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Isolation and identification of protease Producer bacterial isolated from diabetic foot ulcer

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Abstract
A total of 76 clinical samples belonged to patients suffering from diabetic foot infection (DFI) were collected from Al- Kindy Teaching Hospital in Baghdad for the period from July2016 to February 2017. The range of patient ages was between 28 to 70 years; 64% of them were malesand36%females.The primary diagnostic results showed that 80 (88.9 %) of the bacterial isolates were Gram negative and 12 (13.3%) Gram positive. Depending on the results of cultural and microscopic examination, these seventy-six isolates were distributed on the genera as 20 to *Proteus*, 18 to *Escherichia*,16 to *Pseudomonas*, 12 to *Klebsiella*, 10 to *Staphylococcus*, 5 to *Morganella*, 4 *Acinetobacter*, 4*Stenotrophomonas*, 2*Kocuria kristnae* and 1 *Enterococcus*. When all bacteria lisolates were tested for their ability to produce protease enzymes. results showed that all the isolates were protease producer; among them five bacterial isolates belonging to (*Proteus mirabilis*, *Staphylococcus haemolyticus*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Kocuria kristnae*) were the most efficient in protease production with enzyme specific activities of (160, 100, 90, 87, 75, and 60U/mg protein), respectively.

INTRODUCTION

A diabetic foot infection (DFI) an increasingly common problem related to the duration of diabetic disease, and therefore the likelihood of diabetic complications. Infection is best defined of an invasion and multiplication of microorganisms in host tissues that induces host inflammatory response, usually followed by tissue destruction[1].These infections usually begin with a break in the protective cutaneous envelope, typically in a site of trauma or ulceration [2]. Protease enzymes constitute two third of the total enzymes used in various industries[3].They are used in many fields like pharmaceutical, food, detergent industries, waste treatments and others[4][5]. In the study Sharmin and Rahman (2007) declared that protease enzymes which are used in the medicine field are produced in small amounts but require extensive purification before they can be used. Treatment of chronic conditions such as diabetic foot infection (DFI) is a challenge due to the increase in the susceptibility for infection and wound healing delay[6]. Factors such as microbial resistance, complexity of existing therapy and adverse effects emphasize the need for an alternative approach toman age DFI [7]. In the past few years, probiotics are used in prevention and treating various health disorders as well as their use in the applications of gastroenterology and oral health [8][9] . Meurman stated that probiotics could be useful in preventing and treating infections[10]. There is every reason to believe that the putative probiotics mechanisms of action are the same in the peripheral wounds like DFI as they are in other parts of the body[11].

MATERIALS AND METHODS

1. Sampiling

Samples were collected from patients suffering from diabetic foot ulcer infections of various ages and both sexes.**(**A special form was designed to be filled with the name, gender, age, sampling date and previous treatment of each patient). The samples were taken by disposable cotton swabs, and cultured individually into nutrient broth before incubation at 37 ºC for 24h.After incubation, they were subcultured on blood agar and McConkey's agar, and then the suspected isolates were subjected for final identification.

2.Identification of pathogenic bacterial isolates

- Blood Agar Medium[12]

It was prepared by autoclaving blood base agar (pH to 7.0), after cooled to 45°C, 5% blood plasma was added and mixed well . The formation of clear zones or green around the colonies represented the haemolysis of blood.

- Vetic2 Identification [13]

A sterile swab or applicator stick is used to transfer a sufficient number of colonies of a pure culture and to suspend the microorganism in 3.0 mL of sterile saline (aqueous 0.45% to 0.50% NaCl, pH 4.5 to 7.0) in a 12 x 75 mm clear plastic (polystyrene) test tube. The turbidity is adjusted and measured using a turbidity meter called the DensiChek**.** A test tube containing the microorganism suspension is placed into a special rack (cassette) and the identification card is placed in the neighboring slot while inserting the transfer tube into the corresponding suspension tube. The filled cassette is placed into a vacuum chamber station. After the vacuum is applied and air is re-introduced into the station, the organism suspension is forced through the transfer tube into micro-channels that fill all the test wells. A transmittance optical system allows interpretation of test reactions using different wavelengths in the visible spectrum. Calculations are performed on raw data and compared to thresholds to determine reactions for each test. On the VITEK 2 Compact, test reaction results appear as +, –, (–) or (+). Reactions that appear in parentheses are indicative of weak reactions.

