رقم البحث (17)

PREVALENCE OF PSEUDOMONAS AEROUGINOSA ISOLATED FROM PNEUMONIC AND APPARENTLY NORMAL FARM ANIMAL AND DETECTION OF EXOTOXIN A (ETA) GENE BY USING PCR

BY

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ABSTRACT

The present study was carried out on 419 (nasal, tracheal and lung) samples collected from pneumonic and apparently normal cows and 131 (nasal, tracheal and lung) samples collected from pneumonic and apparently normal buffaloes. The bacteriological examination revealed that *Pseudomonas aeruginosa* was isolated from 172(41.1%) out of 419 cows samples and from 46(35.1%) out of 131 buffaloes samples. The positive strains biotyped and tested for its virulence by using congored and toxicity tests. Congored test revealed that 121 out of 218 (*P.aeruginosa* positive strains isolated from cow and buffalo samples) were more virulent and 41strain were toxigenic for mice. PCR technique revealed that presence of exotoxin A (ETA) gene in toxigenic strain.

Keywords, *P .aerouginosa,* Toxin, PCR, Congored.

INTRODUCTION

Respiratory diseases are one of the major problems specially pneumonia, which causing significant mortalities and high coasts are required to reduced mortality rate (**Ajoy et *al.*, 2005; Cassirer *et al.*, 2007, and Goodwin *et al.*, 2008**). Different strains of bacteria cause

pneumonia such as *Pasteurella* species (*P. multocida*), *Pseudomonas* Sp. and Klebsiella *pneumonia* (**Hamad and Al-Attar, 2006**), *P.aeruginosa* is an opportunistic Gram-negative rod which is an important cause of nosocomial infection leading to septicemia **(Ali *et al.*, 2009 and Thet *et al.*, 2013*).*** *P. aeruginosa* is highly pathogenic as it produces large number of toxins; these toxins are important factors in the pathogenicity of *P. aeruginosa*. **(Wolska *et al.*, 2002)**. ETA is considered as the most toxic virulence factor secreted by *P. aeruginosa*. The toxin was first discovered and purified by **Liu (1973)** who reported that *P. aeruginosa* PA103, a protease-deficient strain, produces more toxin than do strains of *P. aeruginosa* which actively produce proteases **(Stephen *et al.*, 1988; Susan *et al.*, 2005).** Exotoxin A appears to mediate both local and systemic disease processes caused by *P. aeruginosa*. It has necrotizing activity at the site of bacterial colonization. Toxigenic strains cause a more virulent form of pneumonia than non-toxigenic strains. In terms of its systemic role in virulence, purified ETA is highly lethal for animals **(Boudenet *et. al.*, 2004).**

Although conventional microbiological methods for identifying *P. aeruginosa* are reliable yet they require several days to complete. Due to the nature of P. *aeruginosa* it is important that rapid and early biomedical diagnostic techniques are developed in order to detect it early which enhances the chance and the rate of recovery. PCR procedure can rapidly and specifically detect. *P. aeruginosa* strains in environmental samples by amplifying the ETA structural gene **(Saiki *et al.*, 1988, Ashraf and Carl, 1994, and Amany, 2004)**.

The objective of this work was isolation and identification of *P. aeruginosa* from pneumonic and apparently normal cows and buffaloes also detection to exotoxin A (ETA) which is considered as the most toxic virulence factor secreted *P. aeruginosa* by using PCR technique.

MATERIALAND METHOD

Samples

During this study a total of 550 samples were collected from pneumonic and apparently normal cows and buffaloes as described in table (1). All samples were obtained from different private farms and abattoirs in Dakahlia Governorate and slaughter house in Basateen.

