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SOME MICROBIOLOGICAL STUDIES ON *PASTEURELLA MULTOCIDA* IN RABBITS

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ABSTRACT

To establish this goal, a total of 120 samples of blood and nasal swabs (60 samples from apparently healthy rabbits and 60 samples from diseased ones) were collected from different farms scattered at different localities in Sohag governorate. All samples were submitted for bacteriological examination by conventional culture method. Recovery of *pasteurella* isolates from apparently healthy and diseased rabbits were (16), (37) isolates respectively, with isolation rate of (26.7%), (61.7%) respectively and total isolation rate of (44.2%). Isolation of *pasteurella*, colonial and cellular morphological pictures were studied and recorded Biochemical reaction proved that only 15(28.3%) of isolates had typical biochemical properties of *pasteurella multocida*. Serotyping of (15) *pasteurella* isolates by using ELISA test revealed that only 13(86.7%) out of 15 *pasteurella* isolates were positive for ELISA test. The capsular serotyping of the selected (13) *Pasteurella multocida* isolates were found to be belong to capsular serotype (A). The somatic serotyping proved that (8 isolates) were belonged to serotype 3 with a percentage of (53.3%) followed by (4 isolates) were belonged to serotype 12 with a percentage of (26.7%) and (one isolate) was belonged to serotype 1 with a percentage of (6.7%). The recovered isolates were submitted for molecular identification of KMT1 gene by PCR. Only 14 (93.3%) out of (15) isolates were positive to KMT1 gene and confirmed to be *Pasteurella multocida*. The occurrence of *Pasteurella multocida* of examined samples of apparently healthy rabbits and diseased ones were (8.3%) and (15%) respectively.

Key words: Microbiological, *Pasteurella Multocida*, Rabbits

INTRODUCTION

Pasteurella multocida is a gram-negative, non-spore forming, non-motile and capsulated coccobacillus that is penicillin-sensitive and belongs to the family *pasteurellacae* (Kuhnert and Christensen, 2008). *Pasteurellosis* or snuffles in rabbits is one of the most serious diseases which causes considerable economic losses in rabbit production units (Stelian *et al*., 2011). It is caused by *pasteurella multocida* which may cause death in rabbits or local infection as (rhinitis, otitis media and abscess), pneumonia and septicemia (Deeb *et al.*, 1990). Infection by *Pasteurella multocida* can also appear without any clinical signs manifested (Jaglic *et al*., 2006).

The species of *Pasteurella multocida* is subdivided into four subspecies that include the type strain, *multocida* and three others, gallicides, septic and the recently described tigres (Petersen *et al*., 2001). It has 5 strains i.e. A, B, D, E and F and 16 serotypes (1 - 16) (Azmat *et al.*, 2013). *Pasteurellosis* in rabbits is mainly caused by the capsular type A and, to a lesser extent, capsular type D strains (Vandyck *et al*., 1995; Dabo *et al*., 1999). Diagnosis of the bacterium was traditionally based on clinical findings, culture and serological testing. *Pasteurella multocida* when grow in various media, yielded moderate to dense growth. Good growth is obtained in Brain heart infusion (BHI) and Casein sucrose yeast (CSY) while poor growth is obtained in Nutrient broth (NB) media (Mahmood, 2001). It will not grow on MacConkey agar and the colony growth is accompanied by a characteristic "mousy" odour that is due to metabolic products. On Leishman's staining, heart blood smears and tissue impression smears prepared from liver, spleen and lung revealed characteristic bipolar organisms suggestive of *Pasteurella multocida* (Purushothaman *et al*., 2008).

Pasteurella species have both an oxidative and fermentative metabolism. Glucose and other carbohydrates are catabolised with the production of acid but without gas. Most species are catalase-positive and oxidase-positive; nitrates are reduced to nitrites by almost all species (Health Protection Agency, 2007).

Pasteurella multocida ELISA test was used for rapid serological identification of such pathogen according to Weber *et al*. (1993). As serology can't differentiate

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between current infection and previous exposure, the quickest and most accurate method for confirming an active *Pasteurella multocida* infection is molecular detection using polymerase chain reaction (Miflin and Balckall, 2001).

The aim of this work was carried out to isolation and identification of *Pasteurella multocida* from both apparently healthy rabbits and diseased ones, serotyping of the isolated microorganisms and characterization of *pasteurella* strain by (PCR).

