Annals of R.S.C.B., ISSN: 1583-6258, Vol. 25, Issue 1, 2021, Pages. 6615 – 6623
Received 15 December 2020; Accepted 05 January 2021.

Molecular Detection of *Proteus mirabilis* isolated from Diabetic foot

Hind Ibrahim Khodair and Zainab H.Abood Al-Asady*
Institute of Genetic Engineering and Biotechnology for Post Graduate Studies, Baghdad University, Iraq.
*Corresponding author email: zainabhekmatt@gmail.com

Abstract
Diabetic foot ulcer (DFU) is one of the diabetic complications associated with major morbidity, mortality, and reduced quality of life and is the most serious complication of diabetes mellitus. A total of 118 wound swab samples were collected from diabetic foot ulcer patients attending five hospitals in Baghdad governorate (Al-Yarmouk Hospital, Al-sewaira Hospital, Medicine City/Baghdad Teaching Hospital, Endocrine gland center, AL-kadhimya Hospital) at a period of study from beginning October 2019 to January 2020. Regarding to the age group factor, the age group (50-59) years were more susceptible to the infection is constituting 49(49%) ,followed by the age group(40-49) years with 34(34%) then , the age group (60-69) years with percentage at 15(15)%.and the age 70 years and above with percentage (2%) Also, the study indicated that the Diabetic foot ulcer was disrupted in male (58%) more than female (42 %).

All specimens were cultured on culture media including blood agar and MaCconkey agar. After the growth of bacteria, the isolates were identified by microscopic examination as well as the biochemical tests including the manual biochemical tests that include(oxidase , catalase, Simmon Citrate, Motility , Indole , Urease , Methyl Red, Kligler iron agar (KIA) ,Lactose fermentation ,Voges-Proskauer) .The identification of *P. mirabilis* confirmed by using the systems API 20E and VITEK-2 system . A total of 18 isolates of *P. mirabilis* were identified. Other bacteria obtained were identified as *Escherichia coli*, *Pseudomonas. aeruginosa*, Klebsiella .*pneumoniae* and *streptococcus spp.* in percentage recorded (37%) , (22%) (20.2%) and (11%) respectively. The genomic DNA of *P. mirabilis* isolates were extracted using wizard genomic DNA purification kit, the extracted genomic DNA was analyzed using 1% agarose gel electrophoresis, and then the concentration and purity of the extracted genomic DNA were determined using Nanodrop spectrophotometer device, to detect *P. mirabilis* isolates by molecular methods, the extracted genomic DNA of these isolates was submitted for amplification to detect the specific gene *16S rRNA* and *aadA1* by the singleplex PCR assay.
Keywords: Molecular Detection, *Proteus mirabilis*, Diabetic foot

Introduction

Diabetes mellitus (DM) is a global epidemic, chronic progressive disease that characterized by several complications affect several body systems. Diabetic foot ulcer(DFU) are defined as non-healing or poorly healing full-thickness wound through the dermis below the ankle in individuals with diabetes for more than three months. DFUs are three types: neuropathic, ischaemic and neuroischaemic ulcers (Mendes *et al*., 2012). Peripheral neuropathy, ulceration, infection and peripheral vascular disease are the most important causes for ulcer complication and lower limb amputation in diabetic patients (Pscherer *et al*., 2012). Diabetic foot must be managed by multidisciplinary team. Proper management of diabetic foot infection should be preceded by

Annals of R.S.C.B., ISSN: 1583-6258, Vol. 25, Issue 1, 2021, Pages. 6615 – 6623
Received 15 December 2020; Accepted 05 January 2021.

