSSIONS

Clinical Symptoms: Some of the affected birds have swelling of the face or wattles, discharge from the nostrils, mouth and eyes which might become “cheesy”, other cases showed labored breathing and, in some cases, lack of coordination. Also the face, combs and wattles was cyanotic (turn a bluish color) with depression, loss of appetite, lameness and ruffled feather.

Gross Lesions of Birds Died with Respiratory Infection: Edema of head, neck and trachea.The lungs were congested and showed feature of pneumonia, such as black ended edges of lungs from which, inflamed liquids. For heart blood film specimens were designed to check gram-negative, bipolar staining bacillus through a microscope. No significant variation in mortality pattern was observed.

Prevalence of P. Multocida Samples: The bacteriological investigations of the internal organs from the dead birds revealed thirty isolates were obtained; lungs (8 isolates), liver (8 isolates), heart (8 isolates) and spleen (6 isolates) as show in table (6), isolates grew well on blood agar without hemolysis, but not on MAC. They showed bipolar, gram-negative rods, catalase, oxidase and indole positive, All strains ferment sugars like glucose, sucrose and maltose while lactose were not fermented and negative for Vogues proskauer, methyl red and gelatin liquefaction test and did not hydrolyze urea, based on the morphologic, biochemical and molecular characteristics, all isolates were identified as *P. multocida*.

Table 6: Recovery of P. multocida from the internal organs of poultry

Breed	Type of sample	Total No. of samples	No. of isolates	Percentage %
Layers	Heart	45	3	6.6%
	Lung	45	5	11.11%
	Spleen	45	2	4.4%
	Liver	45	2	4.4%
Total		180	12	6.66%
Broilers	Heart	50	4	8%
	Lung	50	2	4%
	Spleen	50	5	9%
	Liver	50	3	6%
Total	50	200	14	8.12%
Baladi	Heart	15	1	6.6%
	Lung	15	1	6.6%
	Spleen	15	1	6.6%
	Liver	15	1	6.6%
Total		60	4	8.8%
Total		440	30	7.5%

Photo 1: Pathogenicity in mice; Splenomegaly, enlargement of liver and sever hemorrhages

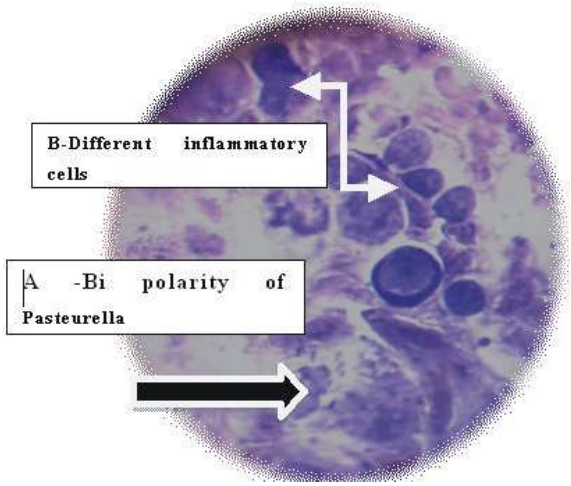

Photo 2: Blood film

Result of Pathogenicity Test in Mice: The entire number (33 out of 33) of inoculated mice died (100%) while control Mice survived, indicating a significantly high virulence causing septicemia in less than 30 hrs. The gross pathology of the disease in mice was characterized by splenomegaly and petechial hemorrhages (Photo 1). The inoculated strains were re-isolated from their organs such as livers and spleens blood film from heart showed bipolarity and different inflammatory cells (Photo 2)

Identification of Capsular Antigen with Multiplex (M-PCR): The 12 isolates *Pasteurella* spp. was subjected to PCR using specific primers for molecular characterization and identification. Polymerase chain reaction with species specific KMT1T7 primers identified all isolates as positive for *Pasteurella* showing amplification of 460-bp (Photo 3). The PCR done by using PM *cap*EF primers could amplify amplicons of 1,044 bp (belonging to type A) (Photo 4).

