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# Bacteriological and Molecular Characterization of *Pasteurella multocida* Isolated from Rabbits by PCR Based Techniques

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# APPROVAL SHEET

This is to approve that the dissertation presented by **Etab Mohamed Sayed Roho Abo Remela** Tanta University Kafr El-Sheikh Branch entitled "**Bacteriological and molecular characterization of *Pasteurella multocida* isolated from rabbits by PCR based techniques"** for the degree of M.V.Sc. "Bacteriology" has been approved by the examining committee.

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# LIST OF ABBREVIATION

| | | |
|---|---|---|
| Bord. | : | Bordetella |
| bp | : | Base pair |
| C.S.F. | : | Cerebrospinal fluid |
| D.D.W. | : | Deionized Distaled Water |
| D.W. | : | Distaled water |
| DEPC | : | Diethylepyrocarbonate |
| DNA | : | Deoxyribonucleic acid |
| dNTP | : | Deoxyribonucleotide triphosphate |
| *E. coli* | : | *Escherichia coli* |
| E.D.T.A | : | Ethylene diamine tetraacetic acid |
| G.O.V.S. | : | General organization of veterinary services |
| I/P | : | Intraperitoneal |
| IHA | : | Indirect haemagglutination |
| P.M | : | *Pasteurella multocida* |
| PCR | : | Polymerase chain reaction. |
| RAPD | : | Random amplified polymorphic DNA. |
| S/C | : | Subcutaneous |
| SDS | : | Sodium dodecyl sulphate |
| SSP | : | Subspecies. |
| T.S.I. | : | Triples sugar iron |
| TAE | : | Tris acetate EDTA |
| *Taq* | : | *Thermus aquaticus* |
| TE | : | Tris-EDTA buffer |
| U.S.A. | : | United States of America |
| U.V. | : | Ultraviolet |
| Y.P.C. | : | Yeast protease cystine |

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# 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 (**Percy *et al.*, 1986 and Dabo *et al.*, 2000**).

*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. (**Zumpt, 1976**).

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 (**Matsumoto and Strain, 1993**). This latter 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 lipopoly saccharides. (**Carter, 1955 and Namioka and Murata, 1961**).

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 antigens (**Rhoades and Rimler, 1990**).

Although serologic typing was useful and accepted in the presumptive diagnosis of *P. multocida*, its encountered short falls and its unsufficient provided informations for epidemiological studies, for which the ability to differentiate phenotypically similar isolates is critically important, a need for improved methods of distinguishing various

*Pasteurella multocida* strains for epidemiologic analysis and disease surveillance was intensified (**Wilson *et al.*, 1992**).

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 (**Chaslus-Dancla *et al.*, 1996**).

On the enlightements of all of the above, the present study was planned with the following prospectives:

1. Investigation the prevalence of *Pasteurella multocida* among apparently healthy and clinically diseased rabbits in Kafr El-Sheikh Governorate in order to determine the percentage of carrier rabbits in the former and the incidence of pasteurellosis in the latter.
2. Phenotypic characterization of the isolated strains of *Pasteurella multocida* by conventional methods. (biochemical and serological typing).
3. Validation the use of recent PCR-based techniques in:
   a) Molecular identification of *Pasteurella multocida* by a specific primer.
   b) Application of random amplified polymorphic DNA assay as a valuable genotypic method for molecular characterization and epidemiological investigation of *Pasteurella multocida* in rabbits.

# 2. REVIEW OF LITERATURE

## 2.1. Incidence of *Pasteurella multocida* organisms in rabbit:

**Flatt and Dungworth (1971a)** mentioned that a percentage of 20% in 8-10 weeks old rabbits showed gross lesion of an enzootic pneumonia. They stated that the most prevalent clinical signs included dyspnea and dried yellowish exudate on the external naris beside loss of condition.

**Fox *et al.* (1971)** isolated *Pasteurella multocida* in pure cultures from 97% of rabbits with otitis media and from the eye and noses of normal appearing rabbits.

**Digiacomo *et al.* (1983)** stated that the prevalence of rabbit pasteurellosis varies considerably depending on the age, sex and health status of rabbits in addition to the technique used for detection. They found that only 50% of infected rabbits has nasal discharge and that the prevalence of *Pasteurella multocida* in the nasal cavities of adult rabbits was 72% based on nasal cultures.

**Holmes *et al.* (1984)** investigated the incidence of *Pasteurella multocida* in various anatomical sites of rabbits that was as follows: nasopharynx 62.5%, middle ear 50% , nasal sinus 37.5% and nasal passage 37.5%.

**Nakagawa *et al.* (1986)** found that the infection rate of *Pasteurella multocida* in suckling rabbits younger than one month old was very low (43%), while reaching nearly 100% in adult rabbits more than five months of age. They added that a rapid increase of the infection rate was observed at two to three months of age.

**Rai *et al.* (1987)** investigated the correlation between the incidence of nasal *Pasteurella* and vaginal *Pasteurella*, they examined bacteriologically nasal and vaginal swabs from 81 healthy rabbits in India. While *Pasteurella multocida* was isolated from 56.8% of all samples, out of 42 breeding does 20 (49.3%) and 18 (42.8%) were positive for nasal and vaginal *Pasteurella* respectively.

**Percy *et al.* (1988)** isolated *Pasteurella multocida* with an incidence of 11.9% from dead rabbits showing various pathological lesions.

**Glass and Beasly (1989)** recorded that *Pasteurella multocida* was isolated from all ages of rabbits and added that the sinus were colonized most often, followed by the trachea, middle ear and lungs, whereas no bacteria could be isolated from the liver.

**Kawamoto *et al.* (1990)** investigated the prevalence of *Pasteurella multocida* in a total of 1147 nasal samples from rabbits in Japan. *Pasteurella multocida* was isolated from 199 (29.8%) of 668 rabbits kept as laboratory animals and from 1 (0.2%) of 479 rabbits in rabbitries. The highest rate of isolation from rabbits was recorded at 10 to 12 months of their housing time.

**Deeb *et al.* (1990)** stated that about 25% of rabbits at weaning had nasal infection with *Pasteurella multocida* and that 75% had infection with *Bordettela bronchiseptica*. They found that the proportion of rabbits with both infection increase with age. They added that 75% of rabbits at about 10 months of age were infected with *Pasteurella multocida*.

**Dillehay *et al.* (1991)** isolated *Pasteurella multocida* A: 3 during an outbreak of pasteurollosis in Flemish Giant (FG) rabbits housed with New Zealand white (NZW) rabbits. They stated that the (FG) rabbits were more susceptible to pasteurellosis than (NZW) rabbits.

**Frymus *et al.* (1991)** isolated 35 strains of *Pasteurella multocida* among 36 animals in a commercial rabbitry with enzootic upper respiratory diseases resembling porcine atrophic rhinitis.

**Mercier (1992)** showed that the carrier rate of *Pasteurella multocida* among normal rabbits in the upper respiratory tract was in between 2-3.9%.

**Katoch *et al.* (1993)** examined for bacterial diseases a total of 115 rabbits from different farms at various ages where four of them were diseased while 71 were dead. Four diseased rabbits exhibiting respiratory infection yielded one *Pasteurella mulltocida* isolate with a percentage of 25%, while 39 dead rabbits exhibiting respiratory infection yielded 16 isolates with percentage of 41%.

**Rai *et al.* (1995)** examined bacteriologically a total of 118 cases of rabbit pneumonia and found that the most common bacterial isolates were *Pasteurella multocida* with a percentage of 68.6%.

**Deeb and Digiacomo (2000)** stated that respiratory diseases are second to gastroenteric diseases in importance in rabbit and that pasteurellosis is the primary respiratory disease affecting domestic rabbits, meanwhile other bacteria as *Bordetella bronchiseptica* and *Staphylococcus* species are significant opportunistic pathogens.

**Marlier *et al.* (2000)** stated that pathogenic bacteria species isolated from lungs of 66 rabbits that died with pulmonary lesions was *Pasteurella multocida*, *Escherichia coli*, *Bordetella bronchiseptica* and *Pseudomonas aeruginosa*, with a percentage of 41, 11, 7 and 6%, respectively.

**Takashima *et al.* (2001)** reported, in Japan, an outbreak of *Pasteurella multocida* infection in rabbits started from sudden death to severe fibrinous and purulent pneumonia with hemorrhage. A large number of *Pasteurella multocida* A: 12 was isolated from the trachea and lung of animals.

## 2.2. *Pasteurella multocida* infection in rabbits in Egypt:

**Fathy (1970)** isolated *Pasteurella multocida* from 74 cases out of 152 cases of rabbits affected with Pneumonia with a percentage of 48.68%.

**Zaher *et al.* (1976)** stated that the most frequent isolated organism from the diseased dead rabbits was *Pasteurella multocida* type III (Roberts) with percentage of 79%. In addition *E. coli* and *Diplococcus pneumonae* were both isolated at an incidence of 10% and 5% respectively.

**Elged *et al.* (1990)** collected 185 samples from rabbits at different private farms of rabbits at Sharkia Governorate showing signs of pneumonia, rhinitis, otitis and osteoarthritis and suspected to be suffered from pasteurellosis. They indicated that the bacteriological examination of the collected samples revealed that incidence of *Pasteurella multocida* was 8.8, 11 and 5% in rabbits suffering from respiratory signs, joint

abscesses and otitis respectively. Moreover, the examined 80 nasal swabs from apparently healthy adult rabbits revealed that *Pasteurella multocida* was the only microorganism isolated with an incidence of 3.8%.

**Mahmoud and Abdel-Baset (1991)** performed a study on 4 rabbit flocks consisting of 85, 56, 60 and 70 does, respectively. They found that the mortality rate due to *Pasteurella multocida* infection was high and ranged from 70-78% in the investigated flocks.

**El-Dirbi (1992)** examined a total of 470 samples. 150 samples were from healthy rabbits and yielded 9 isolates of *Pasteurella multocida* with a percentage of 6%. The other 320 samples were collected from infected rabbits as follow, 312 from internal organs, 8 nasopharyngeal swabs and 10 abscess samples which yielded 43 (11.3%), 7 (87.8%) and 9 (90%), isolates of *Pasteurella multocida* respectively.

**Ibrahim (1993)** examined a total of 100 samples from nasal discharges and abscesses of diseased rabbits from private farms in Giza and Cairo that yielded 12 isolates of *Pasteurella multocida* with a percentage of 12%.

**Nada (1994)** examined a total of 239 samples. 53 samples were from apparently healthy rabbits and yielded two isolates of *Pasteurella multocida* with a percentage of 3.81% while 186 samples were from rabbits with clinical signs of respiratory disease and rhinitis and yielded 17 isolates with percentage of 9%.

**Hussein (2000)** found that the prevalence rate of *Pasteurella multocida* isolated from apparently healthy rabbits was (4.8%), meanwhile, in diseased rabbits was 12.3%.

**El-Shayeb (2000)** examined out a total of 200 rabbits, 55 were apparently normal and 145 were diseased and found that *Pasteurella multocida* was the predominant bacterial species. The organism was found in 19 (36.5%) of the apparently normal and 46.9% of diseased rabbits.

## 2.3. Bacteriology of *Pasteurella multocida*:

### 2.3.1. Morphology:

**Smith (1954)** stated that *Pasteurella multocida* was ovoid or rod-shaped, non-motile, non-sporulated, Gram-negative, bipolar staining organism, specially in preparation made from infected animal tissues stained with Giemsa or methylene blue.

**Merchant and Packer (1956)** noticed that *Pasteurella multocida* was non-motile Gram negative, ovoid or elongated rods and distinctly bipolar when carefully stained with Giemsa stain and that this character was lost with continued subculturing on artificial media.

