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Polymerase chain reaction for differentiation of Pasteurella multocida isolates from turkeys in comparison to strains incorporating in fowl cholera vaccine

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In the present study, polymerase chain reaction (PCR) using random primer (E-20) was used to characterize and identify strains included in this study. Strains included 4 vaccinal reference strains of *Pasteurella multocida*, CU strain and 4 field isolates of *Pasteurella multocida* isolated from diseased turkeys which were identified biochemically and serologically as A:1, A:3, A3x4 and D:11. The obtained results revealed that all strains were reacted positively and in different manner with the E20 primer except the 2 field isolates. The results of these reactions demonstrated in terms of bands of different molecular weight specific to each strain. This can be used as a base for characterization and differentiation of strains involved in the present study as the 2 field strains A:1 and A:3 react with primer. Mouse protection test was performed by vaccination of mice with local fowl cholera oil adjuvant vaccine then challenge with virulent field strains A:1, A:3, D:12 and untypable isolates. Results revealed that the local fowl cholera adjuvant vaccine could protect mice against virulent challenge with A:1, A:3 and D:12 field strains but it could not be protect mice against untypable isolates.

Pasteurella multocida is an important veterinary and opportunistic human pathogen. (Hunt *et al*., 2000). In poultry it produces fowl cholera, a major bacterial disease that is manifested by either an acute septicemia or chronic respiratory infection (Frame *et al*., 1994). High morbidity and mortality rates may result in turkeys, chickens, pheasants and water fowl following infection with certain strain of this bacterium. Many turkey growers in Egypt suffered from epornitics of fowl cholera in their flocks in consecutive years, and it is supposed that this disease is indigenous to such farms (Souror, 1999).

Vaccines such as bacterins have been developed to prevent and control disease in poultry flock. It was found that *P. multocida* bacterin dose not always induce cross-protection (Confer, 1993).This raises the attention to strains with multiple antigenic factors and the possible implication of their presence in the preparation and use of vaccines. Accordingly, the bacterial somatic serotypes present in different farms should be determined to ensure that such serotype(s) is subsequently used in culture bacterin (Curtis, 1979 and Mushin, 1979).

A:1, A:3, A:4 and A:3,4 are present in most commercial bacterins (Glison, 1996). Characterization of Egyptian *P. multocida* turkey isolates is necessary in order to improve the efficacy of vaccine used in Egypt. The principal method for characterization or differentiation of *P. multocida* isolates is a serotyping based on a somatic antigen differentiation as initially described by Heddleston *et al*. (1972). Hence, the objective of this study was to characterize Egyptian *P. multocida* turkey isolates via capsular biotyping, serogrouping, somatic typing and genetic analysis using polymerase chain reaction (PCR) and evaluating potency of fowl cholera oil adjuvant vaccine against these isolates in lab mice.

Materials and Methods

Bacterial strains.

Vaccinal strain. Four vaccinal bacterial strains of *P. multocida* serotypes (A:1, 3, 3x4 and D:11) were used for vaccine preparation.

Live attenuated CU vaccine. Naturally avirulent CU (Clemson University) vaccinal strain

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was used in comparison with turkey isolates. It was commercially purchased under the trade name of Avichol from Schering Cooperation , Omaha, Nebraska, 81185, USA.

Field turkey isolates. Four *P. multocida* isolates were isolated from outbreaks of turkey farms in the last 10 years. They were subjected to cultural, morphological and biochemical identification (Finegold and Martin, 1982; Koneman *et al*., 1992) as well as serological typing using indirect haemagglutination and agar gel precipitation tests. Pathogenicity of the isolates was also determined. *P.multocida* field isolates identity was confirmed on the basis of microscopical examination, colonial morphology, biochemical reactions, pathogenicity to mice and serological tests.

Capsular typing.

Preparation of capsular extract (K-antigen). Capsular extract was prepared according to Carter and Rappy (1962). A 18-24 h heavy seeded growth culture from each field strain of *P. multocida* were harvested from nutrient agar plates, suspended in 5ml normal saline solution and heated at 56°C for 30 min. in water bath to help extract of K-antigen. The treated suspended culture was centrifuged at 3000 rpm for 15 min. The supernatant was collected in sterile clean tubes and used as K-antigen in indirect haemagglutination test (Carter and Rappy, 1962).

Somatic typing. Somatic antigen was prepared following the method of Heddleston (1971). Breifly, 18-24 h heavy seeded growth culture of *P. multocida* was suspended in 1 ml of saline solution containing 0.3 ml standard solution of formaldehyde, heated in water bath at 100°C for 1h. The bacterial cells were sedimented by centrifugation, then the supernatants were collected and used as (O) antigen in the gel diffusion precipitation test (Hofacre and Glisson, 1986).

PCR Pattern.

