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Antibacterial Sensitivity and Detection of Virulence Associated Gene of *Pasteurella multocida* Isolated from Rabbits

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ABSTRACT

The aim of the present work was to determine antibacterial sensitivity and resistance patterns of *Pasteurella multocida* isolated from rabbits in different farms of Assiut Governorate. Also, this study aimed to detect virulence-associated gene (*toxA*) of *Pasteurella multocida*. A total of 40 freshly dead rabbits were used to collect samples from liver, lung and subcutaneous abscess. In addition, tracheal swab samples were collected from 20 diseased rabbits. Bacteriological examination revealed that *Pasteurella* spp. were isolated and phenotypically identified with an incidence rate of 55% (33 out of 60 rabbits). Ten *Pasteurella* spp. isolates were randomly chosen for antibiotic sensitivity testing and molecular identification using PCR. Antibiotic sensitivity test was carried using standard disk diffusion method against 13 antibacterial drugs to determine antibacterial sensitivity and resistance patterns of *Pasteurella* isolates and revealed variable sensitivity and resistance to antibacterial drugs. *Pasteurella multocida* isolates were sensitive to wide variety of antibiotics (norfloxacin, enrofloxacin, ciprofloxacin, florfenicol, doxycycline, gentamycin, cephradine and cefoxitin). Three out of ten isolates were molecularly confirmed to be *Pasteurella multocida* and all of them demonstrated the presence of *toxA* virulence genes. In conclusion, the prevalence of *Pasteurella* infections in rabbits in Assiut Governorate was relatively high.

Key words: Antibacterial resístanse *Pasteurella multocida*, *toxA* gene, virulence genes.

INTRODUCTION

Rabbit pasteurellosis is a serious disease which causes a considerable economic loss in rabbit production units (Stelian et al., 2011). It is caused by *Pasteurella multocida* which may cause pneumonia and septicemia leading to death or local infections such as rhinitis, otitis media, conjunctivitis and abscesses (Deeb et al., 1990).

Pasteurella multocida is an important pathogen of the upper respiratory tract of various wild and domestic animals (Loubinoux et al., 1999). It is a small, Gram-negative, coccobacillus, non-motile, non-spore forming and facultative anaerobe which belongs to family Pasteurellaceae. *Pasteurella multocida* has been isolated from all ages of rabbits and colonized most commonly in the sinus, middle ear, trachea and lungs (Quinn et al., 1994).

Pasteurella multocida is the most common pathogen isolated from rabbits, its prevalence rate has been recorded to range from 7-100% (Nakagawa et al., 1986; Deeb, et al., 1990; Kawamoto et al., 1990). It is considered an opportunist or secondary pathogen which can be found in the respiratory tract of both healthy and diseased animals. In rabbitries, it could emerge as a major pathogen that causes upper respiratory tract infections resulting in considerable economic losses (Deeb and DiGiacomo, 2000). Many researchers have distinguished various cases of non-infected, resistant and chronically infected animals or even healthy carriers (DiGiacomo, et al., 1983; Deeb, et al., 1990). *Pasteurella multocida* has been shown to adhere to the mucosal epithelium of the nasopharynx of rabbits by fimbriae (pili) which correlated to the virulence of the organism (Glorioso et al., 1982). A complex pathogenicity of *P. multocida* has been reported, and several virulence factors including hemagglutinins, fimbriae, lipopolysaccharides, hyaluronidase, iron regulated protein, capsule, iron acquisition proteins and a dermonecrotic toxin have been identified previously (Deeb and DiGiacomo, 2000). Previous reports could not detect *toxA* gene in *P. multocida* isolated from rabbits (Ferreira et al., 2012). In addition, other investigators mentioned that

toxA gene is not commonly found in *P. multocida* strains isolated from rabbits (Ewers et al., 2006; García-Alvarez et al., 2015; Massacci et al., 2018).

Pasteurellosis in rabbits could be controlled through treatment with antibiotics and the slaughter of infected animals. Treating infected animals, however, only alleviates clinical signs and slows the progression of the disease, but it does not eliminate the infection (Deeb and DiGiacomo, 2000). Vaccination of *P. multocida* have been also reported using inactivated formalized *P. multocida* vaccine (Nassar et al., 2013). Methods of detection and diagnosis of *P. multocida* infections relied on microscopic detection of the pathogen via stained smears, isolation by culturing on selective media, then phenotypic or characterization serologically (Christensen and Bisgaard, 2010).