**Gordon and Jordan (1982)** mentioned that *Pasteurella multocida* is a Gram negative non-motile, non-spore forming and rodshaped bacteria that are arranged singly or less frequently in pairs or short chains.

### 2.3.2. Media for cultivation of *Pasteurella multocida* organism:

**Das (1958)** described a selective medium containing crystal violet, aesculin and cobalt chloride for isolation of *Pasteurella multocida*.

**Namioka and Murata (1961)** prepared Y.P.C. agar medium containing yeast extract, protease, peptone, L-cystine, glucose, sucrose, sodium sulphite, potassium diphosphate and on which the organism grew well.

**Bain (1963)** stated that tryptose agar medium is good for the demonstration of the colonial morphology of *Pasteurella* organisms.

**Garlinghouse *et al.* (1981)** prepared an expensive culture medium for the isolation of *Pasteurella multocida* and *Bordetella bronchiseptica* from the nares of laboratory rabbits by incorporating 2 micrograms/ml clindamycin into standard blood agar.

**Mohamed (2003)** concluded that blood agar with clindamycin 2 mg/liter was the best medium where it was easy to isolate *Pasteurella multocida* in a pure culture and with little contamination.

**2.3.3. Culture characters:**

**Webster and Burn (1924)** described three types of *Pasteurella* colonies on solid media, the first consist of fluorescent or iridescent colonies; moderate in size, whitish, opaque generally, unstable and pathogenic, usually recovered from acute infection. The second type consist of blue colonies; smaller, dew drop like, rough in appearance, relatively of low virulence, and is mostly recovered from chronic infection, The third type consist of mucoid or intermediate colonies; large, slimy in appearance colonies that range in virulence between fluorescent and blue variants.

**Carter (1952)** reported that the existence of capsular substance in a primary culture of *Pasteurella multocida*, was rapidly lost when subculture was made on artificial media. He nominated the encapsulated type as the fluorescent type and the unencapsulated type the blue type.

**Wilson and Miles (1975)** reported that *Pasteurella multocida* grows fairly well on nutrient agar forming circular colonies about one millimeter in diameter after 24 hours at 37°C. There were different colonial forms; a smooth form, virulent for rabbit forming smooth, moderately opaque iridescent colonies on serum agar, a rough form forming translucent bluish colonies completely avirulent for rabbit and a mucoid from of intermediate virulence.

**El-Ghawas (1980)** reported that cultures of *Pasteurella multocida* on blood agar showed colonies about 1 mm in diameter, smooth, round with convex and translucent surface and that most of them have a fluorescence appearance.

**Waltman and Horne (1993)** reported that *Pasteurella multocida* grown on 5% sheep blood agar were smooth, iridescent, glistening, dew drop, convex and highly virulent.

**2.3.4. Biochemical characters:**

**Brigham and Rettger (1935)** showed that *Pasteurella* strains did not grow on MacConkey agar and did not liquefy gelatin. All strains produced indole and fermented glucose, sucrose, mannitol with production of acid only while the methyl red and Voges Prauskaur tests were negative.

**Smith (1958)** reported minor difference in the fermentative ability between strains from healthy and diseased animals.

**Heddleston (1976)** studied the biochemical activities of *Pasteurella multocida* cultures from various hosts over a period of ten years and found

that most strains fermented galactose, glucose, manitol, fructose and sucrose. They produce hydrogen sulphide and indole and reduce nitrate whereas inactive in fermentation of dulcitol, lactose, inulin, maltose and salcin. They do not grow on MacConkey and were urease and gelatinase negative.

**El-Ghawas (1980)** studied the biochemical characters of 18 isolates of *Pasteurella multocida* isolated from septicemic cases of animals. She found that all isolates were indole positive and were non-motile. 14 isolates fermented arabinose but did not ferment either xylose or dulcitol.

**Clemons and Gadberry (1982)** tested 96 isolates of *Pasteurella multocida* from man, animals and birds for indole production. They found that all isolates were indole positive within 18 to 24 hours and adviced for *Pasteurella multocida* to use the supplement of 2% peptone broth for the detection of indole.

**Digiacomo *et al.* (1991)** characterized isolates of *Pasteurella multocida* isolated from nasal swabs from a closed colony of rabbits. One strain had mucoid colonies, fermented few carbohydrates and was serotype A:5. The other strain had smooth iridescent colonies, with untypable capsular antigen and type 3 somatic antigen, fermented many carbohydrates.

**El-Dirbi (1992)** reported that the isolates of *Pasteurella multocida* were able to ferment glucose, mannitol and mannose, but were unable to ferment, lactose, salicine, glycerol and maltose, while xylose, dulicitol and arabinose were variable. All strains were indole positive and oxidase

positive, while methyl red, voges prauskeur's urease citrate, gelatin and hydrogen sulphide reactions were negative.

**Ibrahim (1993)** found that all isolates from rabbit pasteurellosis produce acid only from glucose, sucrose, sorbitol, mannose and xylose. Five strains only were positive for arabinose and one for dulcitol. All isolates were negative for lactose, and positive for indole, while citrate utilization, hydrogen sulphide, urea hydrolysis were negative. No growth occur on MacConkey's agar and haemolysis was not produced on blood agar.

**Hussein (2000)** found that all *Pasteurella multocida* isolated from rabbit, were positive for indole, nitrate reduction, catalase and oxidase tests and that all isolates produced acid on triple sugar iron agar. On the other hand, all tested isolates were negative for methyl red, voges proskaeur, citrate and urea test.

**2.3.5. Antigenic characters and serotyping systems:**

**Roberts (1947)** classified *Pasteurella multocida* into four immunological types I, II, III and IV by using mouse protection test.

**Carter (1955)** used haemogglutination test for identification of serological types of *Pasteurella multocida.* He recognized four capsular types A, B, C and D.

**Namioka and Murata (1961)** used a slide agglutination test for somatic typing of *Pasteurella multocida* and found that Carter's capsular antigen type could be divided into several somatic antigen groups and said

that *Pasteurella multocida* organisms serological typing should be made by combining Carter's capsule groups with somatic groups.

**Carter (1967)** found that serotype A and D were the most commonly isolated *Pasteurella multocida* strains from rabbits.

**Namioka (1970)** stated that *Pasteurella multocida* could be classified on the basis of capsular antigens into four serotypes A, B, D and E by the slide agglutination test.

**Heddleston *et al.* (1972)** used gel diffusion precipitin test for serotyping *Pasteurella multocida* recorded from avian species on the bases of somatic antigen and recognized 16 seroptypes.

**Brogden (1980)** among 48 *Pasteurella multocida* culture collected for rabbit over a 56 years period in U.S.A. found that serotypes 3 (25%), and 12 (66.7%) were the most prevalent serotypes.

**El-Ghawas (1980)** isolated 18 isolates of *Pasteurella multocida* from septicemic cases of domestic rabbits and found that 11 isolates belonged to serotype A:8, 2 isolates to A:1 and 4 isolates to D:2 and added that A:8 and D:2 and A:1 are pathogenic to mice while A:1 was not pathogenic for chicken and ducks.

**Digiacomo *et al.* (1983)** reported that serotyping of strains of *Pasteurella multocida* isolated from infected rabbits revealed that 93%were somatic antigen type 12.

**Lu *et al.* (1983)** in U.S.A. studied 111 *Pasteurella multocida* isolates recovered from healthy and diseased rabbits. They were typed for

capsular and somatic antigens by typing systems of Carter and Heddleston, respectively. The major serotypes of 48 *Pasteurella multocida* strains isolated from nasal cavities of healthy rabbits were as follows: 33% A:12, 50% were non-typable A while 10% where non-typable D. Meanwhile, the major of all isolates were as follows; 32% serotype A:12, 30% non-typable: A and 16% were A:3.

**Cary *et al.* (1984)**reported that the most commonly somatic serotypes isolated from rabbits in the United States are 1, 3, 4, 12 and 15.

**Lin *et al.* (1984)** found that their twelve virulent isolates of *Pasteurella multocida* from rabbits were capsular antigen type A and Somatic antigen type 5.

**Klaassen *et al.* (1985)** isolated strains of *Pasteurella multocida* from adult rabbits with mucopurrlent rhinits and identified these strains as serotype 12 by the gel diffusion precipitation test and as capsular type A by staphylococcal hyaluronidase.

**Holmes *et al.* (1986)** found that serotyping of strains isolated from nasopharynx of rabbit were estimated to be serotype 3 or 12.

**Lukas *et al.* (1987)** used the gel diffusion precipitin test for serotyping of 44 *Pasteurella multocida* isolates and found that 57% were serotype 4, 27% were serotype 12 and 16% were serotypes 3.

**Digiacomo *et al.* (1989)** reported that isolates of *Pasteurella multocida* from rabbits with turbinate atrophy were serotype A: 12.

**Kawamoto *et al.* (1990)** concluded that type A: 12 is the predominant serotype in rabbit and their environment in Japan.

**Suckow *et al.* (1991)** isolated 147 *Pasteurella multocida* isolates from rabbits and found that 109 isolates were capsular type A, 19 isolates capsular type D, while 19 isolates were non-typable.

**Ibrahim (1993)** used indirect haemagglutination test for serotyping of twelve isolates of *Pasteurella multocida* from rabbits. He recorded that 9 strains belonged to capsular type A and 3 strain to capsular type D. By using the agar gel precipitation test, he found that all isolates belonged to somatic type 3, 11 and 12.

**El-Dirbi (1992)** studied the serotyping of 70 *Pasteurella multocida* isolates from rabbits. Six isolates were A:1, 15 isolates were A:4, 6 isolates were A:7, 8 isolates were A:9, two isolates were D:4 and one isolate was D:12. She concluded that 93% of all isolates were capsular type A and that 7% of isolates were cpasular type D. On the other hand, somatic typing revealed seven groups 1, 3, 4, 7, 9, 12 and 18.

**Nada (1994)** isolated *Pasteurella multocida* from apparently healthy and diseased rabbits and found that *Pasteurella multocida* serotype A:3 was the most predominant with a percentage of 42.1% followed by serotype A:12 with a percentage of 31.6% while serotype D:15 represented a percentage of 10.5%.

**Opacka *et al.* (1995)** found that the major serotypes among 145 *Pasteurella multocida* strains obtained from rabbits were serotypes 12 and

3 with a percentage of 54.5 and 40%, respectively. The capsular antigens type A and D occurred in 78.6% and 4.1% of the isolates respectively.

**Chaslus-Dancla *et al.* (1996)** examined forty one strains of *Pasteurella multocida* isolated from the middle ear of breeding does suffering from internal otitis, pneumonia, mastitis and metritis. Strains were of serotypes; A: 3 (27 strain), A: 5 (7 strain), A: 9 (4 strains) and A: 7 (3 strains).

**El-Shayeb (2000)** revealed that 61 strains of *Pasteurella multocida* with a percentage of 69% belonged to serotype A: 1 while 9 isolates (21.59%) belonged to type D, meanwhile 8 isolates (9.09%) were untypable.

**Takashima *et al.* (2001)** reported in Japan an outbreak of *Pasteurella multocida* infection in rabbits starting from sudden death to severe fibrinous and purulent pneumonia with hemorrhage. A large number of *Pasteurella multocida* A: 12 was isolated from the trachea and lung of rabbits.