Genomic DNA extraction. *P. multocida* genomic DNA was extracted as described by Bridge (1996). Briefly, a 1.5 ml quantity of culture from each *P. multocida* strain was centrifuged at 13000 rpm for 2 min. The cell pellets were resuspended in 567 µl of TE buffer (Tris 10 mM, EDTA 1 mM, pH 7.5), then 30 µl of 10% SDS and 3 µl of 20 mg/ml of proteinase-K enzyme were added and incubated at 37°C for 1h. The solution was then mixed after incubation with 100 µl of 5 M NaCl. The solution was then mixed with 80µl of CTAB (20 mM EDTA, 100 mM Tris-HCl, pH 8.0, 1.4 M NaCl, 20% cetyl-tris methyl ammonium bromide and 0.2% 2-mercaptoethanol) was added, mixed and incubated at 65°C for 10 min. The lysate was 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 minutes the precipitated DNA was pooled out, rinsed in 70% ethanol and dissolved in 50 µl of Tris-EDTA buffer (pH 8.0).

Arbitrary primer. DNA marker oligonucleotides primer were obtained from Operon Technologies Inc. (USA), for the amplification of RAPD sequence. E-20 5` AAC GGT GAC C -3`

RAPD amplification. PCR amplification reactions were prepared in a volume of 25µl containing 10x PCR buffer (500 mM MCl, 200 mM Tris, pH 8.4) 0.2mM of dNTP (deoxyribonucleotide triphosphates), 2.5 unit of Taq polymerase (Promega Corp., Modison, USA), 0.1 µM of primer, 1.5 mM $MgCl_2$ and 100 ng of purified DNA. The reaction was over laid with 10 µl of nuclease free light mineral oil (Sigma). The amplification was performed in a Perkin-Elmer cetus DNA thermal cycler programmed for 30 cycles of 1min. at 94°C, 1 min. at 35°C, 2min at 72°C for denaturation, annealing and primer extension, respectively and one cycle at 72°C for 7 min. for final extension.

Agar gel electrophoresis. The amplified product was analyzed on 2% agar gel and 0.5 µg of ethadium bromide per ml in 1 x Tris acetate EDTA (TAE) buffer using DNA marker 100 base pair (Cat # 1010, USA). The PCR product was then visualized using ultraviolet light and photographed.

Potency test. A total of 400 Swiss Albino mice weighing of about 18-20 g were used for potency test of fowl cholera vaccine (Aerobic Bacterial Dept., Veterinary Serum and Vaccine Research Institute, Abbasia, Cairo) against turkey field isolates for detection of LD_{50} of the examined strains (Ose and Muenster, 1968).

Results and Discussion

The obtained field *P. multocida* strains identity were confirmed on the basis of growth in broth, microscopical examination, colonial morphology, biochemical reactions, pathogenicity to mice and serological tests.

Serotype of these strains were found to be belonging to capsular and somatic type A:1, A:3 and D:12 and one untypable isolate.

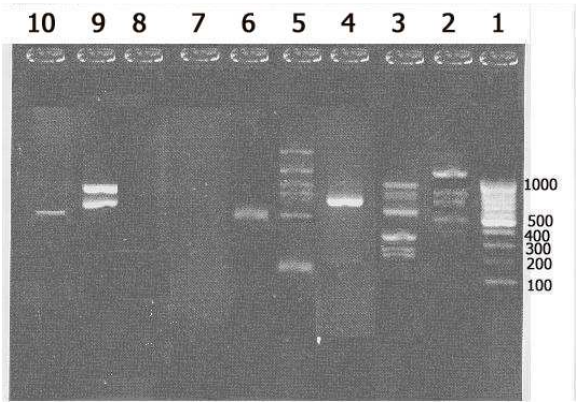

Fig. (1): DNA banding pattern following amplification with primer E2O

Lane (1): Marker, Lane (2): A:3 (vaccinal strain), Lane (3): D:11 (vaccinal strain), Lane (4): A:3 (field strain), Lane (5): A:1 (vaccinal strain) Lane (6): A:1 (field strain), Lane (7): D:12, Lane (8): Untypable field strain, Lane (9): A3x4 (vaccinal strain),Lane (10): CU.

Avian cholera produces great economic losses in poultry industry allover the world (Carpenter *et al*., 1988; Morris and Fletcher, 1988). Despite extensive vaccination, fowl cholera outbreaks continue to occur in commercial poultry industry (Aye *et al*., 2000). PCR technology can be applied to distinguish among *P. multocida* strain.

Studies directed towards the distribution and copy number of prokaryotic repetitive sequences have led to the development of a method that generates specific DNA fingerprints by repetitive sequence based primers in the polymerase chain reaction (PCR) (Versalovic *et al*., 1991).

A sensitive polymerase chain reaction (PCR) based on the method of DNA fingerprinting, called the randomly amplified polymorphic DNA (RAPD), has been used to study heterogenicity in closely related organisms. This method detects differences in the DNA sequence at sites in the genome that are defined by the primers used. Sequence variation is revealed by the number and length of amplified products, which may be phelogenitically conserved (Caetane-Anolles *et al*., 1991). This method is advantageous for strain or isolate identification (Fan *et al*., 1995). Figure (1) showed the assay of bands resulting from segment amplification with the used primer (E20) which amplify segments of *P. multocida* genomic DNA.