Result of Experimental Infection in Chicken: The effects of experimental inoculation of P. multocida isolates in chickens in all groups recorded depended on the duration time and severity of lesions produced, characteristics changes were in heart, liver and spleen. The chickens were died within 3 days after challenging with P. multocida Type A. In post-mortem examination, marked septicemic lesions consisting of white necrotic foci and hemorrhages in heart, liver and spleen (Photo 5), (Photo 6) The observable damage included small number

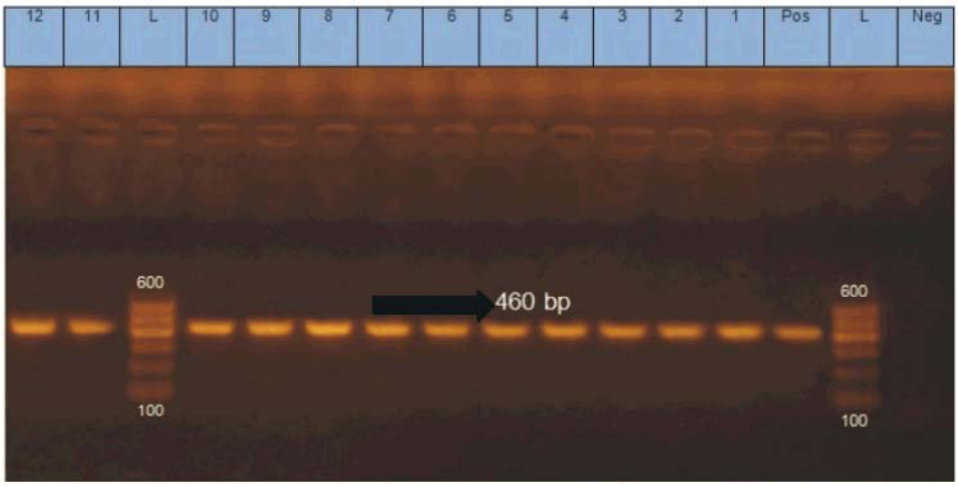

Photo 3: Amplified DNA products (460 bp) of all positive serogroup strains of *P. multocida*
Lane 1: Marker, 100 bp DNA ladder

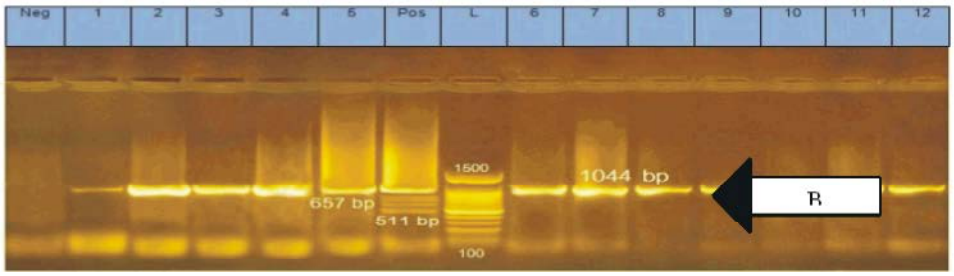

Photo 4: Multiplex capsular PCR (M-PCR) typing system for *P. multocida*.
The arrow B indicated the amplified DNA products of capsular type a serogroup (1,044 bp)

