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COMPARISON BETWEEN TRADITIONAL METHODS AND REAL TIME PCR FOR DIAGNOSIS OF PASTEURELLA MULTOCIDA FROM DISEASED RABBITS

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A B S T R A C T

New-Zealand diseased rabbits (n=80) of different ages (16 suckling, 22 weaned, 31 growing and 11 adult) were obtained from three different rabbit farms at Kaliobeya governorate and were examined for *Pasterulla multocida* (*P. multocida*) microorganism. All rabbits were subjected to clinical and postmortem examination. Samples were collected aseptically from lungs, liver, spleen, heart-blood and nasal swabs. Bacteriological examination revealed that *P. multocida* was isolated from liver, lungs, spleen, heart-blood and nasal swabs of rabbits with an incidence 18.75%, 35.00, 21.25, 26.25 and 33.75%, respectively. Molecular detection by real-time PCR showed that *P. multocida* was verified in liver, lung, spleen, heart-blood and nasal swabs samples from diseased rabbits with an incidence 22.50, 37.50, 21.50, 30.00 and 37.50%, respectively. Comparing the results obtained by real-time PCR and traditional methods, all positive samples for *P. multocida* by traditional methods were also positive in real-time PCR assay, whereas 11 out of 292 the negative samples from the traditional methods were positive using the real-time PCR assays. Current results indicated that real-time PCR is more sensitive and specific for detection of *P. multocida*.

KEY WORDS: Bacteriological examination, *Pasterulla multocida*, PCR Assay, Rabbit,

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1. INTRODUCTION

Pasteurella multocida (P. multocida) is a non-motile, facultative anaerobic, Gram-negative bacillus associated with a spectrum of animal diseases. Diseases caused by *P. multocida* include fowl cholera in birds; atrophic rhinitis in pigs; hemorrhagic septicemia in ungulates; enzootic pneumonia in cattle, sheep, and goats; and snuffles in rabbits [2, 17].

Pasteurellosis is one of the most significant bacterial diseases of rabbits and causes considerable economic loss in large production units throughout the world. This bacterial species is considered an opportunist pathogen and can be found in the respiratory tract of healthy and diseased animals [5, 12, 15]. The disease is characterized by various clinical symptoms as respiratory distress (snuffles), genital affections, abscesses and septicemia but also infection by *P. multocida* can also appear without any clinical signs [6].

Pasteurellosis is a highly contagious disease of rabbits caused by *P. multocida* [8, 22] with prevalence rate has been reported to be between 70% and nearly 100% [5]. It can be transmitted by direct and indirect contact. More than 50% of adult rabbits either die or are culled due to *P. multocida* [15].

Accurate laboratory diagnosis of *P. multocida* depends on the isolation and identification of suspect bacterial causative agent by microscopy and biochemical tests. Extensive sub-culturing is required to obtain a pure culture of the causative organism required for *P. multocida* serotyping [19]. In recent years, genotypic methods of bacterial identification have proved beneficial in overcoming some limitations of traditional phenotypic procedures. Nucleic acid-based assays allow the detection of organisms directly from clinical samples or from small amounts of cultured bacterial cells, thus improving the sensitivity and decreasing the time required for bacterial identification [7, 18].

The purpose of the study was detection and characterization of *P. multocida* strains that caused different outbreaks of rabbit pasteurellosis and comparison between traditional methods and real time PCR for diagnosis of *P. multocida* from diseased rabbits and recently dead rabbits.

2. MATERIALS AND METHODS

2.1. *Animals and sampling*:

A total number of 80 New Zealand diseased rabbits of different ages (16 suckling, 22 weaned, 31 growing and 11 adult) were obtained in three different rabbit's farms at Kaliobeya Governorate for *P. multocida* (table 1). All rabbits were subjected to clinical and postmortem examination.

