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Journal Home Page: <https://tjphs.tu.edu.iq> -- Email: tjops@tu.edu.iq**Detection of Biofilm Producing Bacteria causing Co-infections with Leishmania Ulcers and their Relation with Matrix metalloproteinase-13 Enzyme**Abrar Kareem Dhari¹, Suha Maher Abed¹, Ali Mohammed Abed^{1*}¹⁺²⁺³ Department of Biology, College of Science, Tikrit University, Tikrit, Iraq**Keywords:**Cutaneous Leishmaniasis,
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<http://doi.org/10.25130/tjphs.2024.18.1.4.31.38>**Abstract****Background:** Protozoan parasite causes Cutaneous Leishmaniasis (CL), dermatological disorder. Infected female phlebotomus sand flies spread these parasites. Secondary bacterial infection can worsen tissue damage and scarring.**Objectives:** In order to accurately ascertain the incidence of secondary bacterial infection caused biofilm formation in cutaneous leishmaniasis, it is imperative to investigate the significant immunological alterations by quantifying the levels of human Matrix metalloproteinase -13 (MMP-13).**Materials and Methods:** This study included 90 samples (20 controls and 70 CL sufferers). Study participants ranged in age from 3 months to 65 years, including both genders. Human Matrix metalloproteinase -13 measured by ELISA technique. Ulcer exudates were collected with sterile swabs after non-ulcerated lesions scraped. The specimens were cultured on Blood Agar, MacConkey Agar, Mannitol Salt Agar, and Hi Chrom Acinito. Laboratory methods were identified biofilm producing microorganisms.**Results:** Biofilms- forming secondary bacterial infections were found in 30 (42.8%) of 70 cutaneous Leishmaniasis patients. Bacterial from lesions *Acinetobacter baumannii* (8) cases (26.6 %), *Acinetobacter lwoffii*, *Crobactor*, *Klebsiella*, *Proteus mirabilis*, *Proteus vulgaris*, *Pseudomonas Luteola*, *Raoultella*, *Serratia*, *Sphingo*, (1) cases (3.3 %) for each spp. *Pantoea* (2) cases (6.6 %), *Pseudomonas fluorescence* (3) cases (10 %), and the study found no differences in MMP-13 levels in CL patients (5.7 + 0.2) compared to the control group.**Conclusions:** This study observed no differences in the average concentration in level of MMP-13 patient with CL compared with control groups.

الكشف عن البكتيريا المنتجة للأغشية الحيوية المسببة للعدوى المصاحبة لتقرحات الليشمانيا وعلاقتها بانزيم المصفوفة ميتالوبروتينيز-13

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الخلاصة

الطفيلي الأولي تسبب داء الليشمانيات الجلدي. تنتشر هذه الطفيليات عن طريق إناث ذباب الرمل الفاصدة المصابة. يمكن ان تؤدي العدوى البكتيرية الثانوية إلى تفاقم تلف الأنسجة وتندبها. من أجل التأكد بدقة من انتشار العدوى البكتيرية الثانوية التي تسبب تكوين الأغشية الحيوية في داء الليشمانيات الجلدي، فمن الضروري التحقيق في التغيرات المناعية الهامة عن طريق قياس مستويات مصفوفة ميتالوبروتينيز. شملت هذه الدراسة 90 عينة (20 شخصاً سليماً و 70 مصاباً بداء الليشمانيات). تراوحت أعمار المشاركين في هذه الدراسة من 3 أشهر إلى 65 سنة، ومن كلا الجنسين. تم قياس مصفوفة ميتالوبروتينيز بواسطة تقنية الأليزا. تم جمع إفرازات القرحة باستخدام مسحات معقمة بعد كشط الاصابات غير المتقرحة. تمت زراعة العينات على أجار الدم، الماكونكي، اكار ملح المانيتول، وكروم الاسينيتو. تم تحديد الطرق المختبرية للكائنات الحية الدقيقة المنتجة للأغشية الحيوية. ظهرت العدوى البكتيرية الثانوية المكونة للأغشية الحيوية في بعض المرضى المصابين ب داء الليشمانيات الجلدي. فيما يتعلق بمصفوفة ميتالوبروتينيز، لم تكن هناك اختلافات في المرضى الذين يعانون من داء الليشمانيات الجلدي والذين يعانون من عدوى بكتيرية ثانوية بسبب داء الليشمانيات.

