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Research article

Molecular detection of $bla_{CTX\text{-}m\_2}$ in *Proteus mirabilis*

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ABSTRACT

From 152 different clinical samples collected from patients in Baghdad, 20 isolates of *Proteus mirabilis* were identified by bacteriological and biochemical assays and confirmed by vitek 2 identification system. It was found that 20 (66.6%) isolates were identified as *P. mirabilis* and 10 (33.3%) isolates were *P. vulgaris*. Susceptibility of *P. mirabilis* to cefotaxime was done, Notable, 13 (65%) *P. mirabilis* isolates were resistant to cefotaxime, whereas, 6 (30%) *P. mirabilis*isolates were extended spectrum beta lactamases producers. MIC value of cefotaxime was estimated to 7 isolates (512 µg/ml), and the MIC was 4096 µg/ml for other isolates, One isolate had MIC equal to 128 µg/ml while, another isolate showed MIC equal to 8192 µg/ml to cefotaxime. DNA was extracted from 8 *P. mirabilis* isolates that was resistant to cefotaxime and 2 isolates were sensitive to cefotaxime. The $bla_{CTX\text{-}M\text{-}2}$ *gene* was detected by polymerase chain reaction (PCR). The 8 cefotaxime resistant *P. mirabilis* isolates and one cefotaxime sensitive isolate carried $bla_{CTX\text{-}M\text{-}2}$ gene, while the cefotaxime sensitive isolate did not harbored the gene. Furthermore, this study confirmed that $bla_{CTX\text{-}M\text{-}2}$ gene was carried on chromosomal DNA, by extraction the DNA from 10 *P. mirabilis* isolates having $bla_{CTX\text{-}M\text{-}2}$ gene. DNA profile showed chromosomal DNA band only. Curing of $bla_{CTX\text{-}M\text{-}2}$ gene was done, 4 cefotaxime-resistant *P. mirabilis* isolates were treated with ethidium bromide as a chemical curing agent in different concentrations. The results confirmed that $bla_{CTX\text{-}M\text{-}2}$gene was carried on chromosomal DNA.

Keywords: **β-lactamases, *Proteus mirabilis*, $bla_{CTX\text{-}M\text{-}2}$, cefotaxime.**

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INTRODUCTION

Proteus mirabilis is often found as free-living organisms that is often found in soil, water, and the intestinal tract of many mammals, including humans [1, 2]. *P. mirabilis* is the third most common (after *Escherichia coli* and *Klebsiella pneumoniae*) cause of complicated UTI (causing 12% of infections) and the second most common (after *Providencia stuartii*) cause of catheter-associated bacteriuria in the group of long-term catheterized patients (causing 15% of infections) [2]. the group of long-term catheterized patients (causing 15% of infections) [2]. Besides UTI, *P. mirabilis* was described as opportunistic etiological agents in infections of the respiratory tract and wounds, burns, skin, eyes, ears, nose, and throat, as well as in gastroenteritis resulting

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from the consumption of contaminated meat or other food [3, 4]. β- lactamases are the commonest cause of bacterial pathogenesis. New enzymes and new modes of production of old enzymes now threaten the value of extended-spectrum cephalosporins against *Enterobacteria*. β-lactamases have been classified by their hydrolytic spectrum, susceptibility to inhibitors, and whether they are encoded by the chromosome or plasmids [5]. A major reorganization was proposed by Bush in 1989, with further updating in 1995. The revised Bush scheme classified β-lactamases by their substrate preference among penicillin, oxacillin, carbenicillin, cephaloridine, expanded-spectrumcephalosporins, and imipenem and by their susceptibility to inhibition by clavulanate [6]. A molecular classification was proposed by Ambler in 1980, which took into consideration the amino acid sequence of the β-lactamases, they are divided into four classes: A, B, C, and D, each includes types that are usually plasmid-mediated or chromosomal.Ambler's molecular classification appears to be widely accepted more than the Bush's phenotypic classification due to its simplicity and phylogenetic relationships among the enzymes [7, 8].

In 2010, Jung Hune Lee and others proposed a broadened definition of extended spectrum beta-lactamases (ESBLs). The Ambler Class A ESBLs were named as ESBLs, class C enzymes with extended spectrum as cESBLs, and class D extended-spectrum variant of OXA enzymes as dESBLs. As per this definition a ESBLs include TEM-ESBLs (Temoniera, the name of a patient whom bacteria resistant to this type isoaltes), SHV-ESBLs (sulphydryl variable adescriotion of biochemical properties of this type of β- lactamases), CTX-M-ESBLs, GES-ESBLs (Guiana extended spectrum), and VEB-ESBLs (4 month old vietnamase boy which the first time this type isolated from). cESBLs include AmpC-ESBLs (first type of penecillinase discoveredin *E. coli* and classified in A,B and C according to mutations in these genes) and dESBLs include OXA-ESBLs hydrolyzed to oxacillin and cloxacillin) [8].

