ISOLATION AND IDENTIFICATION OF *BURKHOLDERIA PSEUDOMALLEI* FROM COWS ,GOAT'S MILK AND THEIR SURROUNDING ENVIRONMENT IN BASRAH PROVINCE

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ABSTRACT

Two hundred samples (cow's and goat's milk75 for each,their farm soil 25and 25water samples)from different origin have been cultured on Ashdown selective agar which gave a higher percentage (36.5%)for *B. pseudomallei* isolation , 64% was observed in farm soil followed by water samples 48% than cow's milk33.33% and finally the goat's milk 26.66%. According to L-arabinose biotyping , 39.72% of the *B.pseudomallei* isolates were positive for arabinose (Arb^+) and 60.3% of isolates were negative for arabinose (Arb^-).The virulence testing revealed that most bacterial isolates yield haemolysin, protease, lipase and lecithenase in a percentage of 97.3% , 82.2%, 89.04% and 84.9% respectively.The Arb^- biotypes were more virulent than Arb^+ biotypes with significant differences ($P< 0.05$).All bacterial isolates were characterized by their ability to form capsule(100%),.This study confirmed the presence of casein proteolytic and necrotizing enzymes in the suspension of *B.pseudomallei* .Proteins concentration in this suspension was 0.00248 mg/ml .

INTRODUCTION

Burkholderia pseudomallei is the causative agent of melioidosis, as an ever disease of humans and animals[1]. Melioidosis can present in a number of forms described as acute septicemic, acute established with the subsequent ability to become septicemic. The factors influencing the outcome of disease are not known, although it has been suggested that differences in the virulence of different strains [2] might contribute to the clinical outcome of disease. In addition, differences in the immunological status of the host might also influence the outcome of the disease. Infection by *B. pseudomallei* occurs via inhalation of contaminated dust or when contaminated soil comes in contact with an abraded area of the skin[3] . The infection commonly begins in the lungs, where a pus abscess may form, or it may spread from the skin through the

blood to affect the heart, brain, liver, kidney, joints, and eyes. The pathogenesis of Melioidosis is poorly defined. *B. pseudomallei* can invade both cultured phagocytic and nonphagocytic cells. In these cells, bacterial invasion is followed by intracellular multiplication and induction of cell fusion and multinucleate giant cell formation. The ability of *B. pseudomallei* to adopt a facultative intracellular existence may be an important property in the pathogenesis of both acute and chronic infections.[4]. *B. pseudomallei* is also capable of producing secreted and cell-associated antigens, but the roles of these products in the pathogenesis of disease remain unclear [3]. The flagella and motility, as well as the resistance of the organism to the bactericidal action of normal human serum, are believed to play roles in the ability of the bacterium to disseminate from sites of localized infection, such as the lungs or skin, to virtually any other organ of the body via the blood circulatory system [5]

This study aimed to detect the distribution of *B. pseudomallei* in the cow's and goat's milk and their environment including farm soil and water.

MATERIALS AND METHODS

Samples Collection:

Two hundred samples including cows and goats milk, 75 for each, their farm soil 25 and 25 tap , river and farms trough water samples have been collected. The investigated animals were a lived bovine in different age group and of both sexes, in a period that extends from October to November 2007, present in different places of Basrah Province.

Isolation and Identification:

In the bacteriological analysis of milk and water samples ,methods of Inglis *et al*.,[6] were performed respectively in the isolation of *B. pseudomallei* while in case of soil samples the methods of [7] was performed. .The Ashdown's selective broth (ASB) and Ashdown's selective agar (ASA)were used in the cultivation of these bacteria.

The identification:

1- Morphological tests:
   a- Gram's staining: this staining was conducted according to method Collee *et. al*[8].
   b- Methylen blue staining : was done according to [9] method this stain was used to clarify the bipolar Staining of *B.pseudomallei*.

2- Biochemical and growth tests including , Catalase production oxidase production , oxidation-fermentation , gelatin liquefaction , dehydration (arginine), decarboxylase

(lysine and ornithine) , growth on MacConckey agar , growth on mannitol salt agar , growth at 37°C and 42°C, motility on semisolid medium were performed according to [8]..