**El-Tayeb *et al.* (2004)** characterized 39 isolates of *Pasteurella multocida* recovered from 553 apparently healthy rabbits with a percentage of 7%. Capsular typing showed that 31 isolates with percentage of 79% were capsular type A and 8 isolates (21%) were untypable. Somatic typing was as following; 19 isolates were somatic serotype 3 (49%), 12 were serotype 1 (31%), one isolate were serotype 2, two isolates were serotype 5, two were serotype 4 and two of the isolates (5%) were untypable.

**2.3.6. Natural and experimental infection in rabbit:**

**Watson *et al.* (1975)** infected rabbit of specific pathogen free colony intranasally with cultures of *Pasteurella multocida*. They noticed that inoculated rabbits developed a mucopurulent nasal discharge 4-7 days post inoculation.

**Bayoumi *et al.* (1984)** experimentally infected 25 rabbits with *Pasteurella multocida* alone or associated with *Diplococcus pneumonae, E. coli, Bordetella bronchiseptica* and tried reisolation of these organisms after postmortum examination. They indicated that such bacterial agents not only play an important role in initiating of rabbit pasteurellosis but also shared in severity of the disease.

**Percy *et al.* (1986)** experimentally inoculated domestic rabbits with *Pasteurella multocida* A:3 or D:3 either by aerosol or by intravenous or intratracheal inoculation. Animals either died or killed after 14 days were examined macroscopically and microscopically. They found that pneumonic lesions were most produced in rabbits inoculated intratracheally with serotype A:3, pulmonaryand pleural lesions were observed in some animals inoculated intravenously with serotype type A: 3, lesions were minimal in rabbits inoculated with serotype D: 3 and concluded that the intratracheal route appeared to be the best method to produce *Pasteurella* associated lesions in the lower respiratory tract.

**Glavits and Magyar (1990)** experimentally infected group of female New Zealand rabbits of 8-10 weeks old intranasal with three different *Pasteurella multocida* serotypes A: 3, A: 4 and A: 12. They found that seven out of 18 rabbits died and their necropsy and histology

revealed severe pleuritis with accumulation of fibrinopurulent exudate in the thoracic cavity, in addition to serious rhinitis and trachitis.

**Redonde *et al.* (1993)** infected rabbits experimentally with *Pasteurella* type A. The appeared symptoms were characteristics of the acute respiratory syndrome.

**2.3.7. Recent PCR-based techniques in molecular characterization of *Pasteurella multocida*:**

**Wilson *et al.* (1993)** observed that DNA fingerprint profiles of 50 *Pasteurella multocida* isolates from avians did not match profiles of the somatic type reference strains. They concluded that DNA fingerprinting is useful for accurate identification and epidemiologic study of *Pasteurella multocida* isolates.

**Chaslus-Dancla *et al.* (1996)** used random amplified polymorphic DNA (RAPD) assay to characterize 41 isolates from rabbits. They found that there was a relatively large amount of genetic hetrogenecity that existed among *Pasteurella multocida* isolated from rabbits and validate the use of RAPD assay for epidemiological studies of *Pasteurella multocida* strains.

**Zucker *et al.* (1996)** applied PCR fingerprinting technique to subtype 44 *Pasteurella multocida* subspecies multocida (P.M.SP.M) isolates from the respiratory system of pigs. Their results suggested that PCR fingerprinting is an efficient technique to detect DNA polymorphism within the species and added that this technique could be useful to differentiate *Pasteurella multocida* of the same capsular serotype.

**Schuur *et al.* (1997)** cultured *Pasteurella multocida* from the cerebrospinal fluid (CSF) of 4-month-old infant who was presented with meningitis after being scratched on its head by a cat. Culture of the cat's claws also yielded *Pasteurella multocida* and both isolates had identical biochemical patterns. Analysis of both strains by random amplification of polymorphic DNA (RAPD) and their comparison with *Pasteurella multocida* strains isolated from other cats showed that the two strains were identical and completely different from the unrelated isolates. They concluded that RAPD analysis provided strong evidence for the causal relationship between the cat scratch and patient's meningitis.

**Townsend *et al.* (1998)** analyzed *Pasteurella multocida* isolates by using a pair of primer that produced an amplification product unique to all *Pasteurella multocida* isolates. They also showed that PCR amplification performed directly on bacterial colonies or cultures represents an extremely rapid, sensitive method for *Pasteurella multocida* identification.

**Al-Haddawi *et al.* (1999)** used random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) to characterize forty isolates of *Pasteurella multocida* from healthy (17 isolates) and diseased (23 isolates) rabbits. They recorded that RAPD-PCR results showed the presence of a wide heterogenicity within *Pasteurella multocida* isolates. They concluded that RAPD-PCR is an efficient technique to detect the DNA polymorphism and that it could be used to discriminate *Pasteurella multocida* of rabbit isolates in association with serologic typing.

**Dabo *et al.* (1999)** used polymerase chain reaction (PCR) aiming to characterize rabbit *Pasteurella multocida* isolates. They concluded that

single primer PCR fingerprinting provide a simple and rapid mean of typing of *Pasteurella multocida* isolates from laboratory rabbits and recommended combination of conventional and molecular typing for differentiation among *Pasteurella multocida* isolated from rabbits with pasteurellosis.

**Dabo *et al.* (2000)** evaluated a rapid polymerase chain reaction (PCR) fingerprinting technique for discriminating among 33 *Pasteurella multocida* isolates from rabbits with clinical pasteurellosis and concluded that PCR is an efficient and reproducible method for discrimination of *Pasteurella multocida* from rabbits and could be performed directly using boiled bacterial extract as a source of template DNA.

**Dziva *et al.* (2001)** typed 81 *Pasteurella multocida* isolates from a variety of diseases in animals in Zimbabwe by both capsular typing and RAPD analysis. They found nine different groups of strains with identical RAPD profiles (100% similarity). Their finding showed no significant relationship between RAPD pattern and capsular serogroup. They suggested that RAPD analysis is a useful tool for strain differentiation.

**Huber *et al.* (2002)** used random amplified polymorphic DNA (RAPD) analysis and amplified fragment length polymorphism (AFLP) finger printing for comparing and analyzing between 61 isolates of *Pasteurella multocida* recovered from Turkeys that died of fowl cholera after being vaccinated with M-9 vaccinal strain. They found that both genetic techniques used, effectively showed genomic differences among the causative agent and they showed that pathogenic isolates from vaccinated Turkeys were more genetically similar to M-9 vaccine strain

than isolates from non-vaccinated Turkeys. Statistical analysis revealed that this relationship could not be determined by serotyping alone, indicating the value of RAPD and AFLP analysis in the characterization of diseased causing strains.

**Rocke *et al.* (2002)** used a serotype-specific polymerase chain reaction for identification of *Pasteurella multocida* serotype I and compared the sensitivity of this test with traditional culturing and serotyping techniques. They found that PCR could detect and identified bacteria in 44 cases compared with 45 cases by direct isolation as gold standard. They stated that serotype-specific PCR was much faster and less labor than traditional culturing and serotyping procedures and could result in diagnosis of serotype I pasteurellosis within 24 hours of specimen submission.

**Dziva *et al.* (2004)** used random amplified polymorphic DNA (RAPD) and ribotyping to determine the relationships between 21 *Pasteurella multocida* isolates from cases of atrophic rhinitis in pigs at Zimbabwe. Their study revealed three main clusters, however subclusters were also noted for each RAPD cluster. They stated that RAPD is an effective tool for discrimination of strains than ribotyping.

**Ozbey *et al.* (2004)** used random amplified polymorphic DNA (RAPD) assay to determine genetic differences among a total of 46 *Pasteurella multocida* strains obtained from lungs of cattle, sheep and goats. They obtained distinct band profiles among these strains and results of this study indicated that little genetic heterogeneity exists among *Pasteurella multocida* isolates from cattle and sheep.

# 3. MATERIAL AND METHODS

## 3.1. Material:

### 3.1.1. Samples:

A total of 428 samples were collected from apparently healthy as well as clinically diseased rabbits, and recently dead rabbits.

#### 3.1.1.1. Samples collected from apparently healthy rabbits:

A total of 112 nasal swabs collected from apparently healthy rabbits of different age, sex and localities in Kafr El-Sheikh Governorate as shown in table (1)

#### 3.1.1.2. Samples collected from clinically diseased rabbits:

A number of 278 nasal swabs, 34 lung tissues, two subcutaneous abscesses swabs, one liver tissue sample and one endometrial swab were collected from clinically diseased rabbits with a total number of 316 samples as shown in table (2).

Samples were transferred to the laboratory in ice tank without delay for bacteriological examination.

**Table (1):** Samples collected from apparently healthy rabbits in different localities in 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 Governorate.

| Locality | No. of samples | | Types of samples | Symptoms |
|---|---|---|---|---|
| **I. Governmental farms** | | | | |
| Sakha Animal Production Research Institute | 46 | 26 | Nasal swabs | Rhinitis |
| | | 20 | Lung tissues | congested lung |
| Fac. Agric., Kafr El-Sheikh Experimental Farm | 23 | 10 | Nasal swabs | Purulent exudate from nose |
| | | 12 | lung tissues | Congested lungs |
| | | 1 | Liver tissues | Congestion |
| **II. Private farms** | | | | |
| El-Hamoul | 95 | 94 | Nasal swabs | Rhinitis |
| | | 1 | Endometrial swab | Yellowish thick pus from vagina |
| Bialla | 32 | 30 | Nasal swabs | Rhinitis, emaciation |
| | | 2 | Lung tissues | consolidation |
| Sedi-Salem | 51 | 50 | Nasal swabs | Severe rhinitis |
| | | 1 | Subcutaneous abscess swab | Abscess + pus |
| Desouq | 47 | 46 | Nasal swabs | Rhinitis |
| | | 1 | Abscess swab | Abscess + pus |
| Ariamon | 22 | 22 | Nasal swabs | Rhinitis |
| Total | 316 | 316 | | |

### 3.1.2. Media:

#### 3.1.2.1. Media used for isolation of pasteurella:

**3.1.2.1.1. Nutrient broth (Oxoid):**

It was used as enrichment fluid media during collection of swabs samples and used for growth and multiplication of pure culture.

**3.1.2.1.2. 0.5% Semisolid soft agar (Bailey and Scott, 1974):**

It was used for preservation of the isolated *Pasteurella multocida*.

**3.1.2.1.3. Blood agar (Cruickshank *et al.*, 1975):**

Nutrient agar base plus 7% defribrinated sheep blood was used as enriched medium for the isolation of *Pasteurella multocida*.

**3.1.2.1.4. MacConkey's agar medium (Cruickshank *et al.*, 1975):**

It was used as selective and indicator medium to differentiate between strains of *Pasteurella multocida* and *Pasteurella haemolytica* and member of family enterobacteriaceae.

**3.1.2.1.5. Tryptose agar (Difco):**

It was used for demonstration of colonial morphology of *Pasteurella multocida*.

#### 3.1.2.2. Media used for biochemical reactions:

The following media were used according to **Cruichshank *et al.* (1975)**.

**3.1.2.2.1. Peptone water medium 2%:**

It was used for the detection of indole production.

**3.1.2.2.2. Simmon's Citrate agar: (Oxoid)**

It was used for citrate utilization test.

**3.1.2.2.3. Christensen's urea media (Difco):**

It was used for testing the urease enzyme activity of isolated *Pasteurella* species.

**3.1.2.2.4. Triple sugar iron agar media (Gibco):**

It was used for detection of hydrogen sulphide production as well as the fermentation of glucose, lactose and sucrose.

**3.1.2.2.5. Sugar media:**

Using 1% peptone water containing andrade's indicator to which 1% of the following sugar were added; glucose, lactose, sucrose, mannitol dulcitol; salicin; sorbitol and arabinose.

