Bacteriological And Molecular Characterization Of *Pasteurella Multocida Isolated* From Rabbits By PCR Based Techniques

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ABSTRACT

In this study a total of 428 samples were collected from apparently healthy, clinically diseased and recently died rabbits of different age and sex from different farms scattered at different localities in Kafr El-Sheikh Governorate to determine incidence of *Pasteurella multocida* in rabbits.

Out of 428 samples examined 55 (12.9%) isolates of *Pasteurella multocida* were recovered. They including 112 nasal swabs from apparently healthy rabbits yielded 6 (5.3%) isolates and 316 samples from diseased rabbits yield 49 (15.5%) isolates. These samples including 278 nasal swabs yielding 42 (15%) isolates, 34 lung samples yield 3 (8.8%) isolates, one liver specimen yielded one isolate, one isolate from one endometrium specimen, two isolates from two subcutaneous abscesses swabs.

The isolated strains were identified on the basis of traditional phenotypic procedures as colonial morphology, microscopical examination, biochemical reaction, pathogenicity to mice and serological identification.

The serotyping of *Pasteurella multocida* revealed that 32 (58%) and 16 (29%) isolates out of 55 isolates have capsular group A and capsular group D respectively meanwhile somatic types were 9, 5 and 2. Only 7 (12.7%) isolates were untyped.

The sensitivity and specificity of a *Pasteurella multocida* species specific primer was evaluated through a PCR based assay. All examined *Pasteurella multocida* isolates gave a single amplified product of the expected size (460 pb).

Random amplified polymorphic DNA (RAPD). PCR has been used for characterization of *Pasteurella multocida* isolates recovered from rabbits. An unique banding pattern for individual serotypes was obtained where analysis of the obtained fingertyping results was performed between isolates from the same farm, those from different localities and finally between vaccinal strains and field strains.

INTRODUCTION

The deleterious effect of pasteurellosis in rabbits still remains a common and serious problem for rabbit breeders and researchers. Mucopurulent rhinitis or "snuffles" is by far the major clinical manifestation of chronic pasteurellosis, while more serious forms of the disease include pneumonia, otitis media, septicemia, meningitis and localized abscesses ***(1,2)***. *Pasteurella multocida* the causative organism responsible of these various clinical manifestations of the disease, was estimated to be the most common bacterial pathogen of rabbits ***(3)***.

Until a short period of time since its first isolation in 1881, identification and characterization of *Pasteurella multocida* have depended on its phenotypic characteristics such as morphology, biochemical and serological typing ***(4)***. This later identified *Pasteurella multocida* serovars as a five capsular types designated A, B, D, E and F and 16 somatic types on the basis of antigenic differences in their lipopolysaccharides ***(5,6)***. Because of the antigenic complexity of *P. multocida*, its serotyping was encountered by short falls involving the occurrence of either untypable isolates or those expressing multiple major somatic antigen ***(7)***.

Although serologic typing was useful and accepted in the presumptive diagnosis of *P. multocida* it provided insufficient information for epidemiological studies for which the ability to differentiate phenotypically similar isolates is of critically important. A need for

improved methods of distinguishing various *Pasteurella multocida* strains for epidemiologic analysis and disease surveillance was intensified ***(8)***. Genotyping techniques based upon their high discriminatory power in characterization and differentiation between isolates of the same serotype have been proposed to overcome the uncertainties of phenotypic characterization and to improve the knowledge of a given organism and its epidemiology.

During the last decade the application of new PCR-based techniques has had a revolutionary impact either on the molecular detection or on the characterization of an infectious agent and thus has improved our knowledge of an organism and its epidemiology ***(9)***. Also PCR-Based techniques used for detection of virulant genotype of *Pasteurella multocida* strains isolated from different hosts with various disease status ***(10)***. Rapid polymerase chain reaction (PCR), fingerprinting technique was evaluated for discriminating among 33 *Pasteurella multocida* isolates from rabbits with clinical pasteurellosis and it was proved that it is an efficient and reproducible method for discrimination of *Pasteurella multocida* isolated from rabbit ***(2)***.

Random amplified polymorphic DNA (RAPD) assay was used to determine genetic differences among a total of 46 *Pasteurella multocida* strains obtained from lungs of cattle, sheep and goats. It's result was distinct band profiles among these strains and little genetic heterogencity exists among *Pasteurella multocida* isolated from cattle and sheep ***(11)***.

On the enlightenment of all the above, the present study was planned with the following prospective. Investigation the prevalence of *Pasteurella multocida* among apparently and clinically diseased rabbits, phenotypic characterization of the isolated strains by conventional methods (biochemical and serological typing) and validation the use of recent PCR-based techniques in molecular identification of *Pasteurella multocida* by a specific primer and application of random amplified polymorphic DNA assay as valuable genotypic method for molecular characterization and epidemiological investigation of *Pasteurella multocida* in rabbits.

MATERIAL AND METHODS

A total of 428 samples were collected from healthy as well as clinically diseased and recently dead rabbits from both governmental and private farms in different localities at Kafr El-Sheikh governorate. The number of samples, locatities and sources are shown in Tables 1 and 2.

Table 1. Samples collected from apparently healthy rabbits in different localities at Kafr El-Sheikh Governorate

Locality	Number of sample	Type of sample
I. Governmental farms		
Sakha (Animals Production Research Institute)	15	Nasal swab
Faculty of Agriculture Farm	14	
II. Private farms		
El-Maraska	31	Nasal swab
Kafr El-Sheikh	14	
Desouq	15	
Sedi-Ghazy	23	
Total	112	

Table 2. Samples collected from diseased rabbits in different localities in Kafr El-Sheikh Govrnorate.

