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USING OF THE ELISA FOR DIAGNOSIS OF OEDEMATOUS SKIN DISEASE IN BUFFALOES

By

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SUMMARY

This study was carried out on a total 93 serum samples of buffaloes, they were as follow: serum samples of 56 buffaloes showing clinical signs of Oedematous Skin Disease (O.S.D.), 25 apparently healthy in contact buffaloes and 12 control negative buffalo-calves. Out of 56 bacteriologically examined sanguineous fluids which were collected from O.S.D. lesions, 35 isolates of ***C. pseudotuberculosis*** were recovered.

Pathogenicity test showed that all isolates (35) were pathogenic to guinea pigs. Modified CAMP test revealed that all ***C. pseudotuberculosis*** isolates produce phospholipase D (PLD) and all isolates showed zones of haemolysis with diameters of 10 mm. or more. The sensitivity tests revealed that isolates were highly sensitive to trimethoprim + sulphamethoxazole, amoxycillin, gentamicin and enrofloxacin. When ELISA was applied on the collected serum samples, 48 (85.71%) and 31 (55.36%) out of 56 (100%) serum samples which were collected from buffaloes showed clinical signs of O.S.D. were found positive by using concentrated exotoxin antigen and sonicated ***C. pseudotuberculosis*** antigen respectively. 18 (72%) and 9 (36%) out of 25 (100%) serum samples which were collected from apparently healthy in contact buffaloes were found positive by using concentrated exotoxin and sonicated ***C. pseudotuberculosis*** antigens, re-

spectively. On the other hand, control negative buffalo-calve sera were negative when examined by using the same antigens.

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 and dewlap. This swelling may reach the size of a small watermelon and usually involves the regional drainage lymph nodes (**Selim, 2000**). It was firstly described in Egypt by **Carpano (1934)**, then, it was reported in different governorates of north and south Egypt (**Soliman et al., 1963; Zaghawa and El-Gharib, 1996 and Sayed, 2001**). O.S.D. causes significant economic losses mainly, decrease in milk and meat production, low quality of hide and highly expensive medical treatment (**Shpigel et al., 1993**).

Yeruham et al. (1997) and **Maarouf (2003)** reported that ***Corynebacterium pseudotuberculosis*** is the main isolate in cases of O.S.D. of buffaloes and treatment of these cases takes long time as the bacteria is a facultative intracellular microorganism. Many investigators studied the immunological mechanism of protection against infection with ***C.pseudotuberculosis*** as **Hodgson et al. (1994)** who reported that immunity against ***C.pseudotuberculosis*** depends mainly on humoral immune response. Meanwhile, **Johnson et al. (1993)** reported that protection against ***C.pseudotuberculosis*** is a matter of cell mediated immune response. On the other hand, **Cameron et al. (1998)** reported that there is development

of both types of immune response. The aim of this study is directed to the following :

1- Isolation and identification of the causative agent of O.S.D.

2- Pathogenicity of ***C.pseudotuberculosis*** in guinea pigs.

3- Detection of the synergistic haemolytic activity by modified CAMP test.

4- Determination of antibiogram of the isolated ***C. pseudotuberculosis*** strains.

5- Evaluation of the reliability of ELISA used for diagnosis of O.S.D.

MATERIALS AND METHODS

Samples:

Bacteriological samples:

Sanguineous fluids were collected from oedematous swellings of 56 buffaloes with lesions suspected to be O.S.D., using sterile syringes and Mac-Cartney bottles. All samples were sent to lab. in an ice box with a minimum of delay. Isolation and identification of isolates were done according to **Koneman et al. (1992)** and **Quine et al. (2002)**.

Serum samples:

93 serum samples were collected as follows: 56 serum samples were collected from buffaloes showing clinical lesions suspected to be O.S.D., 25 serum samples were collected from apparently healthy buffaloes in contact with diseased ones, and 12 control negative serum samples were collected from 6 months old buffalo-calves.

Media used for isolation of *C. pseudotuberculosis*:

10% sheep blood agar, tryptone soya agar and brain heart infusion broth with tween 80 were used.

Pathogenicity of *C. pseudotuberculosis* isolates:

Isolates from buffaloes suffering from O.S.D. were tested for pathogenicity according to **Cameron and Buchan (1966).** 72 guinea pigs with average weight of 250-300 gm per animal were used for detection of the virulence of 35 isolates, 2 guinea pigs for each isolate were inoculated subcutaneously with 0.1 ml of a 10^{-3} dilution of a cell suspension containing 0.1 ml packed cells per 100 ml. sterile saline solution. At the same time 2 guinea pigs were inoculated with sterile saline solution, using the same dose and route. Guinea pigs were kept under observation for 7 days. Postmortem examination of dead guinea pigs was carried out. Cultures on broth then on blood agar media were made from lesions.Then incubated at 37c° for 24 hours. Smears were prepared from cultures, stained and examined (Figure, 1).Catalase and other biochemical tests were done on each isolate for confirmation.