This study aimed to detect and identify *P. multocida* by phenotypic characterization and molecular identification in rabbits as well as to determine its antibacterial sensitivity and resistance patterns. In addition, this study investigated the occurrence of *toxA*, a gene associated with the virulence, in *P. multocida*.

MATERIALS AND METHODS

Ethical approval

The research protocol was reviewed and approved by Institutional Animal Care and use Committee (Vet CU20022020160).

Sample collection

A total of 40 recently dead rabbits with a previous respiratory manifestation (snuffling) were used to collect samples from liver, lung and subcutaneous abscesses. In addition, 20 diseased rabbits were used to collect tracheal swabs. All rabbits used ranged from 8 to 48 weeks old, and were obtained from different farms in Assuit Governorate, Egypt. Samples were collected under complete aseptic conditions in sterile tubes containing nutrient broth, transferred immediately to the lab in an icebox for bacteriological examination.

Isolation of *Pasteurella multocida*

The collected samples were inoculated into brain heart infusion broth (BHI) and incubated for 24 hours at 37°C. Sub-culturing was carried out on sheep blood agar (5%) then incubation for 24 hours at 37°C. Growth was examined for typical *P. multocida* colonies.

Phenotypic identification

To confirm the presence of *Pasteurella* on suspected samples, Gram's stain was used for staining films from bacterial isolates for morphological characters and staining reaction as a first step for isolates identification and differentiation. In addition, biochemical reactions; including catalase, urease and indole tests and fermentation of sugars tests including dextrose, sucrose, maltose, dulcitol, arabinose, xylose, lactose, mannitol, galactose and salicin (Cruickshank et al., 1975) were used. *Pasteurella* isolates were preserved at 30% glycerol sterile solution (Biyashev et al., 2014).

Antibacterial sensitivity and resistance patterns

Sensitivity of *Pasteurella* isolates to antibacterial agents was determined using standard disk diffusion method. The criteria proposed by the National Committee for Clinical Laboratory Standards (CLSI, 2013) was used to determine susceptibility rates. The antibiotic discs (Oxoid) used in current study were: erythromycin (15µg), amoxicillin (30 µg), cephradine (30 µg), colistin (10 µg), ciprofloxacin (5 µg), enrofloxacin (5 µg), cefoxitin (30 µg), gentamicin (10 µg), neomycin (10 µg), streptomycin (10µg), florfenicol (15 µg), doxycycline (30 µg), and norfloxacin (10 µg). Interpretation of results was performed according to Quinn et al. (1994) and Koneman et al. (1997).

Multidrug resistant Index

The multi-drug resistance index (MDRI) was determined for every isolate using the following equation according to Chandran et al. (2008):

$$\text{MDR index} = \frac{\text{Number of antibiotics resisted}}{\text{Total number of antibiotics used}} \times 100$$

When an isolate was resistant to more than three antibiotics, it was considered as multidrug resistant. Isolates were considered highly resistant when they had MDRI values of more than 0.2 (20%).

Molecular identification of *Pasteurella* isolates

Ten randomly selected phenotypically identified *Pasteurella* isolates (five from dead rabbits and five from diseased rabbits) were subjected to molecular identification using PCR as following:

DNA extraction

DNA was extracted from the samples by using QIAamp DNA Mini kit (Qiagen, Germany, GmbH)

according to manufacturer's recommendations. In brief, 200µl of the sample suspension were incubated with 10µl of proteinase K and 200µl lysis buffer at 56^{O}C for 10 minutes. Then after, 200µl of 100% ethanol were added to the lysate. The sample was then washed and centrifuged. Nucleic acid was eluted in 100µl of elution buffer that was provided in the kit.

Oligonucleotide primer

The used primers (table 1) were supplied from Metabion (Germany).