Table (1) : Number and types of examined samples

Animal species	Type of samples	Apparently normal	Diseased	Total
Cows	Nasal swab	65	174	239
	Tracheal swab	10	30	40
	Lung tissues.	20	120	140
Total		**95**	**324**	**419**
Buffaloes	Nasal swabs.	20	67	87
	Tracheal swabs.	9	13	22
	Lung tissues.	5	17	22
Total		**34**	**97**	**131**
Overall total		**129**	**421**	**550**

- **Bacteriological examination.**

An inoculum from broth containing the sample was cultivated onto the following media in duplicate including: Pseudomonas agar base with C-N (cetrimide nalidixic acid) supplement, blood agar, MacConkey's agar and nutrient agar plates. All inoculated plates were incubated aerobically at 37^{o}C for 24-48 hrs then examined.

- **Identification of the isolates.**

Suspected colonies were described for their appearance, haemolytic activity, and colonial characters according to **Koneman *et al.* (1992).**

Biochemical examination: (MacFaddin, 1980):

Such as oxidase test**,** motility test**,** sugar fermentation test, nitrate reduction test, urease activity test**,** aesculin test.

- **Serological identification of the isolates:** Unheated viable cells were used as an antigen.

1- The pure, biochemically identified culture of *P. aeruginosa* was streaked on a petri dish of nutrient agar medium using a bacteriological loop, in such a manner as to obtain individual isolated colonies.

2- The inoculated plates were incubated in an inverted position for 18-24 hours at 37^{o}C.

3- Pasteur pipette was used to place droplets of Bacto- *P. aeruginosa* antisera (diluted 1:10) on the appropriate squares of the glass plate.

4- Using a bacteriological loop, suspend part of an isolated colony, into the droplet of antisera. Care must be taken not to cross mix the antiserum droplet.

5- The plate was rotated by hand for about a minute and then observed for the presence of agglutination.

It was advisable to employ a 1:10 dilution of normal rabbit serum for use as a negative control for the antigen. Such as a negative control rules out a rough antigen which might agglutinate spontaneously.

- **Congo red binding activity: (Beckhoff and Vinal, 1986)**

P. aeruginosa strains were cultured onto Congored medium. The reactions were best seen after 24 hours of incubation at 37^{o}C and then left at room temperature for additional 2 days.

- **Determination of toxicity:**

The toxicity was determined according to **Liu and Hsieh, (1973)** as follows: the prepared toxins were diluted in 0.01 M tris-buffer at pH 8.00. 1 ml was injected I/P into mice weighting about 20 grams (3 mice for each).

All inoculated mice were kept under observation of 5 days. The results of mortality and recovery of inoculated toxin were recorded

- **Detection of the ETA gene of *P. aeruginosa* by PCR:**

1. **Isolation of bacterial DNA** was performed according to **(Sarnbrook *et al.*, 1989).**
2. **Determination of isolated DNA.** Extracted DNA was determined by using UV spectrophotometer at 260 nm and 280 nm to measure the concentration of DNA and determine the degree of purity.
3. **Polymerase Chain Reaction (PCR):** PCR was performed according to the method described by **Ashraf and Carl (1994)**
4. **Identification of PCR products:** Following amplification, a 10 µl of the PCR product was mixed with 2 µl of 6 x loading buffer and taken for electrophoresis on a 2% (wt/vol) agarose gel. The experiment was evaluated after being stained with ethidium bromide under UV lamp. A visible band being sized by DNA molecular marker was considered as positive sample.

Nucleotide sequence for ETA according to **(Belaaouaj *et al.*, 1994)**.

(5'-ATGAGTATTCAACATTTTCGTGC-3') 5'CCAATGCTTAATCATGAGGCACC-3'

RESULT

Table (2): Incidence of *P. aeruginosa* obtained from examined diseased and apparently normal cows and buffaloes.

