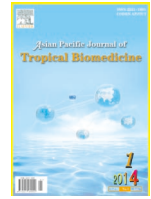




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Molecular analysis of *Pasteurella multocida* strains isolated from fowl cholera infection in backyard chickens

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PEER REVIEW

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Comments

The study is up-to-date and an interesting valuable research work in which authors have demonstrated the clonality of bacteria isolated from backyard chickens that improve the measures for prevention esp. for making vaccines and even in treatment.

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ABSTRACT

Objective: To characterize *Pasteurella* isolated from backyard chickens using whole cell protein lysate profiles and random amplified polymorphic DNA (RAPD) techniques to show their genetic relationship because *Pasteurella multocida* (*P. multocida*) is an important cause of fatal infections in backyard chickens.

Methods: Twenty one *P. multocida* isolates were recovered previously from clinical cases of fowl cholera belonging to individual owners and phenotypically analyzed using biochemical tests and serotyping were used for the genetic characterization.

Results: Phylogenetic study based on both methods revealed that the recovered population of *P. multocida* isolated from backyard chickens differs markedly, constituting a well-separated cluster and appearance of 3 distinguishing lineages with greater discrimination shown by RAPD-PCR that resulted in two subclusters in cluster A and three subclusters in cluster B and were related greatly with capsular serogroups for the examined strains. The whole cell protein revealed the presence of dominant protein bands at approximately 41 and 61 kDa in all of the examined isolates that may be a virulent proteins share in the increasing of its pathogenicity. Clear distinctive bands ranged from 123 to 1554 bp.

Conclusions: Based on the previous findings, there are three spreading clusters that may indicate the association of a small number of *P. multocida* variants with the majority of cases suggesting that certain clones of *P. multocida* are able to colonize the examined backyard chickens. Also, the ease and rapidity of RAPD-PCR support the use of this technique as alternative to the more labour-intensive SDS-PAGE system for strain differentiation and epidemiological studies of avian *P. multocida*. Further application of RAPD technology to the examination of avian cholera outbreaks in commercially available flocks may facilitate more effective management of this disease by providing the potential to investigate correlations of *P. multocida* genotypes, to identify affiliations between bird types and bacterial genotypes, and to elucidate the role of specific bird species in disease transmission.

KEYWORDS

Pasteurella multocida, Chickens, Variation, Molecular characterization

1. Introduction

Fowl cholera, caused by *Pasteurella multocida* (*P. multocida*), occurs sporadically or enzootically in most countries of the world wherever intensive poultry production

occurs, and is known as a bacterial disease with major economic importance due to its high mortality^[1]. *P. multocida* is a heterogeneous species that pathogenicity of individual strains is highly variable and susceptibility to these bacterial strains varies considerably among avian

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species[2].

Generally, diagnosis of the disease in natural outbreaks largely depends on conventional methodologies comprising bacterial isolation and identification by serotyping and biochemical characterization, which reveal the presence of variable serogroups/types in different geographical regions[3]. However, it has been observed that conventional characterization is not sensitive enough to identify and differentiate each strain involved in natural infections[4,5].

The limitations of currently employed techniques have led to significant problems in understanding the disease outbreaks, origin and transmission of pathogens, the virulence characteristics of the organism as well as determining disease incidence and economic importance[5].

Alternatively, DNA-based methods have been applied for rapid identification and differentiation of avian strains of *P. multocida* originating from different regions[6,7].

A number of genotyping and genetic methods represent the major techniques for the characterization of *P. multocida*. Electrophoretic separation of whole cell and outer membrane proteins, or lipopolysaccharides by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and DNA-based techniques such as polymerase chain reaction (PCR) based fingerprinting techniques based on either random amplification of polymorphic DNA (RAPD) by use of short and arbitrarily chosen DNA primers or detection of variable repetitive DNA regions, have been adapted to detect inter- or intra-strain variations which are rapid, specific and highly sensitive, and efficiently employed for differentiation of various strains of different micro-organisms from single/different outbreaks[4,6,8].