proper clinical diagnosis, assessment of severity of infection and wound classification, control of blood sugar, debridement, obtaining adequate specimen for culture, adequate selection of antibiotics, proper timing and selection of surgical intervention with adequate wound care (Armstrong *et al*., 2018). Surgical debridement is cutting away of all necrotic tissue, infected material from the wound and a surrounding callus until a healthy bleeding edge is reached. The benefit of surgical debridement is the change of infected ulcer into an acute ulcer (Joseph and Armstrong, 2012). Minor amputation, is amputation at the level of the foot without the need for prosthesis for walking while major amputation is amputation above the level of the ankle (Yu *et al*., 2019). The number of DFU patient in the world was around 55 million people in 2011, and this number is expected to increase to 83 million by 2030.4. The prevalence of diabetes in Iraq is increasing (Mansour and Al Douri, 2015). Diabetic foot infections are often polymicrobial , may involve exposed deeper structures and also important in that they often the primary indication for amputation (Livesly *et al.*, 2002). The diabetic foot wound is often infected with many bacteria, fungi which may be both aerobic and anaerobic organisms (Rehm,2006).Predominant aerobic microorganisms including *S. aureus*, *S. epidermidis*, *E.coli*, *Klebsiella* spp., *Proteus* spp. *Enterococcus spp*. *P. aeruginosa*, *Enterococcus spp* .coliform bacteria and other species of *Enterobacteriaceae. Peptostreptococcus* species, *Bacteroides* species and others (Gadepalli *et al*., 2006; Jeber and Saeed , 2013). *Proteus* species are part of the gram-negative bacilli involved in wounds, particularly in diabetic wounds (Hegazy., 2016). They cause serious infections, that are hard to eradicate, particularly in highly infected wounds. *Proteus* species that colonize the intestinal tract differ from those colonizing wounds in terms of their ability to possess antibiotic resistance genes (Tom *et al*., 2018). *Proteus mirabilis* is a common etiologic agent of diabetic foot infections**.** *P. mirabilis* multidrugresist (MDR) prevalence was very high in diabetic foot patients .Most were resistant to three or more antibiotic classes, by producing beta-lactamases like extended-spectrum beta-lactamases (ESBLs) (Caubey and Suchitra .,2018), which may be a consequence of the indiscriminate use of antibiotics. The development of drug resistant pathogens in patients is increasing day by day. It is the matter of debate globally.

Materials and methods

Samples collection

During the period of study from beginning October 2019 to January 2020, A total of 118 wound swab samples were collected from diabetic foot ulcer patients attending five hospitals in Baghdad governorate (Al-Yarmouk Hospital, Al-sewaira Hospital, Medicine City/Baghdad Teaching Hospital , Endocrine gland center, AL-kadhimya Hospital). Samples were taken from the patients under sterile conditions and immediately transferred to the laboratory to inoculate into brain heart infusion broth for 4-6 hours, then inoculated on MaCconkey Agar and Blood agar (Hi media, India) at 37°C for 24 hrs then direct exam by Gram stain under light microscope (40x) followed by biochemical tests API20E ,VITEK 2 system and molecular methods.

DNA extraction

Genomic DNA was extracted from the *P. mirabilis* isolates using a commercial wizard genomic DNA purification kit according to manufacturer's instructions (Promega, USA) with some modification. Briefly, 1 ml of an overnight *P. mirabilis* culture grown at 28°C in nutrient broth

Annals of R.S.C.B., ISSN: 1583-6258, Vol. 25, Issue 1, 2021, Pages. 6615 – 6623
Received 15 December 2020; Accepted 05 January 2021.

(Sigma, USA) was transferred to a 1.5 ml micro centrifuge tube. The microcentrifuge tube was centrifuged at 14,000 rpm for 3 minutes to pellet the cells and the supernatant was removed. 600 µl of nuclei lysis solution (wizard genomic DNA purification kit) was added and gently pipet until the cells is re-suspended. The microcentrifuge tube was incubated in water bath at 80°C for 5 minutes to lyse the cells; then cool to room temperature. 3 µl of RNase solution (wizard genomic DNA purification kit) was added to the cell lysate and the microcentrifuge tube was inverted for 5 times to mix. The microcentrifuge tube was incubated at 37°C for 60 minutes and cool to room temperature. 200 µl of protein precipitation solution (wizard genomic DNA purification kit) was added to the RNase-treated cell lysate and vortex vigorously at high speed for 20 seconds to mix the protein precipitation solution with the cell lysate. The microcentrifuge tube was incubated on ice for 5 minutes and centrifuged at 14,000 rpm for 5 minutes. The supernatant containing the DNA was transferred to a clean 1.5ml microcentrifuge tube containing 600 µl of room temperature isopropanol. The microcentrifuge tube was gently mixed by inversion until the thread-like strands of DNA form a visible mass and centrifuged at 14,000 rpm for 5 minutes. The supernatant was carefully pour off and the microcentrifuge tube was drained on clean absorbent paper. 600 µl of room temperature 70% ethanol was added and then the microcentrifuge tube was gently inverted several times to wash the DNA pellet. The microcentrifuge tube was centrifuged at 14,000 rpm for 2 minutes and the ethanol was carefully aspirated. The microcentrifuge tube tube was drained on clean absorbent paper and the pellet was allowed to air-dry for 15 minutes. 100 µl of DNA rehydration solution (wizard genomic DNA purification kit) was added to the microcentrifuge tube and the DNA was rehydrated by incubating at 65°C for 1 hour. The solution was periodically mixed by gently tapping the microcentrifuge tube and the DNA sample was stored at - 20°C until use.