Locality	Types of samples	No. of samples	Symptoms
I. Governmental farms			
Sakha Animal Production	Nasal swabs	26	Rhinitis
Research Institute	Lung tissues	20	congested lung
Fac. Agric. Farm, Kafr El-Sheikh	Nasal swabs	10	Purulent exudate from nose
	lung tissues	12	Congested lungs
Experimental Farm	Liver tissues	1	Congestion
II. Private farms			
El-Hamoul	Nasal swabs	94	Rhinitis
	Endometrial swab	1	Yellowish thick pus from vagina
Bialla	Nasal swabs Lung tissues	30 2	Rhinitis, emaciation consolidation
Sedi-Salem	Nasal swabs	50	Severe rhinitis
	Subcutaneous abscess swab	1	Abscess + pus
Desouq	Nasal swabs	46	Rhinitis
	Abscess swab	1	Abscess + pus
Ariamon	Nasal swabs	22	Rhinitis
Total		316	

Swabs samples were directly inoculated onto blood agar, tryptose agar and MacConkey's agar plate media then incubated aerobically at 37°C for 24-48 hours. The surface pneumonic lung tissues and liver were seased by red hot spatula for sterilization and grinding in sterile mortar with sterile tryptose soya broth and a dropfull of grinded solution was cultured on sheep blood agar and MacConkey's agar incubated at 37°C for 24 hours.

Smears from isolates were stained with Gram's method then microscopically examined. The suspected colonies were picked up and subjected to traditional methods of identification by morphological and biochemical criteria ***(12)***, pathogenicity test ***(13,14)***. Genotypic characterizations were determined by both polymerase chain reaction ***(15- 18)*** and random amplified polymorphic DNA (RAPD) analysis ***(9)***.

For PCR technique our strains (Kafr El-Sheikh strains) were compared with different previously isolated (5 strains) by veterinary serum and vaccine, vaccinal strains field strains and strain isolated from Sharkia, as well as DNA marker (100 bp marker).

Chemicals, reagents and buffers used in molecular studies of *Pasteurella multocida* *(15-18)*:

Reagents:

Tris-HCl buffer, one mm EDTA, Tris EDTA buffer. SDS (sodium dodecyl sulfate) solution. Proteinase K solution (Sigma), 5 M NaCl, Buffer saturated phenol, chloroform /isoamyl alcohol (sigma), Phenol chloroform/isoamyl alcohol, hexadecyle trimethyl ammonium bromide 10%, Sodium chloride 0.7, isopropanol (sigma), 70% ethanol, Tris acetate EDTA (TAD) Electrophoresis buffer (50xstock), thedium bromide solution (stock solution), gel loading buffer, RAPD marker, super ladder DW 100pb obtained from Abgene, UK, single primer used in RAPD assay. Code D14803, primer sequence (5`-3`) 5-AAACGGTTGGGTGAG-3':

Specific primer for *Pasteurella multocidia*

-Forward primer

Cde KMTLsP 6 sequence 5'GCTGTAAACGAACTCGCCAC.3`.

-Reverse primer:

Code KMTit7 5'ATCCGCTATTTACCEAGTGG3' obtained from MWG Biotech. AG, Berlin, Germany.

These primers were synthesized depending on the unique sequence specific to type B:2 *Pasteurella muloticida* isolates ***(18)***.

- Agarose gel (Sigma)

Chemical used in PCR reaction

Shown in Table 3, while those used in RPD reaction shown in Table 4.

Table 3. Chemical used in one specific PCR reaction

Components	Master mix content (µl)
10x buffer (Promega)	5
dNTP	1
Forward primer	0.5
Reverse primer	0.5
Taq. DNA polymerase (Promega)	1
Template DNA	10
Sterile D.D. water	32
Total volume of each master mix.	50

Table 4. Chemical used in RAPD-PCR reaction

Components	Master mix content (µl)
10x PCR buffer (Promega)	5
dNTP (Promega)	2
D14803 Primer	1
Taq. DNA polymerase (Promega)	2
Template DNA	10
Sterile D.D. water	30
Total volume of each master mix.	50

-Extraction of Pasteurella multocida genomic DNA (8,17)

Preparation of specific PCR reaction

-Specific PCR reaction of DNA from *Pasteurella multocida* was performed ***(18)*** with some modification. The reaction was carried out in a volume 50µl containing 10 µl of genomic DNA solution.

A master mixture for reaction was prepared in a 1.5 microcentrifuge so that each reaction contained.

Component Amount (µl) for one RAPD-PCR reaction

D.DH_2O	32
10 x buffer	5
dNTP	1
Primerl (forward)	0.5
Primer 2 (reverse)	0.5
Taq. polymerase	1
Template DNA solution	10
Total	50

The reaction mixture was over laid with 40 µl of light mineral oil. The mineral oil prevents evaporation of the reaction mixture during thermocycling.

Specific-PCR program and temperature profile

Amplification of the DNA was performed by placing the tubes containing the reaction in a MI research thermal cycler programmed to fulfill 31 cycles.

The temperature profile in the different cycles was: an initial denaturation cycle at 95°C for 4 min. This was followed by 30 cycles each comprising (1) denaturation step at 95°C for 1 min., (2) annealing step at 55°C for 1 min. and (3) extension step at 72°C for 1 min. A final extension cycle at 72°C for 9 minutes was carried out.

Random amplified polymorphic DNA (RAPD) analysis for *Pasteurella multocida* isolates

Extraction of *Pasteurella multocida* genomic DNA *(8,17)*

Preparation of RAPD PCR reactions

Random amplification of DNA for *Pasteurella multocida* was performed according to the method of ***(9)*** with some modification. The reaction were carried out in a volume of 50 µl containing 10 µl of genomic DNA solution.

A master mixture for reaction was prepared in a 1.5 microcentrifuge tube so that each reaction contained.

Component Amount (µl)for one RAPD-PCR reaction

D.DH_2O	30
10 x buffer	5
dNTP mix	2
Primer (single primer)	1
Taq polymerase	2
Template DNA solution	10
Total	50

The reaction mixture was over laid with 40 µl of light mineral oil.

RAPD-PCR program and temperature profile

Amplification of the DNA was performed by placing the tubes containing the reaction in a MI research thermal cycler programmed to fulfill 41 cycles as follows:

1. An initial denaturation cycle at 95°C for 3 min.
2. This was followed by 39 cycles each comprising a denaturation step at 95°C for 30 second, an annealing step at 27°C for 30 second and an extension step at 72°C for 60 second.
3. The final cycle was an elongation cycle performed at 72°C for 10 minutes.

