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MOLECULAR DETECTION OF UREASE GENE IN *PROTEUS MIRABILIS* ISOLATED FROM URINARY TRACT INFECTION AND BLADDER CANCER

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Abstract

Samples Were collected Through the period from July to October 2019, (120) clinical samples of med-stream of urine, from patients suffering from urinary tract infections and stone formation including different ages and from both genders from different three hospitals in Baghdad (AL-Yarmouk hospital, Al-Karama hospital and Al Karkh hospital). Samples were transferred to the lab for isolation and identification of *Proteus mirabilis* by using sterile equipment and media. All samples were streaked on Blood agar, MacConkey agar. The plates were incubated aerobically at 37ºC for 24 hours. All isolates were identified and recognized depending on the microscopically feature by using (Gram stain). to detect their response to stain, shape, size and arrangement In addition, the morphological features on culture media such as Swarming on blood agar, Non lactose fermented growth on MacConkey agar also several and many of biochemical tests were used to identify the Proteus isolates, such as catalase, oxidase tests, indole, methyl red/ Voges Proskauer (MR-VP) test, citrate utilization tests, urea test, motility test, gelatin liquefaction test and triple sugar iron agar test. Then the isolates were diagnosed as those belong to *Proteus mirabilis* using biochemical test, further identification was done by using Vitek 2 system. The results showed that only (80) samples give positive bacteriological culture which represented (66.6%). Among these, (40) urine samples were identified as belong to genus *Proteus* which represented (50. %) from those *P. mirabilis* only 75 % (30) isolates while *Proteus vulgaris* were 10 isolates (25%) while the results indicated that isolation percent of *Proteus mirabilis* from females was (68%) which was higher than that of males (32%).

Ten isolates were selected that belong to *P. mirabilis* that show highest urease activity. A genetic investigations for selected isolates were done by the isolation of their using conventional PCR, amplification of specific genes that are virulence *Ure C gene* that responsible for urease production, and *16 Sr RNA* gene that responsible for phylogenic trait for these isolated were amplified specify urease determination accordingly, by using conventional PCR.

Key words : urease gene, *Proteus mirabilis*, urinary tract infection, bladder cancer.

Introduction

Urinary tract infections (UTIs) are the inflammatory disorders of the urinary tract caused by the abnormal growth of pathogens (Prakash, 2013; Amali *et al.,* 2009). Urinary tract infection is known to cause short-term morbidity in terms of fever, dysuria, and lower abdominal pain (LAP) and may result in permanent scarring of the kidney (Hoberman *et al.,* 2003). Urinary tract infections can be community acquired or nosocomial.

Urinary tract infections may be asymptomatic, acute, chronic, and complicated or uncomplicated, and the clinical manifestations of UTIs depend on the portion of the urinary tract involved, the etiologic organisms, the severity of the infection, and the patient's ability to mount an immune response to it. Both asymptomatic and symptomatic UTIs pose a serious threat to public health care, hence reducing the quality of life and resulting into work absenteeism (Olowe *et al*., 2015). The symptoms of UTIs such as fever, burning sensations while urinating, LAP, itching, formation of blisters and ulcers in the genital area, genital and suprapubic pain, and pyuria generally depend on the age of the person infected and the location of the urinary tract infected (Amali *et al*., 2009).

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Several factors such as gender, age, race, circumcision (Conway *et al*., 2007; Dias *et al.,* 2010), HIV (Banu , 2013; Ibadin *et al*., 2006). diabetes, urinary catheter, genitourinary tract abnormalities (Mladenovic *et al*., 2015; Yuyun *et al*.,), pregnancy, infants, elderly (Nicolle, 2008; Nelson, 2015)., and hospitalization status (Adukauskiene *et al*., 2006). bear significant risk for recurrent UTIs. The commonest pathogenic organism isolated in UTI is *E. coli* followed by *K. pneumoniae, Staphylococcus, Proteus, Pseudomonas, Enterococcus* and *Enterobacter* (Manges *et al*., 2006; Mirsoleymani *et al*., 2014). About 150 million people suffer from UTIs each year globally which results in greater than 6 billion dollars in direct health care (Stamm, 2001).

Proteus mirabilis, a gram-negative enteric bacterium, occurs as vegetative swimmer cells and hyper flagellated swarmer cells (Belas,1996)**.** Individuals suffering from urinary tract infections (UTI) caused by *P. mirabilis* often develop bacteriuria, kidney and bladder stones, catheter obstruction due to stone encrustation, acute pyelonephritis, and fever **(**Johnson *et al.,* 1993; Mobley, 1996 and Mobley *et al.,* 1987). *P. mirabilis* is one of the most common causes of UTI in individuals with long-term indwelling catheters, complicated UTI, and bacteremia among the elderly (Mobley *et al.,* 1991; Mobley *et al.,* 1987). As the aging population expands, more individuals will be at risk for *P. mirabilis* UTI (Warren *et al*., 1982).

