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Molecular Diversity of *Pasteurella multocida* Isolated From Different Rabbit Outbreaks at Zagazig Suburbs, Egypt

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Abstract: This study aimed to determine the diversity of molecular fingerprints of *Pasteurella multocida* strains isolated from different outbreaks in rabbit. Thirty four cases suffering from pasteurellosis were subjected to clinical and bacteriological examination. Rabbits showed symptoms of rhinitis, conjunctivitis, otitis media, subcutaneous abscesses and mortality. The predominant lesions recorded were nasal mucosa and lungs affection. Different swab samples were cultured and identified biochemically as standard methods. From 34 clinical cases, 204 swab samples were taken (nasal, tracheal, conjunctival, lung, liver and heart blood), 97 swabs (47.5%) were *P. multocida* positive (27, 20, 19, 14, 9 and 8), with percentage of 79.4, 58.8, 55.9, 41.2, 26.5 and 23.5, respectively. Eight representative isolates were assayed by PCR – fingerprinting using M13 core primer. The amplified bands grouped the total examined 8 isolates of *P. multocida* (2/each outbreak) based on the presence or absence of the major 4 bands (bands A at 1200 bp; B at 600-700 bp; C at 200 bp and D at < 200 bp). The examined representative isolates were separated into 3 genotypes. The genotype I (4/8) shared bands A, B and D were found in outbreaks a (2/2), b (1/2) and c (1/2). The genotype II isolates (3/8) shared bands A, B, C and D were found in outbreaks b (1/2) and d (2/2). The genotype III isolate (1/8) shared bands B and D was found in outbreak c (1/2). PCR-fingerprinting in the present study is effective and reproducible in *P. multocida* strains genotyping.

Key words: Pasturellosis · Rabbit · Post mortem · PCR · Fingerprinting.

INTRODUCTION

Pasteurella multocida is one of the most serious pathogens of rabbits; it causes considerable economic losses in rabbit production units [1]. Infection most likely occurs immediately after birth and the prevalence of carrier rabbits increase to over 90% at 5 month age. The variability in disease course is influenced by different bacterial virulence factors such as capsule, fimbriae, neuraminidase [2]. *P. multocida* are predominant causes of death in rabbits. Chronic healthy carrier rabbits with local infection (rhinitis, otitis media, conjunctivitis, abscesses), pneumonia and septicemia can be distinguished in pasteurellosis [3]. Respiratory disease due to *P. multocida* has been identified as a major cause of financial loss to the rabbit industry. Pasteurellosis can be transmitted by direct and indirect contact mainly aerosol infection. More than 50% of adult rabbits either die or are culled due to *P. multocida* infection. In post mortem of dead rabbits, the fluid and mucous accumulation in the trachea and lungs revealed snuffling sounds on auscultation and a morbidity rate of 35-40% with mortality of 23% were recorded [4]. Stress and immunodeficiency play a major role in the onset of auto infection for pasteurellosis in rabbits [5].

Molecular techniques allow a more discrimination among rabbit isolates of *P. multocida* and improve the epizoological study of the disease [6]. There is a lack of literature about molecular diversity of *P. multocida* strains isolated from rabbits in Egypt. PCR fingerprinting provides reliable means for differentiating *P. multocida* strains [7]. PCR- typing of *P. multocida* isolates from rabbits was carried out by Stahel *et al*. [8] who detected genetic heterogeneity among different clones. Studying the molecular fingerprinting will help to trace the sources and reservoirs of infections.

The purpose of the study was detection and characterization of *P. multocida* strains that caused different outbreaks of rabbit pasteurellosis at Zagazig suburbs by using PCR fingerprinting technique.

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MATERIALS AND METHODS

Animals: Thirty four non vaccinated rabbit cases suffered from clinical symptoms of pasteurellosis were examined in the Veterinary Hospital, Zagazig University during winter season 2011. The examined cases were representing 4 different outbreaks (a, b, c and d) from Zagazig surrounding suburbs. The clinical cases were admitted to the clinic by the owners. Post mortem examination was carried out.

Isolation and Identification of *P. multocida*: Nasal, tracheal, conjunctival, lung, liver and heart blood swabs; 34 each were sampled. Each swab was streaked onto blood agar and MacConkey agar and incubated at 37°C for 24 hours. The identification of *P. multocida* was based on the criteria stated by Carter and Cole [9].