3- Biotyping:

According [10], the *B. pseudomallei* biotypes were determined briefly, Arabians agar was used for detection of L-arabinose utilization. A loop full of 24hr. Nutrient broth culture of each *B. pseudomallei* isolates was spotted on Arabinose agar and incubated at 35°C for 48hr. The appearance of growth in this medium considered as Arb^+ biotype and absence of growth indicate that these isolates were Arb^- biotype. Another medium contained glucose instead of arabinose, used as a control medium for cultivation of all tested isolates.

***In vitro*-testing of virulence factors**:

A- Capsule formation: the isolates of *B. pseudomallei* were tested for the formation of capsule according to [8]... An iodine ink stained smear were prepared for the growth of 48hr. NB culture of *B. pseudomallei* and examined microscopically for capsule formation.

B- Enzymes production: The enzymes that produced by *B. pseudomallei* isolates including, Protease , Haemolysin, Lipase and Lecithinase were detected according to[11,12,13,15] respectively by using dialyzed brain heart milk, blood agar , tween8 agar and egg-yolk agar respectively in the cultivation of the tested isolates.

4- The activity of exoenzymes and toxin in the *B. pseudomallei* suspension:

a- Preparation of bacterial suspension supernatant:

According to [15] , the glycerine brain heart infusion broth (4% glycerin and 36 gm brain heart infusion broth in 1 liter DW.) was used in the cultivation of *B. pseudomallei* at 32°C for 7 days. After that culture was filtrated by 0.45 µ Millipore filter paper to obtain crude culture filtrates.

b- The activity of casein proteolytic enzyme:

Four test tubes were used in this test. To the first one, the supernatant of bacterial suspension (1ml), 1ml of casein solution (2%) and 1ml of normal saline were added. 1ml of a diluted bacterial suspension supernatant (1:1), 1ml of 2% casein solution and 1ml of normal

saline were added to the 2nd test tube. While to the 3rd test tube, 2 ml of concentrated bacterial suspension supernatant test tube, 2ml of the concentrated bacterial suspension supernatant (1ml) of 2% casein solution and 1ml normal saline was added. A standard solution was prepared in the 4th test tube which is a % casein solution and 1ml of normal saline.

The four test tubes were incubated at 35°C for 2hr. After that to each test tube 3ml of 5% trichloroacetic acid solution was added, mixed well and left for 1hr. then centrifuged at 10000rpm. For 15 min. by cooled centrifuged (MSE-England). The amount of digested casein in the four test tubes was estimated by spectrophotometer (APLE,PD-303UV, Japan) depending on the optic density values at 280 nm. [15].

c- The necrtoxin activity:

According to [15] , the activity of nectrotoxin which is present in the supernatant of bacterial suspension was estimated by intradermal injection of this supernatant (0.1ml) into a mature rabbit as follows [concentrated ,diluted (1:1), the heated at 70°C for 10min.), and then heated at 70°C for 30min.) and boiled bacterial supernatant]. The different sites of injection were observed after 24hr.

d- The extraction of the crude active substance:

The active substances (casein protolytic enzyme and necrotoxin) were extracted by precipitation method as absolute ethylalcohol (100ml) was added to 100ml of the bacterial suspension supernatant and this mixture was left under overnight refrigeration. After that the precipitate was collected after centrifugation at 10000rpm for 15 min. [15].Finaly the concentration (mg/ml) of protein was estimated spectrophotometer at 280 and 260 nm according to [16], by the formula(Protein concentration mg/ml= $1.55\times A_{280}$- $0.77\times A_{260}$).

5- **Statistical analysis:**

Statistical analysis was done by using SPSS software version 9.0 and analysis of variance (ANOVA). The SND test was used as a test of significance.

RESULTS

1- Isolation and identification:

B. pseudomallei was identified in 73 samples out of 200cow's and goat's milk and their surrounding environment (Table-1). As it was able to exchange the ASB medium color from violet to red and yellow color (figure1). On as A after 48hr., incubation the colonies were dark pink, convex and circular with an entire edge. After 72hr. The incubation dark spot appears in the center of pink colony, the growth had a characteristic musty odor. On NA the colonial growth varies from white to cream mucoid to rough, wrinkled. Table (1) showed the results of biochemical and growth testing of all isolates .

