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Research Article

Detection of *CTX-M* genes from β-lactam Resistance *Proteus mirabilis* associated with Urinary Tract Infection in Holy Karbala province, Iraq

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ABSTRACT

A total of 325 urine samples were collected from all ages and for both sexes from patients suffering from urinary tract infections in the period from (15 February to 15 May, 2017) from Al Hussein Hospital in Karbala , The number of samples that gave a positive result for laboratory culture was 227 samples (84.69%) .The isolates were then identified according to the physiological and biochemical tests , It was found that the number of samples that were Gram negative was (59.9%) and the *Proteus spp*. obtained were (31.61%) of the total negative bacteria isolates. *Proteus mirabilis* isolates obtained from all isolates of *Proteus spp*. were 38 isolates formed ratio (88.37%) ,These isolates were initially diagnosed by culture them into the MacConkey agar and blood agar followed by a number of morphological and biochemical tests. The isolates were finally diagnosed using the API 20E system. *Proteus mirabilis* isolates obtained from urine samples of female were 27 isolates (71.05%) and 11 isolates (28.9%) from urine samples of male. The isolates were tested for 12 different types of β-Lactam antibiotic to determine the most effective antibiotic toward these bacteria (Ampicillin, Piperacillin, Oxacillin, Cefazolin, Cephalothin , Cefoxitin , Ceftazidime , Ceftriaxone , Cefepime , Ertapenem , Imipenem and Aztreonam). The isolates of *P. mirabilis* showed a clear sensitivity to the Erytapenem , Imipenem , and Aztreonam , the sensitivity ratio was 97.3% , 100% and 100% respectively. The polymerase chain reaction technique was also used to detect *CTX-M* gene which represents one of the three most frequent gene (*TEM*, *SHV*, *CTX-M*) in this bacteria The results of polymerase chain reaction showed that (17) isolates formed (44.74%) carrying the *bla $_{CTX-M}$ gene.* This study came to achieve the following objectives:

Keyword: *Proteus mirabilis, bla $_{CTX-M}$.*

INTRODUCTION

The urinary system, which is composed of kidneys, ureters and urethra, is one of the most important devices in the human body. Its work is done by purifying the blood from harmful substances and substances that exceed the need of the body and disposing of them in the form of urine. The urine, its specifications and its contents are good indicators that reflect the natural physiological condition or pathological for people and its consider free of any bacterial, viral or fungal infection[1]. Inflammation occurs in the urinary tract when the bacteria reach the digestive system in the anus and very close to the opening of the urinary tract, which begins to grow and multiply[2]. The inflammation may result from some type of bacteria such as *E.coli* and *P.mirabilis*. Inflammation from the tube of the penis, then transferred to the bladder and if not treated, is transferred to the ureters and is transmitted to the kidneys and may be transmitted in other ways where the bacteria can pass from the blood to the kidneys or may pass from the intestine to the bladder through the lymphatic vessels[3].

Proteus mirabilis is a member of Enterobacteriaceae family and its Gram negative, nonspore forming bacilli and actively motile via peritrichous flagella, causing a swarming phenomenon on solid media specially can be detected on blood agar. It has characterized virulence factors which includes urease enzyme production[4]. This bacteria considered the second most common cause of urinary tract infections and also nosocomial infections. It produce urinary tract infections in human after leaving the intestinal tract and the rapid motility may contribute to its invasion of the urinary tract. *Proteus mirabilis* has also been shown to be resistant to many β-lactam antibiotic such as penicillins , cephalosporins , carbapenems and monobactam for various reasons such as the production of β-lactamase enzymes which are important defense enzymes produced by bacterial strains to overcome the effect of β-lactam antibiotic and protect their cells from lysis by penicillins , cephalosporins and other antimicrobial agents and considered *bla$_{TEM}$* , *bla$_{SHV}$* and *bla $_{CTX-M}$* are the most important genetic families encoded for β-lactamase enzymes[5].

The most common group of ESBLs not belonging to the

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Table 1: show the primers used in PCR.