Modified CAMP test:

This test was applied on 35 ***C. pseudotuberculosis*** isolates according to **Songer et al. (1990)** using Luria-Bertani (LB) agar containing 5% sheep blood and 10% ***Rhodococcus equi*** (R. equi) filtrate(Figure. 2).

Antibiogram of the isolated strains:

The antibiotic sensitivity test was done according to **Finegold and Martin (1982)** using the following discs : amoxycillin (10), cefadroxil (30), chloramphenicol (30), enrofloxacin (10), erythromycin (15), gentamicin (30), oxytetracycline (30) and trimethoprim + sulphamethoxazole (1.25 + 23.75).

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

The test was carried out by inoculation of culture filtrate in skin of rabbit according to **Doty et al. (1964)**. (Figure, 3). It was carried on 5 culture filterates of the isolates chosen on the bases of pathogenicity test and synergistic haemolytic activity.

Enzyme linked Immunosorbent Assay (ELISA):

The test was applied on 93 collected serum samples using sonicated *C. pseudotuberculosis* antigen and concentrated exotoxin antigen which were prepared according to **Maki et al. (1985)** and **Knight (1978)**, respectively.

A serum dilution was considered positive if it yielded a mean optical density (OD) of each group equal to/or greater than the cut off value **(Dimitri and Mikhail, 1996)**.

Cut off value was estimated as double or more fold of the mean OD of negative serum **(Bassiri et al., 1993)**.

Preparation of hyperimmune serums:

1- Hyperimmune serum against ***C. pseudotuberculosis*** isolate. It was prepared according to **Cameron and Buchan (1966).**

2- Specific antitoxin was prepared according to **Doty et al. (1964).** They were used as control positive serum for ELISA.

Materials used for ELISA:

- Anti-bovine IgG peroxidase conjugate (Sigma)
- Anti-Rabbit IgG peroxidase conjugate (Sigma)
- Flate bottom microtiter plates.
- ABTS (sigma).

Figure (1): Severe congestion of internal organs in guinea pigs infected with *C.pseudotuberculosis*

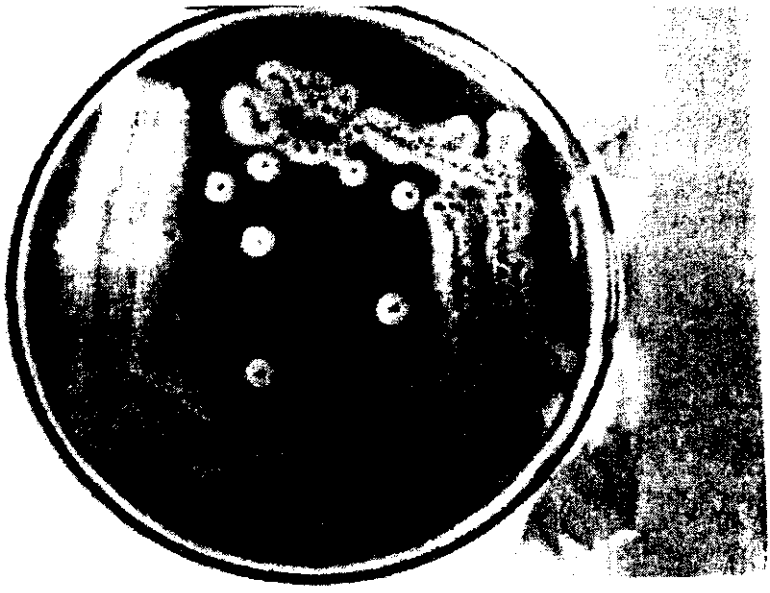

Figure (2): Modified CAMP test.

Figure (3): Determination of the minimum reacting dose.

RESULTS

Table (1): Prevalence of *C. pseudotuberculosis* in samples suspect to be O.S.D.