الكلمات المفتاحية: داء الليشمانيات الجلدي، مصفوفة ميتالوبروتينيز، العدوى البكتيرية الثانوية

Introduction:

Leishmaniasis is a vector-borne infection caused by the protozoan parasite of the genus *Leishmania*. The vectors are female sandflies (*Phlebotomus* and *Lutzomyia*).⁽¹⁾ The disease is distinguished by a range of clinical manifestations are mostly determined by the species of parasite and the immunological response of the host. Based on recent estimations, an annual incidence of 1.5 million new cases of cutaneous leishmaniasis (CL) has been reported. Over 90% of cases occur in five Old World countries. Afghanistan, Algeria, Iran, Iraq, and Saudi Arabia. Additionally, two nations in the New World, Brazil and Peru, also experience a significant number of cases⁽²⁾. The lesions induced by *L. major* exhibit a tendency to undergo spontaneous healing within a timeframe of roughly 18 weeks⁽³⁾. Local care, in conjunction with anti-leishmanial therapy, is a crucial component of the treatment for (CL). The management of secondary bacterial infection is crucial for the process of wound healing⁽⁴⁾. Conversely, the secondary bacterial infection of the cutaneous Lesion would augment the extent of tissue damage and subsequently contribute to scar formation⁽⁵⁾. Human Matrix metalloproteinase-13 (MMP-13) is a category of enzymes that rely on zinc for their catalytic activity. The enzymes are accountable for the process of protein break down within the extra cellular matrix. MMP-13 production is elevated in mucosal leishmaniasis, the more severe from *L. braziliensis* infection⁽⁶⁾. Therefore, the aim of this study was to accurately ascertain the incidence of secondary bacterial infections that are caused as a results of cutaneous leishmaniasis and their relation with immunological levels of (MMP-13).

Materials and methods

The study has been conducted from September 2022 to March 2023. The study included 90 samples (70 patients and 20 controls) were collected from both genders of studied participants, their ages ranging from

3 months to 65 years. The patients were referred to dermatology department in the Al Balad General Hospital, Samarra Hospital, Al Dhuluiya General Hospital in Salah Al-Din Governorate. For secondary bacterial isolation, 70 samples were taken from patients infected with cutaneous leishmaniasis. The parasite was diagnosed clinically by a dermatologist. A data collection sheet was applied to all individuals enrolled in this study in order to obtain Information including the location of the lesion, the number of lesion, their size, shape and texture. Inclusion criteria involve any patient with confirm diagnosis of cutaneous leishmaniasis. Exclusion criteria involve any patient with diabetes mellitus, hypertension, renal disease, and recent use of antibiotics.

Collecting blood samples: Five ml of venous blood were taken using a sterile and dry medical syringe, then the blood sample was placed in a gel tube, and the tubes were left at room temperature for 15-20 minutes then placed in a centrifuge at 3000 revolutions per minute for 10 minutes to extract the serum, then transfer the acquired serum into Eppendorf tubes and store it at -20°C until used to the time of the immunological test of MMP-13 that was measured by using Sandwich-ELISA (7) (Sunlong, China). All samples have been brought to room temperature before 30 minute. Examine all samples at one time to avoid repeated freezing and thawing, which may affect the quality of results.

Swap from lesion for bacterial diagnosis: The skin around the lesions of infected participants was disinfected thoroughly with alcoholic iodine-soaked cotton wool, then carefully scraping non-ulcerated lesions, finally the swap was taken⁽⁸⁾. All samples were cultured on blood agar MacConkey Agar, Mannitol Salt Agar then tested biochemically such as catalase, oxidase, Methyl red, Triple Sugar Iron (TSI) agar, Dnase, coagulase, and Cetrinide test were used to identify and categorize the bacteria. Finally diagnosed by the VITEK[®]2 compact system.

