Differentiation between virulent and avirulent strains of Pasteurella multocida using restriction endonuclease analysis

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In this study we used one reference Climson University (CU) strain and 3 virulent field strains isolated from naturally infected chicken and identified as *P. Multocida*. DNA was extracted from all strains and subjected to restriction endonuclease analysis, using EcoRI, HpaII and Hind III revealing that, great similarity between either the reference or local virulent field isolates. The obtained results indicated that the most differentiable restriction endonuclease enzyme was the H*ind* III, which showed different band patterns between different strains.

Avian Pasteurellosis is the most important bacterial disease that affecting poultry breeding in Egypt (Gergis, 1987).

This disease is caused by *P. multocida* organism, which has been historically distinguished by somatic and capsular serotyping (Rhoads and Rimler, 1990), morphology, nutritional requirements, fermentation characteristics and antibiotics sensitivity profiles (Blackall *et al.,* 1995).

The molecular methods characterizing the genomic structure of P. multocida strains have been provided relatedness between strains (Townsend *et al.,* 1997). Recently sensitive modern techniques have been used to study the heterogenicity in closely related organisms (Welsh *et al.,* 1991).

Despite the vaccination programs, avian cholera outbreaks have been identified and continue to occur (Jonas *et al.,* 2001).

Hence the objective of this study was to use restriction endonuclease analysis (REA) for discriminating isolates of *P. multocida* in poultry farming in Egypt and to create a reference database that can be used for epidemiological studies.

Material and Methods

Strains. Three virulent local *P. multocida* isolates recovered from fowl cholera naturally infected chickens. The isolates were identified according to (Carter, 1955 and Heddleston, 1971) using standard reference antisera obtained from National Animal Disease Center, Ames, Iowa, USA. An avirulent strain, Climson University mutant strain obtained from (Cu) (CLEVB Reference Culture Bank).

The strains were streaked for purity on blood agar, incubated at 37°C overnight and the culture was grown in brain heart infusion broth according to (Carter, 1984).

Genomic DNA extraction. (Dancla *et al.,* 1996): Broth culture from each *P. Multocida* strain was grown in brain heart infusion broth (24 hrs / 37°C) then centrifuged at 6,000 RPM for 20 min. at 4°C. Cell pellets were suspended in 567 µl of TE buffer (tris 10 mM, EDTA 1 mM, pH 7.5). 30 µl of 10% SDS and 3 µl of proteinase K enzyme (20 mg/ml) were added and incubated at 37°C for one hour then mixed well with 100 µl of 5 M NaCl and 80µl of CTAB (20 mM EDTA, 10 mM tris HCl, pH 8.0; 1.4 M NaCl, 2% cetyle-tri-methyl ammonium bromide and 0.2 % 2-mercaptoethanol). The mixtures were incubated at 65°C for 10 min. The lysates were extracted once with phenol: chloroform: isoamyl alcohol (25: 24: 1; v/v/v), and once with chloroform isoamyl alcohol (24: 1 v/v). The aqueous phase was mixed with 0.6 volume of isopropanol and incubated at –20°C for 30 min. The DNA precipitate was pelleted out, rinsed in 70 % ethanol, air dried and dissolved in 50µl of TE buffer (pH 8.0).

Restriction endonuclease digestion. Three enzymes were used in this study, HpaII, EcoRI and HindIII (Takara Bio. Inc, Japan). These enzymes were used as described by Aye et al. (2001); Jonas et al. (2001) and Rubies et al. (2002). The digestion reaction with each of three restriction endonuclease was carried out as recommended by the manufacture.