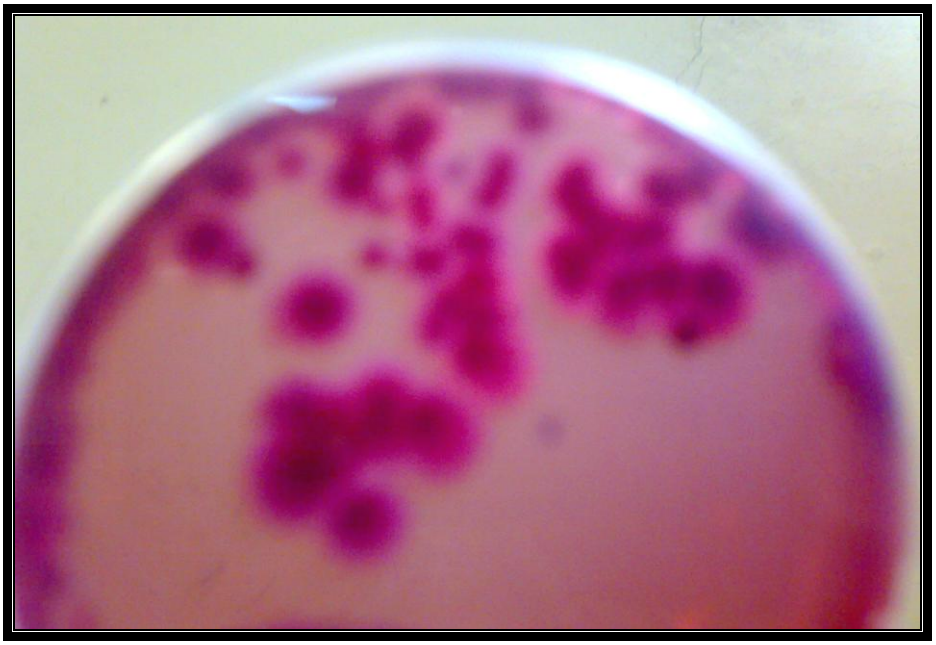


Figure (1): growth of *B.pseudomallei* on ASA after 72hr incubation

Table(1).The morphological, physical and biochemical characteristics of study samples *B. pseudomallei* isolates

Soil		water		Goat's milk		Cow's milk		Tests
Ve^-	Ve^+	Ve^-	Ve^+	Ve^-	Ve^+	Ve^-	Ve^+	
%0	%100	%0	%100	%0	%100	%0	%100	Catalase
%0	%100	%0	%100	%0	%100	%0	%100	Oxides
%0	%100	%0	%100	%0	%100	%0	%100	Gram stain
%0	%100	%0	%100	%0	%100	%0	%100	Methylen blue stain
%0	%100	%0	%100	%0	%100	%0	%100	MacConkey agar
%0	%100	%0	%100	%0	%100	%0	%100	Mannitol salt agar
%0	%100	%0	%100	%0	%100	%0	%100	Motility
%0	%100	%0	%100	%0	%100	%0	%100	G1, La, Ma) oxidase
%0	%100	%0	%100	%0	%100	%0	%100	Glucose fermentative
%12.5	%87.5	%8.33	%91.67	%5	%95	%0	%100	Maltose fermentative
%6.25	%93.75	%8.33	%91.67	%10	%90	%8	%92	Lactose fermentative
%0	%100	%0	%100	%0	%100	%0	%100	Growth 37 °C
%0	%100	%0	%100	%0	%100	%0	%100	Growth 42 °C
%0	%100	%0	%100	%0	%100	%0	%100	Capsule form
%0	%100	%0	%100	%0	%100	%0	%100	Arginin dihydralase
%12.5	%87.5	%16.67	%83.33	%0	%100	%0	%100	Gelatin hydrolase
%6.25	%93.75	%33.33	%66.67	%10	%80	%40	%60	Lysine decarbolas
%0	%100	%0	%100	%45	%55	%60	%40	Ornithine decarbolase

2- Distribution of *B. pseudomallei* isolates and biotypes:

Table-2 display the Distribution of *B. pseudomallei* isolates and biotypes in the tested samples.