Microscopic examination by direct smear: The infection was diagnosed using direct smear according to the method described⁽⁹⁾, whereby a sample was obtained from the edge of an ulcer for detecting amastigote stages of the parasite in fixed phagocytic cells in skin tissue. The smear method was carried out as follows: The skin ulcer was cleaned with ethyl alcohol 70%. The dermal fluid sample was drawn from the edge of the ulcer with a medical syringe with a capacity of 1 milliliter, then it was placed on the tip of a clean glass slide for the purpose of making a thin swab, and the swab was left to dry at room temperature. The dermal fluid smear was stained using Giemsa stain, as the slides were immersed in 70% methyl alcohol for 3 minutes, left to dry at room temperature, then dipped in Giemsa dye and left for 30 minutes. The slides were transferred to tap water to wash off the excess layer and shorten it, with the latter being replaced three to four times. Slides were left to dry at room temperature. The slides were examined by a light microscope and by using an oil lens at a magnification of 1000 to detect the amastigote stage of the parasite, and the sample was considered negative if no such stage was found. It must be pointed out that he had done three replicates for one sample.

Antibiotic Susceptibility Test

Preparation of Culture Media:

Muller-Hinton agar medium was employed for this experiment. The medium was autoclaved, cooled to 45-50°C and poured in the petri dishes. When the medium solidified, the petri dishes were incubated at 37°C for evaporated the excess moisture.

Detection biofilm

Cong red agar:

The plates of Cong red agar prepared then inoculated with the bacterial isolates, and the plates were

incubated in the incubator at a temperature of 37°C for 24-48 hours. After incubation, the color of the developing bacterial colonies was observed, as black colonies indicate that they are productive for the biofilm. While red colonies do not produce biofilm⁽⁸⁾.

Microtiter Plate Titer (MPT):

The microtiter plate (96- well plate) experiment for biofilm formation observes bacterial adhesion to an abiotic surface. This test uses vinyl (U)- Bottom or other 96-well microtiter plates to incubate microorganism. After incubation, planktonic bacteria are rinsed away and biofilms are stained with stained with crystal violet to visualize them.

Ethical approval

This study was conducted based on the ethical standards stipulated in the Declaration of Helsinki No. 12948/63/7. Before taking the sample, the patient's informed written and verbal agreement was obtained, after review and approval of the study protocol and subjects information by the local ethics committee.

Statistical Analysis

SPSS was used for statistical analysis. The continuous variable format was means \pm Stander Error (SE). The link between categorical factors was examined P-values under or equal 0.05 were significant.

Results

Microscopic examination

The investigation found Amastigotes, small, spherical entities (2-4 μ m in diameter) with indistinct cytoplasm, nucleus, and rod-shaped kinetoplast as shown in (Fig. 1). Large nucleus, conspicuous kinetoplast and short axoneme rarely observable by light microscopy. The organism live in host macrophages throughout the body.

