original article

Molecular investigation of extended-spectrum beta-lactamase genes and potential drug resistance in clinical isolates of *Morganella morganii*

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BACKGROUND: Resistance to beta-lactam antibiotics has become more common in *Morganella morganii*, which can cause of outbreaks of bacteremia and septicemia in postoperative patients.
OBJECTIVE: Investigate drug susceptibility of *M morganii*, identify the gene responsible for extended-spectrum beta-lactamase (ESBL) production and explore treatment options.
DESIGN: Descriptive study.
SETTING: Hospitals in An Najaf, Iraq.
METHODS: *M morganii* isolates were identified based on morphology, biochemical tests and VITEK® 2 compact system using (GN-ID) card. *M morganii* isolates were subjected to antibiotic resistance tests using the minimum inhibitory concentration (MIC) technique and an antibiogram was produced. Molecular studies were conducted using the polymerase chain reaction technique.
MAIN OUTCOME MEASURE(S): Minimum inhibitory concentration.
RESULTS: From 395 gram-negative bacteria, only 17 isolates *M morganii* grew on MacConkey agar. *M morganii* isolates strongly resistant to several antibiotics were considered multidrug resistant. All *M morganii* isolates were ESBL producers. Four genes (CTX-M, SHV, TEM and OXA) encoding the β-lactamase enzyme were detected. Meropenem and imipenem were highly active against the *M morganii* isolates.
CONCLUSIONS: All isolates showed resistance to most common antibiotics, which limits options for treatment. This study provided useful information for selecting antibiotics to precisely target infections caused by *M morganii*.
LIMITATIONS: Limited to antibiotic susceptibility and genotype.

Morganella morganii is a rod-shaped gram-negative bacteria, a member of the tribe Proteeae of the family Enterobacteriaceae. The Proteeae, which consists of *Morganella, Proteus* and *Providencia*, are important opportunistic pathogens that cause a variety of nosocomial infections.[1,2] Exposure of bacterial strains to β-lactam antibiotics has altered the dynamics of β-lactamase mutations, leading to an increase in activity against third- and fourth-generation cephalosporins such as cefepime, ceftazidime and cefotaxime as well as aztreonam.[3] Like other Enterobacteriaceae, *M morganii* are naturally resistant to beta-lactam antibiotics, but resistance has become more common in *M morganii*, as demonstrated by the increased production of the so-called extended spectrum β-lactamases (ESBLs).[4,5]

M morganii has been implicated in outbreaks of bacteraemia and septicemia in humans that occur most commonly in postoperative patients.[6,7] The majority of the known gene cassettes confer resistance to antibiotics.[8] The integration of most β-lactamase genes in plasmids and transposons encourages rapid transferance of resistance genes between microbes.[9] The β-lactamase genes are often found within the

integrons with multi-drug resistance cassettes which are involved in resistance to chloramphenicol, aminoglycosides, sulphonamides and macrolides.[10] Third-generation cephalosporins, referred to as extended-spectrum cephalosporins, which include ceftriaxone, ceftazidime, and cefotaxime, were effective against ampicillin hydrolyzing β-lactamases and gained widespread clinical use. However, within a few years of their introduction, hospital-acquired gram-negative bacilli (such as *Klebsiella pneumonia*) started producing a mutated version of β-lactamases called extended spectrum β-lactamases (ESBLs) and plasmid-mediated AmpC β-lactamases that conferred resistance to third-generation cephalosporins.[11] The wide spectrum of β- lactamases represented by TEM-1 and SHV-1 gave rise to the name "extended spectrum" beta-lactamses (ESBL), which later involved CTX-M and OXA-type enzymes.[9] These enzymes are capable of hydrolyzing and inactivating a wide variety of therapeutic β-lactam antimicrobials.[12] The purpose of our study was to investigate drug susceptibility of *M morganii* at hospitals in the city of Najaf, Iraq, identify the gene responsible for extended-spectrum beta-lactamase (ESBL) production and explore treatment options.

PATIENTS AND METHODS

Samples collection

Clinical samples were collected from November 2014 to February 2015. These samples were collected from patients at different hospitals in An Najaf, Iraq (Alsader Teaching Hospital, Al Najaf General Hospital and Al-Furat Al-Awsat hospital). All samples were inoculated onto MacConkey agar plates and incubated at 37°C under aerobic conditions for 18 to 24 hours.

