Electrophoretic pattern of exo-toxins prepared from *Corynebacterium pseudotuberculosis* isolated from Oedematous Skin Disease lesions

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Abstract

Seventy five out of 106 bacteriologically examined sanguineous fluids samples collected from buffaloes Oedematous Skin Disease (O.S.D) lesions, were proved bacteriologically to be *C. pseudotuberculosis* (C.p) ,with an incidence of (70.8%). Sensitivity test revealed that isolates were highly sensitive to trimethoprim + sulphamethoxazole, amoxicillin, gentamicin and enrofloxacin. Diphtheria toxin (DT) produced by *C. pseudotuberculosis* of buffalo was detected using Elek agar immunoprecipitation test, and the obtained results showed that 23 (30.7%) were positive and 52 (69.3%) negative. Virulence of *C. pseudotuberculosis* isolates having both phospholipase D (PLD) and DT, was carried out on 5 culture filtrates (CFs) of 5 isolates which were positive for Elek test and it was assayed by determination of minimum reacting dose (M.R.D), it was 320 M.R.D for 2 CFs and 640 M.R.D. for 3 CFs., Rabbit skin serum neutralization test was applied on the same previous CFs and using of serum from O.S.D. diseased buffaloes and anti. DT serum ,and Detection of DT protein band at 62 K Da. By SDS- PAGe and western immunobloting.

Key words: *Oedematous Skin Disease, C. pseudotuberculosis,* SDS- PAGe, *antibiotics* .

Introduction

Oedematous skin Disease (O.S.D.) is an endemic disease of buffaloes in Egypt characterized by oedematous swellings at the initial site of infection which is usually in the skin of the internal thighs, forelimbs, belly, dewlap and involves the regional drainage lymph nodes **(Selim, 2000).** The disease causes significant economic losses mainly, decrease in milk and meat production, low quality of hide and highly expensive medical treatment **(Shpigel *et al*., 1993).**

Corynebacterium pseudotuberculosis biotype II is the main cause of Oedematous skin Disease of buffaloes in Egypt **(Maarouf, 2003).** It secerets exotoxin (S) including phospholipase – D, which has been implicated as the main player of pathogenesis. This exotoxin is a permeability factor that promote the hydrolysis of ester bonds in sphingomyelin in mammalian cell membranes, possibly contributing to the spread of the bacteria from the initial site of infection to secondary sites within the host **(Tambourgi *et al*., 2002)**.

Rabbit Skin Sensitivity Serum Neutralization test was used for diagnosis of C.p. infection by using sera from infected animals**(Doty *et al*., (1964) and Ammar,(1983**). *Corynebacterium* ulcerans ATCC 9015,C ulcerans from Wales and *Corynebacterium pseudotuberculosis* 992 all produce DT **(Wong and Groman, 1984).** In Egypt, some buffalo isolates of *C. pseudotuberculosis* are infected with b - corynebacteriophage that harbor the entire dt gene. *C. pseudotuberculosis* bacterium which are infected with the entire tox gene (dt tox gene) are responsible for induction of severe Oedematous skin disease (O.S.D) manifestation due to the production of D.T. **(Farida, 2010).**

The aim of this study is directed to the following:

1- Isolation and identification of *Corynebacterium pseudotuberculosis* from buffaloes suffering from Oedematous Skin Disease (O.S.D).
2- Determination of antibiogram of the isolated strains.
3- Detection of Deiphtheria toxin (D.T) producing *C. pseudotuberculosis* isolates by Elek agar immunoprecipitation test.
4- Rabbit skin serum neutralization test using

O.S.D. diseased buffaloes serum and anti-Diphtheria toxin serum.

5- Detection of Diphtheria toxin production from *C. pseudotuberculosis* by SDS-PAGe and western immunoblotting assay .

Material and Methods

Samples:

Sanguineous fluids were collected from oedematous swellings of 106 buffaloes with lesions suspected to be O.S.D. during 2009-2012, using sterile syringes and Mac-Cartney bottles. All samples were sent to laboratory in an ice box with a minimum of delay.

