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Phenotypic and genotypic characterization of *Pasteurella multocida* isolated from chickens

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ABSTRACT

A total of 300 samples from apparently healthy chickens were collected in this study. This samples represented as 100 samples from trachea, 100 samples from lung and 100 samples from heart. They were subjected to morphological and biochemical examination for identification of *P.Multocida*. An incidence of *P. multocida* by cultural examination was 21 (7%);- 0% from tracheal swabs, 13% from lung and 8% from heart while an incidence by biochemical examination was 6 (2%) ; 0% from tracheal swabs - 3% from lung and 3% from heart. All suspected *P. multocida* colonies were subjected to PCR for identification of *P. multocida* isolates and proved that six isolates were found to be positive for *P.multocida* with an incidence of 2% which similar to the results of confirmatory biochemical examination. The drug sensitivity test mentioned that *P. multocida* was sensitive to ciprofloxacin and norfloxacin. Six suspected isolates of *P.multocida* identified by cultural examination were tested by polymerase chain reaction method by using specific primers for *P.multocida* . The *stn* gene was amplified in four isolates giving 617bp, while the *InvA* gene was amplified in four isolates giving 284bp.

1. INTRODUCTION

Poultry Industry provides an important source of animal protein all over the world. Many research works have been guard toward the production of poultry with the minimum of management and feed intake. It is strongly believed that productivity can optimized for a particular breed by improving management and controlling effectively those factors suppressing production. The most important of these factors are infection with bacterial and viral agents. From these bacteria that cause high mortalities in poultry is family pasteurellaceae.

Pasteurella multocida is considered one member of this family (Harper et al., 2014). *Pasteurella multocida* is a Gram-negative, non-motile, coccobacillus, capsulated, non-spore forming bacterium occurring singly, in pairs or occasionally as chains or filaments belonging to the pasteurellaceae family (Akhtar 2013).

In poultry, *P. multocida* often associated with economic loss due to loss of cattle or poultry species (Biswas et al., 2005; Marza et al., 2015).

Fowl cholera is a commonly occurring contagious avian bacterial disease caused by *Pasteurella multocida*, often causing high mortality, thus negative bacterium infects a wide range of animal species, causing diseases such as fowl cholera in poultry (Glisson et al., 2003). resulting in large financial losses in poultry industry.

Therefore, this present work was planned to study prevalence of *P. multocida* among poultry, isolation and identification of *P. multocida* from poultry, estimation of sensitivity of the isolated *P .multocida* strains to different chemotherapeutic agents in vitro and identification of *P. multocida* by PCR.

2. MATERIAL AND METHODS

2.1. Collection of samples:

A total of 300 samples were collected from apparently healthy chickens aging

5- weeks Old, which were maintained in different farms and small-scale breeders. Samples were collected from the internal organs such as trachea, lung and heart of apparently healthy chickens suspected to be suffered from pasteurellosis.

100 samples from each organ. All samples were kept in ice bags and transferred as soon as possible to the laboratory.

2.2. Isolation and identification of P. multocida:

The samples were inoculated directly on the blood agar media as well as the nutrient broth and incubated at 37°C for 18-24 hours then examined for suspected *P. multocida* colonies. The purified colonies were subjected for further identification either microbiologically or morphologically (Gram stain, Motility test or biochemically according to Frank (1988).

2.2. Antibiogram of isolated P. multocida strains:

The disc diffusion technique was adopted according to Cruickshank et al. (1975).

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The pure colonies were selected, and a suspension is made in saline without the incubation. The turbidity of the suspension is adjusted by comparison with, 5 McFarland turbidity standards. The standards and the test suspension are placed in similar 4-6 ml thin glass tubes. The turbidity of the test suspension is adjusted and compared with the turbidity standard against a white background with contrasting black lines, until the turbidity of the test suspension equates to that of the turbidity standard. A sterile, non-toxic swab is dipped into the standardized suspension of the bacteria and excess fluid was expressed by pressing and rotating the swab firmly against the inside of the tube above the fluid level. The swab is streaked in three directions over the entire surface of the Mueller-Hinton agar (Oxoid). The inoculated plates are allowed to stand for 3-5 minutes. The chosen antibiotic discs were placed onto the agar surface using sterile forceps; the discs were placed no closer together than 24mm. Each disc is gently pressed with the point of a sterile forceps to ensure complete contact with the agar surface. The plates were inverted and aerobically at 35°C for 16-18 hours. After incubation the diameters of the zones of inhibition were measured using ruler. The interpretation of the size of the zones of inhibition according to (modified from NCCLS (1990) M2-A4) .