Isolation and identification of the microorganism

M morganii isolates were recovered from clinical samples after culturing on MacConkey agar, which incubated for overnight at 37°C. Identification was based on morphology, gram staining, and biochemical tests (catalase, oxidase, methyl red, citrate, indole, Voges-Proskauer, Kligler iron agar, motility, urease, gelatin liquefaction and lactose). The VITEK® 2 compact system was used to carry out final identification.

Antibiogram testing

Antibiogram testing was carried out using the VITEK® 2 system according to manufacturer instructions using ASTN093 cards. The following antibiotics were tested by ASTN093: piperacillin-tazobactam, piperacillin, ticarcillin, ticarcillin-clavulanic acid, ceftazidime, cefepime, aztreonam, meropenem, imipenem, isepamicin, tobramycin, amikacin, gentamicin, pefloxacin, ciprofloxacin, trimethoprimsulfamethoxazole, minocycline, colistin and rifampicin.

Extended-spectrum β-lactamase production

All bacterial isolates were tested for extend spectrum-spectrum β-lactamase by an initial screening test depending on the MIC results for ceftazidime with the VITEK® 2 compact system. The isolate was considered a potential ESBL producer if the ceftazidime MIC was ≥2 µg/mL (CLSI, 2012). The disk approximation test was used to confirm ESBL production. The test involves 30 µg antibiotic disks of ceftazidime, ceftriaxione, cefotaxime and azetreonam placed 15 mm apart (edge to edge) around a central disk of amoxicillin-clavulanate disk (20:10µg) on Muller-Hinton agar plates inoculated with the organism being tested for ESBL production. The augmentation (increase in diameter of inhibition zone) between the central amoxicillin-clavulanate disk and β-lactam antibiotic disks reflects antibiotic resistance and the organism was recorded as an ESBL producer.[13]

Isolation of plasmid DNA and polymerase chain reaction

Plasmid DNA was extracted by the alkaline method according to manufacturer instructions (G-Biosciences, USA). The polymerase chain reaction (PCR) test was carried out in a final volume of 25 µL containing a 4 µL template DNA, 12.5 µL of 2X KABA2G Robust HotStart ReadyMix, 1.25 µL of forward primer ,1.25 µL of reserve primer and 6 µL of PCR grade water for each sample. The DNA amplification was performed for the *bla*CTX-M gene by an initial denaturation at 94°C for 3 min, denaturation at 94°C for 45 sec, annealing 60°C for 30 sec, extension 72°C for 1 min and final extension 72°C for 3 min followed by 35 cycles of amplification. For the *bla*SHV gene an initial denaturation at 94°C for 3 min, denaturation at 94°C for 1 min, annealing 55 °C for 1 min, extension 72°C for 1 min and final extension 72°C for 7 min was followed by 40 cycles of amplification. For the *bla*TEM gene an initial denaturation at 94°C for 5 min, denaturation 94°C for 1 min, annealing 55°C for 1 min, extension 72°C for 30 sec and final extension 72°C for 5 min was followed by 35 cycles of amplification. For the *bla*OXA gene an initial denaturation at 94°C for 5 min, denaturation at 94°C for 45 sec, annealing 55°C for 45 sec, extension 72°C for 1 min and final extension 72°C for 5 min was followed by 30 cycles of amplification. PCR products were separated on 1 % agarose gel and visualized using a transilluminator (BIO-RAD, USA).

Statistical analysis

The Microsoft Excel (Package 2007) was used to compile the data.

RESULTS

During the study interval from November 2014 to February 2015, clinical samples were collected from 800 patients (**Table 1**). All *M morganii* isolates were resistant to a minimum of three classes of antibiotics to which they were tested, and this were considered multidrug resistant (MDR) (**Figure 1**). All isolates were resistant to piperacillin, piperacillin-tazobactam, and aztreonam, with lower rates of resistance to ticarcillin and ticarcillin-clavulanic acid, with the lowest rates to imipenem and meropenem. **Figure 1** shows the rates of resistance and sensitivity for other antibiotics. The aminoglycosides had a high-to- moderate effect against *M morganii* isolates, while most isolates were resistant to fluoroquinolones, colistin and rifampicin. Results of susceptibility revealed that none of the isolates was fully resistant or susceptible to antibiotics tested.