Table (2): Distribution of *B. pseudomallei* isolates and biotypes in the tested samples:

Source of samples		**Examined No.**	**Positive No. (%)**	**Biotypes**			**SND**
				Examined No.	**Arb$^+$** **N (%)**	**Arb$^-$** **N (%)**	
MILK	Cow	75	25 (33.33)	25	10 (40)	15 (60)	3.535 a
	Goat	75	20 (26.7)	20	9 (45)	11 (55)	1.414 b
Farm soil		25	16(64)	16	6 (37.5)	10 (62.5)	2.828 C
Water	River	12	7 (58.33)	12	4 (33.33)	8 (66.67)	2.82 C
	Tap	5	2 (40)				
	Trough	8	3 (37.5)				
Total		200	73 (36.5)	73	79 (39.72)	44 (60.27)	2.594

SND= stander normal deviation

abc= there is a significant difference among tested samples P<0.05

3- ***In vitro* testing of virulence factors**: Table -3 showed the results of *in vitro* testing of *B. pseudomullei* for the production of virulence factors. Statistically there was no significant difference (P>0.05) in the production of all tested isolates. Virulence factors

Table(3). The virulence factors of *B. pseudomalli*

Virulence factors	No. (%) of positive					Arb^+ n=29	Arb^- n=38
	Cows milk n=20	Goat milk n=20	Farm soil n=16	Water n=12	Total n=73		
Protease	18 (72)	18 (90)	15 (93.7)	9 (75)	60 82.2	21 (72.4)	38 (86.4)`
Lipase	21 (84)	18 (90)	14 (87.5)	12 (100)	65 (82.1)	25 (86.2)	40 (90.9)
Lecithinase	23 (92)	18 (90)	12 (75)	9 (75)	62 (84.9)	16 (55.2)	36 (81.8)
Hemolysin	23 (92)	20 (100)	16 (100)	12 (100)	71 (97.3)	29 (100)	42 (95.5)
capsule	25 (100)	20 (100)	16 (100)	12 (100)	73 (100)	24 (89.65)	44 (100)

The activity of exoenzyme and toxin of *B. pseudomallei* suspension:

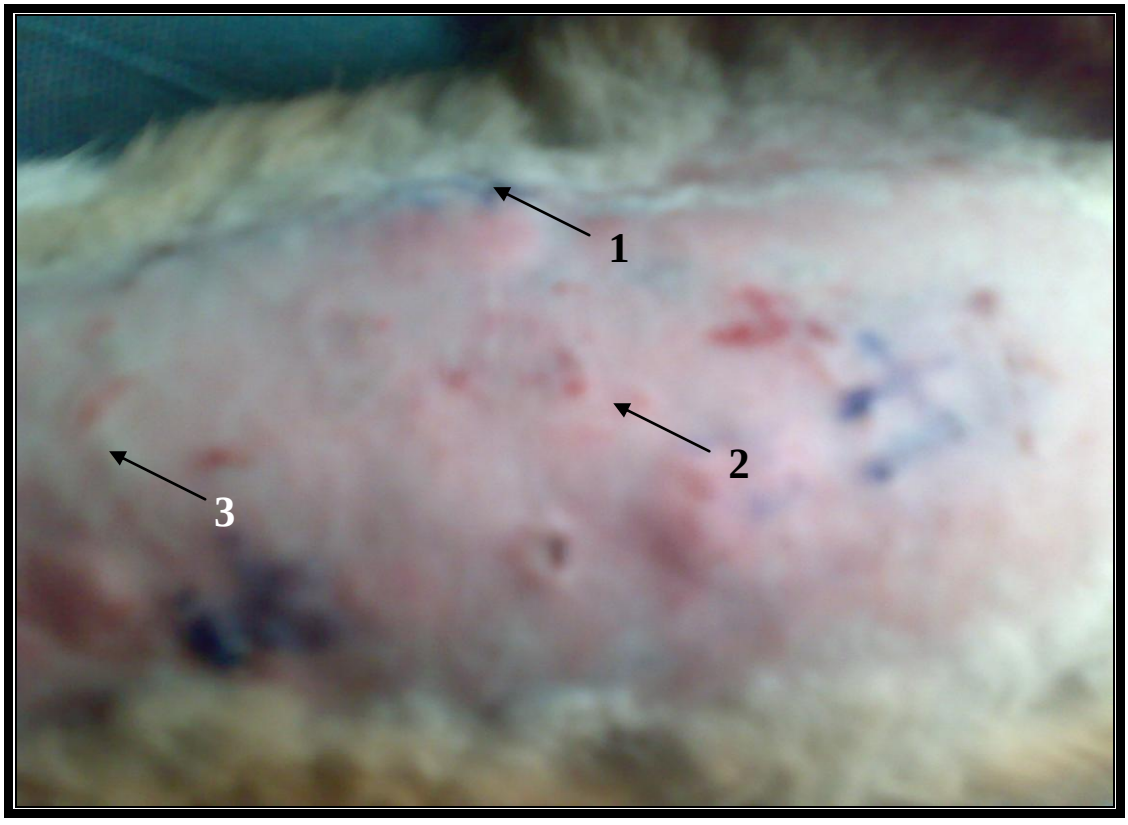
The crude active substance that was extracted and precipitate from the supernatant of *B. pseudomallei* suspension was 0.000212 gm/ml. the protein concentration of the crude active substance was 0.00248 gm/ml.Table -4 showed the spectrophotometric estimated casein proteolytic activity as an optic density value (OD). The OD value increased as the concentration of this enzyme increase which lead to digestion of casein in the trichloroacetic acid.