MATERIALS AND METHODS

1. Animals and sampling:
A total number of 120 apparently healthy and diseased rabbits of different ages (60 of apparently healthy and 60 diseased rabbits) were obtained from three different rabbit farms at Sohag governorate. Samples were collected aseptically from blood and nasal swabs from rabbits in sterile packet and transported to laboratory in an ice box for bacteriological examination as soon as possible.

2. Isolation and identification of *Pasteurella multocida*:
The prepared samples were inoculated under aseptic conditions in enriched nutrient broth incubated for 20-24h at 37°C.nasal swabs samples and a loopful from each blood samples were streaked onto blood agar and casein/sucrose/yeast (CSY) agar containing 5% sheep blood. Suspected colonies were selected and subcultured onto 5% sheep blood agar, CSY and MacConkey then checked for suspected *Pasteurella multocida* colonies. Blood film from heart blood was stained by Leishman stain examined for their bipolarity of *Pasteurella multocida*. Suggestive colonies of *Pasteurella multocida* were subjected to biochemical identification Manasa *et al*. (2013).

3. Serological identification of *Pasteurella multocida* by ELISA:
The commercial *Pasteurella multocida* ELISA test kits (Glory Science Co., Ltd, China Manufacturers, China) were used for rapid serological identification of such pathogen according to Weber *et al*. (1993) then using indirect - haemagglutination test for capsular serotyping and agar gel precipitation test for somatic serotyping.

4. *Pasteurella multocida* specific polymerase chain reaction (PM-PCR):
PCR has been proved to be useful in the detection of DNA of *Pasteurella multocida* (Purushothaman *et al*., 2008; Townsend *et al*., 1998). The success of PCR depends on the method of DNA extraction. The addition of Guanidine thiocyanate, Cetyltrimethyl ammonium bromide, Phenol extraction to the specimen is one of the options for DNAextraction. In the present study, simple boiling method was used to extract the DNA of *Pasteurella multocida* which makes the PCR technique an even more rapid and effective technique for *Pasteurella multocida* identification. Amplifying DNA fragment with in KMT1 gene using the Primers (KMT1SP6 and KMT1T7). In comparison with standard molecular weight marker (100bp), the molecular weight of the PCR products of all the isolates were found to be 460bp specific for *Pasteurella multocida* and confirmed the isolates as *Pasteurella multocida*.

RESULTS

Table 1: Observations of cultural and morphological characters of *Pasteurella multocida*.

Test Performed	Results
Colonial morphology.	Smooth, glistening, translucent colony with circular edge and non-haemolytic colonies on blood agar.
Colony diameter.	Approximately 1 mm in diameter, on blood agar. Colonies grown on Casein/sucrose/yeast agar (CSY) agar are larger.
Staining with gram and leishman's stain.	Negative Pink/ Red short rods with bipolarity.
Capsule stain.	Capsule was observed around cell wall.

Table 2: Biochemical activity of *Pasteurella multocida* isolated from apparently healthy rabbits and diseased ones.

Suspected isolates		No. of biochemically positive isolates	
Origin of isolates	**No.**	**No.**	**%**
Apparently healthy	**16**	**6**	**37.5**
Diseased	**37**	**9**	**24.3**
Total	**53**	**15**	**28.3**

Table 3: Biochemical characterization of *Pasteurella multocida*.

Test	Reaction
Motility test	**Non motile**
Catalase test	**Positive (+)**
Oxidase test	**Positive (+)**
Indol test	**Positive (+)**
Urease test	**Negative (-)**
Sugar fermentation test	
Glucose	**Positive (+)**
Mannitol	**Positive (+)**
Lactose	**V-**

Table 4: Serological typing of *Pasteurella multocida* isolated from rabbits.