-Skim milk agar medium [14]

This medium was prepared by dissolving 5 g of skim milk in 50ml D.W and sterilized by autoclaving. A quantity of 2 g agar was dissolved in 50ml D.W., sterilized by autoclaving and cooled to 45°C. The two were mixed together before distributed into sterilized plates.

3.Screening isolates for protease production- Semi quantitative screening[14]

Each clinical isolate streaked on nutrient agar medium and incubated at 30 °C for 24h. A single colony was then taken and placed on the center of skim milk agar medium plate.The plate was incubated at 37°C for 24h. Ability of each bacterium for protease production was measured based on presence of clear halo zone around each colony.

RESULTS AND DISCUSSION

1. Isolation of bacteria

A total of 76 swab samples were collected from patients referred to Al-Kindy hospital in Baghdad suffering from diabetic foot infection (DFI). As shown in table (3-1), 42% of the samples were taken from sole of the foot, while 20%, 14%, 11%, 9%, 3% and 1% were from the big toe, heel, 2nd toe, 3rd toe, 5th toe and 4th toe, respectively. In this regard, Reiber *et al.*,(1998) stated that DFI is developed at pressure points on the plantar surfaces, over the metatarsal heads, on the big toe, and on the heels[15]

Same table shows that among DFI cases, (64%) were from male patients and the rest (36%) from females. The highest percentage(69%) of infection from sole of the foot was recorded by the male patients compared to only. (31%) by females. Infection of big toe, 2nd toe, and heel were also higher (66%, 62%, 55%) in males, than in female (33%,.38%, 45%), respectively. Adversely, cases of the 3rd toe infection were more common in male than in female patients with percentages of (57%) and (43%), respectively. While the 5th toe infection had the same occurrence percentage (50%) in both genders. On the other hand, the 4th toe infection was recorded only in male. The age group of the diabetic foot patients were ranging between 28-75 yrs. Results in figure (1) shows that age group of 58-75 yrs..was the most affected by diabetic foot infection, while age group 40-55 was the lowest infected. Such findings came in accordance with the results of Frykberg when they found that diabetic foot infection was most common among patients of age group 60-70 yrs. They added that the duration of diabetes mellitus was between 4 to 35 yrs, while that of infection was from 1 week to 20 yrs.[16]

When Wagner classification system was used to classify ulcers of the diabetic foot patients, results in figure (2) show that grade 2 (deep ulcer) was recorded in 36 patients, followed by grade1(superficial ulcer) in 25, grade 3 (abscess otitis) in 11, and grade 4 (gangrenous forefoot) in 4 patients, respectively. While grade 5 (whole foot) and grade 0 (no lesion) ulcers were not recorded in any of the patients. These results are different from those of Dhorod (2010) who recorded that most of the bacterial isolates were obtained from grades 2, 3, 4 of the diabetic foot patients.[17].

Table(1): Numbers and percentages of diabetic foot infection cases distributed according to site of infection and gender of patients.

Isolation source	Male		Female		Total	
	No.	%	No.	%	No.	%
Sole of foot	22	69	10	31	32	42
Big toe	10	66	5	33	15	20
Heel	6	55	5	45	11	14
2nd toe	5	62	3	38	8	11
3rd toe	4	57	3	43	7	9
4th toe	1	50	0	50	1	3
5th toe	1	100	1	100	2	1
Total	49	64	27	36	76	100