In addition, molecular techniques could also be an important tool to reveal epidemic patterns, trace sources of infection and aid the development of reasonable intervention strategies to reduce the presence and spread of *Pasteurella* infections in animals[9].

Due to the variability of *P. multocida* in general and lack of knowledge on isolates circulating in the upper Egypt, the aim of this work was to characterize the avian strains of *P. multocida* that recovered from cases of fowl cholera by comparative analysis of their whole cell protein (WCP) and RAPD profiles to gain deeper insight into the current *P. multocida* population to better understand the inter-strain relatedness, so as to be able to recommend epidemiology well-defined vaccines in the future.

2. Materials and methods

2.1. Bacterial strains and culture conditions

A total of 21 avian isolates of *P. multocida* were used

in this study, including 12 strains serotype–1, 4 isolates serotype–3 and 5 ones untypable (Table 1).

Table 1

Distribution of the examined *P. multocida* isolates according to serotypes, place of isolation, SDS–PAGE and RAPD profiles.

No. of isolate	Serotype	Origin (place)	SDS–PAGE Cluster	RAPD–PCR Cluster
P46	A:1	Qena	1	–*
P47	A:1	Qena	1	1
P83	A:1	Qena	1	2
P87	A:1	Qena	1	1
P110	A:3	Qena	1	–
P120	A:3	Qena	1	1
P132	A:1	Qena	1	1
P145	A:1	Qena	1	1
P152	A:1	Qena	1	1
P265	A:1	Qena	1	1
P166	D	Qena	2	3
P50	D	Qena	2	–
P73	A:1	Qena	2	1
P108	A	Qena	2	2
P20	A:3	Qena	3	–
P259	A:3	Qena	3	–
P175	A	Sohag	2	2
P185	A:1	Sohag	1	1
P184	A:1	Sohag	1	1
P214	A:1	Aswan	1	–
P205	D	Aswan	3	–

The isolates were collected from cases of fowl cholera in chickens on various provinces around upper Egypt (Qena, Sohag and Aswan provinces). All of these isolates were previously characterized using carbohydrate fermentation profiles, serology and PCR typing[10]. These strains were stored at -80°C in 80% (v/v) glycerol in brain heart infusion broth until further use, and before used they were cultivated on tryptic soy yeast extract (TSYE) agar, supplemented with 5% sheep blood, and incubated for 18 h at 37°C and 7% CO_2 .

2.2. SDS–PAGE analysis for whole cell protein

2.2.1. Preparation of isolates for protein extraction

Whole cell lysates were prepared by a method adapted from Lammeli[11]. Briefly, a colony was grown in trypticase soy broth for 24 h at 37°C ; cells were harvested by centrifugation (3000 r/min for 5 min) at room temperature, washed at least three times with, and resuspend in, 10 mL of phosphate buffer solution (0.9 g NaCl, 0.02 g KCl, 0.02 g KH_2PO_4 , 0.29 g NaH_2PO_4 , distilled water to 100 mL, pH 7.2).

2.2.2. Gel Electrophoresis

Cells from 1.5 mL of the washed culture were harvested in a microfuge tube, resuspended in 100 μL of single strength SDS–PAGE sample lysis buffer (62.5 mmol/L Tris–HCl, 2% SDS,

6% 2-mercaptoethanol, 10% glycerol, 0.1% bromophenol blue, pH 6.8) and heated at 100 °C for 10 min. The lysed samples were then centrifuged (5000 r/min for 10min) at room temperature, and 10 µL of the filtrates were loaded on a 4% polyacrylamide (in a low voltage of 50 V) and separated with 12.5% polyacrylamide and electrophoresed using Tris–Glycine–SDS buffer system (25 mmol/L tris, 192 mmol/L glycine, 0.1% SDS, pH 8.3).