PCR amplification

PCR primers were used in a 25-µl reaction that contained 12.5µl of EmeraldAmp Max PCR Master Mix (Takara, Japan), 1µl from each primer of 20 pmol concentration, 4.5µl of water, and 6µl of DNA template. The reaction was then carried out in an applied biosystem 2720 thermal cycler.

Analysis of the PCR products

By electrophoresis, the products of PCR were separated on 1% agarose gel (Applichem, Germany, GmbH) at room temperature in 1xTBE buffer using gradients of 5V/cm. 20µl of the products was loaded in each gel slot for gel analysis. Gelpilot 100bp DNA ladder (Qiagen, Germany, GmbH) was then used to determine the sizes of fragments. Photographing of the gel was done on a gel documentation system (Alpha Innotech, Biometra) and the data were then analyzed by computer software.

Detection of *toxA* gene

Ten samples of *Pasteurella* spp. which were previously identified, were subjected to PCR to detect *toxA* gene. DNA extraction from samples was carried out as previously mentioned. The used primers (Table 1) were supplied by Metabion (Germany). The PCR reaction was performed according to Townsend et al. (1998).

RESULTS

Isolation rate of *Pasteurella multocida*

Examination of individual diseased (20) and freshly dead (40) rabbits showed typical clinical signs and post mortem lesions of pasteurellosis. The results revealed 33 *Pasteurella* isolates were obtained from 60 examined rabbits with overall incidence of 55%. The isolation of *Pasteurella* from lung, liver and subcutaneous abscesses samples from freshly dead rabbits revealed that 25 rabbits were positive for *Pasteurella* (62.5%) while the examination of tracheal swab samples from diseased rabbits showed an isolation rate of 40% (8 out of 20).

Phenotypic identification of *Pasteurella* isolates

The growth of suspected *P. multocida* on brain heart infusion (BHI) agar, appeared as round gray large mucoid colonies. On 5% sheep blood agar, colonies were non-hemolytic dew drop like. Gram stained slides of suspected colonies showed gram negative rods. The suspected *P. multocida* colonies were positive for catalase and indole, but negative for urease. However, sugar fermentation revealed that *P. multocida* colonies were positive with lactose, sucrose, dextrose, mannitol and galactose, but negative with dulcitol, salicin, arabinose, maltose and xylose.

Antibacterial susceptibility and resistance patterns

Results revealed that all *Pasteurella* spp. samples were sensitive to norfloxacin, enrofloxacin, ciprofloxacin, florfenicol, doxycycline, gentamycin, cephradine and cefoxitin. The isolates showed MDRI rang from (15.35% to 42.86%). The Sensitivity and resistance of *P. multocida* isolates to erythromycin, amoxicillin, colistin sulfate, neomycin and streptomycin were variable, results were summarized in table 2.

Molecular identification of *Pasteurella multocida* suspected isolates

Total ten samples were randomly chosen for molecular identification using PCR. Three were confirmed as *P. multocida* and seven isolates were not confirmed (Figure 1).

Detection of *toxA* virulence gene

Expression of *toxA* virulence gene was demonstrated in the three isolates that confirmed molecularly as *P. multocida* (Figure 2).

Table 1. Primers sequences, target genes, amplicon sizes and cycling conditions

Target gene	Primers sequences (5'- 3')	Amplified segment (base pair)	Primary denaturation	Amplification (35 cycles)			Final extension	Reference
				Secondary denaturation	Annealing	Extension		
Kmt1	**F:** ATC-CGC-TAT-TTA-CCC-AGT-GG **R:** GCT-GTA-AAC-GAA-CTC-GCC-AC	460	94˚C 5 min.	94˚C 30 sec.	55˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	OIE (2012)
toxA	**F:** CTTAGATGAGCGACAAGG	864	94˚C 5 min.	94˚C 30 sec.	55˚C 40 sec.	72˚C 45 sec.	72˚C 10 min	Townsend et al. (1998)

F: forward, R: reverse

Table 2. Antibacterial sensitivity test of *Pasteurella multocida* isolated from rabbits by disk diffusion method