Electrophoresis of PCR products

When the PCR program was terminated the

PCR products were analyzed by electrophoretic separation in a 2% agarose gel containing ethedium bromide (0.5 µg/ml) with 1 x buffer. Twelve µl of each PCR product were mixed with 3 µl loading buffer and loaded into the wells of the gel. The gels were run at 100 volt for 1 hour.

Visualization and photography

After electrophoresis the RAPD patterns and specific PCR patterns were visualized with U.V. and transilluminator the gels were photographed using Polaroid camera.

Computer analysis of RAPD-PCR results

Was done using Gel Pro Software, USA.

RESULTS

The bacteriological examination of 428 samples collected from apparently healthy, clinically diseased and recently dead rabbits revealed isolation of 55 (12.9%) isolates. They were 6 (5.3%) isolates from apparently healthy rabbits as shown in Table 5 and 49 (15.5%) isolates from diseased rabbits. They including 42 (15%) from nasal swabs, 3 (8.8%) from lungs specimens, one, two and one isolates were recovered from liver, subcutaneous abscesses and endometrium samples respectively (Table 6).

Morphological identification of the isolates showed rounded, grayish colonies with glistening surface and non haemolytic on blood agar. Microscopically they were Gram-negative cocobacilli. All examined isolates failed to grown on MacConkey's agar.

Biochemical activities of *Pasteurella multocida* isolates as shown in Table 7 revealed that all the examined strains were indole, catalase and oxidase positive, all were urea hydrolysis and citrate utilization test negative and all tested isolates produced acid on Triple sugar iron (TSI), yellow butt and yellow slant and no H_2S production (Hydrogen sulphide production).

Table 5. Prevalence of *Pasteurella multocida* from nasal swabs of apparently healthy rabbits in different localities at Kafr El-Sheikh Governorate

Locality		Number of examined samples		Number of positive cases		Incidence percentage	
		No.	Total	No.	Total	%	Total
I. Governmental farms	Sakha Animal Production Research Institute	15	29	2	4	13.3%	13.7%
	Faculty of Agriculture Farm	14		2		14.3%	
II. Private farms	El-Maraska	31	83	2	2	6.5%	2.4%
	Kafr El-Sheikh	14		0		0%	
	Desouq	15		0		0%	
	Sedi-Ghazy	23		0		0%	
Total		112			6	5.3%	

Table 6. Prevalence of *P. multocida* from diseased and dead rabbits in different localities in Kafr El-Sheikh Governorate

	Locality	No. of sample		Type of samples	No. of positive cases		Incidence percentage
I. Governmental farms	Sakha Animal Production Research Institute	26 20	46	Nasal swabs Lung tissues	2 1	3	6.5%
	Kafr El-Sheikh collage Agriculture Farm	10 12 1	23	Nasal swabs Lung tissues Liver tissues	1 1 1	3	13%
II. Private farms	El-Hamoul	94 1	95	Nasal swabs Endometrial swabs	18 1	19	20%
	Bialla	30 2	32	Nasal swabs Lung tissues	13 1	14	43.8%
	Sedi-Salem	50 1	51	Nasal swabs Subcutaneous abscess	3 1	4	7.8%
	Desouq	46 1	47	Nasal swab Subcutaneous abscess	3 1	4	8.5%
	Ariamon	22	22	Nasal swab	2	2	9%
Total		316	316			49	15.5%

Table 7. Biochemical reactions of *Pasteurella multocida*.

Biochemical tests	Total No. of isolates	+ve isolates		-ve isolates	
		No.	%	No.	%
1- Oxidase Test	55	55	100	0	0
2- Catalase	55	55	100	0	0
3- Indole	55	55	100	0	0
4- Urea hydrolysis	55	0	0	55	100
5- Citrate utilization	55	0	0	55	100
6- TSI (yellow butt & slant)	55	55	100	0	0
7- H_2S production	55	0	0	55	100
8- Growth on MacConkey's agar	55	0	0	55	100
9- Haemolysis on blood agar	55	0	0	54	100

With regard to the result of pathogenecity test in white mice. Mice inoculated with the suspected *Pasteurella multocida* isolates died within 24-72 hours, showing generalized septicaemia with highly congested trachea and lungs, pure cultures of the inoculated isolates were obtained from the heart blood of the dead mice.

Stained smear from the heart blood demonstrated a large number of bipolar organisms when stained with Leishman's and Giemsa stain as shown in Figs. 1 and 2.

Serological typing of isolated *Pasteurella multocida* strain by capsular typing revealed that 32 isolates were found to be serologically related to group A, 16 related to group D and 7 isolates untypable.

Meanwhile serological typing of the isolated strains by somatic typing using tube agglutination test revealed that 21 isolates related to type 9, 11 to type 5 and, 16 to type 2.

Table 8 demonstrated the results of capsular somatic typing of isolated *Pasteurella multocida* strain. PCR based assay for molecular detection of *Pasteurella multocida* using species specific primer was depicted in Fig. 3 and RAPD-PCR assay for DNA fingerprinting of reference and field strains of *Pasteurella multocida* as was depicted in Fig. 4 and 5 and analysed in Tables 9 and 10.

Table 8: Capsular and somatic typing of isolated *Pasteurella multocida* strains

Titre of HA test	No of positive isolates	%	Capsular type	Somatic type
1280	11	22.9%	A	5
640	21	43.8%	A	9
320	8	16.6%	D	2
80	5	10.4%	D	2
40	3	6.3%	D	2
Total	48	100%		

Table 9: The molecular weight of the different amplified bands of vaccinal and field *Pasteurella multocida* strains examined by RAPD-PCR in Fig. 4.