Isolation and identification of isolates: according to **Quinn *et al.* (2002).** Samples were cultured onto brain heart infusion broth with tween 80 for 24 hours at 37 ºc and then a loop-full was taken and cultured onto 10% sheep blood agar and brain heart agar .All inoculated plates were incubated at 37ºc for 24 hrs. then colonies were identified Gram stain and biochemical tests were applied. The isolated strains were kept in soft agar.

Antibiogram of the isolated strains :

The antibiotic sensitivity test was done according to **Finegold and Martin, (1982)** using the following discs: amoxicillin (10 µg), cefadroxil (30 µg), enrofloxacin (10 µg), erythromycin (15 µg), gentamicin (30 µg), oxytetracycline (30 µg) and trimethoprim + sulphamethoxazole (1.25 + 23.75 µg). The sensitivity of C .p. to chemotherapeutic agents must be applied on all isolated strains to avoid presence of drug resistance by strains.

Detection of diphtheria toxin (D.T) producing *C. pseudotuberculosis* isolates by Elek agar immunoprecipitation test:

Elek agar was prepared according to **Reinhardt *et al.* (1998).** Newly born bovine serum (0.5ml) was added to 2.5 ml of molten Elek agar at 45°C, and poured in 4.5 cm – diameter plate. A central well was filled with Diphtheria toxin anti-serum (it was obtained from holding company of Vaccine and sera (VAC SERA). While *C. pseudotuberculosis* isolates were cultured around the well separately. The plates were kept at 37°C for 24 hours.

Determination of minimum reacting dose (M.R.D):

The test was carried out according to **Doty *et al.*, (1964).** It was carried out on 5 culture filtrates (CFs)of 5 isolates which were positive for Elek test.

Rabbit skin serum neutralization test:

It was done according to **Doty *et al.*, (1964).** A volume of 0.2ml of M.R.D. of each CFs were mixed with 0.2ml of serum from OSD diseased buffaloes. Another quantities of the same CFs (M.R.D) were mixed with 0.2ml of anti-DT serum. All the previous neutralized mixtures were incubated at 37°C for one hour. In shaved back rabbit, the previous neutralized preparations were injected intradermally in separate rows. A volume of 0.2ml of normal saline was I/D injected in a separate square as a negative control.

Detection of Diphtheria toxin production from *C. pseudotuberculosis* by SDS-PAGe and western immunoblotting: It was applied according to **Laemmli (1970)** and **Towbin *et al.*, (1979).** The test was applied on 5 culture filterate of 5 isolates which were positive for Elek test. A control positive diphtheria toxin was obtained from VAC SERA, Egypt.

Results

Table (1): Percentage of *C. pseudotuberculosis* isolated from samples suspected to be O.S.D.

No. of examined Samples	No. of +ve	%	No. of –ve	%
106	75	70.8	31	29.2

* The percent was calculated according to the number of examined samples

Table (2): Antibiogram of the isolated strains.

Antibiotic disc used (µg / disc)		Sensitivity		
		Highly sensitive	Moderately sensitive	Resistant
Amoxicillin (10)	No.	63	9	3
	%	84	12	4
Cefadroxil (30)	No.	6	8	61
	%	8	10.7	81.3
Enrofloxacin (10)	No.	57	11	7
	%	76	14.7	9.3
Erythromycin (15)	No.	2	12	61
	%	2.7	16	81.3
Gentamicin (30)	No.	62	8	5
	%	82.7	10.7	6.7
Oxytetracycline (30)	No.	12	23	40
	%	16	30.7	53.3
Trimethoprim + sulphamethoxa-zole (1.25 + 23.75)	No.	69	6	0
	%	92	8	0

* The percent was calculated according to the total number of isolates (75).

The researcher gave an advice to owners of infected animals with O.S.D. to use the highest sensitive therapeutic agents in treatment of infected animals ,and these therapeutic agents gave good results in treatment of O.S.D.