Isolate	Results													MDRI
	ER	AX	CT	CP	EN	CO	CN	NO	SP	FFC	DO	NR	CE	
1	R	R	S	S	S	S	S	R	R	S	S	S	S	30.07%
2	S	R	R	S	S	S	S	R	R	S	S	S	S	30.07%
3	R	S	R	S	S	S	S	S	S	S	S	S	S	15.35%
4	S	R	S	S	R	S	S	S	R	R	S	S	R	35.71%
5	R	S	R	S	S	R	S	R	S	S	R	S	R	42.86%
6	S	R	S	S	S	S	R	R	S	R	S	S	S	30.07%
7	R	S	S	R	S	R	S	S	S	S	R	S	S	30.07%
8	R	R	R	S	S	S	S	R	S	S	S	R	S	35.71%
9	R	S	S	R	S	S	S	R	S	S	S	S	S	15.35%
10	S	R	R	S	R	S	R	S	S	R	S	S	S	35.71%

ER: erythromycin, AX: amoxicilline, CT: colstine sulfate, CP: ciprofloxacin, EN: enrofloxacin, CO: cefoxitin, CN: gentamycine, NO: neomycine, SP: sterptomycine, FFC: florfenicol, DO: doxycycline, NR: norfloxacin, CE: cephradine, MDRI: multi-drug resistance index. S: sensitive, R: resistance.

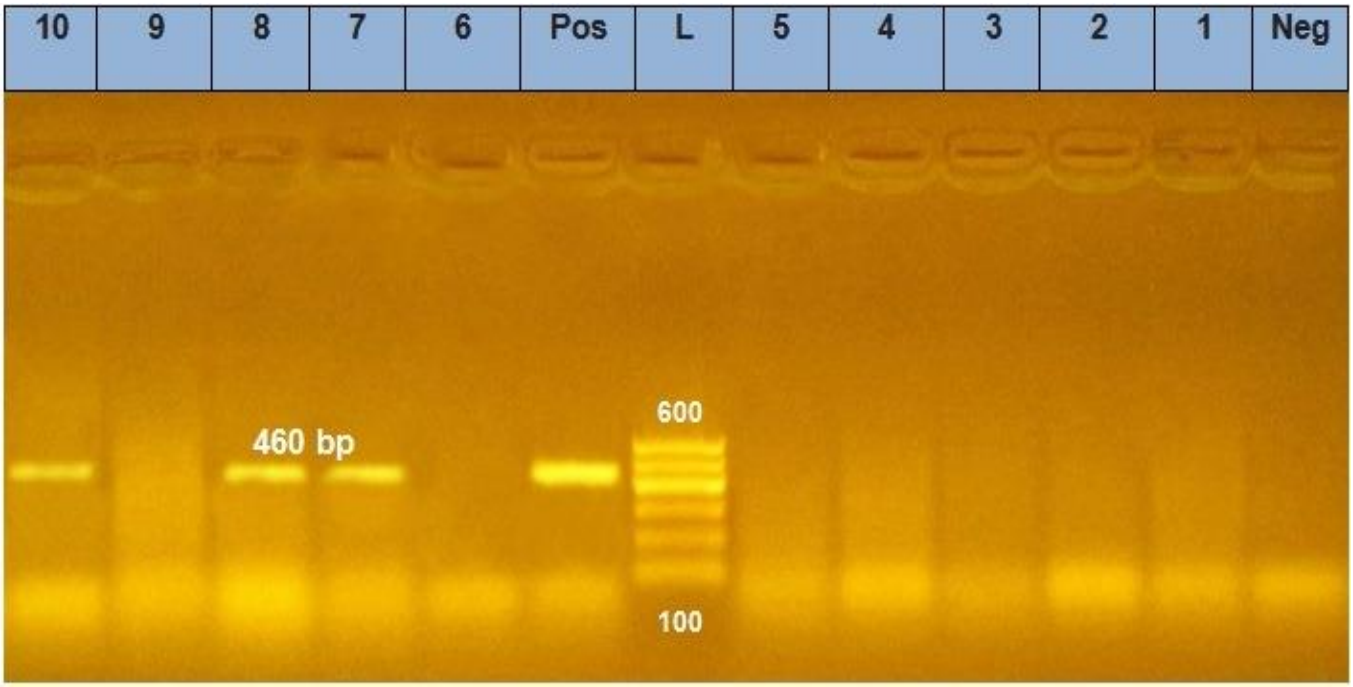

Figure 1. Agarose gel electrophoresis showing amplification of 460 bp fragments specific for *Pasteurella multocida* of 10 samples isolated from rabbits. Lanes 1-6 and 9 are negative, Lane 7, 8 and 10 are positive samples for *kmt1* gene. L: 100bp ladder (100-1000bp). Pos: positive control, Neg: negative control