Animal	Samples	No. of examin samples	Positive		Negative	
			No.	%	No.	%
Cows	**Nasal swabs**	239	89	37.2	150	62.8
	Tracheal swabs	40	15	37.5	25	62.5
	Lung tissues	140	68	48.6	72	51.4
	Total	**419**	**172**	**41.1**	**247**	**58.9**
Buffaloes	**Nasal swabs**	**87**	**34**	**39**	**53**	**60.9**
	Tracheal swabs	**22**	**3**	**13.6**	**19**	**86.36**
	Lung tissues	**22**	**9**	**40.9**	**13**	**59.1**
	Total	**131**	**46**	**35.1**	**85**	**64.9**

Percent was calculated to total number of cows (419) and (131) buffaloes.

As shown in table (2) among a total of 419 cows samples (nasal,tracheal and lung) only172(41.1%) were positive for *P. aeruginosa* while the result of buffaloes samples showed that out of 131 samples(nasal, tracheal and lung) only 46(35.1%) were positive for *P. aeruginos.*

Table (3): Results of serotyping of *P. aeruginosa*.

Animals	Total positive strains	Type1	Untypable
Cows	**172**	**144**	**28**
Buffaloes	**46**	**40**	**6**

The results of serological identification of isolated strains of *P. aeruginosa* from cows showed that 144 isolates were belonging to serotype (1) and 28 isolates were untypable and the results of serological identification of isolated strains of *P. aeruginosa* from buffaloes showed that 40 isolates were belonging to serotype (1) and 6 isolates were untypable.

Table (4): Congored binding activity of *P. aeruginosa* recovered from different sources**.**

Origin of strains	No. of tested strains	* C.R^{+}		* C.R^{-}	
		No.	%	No.	%
Cattle	172	89	51.7	83	48.25
Buffaloes	46	32	69.56	14	30.4
Total	**218**	**121**	**55.5**	**97**	**44.5**

C.R.$^{+}$ = Congo Red positive.C.R.$^{-}$ = Congo Red negative.

Table (4) demonstrates the congo red activity of 218 *P. aeruginosa* isolates recovered from cows and buffaloes. There were 121(55.5)strains congo red positive from both cows and buffaloes also The highest CR+ rate (69.56%) was observed in *P. aeruginosa* isolates of buffalo origin; followed by that of cow origin (51.7%).

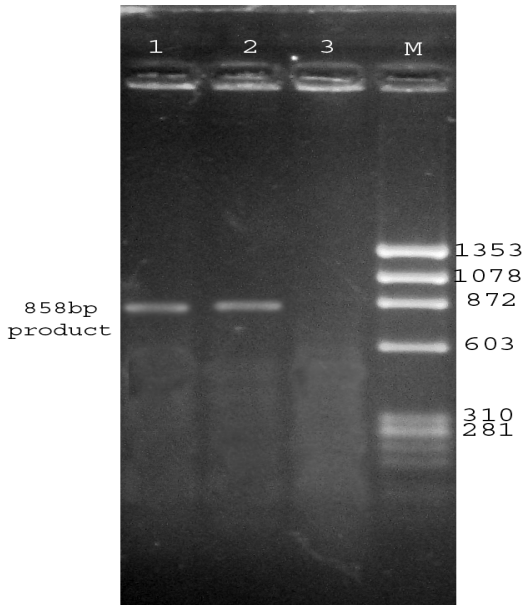
Table (5): Toxic effect of *P. aeruginosa* exotoxin in mice.

Tested strains (+ve) congo red	Dilution of toxin	Number of inoculated mice	Dead mice	Survival rate	% of survival	Toxigenic strain
121	1 : 32	363	129	234	64.4	41

The prepared toxins are protein in nature, heat labile and highly toxic when injected in mice. As shown in table (5) 129 inoculated mice were died within 2 days according to tested dilution of toxin and there were 41(33.9%) isolates out of 121 Congo red positive strains proved to be toxigenic for mice

Results of PCR analysis

As shown in photo (1). The ETA gene of *P. aeruginosa* isolated from clinical samples was detected also the ETA gene was detected for the representing strain for untypable strains and ETA gene not detected for the strain represent the negative toxigenic strains. ETA gene product was detected on 858bp.