DNA quantification

The extracted DNA from the *P. mirabilis* isolates was quantified spectrophotometrically at O.D. 260/ 280 nm with ratios 1.4-1.5. The sensitivity of the *P. mirabilis* -F and *P. mirabilis* -R primers was evaluated by PCR amplification for serial diluted concentrations (10-100 ng) of purified genomic DNA isolated from *P. mirabilis* .

Primers selection

The primers for *aadA1* gene of *P. mirabilis* as the target gene for this study were selected according to ((Drum,2003). This set of primers was designed based on the conserved region in *P. mirabilis* , primers were synthesized by Alpha DNA, Kanda. The primers sequence of *aadA1* gene and their size of product are shown in (Table 1).

Table(1): The primers sequences of *aadA1* gene of *P. mirabilis* and their product size((Drum,2003).

Primer Name	Seq.	Tm	Size of product
aadA1 –F	5'- GCACGACGACATCATTCCG -3'	**58**	~300bp
aadA1–R	5'- ACCAAATGCGGGACAACG -3'		

Annals of R.S.C.B., ISSN: 1583-6258, Vol. 25, Issue 1, 2021, Pages. 6615 – 6623
Received 15 December 2020; Accepted 05 January 2021.

Singleplex PCR master mix

The singleplex PCR reaction of *aadA1*gene detection of *P. mirabilis* was performed in 25 µl volumes containing 5.5 µl of nuclease free water, 12.5 µl of GoTaq Green Master Mix 2X containing (GoTaq DNA polymerase supplied in 2X Green GoTaq reaction buffer (pH 8.5), 400 µM dATP, 400 µM dGTP, 400 µM dCTP, 400 µM dTTP, 3 mM MgCl2, yellow and blue dyes which function as loading dyes when reaction products are analyzed by agarose gel electrophoresis), 2.5 µl of 20 pmol *P. mirabilis* -F primer and 2.5 µl of 20 pmol *P. mirabilis* -R primer and 2 µl of the genomic DNA sample. The mixes were overlaid with 2 drops of mineral oil Table (2).

Singleplex PCR program

Singleplex PCR was carried out in a thermal cycler (Applied Biosystem, 9902, Singapore) according to the PCR program described by (14), with some modification. Briefly, the amplification of *aadA1* gene of *P. mirabilis* was carried out with initial denaturation at 95°C for 5 minutes, followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 58°C for *P. mirabilis* -F and *P. mirabilis* -R primers for 30 seconds, and extension at 72°C for 60 sec. The thermal cycles were terminated by a final extension for 3 minutes at 72°C Table (3).

Singleplex PCR products analysis

The analysis of singleplex PCR products of *aadA1* gene of *P. mirabilis* were performed on 1% agarose gel. The 100 bp DNA ladder (Promega, USA) was used and the gel was run at 100 volt for 45 minutes at room temperature. The singleplex PCR products were stained with ethidium bromide and visualized by an image analyzer (ChemiImager 5500, Alpha Innotech, USA).

Table(2): Singleplex PCR master mix to detect the *aadA1* gene of *P. mirabilis* isolates.

Component	Concentration	Amount (µl)
GoTaq Green Master Mix	2X	12.5
aadA1 -F primer	10 µM/ µl	2
aadA1-R primer	10 µM/ µl	2
Nuclease free water	-	4.5
DNA sample	-	4
Total volume	-	25

Table (3): Singleplex PCR program to detect the *aadA1* gene of *P. mirabilis* isolates.