Widespread use of antimicrobial agents leads to selection of bacteria producing extended spectrum β-lactamases (ESBL), the genes coding for ESBLs are often located on self-transmissible or mobilizable broad-host range plasmids [9]. Most ESBLs are evolved by genetic mutation from native β-lactamases, particularly TEM-1, TEM-2, and SHV-1 [10]. ESBLs are characterized by their ability to hydrolyze peniclillins, early cephalosporins, oxyimino-thiazolyl cephalosporins (including third and fourth generation cephalosporins) and monobactams but not cephamycins or carbapenems. They are inhibited by suicide-inhibitors such as clavulanic acid, tazobactam and sulbactam [11].

A high prevalence of ESBLs among *P. mirabilis* caused invasive infections. Antimicrobial agents resistant problem such as ESBLs can vary greatly depending on the patients' populations, time periods, geographic regions as well as methods of collection and testing strains [12].

Indeed, CTX-M is considered as a new family of plasmid-mediated ESBLs, called CTX-M. The first clinical isolate with a cefotaximase property was report-ed from *E. coli* grown from ear discharge of a 4-month child in Munich, Germany, in 1989. It was named CTX-M-1 (CTX for cefotaximase and M for Munich) [13].

bla_{CTX-M} is preferentially hydrolyze oxy-amino cephalosporins, especially third and fourth generation, and monobactam. It hydrolyzes cefotaxime more rapidly than ceftazidime but not cephamycins such as cefoxitin and carbapenems including imipenem, ertapenem, meropenem, or doripenemIn addition. They are gene-rally susceptible to β-lactamase inhibitors such as clavul-anic acid, sulbactam, and tazobactam [7]. Organisms that produce CTX-M enzymes have become the most prevalent type of ESBLs described during the past 5 years. At present, the *CTX-M* family comprises more 40 enzymes [14]. CTX-M β-lactamases are typical ESBLs that belong to Bush's group 2be and Ambler's class A [15]. Choromosomal β- lactamases genes from some *Kluyvera* species (widely distributed in the environment) have been suggested as the ancestors of each CTX-M cluster and mobilization of bla_{CTX-M}genes to another bacterial genera have occurred through recombinatorial events mediated by genes such as IS*CR1,* IS*Ecp 1* or phage related elements [16] and this is the cause of rapid and widespread of CTX-Menzymes worldwide is mainly due to the frequent association of bla_{CTX-M}genes with genetic mobile elements, such as epidemic plasmids, transposons, and/or insertion sequences located upstream bla_{CTX-M}also responsible for the expression of gene. Classically, genetic location of the corresponding β-lactamase genes (*bla*) in plasmids or chromosome was also used to differentiate these enzymes. Nevertheless, this genetic characteristic is no longer used, as chromosomal *bla* genes can be mobilized and integrated into plasmids or transposons, but a reverse situation of initially describes plasmid-mediated β-lactamases into the chromosome is increasingly found. In addition, protein regulation characteristics (i.e., constitutive or inducible expression) are also noted when referring to different β-lactamases groups but this trait depends on the surrounding genes. In Gram-negative organisms, inducible expression of β-lactamases is commonly found in chromosomal β-lactamases, whereas plasmid-mediated enzymes are generally constitutively expressed. Enhancement expression of their hydrolytic activity is often regulated by promoters present in upstream genes [17]. There are at least 128 of CTX-M types that have been described by using amino-acid sequence relatedness, and then phylogenic tree of CTX-M β-lactamases has been constructed. They are divided into five clusters (CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9 and CTX-M-25). Naming of the cluster was based on the first member of the group that described by Rossolini *et al*, (2008).

Members within a group have >94% amino acid relatedness and ≤90% relatedness across the groups.The bla_{CTX-M} gene encodes a 291 amino acid enzyme. A single amino acid change in bla_{CTX-M} constitutes a new CTX-M type [14]. In current study we try to detect $\boldsymbol{bla_{CTX-m\_2}}$ in *P. mirabilis* by PCR technique.