Lane M: Molecular weight marker.
Lane 1: +ve strain for ETA gene where the amplification fragment 858bp
Lane 2: +ve strain (represent untypable strains) for ETA gene where the amplified fragment 858bp.
Lane 3: -ve strain (-vetoxogenic strains).

Photo No. (1): Electrophoretic pattern of PCR products (858bp specific for ETA gen) in 2% agrose gel stained with ethidiumbromid.

DISCUSSION

P. aeruginosa is an environmentally ubiquitous bacterium that causes severe infections among immunocompromised hosts (**Marlier *et al.*, 2000).** Thus, particular attention has been directed towards *P. aeruginosa* due to its gradual emergence as a significant animals, birds as well as human pathogens, fewreports dealing with the comparative studies on various strains of *P. aeruginosa* isolated from cattle and buffaloes in Egypt, so the questions about their incidence, serotyping, virulence factors and pathogenicity are still in question

A variety of cultural media have been applied in the present work to improve the isolation, then complete identification of the isolates from examined different clinical samples and apparently normal sample, in order not to miss the isolates. All isolated strains belonging to *P. aeruginosa* were extensively studied for their morphological, cultural, biochemical and serological characteristics were done.

It is worthy to note that the incidence of *P. aeruginosa* among examined cattle and buffaloes were 41.1% and 35.1% respectively as shown in table (2). These findings go hand with the observations of **Fuchs (1970); Hirsh *et al.*, (1979); Rajasekhr *et al.*, (1992) and**

Manal Bahaa (2004) who isolated *P. aeruginosa* from cows in higher incidence (15.4%) than in buffaloes (10.5%).

The results of Serological typing of 172 *P. aeruginosa* strains isolated from cows, 144 strains were belonging to serotype (1) and 28 strains were untypable with the available antisera and concerning serological identification of 46 strains of *P. aeruginosa* isolated from buffaloes, 40 strains were belonging to serotype 1 and 6 strains were untypable with available antisera as shown in table (3). Similar findings were reported by, **Riad (1994)** differentiated *P. aeruginosa* strains into nine serotypes and untypable groups and the most prevalent was serotype "1" (34.1%) and 2 strains were untypable serologically. Meanwhile, **Hariharan *et al.* (1989)** identified serologically *P. aeruginosa* recovered from domestic animals and found that serotype "6" was the most predominant followed by serotype "8" and "1". Also on the other hand, **He *et al,* (1998)** isolated different serological type from 101 strains of P. *aeruginosa with* the typed rate of (8)4.2%. Among them type (7) 23.7%, type (1) 13.9% and another strains 15.8% could not be differentiated and so named untyped *P. aeruginosa* group.

The present work was carried with the aim to determine the several virulence factors possessed by *P. aeruginosa* strains isolated from cows and buffaloes. Firstly, in this work, it was started by Congo red activity to distinguish between virulent *P. aeruginosa* and those virulent strains. It was suggested by **Kantor *et al.* (1975)** who used the Congo red dye binding as a phenotyping marker to distinguish between invasive, toxigenic and non invasive, non toxogenic isolates. Data represented in table (4) showed that Congo red positive *P. aeruginosa* constituted 55.5% of the strains isolated from cows and buffaloes. This significant result agreed with the result of **Kumaris*et al.,* (1974); Fuchs (1981) and Jeppesen (1995)** who found that about 60% of examined *P. aeruginosa* strains were Congo red positive. Accordingly, it was suggested that there was a relationship between virulence and Congored activity.