Primer	Sequence		Produt size
CTX-M	F	5′-CGCTTTGCGATGTGCAG-3′	424 bp
	R	5′-ACCGCGATATCGTTGGT- 3′	

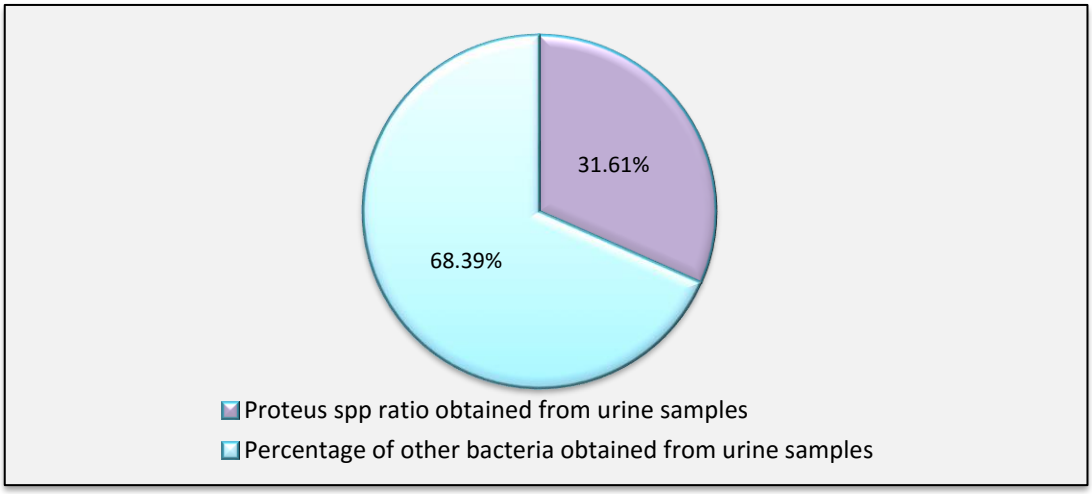

Figure 1: Percentage of isolates of bacteria *Proteus spp.* of urine samples

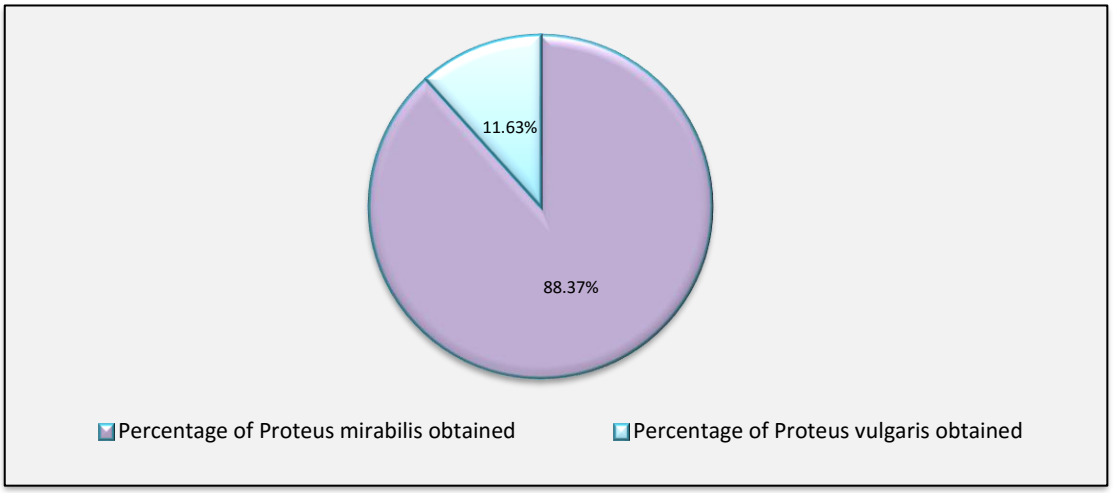

figure 2:Percentage of isolates of *P. mirabilis* compared to *P. vulgaris*

TEM or SHV families is the CTX-M type β-lactamases which constitute a rapidly growing cluster of enzymes that have disseminated geographically. The name CTX-M reflects the potent hydrolytic activity and substationally higher MIC of these β-lactamases against Cefotaxime and more efficiently than Ceftazidime[6].

In 2005 this enzyme was described in *P.. mirabilis* in Moscow, which is encoded with $bla_{CTX\text{-}M\text{-}116}$ and then tested to show that these bacteria carry other types of this enzyme such as *CTX-M-14*, *CTX-M-3* and are transmitted through the plasmid conjunctivitis and found in chromosome[7].

Aim of study

Study the resistance of bacterial isolates toward some (β-lactam antibiotic) commonly used to treat urinary tract infections. Investigate the presence of β-lactam antibiotic resistance $bla_{CTX\text{-}M}$ gene in these bacteria.