Table (3): Detection of Diphtheria toxin produced by *C. pseudotuberculosis* isolates by using Elek agar immu tion test.

Total number	Positive		Negative	
	No.	%	No.	%
75	23	30.7	52	69.3

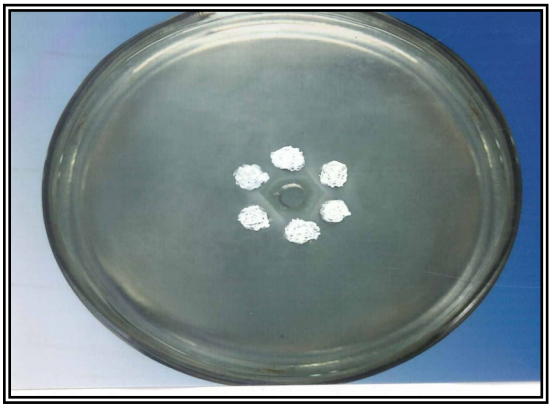

Figure (1): Elek agar immunoprecipitation test for *C. pseudotuberculosis* isolates using anti-DT serum.

- The percent was calculated according to the total number of isolates(75).

Results of (rabbit dermonecrotic test) minimum reacting dose (M.R.D) in skin of rabbit: it was 320 M.R.D. in 2 isolates and 640 M.R.D. in 3 isolates.
A graded series of reactions were produced after 24 hours, ranging from a large swelling with marked necrosis, terminating in abscess formation, to a red flush in a small area.
Results of rabbit skin serum Neutralization test: the mixed sera neutralized the reaction of the culture filtrates.

Results of Diphtheria toxin production from *C. pseudotuberculosis* by SDS-PAGe and western immunoblotting:

SDS-PAGe revealed several bands at different molecular weights (167, 135, 94.7, 62 and 31 KDa), Dipthheria toxin protein band appear at 62 KDa in the control standard DT. (lane-2) and in the five concentrated culture filtrates (Conc. CFs) at lane3-7.
PLD protein band appear at 31 KDa in all conc. CFs immunoblotting of conc. CFs revealed Diphtheria toxin protein band at 62KDa and PLD protein band at 31 KDa.

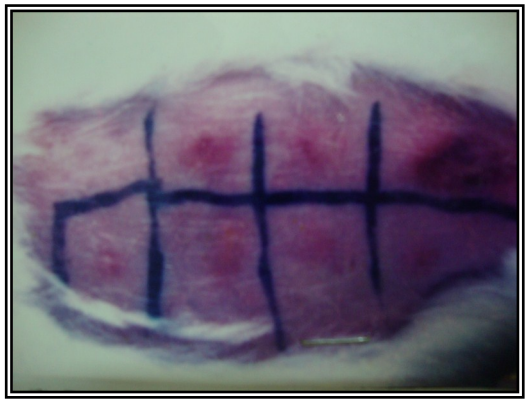

Figure (2): Determination of the minimum reacting dose

Figure (3): determination of rabbit skin serum neutralization test.

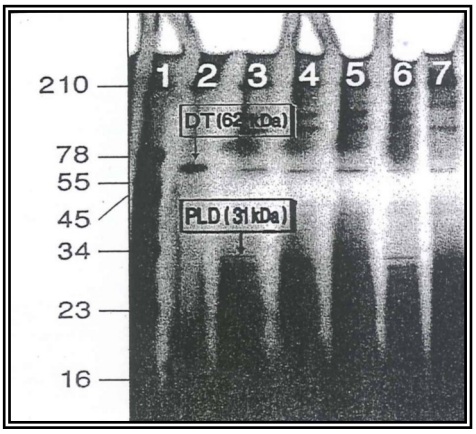

Figure (4): Silver stained SDS-PAGE of concentrated culture filterates (conc. CFs) : of *C. pseudotuberculosis* isolates. Showing Dt at 62.0 k Da and PLD at 31.0 KDa
Lane -1: Broad range protein marker.
Lane -2: Control standard DT. (62.0KDa).
Lane 3-7: Conc. CFs. of isolates.