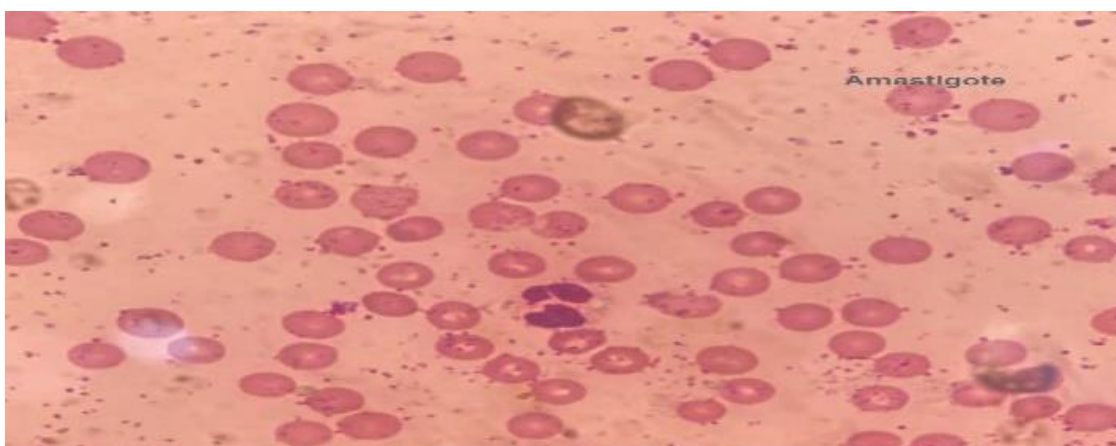


Figure 1: Amastigote stage of Leishmania stained by Giemsa stain 40x

Bacteriological results

The results of the biochemical tests (Figure 2-7) were compared with the characteristics of secondary bacterial infection documented by others.

Methyl red test is used to ascertain the specific fermentation pathway employed for glucose utilization. In the event that the culture lacks acetone, it will exhibit a color transition from brownish- green to yellow, as depicted figure 2. *Klebsilla pneumoniae* exhibits the phenotypic characteristics of being Methicillin-resistant (MR) and Voges- Proskauer (VP) tests positive (VP+). *Pseudomonas aeruginosa* is classified as a glucose non-fermenting bacterium, resulting in a negative reaction for both the methyl red (Fig.2) and Voges-proskauer (VP) tests. Mannitol Salt Agar favors salt – tolerant *Staphylococcus aureus*. *S.aureus* ferments mannitol to produce acid and the production of acid lowers the PH of the medium,

causing the phenol red indicator to turn from red to yellow (Fig.3).

Triple sugar iron (TSI) is most often used to identify Enterobacteriaceae, although it can also identify other Gram negative bacteria. *Klebsilla* spp. produce acid\acid reactions with or without gas. *Acinetobacter* and *Pseudomonas* produce alkaline \ alkaline or K\NC. *Citrobacter* spp. and *Proteus mirabilis* produce H₂S-alkaline\Acid. *Proteus mirabilis* and *Proteus vulgaris* produce acid\acid with H₂S (Fig.4). Dnase positive organisms like *Staphylococcus aureus* and *Serratia* spp. (Fig.5).Coagulase clots plasma detects Coagulase positive *Staphylococcus aureus* in Gram positive, catalase – positive organisms. *Pseudomonas aeruginosa*, exhibits a positive oxidase reaction (Fig.7). When cultivated on Cetrimide agar, *Pseudomonas aeruginosa* will yield blue fluorescent colonies on this agar.

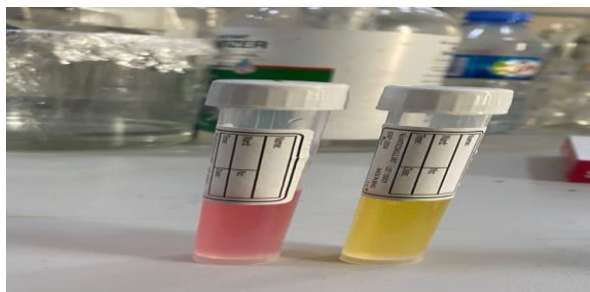


Figure2: Methyl red test



Figure3: Mannitol Salt Agar, positive for *Staphylococcus aureus*



Figure4: Triple Sugar Iron



Figure 5: DNA Test, positive for *Staphylococcus aureus*

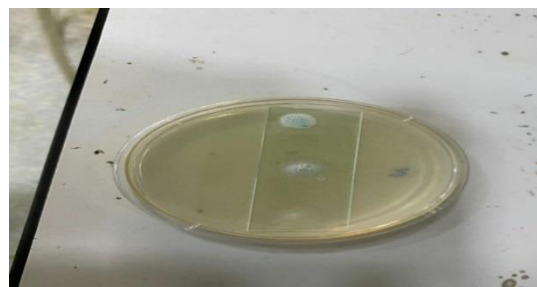
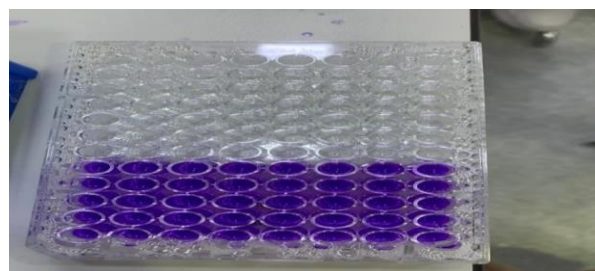