MATERIALS AND METHODS

Isolation and identification of *P. mirabilis*

Through the period from September 2013 to March 2014, 152 clinical samples of urine, wounds, and burns were collected from patients attending to several hospitals in Baghdad, Iraq. The study was conducted following approval from the scientific and ethics committee of College of Sciences, University of Baghdad. Samples were transferred to the laboratory by using transport media for isolation and identification of *Proteus* by using sterile equipment and media. The samples were streaked on blood, MacConkey and XLD agar plates. The plates were incubated aerobically at 37° C for 24 h. The isolates were identified bacteriologically, bioche-mically according to [18]. In addition, the morphological features on culture media such as Swarming on blood agar, Non lactose fermented growth on MacConkey agar and colourless growth on Xylose lysine deoxychoclate agar (XLD) agar were examined, then identification of bacteria was confirmed by using Vitek 2 identification system [19].

Antibiotic susceptibility test

Kirby-Bauer method was done according to [19] to carry out antibiotic susceptibility test for cefotaxime and Ceftazidime. 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). MIC value for *P. mirabilis* isolates to cefotaxime according to method described by Islam *et al* (2008) was detected by using various concentrations of cefotaxime ranged from 2 to 8192 µg/ml [20]. by preparation double folded serial dilutions from 2 to 8192 µg/ml of cefotaxime antimicrobial agents, each dilution of cefotaxime was added to empty sterile Petri-dishes, Mueller Hinton agar at 50°C was poured into Petri-dishes, mixed well and left to solidify at room temperature, then volume of 5 µl of the third dilution of 18 h. Brain Heart Infusion culture of *P. mirabilis* that matched the turbidity of the 0.5 McFarland turbidity standards (1.5 x 10^8 CFU/ml), was drawn by micropipette on the surface of Mueller Hinton agar of each cefotaxime concentration.The plates were left for 10 min., then incubated at 37°C for 24 h, finally the MIC of cefotaxime was recorded.

Detection of ESBLs

Double-disk synergy test method was used to detect ESBLs-producing isolates [10]. A sterile cotton swab was submerged into bacterial suspension standardized to match the turbidity of the 0.5 McFarland turbidity standard (1.5 x 10^8 CFU/ml) by preparing serial dilutions of 18 h. Brain heart infusion culture of tested bacteria, and the third dilution was used after comparing with 0.5 McFarland turbidity standard. The surface of Mueller Hinton agar plates were spread into four directions by the bacterial suspension, the plates were left for 10 min to dry. An amoxicillin/clavulanic acid (30 µg) disc was placed in the middle of Mueller Hinton agar plate, and then the discs of cefotaxime, ceftazidime and aztronam were arranged around the amoxicillin/clavulanic acid (30 µg) disc within 2-3 cm distance. The plates were inverted and incubated at 37°C for 18 -24 h. After incubation, synergism activity between the central disk and any one of surrounding antimicrobial discs was noted to detect the ESBLs producing isolates.

Extraction of DNA from *P. mirabilis*

DNA was extracted from 10 isolates of *P. mirabilis* by using a commercial purification kit (Presto Mini Genomic DNA Kit, (Geneaid, Thailand)). The Gram negative bacteria extraction protocol was used as manufacture protocol. The extracted genomic DNA was confirmed by using gel electrophoresis in 0.8% agarose gel after staining with ethidium bromide and by UV transillu-minator documentation system and photographed with digital camera for documentation of the results.

Estimation of concentration and purity of extracted DNA

From each DNA extracted sample, 2 µl was added to the specialized measuring lens of the Nano- drop system after swabbing the lens with distilled water wetted cotton swab to measure the concentration and purity of extracted DNA sample at 260 nm. The results were recorded computerization.

Amplification of blaCTX-M-2 by Mono Polymerase Chain Reaction

Monoplex PCR was performed for detection of304-1179 *P. mirabilis* $_{blaCTX-M-2}$ gene by using specific set of primers, forward primer (5- ACG CTA CCC CTG CTA TTT -3) and the reverse primer (5- CCT TTC CGC CTT CTG CTC -3) to amplify 780 bp region from the whole genome 875 bp. After extraction and detection of genomic DNA samples, PCR reactants and amplification program were prepared in final concentration of 50 µl, as follows: Template genomic DNA < 250 ng (5 µl), Forward and reverse primer 10 pmol/ µl (5 µl), Go Taq green Master mix 2X (25 µl), de-ionized distilled water (10 µl), while the PCR reaction was performed according to the following conditions: Initial denaturation at 94 °C for 2 min., denaturation at 94 °C for 1 min., annealing for 54 °C for 1 min., extension at 72 °C for 1.5 min. and final extension for 72 °C for 5 min. repeated for 30 cycles.The PCR product was confirmed by using gel electrophoresis in 1.5 % agarose gel after staining with ethidium bromide and by UV transilluminator documentation system and photographed with digital camera for documentation of the results.