The gels were stained with 0.1% (w/v) coomassie brilliant blue R-250 (Merck, India) in 45.4% (v/v) methanol and 9.2% (v/v) glacial acetic acid and destained with 5% (v/v) methanol and 7% (v/v) glacial acetic acid until clear background was obtained. Approximate molecular weights were determined by comparing mobility pattern of samples with that of molecular weight standard marker.

2.3. RAPD analysis

DNA for PCR analysis was prepared by a boiling procedure. Bacteria from overnight 5 mL cultures were suspended in 200 µL deionized water and frozen at –20 °C for 10 min. Afterwards the suspension was heated at 98 °C in a water bath for another 10 min and then centrifuged for 10 min at 12000 r/min. The supernatant was transferred into a clean 0.5 mL Eppendorf tube and stored at –20 °C until used.

PCR was carried out in a final reaction volume of 50 µL using 0.2 mL thin wall PCR tube with arbitrary short primer (Bioneer, Daejeon 306–220, Korea) containing 10 nucleotides (5′–CGT GGG GCC T–3′).

Reactions were carried out in a thermal cycler PCR system (Techne cyclogene) under the following conditions: an initial denaturation for 10 min at 94 °C; 35 cycles for 3 min at 35 °C, 1 min at 72 °C and 30 second at 94 °C; followed by a final extension of 10 min at 72 °C. Following PCR termination, 10 µL of the reaction mixture was analyzed by electrophoresis in a 2% agarose gel containing 500 ng/mL ethidium bromide. After the electrophoresis run was complete, the gel was analyzed using SynGene program (Gene tools: product version 4.00: File version: 4.00.00 Cambridge, England).

3. Results

3.1. Analysis of *P. multocida* isolates using SDS–PAGE

A comparative analysis of WCP variation in 21 *P. multocida* strains of the different serotypes (A1, A3, A capsular group and D ones) has been undertaken. Visual inspection of the protein profiles showed the presence of 8 to 13 clearly visible protein bands with different molecular weights ranged from 33.6 to 102.5 kDa (Figure 1 A, B and C).

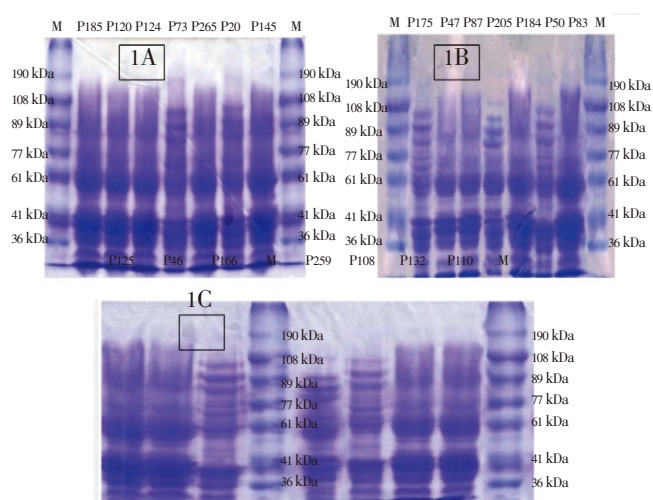


Figure 1. (A, B and C) Fingerprinting analysis using SDS–PAGE shows the electrophoretic pattern of *P. multocida* whole cell protein on the 12% polyacrylamide.

Whole cell protein profiling categorized 21 strains into three profiles (Figure 2) and the majority of isolates, 13 strains capsular type A were clustered in group I. We could not find more distinct patterns serogroup specificity in the other two clusters; cluster 2 contained three serogroup A isolates (P73, P108 and P175) and two strains of serogroup D (P50 and P1166) as well as cluster 3 having 2 isolates (P20 and P259) belonged to serotype A and one strain (P205) belonged to the D capsular serotypes of *P. multocida* (Table 1 and Figure 2).