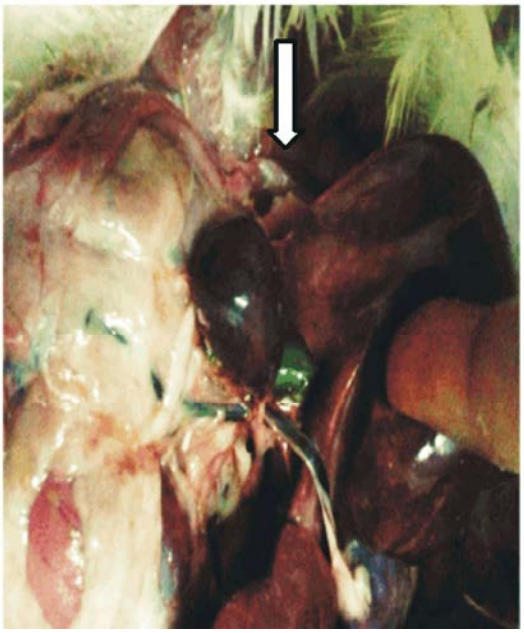

Photo 5: Enlarged and congested liver and spleen

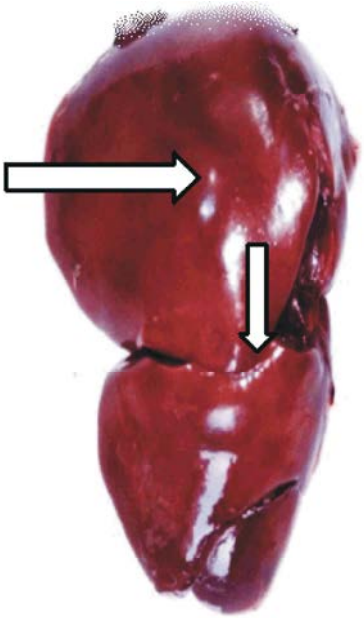

Photo 6: The liver swollen, Showed multiple small necrotic foci

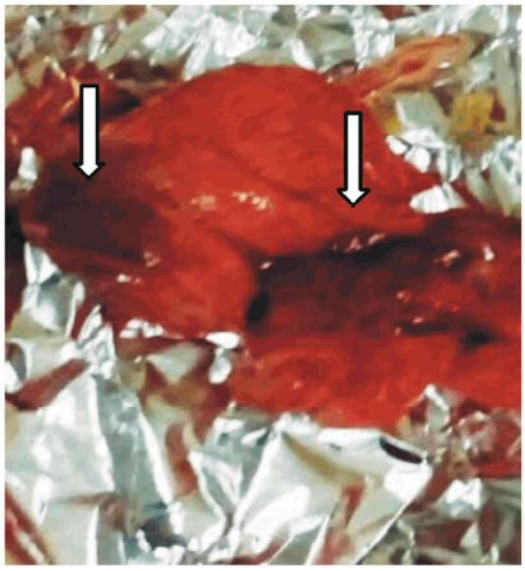

Photo 7: Sever congestion of trachea

Photo 8: Enlargement and sever congestion of lung

of necrotic and inflammatory foci on the liver surface, severe congestion at the throat (Photo7), also pneumonia and pulmonary hyperemia fibrinous exudation on the lung surface (photo 8) also increased amounts of peritoneal and pericardial fluids. The control chicken showed neither mortality nor lesions.

The results of challenge exposure demonstrated that the experimentally prepared fowl cholera vaccines conferred excellent protection in all groups while all the unvaccinated control birds were infected following challenge infection. In post challenge observations, control birds showed characteristic clinical signs and symptoms of fowl cholera like dullness, depression, anorexia, hyperthermia, labored breathing, lameness and greenish diarrhea and ultimately death occurred: P. multocida could not be recovered from immunized chickens, which survived the challenge while it was isolated from all dead control chickens (Table 7)

Table 7: The survivability rate of chicken at challenge infection after 15 days of booster vaccination

Groups	Route of vaccination	Total birds	No. of birds survived	No. of birds died	Percentage of survivability
Group 1	IM	5	5	-	100%
Control (un vaccinated	IM	5	0	5	0%

DISCUSSION

The FC is a highly contagious bacterial disease of domestic and wild birds; the occurrence of fowl cholera in commercial layers and breeder flocks has been reported as the major concern in the poultry industry by other workers [27- 29]. In this study, the clinical symptoms recorded were noticed by Rhoades and Rimler [30].The gross lesions observed in our study are similar to the lesions observed during an acute form of fowl cholera described by Heddleston and Rhoades [31] and others [28, 32, 33].