Figure 6: Oxidase Test, positive for *Pseudomonas aeruginosa*



A- Congo red agar



B- Micro titer (MTP)

Figure 7: Biofilm formation of isolated bacterial

Biofilm formation in patients with CL and secondary bacterial infection from clinical samples:

Approximately 30 (42.8%) patients exhibited positive cultures indicating the presence of secondary bacterial infection biofilm formation. Bacterial isolates from the lesion using the Congo red methods and micro titer plate in the gram positive and negative bacteria were as follows: *Acineto baumannii* (8) cases or 26.6%, *Acinetobacter lwoffii*, *Klebsiella*, *Crobactor*, *proteus vulgaris*, *proteus mirabilis*, *pseudomonas-luteeola*, *sphingomonas*, *Raoultella*, and *Serratia*, (1) case or 3.3% for each spp. *Pantoea* (2) cases or 6.6%. *pseudomonas fluorescense* (3) cases or 10%, and *S.aureus* with *S.sciuri* (4) cases or 13.3% for each spp, samples were strong for biofilm producer as done by Congo red agar and MTP method as seen in figure 7-A.

Micro titer Plate The biofilm forming potential of 60 bacterial isolates was measured using crystal violet staining in 96 well Micro titer plates that observed bacterial adhesion to an abiotic surface as shown in (Figure 7-B).

Antimicrobial susceptibility testing

The antibiotic sensitivity of the bacterial isolates was assessed using the disk diffusion methods, as outlined by the(9).The test organisms were suspended in sterile normal saline solution in order to achieve a turbidity standard of 0>5 McFarland. The suspended organisms were distributed on to a Mueller-Hinton Agar plate (oxid Ltd, Baisngstoke, United Kingdom with the recommendation established by the Clinical and Laboratory standard Institute (CLSI)(10).The antibiotics utilized in this study were sourced from oxid. Fluoroquinolones (Ciprofloxacin 10mcg, Sparfloxacin 5 mcg), Cephalosporin (Ceftazidime30 mcg, cefotaxime 30 mcg), Cefotaxime, Macrolide (Azithromycin 15 mcg, Clarithromycin 15 mcg) Carbapenems (Meropenem 10 mcg), Glycopeptides (Vancomycine 30mcg), fluoroquinolones (Levofloxacin 2 mcg), Aminoglycoside (Gentamycin 10 mcg), Tetracycline (Cloxacillin) were tested as show in (fig 8). The results of antibiotics sensitivity tests are shown in table 1.

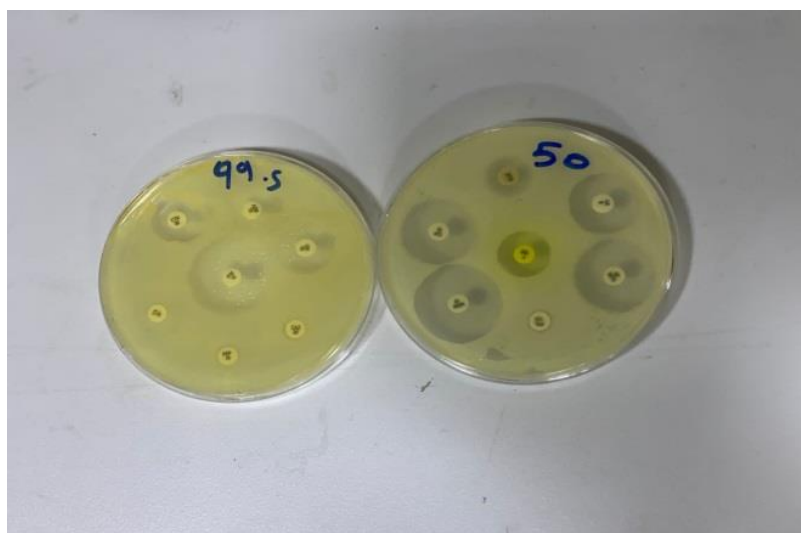
Fig(8): Antimicrobial susceptibility testing of *S.sciuri*