Seven reproducible DNA profiles were produced by the arbitrary primer E20. The primer could not react with the sequence of untypable strain and D:12 (lane 7, 8). This study has shown that *P. multocida* field isolates of turkeys A:1 and A:3 (lane 6, 4) are more genetically similar to both A:1 and A:3x4 vaccinal strains (lane 3, 9) used in fowl cholera oil adjuvant vaccine, as A:1 field isolate shared a common band with A:1 and A:3 vaccinal strain, so the vaccinal strain could protect mice against virulent challenge with field isolate A:1 with a log protection 2.2 (Table 1), same results observed in A:3 field isolate shared with A:3x4 vaccinal strain in one band and mice protected against virulent challenge with A:3 field isolate with log protection 2.4, while there were no similarities between the vaccinal strain A:1 and D:11 vaccinal strains which showed no identity, this result was in agreement of the finding of Abbas *et al*. (2000) who used primers OP-G4 and OP-E3 for discrimination and verification of Pasteurella strains. CU and A:3 vaccinal strains shared in a common DNA band. Vaccinal D:11 strain showed 6 unique bands differ than other P. multocida vaccinal strains. This explains the efficacy for including type D strain improved fowl cholera in Egyptian vaccine (Azzam *et al*., 1992). Even though there was a significant association between the serotype and genetic profile (Aye *et al*., 2000).

Each 4 vaccinal strain used in fowl cholera oil adjuvant vaccine A:1, A:3, A:3x4 and D:11 (lanes 3, 2, 9, 5) showed distinct genetic differences indicating district relationship between genetic differences and antigenic composition. This indicating the importance for including of these vaccinal strains in fowl cholera vaccine formula.

Both untypable and D:12 can not be identified by this method. Same results could be observed by Aye *et al*. (2000) as they stated that isolates belonging to capsular type B, E and untypable capsule can not be identified by the use of restriction endonuclease as the enzyme do not polymerize them, but the locally prepared fowl cholera oil adjuvant vaccine could protect mice against virulent challenge with D:12 field isolate, but it could not protect mice against challenge untypable isolate.

Results shown in table (1) indicated that the locally prepared fowl cholera oil adjuvant vaccine could protect mice against virulent challenge with 3 field isolates (A:1, A:3 and D:12) as log protection were 2.2, 2.4 and 2.6, respectively, while untypable strain could not protect mice against virulent challenge as log protection was 0.3. From these results it could be recommended that addition of untypable isolate to the local fowl cholera oil adjuvant vaccine is

Table (1): Comparative log protection post challenge in mice vaccinated with fowl cholera oil adjuvant vaccine expressed in log_{10} protection.

LD_{50} after challenge	Vaccinated mice challenged with field strains			
	Strain A:1	Strain A:3	Strain D:12	Untypable isolate
Vaccinated	$10^{-7.8}$	$10^{-7.6}$	$10^{-6.9}$	$10^{-10.5}$
Control	10^{-10}	10^{-10}	$10^{-9.5}$	$10^{-10.2}$
Log protection	2.2 **	2.4 **	2.6 **	0.3 *

* Not protective
** Protective

necessary.

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تطبيق تفاعل البلمرة لتحديد بصمة عترات الباستريلا ملتوسيدا المعزولة من الرومى بالمقارنة بالعترات الموجودة فى لقاح كوليرا الطيور

تم توصيف وتعريف العترات المختلفة التى تضمنتها هذه الدراسة باستخدام اختبار تفاعل البلمرة المتسلسل وذلك باستخدام بادئ عشوائى E20 وعترات الباستريلا ملتوسيدا المرجعية التحصينية A:1, A:3, A3x4, D:11, CU و ٤ عترات باستريلا ملتوسيدا معزولة من دجاج رومى مصابة بالمرض وهى معرفة سيرولوجياً وبيوكيميائياً كعترات A:1, A:3, D:12, untypable. وجد أن البادئ الجينى تفاعل إيجابياً مع كل العترات التى تضمنتها هذه الدراسة ماعدا العترتين untypable, D:12 المعزولتين من الحقل البيطرى وكان ناتج التفاعل أوزان مختلفة من شظايا الحامض النووى خاصة بكل عترة يعتمد عليها فى التفريق بين العترات المختلفة.

تم إجراء اختبار الحماية النشطة فى الفئران التى تم تحصينها باستخدام اللقاح المحلى المحضر من عترات A:1, A:3, A3x4 and D:11. وتم إجراء اختبار التحدى فى هذه الفئران باستخدام ٤ عترات معزولة من دجاج رومى مصاب وهذه العترات معرفة سيرولوجياً وبيوكيميائياً وعترة أخرى غير معرفة سيرولوجياً وقد تم استخدام كل عترة على حدة. وقد وجد أن اللقاح المحلى نجح فى حماية الفئران من العدوى من العترات الضارية وهى A:1, A:3, D12.