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Risk Factors Associated with Community-acquired CTX-M Producing *Klebsiella pneumoniae* Typing by Rep-PCR in Sanandaj, Iran

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The CTX-M producing organism is emerging as a resistance source to oxyimino-cephalosporin like ceftriaxone and ceftazidime. However, the laboratory detection of this gene has not yet been well defined in Sanandaj, Iran. The purpose beyond this study is identifying the prevalence of CTX-M and its risk factors in the community-acquired *Klebsiella pneumoniae* infections in Sanandaj. In this case-control study, 100 community-acquired *Klebsiella pneumoniae* strains were used. CTX-M gene was detected using PCR. The probable clonal relation among strains was determined via Rep-PCR. Risk factors associated with CTX-M positive were examined through the univariate logistic regression, Student's t-test, and Mann-Whitney U test. The polymerase chain reaction test, used to identify *CTX-M* gene, showed that 37 (37%) Samples, out of 100 were positive. Based on Rep-PCR, 31 genotypes were identified among 37 Samples of *CTX-M* positive isolates. According to the statistical analysis, the following were the most important independent risk factors in this study: Gestation (p value= 0.036), previous exposure to antibiotic within 3 months (p value= 0.016), having relatives who work in a hospital (p value= 0.001, and distance of under 2 Km from home address to hospital (p value< 0.001). Regarding the increased value of ESBLs-producing strain, it is strongly recommended to use appropriate curative protocols based on the antibiogram. The results of Rep-PCR experiment refute the hypothesis of the clonal spread of one epidemic strain *Klebsiella*; meaning that not all CTX-M-producing species originate from the same strain and that the gene has extended among various strains. Hence, hospitals and their worker have to have better hygiene, hospital wastes have to be disposed properly, and antibiotics use may help prevent the spread of ESBL resistance only in case of being prescribed by a doctor.

Key words: *CTX-M*, Community-acquired, *Klebsiella pneumonia*, Risk factors, Rep-PCR.

Since first report of extended-spectrum beta-lactamases (ESBLs) in *K. pneumoniae* from Germany (1983)[1] the CTX-M family of ESBLs is one of the serious danger to global hygiene[2] to the extent, that in the previous decade it was described as a pandemic.[3] CTX-M is the most widespread ESBLs among Enterobacteriaceae that are agents of hospital and community-acquired.[2,4] This increased resistance is largely due to the spread of *Escherichia coli* and *Klebsiella pneumoniae* containing *CTX-M* gene.[5] *Escherichia coli* and *K. pneumoniae* containing *CTX-M* are increasingly prevalent in community[6] and nosocomial infections.

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One of the foundations of infection control is the knowledge obtained from the surveillance studies. According to reports from patients who have CTX-M-producing bacteria, the following risk factors were identified: history and time-span of hospitalization, disease intensity, hospitalization time-span in ICU, catheter and previous exposure to antibiotic.[7,8] A rapid typing method can help us greatly to examine epidemiology and determine the genetic relations of resistant strains. An appropriate method is proliferation of Repetitive sequences of bacterial DNA through PCR (Semi-automated Repetitive sequence-based polymerase chain reaction) which has these advantages: 1) low expense 2) high discrimination 3) high speed and 4) a reliable device for taxonomy and typing a wide spectrum of Gram negative bacteria and several gram positive bacteria.[9,10] This typing method has proved to be able to determine typing more than 100 isolates in a week.[11,12]

The increased incidence of ESBL attracted our attention to study risk factors in patients who have community-acquired ESBL-producing *K. pneumoniae* and the genetic relation between strains by the Rep-PCR typing method.

MATERIALS AND METHODS

Sampling

During the 10-month study period, we collected 100 samples of *K. pneumoniae* from Outpatients in Sanandaj laboratories and origin of isolation was from urine, blood, wound, sputum and other respiratory samples. Patient information forms were collected in questionnaires. Items in the questionnaire included demographic data (age at the time of study entry and sex), gestation, underlying disease, having relatives (People leaving in the same house) who work in a hospital, distance of home address from hospital and urinary catheter, antibiotic exposure, previous hospitalizations, within the 3 months prior to study entry. The specimens were collected and processed following conventional microbiological procedures for correct management of clinical samples. Finally bacterial strains were storage in LB broth containing glycerol (15%, v/v) for optimal viability.