Strain no and serotype Bands molecular weight in bp	Lane 1 DNA bands of the marker DNA	Lane 2 DNA bands of P M. (A:5) vaccinal strain No. 1	Lane 3 DNA bands of P.M. (A:9) vaccinal strain No. 2	Lane 4 DNA bands of P.M. (D:2) vaccinal strain No 3	Lane 5 DNA bands of P.M. (A:3) field isolate No.4	Lane 6 DNA bands of P.M. (D:2) field isolate No.5	Lane 7 DNA bands of P.M. (A:9) field isolate No.6	Lane 8 DNA bands of –ve control No.7	Lane 9 DNA bands of P M. (A:9) field isolate No.8	Lane 10 DNA bands of P.M. (A:9) field isolate No.9	Lane 11 DNA bands of P.M. (A:9) field isolate No.10	Lane 12 DNA bands of P.M. (A:9) field isolate No.11	Lane 13 DNA bands of P.M. (A:9) field isolate No.12
Band 1	1000	1089						-					
Band 2	900	924						-					
Band 3	800							-					
Band 4	700	692		729		714	714	-	724	724	724	724	724
Band 5	600	648		648									
Band 6	500	492	488	488	488	488	488		488	488	488	488	488
Band 7	400		368	379	391	374	368	350	388 324	388 324	388 324	388 324	388 324
Band 8	300	324	326				326 397						
Band 9	200	254	252	252	252	252	252 204		252	252	252	252	252
Band 10	100	138	138	138	138	138	138		138	138	138	138	138
Band 11		68	68	68	68	68	68		68	68	68	68	68

Table 10. The molecular weight of the different genomic DNA bands of vaccinal and field *Pasteurella multocida* strains examined by RAPD-PCR in Fig. 5.

Strain no. and serotype / Bands molecular weight in bp	Lane 1	Lane 2	Lane 3	Lane 4	Lane 5	Lane 6	Lane 7	Lane 8	Lane 9	Lane 10	Lane 11	Lane 12	Lane 13
	DNA bands of the marker DNA	DNA bands of P.M. (A:5) vaccinal strain No. 1	DNA bands of P.M. (A:9) vaccinal strain No. 2	DNA bands of P.M. (D:2) vaccinal strain No.3	DNA bands of P.M. (A:3) field isolate No.4	DNA bands of P.M. (D:2) field isolate No.5	DNA bands of P.M. (A:9) field isolate No.6	DNA bands of +ve control No.7	DNA bands of P.M. (A:9) field isolate No.8	DNA bands of P.M. (D:2) field isolate No.9	DNA bands of P.M. (A:9) field isolate No.10	DNA bands of P.M. (D:2) field isolate No.11	DNA bands of P.M. (A:5) field isolate No.12
Band 1	1000	1089						-			1075	1075	1694 1075
Band 2	900	924						-			930	930	930
Band 3	800							-					
Band 4	700	692		729		714	714	-	724	784	758	738	685
Band 5	600	648			648			-		620	652	641	645
Band 6	500	492	488	488	488	488	488	-	488	488	488	992	568
Band 7	400		368	379	391	374	368	-	388 324			400	488
Band 8	300	324	326				326 297	-		324	324	324	324
Band 9	200	254	252	252	252	254	252 204	-	252	295 263	252	255	252
Band 10	100	138	138	138	138	138	138	-	138	138	138	138	138
Band 11		68	68	68	68	68	68		68	68	68	68	68

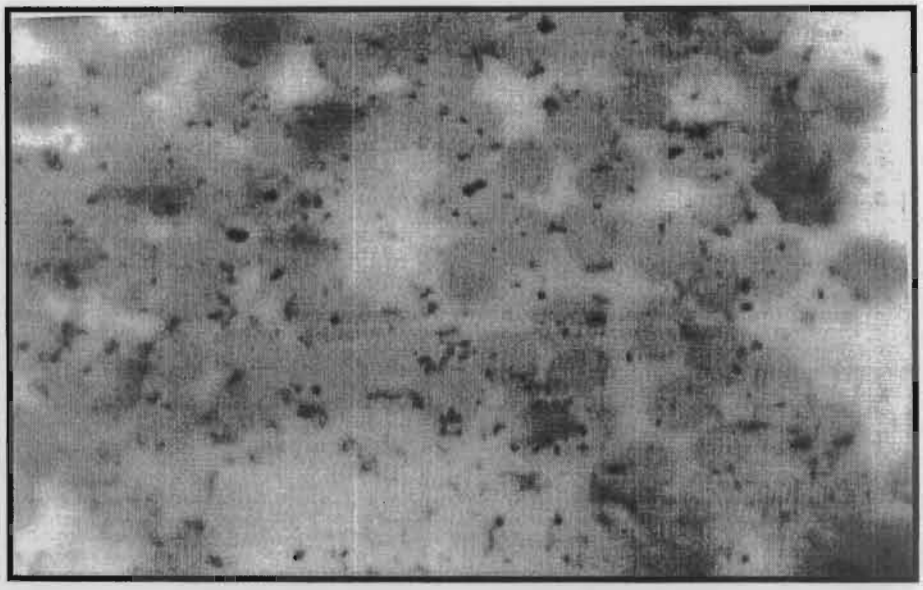

Fig. 1. *Pasteurella multocida* stained by Leishamn's slain showing bipolarity in blood smear (x 3000).

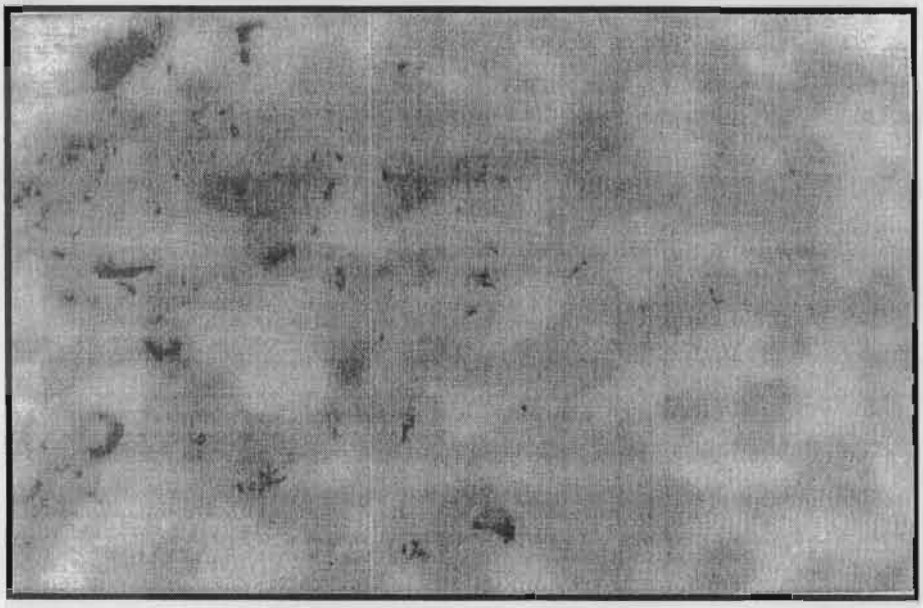

Fig. 2. *Pasteurella multocida* stained by Giemsa stain showing bipolarity in blood smear (x 3000).

