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STUDIES ON SOME BACTERIAL CAUSES ASSOCIATED WITH OEDEMATOUS SKIN DISEASE IN BUFFALOES IN SOHAG GOVERNORATE

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ABSTRACT

As oedematous skin disease (OSD) became an endemic disease in buffaloes in Egypt. This study was carried out on 75 buffaloes from a private farms at Sohag Governorate to determine occurence, clinical finding, age and sex susceptibility, ELISA, molecular diagnosis and antibiogram of *Corynebacterium pseudotuberculosis*. Bacteriological examination of the samples revealed that 44 cases (58.7%) were positive, 30 cases of them (68.2%) were in form of the closed lesions and 14 (31.8%) were open lesions. The main clinical signs of the infected buffaloes had been described. Isolated bacteria were subjected for morphological and biochemical identification. The results obtained revealed that 38 isolates (86.4%) were *Corynebacterium pseudotuberculosis* in single infection and mixed infection with both *Staph. aureus* 3 isolates (6.8%) and *strept. Pyogens* 3 isolates (6.8%). Pathogenicity test for *C.pseudotuberculosis* in guinea pigs indicated that all isolates were pathogenic and cause death of inoculated animals. The OD of ELISA above the cutoff point (0.25) was recorded in serum samples. PCR identification of *Pld* gene revealed positive isolates. Results of antibiograms indicated that most of isolates were highly sensitive to Rifampicin, Ciprofloxacin, Enrofloxacin, Gentamycin, Penicillin and streptomycin. Treatment by effective antibiotics, antihistaminic and eradication of the flies achieved the recovery from the disease.

Key words: *Oedematous skin disease, Bacterial causes, buffaloes, antibiogram*

INTRODUCTION

Oedematous skin disease (OSD) is an endemic disease of buffalo in Egypt, which appears as outbreaks during the summer months, although sporadic cases may appear during other months of the year. It is widely spread in the governorates of Lower Egypt (Nile Delta) and in governorates around Cairo in which the temperature during the summer months is around 32°C and relative humidity is high Abd- El- Hakeim (2005).

Oedematus skin disease (OSD) in buffaloes nowadays becomes an endemic disease in Egypt (Selim, 2001). It was fully studied and discussed through several investigations in different governorates which established that the etiological agent was *Corynebacterium pseudotuberculosis;* (Zaghawa and El-Gharib, 1996 and Ghoneim *et al.,* 2001).

The disease was characterized by low mortality and high morbidity (Abd El Lattif 2011) and clinically by hot painful inflammatory swelling that appeared at different areas of the skin and the lymph vessels draining the inflammed area appeared as cord, also swollen local lymph node. The condition of diseased animals was fair with little change in appetite, decreased milk yield and slight rise in body temperature (Pandey *et al.,* 2007 and Fontaire and Baird, 2008). The disease has a prolonged course and is highly expensive for treatment *(*Abd El-Hakeim 2005; Sayed *et al.,* 2007). Several investigations in different governorates which established that the etiological agent was *C. pseudotuberculosis*as alone or in mixed infection with other pathogen as *Staphylococcus spp.* and *Streptococcus spp,* Sayed *et al.* (2007) and Abd El-Ghafar, (2009).

C.pseudotuberculosis serotype II is the main cause of OSD and exotoxin phospholipase D and its lipid contents of the cell wall are the major cause of pathogensis (Selim, 2001). Several authors suggested that the route of transmission of OSD is through the mechanical way only either by contamination of external environment (soil and water) with *C.pseudotuberculosis* or by external parasites (Hippobosca equina) Sayed *et al.* (2007).

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The aim of the present work was to determine occurence, clinical finding, age and sex susciptability, ELISA, molecular diagnosis and antibiogram of *C. pseudotuberculosis* in buffaloes in Sohag Governorate.

MATERIALS AND METHODS

Animals:
A total number of 75 buffaloes aged from 6 months up to 5 years from a private farms in Sohag Governorate during summer season, were investigated in this study. Diseased buffaloes were suffering from skin lesions either closed or opened in the form of diffuse oedematous swelling in different parts of the skin, ulceration and nodular lesions, were also present.