No.	Step	Temperature	Time	No. of Cycles
1	Initial denaturation	95°C	5 min.	1
2	Denaturation	95°C	30 sec.	

3	Annealing	58°C	30 sec.	30
4	Extension	72°C	60 sec.	
5	Final extension	72°C	3 min.	1
6	Storage	4°C	∞	-

RESULTS

Conventional methods

The conventional methods include culture, Gram staining and biochemical tests showed positive results in 18(15.2%) out of 118clinically samples diagnosed with Diabetic foot ulcer infection,

On MacConkey agar *P.mirabilis* colonies appeared as a pink in color because of the lactose-fermentation, large size, round, regular edge, mucoid texture due to the presence of the heavy capsule in their outer membrane with large size (1.5-2.5 mm) in diameter (Figure 1), While,on blood agar the colonies appeared as grey-white, mucoid and non-hemolytic colonies(Figure 2). The Gram staining of *P. mirabilis* was showed a small straight rods and arranged singly but messily in pairs under the compound light microscope. (Figure 3). The results of biochemical tests were used for further identification of *P. mirabilis* isolates showed positive reactions for indole, catalase, citrate utilization ,urease, capsule stain, voges-proskauer (VP), and motility tests. but was negative for Kligler Iron Agar (KIA) test, motility, oxidase, H2S production.

Analysis of extracted DNA of *P. mirabilis* isolates

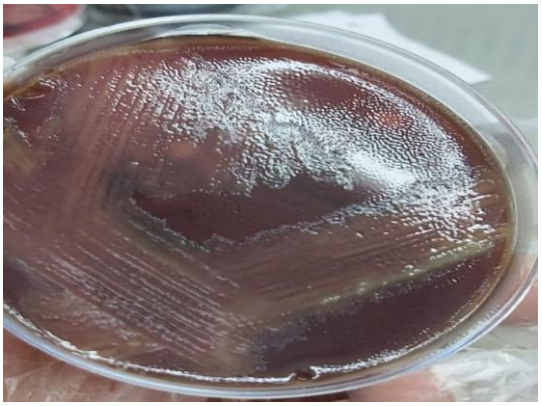
After performing of the DNA extraction from *P. mirabilis* isolates, agarose gel electrophoresis was adopted to confirm the presence and integrity of the extracted DNA using 1 % agaros gel at 7 volt/ cm for 45 minutes (Figure 4).

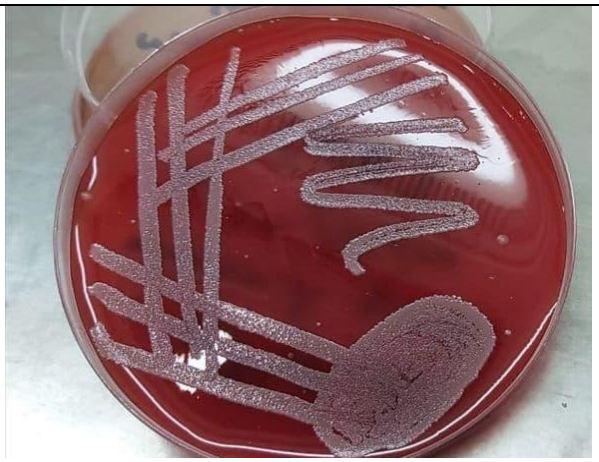
Analysis of singleplex PCR products of *aadA1* gene for *P. mirabilis*

On the basis of the *aadA1* gene sequence, a product of ~300 bp was amplified by singleplex PCR with *P. mirabilis* -F and *P. mirabilis* -R primers. In 50 clinically diagnosed with UTI infection , the singleplex PCR method detected positive results in 18(15.2%) out of 118samples that were positive by the conventional methods include culture Gram staining and biochemical tests. The singleplex PCR products and 100 bp DNA ladder were resolved by electrophoresis. 5µl of the singleplex PCR product were loaded on 1.5% agarose gel and run at 100 volt/ cm for 60 minutes. The gel was stained with ethidium bromide solution (0.5 µg/ ml) for 15-30 minutes; finally, bands were visualized on UV transiluminator at 740 wave length and then photographed by using photo documentation system. The singleplex PCR result was considered positive for *P. mirabilis* when there was presence of ~300 bp singleplex PCR product band of *aadA1* gene for the *P. mirabilis* on the agarose gel electrophoresis, no amplification was observed with negative control (Figure 5).