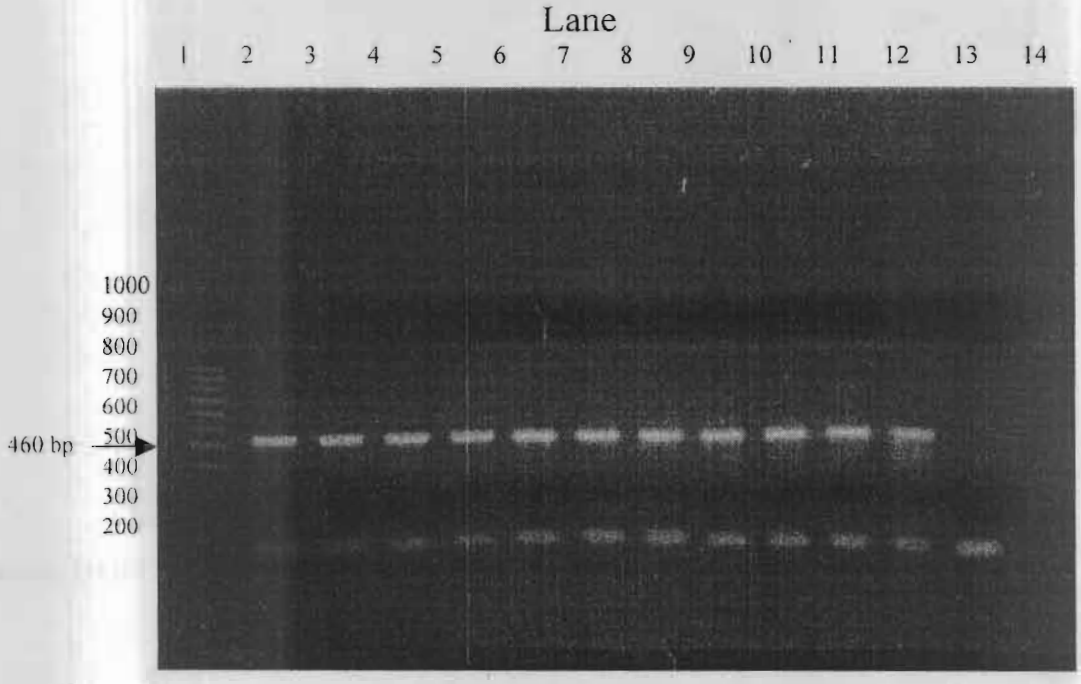

Fig. 3. Electrophoretic pattern of PCR production (460 bp specific for *Pasteurella multocida)* in 1.5% agarose gel stained with ethidium bromide.

Lane 1: DN A marker (100 bp marker)
Lane 2: PM A: 5
Lane 3: PM A: 9 — Reference vaccinal strain
Lane 4:PMD:2
Lane 5: PM A: 3 → Field strain
Lane 6: PM D:2
Lane 7:PMA:9 — Field strain from Kafr El-Sheikh Governorate
Lane 8: PM A: 9 → Field strain from Sharkia Governorate
Lane 9: PM D:2
Lane 10: PM A:9
Lane 11:PMD:2 — Field strain
Lane 12: PM A:9
Lane 13: Negative control

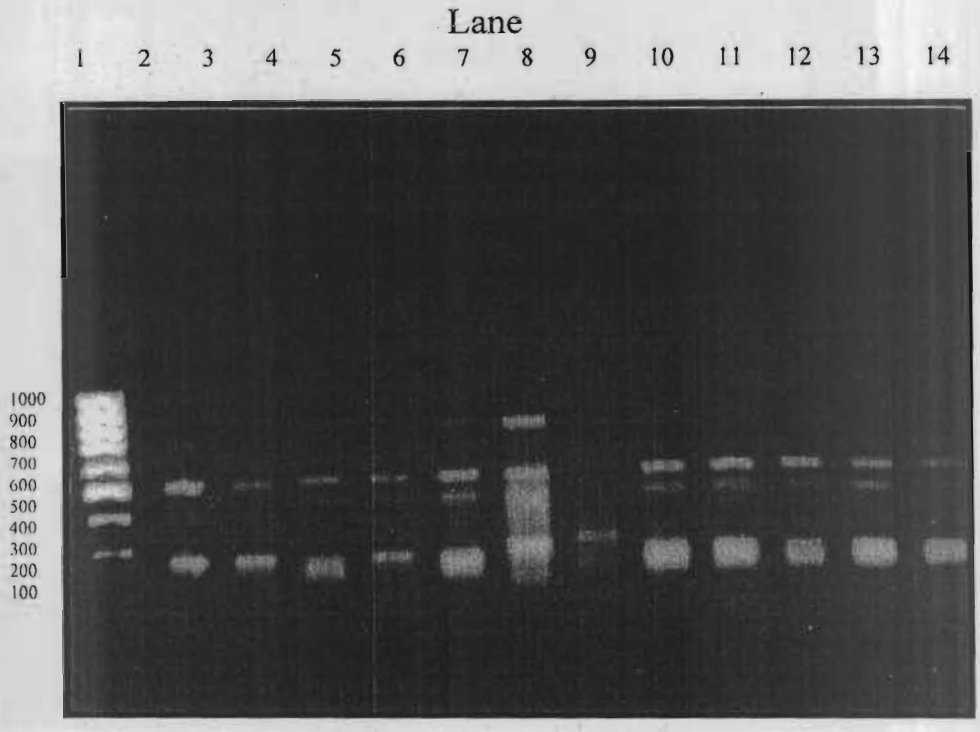

Fig. 4. The electrophoretic profile of RAPD-PCR of field and standard *Pasteurella multocida* genomic DNA as amplified by D14803 primer.