MATERIALS AND METHODS

Collection of Samples

Samples were collected from the patients who visited Al Hussein Hospital in Karbala for the period from (15 February to 15 May, 2017) and organized a questionnaire to collect information from patients including (patient name, age, sex, Type of treatment) where urine samples from the laboratory were collected and transferred to the microbiology laboratory

Bacterial isolation and identification

Urine samples were taken and planted on appropriate culture media, such as blood agar and MacConkey agar. All of the urine samples were cultured to isolate the bacteria causing urinary tract infection and were then diagnosed using a number of traditional tests[8].

Antibiotic sensitivity test

Table 2: shows the sensitivity of P. mirabilis isolates to β-Lactam antibiotic.

Type of antibiotic	Situation	Number	Ratio
Ampicillin	resistant	37	%97.37
	sensitive	1	%2.63
Piperacillin	resistant	37	%97.37
	sensitive	1	%2.63
Oxacillin	resistant	36	%94.74
	sensitive	2	%5.26
Cefazolin	resistant	38	%100.00
Cephalothin	resistant	38	%100.00
Cefoxitin	resistant	35	%92.11
	sensitive	3	%7.89
Ceftazidime	resistant	24	%63.16
	sensitive	14	%36.84
Ceftriaxone	resistant	18	%47.37
	sensitive	20	%52.63
Cefepime	resistant	2	%5.26
	sensitive	36	%94.74
Ertapenem	resistant	1	%2.63
	sensitive	37	%97.37
Imipenem	sensitive	38	%100.00
Aztreonam	sensitive	38	%100.00

Antibiotic sensitivity test was carried using Kirby Bauer method. The following antibiotics were used Ampicillin, Piperacillin, Oxacillin, Cefazolin, Cephalothin , Cefoxitin , Ceftazidime , Ceftriaxone , Cefepime , Ertapenem , Imipenem and Aztreonam . the plates are incubated overnight at 37ºC and the isolate was interpreted as susceptible, intermediate or resistant to particular antibiotics by comparison with standard inhibition zones according to Clinical Laboratories Standards Institute (CLSI)[9].

DNA Extraction and Genes Amplification

Isolates were grown on macConkey agar (37˚ C, over night). A single colony was inoculated to 5 ml of brain heart infusion broth and grown in a shaking incubator at 37˚C for 16–18 h. Genomic DNA was then extracted using the QIAGEN genomic DNA extraction kit (QIAGEN, USA) according to the manufacturer's recommendation.

PCR Amplification of bla_{TEM} gene and Qnr Genes

Amplification of *bla* $_{CTX-M}$ genes were performed in thermal cycler (MJ Reasearch USA) using primers illustrated shown in table (1) were provided by (Bioneer Company, Korea). Briefly each reaction was carried out in 25µl reaction volume using 12.5µl of Accustart ™ Taq PCR Super Mix (VWR-USA), 1µlof primers, 2µl of DNA template, and 8.5µl of Nuclease free water (ddH2o). Thermocycling parameters were as follows: an initial denaturation of 95 ˚C for 5 min, 30 cycles of denaturation at 95 ˚C for 45 s, primer annealing 62 ˚C for 30 s, and extension at 72˚C for 45s. Finally one extension step at 72˚C for 7 min. The amplicons were separated by 1.2% agarose gel electrophoresis at 70 V for 1 h. After electrophoresis, fragments were stained by ethidium bromide and visualized by using ultraviolet ligh[10].

RESULTS AND DISCUSSION

The number of *Proteus spp.* isolates obtained from all samples is 43 isolates, representing 31.61% (figure 1-1) and the number of *Proteus mirabilis* bacteria obtained from all isolates of *Proteus spp.* is 38 isolates forming 88.37% while the number of isolates of the other *Proteus spp*. is 5 isolates, They were all of a species *Proteus vulgaris*, accounting for 11.63% and this is close to what he reached[11] (Figure 1,2).

These isolates were initially identified by planting them on macConkey agar and the blood agar. This was followed by a number of morphological and biochemical tests. The isolates were then definitively identified using the diagnostic system (API 20E) and Vitek 2 identification system[13]. Microscopy Examination of the slides was performed using an optical microscope using the ocular lens. The interaction of the bacteria was observed with the Gram stain, its form, its regularity and its collection. *Proteus mirabilis* was shown as a negative cell of Gram stain in the form of a single cocco bacilli (Singly) or short chains, especially when swabs are taken from modern culture with a length of (1.0 - 3.0 µ) and a width of (0.4-0.8 µ), and this is close to what he reached[12]. A variety of differential cultures were used to observe the growth of *Proteus mirabilis*, such as the blood agar medium, where these bacteria appeared as large, slightly raised, opaque grayish colonies, Swarmming is the primary diagnostic characteristic of these bacteria. MacConkey agar colonies have pale brown to medium-sized bacteria with smooth edges that emit the smell of moldy fish unable to ferment Lactose, which is characterized by the presence of the neutral red directory.