No. of examined samples	No. of +ve	%	No. of -ve	%
56	35	62.5	21	37.5

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

Results of pathogenicity in guinea pigs:

67 of the infected guinea pigs died while 3 guinea pigs and the control ones survived and were sacrificed after 7 days, then postmortem lesions were recorded as follows :

All infected guinea pigs showed local oedema and abscesses with different sizes at the inoculation sites, congestion of livers, spleens and prefemoral lymph nodes. Orchitis in 19 of them. Lungs were congested and hepatized. Hearts and kidneys were pale in colour. Congestion of the internal organs was mild in 3 sacrificed guinea pigs. Isolation and identification of *C. pseudotuberculosis* from the inoculation sites and affected lesions was done.

Results of Modified CAMP test:

All isolates showed zones of haemolysis with diameter of 10mm. or more.

Results of minimum reacting dose (M.R.D.) in skin of rabbits.

It was 320 M.R.D. in 3 isolates and 640 M.R.D. in 2 isolates. One isolate with 640 M.R.D. was used for preparation of ELISA antigens.

Table (2): Antibiogram of the isolated strains.

Antibiotic disc used (mg/disc)		Sensitivity		
		Sensitive	Moderately sensitive	Resistant
Amoxycillin (10)	No.	30	3	2
	%	85.7	8.6	5.7
Cefadroxil (30)	No.	1	5	29
	%	2.9	14.3	82.9
Chloramphenicol (30	No.	0	0	35
	%	0.0	0.0	100
Enrofloxacin (10)	No.	26	5	4
	%	74.3	14.3	11.4
Erythromycin (15)	No.	2	9	24
	%	5.7	25.7	68.6
Gentamicin (30)	No.	28	4	3
	%	80.0	11.4	8.6
Oxytetracycline (30)	No.	4	12	19
	%	11.4	34.3	54.3
Trimethoprim + Sulphamethoxazole (1.25+23.75)	No.	31	4	0
	%	88.6	11.4	0.0

The percent was calculated according to the total number of isolates (35).

Table (3). Results of ELISA on buffaloes sera, using conc. exotoxin antigen.

Serum samples from	Titer	Total No.	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1024	Total + ve	Total - ve
1 Buffaloes showing	No.	56	3	6	10	15	9	5	-	-	-	48	8
clinical lesions of O.S.D.	%	100	5.36	10.71	17.85	26.79	16.07	8.92	-	-	-	85.71	14.29
2. Apparently healthy in	No.	55	3	7	5	3	-	-	-	-	-	18	7
contact buffaloes.	%	100	12	28	20	12	-	-	-	-	-	72.0	28.0
3. Control negative	No.	12	-	-	-	-	-	-	-	-	-	-	12
buffalo-calves.	%	100	-	-	-	-	-	-	-	-	-	-	100
Control + ve serum.	1/1024												

* The percent was calculated according to the total number of each.
* The mean optical density of control negative serum was 0.133
* Sensitivity in relation to group 1& 2 was 81.48%.
* Specificity in relation to group 1& 2 was 18.51%.
* Sensitivity in relation to group 3 was 0.0%.
* Specificity in relation to group 3 was 100%.

Table (4). Results of ELISA on buffaloes sera, using sonicated *C. pseudotuberculosis* antigen.

Serum samples from	Titer	Total No.	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1024	Total + ve	Total - ve
1. Buffaloes showing	No.	56	-	4	-	8	6	5	5	3	-	31	25
clinical lesions of O.S.D.	%	100	-	7.14	-	14.29	10.71	8.93	8.93	5.36	-	55.36	44.64
2. Apparently healthy in	No.	25	2	1	1	1	3	-	-	1	-	9	16
contact buffaloes.	%	100	8	4	4	4	12	-	-	4	-	36	64
3. Control negative	No.	12	-	-	-	-	-	-	-	-	-	0.0	12
buffalo-calves.	%	100	-	-	-	-	-	-	-	-	-	-	100
Control + ve serum.	1/512												

* The percent was calculated according to the total number of each.
* The mean optical density of control negative serum was 0.129
* Sensitivity in relation to group 1& 2 was 49.38%.
* Specificity in relation to group 1& 2 was 50.62%.
* Sensitivity in relation to group 3 was 0.0%.
* Specificity in relation to group 3 was 100%.

DISCUSSION

O.S.D. is a seasonal disease, appears often in summer and causes sever economic losses through low quality of hide, decrease in meat and milk production as well as long course of treatment **(Effat, 1995)**.

C. pseudotuberculosis was considered the main aetiologic agent of O.S.D. causing recurrent outbreaks among buffaloes in Egypt **(Zaki, 1999)**, since, it survives for long periods in soil contaminated by pus **(Knight, 1969)**.