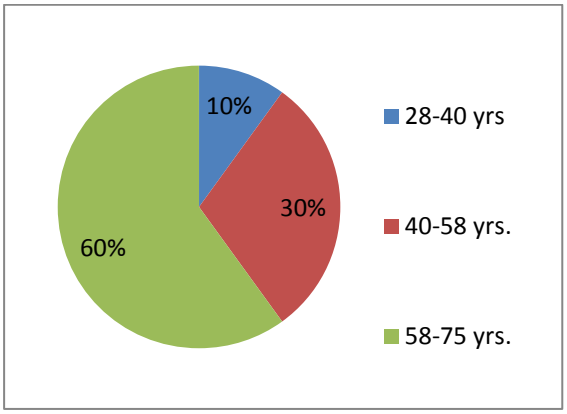

Figure (1):Percentage of DFI between age groups

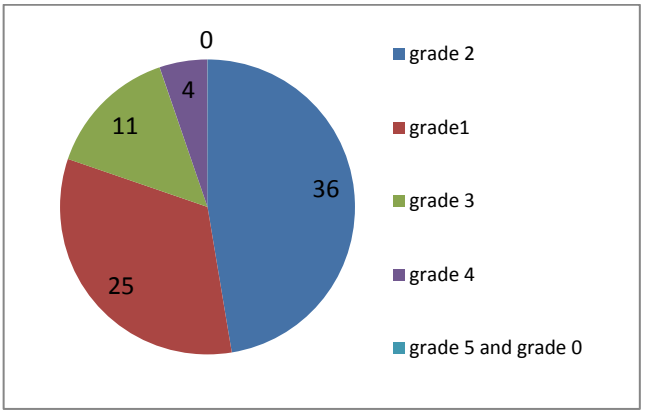

Figure (2):Percentage of DFI according to Wagner classification system

Table (2): Numbers and percentages of bacterial isolates obtained from patients with diabetic foot infection (DFI).

Isolation source	DFI cases			No. of bacterial isolates
	Total	positive for bacteria		
		No.	%	
Sole of foot	32	30	93.75	37
Big of toe	15	13	86.66	18
Heel	11	10	90.90	12
2nd toe	8	8	100	10
3rd toe	7	6	85.71	9
4th toe	1	1	100	2
5th toe	2	2	100	4
Total	76	70	92.10	92

Table (3): Numbers of occurrence of bacterial types present on each patients foot site.

No. of bacterial types	Big toe	2nd toe	3rd toe	4th toe	5th toe	Heel	Sole of foot	Total	
								No.	%
One type	3	3	3	0	0	3	9	21	30
Two types	9	5	3	1	2	7	20	47	67
Three types	1	0	0	0	0	0	1	2	3
Total	13	8	6	1	2	10	30	70	100

As illustrated in table (2), among the 76 patients of diabetes foot infection, 70 of them gave positive results for bacterial occurrence when a total of 92 bacterial isolates was obtained from them.

The highest occurrence of bacterial growth was recorded in the sole of diabetes foot patients when 37 of the 92 isolates were detected among its 32 patients. while18 ,12, 10, 9, 4 and 2 isolates, were obtained from the big toe, heel, 2nd toe, 3rd toe, 5th and4th toe, respectively. Results in table (3) show that one type of pathogenic bacteria was detected in 21 (30%) of the 70 diabetes foot infected patients, while 47 (67%) of the patients were infected with two types (Polymicrobial infection) and 2(3%) with three types of pathogens. On the other hand, no bacterial isolate was detected in 6 (8%) of the diabetes foot patients. Several researchers observed that the polymicrobial infection was the most common among the diabetes foot patients included in their studies[18][19][20][21][22]. Adversely, Viswanathan and Raga reported only one type of bacteria in the DFI patients[23][24]. Such this differences in this regard may be related to the country where the study is performed, and to the intensity of ulcer accompanying the infection[25][26].

Gram (-) bacteria in this study were the predominant pathogens among the patients of diabetic foot infections, *Proteus*, comparable findings were also recorded by various studies such as[21][25][24][27]. But in the deferent studies found that Gram (+) bacteria was the predominant organisms in the diabetic foot infections[28][29][30].