**3.1.2.2.6. Glucose phosphate broth:**

It was used for both methyl red reaction and Voges Proskauer Tests.

**3.1.2.2.7. Gelatin liquifaction media:**

It was used for detection of gelatin liquefaction at 22°C.

**3.1.2.3. Media used for preparation of genomic DNA:**

1. Brain heart infusion broth medium (Oxoid) it was used for making heavy culture of pasteurella strains.
2. Brain heart infusion agar medium (Oxoid).

### 3.1.3. Reagents and solutions:

**3.1.3.1. Kovac's reagent for indole test:**

| | |
|---|---|
| Pure isoamyl alcohol. | 150 ml |
| D-dimethyle amino benzaldehyde | 50 g |
| Concentrated HCl | 50 ml |

**3.1.3.2. Methyl red: (pH indicator for methyl red test):**

Methyl red (0.1 g) was dissolved in 300 ml of 95% ethyl alcohol (then 200 µl of distilled water were also added).

**3.1.3.3. Voges-proskauer reagent for Voges proskauer test:**

a. Alpha naphthol (5% in absolute ethyl alcohol 5 g).

b.

| | |
|---|---|
| Potassium hydroxide (40%) | 40 g |
| Creatine (3%) | 0.3 g |
| Distilled water | 100 ml |

**3.1.3.4. Hydrogen peroxide ($H_2O_2$):**

30% $H_2O_2$ Merck-Schucharadt, F. Germany (lot NO. 631 K2865597. Art 8597) it was used for performing catalase test.

**3.1.3.5. Oxidase reagent:**

1% solution of tetramethyl p. phenylene diamine dihydrochloride used as reagent in oxidase test.

The reagent was freshly prepared for use.

**3.1.3.6. Normal saline:**

A total of 8.5 g sodium chloride dissolved in 1 liter distilled water and autoclaved at 121°C for 15 minutes. It was used in the indirect haemaglutination test for serum dilution.

**3.1.4. Stains used:**

The following stains were performed according to **Cruickshank *et al.* (1975)**.

**3.1.4.1. Gram's stain:**

To differentiate between the isolated organisms into the classical Gram positive and Gram negative isolates.

**3.1.4.2.1. Leishman's stain, Giemsa stain:**

They were used to detect the pasteurella microorganisms bipolarity in blood smears.

**3.1.5. Strains of *Pasteurella multocida*:**

**3.1.5.1. References *Pasteurella multocida* vaccinal strains:**

A:5, A:9 and D:2 were obtained from Veterinary Serum and Vaccine Research Institute, Abbassia, Cairo.

**3.1.5.2. Lyophylised field strain:**

The following field strains (A:3, A:9, D:2, A:9, D:2, A:5) were isolated over a period of ten years from different outbreaks of rabbits pasteurellosis at different localities in Egypt. They were obtained kindly from the Bacterial Vaccine Department, Veterinary Serum and Vaccine Research institute, Abbassia, Cairo.

**3.1.6. *Pasteurella* antisera**

**Anti-K-sera:**

Used for identification of the capsular types of the isolates strains of *Pasteurella multocida* using indirect haemagglutination test (IHA) as described by **Carter and Rappy (1962).**

**Anti-O-sera:**

Used for identification of "O" group of the isolated *Pasteurella multocida* stains using agglutination test as described by **Namioka and Murata (1961)**. Both anti-K and anti-O sera were obtained from the aerobic bacterial Vaccine Department, Veterinary Serum and Vaccine Research Institute, Abbasia, Cairo, Egypt.

### 3.1.7. Laboratory animals:

#### 3.1.7.1. White mice:

300 white mice of 18-25 grams were used for purification and pathogenicity of *Pasteurella multocida*. These mice were obtained from the mice farm at Vet. Serum and Vaccines Research Institute, Abassia, Cairo.

#### 3.1.7.2. Rabbits:

50 native rabbits ranging in weight between 1-1.5 kg were used for passage of *Pasteurella multocida* strains.

Two month old rabbits of both sex ranging in weight between 1.5-2 kgs were used for the preparation of antisera against capsular and somatic antigens of *Pasteurella multocida*. All rabbits were from Vet. Serum and Vaccine Research Institute Abassia, Cairo.

### 3.1.8. Chemicals, reagents and buffers used in molecular studies of *Pasteurella multocida*:

#### 3.1.8.1. Chemicals, reagents and buffers used for DNA extraction:

All chemicals, reagents and buffers were prepared according to **Sambrook *et al.* (1989), Wilson *et al.* (1992) and Ausubel *et al.* (1999)**.

#### 3.1.8.2. Tris-HCl:

Tris buffer [0.05 M] of pH (7.1-8.9) was prepared by mixing 50 ml of 0.1 M Tris with the indicated volume of 0.1 N HCl and the volume of mixture was then completed to 100 ml with double distilled water (dd $H_2O$). It was used in the preparation of Tris-EDTA [TE] buffer.

**3.1.8.3. 1 mM EDTA:**

0.5 M EDTA of pH 8.0 was prepared by dissolving 186.1 g $Na_2$ EDTA. $2H_2O$ in 700 ml d.d $H_2O$. Adjusted pH to 8.0 with 10.0 M NaOH [50 ml]. Then the mixture was completed to 1 liter by adding d.d $H_2O$.

**3.1.8.4. TE buffer:**

It was prepared as follow:

| | |
|---|---|
| Tris-HCl (pH 8.0) | 10 mM |
| EDTA | 10 mM |

It was used for dissolving of DNA.

**3.1.8.5. Sodium dodecyl sulfate [SDS] solution:**

Stock solution [SDS 10%, w/v] was prepared by dissolving 10 g of SDS in 100 ml double distilled water. The solution was stored at room temperature. It was used for lysis of the bacterial cells.

**3.1.8.6. Proteinase "K" solution [Sigma]:**

It was used in a concentration of 20 mg/ml of d.d $H_2O$, for extraction of DNA. The solution was stored at -20°C.

**3.1.8.7. 5M NaCl:**

It was prepared by dissolving 292 g of NaCl in 12 liter of d.d $H_2O$.

**3.1.8.8. Buffered saturated phenol:**

It was used in purification of nucleic acids. It was saturated with 10 mM Tris of pH 8.0.

**3.1.8.9. Chloroform/isoamyl alcohol [Sigma]:**

It was used for extraction of nucleic acids. It was prepared by adding 24 ml of chloroform to 1 ml of isoamyl alcohol.

**3.1.8.10. Phenol, Chloroform, Isoamyl alcohol**

It was used for purification of DNA. It was prepared as a mixture of the phenol, chloroform, isoamyl alcohol at a concentration of 25: 24: 1.

**3.1.8.11. CTAB/NaCl solution:**

It was prepared as follow:

| | |
|---|---|
| Hexadecyle trimethyl ammonium bromide | 10% |
| NaCl | 0.7 M |

It was used for elimination of cell wall debris, polysaccharides and denatured proteins by selective precipitation.

**3.1.8.12. Isopropanol/[Sigma]:**

It was used for precipitation of DNA.

**3.1.8.13. 70% Ethanol:**

It was used for washing of DNA. It was prepared by adding 70 ml of absolute ethanol to 30 ml of d.d $H_2O$.

**3.1.8.14. Tris acetate EDTA (TAE) electrophoresis buffer (50xstock):**

| | | |
|---|---|---|
| Trisbase | 242.0 g | 50 x |
| Glacial acetic acid | 57.1 ml | |
| EDTA 0.5 M (pH 8.0) | 100.0 ml | |
| D.DW To | 1000 ml | |

Dilute to 1x in DDW and cool before electrophoresis use 1 x buffer for preparation of gels.

**3.1.8.15. Ethedium bromide solution (Stock solution):**

| | |
|---|---|
| Ethedium bromide powder | 10 µg |
| DEPC treated sterile D.W. | 1.0 µl |
| Mix and store covered at -4°C put in | |
| Melted agarose. To reach a final | |
| Concentration of 1-0.5 µg/ml. | |

It was prepared as stock of 10 mg/ml. It was used in concentration of 0.5 mg/ml for staining of DNA in agarose gel. The solution was stored at room temperature.

**3.1.8.16.Gel loading buffer (6 x stock)**

| | |
|---|---|
| Bromophenol blue | 0.25% |
| Xylene cyanol FF | 0.25% |
| Glycerol | 30.0% |

Component are dissolved in sterile D.D.W. and stored covered with aluminum foil at room temperature.

**3.1.8.17. RAPD Marker:**

Super ladder DW 100 bp obtained from Abgene, UK.

**3.1.8.18. Single primer used in RAPD assay**

Code D14803

Primer sequence (5'-3')

5'-AAACGGTTGGGTGAG-3'

**3.1.8.19. Specific primer for *Pasteurella multoicida***

**3.1.8.19.1. Forward primer**

Code KMTIsP6 sequence

5'GCTGTAAACGAACTCGCCAC.3'

**3.1.8.19.2. Reverse primer:**

Code KMTiT7 5'ATCCGCTATTTACCEAGTGG3' obtained from MWG Biotech. AG, Berlin, Germany.

These primers were synthesized according to **Townsend *et al.* (1998)** depending on the unique sequence specific to type B:2 *Pasteurella multocida* isolates **(Townsend, *et al.*, 1996)**.

**3.1.8.19.3. Agarose gel (sigma):**

Prepared by dissolving 2 g of agarose in100 ml of 1 x TAE buffer (Tris acetate EDTA) electrophoresis buffer. It was used for electrophoretic separation of amplified nucleic acid segments (DNA).

**Table (3):** Chemicals used in one specific PCR reaction.

| No. | Components | Master mix content |
|---|---|---|
| 1 | 10x buffer (Promega) | 5 µl |
| 2 | dNTP | 1 µl |
| 3 | Forward primer | 0.5 µl |
| 4 | Reverse primer | 0.5 µl |
| 5 | *Taq*. DAN polymerase (Promega) | 1 µl |
| 6 | Template DNA | 10 µl |
| | Sterile D.D. water | 32 µl |
| | Total volume of each master mix | 50 ul |

x = means conc.

**Table (4)**: Chemical used in one RAPD-PCR reaction.

| No. | Components | Master mix content |
|---|---|---|
| 1 | 10 x PCR buffer (Promega) | 5 µl |
| 2 | dNTPS (Promega) | 2 µl |
| 3 | D14803 Primer | 1 µl |
| 4 | *Taq.* DNA-polymerase (Promega) | 2 µl |
| 5 | Template DNA | 10 µl |
| 6 | Sterile D.D. water | 30 µl |
| | Total volume of each master mix | 50 µl |

x= mean conc.

## 3.2. Methods:

### 3.2.1. Collection of samples for bacteriological examination:

#### 3.2.1.1. Nasal swabs:

The samples were collected under aseptic condition by means' of sterile cotton swabs and transported to the laboratory as quickly as possible in peptone water (1%).

#### 3.2.1.2. Recently dead rabbits:

Internal organs; heart, liver, lung were collected under aseptic condition. In sterile plastic bags and transported with minimum delay to the laboratory for bacteriological examination.

### 3.2.2. Isolation of *Pasteurella multocida*:

#### 3.2.2.1. From nasal swabs:

The samples (Nasal swabs) were inoculated directly into blood agar media, tryptose agar media and MacConkey's agar plate media.

Then examined for suspected *Pasteurella multocida* colonies.

Between different colonies which appeared on the surface plate, suspected colony (non-haemolytic dew drop like colonies) were selected and subcultured onto 7% sheep blood agar and MacConkey's agar and films were made from grown colonies and stained with Gram's stain and examined microscopically.