Confirmation of ESBL production

Confirmatory combination disc diffusion test was carried out for all isolates. A disc ceftazidime (30 ìg) alone and ceftazidime plus clavulanic acid (30 ìg/10 ìg) were placed at a distance of 25 mm on Mueller-Hinton Agar plate inoculated with a bacterial suspension of 0.5 McFarland turbidity standards. After overnight incubation at 37°C, a positive result was interpreted as e" 5 mm increase in a zone diameter for ceftazidime in combination with clavulanic acid versus its zone when tested alone.

DNA extraction

Bacterial genomic DNA was extracted via AxyPrep Bacterial Genomic DNA Miniprep Kit (Axygen Biosciences, USA ([13] and the extracted DNA was checked by agarose gel electrophoresis.

Detection of CTX-M gene

In this study, we utilized primer *CTX-M*-F (5'- ACGCTGTTGTTAGGAAGTG-3'), and *CTX-M*-R (5'- TTGAGGCTGGGTGAAGT-3').[14] This primer proliferates different sorts of CTX-M (CTX-M-1,-3,-12,-15,-22,-30,-32,-33,-38,-52,-57,-58,-60,-61). The size of the final PCR product for this primer is 759 bp.

PCR were performed with initial denaturation (5 minutes at 94 °C) and then 35 cycles of denaturation (45 seconds at 94 °C), annealing (45 seconds at 58 °C), extension (1 minute at 72 °C). Final products were extended by incubation for 7 min at 72°C.PCR product was analyzed by agarose gel electrophoresis (%1.5) and100 bp DNA ladder was used; finally visualized under UV light after staining with ethidium bromide (50 µg/ml).

Rep-PCR

In order to identify the genetic relation between the 100 existing samples we used the typing technique of Rep-PCR for its simplicity and high speed. Rep-PCR reaction was done in the final volume of 25 microliters including 12.5 microliters of PCR Master Mix, 1 microliter of DNA sample, 1 microliter of every primer, and 9.5 microliters of distilled water. The primer pairREP1 (5'-IIIGCGCCGICATCAGGC-3') and REP2 (5'-ACGTCTTATCAGGCCTAC-3') was used to amplify putative Rep-like elements in the genomic bacterial DNA.[15]

Amplification reactions were carried out in Eppendorf thermal cycler, with an initial denaturation (2 minute 95 °C) then 35 cycles of denaturation (1 minute 92 °C), annealing (1 minute

40 °C), extension (8 minute 65 °C), with final extension of 8 min at 65 °C.

Rep-PCR products were electrophoresis in 1.5 %agarose gel. The bonds were detected after staining with by ethidium bromide (50 µg/ml), and photographed by ENDURO™ GDS Gel Documentation System.

To do a fingerprinting analysis, a matrix with 1 for the existence of bond and 0 for its absence were made and the relevant dendrogram was drawn by NTSYS v2.02e software using algorithm of the Unweighted Pair-Group Method (UPGMA).

Statistical analysis

In this study, qualitative variables were evaluated with 95% confidence interval using univariate logistic regression. Quantitative variables were analyzed with Student's t-test, and Mann-Whitney U test. P valued"0.05were considered statistically significant. The statistical analysis software employed was SPSS v20.

RESULTS

In this case-control study, *CTX-M* gene was detected in 37 out of 100 samples, which is a considerable percent of resistance to beta-lactamases.

Risk factors associated with CTX-M spread

Based on statistical analysis we found risk factors associated with *CTX-M* spread that is

Table 1. Univariate analysis of risk factors associated with increased risk of acquisition of ESBL-producing *K. pneumoniae*