Annals of R.S.C.B., ISSN: 1583-6258, Vol. 25, Issue 1, 2021, Pages. 6615 – 6623
Received 15 December 2020; Accepted 05 January 2021.

Figure(1)Colonies morphology of *P.mirabilis* culture on MacConkey agar medium at 37ºC for 24hrs.





Figure(2)Colonies morphology of *P.mirabilis* culture on blood agar medium at 37ºC for 24hrs.

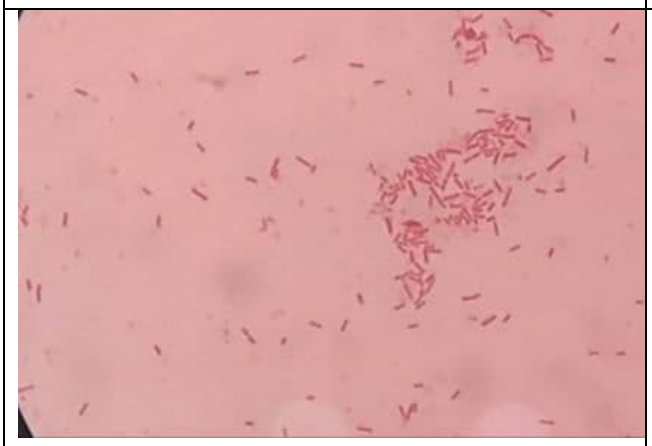


Figure (3): Microscopic examination of *P. mirabilis* (Gram stain).

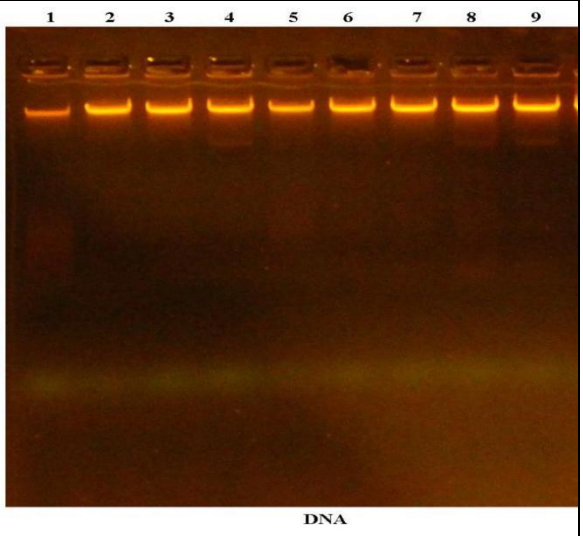


Figure (4): Gel electrophoresis of extracted genomic DNA of *P. mirabilis* isolates using 1% agarose gel at 7volt/cm for 1 hour. Lane 1-10: Extracted genomic DNA.

Annals of R.S.C.B., ISSN: 1583-6258, Vol. 25, Issue 1, 2021, Pages. 6615 – 6623
Received 15 December 2020; Accepted 05 January 2021.

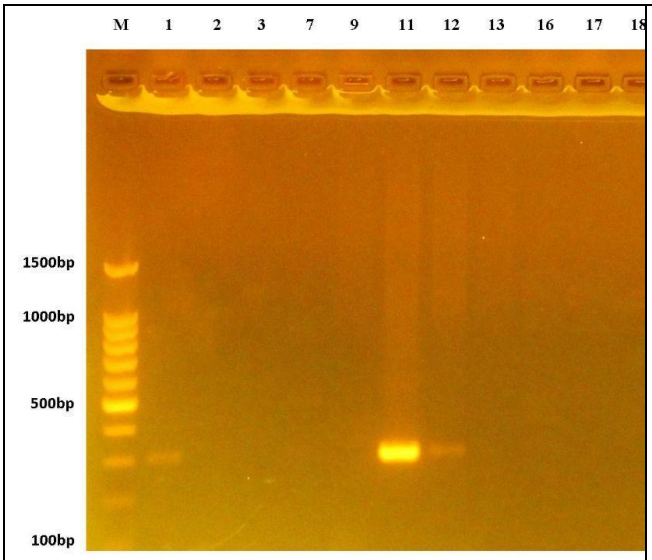


Figure (5): Agarose gel electrophoresis of PCR products for the *aadA1* gene . Lane M: 1500bp DNA ladder; lanes 1,11: positive result with positive bands of 300 bp *P. mirabilis* (70V for 2hr).