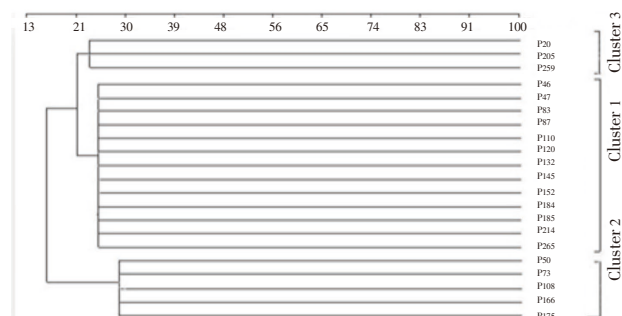


Figure 2. Dendrogram grouping based on the SDS–PAGE WCP lysate profiles.

3.2. DNA fingerprinting of avian isolates of *P. multocida* using RAPD–PCR

For a more general level of genetic analysis of for the 14 examined *P. multocida* isolates, RAPD–PCR was conducted. Clear generated DNA fragment patterns from 8 to 12 bands were observed (Figure 3) which varied in molecular weight from 150 bp to 4000 bp. The banding pattern revealed that three major clusters were observed illustrating genetic relationships among the isolates (Figure 4). Cluster I was the largest comprising of two subclusters A1 and A2 followed by cluster B comprising of three miniclusters B1, B2 and B3 then C (Figure 4).

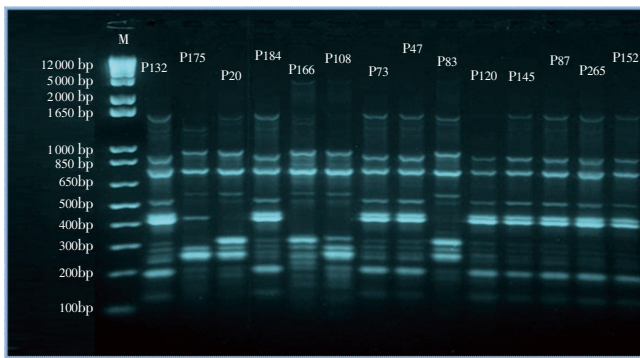


Figure 3. RAPD-PCR fingerprinting profiles of *P. multocida* strains. Lane M: 100 bp DNA ladder plus marker; Lanes 1 to 14: strains from fowl cholera cases.

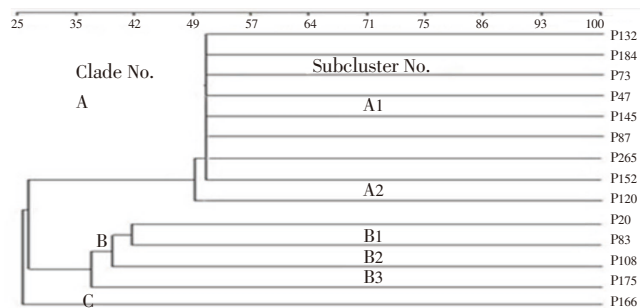


Figure 4. UPGMA dendrogram of genetic relatedness of 14 *P. multocida* isolates recovered from backyard chickens using RAPD-PCR analysis.

There appeared to be a genotypic relationship as defined by RAPD analysis and capsular serogroups that cluster A included *P. multocida* type A as well cluster B included most of the A serotype except one isolate belonged to serogroup D and cluster C contained one strains belonged to serogroup D (Table 1 and Figure 4).

Also the analysis of results indicated the superiority of RAPD-PCR in discrimination than SDS-PAGE which generated two subclusters A1 and A2 and three subclusters B1, B2 and B3 within cluster A and B respectively (Figure 3).

4. Discussion

The epidemiology of fowl cholera outbreaks is complex[2]. Traditional methods are only of limited use in studies of such outbreaks especially where with an involvement of multiple strains of *P. multocida*. This study was undertaken to examine the diversity of south valley *P. multocida* isolates originating from backyard chickens in different locations using two different typing methods (SDS-PAGE and RAPD-PCR).