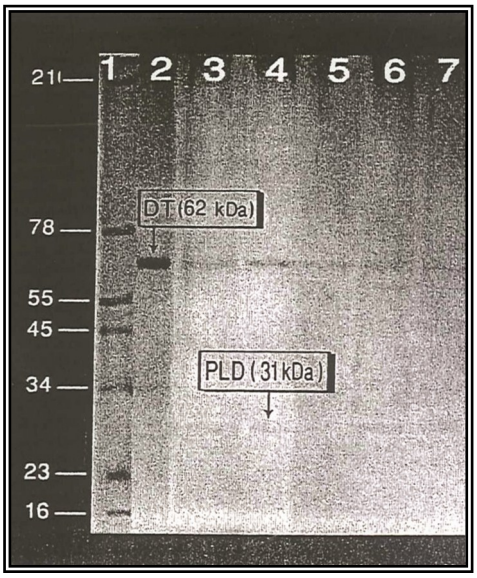

Figure (5): Immunoblotting of concentrated CFs. of *C. pseudotuberculosis* isolates, using anti-DT serum. Showing DT bands at 62.0KDa. and PLD bands at 31.0 KDa.
Lane -1: Broad range protein marker.
Lane -2: Control standard DT.
Lane 3-7: Conc. CFs. of isolates.

Discussion

C. pseudotuberculosis is classified into two biotypes based on nitrate reducing activity. Biotype-I, is a nitrate negative, isolated from sheep and goat, and biotype-II, is a nitrate positive, isolated from horses and buffaloes **(Barakat *et al*., 1984).** The great variation in the pathogenesis of *C. pseudotuberculosis* from different host species may be attributed to the presence of virulence factor (s) other than the phospholipase-D (PLD) in the exotoixn, one of these factors may be the diphtherial toxin (DT) **(Farida, 2010).**

Table (1) showed the prevalence of *C. pseudotuberculosis* in samples suspected to be O.S.D., out of 106 examined samples, 75 (70.8%) isolates of C.p. were recovered. While, Abd El – Latif (2011) revealed that from buffaloes suffered from O.S.D., 30 isolates (83.3%) were *C. pseudotuberculosis* in single infection, 4 isolates (11.1%) in mixed infection with both *Staph. aureus* and 2 isolates (5.6%) with *Strept. pyogenes*. The bacteriologically negative samples for C.p. were 31 (29.2%), this may be resulted from that the animals were under therapeutic treatment.

Antibiogram of 7 chemotherapeutic agents on 75 isolates from buffaloes suffered from O.S.D. were represents in Table (2) there were differences in isolates susceptibilities and zones of inhibition to different chemotherapeutic agents. All isolates were sensitive to trimethoprim + sulphamethoxazole, amoxicillin, gentamicin and enrofloxacine. It was clear that many isolates showed resistant to many antibiotics, this may be attributed to wrong dose, duration of drugs and route of administration or plasmid resistant. In this concern, **Abd El-Latif (2011)** indicated that most of the C.p. isolates were highly sensitive to gentamicin, penicillin, ciprofloxacin and streptomycin.

Results illustrated in Table (3) showed *C. pseudotuberculosis* isolates producing the diphtheria toxin by using Elek agar immunoprecipitation test. Out of 75 isolate, 23 (30.7%) were positive to diphtheria toxin production. While 52

(69.3%) were negative to diphtheria toxin production. In this concern, **Ahmed, (2007)** who could detect DT produced from 10 (55%) strains out of 17 isolates of *C. pseudotuberculosis* of buffalo origin. Moreover, isolates that produced precipitation lines which appeared through 48 hours against the standard anti-DT serum are considered as Elek positive **(Reinhardt *et al*., 1998).**