The ceftazidime MIC results from the VITEK® 2 compact system showed that all *M morganii* isolates (n=17) were ESBL producers during the initial screening (**Table 2**). The phenotypic confirmatory test using the disk approximation method confirmed the ability of *M morganii* isolates to produce ESBLs. All *M morganii* isolates (n=17) were ESBL positive on the phenotypic confirmatory test (**Figure 2**).

Table 3 shows the specific primers used to detect extended-spectrum β-lactamase genes (CTX-M, SHV, TEM and OXA) from the 17 isolates of *M morganii*.[14-17] Of the 17 *M morganii* isolates, 16 carried at least one type of blagene (**Figures 3, 4, 5 and 6**). However, the most commonly identified ESBL gene was *bla*CTX-M type in 15 of the isolates (**Figure 3**). PCR amplification using specific primers of the *bla*SHV gene found the gene in 10 isolates (**Figure 4)**. The *bla*TEM gene was detected in six isolates (**Figure 5**). Finally, only two of the examined isolates harbored a gene for the OXA type enzyme (**Figure 6**).

Table 1. Sources and distribution of clinical samples according to type of infection.

Infection type	Samples No.	Bacterial growth No. (%)	Yielded no growth No. (%)
Urinary tract infection	400	207 (51.8)	193 (48.2)
Ear infection	200	119 (59.5)	81 (40.5)
Respiratory tract infection	200	69 (34.5)	131 (65.5)
Total (%)	800	395 (49.4)	405 (50.6)