Out of 56 examined bacteriological samples collected from oedematous swellings in buffaloes, 35 (62.5%) isolates of ***C.pseudotuberculosis*** were recovered as showed from data in Table (1). While **Zaki (1999)** recovered ***C. pseudotuberculosis*** isolates with a percentage 41.5% from O.S.D. lesions in buffaloes. Moreover, **Maarouf (2003)** found that the incidence of isolation of ***C. pseudotuberculosis*** was 100% in buffaloes suffered from O.S.D. symptoms and reported that ***C. pseudotuberculosis*** is the main isolate in cases of O.S.D. in buffaloes. The bacteriologically negative samples for ***C. pseudotuberculosis*** were 21 (37.5%), this may be resulted from that the animals were under therapeutic treatment.

The postmortem lesions after infection of guinea pigs come in accordance with those obtained by **Galila (1998)** who stated that, infected guinea pigs showed abscesses at the site of inoculation with congestion of the internal organs. Mean while, **Maarouf (2003)** reported that all ***C. pseudotuberculosis*** isolates were pathogenic.

Modified CAMP test is used for the detection of the PLD production by ***C. pseudotuberculosis*** isolates, this was allowed by detection of synergistic haemolytic activity of PLD of ***C. pseudotuberculosis*** which is an important feature of the organism with phospholipase C of ***Rhodococcus equi.*** All isolates in this study showed zones of haemolysis with diameters of 10 mm. or more. This result agree with **Egen et al. (1989)** who suggested that the synergistic haemolytic assay is at least predictive for the presence of PLD activity, and they mentioned that all known isolates of ***C.pseudotuberculosis*** produce a toxic PLD that lyse sheep RBCs in synergy with ***R. equi*** filterate.

It was noticed that there was a correlation between the diameter of the haemolytic zone resulted from synergistic haemolysis for each isolate and its virulence in guinea pigs, since, guinea pigs died quickly with pronounced signs of toxaemia as the diameter of haemolytic zone increase.

Antibiogram of 8 chemotherapeutic agents on 35 isolates from buffaloes suffered from O.S.D. were presented in Table (2) all isolates were completely resistant to chloramphenicol. There were differences in isolates susceptibilities and zones of inhibition to different chemotherapeutic agents. All isolates were sensitive to trimethoprim + sulphamethoxazole, amoxycillin, 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. The obtained results coincided to large extent with that of **Selim et al. (1998)** who

recognized that all Gram positive isolates were sensitive to penicillin, enrofloxacin, ampicillin, flumequine, erythromycin and trimethoprim + sulphamethoxazole. Also, nearly similar results were obtained by **Abou-Zaid and Hammam (1994)** and **Sayed (2001)**.

The obtained results coincided with **Barakat et al. (1980)** who measured toxin potency by the minimal reacting dose in rabbits.

Results illustrated in Table (3) showed results of ELISA on buffaloes sera, using conc. exotoxin antigen. It was clear that serum samples collected from buffaloes showed clinical symptoms of O.S.D. gave the highest positive result which was 48 (85.71%), followed by serum samples collected from apparently healthy in contact buffaloes, it was 18 (72%), while serum samples of the control negative buffalo-calves were completely negative. The seronegative serum samples were 8 (14.29%) collected from buffaloes showed clinical signs of O.S.D. may be attributed to the interval between exposure to infection and time of collecting samples (**El-Seedy et al., 2005**). While seropositive serum samples collected from apparently healthy in contact buffaloes, these animals may be in the incubation period, taked subclinical infection and self cured due to immune status of the infected animal, age, route and extent of exposure or previously diseased with O.S.D.

This suggestion come in agreement with that of **El-Seedy et al. (2005)** who mentioned that there is inter-host variability including the age and

immune status of the infected animal, the route and extent of exposure to the pathogen. Also, this explained why the control negative buffalo-calves were seronegative, due to age and immune status of animal. The sensitivity of the test in relation to the diseased and incontact apparently healthy buffaloes was 81.48%, this good sensitivity in detecting antitoxins, while specificity of the test was 18.51%, this is very poor in detecting absence of antitoxins thus, ELISA using conc. exotoxine could be used to say that a seropositive animal has the disease, but the reverse is not true.

The sensitivity of the test in relation to the control negative buffalo-calves was 0.0% while specificity was 100%, this excellent specificity could be used to say that a seronegative animal has not the disease. This means that the test using conc. exotoxin antigen could be used with reasonable accuracy to evaluate the seropositive animals and seronegative ones also.