.

Discussion

Identification of this bacteria by using the conventional methods include culture, Gram staining and biochemical tests which were go together with study conducted by (Colee *et al*., 1996)**.** The other bacterial isolates bacterial isolated were *Escherichia coli* (37%), *Pseudomonas aeruginosa*(22%) ,*Klebsiella pneumoniae*(20.2%)and *streptococcus spp*.(11%).On MacConkey agar , *P.mirabilis* colonies appeared as a pink in color because of the lactose-fermentation, large size, round, regular edge, mucoid texture due to the presence of the heavy capsule in their outer membrane with large size (1.5-2.5 mm) , such characteristics came in accordance with guide of practical medical microbiology of (Collee *et al*., 1996). Also,The results of *P.mirabilis* culture on blood agar appeared as grey-white, mucoid and non-hemolytic colonies.These results came in accordance of practical medical microbiology of (Collee *et al*., 1996). In addition, the result of Gram staining of *P.mirabili* goes together with result of exhibited that in a typical Gram film, the organism appears as a Gram-negative. Non-motile, Small straight rod- shaped arranged single or in pairs under the compound light microscope results as agree with (Garrity, 2005). The results of biochemical tests were used for further identification of *P.mirabilis* isolates showed positive production Catalase test, Urease test, Citrate utilization test, Motility test, Methyl Red, Kligler iron agar (KIA), but was negative result for Oxidase test, Indole test, Lactose fermentation, Voges-Proskauer(VP), The results of biochemical tests of current study agree with study conducted by(Collee *et al*., 1996). In addition, the automated biochemical tests such as Api-20E and VITEK 2 system identification revealed that 13 of isolates were belonged to the *P.mirabilis*.The manual biochemical tests are largely used for bacterial identification in clinical laboratories, the advantages of conventional methods were non costly but the disadvantages of those methods were consuming time, contamination

present, false positive result and require a large amount of sample, while the automated biochemical tests such as Api-20E and VITEK 2 system. The API 20E system is faster still time-consuming to set up and read, requires up to 48 h of incubation, and gives results while VITEK 2 system used in many previous studies was detected bacteria faster, efficient and away from the contamination that may prevent detection of the pathogen. In addition confirmation the biochemical tests. In current study, the singleplex PCR method was used for detection of *P.mirabilis* by using pair primers targeted the *aadA1* gene (~300 bp) showed a positive result in 18(15.2%) out of 118 samples. The benefits of molecular methods are more sensitive, timesaving, specific, and cost-effective ways for the identification of *P. mirabilis*. These explanations made molecular methods relatively more accurate than conventional methods(Hamid *et al*., 2020). Conventional studies have highlighted the difficulties in identifying *P.mirabilis* strains based on commercial phenotypic identification systems. Identification of *P.mirabilis* has long been based on phenotypic and biochemical test, these physiological and biochemical tests were performed on selected isolates using API20E ,Vitek 2 system and PCR method(Hamid *et al*., 2020).This explains that the molecular diagnosis of *P.mirabilis* by the singleplex PCR method was more sensitive and efficiency than the diagnosis of these bacteria by conventional methods**.** This data agrees with the study by(Mukhtar *et al*., 2018)who confirms the efficacy of the PCR assay compared to conventional methods of diagnosis in the clinical setting.

CONCLUSION

Many evidence indicate that *P.mirabilis* are based upon inaccurate isolate identification, resulting from inadequate identification conventional methods include culture, Gram staining and biochemical tests that lack the resolution needed to discriminate *P.mirabilis* isolates, on the other hand, *aadA1* gene appeared to be useful genetic marker for determination of *P.mirabilis* and singleplex PCR using species-specific primers could be represented rapid, sensitive and specific molecular method for detection of this bacteria in different human infections.