Lane 1: DNA marker (100 bp marker)
Lane2:PMA:5
Lane 3: PM A:9 } Reference vaccinal strain
Lane 4:PMD:2
Lane5:PMA:3 → Field strain
Lane 6:PMD:2
Lane 7: PM A:9 } Field strain from Kafr El-Sheikh Governorate
Lane 8: *E. coli* → Field strain
Lane 9: PM A: 9
Lane 10:PMA:9
Lane 11: PM A:9 } Field strain from Sharkia Governorate
Lane 12:PMA:9
Lane 13:PMA:9

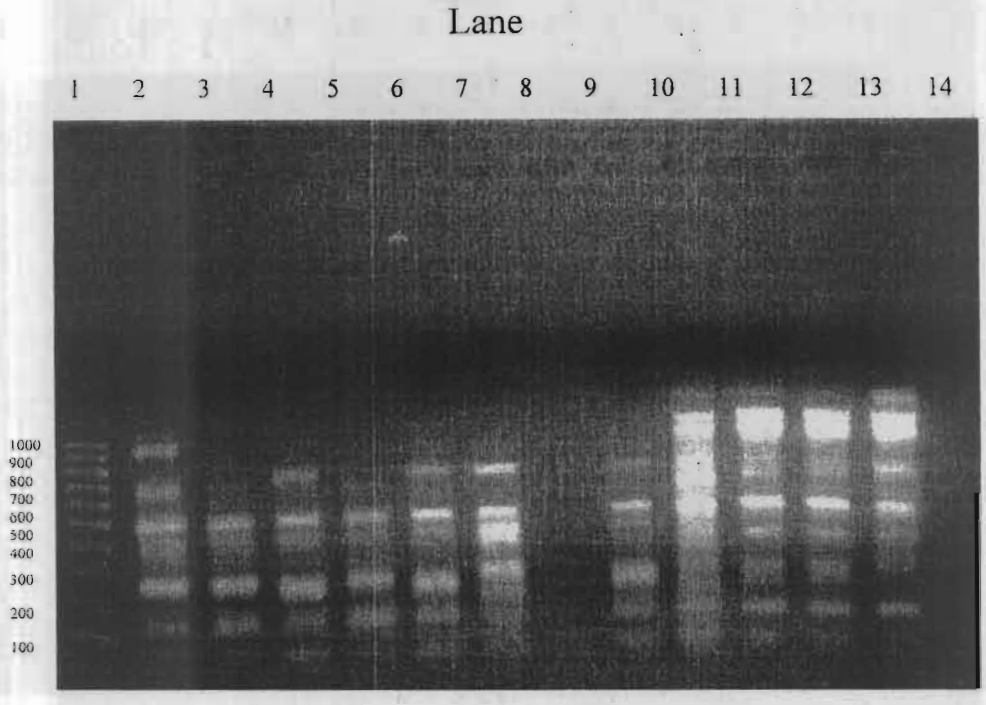

Fig. 5. The electrophoretic profile of RAPD-PCR of field and standard *Pasteurella multocida* genomic DNA as amplified by D14803 primer

Lane 1: DNA marker (100 bp marker)
Lane 2: PM A: 5
Lane 3: PM A: 9 } Reference vaccinal strain
Lane 4:PMD:2
Lane 5: PM A: 3 → Field strain
Lane 6: PM D:2
Lane 7- PM A 9 } Field strain from Kafr El-Sheikh Governorate
Lane 8: Negative control
Lane 9: PM A: 9 Field strain from Sharkia Governorate
Lane 10: PMD:2
Lane 11: PM A:9 } Field strain from Kafr El-Sheikh Governorate
Lane 12:PMD:2
Lane 13:PMA:5

DISCUSSION

Rabbit pasteurellosis continues to be a major concern for the rabbit industry. The Gram-negative bacterium, *Pasteurella multocida* the causal agent of common and serious manifestations of the disease in rabbits that include mucopurulent rhinitis, pneumonia, otitis media, septicemia, meningitis and localized abscesses. It was estimated to be the most common bacterial pathogen of rabbits isolated from either diseased or apparently healthy animals ***(1,19)***.

In the present investigation 55 isolates of *Pasteurella multocida* were isolated out of a total of 428 samples, 6 isolates of *Pasteurella multocida* from 112 samples of nasal swabs collected from apparently healthy rabbits with an isolation rate of 5.3% and 49 isolates of *Pasteurella multocida* were recovered from 316 samples of diseased rabbits with an isolation rate of 15.5% , and a total incidence of 12.3%. These results are similar with that obtained previously ***(20)*** which indicated that out of 259 nasal swabs collected from apparently healthy and diseased rabbits from different governmental and private Sharkia farms, 24 isolates were obtained with a total incidence of 9.3%. Also, these results nearly agree with those obtained by ***(21)***, who found that out of 239 nasal swabs collected from apparently healthy and diseased rabbits, 19 rabbits were positive for *Pasteurella multocida* with an incidence of 7.9%.

Among apparently healthy rabbits *Pasteurella multocida* rate was 5.3%. These results nearly consistent with those cited by several investigators, as they isolated *Pasteurella multocida* from nasal swabs collected from apparently healthy rabbits with incidence of 3.8% ***(22)*** and 6% ***(23)*** .

On the other hand, conflicting results have been reported ***(24)*** the isolation of *P. multocida* from nasal and vaginal samples collected from 81 apparently healthy rabbits with incidence 56.8 and 49% respectively.

In the present study the incidence of *P. multocida* among diseased rabbits was 15.5%. *Pasteurella multocida* was isolated from nasal swabs of rabbits with clinical signs of respiratory disease with an incidence of 19% ***(21)*** and 9.1% ***(23)*** respectively.

On the other hand, other studies isolated *Pasteurella multocida* from diseased rabbits with an incidence of 79% (24) and 72% (25) ***(25,26)*** which are higher than that obtained from our investigations.