Bacterial plasmid curing

Four *P. mirabilis* isolates were used for plasmid curing to investigating the presence of $bla_{CTX\text{-}M\text{-}2}$on the plasmid or chromosomal DNA using ethidium bromide as a curing agent. Two methods were used to extract bacterial plasmid DNA then performed by gel electrophoresis technique (0.8% agarose) in order to obtain the pattern and number of plasmid DNA bands: Salting out method and commercial kit (pureyeild ^{TM} plasmid Miniprep system, Promega, USA). Curing for bacterial plasmid DNA was examined for4 isolates of *P. mirabilis* resistant to cefotaxime, by using graduated concentration of ethidium bromide dye, using several concentrations (10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 125, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375 and 400 µg/ml).

RESULTS AND DISCUSSION

Based on common characters, out of 152 samples, 30 isolates of ***Proteus*** were identified by using three differential media (blood, MacConkey, XLD agar) in addition to microscopic examination by using Gram stain showed that all isolates were appeared polymorphic Gram negative rods [1,18]. Moreover, vitek 2 identification system (Biomerieux/ France) was used to confirm the identification of 30 ***Proteus*** isolates. The results represented that 20 specimens (66.6%) of different clinical specimens were identified as *P. mirabilis*, and 10 specimens (33.3%) were identified as *P. vulgaris* from different clinical samples.

The susceptibility of *P. mirabilis* isolates to cefotaxime, the third generation of cephalosporins was investigated. It was noticed that 13 of 20 (65%) isolates were resistant to above antibiotics (**fig 1**).

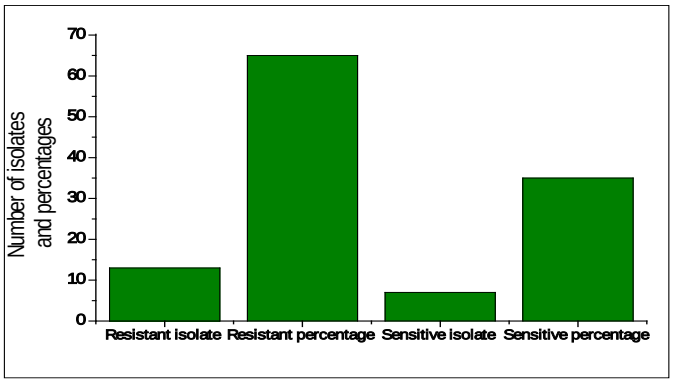

Fig 1. Susceptibility of *P. mirabilis* isolates to 10 µg cefotaxime.

This result was higher than that observed by Yasien (2008) and Fegla (2010), who reported that percentage of resistant was (16.2%) and (35%) respectively [21,22]. This confirmed the increasing of cefotaxime resistant producing bacteria epidemiology, including ***Proteus*** across wide regions was around the world with passing time, so it is worthily to note that the results of this study were higher than what recorded in the recent years.In addition, the present results indicated that 30% of isolates were resistant to ceftazidime, this agreed with Turner (2005) and Rossilini (2008) results, who refered that CTX-M producing bacteria showed resistance to cefotaxime, have greater activity against cefotaxime more than ceftazidime [11,15]. The importance of these two antimicrobial agents belong to the fact that they belonge to the third generation of cephalosporins, which theoretically have wide activity against Gram negative bacterial infection, especially ***Proteus*** [23] (**fig.2, fig 3**).

On the other hand, the results of MIC showed that the value 512 µg/ml was estimated in 7 isolates, the next was 4096 µg/ml was recorded in 4 isolates, one isolate had MIC equal to 128 µg/ml, and another isolate showed an MIC value equal to 8192 µg/ml.

In this study it revealed that 6 among 20 (30%) isolates of *P. mirabilis* were able for production ESBLs. These results were higher than other studies [13,24] but Harada *et al.* (2011) reported that from 28 *P. mirabilis* isolates producing ESBLs except one harbored $bls_{CTX\text{-}M\text{-}2}$ [25]. The presence of high percentage of isolates that expressed ESBLs may due to the high proportion of antimicrobial agents usage. This refers that the ESBLs producing *P.* may not belong to CTX-M ESBLs producing type, it may return to other types of ESBLs, such as SHV or TEM.