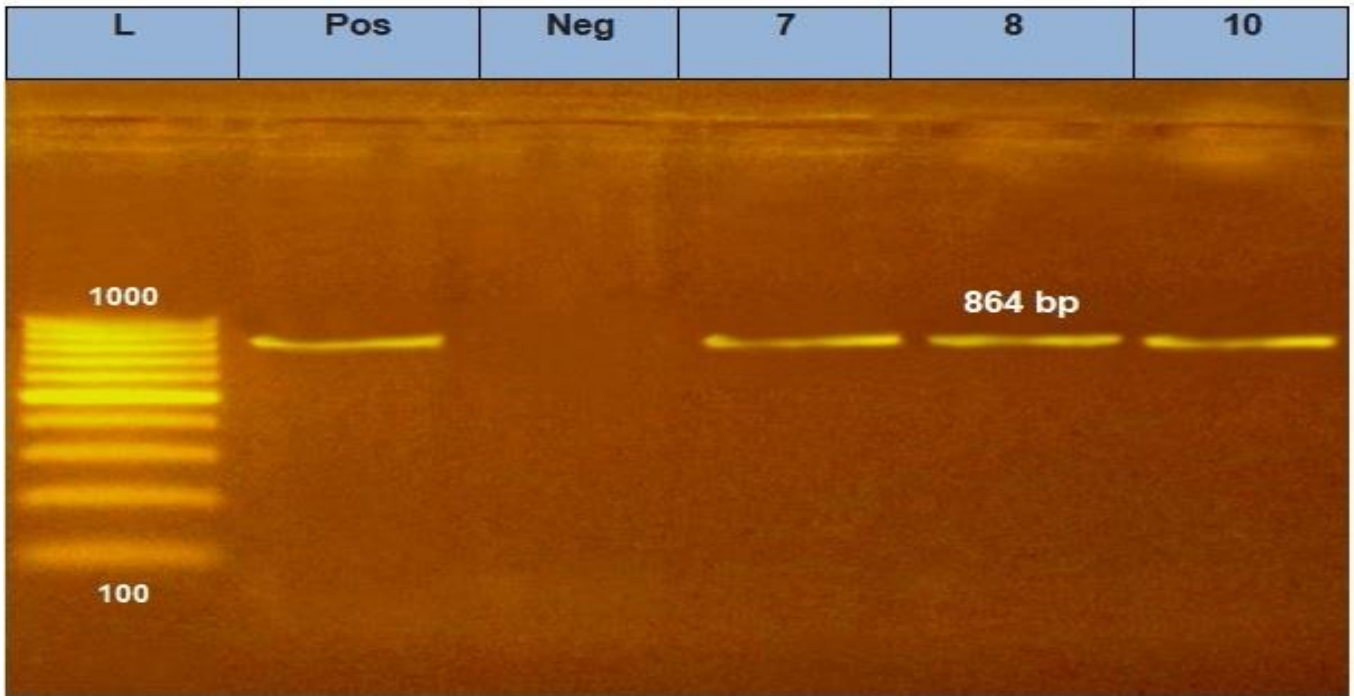

Figure 2. PCR results for 864bp *toxA* gene of *Pasteurella mutocida* showing positive amplification of the gene in tested samples isolated from rabbits. Lanes 7, 8, and 10 are positive samples for *toxA* gene, Pos: positive control, Neg: negative control. L: 100bp ladder (100-1000bp).