Table (2) shows the expression of *P. mirabilis* isolate isolates under study resistance and sensitivity to some β-lactam antibiotic. The percentage of resistance to ampicillin was 97.37% and P. mirabilis isolates showed clear resistance Of Piperacillin is similar to that observed by Ampicillin 97.8% and is close to what he reached[14]. *P. mirabilis* showed significant resistance to oxacillin with a resistance of 94.8%. It was observed that isolates of *P. mirabilis* showed 100% total resistance to Cefazolin and the resistance of these bacteria to Cephalothin was 100% and *P. mirabilis* isolates showed a clear resistance to Cefoxitin 92.2% while the resistance of these bacteria to Ceftazidime was 63.2% and the ratio of resistance to Ceftriaxone was 47.3% and this is close to what he reached[15]. The susceptibility test for cefepime isolates of P. mirabilis isolates was found to be 94.7% and P. mirabilis isolates showed a clear sensitivity to Ertapenem that the sensitivity of this antibiotic was 97.3% In contrast, Imipenem was 100% sensitive, while aztreonam was 100%

The results showed that after the samples were removed from the PCR and placed in the electrical relay device described in Figures (3), (4) and (5), 17 isolates of *P .Mirabilis* was a carrier of *bla* $_{CTX-M}$ gene (44.74%) and product size (424 bp) and 21 isolates were non-carrying this gene. and thus agreed with the percentage obtained[16].

Table (3) showed that there were 17 isolates with 44.7% carrying this gene and resistance to Ampicillin and there

Table 3: Shows the presence of the *bla* $_{CTX-M}$ gene in *P.mirabilis* bacteria and its effect on the antibiotic resistance mechanism under study.

Ampicillin				
Gene found	Resist	Resistant precentage	Sensitive	Sensitive precentage
Not harbouring	20	52.6%	1	2.6%
	17	44.7%	0	0.0%
Piperacillin				
Not harbouring	20	52.6%	1	2.6%
	17	44.7%	0	0.0%
Oxacillin				
Not harbouring	19	50.0%	2	5.3%
	17	44.7%	0	0.0%
Cefazolin				
Not harbouring	21	55.3%	0	
	17	44.7%	0	0
Cephalothin				
Not harbouring	21	55.3%	0	0
	17	44.7%	0	0
Cefoxitin				
Not harbouring	20	52.6%	1	2.6%
	15	39.5%	2	5.3%
Ceftazidime				
Not harbouring	13	34.2%	8	21.1%
	11	28.9%	6	15.8%
Ceftriaxone				
Not harbouring	7	18.4%	14	36.8%
	11	28.9%	6	15.8%
Cefepime				
Not harbouring	0	0.0%	21	55.3%
	2	5.3%	15	39.5%
Ertapenem				
Not harbouring	0	0.0%	21	55.3%
	1	2.6%	16	42.1%
Imipenem				
Not harbouring	0	0	21	55.3%
	o	o	17	44.7%
Aztreonam				
Not harbouring	0	0	21	55.3%
	o	o	17	44.7%

were 20 isolates with 52.6% non carrying this gene and only one isolating of these bacteria was 2.6% sensitive for this antibiotic and not carrying the gene, without indicating the presence of any sensitive isolation of this antibody be carrier of this gene. The table also showed the same results for Piperacillin, which was obtained for Ampicillin , while Oxacillin showed that there were 17 isolates by 44.7% carrying the gene and resistance to this antibiotic while there were 19 isolation of 50% non carrying this gene and resistance to this antibiotic and there were only two isolates of these bacteria by 5.3% sensitive to this antibiotic is not carrying the gene and did not indicate any sensitive isolation of the antibiotic that carrying the gene This was explained by[17] in the role of this gene in the resistance of these antibiotics . The table also showed that 17 isolates 44.7% have the gene and are resistant to Cefazolin while there were 21 isolated by 55.3% non-carrier of this gene and resistant to this antibiotic, while no sensitive isolation of these bacteria towards this carrier or non-carrier of the gene and there were 15 isolates with 39.5% gene-carrying and cefoxitin resistance while 20 isolates with % 52.6 resistance to this antibiotic were not carrying the gene and only one isolation was recorded 2.6% was sensitive to this antibiotic and not carrying the gene, compared with two isolates 5.3% Gene- carrying and sensitive to this antibiotic . Unlike Ceftazidime, it was observed that there were 11 isolates with 28.9% carrier of this gene and resistant to this antibiotic while there were 13 isolates by 34.2% non-carrier the gene and resistant to this antibiotic while 6 isolates and 15.8% were gene-carrier and sensitive to the antibiotic As opposed to 8 isolates with 21.1% non-carrier the gene and sensitive to ceftazidim.