#### 3.2.2.2. From internal organs:

The surface of pneumonic lung tissues and liver were seased by red hot spatula for sterilization and grinding in sterile morter with sterile tryptose soya broth and a dropfull of grinded solution were inoculated on sheep blood agar and MacConkey's agar inoculated at 37°C for 24 hours.

The suspected colonies were picked up and subcultured for further identification.

### 3.2.3. Purification of the isolated *Pasteurella multocida* strains:

Each culture showing gram negative coccobacilli and not give growth on MacConkey's agar were inoculated in 3 white mice each mouse was inoculated by 0.2 ml of the suspected culture subcutaneous (S/C). The mice were kept under observation for 72 hours after inoculation. Dead mice were necropsied and by using sterile Pasteur pipette. Aspirated blood from heart was inoculated in tryptose soya broth and blood agar to isolate *Pasteurella multocida* from heart blood. Blood films were prepared from the blood of dead mice and stained with Leishmain's stain or Giemsa stain after fixation by methyl alcohol, films were examined for the presence of bipolar organism.

### 3.2.4. Biochemical identification of the obtained isolates:

It was done according to **Cruickshank *et al.* (1975)**.

#### 3.2.4.1. Catalase test:

A loopfull of hydrogen peroxide was put on a clean glass slide and then a loopfull of each isolate was added. The positive result was detected by foaming.

#### 3.2.4.2. Indole test:

To 24 hours peptone water cultures about 0.5 ml of Kovac's reagent was gently trickled drop by drop down the side of the tube. The development of rosy ring at the surface indicated positive result. No change (yellowing) at the surface indicate negative result.

**3.2.4.3.Oxidase test (Wolf *et al.*, 1975):**

Filter paper (6 $cm^2$ of filter paper) was placed in a petri dish and 2-3 drops of oxidase reagent were added to the center of the paper with a glass loop, a loopfull of one colony was streaked on the reagent impregnated paper. A positive blue color occur within 5-10 second.

**3.2.4.4. Urea utilization test:**

The Christensen's medium was heavily inoculated with the isolates and inoculated at 37°C the medium was examined after 24-18 hours up to 96 hours. Urea positive organisms produced a purple pink color due to splitting of ammonia (alkaline).

**3.2.4.5. Citrate test:**

Simmon's citrate medium was inoculated from bacterial culture of the organism to be tested, over the slope surface of the medium and incubated for 24 hours at 37°C. Positive reaction indicated by growth of culture and change the colour of the medium from green to blue.

Negative reaction no growth nor change in colour of medium.

**3.2.4.6. Triple sugar iron agar (T.S.I.):**

Double inoculation were made from a purified solid culture medium by stabbing into the depth of the medium (butt) and by streaking on the surface of slant. Tubes were incubated at 37°C for 18-24 hours. A positive reaction was indicated by presence of black coloration at the butt of the slope. Absence of black color indicated negative $H_2S$ and yellow coloration indicated sugar fermentation

**3.2.4.7. Sugar fermentation:**

The peptone water with 1% of different types of sugars were inoculated with the organism and incubated for about 1-7 days at 37°C results were recorded daily for the production of acid which appear as pink

colour (Andrad's used as indicator) and gas production (this was determined by Durham's tubes.

**3.2.4.8. Gelatin liquifaction test:**

The medium was stabbed with a bacterial growth from 24 hours pure culture broth to a depth of 1/2 inch. A control tube was set up (uninoculated). The inoculated tubes were incubated at 35°C for 1-14 days every 24 hours both tubes were placed in refrigerator for 2 hours to determine whether gelatin was liquified or not. Other tubes were checked daily for up to 2 weeks.

### 3.2.5. Pathogenicity test (according to Ali, 1991):

Twenty four hours pure culture of each isolate of *Pasteurella multocida* was suspended in sterile saline, then the turbidity was compared with standard turbidity tube (MacFarland 0.5 barium sulphate). The bacterial suspension containing approximately 7 x $10^8$ viable organisms per ml. (0.2 ml of each isolate of *Pasteurella multocida* suspension) was injected intraperitoneall in 2 white mice. The control group was injected with 2 ml sterile saline I.P. Mice were observed for 24-72 hours where dead mice were dissected and smears from the heart blood and parenchymatous organs were stained with Leishman's stain for the demonstration of bipolarity as well as reisolation of the causative organisms onto 10% sheep blood agar was done.

### 3.2.6. Serological typing of *Pasteurella multocida*:

**3.2.6.1. Capsular typing of *Pasteurella multocida*:**

It was performed according to **Carter and Rappy (1962)** for capsular typing using indirect haemagglutination test as follows:

**a. Preparation of formalized erythrocytes:**

1. Fresh sheep blood was carefully washed five times with 0.85% cold saline and the cells were packed after the final wash (15 minutes at 3000 rpm).
2. The washed packed cells were then resuspended in eight volume of phosphate buffer saline at pH 6.8-6.9 in a 500 ml conical flask.
3. About 50 ml of 40% formaldehyde solution pH 5.5-6.0 was introduced into a length of dialysis tubing and tied off so that the tubing was only 2/3 full, but the air was excluded. The dialysis bag was placed in a flask which was then agitated at room temperature on a mechanical shaker for two hours at a speed that provide the most vigorous mixing action with minimal foaming.
4. After agitation for two hours, the contents of the dialysis sac was poured into the flak and the shaking was continued for an additional 16-18 hours. The suspension was then filtered through gauze to remove cell debris.
5. Half volume of saline was added to filtered cell suspension, after which the cells were washed six times in ten volumes of saline by centrifugation at 3000 rpm for 10 minutes.
6. Finally, the packed cells were resuspended in 0.85% saline to make 50% suspension. The cells were then bottled in convenient amount and freeze dried.

**b. Preparation of capsular extracts of *Pasteurella multocida*:**

The 18-24 hours confluent growth from a blood agar plate was washed off with 4-5 ml of physiological saline and heated at 56°C for 30

minutes. The bacterial cells were then separated by centrifugation and the supernatant fluid was transferred to another tube.

**c. Sensitization of formalinized erythrocytes:**

To three ml of bacterial extract 0.2 ml of packed red ells were added and after thorough mixing incubated at 37°C for two hours and were then separated by centrifugation. The treated erythrocytes were then washed three times with 10 ml amounts of saline after which sufficient saline was added to give a 1% suspension.

**d. Preparation of immune serum (Carter, 1955):**

All immune sera were adsorbed by addition of 0.2 ml of packed washed formalinized red cells to each 1.5 ml serum to remove non-specific haemagglutinins. After incubation for several hours, the cells were removed by centrifugation.

**e. Test procedure:**

1. Doubling dilutions of the anti-K-sera were made in the wells of multi-hole lucite perspex plate. The dilutions used were obtained with the following amounts.
2. The two controls used were:

a. 0.4 ml of 1% suspension of treated cells + 0.4 ml saline.

b. 0.4 ml of 1: 5 dilution of the serum used + 0.4 ml of 1% suspension of untreated red cells.

3. The plates were shaken for 10 minutes, then left at room temperature for approximately two hours at which a reading was taken. A second reading was taken after overnight incubation in the refrigerator. Positive reactions consisted of a marked diffuse haemagglutination

while a negative pattern consisted of compact sharply demarcated disc of sedimented cells (button like).

**3.2.6.2. Somatic typing of *Pasteurella multocida*: According to Namioka and Murata (1961):**

**Preparation of somatic antigens**:

Each culture was seeded on a YPC agar plate and incubated at 37°C for one hour. Growing germs were collected in saline and washed once. The packed cells from each plate were treated as follows:

a. They were suspended in sufficient 0.3 percent formalinized buffered saline to produce a turbidity corresponding to tube no. 4 or McFarland's nephelometer.

b. The antigen was then heated at 75 °C for 1 hour, at 100 °C for 1 hour, and at 121°C for 2 hours.

c. Packed cells collected from a YPC agar plate were suspended in 2 ml of buffered saline. To the suspension, was added an equal volume of absolute alcohol. The mixture was incubated at 37 °C for 18 hours in a plugged tube. Then, it was centrifuged and the packed cells were resuspended in buffered saline so that the resulting suspension equaled the turbidity of tube no. 4 of McFarland's nephelometer.

d. Packed cells obtained by seeding a 16-hour-old culture (200 mg in weight) on a YPC agar plate were suspended in 10 ml of N HCl saline incubated in a plugged tube at 37 °C for 16 to 18 hours. After the incubation, the suspension was centrifuged and the sedimented organisms were washed twice with 0.3 percent formalinized buffered saline. The packed cells were resuspended in 20 ml of buffered saline. The final pH of the suspension was adjusted to 7.0 with a 10 percent

solution of sodium bicarbonate (HCl antigen). When auto agglutination was observed in the suspension at pH 7.0, the culture employed was antigenically rough. Such cultures were discarded.

**Antisera**:

Antisera were prepared with formalinized antigen (a), boiled antigen (b), and HCl antigen (d), respectively, at a turbidity to match tube no. 4 of McFarland's nephelometer. Rabbits received 15 or more intravenous injections in amounts of 0.5, 1, 2, 4, 6, 6, .....6 ml, respectively, at 5-day intervals. The suitable weight of a rabbit for immunization was 2000 g or less. It should be emphasized that it was somewhat difficult to obtain high-titer serum with HCl antigen. If the antisera prepared were not satisfactory in titer, more injections were necessary. Blood was collected when the serum titer was 320 or more.

**Tube agglutination tests:**

Tube agglutination tests were employed. The tests were carried out with fomalinized, heated, alcohol, and HCl antigens, at the turbidity of tube no. 1 of MacFarland's nephelometer. Results of tube agglutination tests were read after incubation at 37 °C for 18 hours.

**3.2.7. Method of genotypic characterization:**

**3.2.7.1. Polymerase chain reaction for identification of *Pasteurella multocida* isolates:**

**3.2.7.1.1. Extraction of *Pasteurella multocida* DNA:**

A. Extraction of *Pasteurella genomic* DNA:

DNA was purified as described by **Wilson (1987) and Ausubel *et al.* (1999)** as follow:

1. *Pasteurella* species strains were grown into brain heart infusion broth for 24 hours at 37°C with shaking.
2. A 1.5 ml quantity of culture from each *Past. multocida* strain was centrifuged in a microcentrifuge at 2000 rpm for 5 minutes at 4°C.
3. The pellet was resuspended in 567 µl of TE buffer [10 mM Tris-HCl, 1 mM EDTA (pH 7.5)].
4. Bacteria were lysed by the addition of 30 µl of 10% SDS and 3 µl of proteinase K (20 mg/ml) followed by incubation for 1 hour at 37°C.
5. Cell wall debris, polysaccharides and denatured proteins were eliminated by precipitation with addition of 100 µl of 5 M NaCl, 80 µl of CTAB/NaCl (10% hexadecyltrimethyl ammonium bromide in 0.7 M NaCl), mixed and incubated at 65°C for 10 minutes.
6. Seven hundred microliter of chloroform/isoamyl alcohol (24: 1) was added to each sample then centrifuged at 12000 rpm for 4 minutes.
7. The aqueous viscous supernatant was removed to a fresh microcentrifuge tube and equal volume of phenol/chloroform/isoamyl alcohol (25: 24: 1) was added to each sample, then centrifuged in a microcentrifuge at 12000 rpm for 5 minutes.
8. The aqueous phase was transferred to a new tube, and the DNA was precipitated with 0.6 ml volume Isopropanol and pelleted by centrifugation at 12000 rpm for 30 minutes at 4°C.
9. The pellet was washed with 70% ethanol to remove any residue of CTAB and recentrifuged for 5 minutes at room temperature to repellet it.
10. The supernatant was decanted and the tube inverted on absorbent paper for 3 minutes, to dry and then dissolved in 100 µl of Tris-EDTA buffer (pH: 8.0).