Serotyping of *Pasteurella.multocida* isolates indicated that 32 isolates with a percentage of 58% were capsular group "A" and 16 isolates with a percentage of 29% were group "D" and 7 isolates were untypable with a percentage of 12.7% using the indirect haemagglutination. On the other hand, somatic type were 9, 5 and 2 using tube agglutination test. Several previous studies ***(27-29)*** also revealed that capsular antigen type "A" and type "D" were predominant between the virulent isolates of *Pasteurella multocida* isolated from rabbit.

Somatic serotyping showing some similarity and differences which may be due to differences in geographical location and uncontrol treatment with different antibiotics.

Conventional methods for isolation and identification of *Pasteurella multocida* involve the exhaustive task of obtaining pure cultures, which is particularly time-consuming when presented with samples as nasal swabs that commonly contain mixed bacterial flora. Additionally, suspected isolates as *Pasteurella multocida* involves subjecting the isolates to range of biochemical tests where isolates with aberrant biochemical properties that can be particularly perplexing which makes identification difficult ***(30)***.

The recent development of species-specific PCR assays for *Pasteurella multocida* has provided rapid presumptive identification of the organism. One of the objective of this study was to evaluate through a PCR based assay the sensitivity and specificity of *Pasteurella multocida* species specific primer as a rapid method for the identification of *Pasteurella multocida* isolates recovered from rabbits aiming to overcome the limitations

associated with the conventional methods for isolation and identification.

As shown in Fig. 3, all examined *Pasteurella multocida* isolates gave a single amplified product of the expected size of 460 bp as cited previously ***(18),*** which emphasizing the sensitivity of PCR in identification of all examined *Pasteurella multocida* strains, that consisted of field strains with geographical and genetic diversity [Lane (5-12)] as well as reference vaccinal strains [Lane 2-4]. On the other hand, the specificity of the PCR assay was assumed by the negative control DNA sample, [Lane 13] that didn't show any amplification products. The PCR assay enables confirmation of a suspect colony of *Pasteurella multocida* in less than five hours whereas confident identification of a suspect colony by conventional phenotypic methods requires up to five days.

Furthermore, the possibility of direct use of the confluent growth culture regardless of the purity of the sample hence, overcoming the possibility of some isolates to be non pathogenic for mice will ensure rapid detection of *Pasteurella multocida* ***(2)***.

In epidemiology, it is critically important to discriminate phenotypically similar isolates, particularly when establishing bacterial vaccine strain where in this context, the limited characterization provided by phenotypic techniques intensified the need for improved methods for epidemiologic analysis and disease surveillance ***(31)***.

Genotyping techniques, based upon their high discriminatory power in characterization and differentiation between isolates of the same serotype have been proposed for conducting molecular epidemiologic analysis and outbreak investigations aiming to improve the knowledge of a given organism and its epidemiology (by tracing the source of infection).

The random amplified polymorphic DNA (RAPD) has been successfully used in the characterization of several organisms and additionally was proven to be a useful technique for studies in rabbit pasteurellosis ***(32)***.

RAPD doesn't require previous detailed knowledge of the DNA to be analysed furthermore, the availability of commercial kits makes it easy to apply this technique in molecular typing of bacterial isolates ***(9)***.

Characterization and differentiation of *Pasteurella multocida* isolates through DNA-fingerprinting by RAPD-PCR assay was one of the main objective of this study aiming to investigate through molecular epidemiologic analysis the correlation between *Pasteurella multocida* isolates and geographic locations.

RAPD-PCR was performed in this study on several *Pasteurella multocida* strains either isolated from Kafr El-Sheikh Governorate or from other locations in Egypt, in addition to three reference vaccinal strains.

Analysis of the obtained DNA fingerprint results was subsequently performed, through several dimensions. First between *Pasteurella multocida* isolates from the same farm, second between field isolates from different localities and finally between reference vaccinal strains and field strains.

Fragments generated by this primer ranged from 68 to 1694 bp in size in all isolates while the number of bands ranged from 6 to 11 bands.

In spite of the obvious variability showed by RAPD between *Pasteurella multocida* isolates, which emphasize the heterogemcity existing among *Pasteurella multocida* strains isolated from rabbits, all examined *Pasteurella multocida* strains shared two major bands of 68 and 138 bp indicating that a particular band can also be considered to be a characteristic trait or a molecular marker of an organism.

DNA fingerprint patterns of the examined three reference vaccinal strains by RAPD assay revealed Lane 2 containing vaccinal strain A: 5 was differentiated into 9 bands while Lane 3 containing vaccinal strain A: 9 was differentiated into 6 bands meanwhile, Lane 4 containing vaccinal strain D: 2 was

differentiated into 6 bands. This leads to the conclusion that the used technique is reproducible and discriminative among the tested strains of various clones.

Despite the common two major bands (68 and 138 bp) shared by all *Pasteurella multocida* isolates and the 488 bp band shared only by A: 9 and D: 2 vaccinal strain (Lane 3, 4, respectively) a clear heterogenicity was obvious between the three vaccinal strains. This could interpret the fact that G.O.V.S in a polyvalent vaccine of these three vaccinal strains is recommended in Egypt for ensuring a protective immunity against rabbit pasteurellosis, overcoming the heterogenicity of rabbit *Pasteurella multocida* strains allover the country.

Five strains of *Pasteurella multocida* isolated from the same farm at Sharkia governorate were subjected to RAPD. (Fig. 4 and Table 9). The analysis of their electrophoretic pattern revealed that they are genetically indistinguishable on the basis of their identical banding patterns either in size or in number of bands. This supports the suggestion that all five are the same clone strain and demonstrates the utility of RAPD analysis as an effective epidemiological tool in monitoring transmission of a pathogen ***(33)***. These results may be due to the fact that the tested strains were the circulating clone of infection.

In fact, a closed structure system of rabbit production would appear more favourable for a vertical clonal dissemination where a single strain of *Pasteurella multocida* tended to predominate within a herd ***(34)***. This observation agree with the finding of Digiacomo and his Collages ***(35)*** who stated that the spread of *Pasteurella multocida* by direct contact between rabbits is the most important mode of transmission of the organism.