PCR Technique: Two representative strains for each outbreaks (total No = 4x2) were analyzed by PCR fingerprinting according to Gerardo *et al*. [7]. Briefly, several colonies of each isolate were suspended in 1 ml of sterile distilled water to turbidity approximately equal to a number 3 McFarland standard. 200 µl of the cell suspension was pelleted, re-suspended in 200 µl of Insta-Gene Matrix (Bio-Rad, Hercules, Calif.) and then incubated at 50°C for 15 to 30 min. Cell solutions were vortexed and then heated for 8 to 10 min at 100°C. Cell lysate supernatants containing the DNA extract were centrifuged to remove cellular debris and stored at -20°C until use. Each cell lysate supernatant was subjected to PCR amplification in 50-µl volumes containing 25 ml of cell lysate supernatant; PCR buffer with 1.5 mM $MgCl_2$ (final concentration) (Perkin-Elmer Cetus, Norwalk, Conn.); 200 mM dATP, dCTP, dGTP and dTTP (Pharmacia LKB Biotechnology, Piscataway, N.J.); 25 pmol of each primer, M13 core (5`-GAGGGTGGCGGTTCT-3`); and 2.5 U of *Taq* DNA polymerase (Perkin-Elmer, Norwalk, Conn.). Amplification reactions were performed as follows: 40 s at 93°C, 1 min at 50°C and 40 s at 72°C for 35 cycles, followed by a final extension cycle of 6 min at 72°C. Reaction tubes were held at 4°C prior to analysis. Samples were concentrated to approximately 20 to 25 µl each (Speed-Vac; Savant, Halbrook, N.Y.) prior to electrophoretic separation in 1.2% agarose gels (0.5 by 25 by 20 cm) for 5 h at 3V/cm. The molecular weight marker was a DNA ladder 100 bp. Amplified products were detected by the ultra violet trans-illuminator. Data were evaluated as described previously by Claros *et al*. [10].

RESULTS

The incidence of *P. multocida* in rabbit clinical cases at Zagazig surrounding suburbs are shown in Table 1. From 34 clinical cases, 204 swab samples were taken (nasal, tracheal, conjunctival, lung, liver and heart blood;

Table 1: Incidence of *Pasteurella multocida* in rabbit's clinical cases in Zagazig surrounding suburbs

	Clinical cases (No=34)		Total swabs (No=204)	
	Positive		Negative	
Swabs	No.	%	No.	%
Nasal	27	79.4	7	20.6
Tracheal	20	58.8	14	41.2
Conjunctival	19	55.9	15	44.1
Lung	14	41.2	20	58.8
Liver	9	26.5	25	73.5
Heart blood	8	23.5	26	76.5
Total	97	47.5	107	52.5

Table 2: *P. multocida* strains molecular heterogeneity representing 4 outbreaks of rabbits in Zagazig Suburbs

		No of isolate/No of lane in Fig. 1							
Amplified band bp	Band code	1/3	2/4	3/5	4/6	5/7	6/8	7/9	8/10
1200	A	+	+	+	+	+	-	+	+
600-700	B	+	+	+	+	+	+	+	+
200	C	-	-	-	+	-	-	+	+
<200	D	+	+	+	+	+	+	+	+
Genotype		I	I	I	II	I	III	II	II
Outbreak		a		b		c		d	

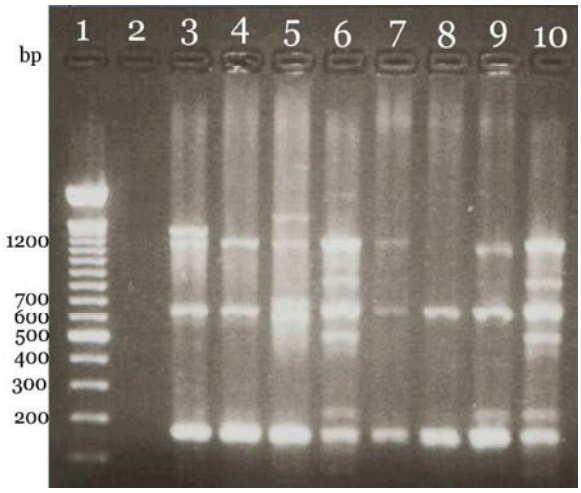

Fig. 1: *Pasteurella multocida* PCR fingerprint profiles.
Lane 1: 100 bp marker (Standard DNA)
Lane 2: Negative control (no DNA template)
Lanes 3-10: *P. multocida* strains representing 4 outbreaks (2/each, respectively)

34 each). *P. multocida* was isolated from a total of 97(47.5%) swabs. Recovered isolates from the examined swabs (nasal, tracheal, conjunctival, lung, liver and heart blood) were 27, 20, 19, 14, 9 and 8, with percentages of 79.4, 58.8, 55.9, 41.2, 26.5 and 23.5, respectively. All positive cases showed symptoms of pasteurellosis (rhinitis, conjunctivitis and otitis media). Recorded post mortem findings were congestion and ulceration of nasal mucosa, hemorrhagic exudates in the thoracic cavity, pneumonia with partial adhesions of pleura to lung surface, congestion of liver and subcutaneous abscesses.