The virulence of the *C. pseudotuberculosis* (5) culture filterates (CFs) were assayed by applying the rabbit dermonecrotic test and rabbit skin serum neutralization test. The obtained results in figure (2) revealed that the undiluted CFs produced the largest diameter of skin lesions (1.9 cm-1.8cm) with a necrotic center. This severe skin reaction indicated that they produced PLD in high amount which has a dermonecrotic effect **(Doty *et al*., 1964)** , also, the CFs contain DT which produce sever dermonecrotic reactions **(Efstratiou *et al*., 1998).**

The obtained results in the rabbit skin serum neutralization test conicided with that of **Doty *et al*., (1964)** who reported that the exotoxins of *C. pseudotuberculosis* had stimulated the production of antitoxins, that are capable of neutralizing the dermonecrotic effect of the exotoxin.

Detection of DT protein production from conc. CFs of 5 *C.pseudotuberculosis* isolates were done by electrophoresis and immunoblot using the anti-DT serum. It is of interest to notice that anti-DT serum revealed two bands with the five conc. CFs, one at 62 KDa and the other at 31 KDa and was faint, (figure 5). These results were completely coincide with those of Elek test.

It could be concluded that, the Oedematous Skin Disease in buffaloes is acute disease of buffaloes in Egypt and caused by *C. pseudotuberculosis* biotype II. Moreover, its virulence depends upon 2 virulence factors which are PLD and DT.. So, the severity of O.S.D. in buffaloes under field condition depends upon the presence of one or the two exotoxins (PLD and DT). A further investigation must be performed on *C. pseudotuberculosis* exotoxins, PLD and DT. Therefore, to prevent this disease it is recommended to take hygienic measures, control of insect vectors, treatment of diseased animals with effective antibiotic.

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النمط الكهربى للسموم الخارجية المحضرة من ميكروب كورينى السل الكاذب المعزول من أفات مرض الجلد الاوديمى

ماجدة فؤاد عيسى

قسم بحوث أمراض الجاموس – معهد بحوث صحة الحيوان – الدقى – جيزة

الملخص العربى

أجريت هذه الدراسة على 106 عينة سائل ارتشاحى من جاموس تبدو عليه أعراض إكلينيكية مشابهة لمرض الجلد الأوديمى. بإجراء الفحص البكتريولوجى على جميع العينات – تم عزل 75 عتره من ميكروب كورينى السل الكاذب.

وبإجراء اختبار الحساسية كانت المعزولات حساسة لترای میثوبریم + سلفامیزوکزازول والأموکسیسلین والجنتامیسین والإنروفلوکساسین. ومن أجل التعرف على عترات كورينى السل الكاذب المفرزة لسم الدفيتريا تم إجراء اختبار إليك آجار للإنتشار المناعى المزدوج على العترات المعزولة ضد المصل المضاد لسم الدفتريا وكانت النتيجة كالتالى: 23 (30.7%) إيجابى، 52 (69.3%) سلبى لوجود السم الدفتيرى. وعلى أساس الاختبار السابق تم قياس ضراوة المعزولات التى تحتوى على كل من الفوسفوليبازاد، والسم الدفتيرى معاً عن طريق استخدام عدد 5 من السموم الخارجية – المحضرة من 5 من المعزولات الإيجابية لإختبار إليك آجار للانتشار المناعى المزدوج فى اختبار تعيين الجرعة المتفاعلة الصغرى (M.R.D) فى جلد الأرنب وكانت النتيجة M.R.D 320 فى عدد 2 من السموم الخارجية، M.R.D 640 فى عدد 3 من السموم الخارجية المحضرة من المعزولات بالاضافة الى اختبار التعادل المصلى فى جلد الأرانب. وأجرى هذا الاختبار على نفس الخمسة سموم الخارجية السابقة باستخدام مصل من الجاموس المصاب بمرض الجلد الأوديمى ومصل ضد سموم الدفتيريا. كما تم إجراء اختبار الفصل الكهربائى على السموم الخارجية السابقة وأيضا اختبار الأميونوبلوتنج وتم تعيين السم الدفتيرى عند 62 كيلودالتون.