As regards to the toxicity of *P. aeruginosa* in mice, the extracted exotoxin when injected in mice, all inoculated mice were died within 2-5 days according to the dilution of tested toxin concerning cows and buffaloes 41 out of 121 tested strains were toxigenic strains as shown in table(4). The same findings were documented by **Amineh and Safaei (2001), Wolska *et al.,* (2002) and Rasmussen *et al.,* (2008)** who proved that, exotoxin (A) is the most important virulence factor associated to *P. aeruginosa* and also the other producing toxins are important factors in the pathogenicity of *P. aeruginosa.*

The main goal of this work is the use of PCR for two purposes one is a complete identification and detection of *P. aeruginosa* isolates, and the second is the confirmation of ETA producer strains from these isolates. The use of gene DNA sequence of the oligonucleotide was Primers with the chosen 3 strains , one represent the whole +ve toxigenic strain with mice, one represent untypable and the last one represent the whole –ve with mice. The use of PCR nowadays greatly provide specific, rapid, simple and highly sensitive detection of *P. aeruginosa* from clinical samples **Hummel and Unger (1988); Schmidt *et al.* (1995).**

Moreover, PCR technique is widely used in veterinary research and this technique is likely to have a strong impact in the epidemiology treatment and prevention of animal infectious disease (**Rodriquez, 1997 and Amany, 2004).**

The result in photo show that the positive sample contain exotoxin A-DNA gene also untypable strains contain exotoxin A-DNA gene and this mean that if there is available antisera present these strain could typable.

These results are in accordance with the result done with method of intra-peritoneal detection of toxin in mice which clear that the PCR confirmation of the presence of DNA gene in the positive samples by I/p test in mice and the –ve by I/p test in mice.

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الملخص العربي

دراسة علي مدي تواجد ميكروب السيدوموناس اريجينوزا المعزوله من حيوانات المزرعه التي تبدوا عليها أعراض التهاب رئوي واخري تبدو سليمه ظاهريا والكشف عن تواجد نوع السموم A باستخدام سلسلة البلمرة التفاعلية

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في هذه الدراسة تم الفحص البكتريولوجي علي عدد ٤١٩ عينة من الأبقار و١٣١ عينة من الجاموس هذه العينات تم جمعها من حيوانات تبدوا سليمة ظاهريا وأخري تظهر عليها أعراض الالتهاب الرئوي وهذه العينات تم فحصها للحصول علي صورة كاملة لميكروب السودوموناس اريجينوزا والسموم التي تفرزها سواء كان الحيوان يبدو سليما ظاهريا أو تظهر عليه أعراض الالتهاب الرئوي.

أثبت الفحص البكتريولوجي أن هناك ١٧٢ عينة ايجابية من أصل ٤١٩ عينة تم جمعها من الأبقار بنسبة ٤١٪ سواء كانت هذه الأبقار تبدوا سليمة ظاهريا أو تبدوا عليها أعراض الالتهاب الرئوي أما بالنسبة للعينات التي تم جمعها من الجاموس فكان هناك ٤٦ عينة إيجابية من أصل ١٣١ بنسبة ٣٥,١٪ .

تم عزل ١٢١ عترة من السودوموناس اريجينوزا كانت موجبه لاختبار الكونجو الأحمر من أصل ٢١٨ عينة إيجابية للفحص البكتريولوجي من كل من الأبقار والجاموس وبإجراء طرق الفحص التقليدية علي هذه العترات تبين أن هناك ٤١ عترة من أصل ١٢١ من كل من الأبقار والجاموس قادرة علي انتاج السموم.

تم التصنيف السيرولوجي للعترات المعزولة من الأبقار وكانت النتيجة أن ١٤٤ عترة تنتمي للنوع السيرولوجي (١) و٢٨ عترة غير مصنفة سيرولوجيا بينما العترات المأخوذة من الجاموس فكانت نتيجة التصنيف السيرولوجي لها ان ٤٠ عترة تنتمي للنوع السيرولوجي (١) بينما ٦ عترات غير مصنفة سيرولوجيا.

فقد اوضح سلسلة البلمرة التفاعلية وجود الجين الخاص بنوع السموم (A) بالإضافة إلي ذلك فإن العينات التي لم يتم تصنيفها تحتوي علي نفس الجين وهذا يعنى أنها لو توافر مضاد المصل لكان من الممكن تصنيفها هى الأخرى.