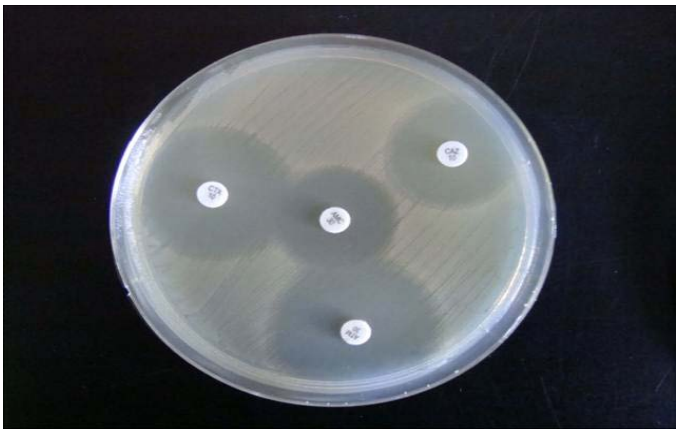

Fig 2. Production of ESBLs by *P. mirabilis* isolate R2 using double disk synergy method. 1, Cefotaxime; 2, Ceftazidime; 3, Aztronam; 4, Amoxicillin/Clavulanic acid.

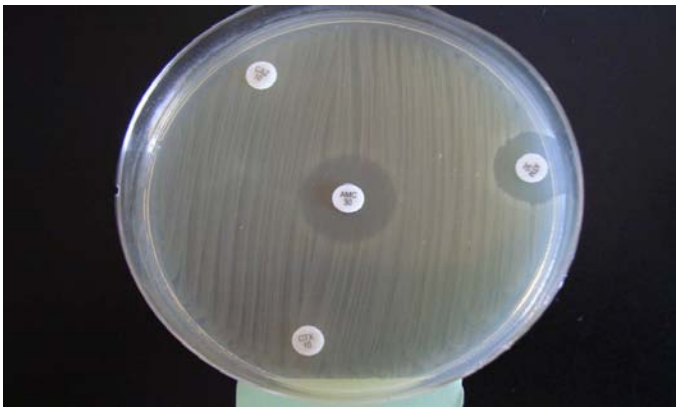

Fig 3. Cefotaxime resistant *P. mirabilis* isolate R5 using double disk synergy method. 1, Cefotaxime; 2, Cetazidime; 3, Aztronam; 4, Amoxicillin/ Clavulanic acid.

Thereafter, 10 isolates of *P. mirabilis* were selected for detection*bla*$_{CTX-M-2}$ gene; 8 isolates resist to cefotaxime and compared with 2 isolates that were sensitive to cefotaxime. DNA was extracted from these isolates by using commercial DNA purification kit (Geneaid, Thailand), and then DNA concentration and purity were determined by using Nano- drop system. The results revealed that the concentrations of DNA was ranged from 45.8 ng/µl to 112.3 ng/µl, while purity was ranged from 1.82to 2.05. The DNA purity was qualified by using gel electrophoresis technique.

Monoplex PCR technique was used for detection of*bla*$_{CTX-M-2}$ gene in 10 *P. mirabilis* isolates susceptible to cefotaxime (8 isolates were resistant, and 2 isolates were sensitive to Cefotaxime). The results showed that this gene with 780 bp was found in 8 isolates of *P. mirabilis*, after confirmed by gel electrophoresis technique with molecular weight of *bla*$_{CTX-M-2}$ according to Nagano *et al.* (2003) by using 100bp DNA ladder [13] (**fig 4**).

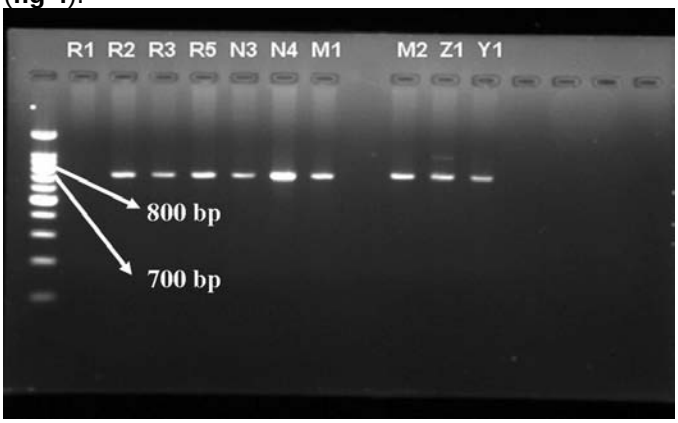

Fig 4. PCR reaction with monoplex set B of primers for *P. mirabilis* isolates (R1, R2 are sensitive to cefotaxime, and R3, R5, N3, N4, M1, M2, Z1, Y1 are resistant to cefotaxime) with DNA marker 100 bp ladder (M), in 1.5 % agarose gel at 5 volt/cm for 1-2 h, visualized by E-graph gel documentation equipped by UV light source