As shown in Fig. 4 and 5 and Tables 6 and 7 the analysis of the electrophoretic pattern of two *Pasteurella multocida* field strains isolated from Kafr El-Sheikh governorate (Lane 6-7) revealed that beside the common two major bands (68 bp and 138 bp), the three bands 254 bp, 488 bp and 714 bp were shared by both isolates, two of these bands (254 bp and 714 bp) weren't shared by any other *Pasteurella multocida* isolates tested by RAPD in this study suggesting that the correlation between fingerprint results among isolates and geographic locations cannot be over emphasized. Moreover, they may be from different clone ancestor.

For investigation and comparing DNA fingerprinting of various *Pasteurella multocida* isolates from different areas, four field strains recovered from rabbits at different localities in Egypt over a period of ten years, were submitted to RAPD in this study as shown in Fig. 5 and Table 7 (Lane 10, 11, 12, 13). The analysis of their electrophoretic pattern showed that although many fragments (324 bp, 930 bp and 1075 bp) appeared common to several strains, the obtained patterns were qualitatively sufficient for accurate strain differentiation.

The present study documented the existence of genotypic differences among strains of the same serotype as shown in Fig. 5. Lane 2 and lane 13 consisting of genomic DNA of serotype A: 5, while lane 3, 9 and 11 consisting of DNA of serotype A: 9 and lane 4, 6,10, 12 consisting of genomic DNA of serotype D: 2. The electrophoretic profiles of these isolates showed genotypic differences that emphasize the ability of the RAPD-PCR assay to discriminate between closely related strains and to establish the relationship of isolates that could not be distinguished by serotyping.

In this study, the reproducibility of the RAPD assay was assessed by repeating the test at least twice, where identical RAPD profiles were obtained (Fig. 4 and 5).

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الملخص العربى

التوصيف البكتيرى والجزيئ لميكروب الباستريلا مالتوسيدا المعزول من الأرانب باستخدام تقنية اختبار البلمرة المتسلسل

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فى هذه الدراسة تم فحص ٤٢٨ عينة جمعت من أرانب سليمة ظاهريا ومن أرانب مريضة وحديثة الوفاة مختلفة العمر والسلالة من مزارع مختلفة بمحافظة كفر الشيخ لعزل ميكروب الباستيريلا مالتوسيدا فى الأرانب.

أجرى الفحص البكتريولوجى على هذه العينات لعزل الباستيريلا مالتوسيدا وأمكن عزل ٥٥ عينة من الباستيريلا مالتوسيدا. من ٤٢٨ عينة التى تم فحصها بنسبة مئوية ١٢,٥٪ وهى تشمل على ١١٢ مسحة أنفية من الأرانب السليمة ظاهريا تم عزل ٦ معزولات بنسبة ٥,٣٪ بينما ٣١٦ عينة من الأرانب المريضة وحديثة الوفاة تم عزل ٤٩ معزولة بنسبة ١٥,٥٪ من بينهم ٤٢ معزولة عزلت من ٢٧٨ مسحة أنفية من أرانب مريضة بنسبة ١٥٪ و ٣ معزولات من ٣٤ عينة رئوية بنسبة ٨,٨٪ كما تم عزل معزولة واحدة من كل من واحد عينة كبدية وأخرى من الرحم كما تم عزل ٢ معزولة من ٢ عينة أخذت من خراريج تحت الجلد. حيث تم تصنيف هذه المعزولات على حسب الطرق التقليدية المعتادة من خواص مستعمراتها والصفات الخلوية والسلوك الكيميائى وضراوتها للفئران والتصنيف السيرولوجى.

وبدراسة الخواص البيوكيميائية للعترات المعزولة من ميكروب الباستريلا مالتوسيدا وجد أنها إيجابية فى انتاج الإندول والكاتاليز والاكسيديز بينما كانت سلبية فى انتاج اليوريز واستخدام السترات وإنتاج كبريتيد الهيدروجين وكذلك سلبية فى النمو على مزارع الماكونكى. أوضح التصنيف السيرولوجى بأن عترات الباستيريلا مالتوسيدا المعزولة من الارانب بتقسيم كارتز ٣٢ معزولة منها تتدرج تحت مجموعة الكابسول "أ" بنسبة ٥٨٪ و١٦ معزولة تتدرج تحت مجموعة الكابسول "د" بنسبة ٢٩٪ والنوع الجسدى كان ٩، ٥ بينما وجد ٧ معزولات لم يتم تصنيفها بنسبة ١٢,٧٪.

فى هذه الدراسة تم دراسة حساسية وخصوصية البادئ الخاص بالباستيريلا مالتوسيدا وقدر من خلال اختبار البلمرة المتسلسل (PCR) كل معزولات الباستيريلا مالتوسيدا التى فحصت أعطت قطعة واحدة مضاعفة وهى ٤٦٠ زوج من النيكليوتيدات بينما الكنترول السلبى لم يعطى أى قطعة مضاعفة أو بصمة وهذا يؤكد على خصوصية اختبار البلمرة المتسلسل (PCR). وكذلك الحمض النووى المتعدد الصور المضاعفة عشوائيا (RAPD) أظهر نجاحا فى هذه الدراسة فى تصنيف الباستيريلا مالتوسيدا المعزولة من الأرانب على أساس أنه طبق على معزولات متعددة من الباستيريلا مالتوسيدا ومن خلال التحليل وجد أن قطع متشابه حصل عليها من نفس العتره بينما هناك اختلاف بين البصمات الحاصل عليها بين المعزولات من نفس المزرعة والأخرى من أماكن مختلفة وبين المعزولات التى تستخدم فى التحصين.

من هذا نستخلص أن البصمات الناتجة عن استخدام الحمض النووى المتعدد الصور المكبرة عشوائيا (RAPD) أعطت لنا تحليل مميز لمعزولات البكتريا وهذا يتغلب على التقصير فى التميز بالطرق المعتادة وتقدم هذه الدراسة أسلوب حديث لتقصى مصادر العدوى بالباستيريلا مالتوسيدا مما يساعد فى إعداد العترات السارية للعدوى لتحضير اللقاح المتخصص لهذا الميكروب.