Table (4). the activity of casein proteolytic enzyme:

No. of test tube	Quantity of added supernatant/ ml	Absorption A280
1	(1:1)diluted ml1	1.722
2	1ml Concentrated	2.450
3	2ml Concentrated	3.21
4	0	2.227

The effect of necrotoxin of the bacterial supernatant:

After 24hr. Of intradermal injection, skin hypersensitivity was developed in the form of red, convexed spots of different sizes. No skin reaction to the heated or boiled supernatant was observed(fig. 2).



Figure(2).The activity of necrotoxin of *B. pseudomallei* suspension

DISCUSSION

The distribution of *B. pseudomallei*:

The present study aimed to detect or investigate the presence of *B. pseudomallei* in 200 samples of milk, farm soil and water. *B. pseudomallei* was diagnosed in an overall rate (36.55%). The presence of this bacteria in Basrah was supported by two studies conducted previously in Basrah [17,18]. The rate of *B. pseudomallei* isolation which was reported in the present study in concern to cow's milk (33.3%) and goat's milk (26.7%) also supported by the study of,[18] who indicate that this bacteria was isolated from the viscera of cows, buffaloes and sheep in the rate of 35%. The isolation of *B. pseudomallei* from healthy and infected goat's milk in the rate of 26.7% in constant with [19] who reported that *B. pseudomallei* was isolated from goat's milk which was found to have mastitis and from healthy ones. The explanation for this result is by the indication of [19] that *B. pseudomallei* is facultative intracellular bacteria. They remain in the host as latent infection for many years. The present results of *B. pseudomallei* isolation from farm soil and water supply in the rate of 64% and 48% respectively in contrast with [17] results as she indicate that the rate of *B. pseudomallei* isolation from water 51.3% and from soil was 43.5%. the difference between these two studies results related to the difference in the percentage of soil moisture which results from the accumulation of animals manure and lack of water from punctured pipes. In the present study *B. pseudomallei* was isolated from water of the river at a rate of 58.3%, tap water (40%) and from farms trough (37.5%), these results were in contrast with [17] in concern with the rate of isolated from the river and tap water (88.8% and 28.6% respectively).

Despite to the same region (Basrah) of the two studies conduction there was a difference in the rate of *B. pseudomallei* from river water, but the tap water isolation rate in the two studies was lower than that of river water. This finding might be related to physical, chemical growth factors in the river which were suitable to the growth of bacteria. On the other hand the present rate of tap water *B. pseudomallei* isolation was higher than the rate (7%) which was reported by [20]. This difference might be related to differences in environmental conditions and the methods of purification and sterilization of water. In concern to farm soil the *B. pseudomallei* was isolated from these source samples in a rate of 64% The present result in contrast with [21,22] who reported (68% and 1.8% respectively). Difference in climate conditions (rain, humidity and temperature) may influence the results of these studies.