DISCUSSION

The relatively high prevalence rate of *Pasteurella* isolation in the current study suggests that it is probably an important pathogen causing high mortality in rabbits. Previous studies revealed variable incidences of *P. multocida* isolation (Lee et al., 1990; Takashima *et al.*, 2001; Stelian et al., 2011). Sanchez et al. (2004) isolated *P. multocida* from clinically healthy animals with a prevalence of 20-90%. However, low values of isolation incidences were also previously reported for *P. multocida* from liver, lungs, spleen, heart-blood and nasal swabs of rabbits with an incidence ranging from 18.75% to 35.00% (Mazed et al., 2013). Lower values of prevalence (3.4% to 9.4%) have been recorded in diseased rabbits by Asran et al. (2016). This discrepancy in prevalence rates of *P. multocida* could be attributed to method of detection employed or the locality in which the study was done.

Antimicrobial resistance test for 10 randomly selected *Pasteurella* isolates reveled that most of them showed multidrug resistant to more than two antibiotics with MDRI ranged from 15.35% to 42.86%. Ferreira et al. (2012) investigated the antimicrobial sensitivity of *P. multocida* isolated from rabbits in Brazil and found that 47.8% of the strains were resistant to at least one of the tested drugs. However, in the current investigation, resistance was common against erythromycin, amoxicillin, colistin sulfate, neomycin and streptomycin. Unlikely, Tang et al. (2009) and Ferreira et al. (2012) reported that the high resistance of the isolates was against sulfonamides and cotrimoxazole. They reported that increased resistance of *P. multocida* against antimicrobials could be attributed to the irresponsible use, overuse, and misuse of antibiotics in rabbitaries.

Molecular identification is the basic method for identifying of *P. multocida* strains. Due to their great discriminatory power, DNA-based identification method has been established as an effective method in characterization of *P. multocida* (Blackall and Miflin, 2000). Using PCR as method for characterization, the current results showed that, three out of ten phenotypically identified *Pasteurella* isolates were confirmed as *P. multocida* (30%). Similar findings have been obtained by Mazed *et al.* (2013), where the molecular detection by real-time PCR of *P. multocida* revealed its occurrence in diseased rabbit samples with an incidence of 29.5%.

Therapy by using antimicrobial is one of the preliminary control measures to reduce morbidity and mortality resulting from *P. multocida* infections in rabbit and antibiotics are still the first choice to prevent and control *P. multocida* infections (Kehrenberg et al., 2001). However, the improper and misuse of antibiotics promotes

the development of drug resistance (Percy et al., 1984; Kehrenberg et al., 2001; Oh et al., 2019). *P. multocida* isolates, in the present study, were sensitive to wide variety of antibiotics (norfloxacin, enrofloxacin, ciprofloxacin, florfenicol, doxycycline, gentamycin, cephradine and cefoxitin). However, sensitivity and resistance of *P. multocida* isolates to erythromycin, amoxicillin, colistin sulfate, neomycin and streptomycin were variable. These findings simulate previous reports that the most effective antibiotics against *P. multocida* are cephalosporins, florfenicol, tetracyclines, and fluoroquinolones (Salmon et al., 1995; Kehrenberg et al., 2001; Yoshimura et al., 2001; Ferreira et al., 2012).

The *toxA* genes is a virulence gene used for detection of the *P. multocida* pathogenicity (Furian et al., 2013). In consistence with Ahmed et al. (2016), who could detect *toxA* gene from rabbit *P. multocida* isolates in Egypt, the current investigation revealed the expression of *toxA* virulence gene of *P. multocida* in 3 out of 10 isolates. Unlikely, Ferreira et al. (2012) could not detect *toxA* gene in a study that performed on 46 *P. multocida* isolates with 0 percentage of presence of *toxA* gene. In addition, some other reports stated that this gene was uncommonly found in strains of *P. multocida* isolated from rabbits (Ewers et al., 2006; García-Alvarez et al., 2015; Massacci et al., 2018). *ToxA* gene has been reported to differentiate nontoxinogenic from toxinogenic strains of *P. multocida* (Lichtensteiger et al., 1996).

DECLARATIONS

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Authors' contributions

Fatma M Mohamed conceived the idea and planned the study. Fatma M Mohamed, Marium F. Mansy and Ahmed K. Hassan participated in the collection of samples, isolation and identification of *P. multocida*. Fatma M Mohamed, and A. K. Hassan wrote the manuscript. A M Abd-Al-Jwad revised the manuscript.

Competing interests

The authors declare no conflict of interests.

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