Bacteriological samples:
A total number of 75 samples were collected from diseased buffaloes by aspiration from closed lesions by sterile syringes after disinfecting the surface using 5% tincture iodine. A sterile disposable syringe filled with 5 ml sterile saline solution was injected and the contents of the lesion were aspirated with the same syringe. Samples from open lesion were taken by sterile cotton swabs.

All samples were taken under complete aseptic conditions and transported as rapidly as possible in ice bag container to the laboratory where isolation and identification of the organisms were performed.

Both aspirated exudates and cotton swabs were inoculated into Nutrient broth media overnight and incubated aerobically at 37°C, then streaked onto 10% sheep blood agar, Nutrient agar and Mac Conkey's agar plates and incubated at 37°C for 48 hours aerobically. Growing colonies were purified and identified morphologically by Gram's stain (Bailey and Scott's, 1990) and biochemically for glucose, sucrose and maltose fermentation, catalase activity, gelatine liquefaction, urea production, methyl red and nitrate reduction were adopted according *to* Koneman *et al.* (1992).

Biotyping of *C. pseudotuberculosis:* (by nitrate reduction test)
*C. pseudoluberculosis*was biotyped according to the presence or absence of nitrate reductase enzyme. Biotype I did not express the enzyme (nitrate reduction test negative) whereas biotype II was capable of producing the enzyme (nitrate reduction test positive) (Quinn *et al.*, 2002).

Staph.aureus and strept. pyogens isolates were identified according to colonial morphology, pigment production, microscopically by Gram stain and biochemically according to (Baily and Scott 1990) using catalase activity, coagulase as well as novobiocin (30mcg) and polymixin sulphate (300µ) sensitivity test for identification of *Staphylococcus sp*.

Pathogenicity test:
Eight guinea pigs of about 250- 350 gm body weight were used in the reisolatson of C. *pseudotuberculosis* (The most causative agent of OSD) as well as determination of its pathogenicity. Six guinea pigs were inoculated subcutaneously (s/c) with isolated *C.pseudotuberculosis* at dose of 0.25ml according to Ibrahim *et al.* (2007). At the same time two, guinea pigs (as control) were inoculated with sterile broth by using the same dose and route of inoculation. From dead guinea pigs showed post mortem changes and reisolation of inoculated isolates according to El- Far (1976) and Rafequ and Mahmoud (2007).

Detection of phospholipase D-antigen:
Which is produced by C. *pseudotuberculosis* cells. The isolates were cultivated in brain heart infusion broth, incubated and agitated at 37°C for 36 hrs. bacteria were precipitated by centrifugation at 5000 rpm for 10 minutes at 4°C in cooled centrifuge and then filtered. Cultuere supernatant fluids were dialyzed overnight.

ELISA detection of C. pseudotuberculosis antibodies:
ELISA according to Menzies *et al.* (1994). Indirect ELIZA performed by coating microtiter plates with phospholipase D (PLD) using test kits supplied by LIA bovine Assay Co, Italy by (ELISA statfax / Model 2100 USA).

Molecular diagnosis of C. pseudotuberculosis

Extraction of DNA
According to the above-mentioned bacteriological isolation and identification, *C. pseudotuberculosis* colonies were grown in BHI broth (BHI; Oxoid) at 37°C for 48–72 h before DNA extraction. Bacterial DNA was extracted using QIAamp DNA Mini Kit (Catalogue no. 51304) according to the prescribed instructions.

Primers, amplification conditions, and agarose gel electrophoresis
The oligonucleotide primers used in this study targeting the *Pld* gene of *C. pseudotuberculosis* were obtained from previously published work by Ilhan (2013).

They have specific Sequence and amplify a specific product of 203 bp. The *Pld* gene forward primer (*PLD-F*) sequence (5′→3′) was ATAAGCGT AAGCAGGGAGCA and the The *Pld* reverse primer (*PLD-R2)* sequence(3′→5′) was ATCAGCGGTG ATTGTCTTCCAGG.