Origin of isolates	Positive for *Pasteurella multocida*	Capsular and somatic serotyping					
		A:3		A:12		A:1	
Apparently healthy	No.	No.	%	No.	%	No.	%
	6	1	16.6	1	16.6	0	0
Diseased	9	7	77.7	3	33.3	1	11.1
Total	15	8	53.3	4	26.7	1	6.7

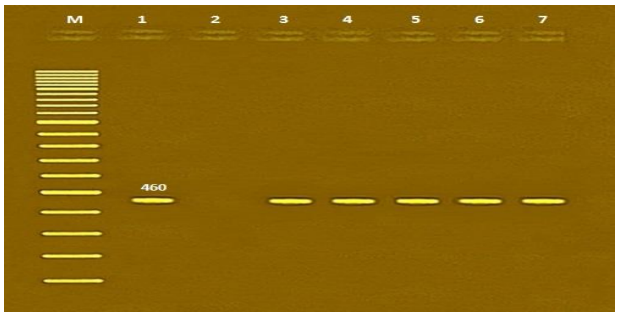

Fig. 1: showed Lane M: 100 bp ladder as molecular size DNA marker, Lane 1: Control positive *Pasteurella multocida*, Lane 2: Control negative DNA free sample and lanes 3 to 7: positive *Pasteurella multocida* and these isolates gave a single amplified product of the expected size of 460 bp.

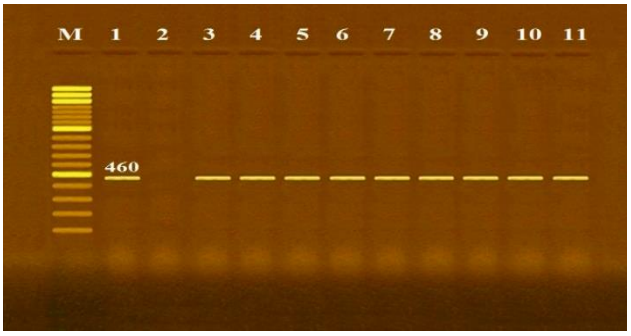

Fig. 2: Showed Lane M: 100 bp ladder as molecular size DNA marker, Lane 1: Control positive *Pasteurella multocida*, Lane 2: Control negative DNA free sample and lanes 3 to 11: positive *Pasteurella multocida*.

Table 5: PCR confirmation of *Pasteurella multocida* isolates using species specific primers.

No. of *P. multocida* isolates	PCR +VE isolates	%	PCR -VE isolates	%
6	**5**	**83.3**	**1**	**16.7**
9	**9**	**100**	**0**	**0**
15	**14**	**93.3**	**1**	**6.7**

Table 6: Occurrence of *Pasteurella multocida* isolated from different samples of apparently healthy rabbits and diseased ones.

Origin of samples	No. of samples	*Pasteurella multocida*	
		No	%
Apparently healthy	60	5	8.3
Diseased	60	9	15
Total	120	14	11.7

DISCUSSION

All isolates were subjected to full phenotypic characterization that showed in Table (1) revealing that all isolates were typical non-haemolytic, smooth, grayish glistening translucent and dew drop like colonies and their diameter was 1mm approximately on blood agar but these colonies were larger when grow on CSY agar. Direct smears from fresh samples that stained with gram's and leishmans stain's show gram negative capsulated cocco bacilli with bipolarity. These results are reported also by Abdullah *et al*. (2013); Bhimani *et al*. (2014).

Biochemical reactions of isolates as recorded in Table (2) revealed that (15) isolates out of all suspected isolates recovered from blood, nasal swabs samples of apparently healthy and diseased rabbits were positive biochemically with apercentage of (28.3%). These results go hand in hand with Ekundayo *et al.* (2008); Manasa *et al*. (2013).

As well as these isolates were non motile and positive for oxidase, catalase and in dole test but negative for urea hydrolysis. Also able to ferment glucose, mannitol and can't ferment lactose. As shown in Table (3) these results go hand in hand with the previous results of Purushothaman *et al*. (2008); Abdullah *et al*. (2013) and Azmat *et al.* (2013).

The recorded date in Table (4) showed that 15 suspected organisms isolated from examined samples were submitted for ELISA test for *Pasteurella multocida* and revealed that *Pasteurella multocida* isolate from rabbits belong to capsular serotype (A) which agreed with the results ofJonas *et al.* (2001) and Tahmatan *et al*. (2014) and somatic serotypes belonged to 3:A (8 isolates) with a percentage of 53.3%, 12:A (4 isolates) with a percentage of 26.7%, 1:A (1 isolates) with apercentage of 6.7% while the remaining two strains (13.3%) were untypable. These results nearly coincided with those obtained by Okerman (1993).

The results illustrated, as in Table (5), that 14 out of 15 isolates were positive *Pasteurella multocida* with apercentage of 93.3% of the examined isolates. The specificity of the primer was confirmed by positive amplification of fragment with the extracted DNA of the bacterial isolates at expected size of 460 bp as shown in Fig. (1) and(2).