The antibiotic sensitivity disks (Oxoid) were used according to (modified from NCCLS (1990) M2-A4), Ciprofloxacin, Norfloxacin, Spectinomycin, Piperacillin, Streptomycin, Doxycycline, Cefoxitin, Amoxicillin, Flucloxacillin, Erythromycin.

2. 4 Identification of P. multocida by PCR according to Sambrook et al., 1989:

2.4.1. Extraction of DNA According to QIAamp DNA mini kit instructions

1. 20 µl QIAGEN protease were pipetted into the bottom of a 1.5 ml micro centrifuge tube.
2. 200 µl of the pooled swabs or the organs wash were added.
3. 200 µl buffer AL were added to the sample, mixed by pulse vortexing for 15 seconds.
4. The mixture was incubated at 56˚C for 10 min.
5. The 1.5 ml micro centrifuge tube were spin 2 times to remove drops from the inside of the lid.
6. 200 µl ethanol (96%) were added to the sample and mixed again by pulse vortexing for 15 seconds. After mixing, the 1.5 ml microcentrifuge tube was briefly centrifuged to remove drops from the inside of the lid.
7. The mixture from step 6 was carefully applied to the QIAamp mini spin column (in a 2ml collecting tube) without wetting the rim. the cap was closed, and centrifuged at 8000 rpm for 1 min. The QIAamp mini spin column was placed in a clean 2 ml collection tube, and the tube containing the filtrate was discarded.
8. The QIAamp mini spin column was carefully opened and 500 ml buffer AW1 were added without wetting the rim. The cap was closed, and centrifuged at 8000 rpm for 1 min. The QIAamp mini spin column was placed in a clean 2 ml collection tube, and the tube containing the filtrate was discarded.
9. 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.
10. The QIAamp mini spin column was placed in a new 2 ml collection tube and the old collection tube was discarded with the filtrate. Centrifugation at full speed for 1 min was done.
11. The QIAamp mini spin column was placed in a clean 1.5 ml microcentrifuge tube, and the collection tube containing the filtrate was discarded. The QIAamp mini spin column was carefully opened and 100 µl buffer AE were added. The QIAamp mini spin column was incubated at room temperature (15-25˚C)for 1 min, and then centrifuged at 8000 rpm for 1 min.

2.4.2. Preparation of PCR Master Mix for PCR according to Emerald Amp GT PCR master mix (Takara):

Each reaction was performed in a final volume of 28 µl each reaction contained a mixture consisted of Emerald Amp GT PCR master mix (2x premix), PCR grade water, Forward primer, Reverse primer and Template DNA as shown in table (1).The ladder was mixed gently by pipetting up and down. 6 µl of the required ladder were directly loaded. Electrophoresis grade agarose (1 g) was prepared in 100 ml TBE buffer in a sterile flask, it was heated in microwave to dissolve all granules with agitation, and allowed to cool at 70˚C, then 0.5µg/ml ethidium bromide was added and mixed thoroughly. The warm agarose was poured directly in gel casting apparatus with desired comb in apposition and left at room temperature for polymerization.The comb was then removed, and the electrophoresis tank was filled with TBE buffer. Ten µl of each PCR product, negative and positive control were mixed with 1µl of loading buffer and mixed. The mixture was placed to the well of agarose .Also 6 µl of DNA molecular size marker were loaded to the gel. The run was stopped after about 30 min of 100 voltages and then the gel was transferred to UV cabinet.

Table 1 The amount of each component of PCR mixture.

Component	Volume/reaction
Emerald Amp GT PCR mastermix (2x premix)	12.5*µl*
PCR grade water	4.5 *µl*
Forward primer(20 pmol)	1.0 *µl*
Reverse primer (20 pmol)	1.0 *µl*
Template DNA	9.0 *µl*
Total	28.0 *µl*

3. RESULTS

The result of biochemical identification of the isolates showed identical biochemical reaction to be *P .multocida* (Table 2).

By cultural examination, 13 lung samples were found to be suspected positive (13%) for *P. multocida* and 8 heart samples were found to be suspected positive (8%) from a total number of 300 examined samples (Table 3 & Fig. 1).

By biochemical examination, a total number of 300 examined samples that were represented as 100 tracheal swabs with an incidence of *P. multocida* (0%), 100 lungs with an incidence of (3%) and 100 hearts with an incidence of (3%) (Table 4).