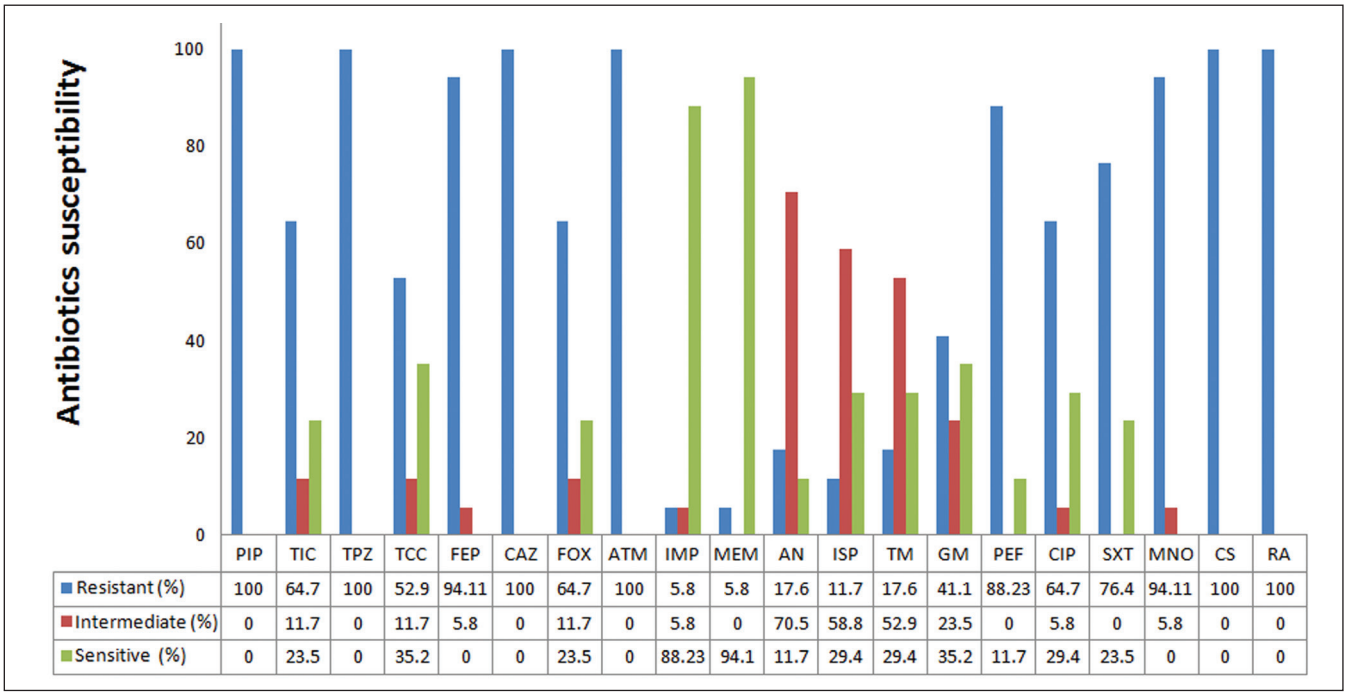

Figure 1. Antibiogram testing of *Morganella morganii* isolates with the automated VITEK® 2 compact system by using AST-N093 cards (n=17). PIP, Piperacillin; TIC, Ticarcillin; TZP, Piperacillin-tazobactam; TCC, Ticarcillin-clavulanic acid; FEP, Cefepime; CAZ, Ceftazidime; FOX, Cefoxitin; ATM, Aztreonam; IPM, Imipenem; MEM, Meropenem; AN, Amikacin; ISP, Isepamicin; TM, Tobramycin; GN, Gentamicin; PEF, Pefloxacin; CIP, Ciprofloxacin; SXT, Trimethoprim-sulfamethoxazole; MNO, Minocycline; CS, Colistin and RA, Rifampicin.

Table 2. Minimum inhibitory concentration (MIC) ranges for each antibiotic for *Morganella morganii* isolates.

Antibiotic	MIC range (µg/ml)	Antibiotic	MIC range (µg/ml)
Cefepime	4 - ≥64	Gentamicin	2 - ≥16
Ceftazidime	32 - ≥64	Pefloxacin	0.5 ≥16
Aztreonam	32 - ≥64	Ciprofloxacin	0.5 ≥4
Imipenem	2 - ≥16	Trimethoprim-sulfamethoxazole	≤20 - ≥320
Meropenem	0.5 - ≥16	Minocycline	8 - ≥16
Amikacin	8 - ≥64	Colistin	≥16
Isepamicin	8 - ≥64	Rifampicin	≥32
Tobramycin	2 - ≥16		

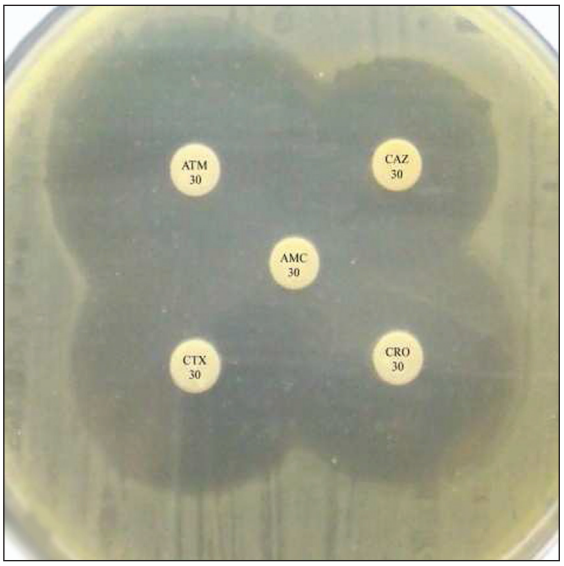

Figure 2. Show positive result of phenotypic confirmatory test for ESBL.

Table 3. Primer sequences for detection of extended-spectrum beta-lactamase genes.

No.	Target gene	Primer sequence	Amplicaon Size (bP)		References
1	*bla*CTX-M	F	CGCTGTTGTTAGGAAGTGTG	569	14
		R	GGCTGGGTGAAGTAAGTGAC		
2	*bla*OXA	F	ATATCTCTACTGTTGCATCTCC	619	15
		R	AAACCCTTCAAACCATCC		
3	*bla*TEM	F	TGCAACAGTGCCTCTCGATA	717	16
		R	CTCGTGCACCCAACTGATCT		
4	*bla*SHV	F	GGTTATGCGTTATATTCGCC	867	17
		R	GGTTAGCGTTGCCAGTGCTC		

DISCUSSION

Much evidence supports the hypotheses that two factors largely contribute to the development and spread of antimicrobial resistance: the transmission of plasmids between microbes and poor selection of antimicrobials for treatment of infections. Plasmid-mediated antimicrobial resistance from horizontal transmission of plasmids is a global threat.[18]