Risk factors	Control (n= 63)	CTX-M positive(n= 37)	OR (95%CI)	*P* value
Gender			1.77(0.761-4.149)	0.184
Male	29	12		
Female	34	25		
Mean age in years	40.75	49.59	-	0.006
Gestation	2	6	5.903(1.125-30.975)	0.036
Previous hospitalizations witin the 3 months prior to study entry	13	28	11.96(4.547-31.491)	<0.0001
Mean duration of hospitalization in days	2	5	-	<0.0001
Previous exposure to antibiotic within the 3 months prior to study entry	13	24	2.806(1.208-6.518)	0.016
Urinary catheter within the 3 months prior to study entry	9	2	0.343(0.070-1.681)	0.187
Having relatives who work in a hospital	2	11	12.904(2.671-62.336)	0.001
Distance of home address from hospital				
Under 2 Km	13	23	4.319(2.564-15.574)	<0.0001
2-8 Km	36	13	0.406(0.175-0.940)	0.035
Over 8 Km	14	1	0.097(0.012-0.773)	0.028

Table 2. Multivariate analysis of underlying disease

Disease	Control (n= 63)	CTX-M positive (n= 37)	OR	*P* value	CI (95%)	
					Lower	Upper
Cardiovascular disease	2	2	1.399	0.764	0.156	12.519
Pulmonary disease	9	5	1.217	0.754	0.357	4.156
Diabetes	2	8	7.715	0.015	1.479	40.247
Dialysis	1	3	4.773	0.203	0.431	52.828
vesical calculus	3	2	0.969	0.976	0.129	7.255
Renal calculus	8	2	0.548	0.474	0.105	2.844

shown in table 1. Moreover, we found statistically significant relation between previous hospitalization within last three months and the presence of *CTX-M*. We also found out that the longer Mean duration of hospitalization the more effective it will be on the extension of CTX-M-producing strains. A list of underlying disease of the patients is presented in table 2 and it was only in the case of diabetes that we saw a significant relation between the two groups.

In this study, having relative who work in a hospital has been examined as a risk factor; what was not observed in past studies. CTX-M-producing *K. pneumoniae* were isolated from 11 out of 13 patients either were workers in a hospital or had some relative working there. This indicates the relation between hospital resistance and resistance in the community. Previous exposure to antibiotic within last three months, and home address distance from hospital were other observed risk factors in this study (see table1).

Rep-PCR

The next stage was trying to determine the genetic relation between the strains. After drawing the dendrogram based on Rep-PCR, we arranged the CTX-M-producing strains: those strains which had 100 percent genetic similarity were put in one pattern and other strains were, each, put in one distinguished pattern, according to which there exist 31 patterns among the 37 CTX-M-producing strains. Thus, 37 strains of *CTX-M* positive have 31 distinctive genotypes (figure 1).

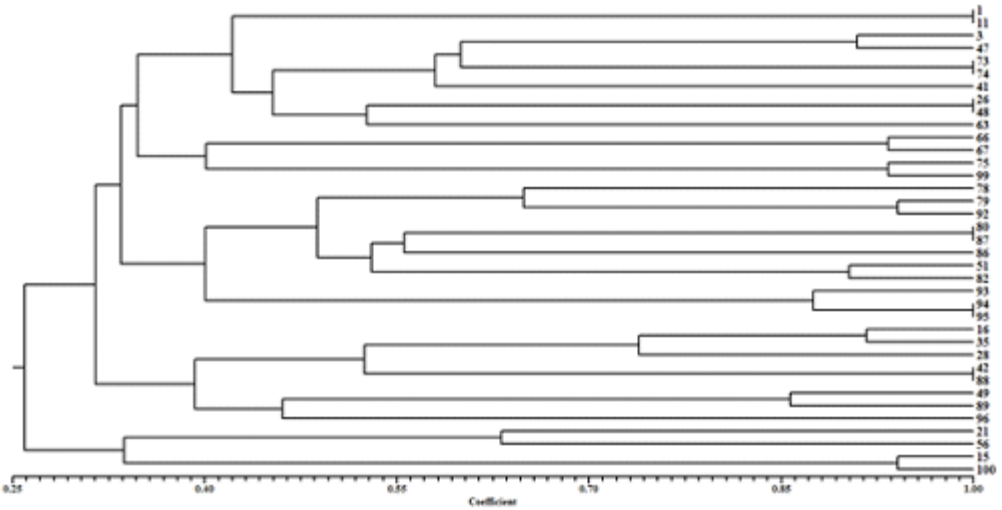

Fig. 1. Rep-PCR analysis. The dendrogram shows 31 genetic patterns in 37 CTX-M-producing strains

DISCUSSION

CTX-M type of ESBL which offers a high resistance to cefotaxime, not to ceftazidime, was described in 1992[16] and 1993[17]. This family of ESBL is of a class and Ambler classification that is attributed to cefotaximase family (CTX-M). As said before, this type of ESBL was described as a pandemic. We need worldwide studies to find a strategy for preventing the spread of this gene any more. Hence, we identified the risk factors, which help extend the CTX-M-producing strains (table 1 and 2).