**3.2.7.1.3. Preparation of specific PCR reaction:**

Specific PCR reaction of DNA from *Pasteurella multocida* was performed according to the method of **Townsend *et al.* (1998)** with some modification. The reaction were carried out in a volume 50 µl 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 for one RAPD-PCR reaction |
|---|---|
| D.D $H_2O$ | 32 µl |
| 10 x buffer | 5 µl |
| dNTP | 1 µl |
| Primer1 (forward) | 0.5 µl |
| Primer 2 (reverse) | 0.5 µl |
| *Taq*. polymerase | 1 µl |
| Template DNA solution | 10 µl |
| Total | 50 µl |

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

**3.2.7.1.4. 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 a (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.

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

**3.2.7.2.1. Extraction on of *Pasteurella multocida* genomic DNA:**

As mentioned in 3.2.7.1.1.

**3.2.7.2.2. Preparation of RAPD PCR reactions:**

Random amplification of DNA from *Pasteurella multocida* was performed according to the method of **Chaslus-Dancla *et al.* (1996)** 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 for one RAPD-PCR reaction |
|---|---|
| D.D $H_2O$ | 30 µl |
| 10 x buffer | 5 µl |
| dNTP mix | 2 µl |
| Primer (single primer) | 1 µl |
| *Taq* polymerase | 2 µl |
| Template DNA solution | 10 µl |
| Total | 50 µl |

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

**3.2.7.2.3. 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.

**3.2.7.2.4. 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. 12 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.

**3.2.7.2.5. Visualization and photography:**

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

**3.2.7.2.6. Computer analysis of RAPD-PCR results:**

was done using Gel Pro Software, USA.

# 4. RESULTS

## 4.1. Prevalence of *Pasteurella multocida* in rabbits located in Kafr El-Sheikh Governorate:

In this trial a total of 428 samples collected from apparently healthy as well as clinically diseased rabbits and recently dead at different farms at Kafr El-Sheikh Governorate.

The presented results revealed the isolation of 55 isolates of *Pasteurella multocida* from nasopharyngeal swabs from apparently healthy and clinically diseased and recently dead rabbits.

Nasopharyngeal swabs (112 samples) collected from apparently healthy rabbits yield 6 (5.3%) *Pasteurella multocida* isolates Table (5).

Nasopharyngeal swabs (278 samples) collected from diseased rabbits yield 42 (15%) *Pasteurella multocida* isolates, Table (6).

Samples collected from lung tissues (34 samples) yield 3 (8.8%) *Pasteurella multocida* isolates.

One isolate from liver tissues, another isolate from endometrium and two from subcutaneous abscess.

These isolates showed non haemolytic colonies on blood agar with rounded, greyish colonies with glistening surface and when examined microscopically they showed gram negative cocobacilli.

**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 | |
|---|---|---|---|---|---|---|---|
| | | Total | No. | Total | No. | Total | % |
| I. Governmental Farms | Sakha Animal Production Research Institute | 29 | 15 | 4 | 2 | 13.7% | 13.3% |
| | Faculty of Agriculture Farm | | 14 | | 2 | | 14.3% |
| II. Private farms | El-Maraska | 83 | 31 | 2 | 2 | 2.4% | 6.5% |
| | Kafr El-Sheikh | | 14 | | 0 | | 0% |
| | Desouk | | 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 | 46 | 26 | Nasal swabs | 2 | 3 | 6.5% |
| | | | 20 | Lung tissues | 1 | | |
| | Kafr El-Sheikh collage Agriculture Farm | 23 | 10 | Nasal swabs | 1 | 3 | 13% |
| | | | 12 | Lung tissues | 1 | | |
| | | | 1 | Liver tissues | 1 | | |
| II. Private farms | El-Hamoul | 95 | 94 | Nasal swabs | 18 | 19 | 20% |
| | | | 1 | Endometrial swabs | 1 | | |
| | Bialla | 32 | 30 | Nasal swabs | 13 | 14 | 43.8% |
| | | | 2 | Lung tissues | 1 | | |
| | Sedi-Salem | 51 | 50 | Nasal swabs | 3 | 4 | 7.8% |
| | | | 1 | Subcutaneous abscess | 1 | | |
| | Desouq | 47 | 46 | Nasal swab | 3 | 4 | 8.5% |
| | | | 1 | Subcutaneous abscess | 1 | | |
| | Ariamon | 22 | 22 | Nasal swab | 2 | 2 | 9% |
| **Total** | | **316** | **316** | | | **49** | **15.5%** |

## 4.2. Phenotypic characterization of isolated strains of *Pasteurella multocida*:

### 4.2.1. Biochemical activities of *Pasteurella multocida* isolates:

As shown in Table (7), all the examined *Pasteurella multocida* strains were indole, catalase and oxidase positive. all were urea hydrolysis and citrate utilization tests negative and all tested isolates produced acid on TSI (triple sugar iron) yellow butt and yellow slant and no $H_2S$ production (Hydrogen sulphide production).

All examined isolates failed to grown on MacConkey's agar and haemolysis was not produced on blood agar.

**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 |

### 4.2.2. Pathogenicity to mice:

Pathogenecity was tested 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 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 Figure (1) and Figure (2).

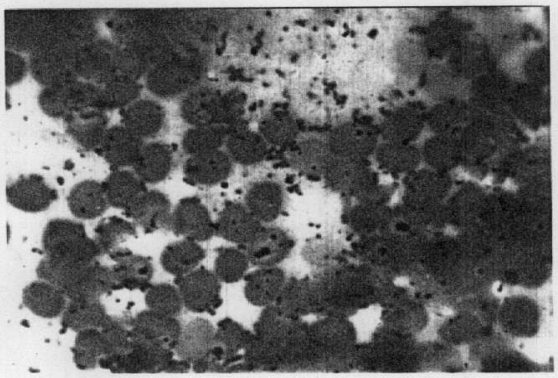

**Fig. (1):** *Pasteurella multocida* stained by Leishamn's stain showing bipolarity in blood smear (x 3000)

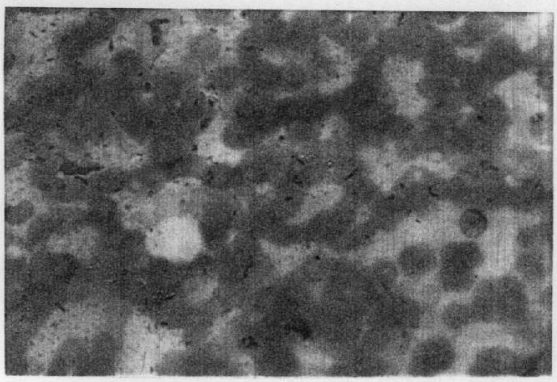

**Fig. (2):** *Pasteurella multocida* stained by Giemsa stain showing bipolarity in blood smear (x 3000).

### 4.2.3. Serological typing of isolated *Pasteurella multocida* strains:

#### 4.2.3.1. Capsular typing of *Pasteurella multocida*:

Capsular typing of the isolated strains of *Pasteurella multocida* was performed by the indirect haemogglutination test **(Carter and Rappy, 1962)**.

32 isolates were found to be serologically related to group A.
16 isolates were found to be serologically related to group D.
7 isolates untypable.

The incidence of positive haemaglutination reaction were 43.8%, 22.9%, 16.6%, 10.4% and 6.3% at titres 640, 1280, 320, 80 and 40, respectively.

The titres of the typable isolated strains of *Pasteurella multocida* are demonstrated in Table (8).

#### 4.2.3.2. Somatic typing of *Pasteurella multocida*:

Tube agglutination test **(Namioka and Murata, 1961)**. was used for somatic typing of the isolated strain of *Pasteurella multocida.*

21 isolates were found to be serologically related to type: 9.
11 isolates were found to be serologically related to type: 5.
16 isolates were found to be serologically related to type: 2.

Table (8) demonstrate the results of a slide agglutination test typing isolates of *P. multocida*.

**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% | | |

**4.2.4. Molecular characterization of isolated strains of *Pasteurella multocida:***

**4.2.4.1. PCR based assay for molecular detection of *Pasteurella multocida* using species specific primer as shown in Fig. (3)**

**4.2.4.2. RAPD-PCR assay for DNA fingerprinting of reference and field strains of *Pasteurella multocida* as shown in Fig. (4 & 5) and analysed in Tables (9 & 10)**

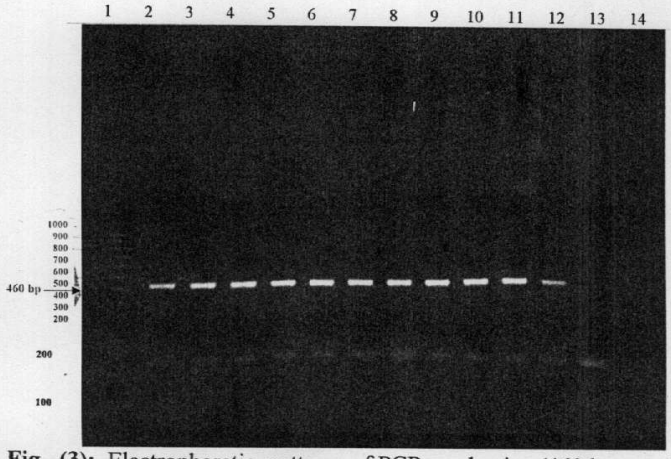


**Fig. (3):** Electrophoretic pattern of PCR production (460 bp specific for *Pasteurella multocida*) in 1.5% agarose gel stained with ethidium bromide.

Lane 1: DNA marker (100 bp marker)
Lane 2: PM A:5
Lane 3: PM A:9
Lane 4: PM D:2 } reference vaccinal strain*
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: PM A:9 → Field srain from Sharkia Governorate
Lane 9: PM D:2
Lane 10: PM A:9
Lane 11: PM D:2
Lane 12: PM A:9 } Field strain*
Lane 13: Negative control

* Veterinary Serum and Vaccine Research Institute, Abbasia, Cairo

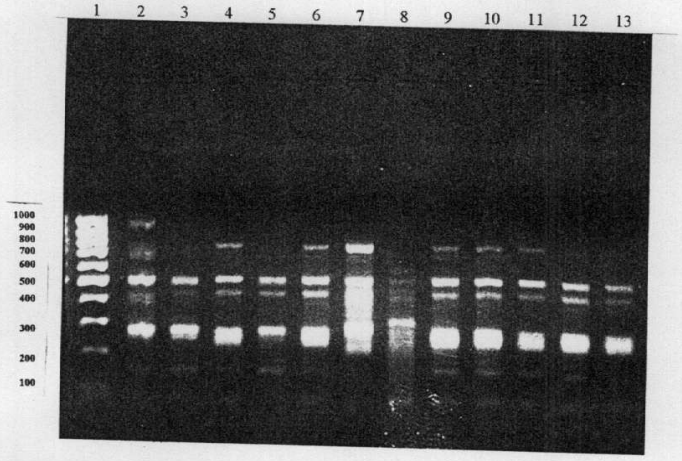


**Fig. (4):** The electrophoretic profile of RAPD-PCR of field and standard *Pasteurella multocida* genomic DNA as amplified by D14803 primer