In the present study, patterns generated by RAPD and SDS-PAGE exhibited little heterogeneity of avian strains of *P. multocida* as indicated by the presence of 3 clusters upon using both. We hypothesize that this pattern resulted from genetic drift leads to divergence of the *P. multocida* isolates cultured from live birds[12]. The association of a small number of RAPD and SDS-PAGE types with the majority of the examined strains suggests that a relatively small number of virulent groups (or clones) are responsible for cases of infection, which is in agreement with that mentioned by Selander and Musser

who noticed in many pathogenic bacteria species[13], the majority of cases of infectious disease are often caused by a small proportion of the total number of extant clones.

Other potential contributor to a little bacterial diversity obtained in this study is the low number of examined chickens as shown by Hotchkiss *et al*[14], no vaccination pressure and little trials for treatment. However, the lack of heterogeneity among the avian population is surprising, particularly in view of the variety that *P. multocida* isolated indicates that outbreaks of fowl cholera in this study are perhaps perpetuated by a stable *P. multocida* genotype. As shown before, the RAPD-PCR is more discriminatory than SDS-PAGE which gives three clusters with five mini clusters within cluster A and B in contrast to three clusters with SDS-PAGE.

The SDS-PAGE, being a stringent and labour-intensive typing method, in contrast to RAPD-PCR offers the considerable practical advantage that it requires no high-quality DNA preparations[15]. Moreover, rapid applicability to bacterial culture lysate/colonies makes it a simpler and rapid method to employ before detailed further investigation of epidemiology[16]. Moreover, many workers have also reported that the use of RAPD-PCR assay is a fast and resource efficient method of classifying and differentiating individual strains on the basis of small genomic differences with high specificity[17].

The present study showed the relationship between RAPD patterns and capsular groups (Table 1 and Figure 4), in spite of their origins of isolation that may be due to the birds movement between markets that resulted in transmission of *P. multocida* strains which could be the probable reason for this phenomenon[1]. Also, our results support the data reported by Dziva *et al.* who observed a relationship between RAPD clustering and capsular groups[17].

Our findings are in contrary to the high degree of heterogeneity among avian *P. multocida* isolates that was demonstrated by Jabbari *et al.* who observed a high degree of heterogeneity among avian *P. multocida* isolates as demonstrated by repetitive extragenic palindrome polymerase chain reaction[18].

In conclusion, we have shown that little diversity exists among *P. multocida* isolates originating from backyard chickens in the South Valley provinces (Qena, Sohag and Aswan provinces) indicates that outbreaks of fowl cholera in this study are perhaps perpetuated by a stable *P. multocida* genotype[19]. In the event of vaccine production our knowledge of the molecular basis of this diversity in *P. multocida* will lead to a better understanding to the development of improved vaccines and disease transmission throughout the entire nation.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

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Comments

Background

This study deals with important bacteria, *P. multocida* that represent an economic burden in many parts of the world to chickens as well as the use of robust technique in molecular epidemiology of this microbe.

Research frontiers

From my viewpoint, this is considered as a good study for investigating the molecular epidemiology of this pathogen (*P. multocida*) in the poultry in Upper Egypt, Egypt.

Related reports

Number of researcher studied the molecular characterization of *P. multocida* in chickens. Markam SK., Khokhar RS, Kapoor S and Kadian SK also conducted the research entitled “Molecular characterization of field isolates of *Pasteurella multocida* by polymerase chain reaction in the state of Haryana”.

Innovations and breakthroughs

The results of this study revealed the little heterogeneity among *P. multocida* isolates. That lead to easier methods for prevention and control especially during vaccine application.

Applications

The data in the article is helpful for the effective planning and implementation of control and prevention strategies for control of this disease. The article shows that backyard poultry is a vehicle of bacterial transfer and may play an important role in diseases prevalence. This study supports and suggests the use of RAPD–PCR for rapid determination of microbe clonality especially when need vaccine manufacturing.

Peer review

The study is up-to-date and an interesting valuable research work in which authors have demonstrated the clonality of bacteria isolated from backyard chickens that improve the measures for prevention especially for making vaccines and even in treatment.

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