The results of the sensitivity of *P. multocida* isolated from lung and heart to 10 different antibiotics (Table 5). All strains isolated from lung were sensitive to ciprofloxacin, norfloxacin and spectinomycin, while all strains were intermediate to piperacillin, streptomycin and doxycycline and resistant to cefotaxime, amoxicillin, flucloxacillin and erythromycin. All strains isolated from heart were sensitive to ciprofloxacin, norfloxacin and spectinomycin. While all strains were intermediate to piperacillin and resistant to

streptomycin, Doxycycline, amoxycillin, flucloxacillin and erythromycin.

Six suspected isolates of *P. multocida* identified by cultural examination were tested by polymerase chain reaction method by using specific primers for *P. multocida*. The *stn* gene was amplified in four isolates giving 617bp (Fig. 3) while the *InvA* gene was amplified in four isolates giving 284bp (Fig. 3).

4. DISCUSSION

Pasteurella multocida can cause per-acute, acute and chronic infections that can be associated with high mortalities (Christensen, Bisyaard 2000). The presumptive *Pasteurella multocida* produced small mucoid dew drop like colonies were observed on blood agar medium after incubation at 37˚C for 24 hrs and appeared as gram negative cocco-bacilli were observed when stained with gram stain, record similar results was obtained by Kiran et al. (2012).

Identification is based primarily on the results of biochemical test shown in Table (1). The isolates that are fermented include glucose, mannose, fructose and sucrose; maltose are not fermented. It produced indole, catalase and oxidase. There was no haemolysis on blood agar. There was no reaction on methyl red test. These results were closely correlated with Abd El-Dayem (1990), Calnek et al., (1997), Bebars (2000), Shiva Chandra (2005) and Kiran et al., (2012).

Table 2 Biochemical reaction of the isolated *Pasteurella multocida* using standard laboratory tests

Biochemical tests	Result
Oxidase	+ve
Catalase	+ve
Indole production	+ve
Growth on MacConkey's' agar	-ve
Urease test	-ve
TSI	+ve
M.R .and V.P. tests	-ve
Haemolysis on blood agar	-ve
Nitrate reduction	+ve
Glucose	+ve
Mannitol	+ve
Arabinose	-ve
Lactose	-ve
Trehalose	-ve

Table 3 Prevalence of isolated *P. multocida* from collected samples in cultural examination and stain reaction.

Type of samples	No.	Cultural and microscopically examination			
		No. of +ve samples	%	No. of –ve samples	%
Tracheal swabs	100	0	0%	100	100%
Lung	100	13	13%	87	87%
Heart	100	8	8%	92	92%
Total	300	21	7%	279	93%

Table 4 Prevalence of *P. multocida* identified by biochemical examination.

Type of samples	No.	Results of biochemical examination			
		No. of +ve samples	%	No. of –ve samples	%
Tracheal swabs	100	0	0%	100	100%
Lung	100	3	3%	97	97%
Heart	100	3	3%	97	97%
Total	300	6	2%	294	98%

Table 5 Results of antibiogram of isolated *P. multocida* strains.

Antibiotics	*P. multocida* isolated from Lung			*P. multocida* isolated from Heart		
	Sensitive	Intermediate	Resistant	Sensitive	Intermediate	Resistant
Spectinomycin	S			S		
Cefotaxime			R			R
Streptomycin		I				R
Piperacillin		I			I	
Ciprofloxacin	S			S		
Flucloxacillin			R			R
Erythromycin			R			R
Doxycycline		I				R
Amoxycillin			R			R
Norfloxacin	S			S		

S = sensitive. I = intermediate. R = Resistant

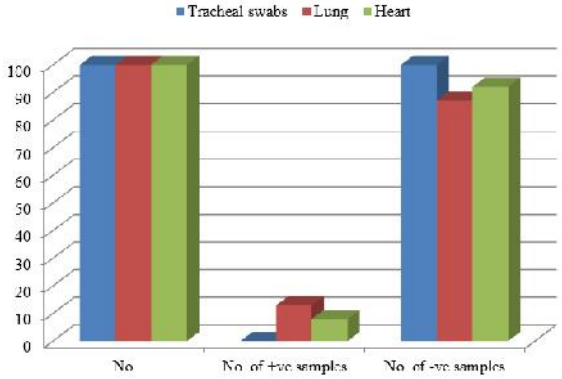

Fig. 1 Prevalence of isolated *P. multocida* from collected samples in cultural examination