Table 1: Antimicrobial susceptibility testing of secondary bacterial infection

Selected organism	Antibiotic	No :-	<i>S.aureus</i>		<i>S. sciuri</i>		<i>Pseudomonas fluorescens</i>		<i>Pseudomonas-luteola</i>		<i>Acineto baumanni</i>		<i>Acineto lowffii</i>		<i>Sphingomonas</i>		<i>Serratia</i>		<i>klepsella</i>		<i>Proteus vulgaris</i>		<i>Raoultella</i>		<i>Crobacter</i>		<i>Pantoea</i>		<i>Porteus mirabilis</i>	
			N= 4	N= 4	N= 3	N= 1	N= 8	N= 1	N= 1	N= 1	N= 1	N= 1	N= 1	N= 1	N= 1	N= 1	N= 1	N= 1	N= 1	N= 1	N= 1	N= 1	N= 1	N= 1	N= 1	N= 1	N= 2	N= 1		
Cephalosporin	CAZ-30	R	3	75%	2	50%	3	100%	1	100%	3	37%	0	0%	1	100%	0	0%	0	0%	1	100%	0	0%	1	100%	1	100%	1	100%
		S	1	25%	2	50%	0	0%	0	0%	5	63%	1	100%	0	0%	1	100%	1	100%	0	0%	1	100%	0	0%	0	0%	0	0%
	CTX-30	R	3	75%	2	50%	2	66%	0	0%	3	37%	1	100%	0	0%	0	0%	0	0%	0	0%	0	0%	1	100%	1	100%	1	100%
		S	1	25%	2	50%	1	34%	1	100%	5	63%	0	0%	1	100%	1	100%	1	100%	1	100%	1	100%	1	100%	0	0%	0	0%
Fluoroquinolones	CIP-10	R	1	25%	3	75%	1	33%	0	0%	2	25%	0	0%	1	100%	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%	1	100%
		S	3	75%	1	25%	2	67%	1	100%	6	75%	1	100%	0	0%	1	100%	1	100%	1	100%	1	100%	1	100%	1	100%	0	0%
	SPX-5	R	0	0%	2	50%	1	33%	1	100%	4	50%	0	0%	1	100%	0	0%	1	100%	0	0%	1	100%	0	0%	0	0%	1	100%
		S	4	100%	2	50%	2	67%	0	0%	4	50%	1	100%	0	0%	1	100%	0	0%	1	100%	0	0%	1	100%	1	100%	0	0%
Macrolide	AZM-15	R	0	0%	1	25%	0	0%	0	0%	3	37%	0	0%	0	0%	0	0%	0	0%	0	0%	1	100%	0	0%	1	100%	1	100%
		S	4	100%	3	75%	3	100%	1	100%	5	63%	1	100%	1	100%	1	100%	1	100%	1	100%	0	0%	1	100%	0	0%	0	0%
	CLR-15	R	1	25%	1	25%	1	33%	0	0%	2	25%	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%	1	100%	0	0%
		S	3	75%	3	75%	2	67%	1	100%	6	75%	1	100%	1	100%	1	100%	1	100%	1	100%	1	100%	1	100%	0	0%	1	100%
Carbapenems	MEM-10	R	0	0%	1	25%	0	0%	0	0%	1	13%	0	0%	0	0%	0	0%	0	0%	0	0%	1	100%	0	0%	1	100%	1	100%
		S	4	100%	3	75%	3	100%	1	100%	7	87%	1	100%	1	100%	1	100%	1	100%	1	100%	0	0%	1	100%	0	0%	0	0%
Glycopeptides	VA-30	R	2	50%	3	75%	3	100%	1	100%	8	100%	1	100%	1	100%	1	100%	1	100%	1	100%	1	100%	1	100%	1	100%	1	100%
		S	2	50%	1	25%	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%
Lienomycin	L-2	R	1	25%	2	50%	2	67%	1	100%	3	37%	0	0%	1	100%	0	0%	1	100%	1	100%	1	100%	1	100%	1	100%	1	100%
		S	3	75%	2	50%	1	33%	0	0%	5	63%	1	100%	0	0%	1	100%	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%
Aminoglycosides	CN-10	R	3	75%	1	25%	2	67%	1	100%	4	50%	0	0%	0	0%	1	100%	1	100%	1	100%	1	100%	1	100%	1	100%	1	100%
		S	1	25%	3	75%	1	33%	0	0%	4	50%	1	100%	1	100%	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%
tetracycline	COX-5	R	3	75%	2	50%	3	100%	1	100%	5	63%	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%	1	100%	1	100%	1	100%
		S	1	25%	2	50%	0	0%	0	0%	3	37%	1	100%	1	100%	1	100%	1	100%	1	100%	1	100%	0	0%	0	0%	0	0%