Identification of bacterial isolates

1.Cultural and microscopic characterization

Identification of the suspected (92) bacterial isolates was performed at first depending on the characteristic s of colonies grown on the surface of both MacConkey and Blood agar, then by microscopic characteristics depending on their Gram reaction. The suspected isolates were cultured on MacConkey agar which contains bile salts and crystal violet to promote growth of *Enterobacteriaceae* and related enteric Gram negative rods, in addition to suppress growth of Gram positive bacteria and some fastidious Gram negative bacteria. Lactose in this medium is the sole carbon source that differentiates between lactose-fermenting bacteria and non-lactose-fermenting bacteria. The lactose-fermenting bacteria are characterized by producing pink colonies due to the conversion of neutral red indicator dye when the pH is below 6.8. Adversely, the non-lactose bacterial growth appears color less or transparent[31]. Blood agar is a bacterial growth medium that contains 5% blood as enrichment factor by providing a rich nutrient environment for many types of bacteria. It is also considered as differential by its ability to distinguish the pathogenic bacteria from others based on its effect of their produced enzymes (known as hemolysins) which lyses the red blood cells[12]. Gram staining procedure shows that 80 (88.9 %) of the 92 isolates were Gram negative compared to 12 (13.3%) as Gram positive. Depending on the results of cultural and microscopic examination, the isolates are distributed on the genera of bacteria as follow: 20 *Proteus*, 18 *Escherichia*, 16 *Pseudomonas*, 12 *Klebsiella*, 10 *Staphylococcus*, 5 *Morganella*, 4 *Acinetobacter*, 4 *Stenotrophomonas*, 2 *Kocuria kristnae* and 1 *Enterococcus.*

2. Biochemical characterization (VITEK 2)

Identification of bacterial isolates was also done by the VITEK 2 system, at the Central Health Laboratory/Ministry of Health, by using the Gram positive and Gram negative cards**.** In general, VITEK 2is an easy-to-handle system that provides a rapid (4 to 15 h) and reasonably accurate means for identification of most commonly isolated species. One of the most important advantages of the VITEK 2 system is its significant reduction of handling time, which will have a positive impact on the work flow of the clinical microbiology laboratory[13]. Different results in the order of most

occurring DFI bacteria were obtained by Umadevi *et al.* (2011) when they found that *Klebsiella pneumoniae* was the most occurred etiological agent in DFI patients, followed by *Pseudomonas aeruginosa*, then *Staphylococcus aureus*.[32] Regarding themless common bacterial species, Citron *et al.* (2007) reported that *Aeromonas hydrophila and Serratia marcescens* were the least occurrence cause of DFI with a percentage of only (0.95%) for each[33]

3.Semi- quantitative screening:

It was achieved by detecting the ability protease production by formation of halo zone of hydrolysis around each colony when grown on skim milk agar. Results shown in figure (3) and table (5) declared that these isolates were able to hydrolyze skim milk agar medium and forming halos of hydrolysis with variable degrees [14][34]. All the isolates in this study were protease producer when they had been grown on skim milk agar and incubated at 37ºC for 24-48 hrs. The proteolytic zone around the colonies was very clear and could be simply detected. The diameters of halos ranged between 4 and 10 mm depending on the isolate. Among them, *Proteus mirabilis* isolate was the most efficient in protease production and have the highest diameter of hydrolysis on skim milk agar while *Kocuria kristnae* have the lowers diameter of hydrolysis, *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus haemolyticus* give different diameters 8, 7, 5 respectively. Gupta *et al.*, (2005) performed isolation of bacterial strains from environmental samples and screened their capability of protease production using skim milk agar and reported that the *Streptomycin sp*. was the maximum producer of protease among the isolated strains[35] .*Bacillus cereus*, *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, *Proteus mirabilis*, *Serratia marcescens*, showed zone formation in skim milk agar, due to proteolytic activity, But *Micrococcus luteus* hydrolyse the milk protein slowly this was reported by Wieldmann *et al*. (2000)[36]. Braun and Fehlhaber (2002) reported that *Bacillus cereus*, *Proteus mirabilis* and *Micrococcus luteus* showed proteolytic activity at different environmental conditions such as temperature at 2 to 37ºC and PH 4 to 7.3. showed proteolytic activity at 37ºC. [37].