Agarose gel electrophoresis. The digested DNA fragments were analyzed on an agarose gel

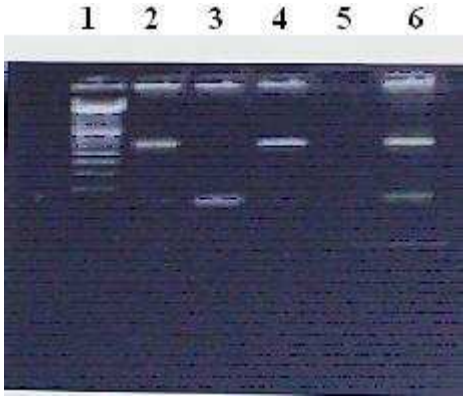

Fig. (1): Gel Electrophoretic analysis of *P. multocida* strains DNA digested with EcoRI restriction enzyme. Lane (1): standard DNA marker, Lane (2): Cu, Lane (3): field isolate (1), Lane (4): field isolate (2) and Lane (5): field isolate (3).

Fig. (2) Gel Electrophoretic analysis of *P. multocida* strains DNA digested with H*pa*II restriction enzyme. Lane (1): standard DNA marker, Lane (2): Cu, Lane (3): field isolate (1), Lane (4): field isolate (2) and Lane (5): field isolate (3).

Fig. (3) Gel Electrophoretic analysis of *P. multocida* strains DNA digested with H*ind*III restriction enzyme. Lane (1): standard DNA marker, Lane (2): Cu, Lane (3): field isolate (1), Lane (4): field isolate (2) and Lane (5): field isolate (3).

0.8 % in TEA (1x) buffer using 1kb DNA ladder (Takara, Bio. Inc., Japan) as standard marker. The gels were stained with ethidium bromide (0.5µg/ml), then visualized under ultraviolet light and photographed (Sambrook *et al.,* 1989).

Results and Discussion

In this study one reference avirulent Cu strain and 3 locally isolated and identified strains were used.

DNA was extracted from the isolated and identified local strains and reference Cu strain of *P. multocida* then subjected to restriction digest with EcoRI, HpaII and HindIII restriction endonuclease to study the heterogenicity in closely related organisms. This method detects the similarity of the DNA after exposure to digestion with restriction enzymes. In the present study the DNA cleavage patterns showed great similarity with all used restriction enzymes. These results are confirmed by (Dancla *et al.,* 1996) who used 40 *P. multocida* strains and reported that all these strains showed more than 90 % similarity. EcoRI restriction enzyme digestion (Fig. 1) identified 3- 4 restriction fragments while HpaII restriction enzyme digestion (Fig. 2) showed the same similarity in patterns except in one band that was not clear in local isolate No. 1.

These results coordinated with that obtained by (Aye *et al.,* 2001) who stated that EcoRI, HpaII, and HindIII restriction enzyme digestions identified three, five and five restriction fragments length polymorphism profiles respectively in *P. multocida* recovered from turkeys outbreaks in Ohio. Rubies *et al.,* (2002) also stated that only 2-3 restriction endonuclease analysis patterns were detected on using HpaII with one clearly prominent pattern.

Concerning the DNA restriction endonuclease pattern obtained by using H*ind*III (Fig. 3), it was the best reliable and demons-trated to more extent either the differences or similarity between the reference and local isolates. It revealed seven restriction patterns with Cu reference strain coordinated with the field isolate (1) except one band at 600 bp while the 2nd field isolate differed in one band at 8500 pb and showed absence of one band at 2500 pb. The 3rd field isolate showed the difference in the presence of one band at 8500 pb and the absence of 2 bands at 2200 pb and 3500 pb.

These results correlated with that obtained by (Jonas *et al.,* 2001) as they demonstrated seven distinct DNA profiles from digestion with the restriction endonuclease Hind III.

The obtained results demonstrated that the restriction endonuclease analysis of DNA of *P. multocida* isolates from chickens showed great similarity between the different isolates, referred to limited number of restriction patterns differences. The exchange of clones of *P. multocida* between avian species rarely happens under normal conditions. It could be concluded that the majority of the *P. multocida* strains distributed across multiple farms and across multiple areas are closely related and can be easily controlled by restricted program including either vaccination or medical interference.

On conclusion, restriction endonuclease analysis for discriminating isolates of *P. multocida* isolated from poultry farms in Egypt can be used.

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