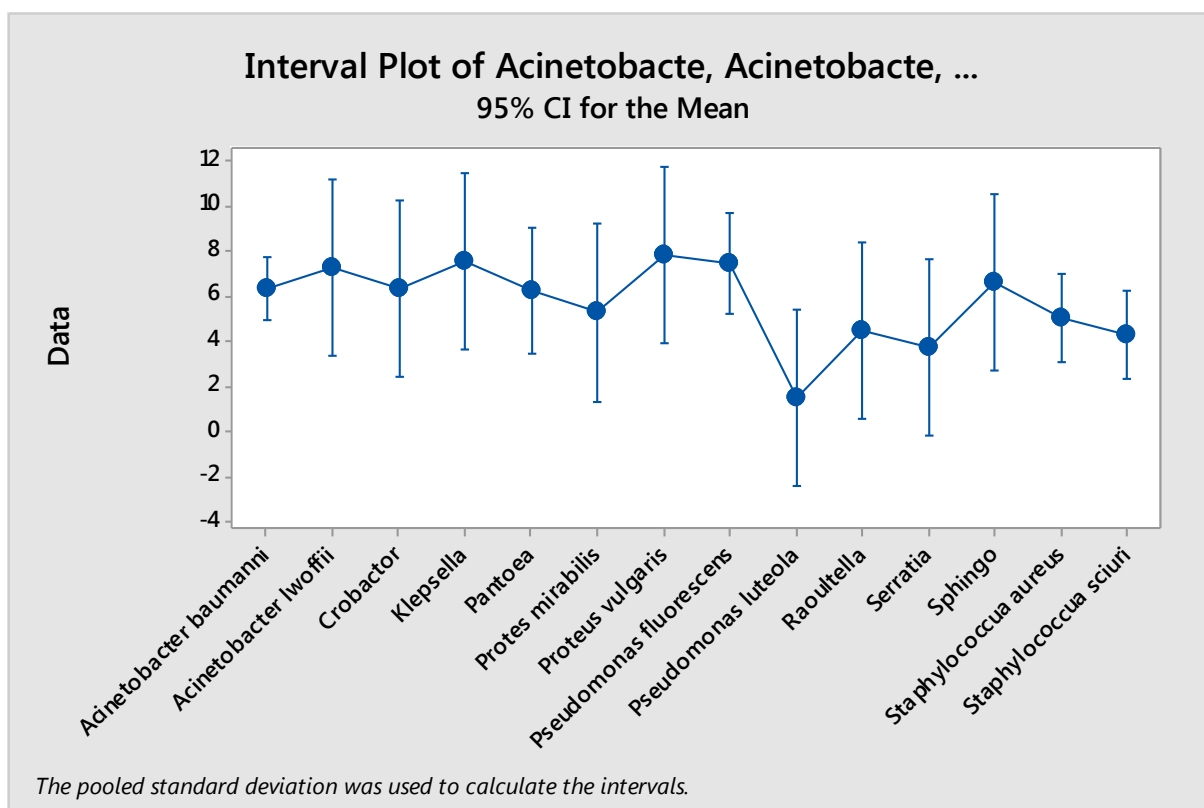
Serum Human Matrix metalloproteinase -13 level in patients with CL and control groups:

The study observed no differences in the average concentration of MMP-13 in infected patients with CL 5.7±0.2 (Pg/ml), compared to control group (6.9±0.6 Pg/ml) as shown in Table2.