These infections of the urinary tract occur in an ascending manner (Bacheller and Bernstein, 1997). Uropathogenic microorganisms contaminate the periurethral area, enter the bladder through the urethra, and establish an initial colony. These bacteria have specific adhesion and motility phenotypes that allow them to ascend to the bladder against the flow of urine, which normally prevents bacterial invasion at low infectious doses. After initial colonization, *P. mirabilis* ascends the ureters and initiates an interaction with epithelial cells of the renal pelvis, which allows colonization of the kidney (Jansen *et al*., 2003). In some cases, bacteria breach the one-cell-thick renal tubular epithelial barrier and enter the bloodstream **(**Esposito *et al.,* 1980).

The persistence of a *P. mirabilis* infection is compounded by the ability of this organism to cause the formation of urinary stones and encrust indwelling catheters (Mobley, 1987), which provide a niche protected from host immune cells and antimicrobial agents. Indeed, the formation of stones around the organisms can make antibiotic treatment ineffective, since antibiotics must penetrate the stones to act. Stone formation requires urease, which catalyzes the hydrolysis of urea into carbon dioxide and ammonia **(**Johnson *et al.,* 1993; Jones *et al.,* 1996; Li *et al.,* 2002). An increased ammonium concentration raises the environmental pH, which mediates precipitation of normally soluble polyvalent ions from the urine (Griffith, 1979). Specifically, precipitation of magnesium, ammonium, phosphate, and calcium ions results in formation of the struvite and carbonate hydroxyapatite crystals that comprise urinary stones (Griffith,1978).

Materials and Methods

Samples collection

The samples were collected during the period from July to October 2019. (120) samples of urine were collected from in patient and out patients who attended to AL-Yarmouk, AL-Karkh and AL- Kindi hospitals in Baghdad. Midstream urine samples were collected in sterile containers from patients that had symptoms of urinary tract infection. The samples were cultured on blood agar media and MacConkey media, which is a selective and differentiation medium used with the other mentioned media to seeking the identification of *Enterobacteriaceae* and incubated at 37! for 24 h then direct exam by Gram stain under light microscope (40x) followed by biochemical tests API20E and VITEC 3 system.

Molecular identification of *P. mirabilis*

DNA Extraction

Genomic DNA was extract from bacterial isolate (*P. mirabilis*) by using commercial purification kit (Genomic DNA Mini Kit) (Geneaid, Taiwan). This kit was designed for the isolation of DNA from Gram positive and Gram negative bacteria. DNA was extracted by this kit using bacterial protocol (for gram negative bacteria) with some modification. Briefly, 1 ml of an overnight *P. mirabilis* culture grown at 28°C in nutrient broth (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 resuspended. 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 (Genaid 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 ofDNA 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 was drained on clean absorbent paper and the pellet was allowed to air-dry for 15 minutes. 100 µl of DNA rehydration solution (Genaid 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.

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*.

Estimation of DNA concentration and purity

The DNA concentration of samples are measured using Nano drop by placing 1µl of the extracted DNA in the instrument to determine the concentration and purity is detected by noticing the ratio of O.D. 260/280 to detect the concentration of DNA samples with protein. The accepted 260/280 ratio for pure DNA, which is 1.8 - 2 DNA quality, could be assessed by 1% agarose gel electrophoresis (Sambrook and Russell, 2012).

DNA concentration (µg/ml) = O.D 260 nm x 50 x Dilution factor

The DNA purity ratio estimate according to this formula:

DNA purity ratio = O.D 260 nm / O.D 280 nm.

Agarose preparation

Agarose gel was prepared by dissolving 1 g of agarose powder in 100 ml of TBE buffer in Microwave, allowed to cool to 50°C and 2.5 µl of ethidium bromide was added (Sambrook *et al.,* 2001). The comb was fixed at one end of the tray for making wells used for loading DNA sample. Comb was removed after hardening of agarose leaving wells..

Agarose gel electrophoresis technique

The agarose was poured gently into the tray, and allowed to solidify at room temperature for 30 min. The comb was then removed gently from the tray. The gel was transferred into electrophoresis machine which contained the TBE buffer that used in preparation of agarose gel. The tray was fixed in an electrophoresis chamber filled with TBE buffer that covered the surface of the gel was done by using TBE (1X) buffer to the electrophoresis tank, tray with agarose was immersed in electrophoresis tank and buffer was about few milliliters above the surface of agarose.