The table also showed that seven isolates were identified as 18.4% non-carrying the gene and resistant to Ceftriaxone and 11 isolates with 28.9% gene-carrying and resistance to this antibiotic while 14 isolates 36.8% were sensitive to this antibiotic and did not carry the gene compared with 6 isolates of 15.8% sensitive to Ceftriaxone and are carriers of the gene. In addition , no isolation of Cefepime resistance was observed do not carry the gene Compared to only two isolates at 5.3% be a carrier of the gene and resist to Cefepime while there were 21 isolates by 55.3% do not carry the gene and sensitive to Cefepime and It was also found that 15 isolates by 39.5% be sensitive to the antibiotic and carrier the gene this was explained by[18] in the role of this gene in the resistance of these antibiotics .The table also shows the relationship between resistance of these bacteria to Carbapenem and bla_{CTX-M} where no isolation was noted was resistance to Ertapenem do not carry the gene compared to only one isolation of 2.6%, which is the carrier of the gene and resistant to this antibiotic and there were also 21 isolates of 55.3% sensitive to the Ertapenem and not carrier of the gene compared with 16 isolates of 42.1% sensitive to this antibiotic and carrier of this gene while there was no sign of isolation Resistance to imipenem not carrier or a carrier of the gene contrary to what was noted , there were 21 isolations of 55.3% sensitive to Imipenem not carrier of the gene and 17 isolates 44.7% are sensitive to this antibiotic and gene-carrying. Table (3) shows the relationship between resistance of these bacteria to Monobactams and bla_{CTX-M} where 21 isolates were isolated by 55.3% sensitive to Aztreonam not carrier of the gene compared with 17 isolated isolates 44.7% were sensitive to this antibiotic are carrier of the gene While no isolating resistance to azithronam Carrier or non-carrier of the bla_{CTX-M} gene.

CONCLUSIONS

The results of the present study showed that *Proteus mirabil*is bacteria as one of the main causes of urinary tract infections. *Proteus mirabilis* isolates showed an enhanced sensitivity to fourth-generation Cephalosporins such as Cefepime, and Monobactam group such as Aztreonam, also Carbapenems group such as Ertapenem

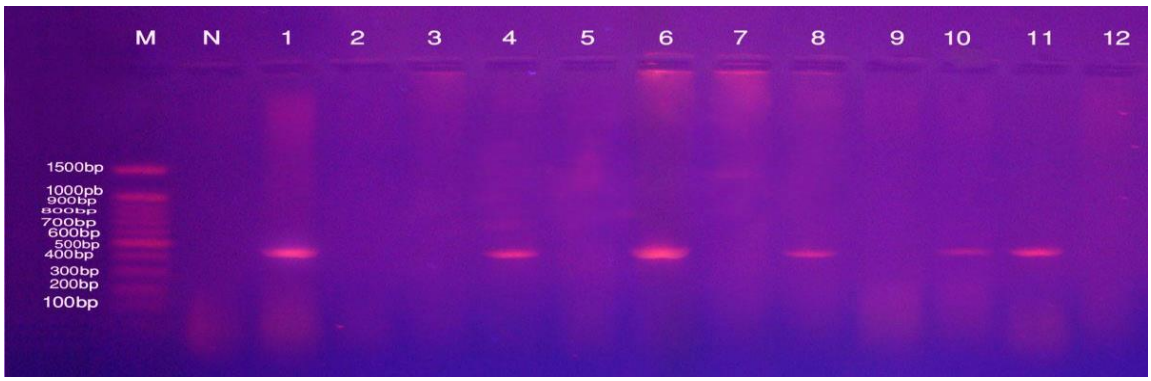

Figure 3: Ethidium bromide-stained agarose gel mPCR of genes examination of antibiotics Group Extended spectrum beta-lactamase *bla$_{CTX-M}$* gene in isolates of *P. mirabilis*. Where an DNA molecular size marker M: Marker ladder 15000-100bp, 6 isolates were positive for the gene beta-lactamase (*bla$_{CTX-M}$*) gene length of 424bp. The electrophoresis performed at 70 volt for 1 hr.