Amplification reaction mixtures were prepared in volumes of 50 µL containing 5 µL of 10× PCR master mix (Fermentas, Vilnius, Lithuania), 5 µl of 25 mM MgCl2, 0.2 µL of 10 mM dNTP mixture (Fermentas), 2 U of Taq DNA polymerase (Fermentas), 1 µmol of 25 mM each primer, and 5 µL of template. PCR was performed in a DNA thermocycler (Thermo Electron Corp., Waltham, MA, USA) and amplifications were performed for gene *PLD.* Primary denaturation 94°C for 5 min. Secondary denaturation 94°C for 30_s . Annealing 56°C for $30_{s..}$ Extension 72°C for $30_{s.}$ Number of cycles 35. Final extension 72°C for 10 min. The negative control contained sterile, DNase, and DEPC (diethylpyrocarbonate)-treated water (Applichem) instead of DNA template. As a positive control, DNA isolated from *C. pseudotuberculosis* Pl 18 strain (isolated strain from buffaloes with OSD. The amplified products were analyzed by electrophoresis on a 2% (w/v) agarose gel against gel pilot 100 bp ladder (Qiagen, USA, Cat. No. 239035). Amplified products were visualized using a gel documentation system, and the data were analyzed through computer software. PCR products with a molecular size of 203 bp (*Pld*) considered positive for *C. pseudotuberculosis*.

In-vitro antimicrobial susceptibility testing:
Antibiogram sensitivity was performed for isolated strains by the agar diffusion technique Quinn *et al*. (2002). The used chemotherapeutic discs were Pencillin (10 1U), Streptomycin (10 ug), Gentamycin (10 ug), Oxytetracycline (30 ug), Ampicillin (10 ug), Amoxycillin (25 ug), Rifampicin (10 ug), Ciprofloxacin (20ug) and Enrofloxacin (5 ug). The degree of sensitivity was determined and interpreted according to Koneman *et al.* (1992).

RESULTS

A- Clinical signs:
The main clinical signs in buffaloes included acute oedematous swellings accompanied with single or multiple abscesses or ulcerative lesions. The lesions were present on the flanks, shoulders, neck, one or more limbs, dewlap and contained either a sero-guineous exudates or blood stained yellowish or greenish pus.

Results of bacteriological examination:
The obtained bacteriological results were tabulated in tables (1-8).

Table 1: Bacteriological examination of the affected buffaloes.

Case of animal	Number of examined buffaloes	Positive samples		Negative samples	
		No.	%	No.	%
Affected buffaloes with closed and open lesions.	75	44	58.7	31	41.3

Table 2: Percentage of clinical forms of oedematous skin disease.

Clinical forms	Number of examined animals	Number of diseased animals	%
Closed lesions	44	30	68.2
Open lesions	44	14	31.8

Table 3: Age susceptibility of *Corynebacterium pseudotuberculosis*.

Age group	No. of animals	No. of infected animals	
	75		(44 case)
		No.	%
6 monthes-1 year	10	0	0
1-2 years	25	14	56
3-5 years	40	30	75

Table 4: Sex susceptibility of *Corynebacterium pseudotuberculosis*

Sex	No. of animals	No. of	infected animals
	75		(44 case)
		No.	%
Female	56	40	71.4
Male	19	4	21.1
Total	75	44	58.7

Table 5: Biochemical activities of C. pseudotuberculosis strains isolated from OSD buffaloes.

Biochemical actvites / Organism	Nitarate reduction	Catalase	Urease	Glucose	Maltose	Sucrose	Gelatin Liquifcation	Methyl red
C. pseudotuberculosis	+	+	+	+	+	-	-	+

Table 6: Prevalence of bacterial species isolated from skin lesions of OSD.

Bacterial species	Forms of the positive OSD buffaloes (44)				Total	
	Closed Lesions (30)		Open lesions (14)			
	No.	%	No.	%	No.	%
C. pseudotuberculosis	30	100.0	8	57.1	38	86.4
C.pseudotuberculosis + Staph. aureus	0	0.0	3	21.4	3	6.8
C.pseudotuberculosis + Strept. Pyogenes	0	0.0	3	21.4	3	6.8

Table 7: Optical denisity (OD) values of buffaloes sera using PLD as coating antigen.