Samples were collected aseptically from lungs, liver, spleen, heart-blood and nasal swabs from dead rabbits during postmortem examination or from diseased rabbits after euthanasia in sterile packet and transported to laboratory in an ice box for bacteriological examination as soon as possible.

2.2. *Isolation and identification of Pasteurella multocida*:

Samples were pierced with a sterile platinum loop and cultured directly into brain heart infusions (BHI) agar, Blood agar, MacConkey agar and Nutrient broth and were incubated at 37°C for 24 hrs. Suspected colonies (very minute and brilliant) were picked up and sub-cultured on slopes and incubated at 4°C for further studies.

Identification of *P. multocida* (non-hemolytic) was carried out through staining by Giema and Leishman stain and examination under microscope to see the bipolarity. Blood film from heart blood was stained by Leishman stain examined for the bipolarity of *P. multocida.* Suggestive colonies of *P. multocida* were subjected to morphological and biochemical identification [3, 11, 16].

2.3. *DNA extraction and quantitative real-time RT-PCR (qRT-PCR)*:

Bacteria were harvested from triplicate BHI cultures. Extraction of genomic of bacteria was done by GeneJETGenomic DNA Purification Kit. #K0721 (Thermo Fisher Scientific, Inc., USA)

2.4. *Real-time PCR:*

Real-time PCR was done using the Stratagen system with SYBRâ Green JumpStartä Taq ReadyMixä #S4438 (Sigma-Aldrich, USA) I detection and Tm analysis. The procedure was optimized with regard to concentrations of primers, and denature/extension temperature.

The optimized reaction was carried out in 20µl final reaction volume containing10µl of kit-supplied SYBR® PCR master mix, 0.4µl concentrations of each forward and reverse primer (each 10 µm), KMT1T7-5'-ATC CGC TAT TTA CCC AGT GG-3 'and KMT1SP6 5'-GCT GTAAAC GAACTC GCCAC-3' [24, 25], 2µl DNA template, and 7.2µl distilled water to final volume 20.0µl. Prior to cycling, the glass capillaries were sealed and centrifuged at 3000 rpm for 10 sec. The thermal profile for the real-time PCR was 95°C for 120 sec, followed by 40 cycles of 95°C for 10 sec, 60°C for 30 sec with two-step.

2.5. *Melting curve analysis of the PCR product:*

Melting curve analysis was performed to measure the specificity of PCR product. After PCR cycling, samples were heated to 95°C for 15 sec, 65°C for 15 sec and then heated to 95°C for 15 sec at a linear transition rate of 0.1°C/sec, and then hold at 16°C. Fluorescence of the samples was monitored continuously while the temperature was increasing. SYBR Green I is released upon denaturation, which resulted in a decreasing fluorescence of the signal. The software calculates the *T*m. All samples were analyzed once.

Table 1 Number of examined rabbits from different rabbit's farms at Kaliobeya Governorate.

Farm number	Locality	Total No. of rabbits in farm	Total No. of ex. Rabbits	%
1	Toukh	2800	36	1.28
2	Kalyoub	1400	28	2.00
3	Sheben Alkanater	1200	16	1.33
Total		5400	80	1.48

% was calculated according to the total No. of rabbits in farm.

3. RESULTS AND DISCUSSION

Pasteurellosis is a highly contagious disease of rabbits caused by .*P. multocida*. Rabbits can become infected with *P. multocida* immediately after birth and the prevalence of *P. multocida* colonization increases with age until about 5 months [14]. *P. multocida* causes aspectrum of conditions including rhinitis (snuffles) with purulent nasal discharge, pneumonia, otitis media, pyometra, orchitis, abscesses, oculoconjunctivitis and septicaemia. It is considered to be a predominant cause of death in rabbits which in turn result in considerable economic losses to the rabbit industry [4, 10, 20].