Refferences

1. **Armstrong,D.G. and Lipsky,B.A.;Lvery,L.A. and Mills,J.L.(2018).**Diagnosis and Management of Diabetic foot complications .
2. **Caubey ,M. and Suchitra,M.S.(2018).**Occurrence of TEM,SHV and CTX-M b lactamasese in clinical isolates of proteus species in a Tertairy Care Center Infect. Disord.drug targets. 18(1):68-71.
3. **Collee, J. G.; Fracero, A. G.; Marmiotro, B. P. and Simmon, A. (1996).** Practical Medical Microbiology. (14th ed.).
4. **Drum ,D.J.V.(2003).**Ecology of antimicrobial resistance among domestic and free range fowel populations.PG.Thesis. The university of Georgia ,Athens,USA.
5. **Gadepalli, R.; Dhawan, B.; Sreenivas, V.; Kapil, A.; A mmini, A .C. and Chaudh, R. R . (2006).** Aclinico - microbiological study of diabetic foot ulcers in an Indian tertiary care hospital. *Diabetes Care .J*. 29(8): 1727-32.
6. **Garrity, G. M. (2005).** Bergey's Manual of Systematic Bacteriology. (2nd ed.). Vol. 2. Williams and Wilkins, Baltimol. London.
7. **Hamid,S.; Taha,A. and Abdulwahid,M.(2020).**Distribution of blaTEM, blaSHV, blaCTX-

M, blaOXA, and blaDHA in Proteus mirabilis Isolated from Diabetic Foot Infections in Erbil, Iraq. Cell Mol Biol (66(1): 88-94.

8. **Hegazy,W.A.H.(2016).**Diclofenac inhibits virulence of Proteusmirabilis isolated from diabetic foot ulcer. African Journal of Microbiology Research.10(21):733-743.
9. **Jeber ,M.A.and Saeed, E.A.(2013).** Isolation and Identification of bacterial causesfrom diabetic foot ulcers.Tikrit Journal of pure Science .18(3):6-9.
10. **Joseph,L.;Leykum,B. and Armstrong, D.G.(2012).**Surgical management of diabetic foot ulcers .1-16.
11. **Livesly, N.J. and Anthy, W.C. (2002).** Infected pressure ulcer in elderly individual. Clinical infectious disease.1390-1396.
12. **Mansour ,A.A. and Al-Douri,F.(2015).**Diabetes in Iraq:Facing in epidemic.A systematic Review .Wulfenia Journal.22(3):258-273.
13. **Mendes,J.J. and Neves,J.(2018).**Diabetic foot infections: current diagnosis and treatment.The Journal of Diabetic Foot Complications .4(2):26-45.
14. **Mukhtar, A.Noha Ahmed Abd Alfadil,A Mohamed,M.; Altayb,H.; Elzaki,S. and Hassan ,M.(2018).** "Identification of *Proteus mirabilis* on Banknotes Using 16s rRNA Gene in Khartoum State," *Sudan Journal of Medical Sciences*. 13(3): 175–186.
15. **Pscherer,S.; Dippel,F.W.; Lauterbach ,S. and Kostey.(2012).** Amputation rate and risk factors in type 2 patients with diabetic foot syndrome under real –life conditions in Germany .Pim care Diabetes. 6(3):241-246.
16. **Rehm, P. (2006).** Licensure.Clinical psychology:science and practice.13(3).
17. **Tom, I.M.; Agbo ,E.;Faruk, U.A.;Umoru ,A.M.; Ibrahim, M.M.; Umar, J.B.; Haruna, A.B.and Aliyu, A.(2018).** Implication of Proteus spp in the pathology of nosocomial wound infection in northeastern Nigeria. I.J.P.R . 9: 1-8.
18. **Yi,R.K.;Leng,L.C,;Kumar,R.L.Zakaria,Z.R. and Khan,Ed Simor.(2019).**Microbiology of diabetic foot infection in three district hospital in Malaysia and comparison with south east Asian countries .24(5):394-398.