Biotyping of *B. pseudomallei*:

According to arabinose utilization *B. pseudomallei* was classified in the present study into Arb^+ 39.7% and Arb^- 60.3%. This result was in contrast with [18a,17] who reported Arb^+ 63.07% and Arb^- 36.93%,Arb^+ 25.58% and Arb^- 74.41% respectively. The explanation for this difference in the results could be explained by the indication of [16] that there was variation in the *B. pseudomallei* biotypes distribution observed in the same or different geographical regions.

Detection of virulence factors:

The present results confirmed the ability of *B. pseudomallei* isolates to produce protease, lipase,lecithinase and hemolysin in an overall rate (82.2, 82.1, 84.9 and 97.3 respectively). These results in contrast with [18] who reported variable rate for isolates in the production of the same four enzymes (protease 75.4%, lipase 89.2%, lecithinase 90.8% hemolysine 96.9%.) Also the present results of the four enzyme production differ from the results of [17] who reported (100%) rate in the production of both protease and lipase,but same rate was reported in case of lecithinase production (85.7%). In concern to the virulence factors of Arb^- and Arb^+ biotypes the present results revealed that both biotypes produced all virulence factors which were investigated in this study but differ significantly in the rate of their production. These results were in agreement with [18,17,23] .The present results that deal with crud active substances which were detected in the *B. pseudomallei* suspension were in agreement with [18,17,24] who mention that the supernatant of *B. pseudomalli* culture contain at least two types of proteolytic, necrotic compounds all of them were heat labile. In conclusion *B.pseudomallei* was isolated and identified in all studied samples.Many virulence factors were observed in these bacteria.

عزل وتشخيص جرثومة *Burkholderia pseudomaleli* من حليب الأبقار والمعز والبيئة المحيطة بها في محافظة البصرة

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الخلاصة

جمعت 200 عينة من حليب الأبقار والمعز والبيئة المحيطة بها فكانت 75عينة كلاً من حليب الأبقار والماعز و25عينة من تربة الحظائر و25 عينة من الماء. زرعت جميع العينات على وسط انتقائي Ashdaown selective agar والذي أعطى أعلى نسبة عزل للجرثومة*Burkholderia pseudomallei* حيث بلغت نسبة عزل 36.5% بواقع 73 عيرة من اصل 200 عينة كما وجد اعلى نسبة عزل في عينات التربة وبنسبة 64% تلتها عينات الماء 48% ومن ثم عينات حليب

الأبقار 33.33% واخيراً حليب المعز 26.66%. اعتماداً على التنميط الحيوي باللارابينوز لجرثومة *B.pseudomallei* وجد ان 39.72% من العزلات كانت ضمن النمط الموجب للارابينوز (Arb^+) و60.27% من العزلات كانت ضمن النمط السالب للارابينوز Arb^- .

تم الكشف عن ضراوة هذه الجرثومة مختبرياً وقد تبين ان معظم العزلات قد أنتجت كل من الإنزيم الحال للدم والإنزيم الحال للبروتين والإنزيم الحال للدهن والإنزيم الحال لليسيثين وبنسب متفاوتة بلغت 97.3% و82.2%و 89.04%و 84.9%على التوالي.وقد تميزت جميع العزلات بقدرتها على تكوين المحفظة capsule 100% .

أوضحت الدراسة الحالية ان العزلات ذوات النمط السالب للارابينوز اشد ضراوة من العزلات ذوات النمط الموجب للارابينوز حيث وجد ان نسبة العزلات ذوات النمط Arb^- التي أنتجت الإنزيمات (الحال للبروتين والدم والليسثين) أعلى من نسب العزلات ذوات النمط Arb^+ ويوجد فرق معنوي $P<0.05$ بين هذين النمطين الحيويين.

تميزت جميع العزلات البكتيريه بتكوين المحفظه وقد أثبتت الدراسة وجود الإنزيم الحال للكازائين وانزيم التنخر في معلق جرثومة*B.pseudomallei* وقد بلغ تركيز البروتين في هذا المعلق 0.00248 ملغم لكل 1مل.

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