It is remarkable to notice that one of 2 isolates of *P. mirabilis* that phenotypically sensitive to cefotaxime was also had *bla*$_{CTX-M-2}$ gene in their whole genome, while the other isolate lack that gene, so it is compatible with phenotype sensitive to cefotaxime. Indeed, the presence of *bla*$_{CTX-M-2}$ gene in the *P. mirabilis* isolate that are sensitive to cefotaxime may due to the gene regulation that effect the expression of *bla*$_{CTX-M-2}$ gene. Cefotaxime as a substrate may induce or inhibit gene expression [26]. The process of switch on or switch of for the *bla*$_{CTX-M-2}$ gene expression may depends on the concentration off cefotaxime which act as a repressor substrate, the repressor concentration of cefotaxime that required to switch off *bla*$_{CTX-M-2}$ gene expression may varies between different strains, so the results showed *P. mirabilis* isolatescontained *bla*$_{CTX-M-2}$ gene but had different response to cefotaxime. The extracted DNA by Salting out method and commercial kit (pureyeild ™ plasmid Miniprep system, Promega, USA) was purified and analyzed by using gel electrophoresis, and the results indicated one band that represented a chromosomal DNA in all 10 isolates in this study (**fig 5**).

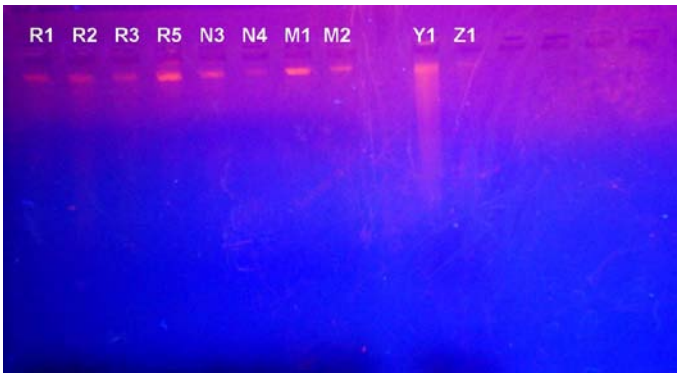

Fig 5. Chromosomal DNA bands from 10 isolates of *P. mirabilis* (R1, R2 are sensitive to cefotaxime, and R3, R5, N3, N4, M1, M2, Z1, Y1 are resistant to cefotaxime), on 0.8 % agarose gel at 5 volt/cm for 1-2 hrs., visualized by E-graph gel documentation equipped by UV light source

Although several studies [10,12,27] confirmed that *bla*$_{CTX-M-2}$locatedon plasmid DNA, other studies [17] indicated that several antibiotics, such as cefotaxime, ceftazidime and ceftriaxone which they are drugs of choice for treatment of serious infections caused by members of family *enterobacteriaceae* are usually encoded in plasmids, transposon and/or integrin. This leads to conclusion the possibility of encoded *bla*$_{CTX-M-2}$ on chromosomal DNA. To confirm this fact (that local isolates of *P. mirabilis* contain only chromosomal DNA which *bla*$_{CTX-M-2}$encoded with) curing of bacterial plasmid DNA by ethidium bromide was carried on 4 isolates of *P. mirabilis* resistant to cefotaxime was done. The treated bacteria were able to grow in nutrient broth containing 400 µg/ml of ethidium bromidewhich indicated the result of pattern of genetic material by extraction which confirmed that *bla*$_{CTX-M-2}$ gene in tested isolates was encoded on chromosomal DNA and there is no presence for plasmids that may be cured with ethidium bromide. Consequently the treated bacteria were not able to grow on nutrient agar medium containing cefotaxime, while good growth was seen on nutrient agar used as control. This result leading to conclusion that *P. mirabilis* isolates sustain the ability to resist to cefotaxime and confirm that the gene is encoding on chromosomal DNA.

Conflict of interest

The authors declare that they have no conflict of interests.

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