Primers selection and preparation

Concerning the conventional PCR reaction, primers were used for detection of the gene. The primer of (*Ure C*) gene (533 bp) described in (Table 1). Primers used in this study was purchased from Macrogen company (South Korea) in lyophilized form, dissolved in free ddH_2O to give a final concentration of (100) picomols/µl as stock solution and kept a stock at -20. To prepare 10 picomols/ µl concentration as work primer suspended, 10 µl of the stock solution in 90 µl of the free ddH_2O water to reach a final volume 100 µl.

Amplification reaction

Using 25µL of PCR reaction The PCR amplification mixture which used for detection nucleated of each gene includes FIREPol® Master Mix 5X (12.5 µl), 3 µl of DNA template, 1 µl (1 mM) of each forward and reversed primers and 7.5 µl of nuclease free water to complete the amplification mixture to (25µl), (Table 2).

Table 1: Sequences of primers used for conventional PCR to detect *URE C* and *16srRNA* gene for *p mirabilis*.

Genesname	Sequences primer	Size (bp)	Ref.
Ure C	F: CCGGAACAGAAGTTG TCG CTGGA R: GGGCTCTCCTAC CGA CTT GATC	533	(D'Orazio *et al.,* 1996)
16S rRNA	F: GTGTAGCGGTGAAAT GCG R: ACG GGCGGTGTG TAC AA	1500	

Table 2: Components of the mixture and their sizes.

Components	Volume
Master Mix	10
Forward primer	1
Reverse primer	1
Nuclease Free Water	5
DNA	3
Total volume	20

Amplification of 16SrRNA gene

PCR was carried out in a thermal cycler (Applied Biosystem, 9902, Singapore) according to the PCR program described by (Stankowska *et al.,* 2008), with some modification. Briefly, the amplification of 16SrRNA gene of *P. mirabilis* was carried out with initial denaturation at 95°C for 6 minutes, followed by 35 cycles of denaturation at 94°C for 1 minutes, annealing at 50°C for 1 minutes, and extension at 72°C for 1 minutes. The thermal cycles were terminated by a final extension for 5 minutes at 72°C (Table 3).

Table 3: PCR program for amplification of 16SrRNA gene (Stankowska *et al.,* 2008).

Step	Temperature	Time	No. of cycles
Initial denaturation	94℃	2 min.	1
Denaturation	94 ℃	1min	30
Annealing	50.2 ℃	1 min	
Extension	72℃	1 min.	
Final extension	72℃	5min.	1

16S rRNA gene is highly conserved within species and among species of the same genus, and hence, can be used as an alternative technique for identification of bacteria to the species level. **(**Relman. 1999; Olsen *et al.,*1993).

Amplification of *ure C* genes

Table 4: PCR program for amplification of *Ure C* gene (Stankowska *et al.,* 2008).

Step	Temperature	Time	No. of cycles
Initial denaturation	94℃	2 min.	1
Denaturation	94 ℃	1min c	30
Annealing	63℃	30 sec.	
Extension	72℃	1 min.	
Final extension	72℃	4min.	1

PCR products analysis

PCR product (10 µl) was pipetted into each well, and 5 µl of (100 bp ladder) added in the first well to use as a molecular marker to estimate the size of the PCR products. Each well was loaded with 10 µl (7µl of sample and 3 µl loading dye) Electric current was set up at 100 Volt and 70 amp for 1h, PCR products invisible bands were visualized using a UV trans-illuminator and photographed using digital camera.

Results

Conventional methods

The conventional methods include culture, Gram staining and biochemical tests showed positive results in 12(24%) out of 50(100%) clinically diagnosed with UTI infection, the results of samples culture on blood base agar showed the bacterial isolates were large, mucoid, white to grey and Non-heamolytic colonies (Fig. 1) and pink colonies, mucoid texture with large size regular edge, round, mucoid texture with large size, were (3-4 mm) in diameter on MacConkey agar. (Fig. 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. (Fig. 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), motility tests, triple sugar iron test showed Alk/A with positive hydrogen sulfide and gas production.. but was negative for Kligler Iron Agar (KIA) test, motility, oxidase, H_2S production (Table 5).

Table 5: Biochemical tests for identification of *Proteus. mirabilis.*

Test	*Proteus mirabilis*
Catalase	+
Oxidase	_
Indole	_
Methyl red	+
Citrate utilization	_
Motility	swarming +
Urease	+
TSI	(+K/K+G)
H_2S production	+
Voges-poskauer	_

(+) positive result (-) negative result ;TSI, triple sugar iron ; G, gas; K, alkaline.