Lane 1: DNA marker (100 bp marker)
Lane 2: PM A:5
Lane 3: PM A:9
Lane 4: PM D:2 } reference vaccinal strain*
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: *E. coli* → Field strain
Lane 9: PM A:9
Lane 10: PM A:9
Lane 11: PM A:9
Lane 12: PM A:9
Lane 13: PM A:9 } Field strain from Sharkia Governorate*

* Veterinary Serum and Vaccine Research Institute, Abbasia, Cairo

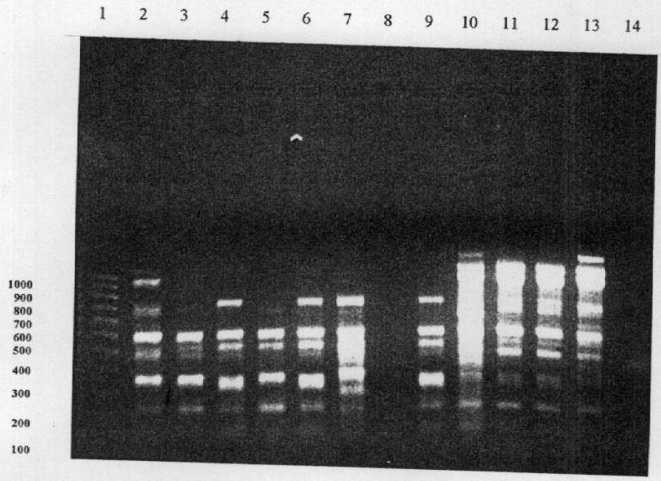


**Fig. (5):** The electrophoretic profile of RAPD-PCR of field and standard *Pasteurella multocida* genomic DNA as amplified by D14803 primer

Lane 1: DNA marker (100 bp marker)
Lane 2: PM A:5
Lane 3: PM A:9
Lane 4: PM D:2 } reference vaccinal strain*
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: PM D:2
Lane 11: PM A:9
Lane 12: PM D:2
Lane 13: PM A:5 } Field strain from Kafr El-Sheikh Governorate

* Veterinary Serum and Vaccine Research Institute, Abbasia, Cairo

**Table (9):** The molecular weights 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<br>324 | 388<br>324 | 388<br>324 | 388<br>324 | 388<br>324 |
| Band 8 | 300 | 324 | 326 | | | | 326<br>297 | - | | | | | |
| Band 9 | 200 | 254 | 252 | 252 | 252 | 254 | 252<br>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 weights 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 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 (D:2) field isolate No. 9 | Lane 11 DNA bands of P.M (A:9) field isolate No. 10 | Lane 12 DNA bands of P.M (D:2) field isolate No. 11 | Lane 13 DNA bands of P.M (A:5) field isolate No. 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Band 1 | 1000 | 1089 | | | | | | - | | 1694<br>1075 | 1075 | 1075 | 1694<br>1075 |
| Band 2 | 900 | 924 | | | | | | - | | 930 | 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<br>324 | | | 400 | 488 |
| Band 8 | 300 | 324 | 326 | | | | 326<br>297 | - | | 324 | 324 | 324 | 324 |
| Band 9 | 200 | 254 | 252 | 252 | 252 | 254 | 252<br>204 | - | 252 | 295<br>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 |

# DISCUSSION

Rabbit pasteurellosis is continue to be a major concern for the rabbit industry for being the major source of economic losses for many rabbiteries **(Digiacomo *et al.*, 1991).**

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, was estimated to be the most common bacterial pathogen of rabbits isolated from either diseased or apparently healthy animals (**Flatt, 1974; Percy *et al.*, 1986).**

The main objectives of this study were to investigate the prevalence of *Pasteurella multocida* in diseased as well as apparently healthy rabbits at different localities in Kafr El-Sheikh Governorate. Also identification and characterization of the isolated strains either by phenotypic methods or by recent PCR-based genotypic techniques was a main purpose of this study aiming to resolve relationships among bacterial isolates for epidemiological studies.

In the present investigation, as shown in Tables (5 & 6) 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% as shown in Table (6) with a total incidence of 12.3%. These results agree with those obtained by **Hussein (2000)** who found that out of 259 samples (nasal swabs) collected from apparently healthy and diseased rabbits from different governmental

and private Sharkia farms, 24 isolates were obtained with an total incidence of 9.3%. Also these results nearly agree with those obtained by **Nada (1994)** who found that out of 239 samples (nasal swabs) collected from apparently healthy and diseased rabbits, 19 rabbits were positive for *Pasteurella multocida* with an incidence of 7.9%. Also, **El-Dirbi (1992)** revealed that isolation of 70 strains of *Pasteurella multocida* of 470 samples collected from infected and apparently healthy rabbits obtained form different localities of Kaluobia governorate with an incidence of 14.8%.

On the other hand, **Digiacomo *et al.* (1983)** reported that the percentage of infection with *Pasteurella multocida* in adult rabbits was 72%. Also, **Deeb *et al.* (1990)** who recorded that at weaning, about 25% of rabbits had nasal infection with *Pasteurella multocida*.

Among apparently healthy rabbits *Pasteurella multocida* rate was 5.3%. These results nearly agree with the findings of **Elged *et al.* (1990)** who examined 80 nasal swabs from apparently healthy adult rabbits, and isolated *Pasteurella multocida* with an incidence of 3.8%. Also, **El-Dirbi (1992)** isolated 9 isolates from 150 nasal swabs from apparently healthy rabbits with an incidence of 6% and **Mercier (1992)** who showed that the carrier rate of *Pasteurella multocida* among apparently healthy rabbits in the upper respiratory tract was in between 2-3.9%. Also **Nada (1994)** examined 53 nasal swabs originated from apparently healthy rabbits, and he isolated *Pasteurella multocida* with an incidence of 3.8%. Finally **Hussein (2000)** among 150 nasal swabs from apparently healthy rabbits isolated *Pasteurella multocida* with an incidence rate of 4.8%.

On the other hand, these results contradicted with that obtained by **Rai *et al.* (1987)** who examined samples of rabbits nasal and vaginal swabs collected from 81 healthy rabbits, and they isolated *Pasteurella multocida* from 56.8 and 49.% of examined vaginal and nasal samples, respectively.

In the present study the incidence of *Pasteurella multocida* among diseased rabbits was 15.5%. This is nearly in accordance with the finding of **Percy *et al.* (1988)** who isolated *Pasteurella multocida* from dead rabbits showing various pathological lesions in an incidence of 11.9%. Also, **Elged *et al.* (1990)** who collected 185 samples from rabbits showing signs of pneumonia, rhinitis, otitis and osteoarthritis at different private farms of rabbits at Sharkia governorate. They indicated that the bacteriological examination of collected samples revealed that the incidence of *Pasteurella multocida* was 8.8, 11 and 5%. **El-Dirbi (1992)** also examined 320 samples form infected rabbits and isolated *Pasteurella multocida* with an incidence of 19%. Also, **Ibrahim (1993)**, examined 100 swabs of nasal discharge and pus collected from diseased rabbits and isolated *Pasteurella multocida* with an incidence of 12%. **Nada (1994)** examined 186 rabbits nasal swabs with clinical signs of respiratory disease and rhinitis and isolated *Pasteurella multocida* with an incidence of 9.1%. Finally **Hussein (2000)** examined 154 samples form diseased rabbits and isolated *Pasteurella multocida* with an incidence of 12.3%.

On the other hand, these results seemed to disagree with **Zaher *et al.* (1976)** who isolated *Pasteurella multocida* from 79% of diseased rabbits and **Digiacomo *et al.* (1983)** who reported that the percentage of infection with *Pasteurella multocida* in adult rabbits was 72%. Also, **Mahmoud and Abdel-Baset (1991)** who found that the mortality due to

*Pasteurella multocida* infection was high and ranged from 70-78% in the investigated flocks.

The isolated 55 strains in this study were subjected to a full phenotypic characterization where they showed smooth irridescent colonies and none of these strains showed a mucoid intermediate or rough colonies. These colonies fail to grow on MacConkey's agar plates, were non-haemolytic on blood agar media and negative for pigment production. Microscopic examination of these strains revealed that all examined strains were Gram-negative, coccobacilli, non-motile, non-sporulated, with round ends. When subjected to different biochemical reactions it was noticed that some reactions were constantly positive while others were constantly negative table (7). Among the constant positive reactions were indole oxidase, and catalase tests while negative reactions were urease and citrate utilization tests. All the examined strains were thus identified as *Pasteurella multocida*. These results were in agreement with those of **Brigham and Rettger (1935), El-Dirbi (1992), Ibrahim (1993) and Hussein (2000)**.

Pathogencity was tested in white mice, where mice inoculated with the suspected *Pasteurella multocida* isolates died within 24-72 hours showing generalized septcaemia with highly congested trachea and lungs. Pure culture of inoculated isolates were obtained from 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 Fig. (1 & 2).

The significance of identification by serotyping of strains of *Pasteurella multocida* which are associated with rabbit pasteurellosis

cannot be over emphasized where neglection of identification by serotyping tends to invalidate some research reports where Pasteurellae are involved.

In this study serotyping of *Pasteurella multocida* isolates indicated that 32 isolates with a percentage of 58% were capsular group "A" and that 16 isolates with a percentage of 29% were group "D" by using the indirect haemogglutination and 7 isolates were untypable with a percentage of 12.7%. On the other hand, somatic type were 9, 5 and 2 by using tube agglutination test. These results agreed with those of **Carter (1967)** who found that serotype A and D were the most commonly isolated *Pasteurella multocida* strains from rabbits and **Lu *et al.* (1983)** who concluded that serotype "A" was predominant from *Pasteurella multocida* isolated from rabbit. These results agreed also with **Lin *et al.* (1984)** who found that all the 12 virulent isolates of *Pasteurella multocida* from rabbits were capsular antigen type "A" and somatic antigen type 5. **Ibrahim (1993)** used indirect haemagglutination test for serotyping 12 isolates of *Pasteurella multocida* from rabbits and recorded that 9 strains belonged to capsular type "A" and 3 strain to capsular type "D". Also, **El-Shayeb (2000)** revealed that (69%) of *Pasteurella multocida* belonged to serotype "A" and (21.59%) belonged to type "D".

On the other hand, somatic serotyping showing some similarity and differences which may be due to differences in geographical location and uncontrol treatment with different antibiotic.

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, a suspected isolate as *Pasteurella multocida* involves subjecting the isolate to a range of biochemical tests, were isolates with aberrant biochemical properties can be particularly perplexing which makes identification difficult (**Kasten *et al.*, 1997a**).

Recently the application of PCR-based techniques has had a revolutionary impact on the diagnosis of infectious disease (**Wilson *et al.*, 1993**). Various nucleic acid-based assays has dramatically improved the sensitivity and versatility of bacterial detection and identification, particularly with regards to *Pasteurella multocida* (**Townsend *et al.*, 1998**). Such techniques facilitated bacterial identification at any level of specificity, with the use of genus and species specific PCR primers. (**Relman and Persing, 1996**).

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 (**Townsend *et al.*, 1998**) 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 did not show any amplification product. 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* (**Dabo *et al.*, 2000**).

In conclusion, the PCR assays used in this study have been shown to be rapid, sensitive and efficient useful diagnostic tool for molecular detection and characterization of *Pasteurella multocida* recovered from rabbits.

Investigation of the epidemiology of *Pasteurella multocida* infections in rabbits is of major importance since one of the main approaches for combating an infectious disease is controlling the dissemination of its causative agent (**Chaslus-Dancla *et al.*, 1996**).

In epidemiology, it is critically important to discriminate phenotypically similar isolates, particularly when establishing the identify of 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 (**Stull *et al.*, 1988**).