The study observed that no significant differences in serum MMP-13 level in patients with CL and secondary bacterial infection was observed (p-value = 0.302) (Fig 9).

Table 2: Serum MMP-13 level in patients with CL and in control groups

MMP-13(Pg/ml)	Patients(N=70)	Control (N=20)	P-value
Mean ±S.E	5.7±0.2	6.9±0.6	^{ns} 0.111



Fig(9): Serum MMP-13 level in patients with CL and secondary bacterial infection

Discussion

Cutaneous Leishmaniasis is a prevalent global health issue that exerts a substantial influence on body mortality and morbidity rates. The clinical phenomenology of CL is thought to be influenced by innate immune mediators, which can either restrict or enhance the spread of the parasite^(1,2). The occurrence of secondary bacterial infection is recognized as a complication associated with CL⁽¹⁾. The presence of secondary bacterial infection has the potential to worsen the condition and contribute to the formation of the permanent scar due to its ability to intensify tissue death and necrosis the manifestation of painful ulcers accompanied by purulent discharges and inflammation in the surrounding area may be observed. Furthermore, the length of the sickness would be extended⁽¹²⁾. In these instance, the proper utilization of antibiotics

might lead to a reduction in the subsequent occurrence of illness. The current study conducted in Iraq has revealed that some patients exhibited positive cultures for secondary bacterial infection and biofilm formation, and several bacteria isolated. Other study identify *S.sciuri* and *S.aureus* the causative agent that isolated from CL ulcers⁽¹⁴⁾. Chicleros Ulcer, a type of CL by *Leishmania mexicano*, were found to include pathogenic microorganism in Yucatan , Mexico. These patients exhibited resistant to antimonial treatment, also the study indicates the necessity of eradicating bacterial infections through antibiotics therapy prior to initiating antimonial administration⁽¹⁵⁾. The current study observed no significant differences in the average concentration of MMP-13 in infected patients with CL, compared to control group. The entire lesions that have secondary bacterial infection

were ulcerated. There were bacterial isolates from the lesions that were ulcerated; also the study concluded that destruction of the epidermis in the ulcerated lesion had predisposed the patients to the secondary bacterial infection⁽¹⁶⁾. High levels of MMP-13 can be considered a biomarker of poor prognosis indicating a high risk of parasite dissemination in the chronic phase of visceral leishmaniasis infection. MMP-13 expression has not been detected in normally healing human skin ulcers but has been detected in abundance. By fibroblasts in chronic skin ulcers, results indicate low levels of the enzyme during normal wound healing and increase in chronic wounds⁽¹⁷⁾.

The topical antiseptic solution need for ulcerated lesions of the CL to prevent secondary bacterial infection that may be accelerated tissue destruction⁽¹⁸⁾. This study disagree with⁽¹⁹⁾, that show high level of MMP-13 in patients with CL which suggested the patients that infected with CL, increased macrophage that led to increase MMP-13 secretion because it is a type IV component of collagen, then the basement membrane of the skin increase, and causes excessive tissue damage through the migration of inflammatory cells to the site of injury⁽¹⁹⁾. The increased and unregulated activity of the enzymes contributed to chronic inflammation and increase tissue destruction that may help pathogen to enter the blood stream and invade different tissue. In addition the result of increase MMP-13 was due to increase enzyme secretion and inhibition gastric protein secretion⁽²⁰⁾.

Conclusions: Biofilm forming secondary bacterial infections were presented in some of patients that were infected with CL. Regarding MMP-13, there were no differences in patients with CL and those with secondary bacterial infection due to leishmaniasis.

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