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 % agarose gel at 7 volt/ cm for 45 minutes (Fig. 4).

Analysis of PCR products of *Ure C* gene for *P. mirabilis*

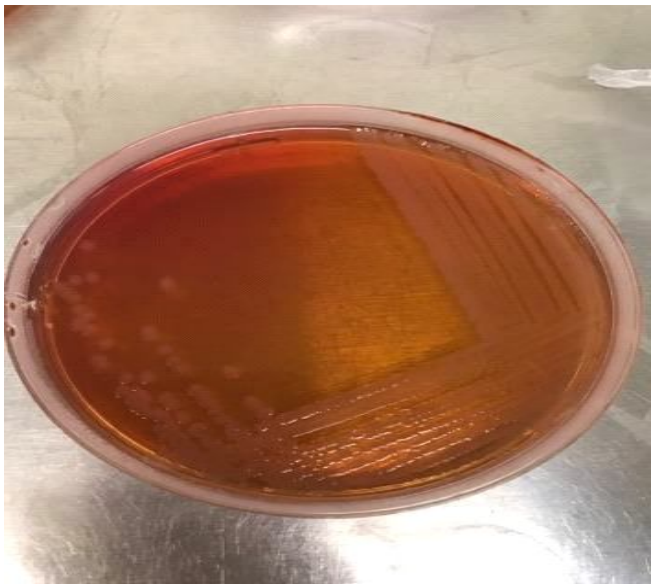

Fig. 1: A: Proteus mirabilis colonies are non-lactose fermentor on MaCconkey agar after incubation at 37° C for 24 h.

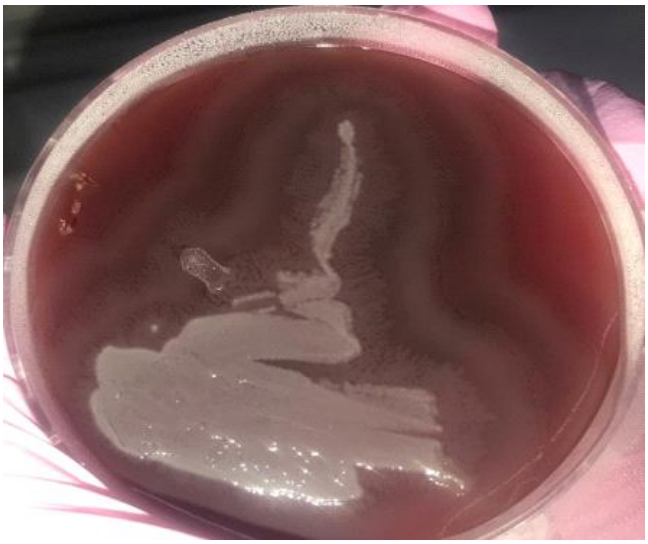

Fig. 2: Swarming phenomena on blood agar base.

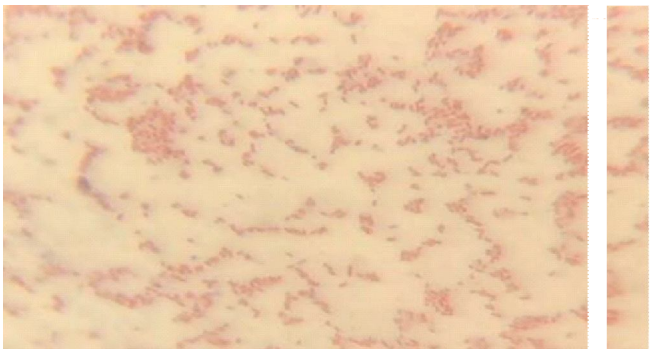

Fig. 3: *Proteus mirabilis*. Stained with gram stain and visualized under light microscope.

On the basis of the *Ure C* gene sequence, a product of ~533 bp was amplified by PCR with *P. mirabilis* -F and *P. mirabilis* -R primers. In 25clinically diagnosed

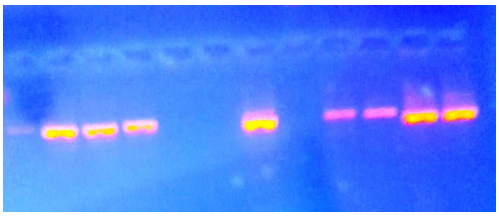

Fig. 4: Agarose gel electrophoresis of genomic DNA extracted from *P. mirabilis* isolates. Electrophoresis was done on 1% agarose gel at 100 volt for 60 min. visualized under UV light source after staining with ethidium bromide.