No. of diseased serum samples	Sero-positive		Sero- negative		Mean of OD ± SD	Cut point
	No.	%	No.	%		
20	16	80%	4	20%	0.625 ± 0.028 (0.750 - 0.325)	0.25

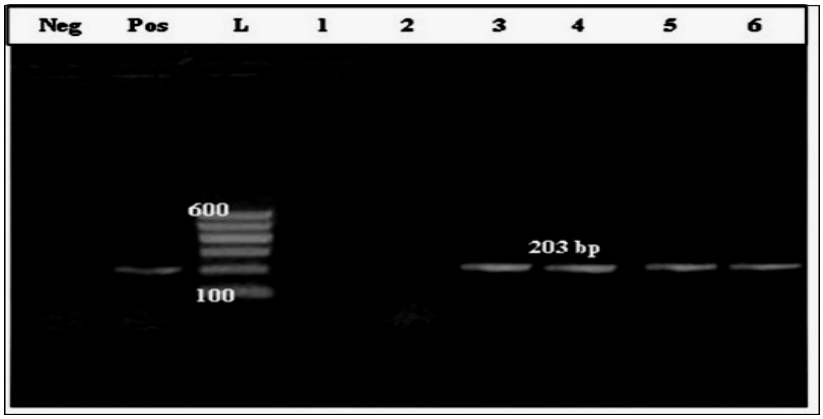

Figure1: Polymerase chain reaction-amplified DNA fragment of 203 bp and specific for the phospholipase D gene of *C. pseudotuberculosis*. Neg,: Control negative, Pos.: Control positive, L: Molecular marker, Lanes 1-2 culture-negative samples, Lanes 3-9 culture-positive samples.

Table 8: Antimicrobial susceptibility testing of bacterial isolates recovered from OSD infected buffaloes.

Organisms / Antimicrobial agent and its potency		C. pseudotuberculosis (44)	Staph. aureus (3)	Strept Pyogenes (3)
Rifampicin	10Ug	+++	+++	+++
Gentamycin	10Ug	+ +	+ +	+ +
Streptomycin	10Ug	+ +	+ +	+ +
Ciprofloxacin	20Ug	+ + +	+ ++	+ +
Penicillin	10Ug	+ +	++	+++
Amoxycillin	25Ug	+ +	++	R
Ampicilin	10Ug	++	++	++
Oxytetracycline	30Ug	+ +	+ +	+ +
Enrofloxacin	5Ug	+ ++	+ + +	+ + +

+ + +: highly sensitive
++ : moderate sensitive
R : resistant

DISCUSSION

Oedematous skin disease (OSD) is an endemic infectious disease that appear mainly among buffaloes and occasionally cows in Egypt (Rafequ and Mahmoud, 2007).

Out of the examined 75 buffaloes 44 showed clinical signs of OSD (Closed and open lesions) representing a morbidity rate of 58.7% whereas no mortalities were recorded (Tables I, 2). These results agree with Zaghawa and El-Gharib (1996) who found that OSD in buffaloes were 44.3%. Higher incidence of OSD was recorded by Sayed *et al.* (2007) who mentioned that morbidity of OSD was 95.5% between buffaloes, while the lower incidence was recorded by Abou-zaid; (2001) and Rafequ and Mahmoud (2007) with percent of 9.7% and 26.1% respectively.

The disease was characterized by low mortality and high morbidity (Khalil *et al.,* 1995) and clinically by hot painful inflammatory swelling appeared at different areas of the skin and the lymph vessels draining the inflamed area appeared as cord. The local lymph node was swollen reaching the size of watermelon. Necrosis and ulceration of the skin were also seen. The condition of diseased animals is fair with little change in appetite, decreased milk yields and slight rise in body temperature. In addition, lameness may be noticed (Al-Gaabary & Ammar, 1999 and Abu Zaid, 2001).

Oedematous skin disease is an acute and seasonal disease appear often in summer and cause redness and swelling of skin in hairless areas, also cause severe economic losses through low quality of skin, decrease in meat production as well as long course of treatment (Ghoneim *et al.,* 2001*;* Zaki, 2004 and Syame, 2006).

Table (3) showed the age susceptibility of *C.pseudotuberculosis* causing Oedematous skin disease. The infection rates of OSD were zero percent in 6 months age till 1 year. These results may related of partial protection by maternal antibodies or because they have not had the same duration of exposure to bacteria or a factor of long incubation period. While 56 % (14 animals), in age group 1-2 years old and 75% (30 animals), in age 3-5 years old.

Concerning the age susceptibility, animals from 1-4 years in both buffaloes and cattle reported by Al-Gaabary and Ammar (1999) and Shpigel *et al.* (1999) who described the epidemiological feature of edematous skin disease in Egypt. On the other hand, Zaghawa and El-Gharib (1996) found that edematous skin disease was more prevalent in animals lower than 2 years in comparison to those more than 2 years.