Results in table 2, showed the incidence of *P. multocida* from diseased rabbits by bacteriological isolation (traditional methods). Out of 400 samples 108 samples (27%) were positive for bacteriological isolates. *P. multocida* was recovered from liver, lung, spleen, heart-blood and nasal with an incidence of 18.7, 35, 21.2, 26.2, and 33.7%, respectively. This result is nearly similar to the previous studies [13, 22] found that the isolation rate of *P. multocida* was 27-31% in diseased rabbits. On the other hand, higher results 77.5% was recorded by Stelian et al. [21]. The variation in distribution frequency may be due to individuality of health or immunological status of the sampled rabbits and environmental conditions [5]. *P. multocida*is considered to be an important pathogenic bacterium of domestic animals. Particularly, outbreaks caused by this species that resulted in considerable economic losses in rabbi tries [12]. It appeared that some virulence factors (i.e. bacterial adhesion to respiratory tract epithelial cells, inhibition of phagocytosis, and toxin production) and vaccine efficacy are related to *P. multocida* capsular sero- groups [1, 2].

Results in table 3, showed the incidence of *P. multocida* from diseased rabbits using real time PCR. Out of 400 samples 119 samples (29.7%) were positive for bacteriological isolates.

Table 2 Incidence of *Pasteurella multocida* from diseased rabbits using traditional methods

Organ	No. of examined	Positive		Negative
		N	%	N
Liver	80	15	18.7	65
Lung	80	28	35.0	52
Spleen	80	17	21.2	63
Heart-blood	80	21	26.2	59
Nasal	80	27	33.7	53
Total	400	108	27.0	29

Recovered *P. multocida* from liver, lung, spleen, heart blood and nasal was at an incidence of 22.5, 37.5, 21.5, 30.0 and 37.5%, respectively.

Table 3 Incidence of *Pasteurella multocida* from diseased rabbits using real time PCR

Organ sample	No. of examined	Positive	
		n	%
Liver	80	18	22.5
Lung	80	30	37.5
Spleen	80	17	21.2
Heart-blood	80	24	30
Nasal	80	30	37.5

Results in Fig 1 showed that all positive samples recorded from the traditional methods after boiling. We can observe that the positive samples appeared from cycles 16 and above the threshold, while the negative appeared below the threshold. After boiling the negative group which recorded from the traditional methods it appeared as positive results but appeared from the 26 cycles.

Results in Fig 2 showed the positive samples melting curve recorded at 84.4°C Comparison the results obtained by real-time PCR and traditional methods . All positive samples for *P. multocida* by traditional methods were also positive in real-time PCR assay, whereas 11 in 292 of the samples negative for *P. multocida* in the traditional were positive in the real-time PCR assays.

A SYBR Green I based quantitative PCR is an excellent diagnostic tool with high sensitivity, specificity, and a fast turnaround time [8, 23]. This system is called real-time PCR because the accumulated amplicons can be monitored directly during the DNA amplification process in closed tube with no post-PCR electrophoresis by a real-time PCR method. In addition, the real-time PCR technique has been shown to provide good sensitivity and a linear relationship between the copy number and cycle threshold (Ct) values. The quantization of DNA is based on the determination of the threshold cycle when the amplified PCR product is first detected. The higher the initial DNA copy number input, the sooner the product of amplification is detected.

SYBR Green I can bind to any double-strand DNA, so the dye can also be used in diagnosis of other bacteria, and most of real-time machines can detect the fluorescence emitted by SYBR Green I. These will lower the diagnosis costs and make the method more applicable and practicable than probe. The real-time PCR detection system complements and extends previous methods for detection and quantization of *P. multocida* [26].