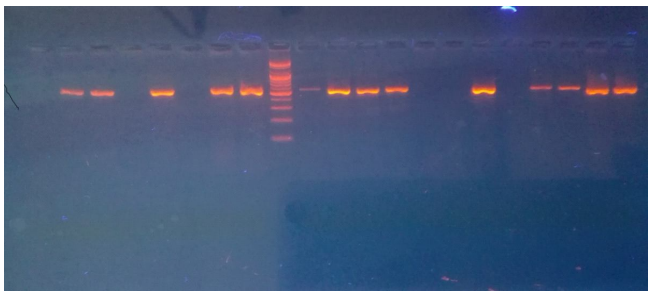

Fig. 5: Gel electrophoresis for amplified of *Ure C* gene of Proteus mirabilis Lane M (100 bp) ladder, Line: 1-10 represent isolates and respectively. The product was electrophoresis on 1% agarose at 100 volt for 60 minutes.

with UTI infection, the PCR method detected positive results in 25(62.5%)%) out of 25(100%) samples that were positive by the conventional methods include culture Gram staining and biochemical tests. The PCR products and 100 bp DNA ladder were resolved by electrophoresis. 5µl of the 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 PCR result was considered positive for *P. mirabilis* when there was presence of ~1500 bp PCR product band of *Ure C* gene for the *P. mirabilis* on the agarose gel electrophoresis, no amplification was observed with negative control (Fig. 5).

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 (Al-Aabideen, 2005) showed that the percentage of positive cultures of urine samples were 63.6%. while (Al-kabby, 2007) found that the percentage of positive culture of urine samples was (28.9%). The reason of this differences in percentage may be owed to

differences in either size of samples or hospital locations as well as to the season and medications before sampling. as previously described by (Liaw *et al.,* 2000) was detected by the conventional methods of culture on blood base agar and MacConkey agar plate, Gram staining, API 20 E and VITEC- 2 system. Also the result of *P. mirabilis* culture on blood agar agrees with results of the same study showed that the *P. mirabilis* colonies on blood agar appear. showed swarming phenomenon on blood agar that looks like concentric rings rising from one center. A distinguishable swarming which is a unique characteristic for genus *Proteus* was observed, which is considered as confirmatory phenomenon for genus *P. mirabilis,* while culture of *P. mirabilis* on MacConkey agar appear pale yellow color with no lactose fermentation colonies. In addition, the result of Gram staining of *P. mirabilis* goes together with result of exhibited that in a a Gram negative rods, non spore former, and motile under the compound light microscope. The results of biochemical tests were used for further identification of *P. mirabilis* isolates showed positive production of positive for catalase, urease, phenylalanine-deaminase, motility and triple sugar iron but was negative for oxidase and indole tests. These results were agreed with the results obtained by Manos and The results of biochemical tests of current study agree with study conducted by Manos and Belas (2006) and (Hawkey, 2016) and the automated biochemical tests such as Api-20E and VITEK 2 system identification revealed that 17 of *Klebsiella* isolates were belonged to the species *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. Ten isolates of *P. mirabilis* which showed highest urease activity were selected for amplification of *Ure C* gene. Accordingly, genomic DNA was extracted using commercial genomic DNA extraction kit (Geneaid, Taiwan), Concentrations and purity of DNA were measured by nanodrop spectrophotometer, all of the isolates had concentrations of DNA ranged to (62.1 - 12.5) ng/µl are, while purity is ranged to (1.8-1.98). That result showed in Table (4-3). DNA from ten isolates were subjected to 1% agarose gel electrophoresis to conform DNA integrity. Results shown in figure (5) Result was shown in figure (5) indicate successful amplification of the gene for all isolates as indicated by the presence of band with molecular size (533 bp), these results are in agreement with a previous study by Pathirana *et al.,* (2018) who indicating the presence of *Ure C* about 100 % isolates obtained from UTI patients. The results of molecular identification showed that 25 isolates (62.5%) were belonged to *P. mirabilis* as the amplicon appeared with molecular weight. This explains that the molecular diagnosis of *P. mirabilis* by the PCR method was more sensitive and efficiency than the diagnosis of these bacteria by conventional methods**.** This data agrees with the study by (Smabrook *et al.,*1982) 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, *Ure C* gene appeared to be useful genetic marker for determination of *P. mirabilis* and PCR using species-specific primers could be represented rapid, sensitive and specific molecular method for detection of this bacteria in different human infections.

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