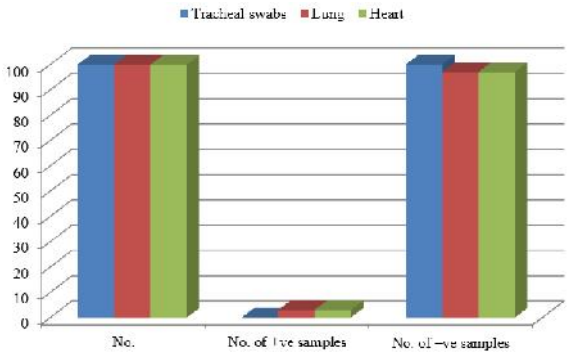

Fig. 2 Prevalence of *P. Multocida* identified by biochemical examination

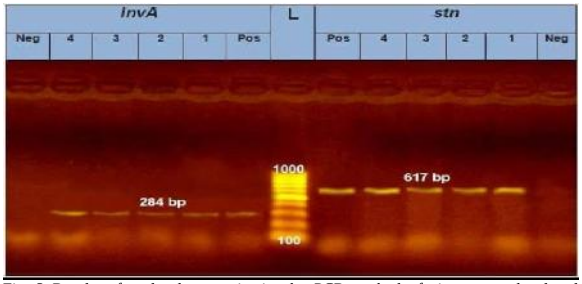

Fig. 3 Results of molecular examination by PCR method of six suspected cultural colonies: Lane L : 100-1000bp DNA ladder. Neg. : Negative control. Pos.: Positive control. Lane (1, 2, 3, 4): Positive samples. Lane (5, 6): Negative samples

While an incidence of *Pasteurella multocida* was 6 (2%) by biochemical examination, 0% of tracheal swabs, 3% lung and 3% heart as shown in table (3) and fig. (2). These results were nearly in agreement with Ahmed (2003) who isolated *Pasteurella multocida* from chickens with a percentage of 1.8%. Also, Everlon et al. (2013) who isolated *Pasteurella multocida* from chicken with incidence

of 3 (3.3%). Also, these results nearly similar to results obtained by Kiran et al. (2012) and Dilara et al .(2018).

The results of antibiogram of isolated *Pasteurella multocida* from chickens table (4) revealed that all strains isolated from lung were sensitive to ciprofloxacin, norfloxacin and spectinomycin, while all strains were intermediate to piperacillin, streptomycin and Doxycycline and resistant to cefoxitin, amoxicillin, flucloxacillin and erythromycin. All strains isolated from heart were sensitive to ciprofloxacin, norfloxacin and spectinomycin, while all strains were intermediate to piperacillin and resistant to streptomycin, Doxycycline, cefoxitin, amoxicillin, flucloxacillin and erythromycin. These results coincide with the results of Xibiao et al. (2009), Yoshimura etal. (2001) and Khrenberg et al. (2001), who indicated that ciprofloxacin and norfloxacin were the most active drugs against *Pasteurella multocida.*

While *Pasteurella multocida* were resistant to amoxicillin, tetracycline and erythromycin. But the findings of our study differ from results noted by Thales et al. (2016), who indicated that *P. multocida* were susceptible to amoxicillin and erythromycin. Increasing multidrug resistance is attributable at least in part to the use of antibiotic additive in animal feed and the extensive use of antimicrobial agents in veterinary medicine Tang (2009).

In this study, PCR was a perfect method for rapid, sensitive and accurate detection of *Pasteurella multocida*. The results of PCR showed that the *stn* gene and *InvA* gene were amplified in four isolates from six suspected isolates of *P. multocida* identified by cultural examination.

The *stn* gene was amplified giving 617bp, while *InvA* gene was amplified giving 284bp. Similar results were reported by Shayegh et al. (2010), Kwaga (2013) and Aktar (2013), who indicated that the PCR amplification could amplify 620 bp size as shown in fig. (3).

5. CONCULSION

Pasteurella multocida is one of the most important pathogenic bacteria in Egypt that can infect the chickens and causing fowl cholera. Detection of fowl cholera considered a good tool in protection of chicken in Egypt. 23 samples out from total 300 samples were positive to *P. multocida* by cultural examination. *P. multocida* can be treated by ciprofloxacin and norfloxacin. The widespread and injudicious use of antibiotics by poultry farmers may result in multidrug resistance of *P. multocida*. Finally, this study provides a starting point for the development of appropriate molecular tools to study more about *P. multocida* in chicken.

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