Regarding to the percentage of isolation rate of: *P. multocida* obtained (7.5%) is higher than the level reported by Mbuthia *et al.* [28]: who recorded a rate of 6.2%. This difference may be due to the number of samples, method of isolation and presence of stress and age of birds, on the other hand it is lower than results recorded by Hasan *et al.* [33], Hossain *et al.* [34], Belal [35] and Panna *et al*.[36]. Hasan *et al.* [32] found 12.05% prevalence in layer chicken and 4.25% in broiler chicken, whereas Hossain *et al.* [33] found 13.04% prevalence in chicken while Panna *et al.* [35] recorded it 11.42% and Belal [34] found 59.72%. The lower rate of *P. multocida* found in this study might be due to differences in age and breeds of the chickens and also for the resistance power of the commercial chicken due to improved management, vaccine and nutrition. The fowl cholera organism was characterized by growing well on blood agar; all the isolated organisms in this study were Gram negative coccobacillary shape in Gram staining method and bipolar characteristics in Leishman's staining method which is in support of the findings of Ievy *et al.* [24], Ashraf *et al*. [37] and Akhtar [38].The morphology, staining and cultural characteristics of the organism in different culture media were studied according to the procedure described

by Quinn *et al.* [17]. Analytical Profile Index results of the isolates are in accordance with Waltman and Horne [39] i.e. excellent to good identification. Furthermore, the system was found quick and easy to perform, as it took less than 24 hrs for biochemical as well as sugar fermentation tests on a single strip.

Multiplex PCR represents an important tool for the diagnosis and study of a large number of samples and reducing time required [40, 41]. Specific primers were used for the identification of *P. multocida*: [41] (Table 2). In comparison with standard molecular weight marker (100pb) the molecular weight of PCR products of all isolates were found to be 460pb specific for *Pasteurella* photo3.the same reported by Akhtar[38], Townsend *et al.* [43], Kumar *et al.* [44], Ranjan [45] and Manasa [46]. All the 12 isolates of *P. multocida* were confirmed to be Type A, indicated by the amplification an amplified DNA band was found at the size of 1,044 bp (Photo 4); similar results were reported by other authors; Akhtar, [38], Shayegh *et al.* [47] and Kwaga [48].

Pathogenicity or virulence of *P. multocida* is complex and variable with endotoxins being produced by virulent and a virulent [8].Clinical signs manifested were depression, in: appetence, ruffled feathers, dyspnea, those continued till death. All deaths in all group occurred within 7days and mortality rates were 100% in all group with different in duration, These findings are collaborated with the findings of Botzler [49] who also observed that clinical signs of fowl cholera in birds vary depending on various factors such as age, species, dose of strain, route of entry of the bacterium and form of the disease. The postmortem findings in the experimentally challenged chickens in the form of congested liver, spleen, lungs as well as petechial and ecchymotic hemorrhages confirmed the findings of Visut *et al.* [50]. The result of this pathogenicity study indicated that all the experimental chickens inoculated with serotypes A with different concentrations came down with the clinical signs of fowl cholera similar to those reported by Rimler and Glisson [51] depending on strain, the gross lesions and could possibly explain the profound debilitation, massive flock morbidity and mortality observed in chickens suffering from the acute form during outbreaks of fowl cholera.

In this study the protective efficacy of vaccine was measured by determining the survivability rate of the birds of each vaccinated group by challenge infection. The results of challenge exposure demonstrated that the experimentally prepared fowl cholera vaccines conferred excellent protection in all groups while all the unvaccinated control birds were infected following challenge infection. In post challenge observations, control birds showed characteristic clinical signs and symptoms of fowl cholera like dullness, depression, anorexia, hyperthermia, labored breathing, lameness and greenish diarrhea and ultimately death occurred: *P. multocida* could not be recovered from immunized chickens, which survived the challenge while it was isolated from all dead control chickens and this finding agrees with that recorded by Zahoor and Siddique [52]

The findings of present study have revealed that chickens were highly susceptible to infections with *P. multocida* serotypes, these serotypes of *P. multocida* were found to cause high morbidity, mortality, clinical, gross and histopathology features in the experimental chickens The protective efficacy of fowl cholera vaccine was measured by determining the survivability rate of the birds of each vaccinated group by challenge infection. The experimental fowl cholera vaccine conferred excellent protection of vaccinated birds in all groups while all the unvaccinated control birds were infected following challenge infection

Recommendations: It is therefore recommended that chicken should be vaccinated against fowl cholera with a polyvalent fowl cholera vaccine consisting of *P. multocida* serotype A to improve the immune status of chickens.

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