The obtained results in Table (6) proved that the occurrence of *Pasteurella multocida* of apparently healthy rabbits was 8.3%. These results nearly coincided with that reported by Eslam (2015), who found an isolation incidence of 9.42%, and Nada (1994), who examined 53 nasal swabs originated from apparently healthy rabbits, and isolated *Pasteurella multocida* with an incidence of 9.1%. In the present work, incidence of *Pasteurella multocida* among diseased rabbits was 15%. These results are nearly in accordance with Ibrahim (1993); Hussein (2000) and Hussein (2004) who isolated pasteurella multocida with an incidence of (12%) , (12.3%) and (12.6%) respectively.

CONCLUSION

Under conditions of this investigation and according to data obtained, it was concluded that:-

1. PCR assay is highly sensitive and specific method for detection and identification of *Pasteurella multocida* than conventional biochemical and serological tests as a rapid technique.

2. *Pasteurella multocida* is one of the most serious pathogens of rabbits; it causes considerable economic losses in rabbits production units.

3. Rabbit's infection of *Pasteurella multocida* represent great harm to human health as the rabbits consider one of the most important source of food for humans.

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بعض الدراسات الميكروبيولوجيه علي ميكروب الباستريلا ملتوسيدا في الارانب

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أجريت هذه الدراسة على بكتريا الباستريلا ملتوسيدا لتشمل ١٢٠ عينة (٦٠ عينة دم و٦٠ عينه من المسحات الأنفية) من الأرانب السليمة والمشتبه في إصابتهم بالميكروب والتي تم جمعها من مصادر مختلفة بمحافظة سوهاج. وقد خضعت جميع العينات للفحص البكتريولوجي. باستخدام الطرق التقليديه في العزل وتم الحصول علي العزل المبدئي في الارانب السليمة والمصابة وكانت (١٦) و (٣٧) معزوله بنسبة (٢٦.٧٪) و (٦١.٧٪) علي التوالي وبذلك فإن العدد الكلي للعزل كان (٥٣) معزوله بنسبة (٤٤.٢٪) وتم دراسة ووصف وتسجيل الاشكال الظاهرية (المورفولوجيه) لخلايا الميكروب بالصبغات المختلفه وكذلك شكل مستعمراته علي المستنبتات المختلفه. كما أظهرت الاختبارات البيوكيميائيه أن هناك ١٥ معزوله فقط من إجمالي العدد الكلى للمعزولات علي انها باستريلا ملتوسيدا بنسبة (٢٨.٣٪). وتم إجراء اختبار (الإليزا) الخاص بعتره الباستريلا ملتوسيدا وكانت عدد المعزولات الموجبة لهذا الاختبار ١٣ معزوله من المعزولات الـ (١٥) الإيجابية بنسبة (٨٦.٧٪) وعدد ٢ من المعزولات لم يتم التعرف عليها بنسبة (١٣.٣٪). وبدراسة تصنيف الـ ١٣ معزوله إعتماداً علي انتيجن المحفظه الخارجية للميكروب وجد أن جميع المعزولات تتبع النوع (A) وبالتصنيف إعتماداً علي انتيجن الجسمي أظهرت النتائج أن (٨) معزولات تتبع النوع (٣) هو الأكثر انتشاراً بنسبة (٥٣.٣٪) ثم يتبعه (٤) معزولات تتبع النوع (١٢) بنسبة (٢٦.٧ ٪) ثم معزوله واحده فقط تتبع النوع (١) بنسبة (٦.٧٪). وتم اجراء اختبار لـ ١٥ عتره المعزولة باستخدام تفاعل البلمره المتسلسل لتحديد الجين الخاص بجنس الباستريلا باستخدام بادئ لتضاعف جين (KMT1) وكانت نسبة تواجد الجين بنسبة (٩٣,٣%) واتضح من ذلك أن تفاعل البلمره المتسلسل يعتبر أكثر دقة وحساسية من الاختبارات التقليدية والسيرولوجيه. تشير نتائج هذا البحث أن تواجد ميكروب الباستريلا ملتوسيدا من كل العينات التي تم فحصها من الارانب السليمة ظاهريا والارانب المصابة كانت بنسبة (٨.٣٪) و(١٥٪) على التوالي.