The result given in Table (4) showed results of ELISA on buffaloes sera, using sonicated ***C. pseudotuberculosis*** antigen, the incidence of seropositive samples in clinically diseased buffaloes with O.S.D., apparently healthy in contact buffaloes and control negative buffalo-calves were 55.36%, 36.00% and 0.0% respectively. These low results may be attributed to the unidentified strain differences may exist between isolates of ***C. pseudotuberculosis*** causing disease and the standard strain that was used as a source of antigens (**Takai et al., 1987**). The sensitivity of the test in relation to the diseased and apparently healthy in contact buffaloes was 49.38% with specificity 50.62%. while, sensitivity in relation to control

negative buffalo-calves was 0.0% and specificity was 100%. This poor sensitivity in detecting antibodies for ***C. pseudotuberculosis*** organisms, also the test has poor specificity. While, specificity of the test in relation to control negative buffalo-calves was 100%, this excellent specificity could be used to say that a seronegative animal has not the disease, but the reverse is not true.

Many investigators concerned with ELISA as a method used for detection of animals infected with ***C. pseudotuberculosis*** as **Dercksen et al. (2000)** who used cell wall antigens or toxin antigen in ELISA, the sensitivity was (94 ± 3%) and the specificity was (98 ± 1%) for diagnosis of ***C. pseudotuberculosis*** infection. They added that ELISA will now be tested for use in eradication and control programmes. Moreover, **KaBa et al. (2001)** who used a bacterial whole cell extract as solid-phase antigen in ELISA for the diagnosis of ***C. pseudotuberculosis*** infections, they proved the reliability of ELISA for the detection of infection. Mean while, **El-Seedy et al. (2005)** applied an ELISA for detection of antibodies directed against somatic and PLD antigens.

It could be concluded that, on the bases of our results, we believe that an ELISA using conc. exotoxin must now used for rapid detection of diseased animals with O.S.D. specially those are bacteriologicaly negative. And we will be carefull in case of seronegative cases, we must take another serum sample with a week interval. Separation of buffaloes affected with O.S.D. clinical signs and following hygienic measures with rapid treatment by the drug of choice are recommended to void outbreaks.

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إستخدام إختبار الإليزا فى تشخيص مرض الجلد الأوديمى فى الجاموس

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أجريت هذه الدراسة على ٩٣ عينة مصل مقسمة كالآتى:

١) ٥٦ عينة مصل من جاموس تبدو عليه إصابات إكلينيكية مشابهة لمرض الجلد الأوديمى.
٢) ٢٥ عينة مصل من جاموس سليم ظاهرياً ومخالط للجاموس الذى عليه أعراض مرض الجلد الأوديمى.
٣) ١٢ عينة مصل سالبة تم تجميعها من عجول جاموس عمر ٦ شهور تعيش فى منطقة خالية من المرض.

بالفحص البكتريولوچى لـ ٥٦ عينة سائل إرتشاحى التى تم تجميعها من الجاموس فى المجموعة الأولى ، تم عزل ٣٥ عترة من ميكروب كورينى السل الكاذب. وبإجراء إختبار الضراوة للعترات المعزولة (٣٥)، كانت جميعها ممرضة لأرانب غينيا. أيضاً تم إجراء إختبار كامب المطور على العترات المعزولة والذى أوضح أن كل العترات تنتج الفوسفولیباز د وتتراوح مناطق تحليل الدم بين ١٠ مم أو أكثر. وبإجراء إختبار الحساسية كانت المعزولات حساسة لـ ترای میثوبریم + سلفا میزوکزازول والأموکسیسلین والچنتامیسین والإنروفلوكساسين. بالفحص السيرولوچى لعدد ٩٣ عينة مصل ممثلة للثلاث مجموعات تحت الدراسة بإستخدام إختبار الإليزا وبإستخدام نوعان من الأنتيچينات وهما أنتيچين السم الخارجى المركز والأنتيچين الجسمى. وجد أن هناك ٤٨ (٧١ . ٨٥٪) عينة إيجابية من المجموعة الأولى عند إستخدام أنتيچين السم الخارجى المركز، و٣١ (٣٦ . ٥٥٪) عينة إيجابية عند إستخدام الأنتيچين الجسمى. أما بالنسبة للمجموعة الثانية، فكان عدد الحالات الإيجابية ١٨ (٧٢٪) عند إستخدام أنتيچين السم الخارجى المركز، و ٩ (٣٦٪) عينات إيجابية عند إستخدام الأنتيجين الجسمى.

أما المجموعة الثالثة فكانت جميعها سالبة بإستخدام ذات النوعين من الأنتيچينات.