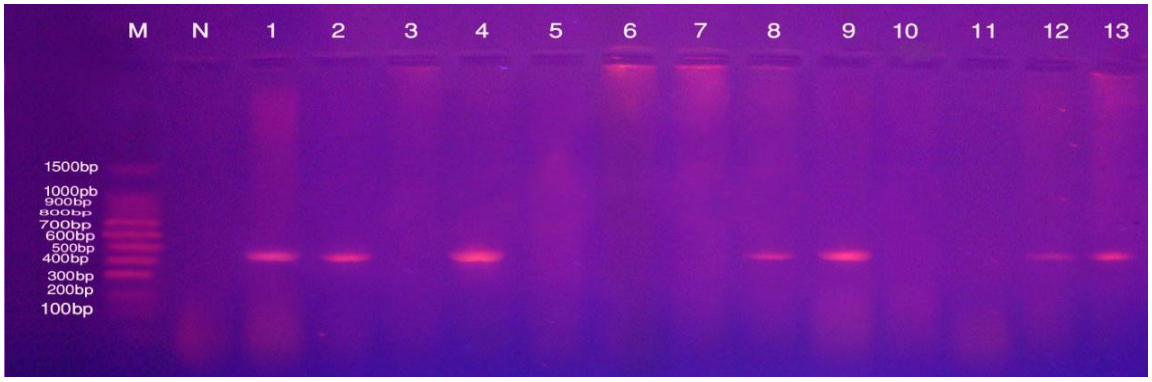

Figure 4: Ethidium bromide-stained agarose gel mPCR of genes examination of antibiotics Group Extended spectrum beta-lactamase *bla$_{CTX-M}$* gene in isolates of *P. mirabilis*. Where an DNA molecular size marker M: Marker ladder 15000-100bp, 7 isolates were positive for the gene beta-lactamase (*bla$_{CTX-M}$*) gene length of 424bp. The electrophoresis performed at 70 volt for 1 hr.

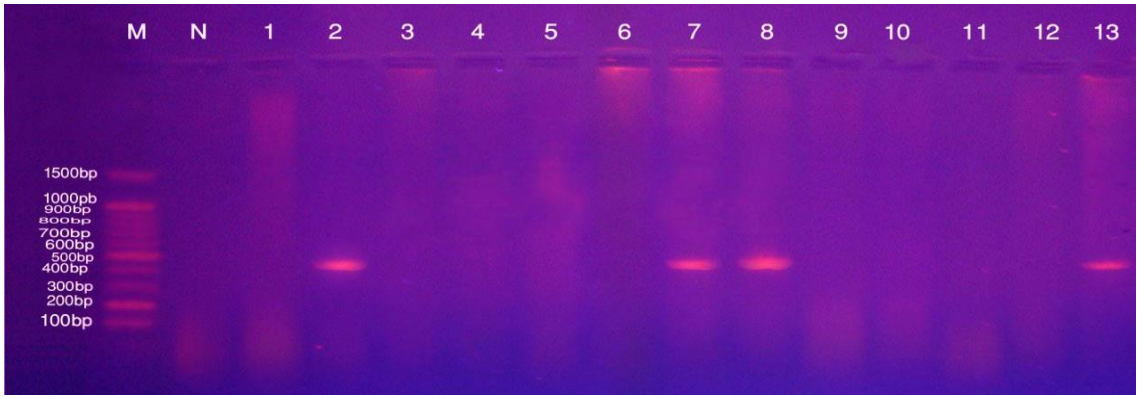

Figure 5: Ethidium bromide-stained agarose gel mPCR of genes examination of antibiotics Group Extended spectrum beta-lactamase *bla$_{CTX-M}$* gene in isolates of *P. mirabilis*. Where an DNA molecular size marker M: Marker ladder 15000-100bp, 5 isolates were positive for the gene beta-lactamase (*bla$_{CTX-M}$*) gene length of 424bp. The electrophoresis performed at 70 volt for 1 hr

and Imipenem The large spread of *bla$_{CTX-M}$* genes that resistance to beta lactam antibiotic

RECOMMENDATIONS

The use of antibiotics Cefepime, Aztreonam, Ertapenem and Imipenem in the treatment of urinary tract infection caused by bacteria *Proteus mirabilis*. Further studies on bacteria using a modern technique such as Real time PCR in the detection of the expression of genes that resistance betalactam antibiotic

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