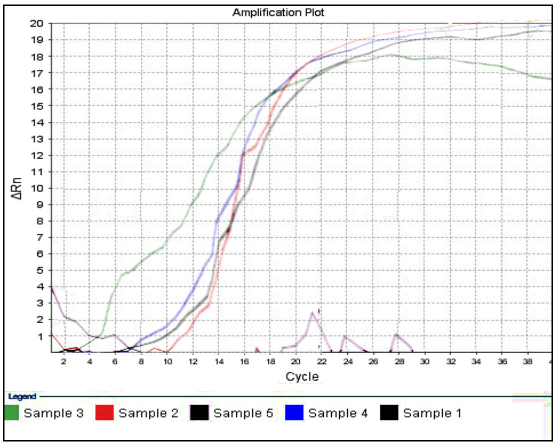

Fig 1 Amplification plots for positive samples. ■ All positive samples resulted in using traditional methods. ■ Group of negative liver samples using traditional methods. ■ Group of negative lung samples using traditional methods. ■ Group of negative heart blood samples using traditional methods. ■ Group of negative nasal samples using traditional methods.

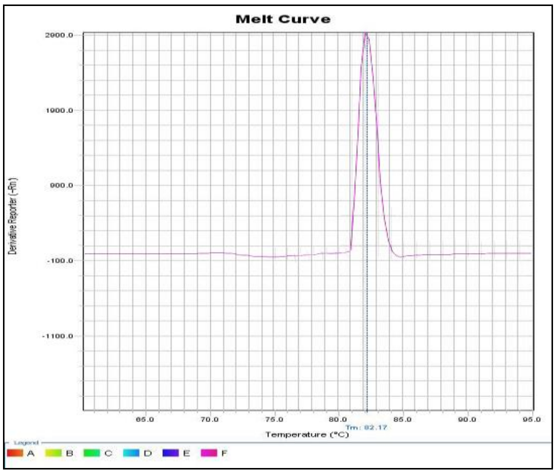

Fig 2 Dissociation curve for positive samples recorded at 82.17˚C.

The melting temperature of *P. multocida* detected at 83.64°C these results nearly to [9, 26] who recorded the melting point at 85.5°C.

The real-time PCR increased the detection of *P. multocida* samples over that achieved by traditional methods. Tests on the reproducibility and specificity of the method suggest that the established real-time PCR system appears to be reliable and stable.

4. CONCLUSION

In conclusion, the established real-time PCR assay was rapid, sensitive and specific for the detection and quantification of *P. multocida* over that achieved by bacteriological isolation from diseased rabbits. This finding helps in the prevention and control of rabbit pasteurellosis.

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مجلة بنها للعلوم الطبية البيطرية

مقارنة بين الطرق التقليدية وتفاعل البلمرة المتسلسل في تشخيص الباستريلا في الأرانب المصابة

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الملخص العربي

اجريت هذه الدراسة على ثمانون من الأرانب النيوزلندية المريضة المختلفة الأعمار (16 رضيعة، 22 مفطومة، 31 يافعة، و11 بالغة) من ثلاث مزارع مختلفة في محافظة القليوبية لبيان مدى اصابتها بميكروب الباستريلا. تم تجميع عينات من الرئتين، الكبد، الطحال، دم القلب، ومسحات الأنف. اظهرت نتائج الفحص البكتريولوجي عزل ميكروب الباستريلا من الأرانب بنسبة 18.75%، 35.00%،21.25%، 26.25%، و 33.75% على التوالي الكبد والرئتين والطحال والقلب ومسحات الأنف. في حين أن إستخدام تفاعل البلمرة المتسلسل بين أن نسبة عزل الباستريلا من الكبد، الرئتين، الطحال، الدم في القلب، ومسحات الأنف للأرانب المريضة كانت 22.5%، 37.5%، 28.7%، 22.5%، و 37.5 على التوالي. بمقارنة النتائج من تفاعل البلمرة المتسلسل وطرق عزل البكتريولوجي التقليدية كانت جميع العينات الأيجابية بالطرق التقليدية ايضاً إيجابية في نفس الوقت باستخدام تفاعل البلمرة المتسلسل، بإستثناء احد عشر عينة من اجمالى عدد العينات الخاضعة للدراسة كانت سلبية بطرق العزل التقليدية و كانت ايجابية باستخدام تفاعل البلمرة المتسلسل.

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