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 analyses and outbreak investigations aiming to improve the knowledge of a given organism and its epidemiology (by tracing the source of infection).

One of the most promising of the molecular typing methods, involves polymerase chain reaction amplification with a single short oligonucleotide primer that amplify certain sections of the genome, to produce identifiable banding patterns, thus offering a detailed fingerprinting of the genomic composition of an organism. This method, termed 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 (**Power, 1996**).

RAPD does not 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 (**Chaslus-Dancla *et al.*, 1996**).

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 obvious variability showed by RAPD between *Pasteurella multocida* isolates, which emphasize the heterogenicity 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 particulare 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 the following; 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 strain. This could interpret the fact that a polyvalent vaccine of these three vaccinal strains is recommended by the G.O.V.S in Egypt, for ensuring a protective immunity against rabbit

pasteurellosis by overcoming the heterogenicity of rabbits. *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). 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 (**Dziva *et al.*, 2001**). These results may be due to the tested strains which are 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 (**Kawamoto *et al.*, 1990**). This observation agree with the finding made by **Digiacomo *et al.* (1987)** who stated that 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), Table (9) and (10), 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 were not shared by any other *Pasteurella multocida* isolate 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. [Fig. (5) and Table (10)]. 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 Fig. (5)].

# SUMMARY

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

Out of 428 samples examined 55 isolates of *Pasteurella multocida* were recovered with total percentage of 12.9%. 112 Nasal swabs from apparently healthy rabbits yielded 6 isolates with percentage of 5.3% while 316 samples from diseased rabbits yielded 49 isolates with a total percentage of 15.5% 278 nasal swabs collected from diseased rabbit yielded 42 isolates with percentage of 15% and 34 lung samples yield 3 isolates with percentage of 8.8%. 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.

Recently a genotypic method of bacterial identification have proven to overcome limitations of traditional phenotypic method procedures.

Biochemically, the isolated strains of *Pasteurella multocida* were indole, catalase and oxidase positive while urea hydrolysis citrate utilization, hydrogen sulphide production tests were negative. They failed to grow on the MacConkey's agar and haemolysis was not produced on blood agar.

The serotyping of *Pasteurella multocida* strains revealed that from 55 isolates; 32 isolates with percentage of 58% belonged to capsular group "A" (Carter) and 16 isolates with percentage of 29% were capsular group "D" while somatic type were 9, 5 and 2 and 7 isolates with a percentage of 12.7% were untyped serological.

In this study, 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 of 460 bp, meanwhile, the negative control DNA sample did not show any amplification product. Based upon its specificity and sensitivity the PCR assay was estimated to be a valuable and effective method for molecular detection of *Pasteurella multocida* from rabbits.

Random amplified polymorphic DNA (RAPD) PCR has been successfully used in this study in the characterization of *Pasteurella multocida* isolates recovered from rabbits as it was performed on several *Pasteurella multocida* strains. A 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.

It was concluded that RAPD fingertyping method provided a stable and high discriminatory analysis of bacterial isolates, thus overcoming the uncertainties of characterization encountered with conventional phenotypic methods.

# CONCLUSION

The obtained results in this study showed that RAPD fingertyping method provided a stable and highly discriminatory analysis of bacterial isolates in contrast to the uncertainties of characterization based on variable phenotypic characteristics. The application of RAPD-PCR assay will enable straight forward genetic typing of *Pasteurella multocida* isolates and will provide a facile means of conducting molecular epidemiologic analysis, outbreak investigations and evaluation the effectiveness of current immunization programs.

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## الملخص العربى

فى هذه الدراسة تم فحص ٤٢٨ عينة جمعت من أرانب سليمة ظاهريا ومن أرانب مريضة وحديثة الوفاة مختلفة العمر والسلالة من مزارع مختلفة بمحافظة كفرالشيخ لتحديد حدوث الباستيريلا مالتوسيدا فى الأرانب.

أجرى الفحص البكتريولوجيى على كل هذه العينات لعزل الباستيريلا مالتوسيدا وأمكن عزل ٥٥ معزوله من الباستيريلا مالتوسيدا من ٤٢٨ عينة التى تم فحصها بنسبة مئوية ١٢,٩% ومن ١١٢ مسحة أنفيه من الأرانب السليمة ظاهريا تم عزل ٦ معزولات بنسبة ٥.٣% بينما ٣١٦ عينة من الأرانب المريضة وحديثة الوفاه تم عزل ٤٩ معزولة بنسبة ١٥.٥% من بينهم ٤٢ معزوله عزلت من ٢٧٨ مسحة انفية من أرانب مريضة بنسبة ١٥% و ٣ معزولات من ٣٤ عينة رئوية بنسبة ٨.٨% حيث تم تصنيف هذه المعزولات على حسب الطرق القديمة المعتاده مثل خواص مستعمراتها والصفات الخلوية والسلوك الكيميائى وضراوتها للفئران والتصنيف السيرولوجى وفى السنوات الأخيرة أصبح التصنيف الجينى يقدم دور مفيد للتغلب على التقصير فى الطرق القديمة المعتادة.

وبدراسة الخواص البيوكيميائية للعترات المعزولة من ميكروب الباستيريلا مالتوسيدا وجد أنها ايجابية فى انتاج الإندول والكاتاليز والأكسيديز. بينما كانت سلبية فى إنتاج اليوريز واستخدم السترات وانتاج كبريتيد الهيدروجين وكذلك سلبية فى النمو على مزارع الماكونكى.

أوضح التصنيف السيريولوجى بأن العترات المعزولة الباستيريلا مالتوسيدا فى الأرانب ٣٢ معزولة منها تندرج تحت مجموعة الكبسول "أ" بتقسيم كارتز بنسبة ٥٨% و١٦ معزوله تندرج تحت مجموعة الكبسول "د" بنسبة ٢٩% والنوع الجسدى ٩ ، ٥ ، بينما وجد ٧ معزولات لم يتم تصنفيها بنسبة ١٢,٧%.

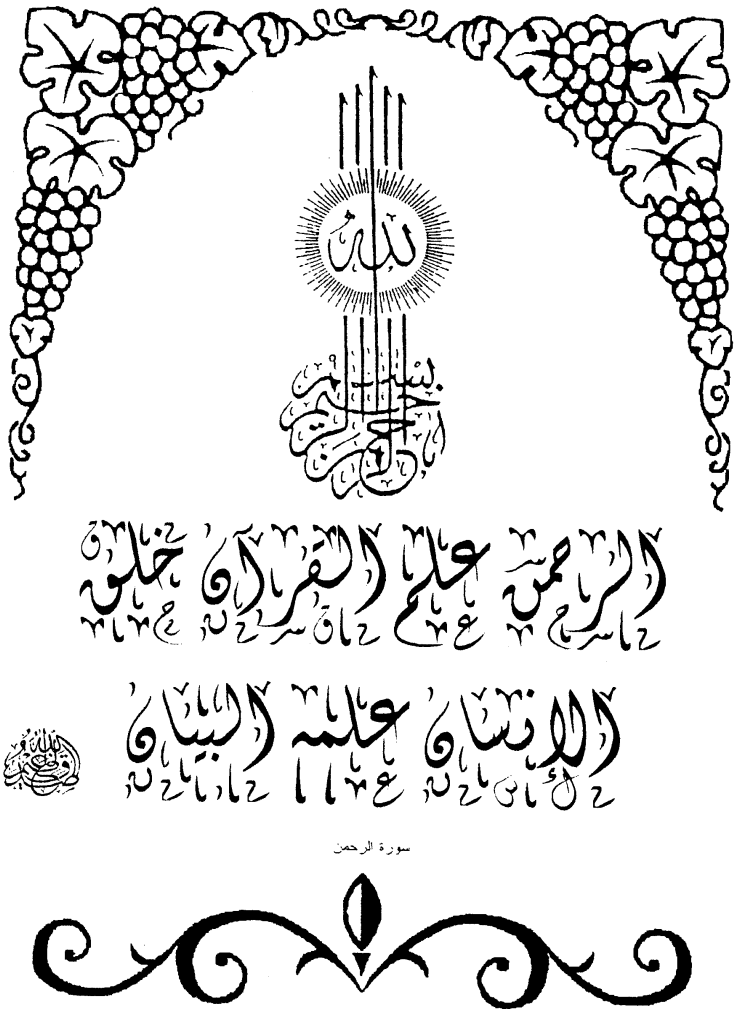
فى هذه الدراسة تم دراسة حساسية وخصوصية البادئ الخاص الباستيريلا مالتوسيدا تقدر من خلال إختبار البلمرة المتسلسل (PCR).

كل معزولات الباستيريلا مالتوسيدا التى فحصت أعطت قطعة واحدة مضاعفة وهى ٤٦٠ زوج من النيكليوتيدات. بينما الكنترول السلبى لم يعطى أى قطعة مضاعفة او بصمة وهذا يؤكد على خصوصية إختبار البلمرة المتسلسل (PCR).

وبناء على خصوصية وحساسية اختبار البلمرة المتسلسل أصبح يقدر بأنه مهم وفعـــال فى التحديد الجينى للباستيريلا مالتوسيدا المعزول من الأرانب.

وكذلك الحمض النووى المتعدد الصور المضاعفة عشوائيا (RAPD) اظهر 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 ان قطع متشـــابهة حصـــل عليها من نفس العترة بينما بتحليل البصمات الحاصل عليها بين المعزولات من نفس المزرعـــة والاخرى من اماكن مختلفة وبين المعزولات التى تستخدم فى التحصين.

من هذا نستخلص ان البصمات الناتجة عن استخدام الحامض النووى المتعدد الصـــور المكبرة عشوائيا (RAPD) أعطت لنا تحليل مميز لمعزولات البكتريـــا وهـــذا يتغلـــب علـــى التقصير فى التميز بالطرق المعتادة وتقدم هذه الرسالة اسلوب حديث لتقصى مصادر العـــدوى بالباستيريلا مالتوسيدا مما يساعد فى إعداد العترات السارية للعدوى لتحضير اللقاح المتخصص لهذا الميكروب.




سورة الرحمن

جامعة طنطا - فرع كفر الشيخ
كلية الطب البيطرى
قسم الميكروبيولوجيا

## قرار لجنة الحكم والمناقشة

قررت لجنة الحكم والمناقشة بجلستها المنعقدة فى يوم الثلاثاء الموافق ٢٠٠٥/٣/٨م بكلية الطب البيطرى بكفر الشيخ ما يلى :-

الرسالة المقدمة من السيدة **طب./ عتاب محمد سيد روحة ابو رميلة** ترقى بها للحصول على درجة الماجستير وتوصى اللجنة بترشيح سيادتها للحصول على درجة الماجستير فى العلوم الطبية البيطرية تخصص(**بكتريولوجيا – فطريات – مناعة**) من قسم الميكروبيولوجيا كلية الطب البيطرى بكفر الشيخ – جامعة طنطا.

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جامعة طنطا
كلية الطب البيطرى
قسم الميكروبيولوجيا

# التوصيف البكتيرى و الجزيئى لميكروب الباستريلا مالتوسيدا المعزول من الأرانب بإستخدام تقنية إختبار البلمرة المتسلسل

رسالة مقدمة من

**ط.ب/عتاب محمد سيد روحة أبو رميلة**
*بكالوريوس العلوم الطبية البيطرية ٢٠٠٠ ــ جامعة طنطا*

للحصول على
درجة الماجستير فى العلوم الطبية البيطرية
(بكتريولوجيا ، ميكولوجيا ، إميفولوجيا)

تحت إشراف

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٢٠٠٥