Table (4) showed the sex susceptibility of *C. pseudotuberculosis* causing Oedematous skin disease. The infection rates of OSD were more prevalent in females 71.4% (40 animals) than males 21.1% (4 animals). Our results supported by (Al-Gaabary *et al.,* 2010). The prevalence of OSD was significantly higher in slaughtered females than slaughtered males.

Table (5) showed the main cause of OSD in buffaloes is *C. pseudotuberculosis* which is a Gram-positive bacilli, small poleomorphic (straight to slightly curved) appears as short chain or clumps resembling Chinese letters, aerobic or facultative anaerobic, non motile, non spore forming and grow slowly on enriched media and produce a toxic phospholipase D Dorella (2006). The organism is capable of surviving within the phagocytes due to its high lipid content on the cell surface. (Pointkowski and Shivers 1998).

C. pseudotuberculosis were Gram stained. Gram-positive colonies were positive for urease and glucose fermentation and negative for sucrose. Biotyping of the isolated strains of *C.pseudotuberculosis* showed positive nitrate reduction test. A result which come in accordance with that mentioned by Sayed (2001); Yeruham *et al.* (2003); Zaki (2004) and Abd El Ghafar (2009) recorded that the most of *C.pseudotuberculosis* isolated from buffaloes were nitrate positive.

Table (6) showed Bacteriological results revealed that the isolation of *C. pseudotuberculosis* from closed lesions was 30(100%) from 30 positive cases and open lesions 8(57.1%) from 14 positive cases. This results are recorded by Sayed (2001) who isolated *C.pseudotuberclosis* from closed lesions in a higher rate than from open lesions with percent of (86.6%) and (58.3%) respectively. On the other hand El-Sayed (2006) isolated *C.pseudotuberclosis* (15%) from closed lesions. *C.pseudotuberclosis* was isolated in pure culture 38 (86.4%) from both closed and open lesions (Table 6). These results agreed with those recorded by *Khalil et al. (1995)* who recorded that *C.pseudotuberclosis* was isolated from OSD infected animals with an incidence of (83.3%). Zaki (2004) and Sayed *et al.* (2007) detected *C. pseudotuberculosis,* in 12.8% and 80.0% of OSD infected buffaloes, respectively.

In the present work *C. pseudotuberculosis* was isolated in mixed culture from open lesions only with *Staphylococcus aureus* 3 (21.4%) and *Streptoccus pyogenes* 3 (21.4%) (Table 6). On the other hand, Sayed (2001) isolated C. *pseudotuberculosis,* with *Staphylococcus aureus*(4 cases), *anthracoid*(3 cases), *Streptococcus pyogenes* (1 case) and *E.coli* (1 case). ALi and Zaitoun (1999) and Sayed *et al.* (2007) found that mixed culture of

C. pseudotuberculosis, with *Staphylococcus spp.* and *Streptococcus spp.* with percent of 10%, with *Staphylococcus spp.* 15.3% and with *streptococcus spp.* 7.5% respectively.

The variation in the disease frequency between the different studies may be attributed to the endemic nature of the disease which leads to variation in animal immunity and the variation in the presence of a few or large numbers of susceptible animals.

Concerning guinea pigs inoculation for studying both pathogenesis and re-isolation, all the inoculated isolates of *C.pseudotuberculosis* killed guinea pigs within 2-5 days post injection where the dead guinea pigs showed congestion and maceration of muscles at site of injection in addition to congestion of the internal organs. This come in agreement with the findings of El-Sawah (2002); Rafequ and Mahmoud (2007). Also Zaki (2004) reported that all isolates of *C.pseudotuberclosis* produced an exotoxin which was lethal for experimental animals usually within 48 hours of inoculation. The postmortem examination revealed congestion of internal organs.

Table (7) results of ELISA revealed that 16 serum samples of diseased buffaloes from 20 (80%) were sero – positive while the rest 4 samples (20%) were sero- negative. Samples revealed (OD) reading above the cut off point (0.25) these results suggest that ELISA is specific, sensitive and relatively inexpensive to set up. It can offer rapid screening of samples with a negative result available in 24 hours and an early indication of a potential positive result for detecting antibodies against *C.pseudotuberclosis* (Abramovits, 2005).