In the present study, *M morganii* isolates were highly resistant to many antimicrobials (cephalosporins, ceftazidime and cefepime). However, resistance to third-generation cephalosporins occurred mainly by mutations in the common group of class A β-lactamases that include TEM, SHV and CTX-M.[19] We found high resistance to third-generation cephalosporins such as ceftazidime that may be due to the production of cefotaximases encoded by the CTX-M gene. These CTX-Ms appear to have more activity toward cefotaxime than ceftazidime.[20]

We found intermediate resistance (47%) toward ticarcillin/clavulanic acid. Although β-lactamase inhibitors prevent the inactivation of the β-lactam antibiotics, they have low antimicrobial activity themselves.[21] Since high susceptibility rates to imipenem and meropenem (88.2% and 94.1%, respectively) were found among *M morganii* isolates, this study shows that those two β-lactam antibiotics are the most effective against *M morganii*, a finding consistent with previous data by researchers from Greece and China.[2,22]

In this study, ceftazidime resistance was chosen to detect ESBL producers because it is the best third-generation cephalosporin substrate for most TEM, SHV and CTX-M derived ESBLs.[23,24] The CTX-M genes are a recent family of plasmid-mediated ESBLs; some of them are part of transposons or constitute gene cassettes in integrons.[25,26] The blaSHV gene was detected in 10 isolates. The SHV β-lactamases enzymes are mainly found in gram-negative bacteria.[27] These enzymes possess variants of substituted serine instead of glycine at position 238 and lysine instead of glutamate at position 240.[28] There are more than 125 SHV varieties described worldwide.[29] The SHV β-lactamases were the predominant ESBL types in Europe and the United States.[30] The blaTEM gene was detected in six of the tested *M morganii* isolates. The TEM-ESBLs are the first plasmid-mediated β-lactamase that are detected in many genera of Enterobacteriaceae such as *Proteus mirabilis* and *Klebsiella pneumonia, E coli;* and also in non-Enterobacteriaceae like *Pseudomonas aeruginosa*.[31] Finally, two of the *M morganii* isolates harbored the blaOXA gene. However, most of the OXA derivative genes are located in plasmids and

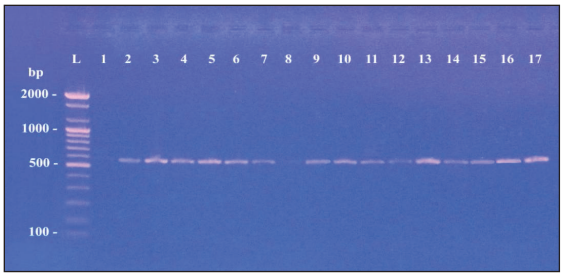

Figure 3. blaCTX-M gene with amplified product 569bp.

Figure 5. *bla*TEM gene with amplified product 717 bp.

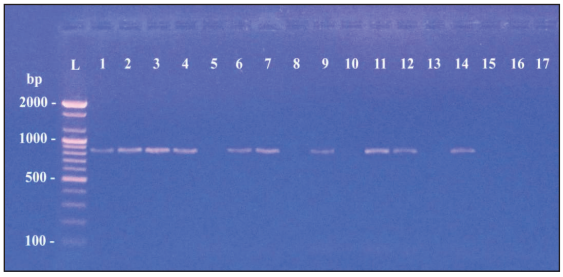

Figure 4. *bla*SHV gene with amplified product 867 bp.

Figure 6. *bla*OXA gene with amplified product 619 bp.

integrons,[32] while some OXA-type β-lactamases are encoded by chromosomal genes that appear to be resident in some microbial genomes such as those in *Pseudomonas aeruginosa*.[33] The integrons that harbor co-resistance genes make it a useful tool for facilitating dissemination.[34,35]

Our study was limited to antibiotic susceptibility and genotype testing. further study using sequencing technology and proteomic analysis is recommended.

In summary, *M morganii* harbors several beta-lactamases genes and shows resistance to most common antibiotics, which limit options for treatment. This study provided useful information for seleccting antibiotics to precisely target infections caused by *M morganii*.

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