4. Quantitative screening

The ninety two clinical local isolates were screened quantitatively to examine their ability for protease production by growing in the production broth medium for 24 hrs at 37°C, then were centrifuged and specific activity of protease in the crude filtrate was determined[38]. Result in table (6) indicates that all isolates possessed the ability to produce proteases with variable degrees but the five isolates in the table give the highest inhibition zones. Specific activity of protease in culture filtrate was ranged between 60 and160U/mg protein for the five highest producing isolates (*Proteus mirabilis*, *Staphylococcus haemolyticus*, *Pseudomonas aeruginosa*, *Escherichia coli*, and *Kocuria kristnae*). Among these isolates, *Proteus mirabilis* was the superior in production by giving 160 U/mg protein specific activity compared to only 60 U/mg produced by *Kocuria kristnae*. a protease activity has been observed in many isolates of *P. mirabilis* and *Proteus vulgaris* [39].

As shown in Table (6), *Staphylococcus haemolyticus* had specific activity 100 U/mg protein, the specific activity of *Pseudomonas aeruginosa* was 90 U/ mg protein and *Escherichia coli* was 87 U/mg protein. When these results were compared with semi-qualitative detection of enzyme Table (5) they were nearly identical. The differences in the ability of the isolates to produce protease may be related to genetic variations of the genes responsible for the production of protease[40] or may be related to site of infection it need protease enzyme to invade tissue due to role of alkaline protease enzyme to resist phagocytosis by cleaving the lgG and inhibited neutrophil function [41]. Sharma *et al*, (2015) reported the maximum protease activity by the bacterial isolate at 37ºC after incubation time of 72 h.[42]. The highest extracellular protease production by *Bacillus sp* without optimization of culture conditions was reported by[43]. In another study, in (2013) reported maximum protease activity exhibited by *Bacillus sp* after 24 h of incubation at 25 ºC using different nitrogen and Carbon sources like yeast extract and sucrose respectively[44]. Smita *et al*., (2012) studied the enzyme activity of the bacterial isolate in a medium PH 7.0 without optimization at 37 ºC for 48h in a shaker[45].

Table (4): **Numbers and percentages of bacterial species isolated from diabetic foot infection patients.**

Species of bacteria	No.	Percentage %
Proteus mirabilis	20	21.73
Escherichia coli	18	19.56
Pseudomonas aeruginosa	16	17.39
Klebsiella pneumoniae	12	13.04
Staphylococcus aureus	6	6.52
Staphylococcus haemolyticus	2	2.17
Staphylococcus lugdunensis	1	1.08
Staphylococcus haemolyticus	1	1.08
Morganella morganii	5	5.43
Acinetobacter	4	4.34
Stenotrophomonas maltophilia	4	4.34
Kocuria kristnae	2	2.17
Enterococcus faecalis	1	1.08
Total	**92**	**99.93**

Figure (3): Proteolytic activity of a *proteus mirabilis* isolate after incubation on skim milk agar at 37°C for 24hrs.

Table (5): Diameters of halos formed around colonies of bacterial isolates grown on skim milk agar after incubation for 24 hours at 37°C.

NO	Isolate	Diameter of clear zone (mm)
1	*Proteus mirabilias*	10
2	*Staphylococcus haemolyticus*	8
3	*Pseudomonas aeruginosa*	7
4	*Escherichia coli*	5
5	*Kocuria kristnae*	4

Table (6): Specific activity of proteases produced by the clinical bacterial isolates after growing in the production broth medium and shaking incubation for 24hrs at 37°C.

NO	Isolate	Specific activity (U/mg protein)
1	*Proteus mirabilis*	160
2	*Staphylococcus haemolyticus*	100
3	*Pseudomonas aeruginosa*	90
4	*Escherichia coli*	87
5	*Kocuria kristnae*	60

CONCLUSIONS

1. Polymicrobial pattern was the most common occurrence in the diabetic foot infection (DFI) of patients included in the study.
2. Majority of pathogenic bacterial isolates obtained from patients with DFI were found to possess the ability of producing proteases.
3. *Proteus mirabilis* which the highest protease enzyme specific activity while *Kocuria kristnae*.

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