Figure (1) showed PCR identification of *pld* gene of *C. pseudotuberculosis* revealed positive result at the length of 203 bp.

The oligonucleotide primers used for PCR were from the *pld* gene of *C. pseudotuberculosis* (Phospholipase D). PLD gene protects the bacteria from killing by phagocytic cells and enables bacteria to escape from neutrophils and impair neutrophils chemotaxis toward the site of infection (Phospholipase D) PLD gene increase vascular permeability and bacterial survival in the host (Hodgson *et al.,* 1999 and Dorella *et al.,* 2006).

Table (8) revealed that the most isolates of *C.pseudotuberculosis* (main cause of OSD) were highly sensitive to while moderate sensitive to Gentamycin, Penicillin and Streptomycin. Similar results were obtained by Hassan (1988) who said that combination of Penicillin with Sterptomycin showed good results in treatment of OSD. High sensitivity of Gentamycin was recorded by Khalil *et al. (*1995); Sayed (2001) and Sayed *et al.* (2007). On other hand, Abou-Zaid (2001) found that *C.pseudotuberculosis* strains were sensitive to Doxycycline, Erythromycin, Gentamycin and Cephalocin.

CONCLUSION

Finally, it could be concluded that, the oedematous skin disease in buffaloes is acute disease occurs in different localities in Egypt and mainly caused by *C.pseudotuberculosis* in single infection or in mixed infection with other bacteria.

PCR confirm *C. pseudotuberculosis* diagnosis, and the best contribution for the epidemiological surveillance of the disease in buffaloes.

RECOMMENDATION

To prevent this disease it is recommended that hygenic measures must be taken in consideration in an area free from infection together with treatment with effective antibiotic as Rifampicin, Ciprofloxacin and Enrofloxacin, Gentamycin, Penicillin, and Streptomycin in addition to control of insect vectors.

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دراسات عن بعض الاسباب البكتيرية المصاحبة لمرض الجلد الاوديمى فى الجاموس فى محافظة سوهاج

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نظرا لظهور مرض الجلد الاوديمى فى الجاموس بصورة متكررة واصبح متوطن فى مصر. اجريت هذه الدراسة على عدد ٧٥ من الجاموس من مزارع خاصة بمحافظة سوهاج لمعرفة مدى تواجد والاعراض الجانبية وأعمار الحيوانات المريضة بالاضافة الى تشخيص الميكروب المسبب باستخدام الاليزا وتفاعل البلمرة المتسلسل. حيث اظهر الفحص البكتريولوجي للحالات عن وجود اصابة بالمرض فى حالة (٥٨.٧٪). منها ٣٠ حالة (٦٨.٢ ٪) في صورة النوع الاوديمى الصديدى المغلق والنوع الصديدى المفتوح ١٤ حالة (٣١.٨٪). وقد اظهرت نتائج الفحص المورفولوجى والبيوكيميائى للمعزولات البكتيريه عن عزل وتصنيف الميكروب الكورينى باكتريام سيدوتيبركلوزس بصورة منفردة فى ٣٨ حالة (٨٦.٤%) وفى صورة مشتركة مع كل من الميكروب العنقودى الذهبى فى ٣ حالات (٦.٨%) ومع الميكروب السبحى الصديدى فى ٣حالات (٦.٨%) وباجراء اختبار العدوى الصناعية لميكروب الكورينى باكتريام سيدوتيبركلوزس فى الارنب الغينى كانت كل المعزولات من النوع الممرض. وتم استخدام اختبار الاليزا وكان تسجيل العينات للكثافة الضوئية عند نقطة (٠.٢٥) واظهرت النتائج ايجابية لتفاعل البلمرة المتسلسل لجين *Pld* للميكروب. أشارت نتائج اختبار الحساسية للمضادات الحيوية إلى أن جميع العترات المعزولة كانت حساسة لكل من الريفامبيسين وسيبروفلوكساسين واينروفلوكساسين والجنتاميسين والبنسللين والستربتومايسين. وبالعلاج باستخدام المضادات الحيوية طبقا لنتائج اختبار الحساسية مع مضادات الهيستامين وكذلك مكافحة الحشرات الطائرة امكن الوصول الى نسبة شفاء عالية من المرض.