In various studies, a relation has been observed between sex and the infection caused by ESBL-producing Enterobacteriaceae[18-21]. This difference might be due to regional variances in prescribing antibiotics associated with sex (for example, the use of antibiotics to treat the urinary tract infections in women).[18] in the present study, sex was not identified as a risk factor. However, Gestation was detected as a factor in extending the infection with strains of CTX-M-producing *K. pneumoniae*; because urinary tract infections, in which *K. pneumoniae* play a role, happen more frequently in pregnant women and this by itself

leads to increasing the use of antibiotics by such women.

As in our study, previous exposure to antibiotic within the 3 months prior to study entry has been detected as a risk factor in the majority of studies.[7,18,19,22-25] By selective pressure, this risk factor may cause eliminating all sensitive strains.

As in other studies, we identified Previous hospitalizations within the 3 months prior to study entry as a risk factor.[8,23,25-27] When someone is hospitalized, there are several possibilities for gene transference: 1) that one becomes infected with bacteria containing resistance genes and this resistance may spread among other members of the society; 2) that during hospitalization, the resistance genes stay on one's skin and ultimately they may be transferred, via transformation mechanism, to those bacteria lacking this gene; 3) this gene goes inside bacteriophages and the hospitalized person carries these viruses outside the hospital and resistance genes get transferred to other bacteria, through transduction mechanism, and the *CTX-M* gene spreads in the community. We also detected longer Mean duration of hospitalization as a risk factor and this confirms our assumptions.

Urinary catheters, especially when used for a long time, are among the most important factors that help develop urinary tract infection and sometimes bacteremia. In this study, urinary catheter has not been identified as a risk factor. In many studies, as in the case of Arslan et al. (2005), urinary catheter was detected as a risk factor for ciprofloxacin resistance[24], and Hayakawa et al. (2013), independent risk factor for CTX-M-producing isolation included presence of a urinary catheter.[28] It is possible that urinary catheters be infected with bacteria or the virus containing the gene and in this way CTX-M-producing infection and even other genes increase.

Moreover, having some relatives working in a hospital could be a risk factor for *CTX-M* extension because the body, especially the hands, of those may be infected with the CTX-M-producing bacteria and this gene can be transferred to other bacteria through the conjugation mechanism; or may be infected with *CTX-M* gene in which case transformation could be an agent of diffusion; or these genes can placed inside the bacteriophage, the health workers will carry these viruses outside the hospital, the resistance genes may be transfer, through transduction, from hospital bacteria to other bacteria and finally *CTX-M* gene would spread in the community.

In the case of short distance, home address from hospitals (under 2 Km) was determined as a significant risk factor. Resistance gene from hospital to environment could be transfer via hospital wastes and sewages contain resistance genes, bacteriophages, bacteria containing these genes and so on. Unfortunately, resistance genes will extend outside the hospitals via various gene transfer mechanisms.

The typing technique was necessary in distinguishing the two hypotheses. First: one epidemic *Klebsiella* strain spread among all patients and so an ancestral strain is responsible for the extension of resistance; this means a relation between all patients. Secondly: *CTX-M* gene spreads among different *Klebsiella* strains. We identified 31 different genotypes among 37 samples of *CTX-M* positive. Thus, the results of this experiment refute the hypothesis of the clonal spread of one epidemic *Klebsiella* strain; meaning that not all species producing CTX-M originate from the same strain and that the gene has extended among various strains. Accordingly, we cannot contradict the possibility that a plasmid or a transposable element containing *CTX-M* has caused the spread of the gene among different *Klebsiella* strains.

CONCLUSION

In conclusion, we determined the existence of *CTX-M* gene and we determined associated risk factors in community- acquired infections. Some of these factors were more interesting like that being live in vicinity of hospitals.

Conflict of interest

The authors have no conflict of interest to declare.

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