PCR grouped the total examined 8 isolates of *P. multocida* (2/each outbreak) based on the presence or absence of the major 4 amplified bands into A at 1200 bp; B at 600-700 bp; C at 200 bp and D at < 200 bp) (Fig. 1). The representative isolates were separated into 3 genotypes. Genotype I (4/8, 50%) shared bands A, B and D are found in outbreaks a (2/2), b (1/2) and c (1/2). Genotype II isolates (3/8, 37.5%) shared bands A, B, C and D are found in outbreaks b (1/2) and d (2/2). Genotype III isolate (1/8, 12.5%) shared bands B and D was found in outbreak c (1/2) (Table 2).

DISCUSSION

This study shows the incidence of *P. multocida* clinical cases in rabbits at Zagazig suburbs, Egypt. The recorded symptoms and lesions are in agreement with Zoran *et al*. [11] who mentioned that the clinically diseased rabbits showed fever and respiratory distress resulting in respiratory failure and its pathological findings were severe fibrinopurulent pleuropneumonia with extensive fibrinous adhesions on the parietal pleura and lung surfaces. Serohemorrhagic exudate in thorax could also be observed and the alveoli (and some bronchioles) were partly or completely filled with fibrin and mixed cellular infiltrate (erythrocytes, leukocytes and desquamated epithelial cells). Al-Haddawi *et al*. [12] reported that the gross findings in the infected rabbits were congestion of the nasal mucosa with mucopurulent exudate in the nasal cavity of one rabbit and catarrhal exudate mixed with yellowish threads of pus in three cases and consolidation of the right and left apical and cardiac lobes of the lung, with adjacent emphysematous areas.

The isolation rate of *P. multocida* from the 204 swabs of the examined clinical rabbit cases in this study was 47.5% which is nearly similar to the results of Percy *et al.* [13] who recorded isolation rate of 51.3% in diseased rabbits. On the other hand, higher results 77.5% was recorded by Kawamoto *et al*. [14], whereas lower results were reported by Amal and Omyma [15], Lee *et al*.[16] and Stelian *et al*. [1] with rates of 36.4%, 31% and 27%, respectively.

The variation in distribution frequency may be due to individuality of health or immunological status of the sampled rabbits and environmental conditions. Moreover, Amna *et al*. [17] mentioned that prevalence of *P. multocida* infection in rabbits housed under laboratory conditions or on breeding farms without respiratory sings has been recorded to be between 15.8% and 94%. The clinical case may be a source of contamination to the rabbitries environment. Kawamoto *et al*. [14] isolated *P. multocida* from environmental samples with frequency of 31 % (37/126). So, the occurrence of pasteurellosis in rabbit farms may increase the risk of biohazards in the environment.

DNA-based typing method has been already established as an effective tool in characterization of *P. multocida* [18]. The obtained results shown in Table 2 and Figure 1 revealed genotyping of the examined 8 clones of *P. multocida*. Gerardo *et al*. [7] succeed to discriminate the *P. multocida* strains isolated from different bite wounds in human cases using M13 core primer in PCR-fingerprinting. Moreover, Zucker *et al*. [19] used the same primer in PCR to distinguish strain- to –strain variation among *P. multocida* pig respiratory isolates. The difference in PCR-fingerprinting among *P. multocida* strains are due to differences in the source of these isolates [7]. The obtained data showed that

PCR-fingerprinting profiling is of considerable epidemiological value. The circulating *P. multocida* strain was genotype I conserved in 3 of the 4 examined outbreaks. So, the local vaccine strain should be prepared from the circulating strain in the region. Norgen *et al*. [20] recorded that the molecular diversity among *P. multocida* may be due to antigenic shift and a consequence of homologous intragenic recombination within the antigen genes. Nevertheless, this kind of intragenic changes has not led to phenotypic changes and may be recognized by PCR-fingerprinting [21]. Further investigation would be useful to clarify the correlation with genotypes and divergent patterns of pathogenicity.

CONCLUSION

It could be concluded from the present study that there are diversity among *P. multocida* isolates from rabbit clinical cases in Zagazig suburb. PCR-fingerprinting helped to distinguish their genotypes and further studies are needed to trace the sources of pasteurellosis in rabbits. This finding may help in establishment of prevention and control studies of rabbit pasteurellosis.

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