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Characterization of bla_{TEM} and bla_{CTX-M} beta-lactam resistance genes in chronic rhinosinusitis

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Abstract

Background Chronic sinusitis is one of the most challenging health problems of contemporary society. Although several treatment methods have been defined, a comprehensive understanding of the underlying causes (e.g., antibiotic resistance) is still elusive. The aim of this study was to characterize two of the main extended-spectrum beta-lactamase genes—i.e., bla_{TEM} and bla_{CTX-M} genes—and investigate antimicrobial resistance in bacteria isolated from chronic sinusitis. Samples from 70 chronic sinusitis patients and 20 healthy individuals (controls) were analyzed for the presence of bla_{TEM} and bla_{CTX-M} resistance genes using the polymerase chain reaction (PCR) test, followed by gene sequence analysis.

Results Phenotypic and genotypic beta-lactam resistance was observed in 58.7% and 61.54% of the gram-negative isolates, respectively, with 38.46% carrying the bla_{TEM} gene and 34.62% harboring the bla_{CTX-M} gene. Sequencing data indicated high heterogeneity in bla_{CTX-M} genes (69–100% similarity to reported sequences) and lower heterogeneity in bla_{TEM} genes (93–99%).

Conclusion Broad-spectrum beta-lactam resistance is a major pathogenesis factor in chronic rhinosinusitis, and careful consideration is required for antimicrobial therapy. High bla_{CTX-M} heterogeneity could mean high horizontal transfer rate of this gene and warrant a surveillance program.

Keyword Chronic rhinosinusitis, bla_{TEM}, bla_{CTX-M}, Broad-spectrum beta-lactamase, PCR

Background

Sinusitis is the inflammation of the mucosal tissue lining the sinuses (hollow cavities in the facial bones), and chronic bacterial sinusitis is one of the most common health problems in the contemporary society. Microbiology of chronic bacterial sinusitis has been explained extensively, encompassing wide variations in terms of geographical areas, patients, laboratory methodologies and sampling methods [1–3].

The nasopharynx is colonized by normal bacterial flora, including potential pathogens. They can spread from the nasopharynx to the sinus cavity during viral respiratory infections and cause various respiratory tract infections, including sinusitis [4]. Emergence of antimicrobial resistance in organisms responsible for chronic sinusitis has called into question the efficacy of antibiotic treatment of these infections [5, 6]. Furthermore, the study of recent trends in antimicrobial resistance of bacterial species involved in rhinosinusitis has revealed that the prevalence of extended-spectrum

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beta-lactamase (ESBL)-producing strains in these infections is on the rise [7]. Therefore, careful consideration of the bacteria involved prior to adopting treatment regimen is paramount [8], especially in the case of beta-lactam and extended-spectrum beta-lactam resistant bacteria in chronic sinusitis, which have further complicated the antimicrobial therapy process [9].

Beta-lactamases are enzymes produced by bacteria, which create resistance to beta-lactam antibiotics, such as penicillins, cephamycins and carbapenems. From a functional viewpoint, three beta-lactamase enzyme groups (groups 1, 2 and 3) have been defined, based on their inhibitor profiles and substrates [10]. In another classification scheme, beta-lactamase proteins have been classified into four classes (classes A, B, C and D) based on amino acid sequences [11]. ESBLs are enzymes that can neutralize extended-spectrum antibiotics such as extended-spectrum penicillins and third-generation cephalosporins as well as aztreonam [12]. They can, however, be overcome by some beta-lactamase inhibitors such as clavulanic acid, tazobactam and sulbactam. ESBL genes are believed to have originated from narrow-spectrum beta-lactamase genes through mutation, and they have managed to spread globally by means of mobile genetic elements, while constantly taking new forms. Two of the most important ESBL genes are the classic bla_{TEM} gene and the more recently spreading $bla_{CTX\text{-}M}$ gene (both class A beta-lactamases) [13, 14].

Like most respiratory tract infections, sinusitis occurs in stages. The first stage is the viral phase, which ends in complete recovery of most patients after one to two weeks [15]. However, in some cases, the viral phase is followed by acute bacterial infection, and delayed or unsuccessful treatment of acute sinusitis can lead to chronic sinusitis [4, 15].

Although the mechanism of inflammation associated with chronic sinusitis is still controversial, the presence of bacteria within the sinuses has been well documented [2].

Streptococcus pneumoniae, Haemophilus influenzae, Moraxella catarrhalis and *Streptococcus pyogenes* have been most commonly isolated in cases of acute bacterial sinusitis. In case of chronic rhinosinusitis, the most common bacterial isolates are *Staphylococcus aureus* and anaerobic organisms such as *Fusobacterium, Prevotella*, *Porphyromonas* and *Peptostreptococcus* spp. *Pseudomonas aeruginosa* and other aerobic and facultative gram-negative rods have also been recovered from patients with underlying health problems. Unfortunately, antibiotic resistance is prevalent among these aerobic and anaerobic bacteria, and a large proportion of them (over a third) produce beta-lactamases. Moreover, most *Staphylococcus aureus* isolates involved in these infections are of the Methicillin-resistant (MRSA) type [2, 4, 8, 16].

Factors affecting the microbiology of sinusitis may include vaccination, history of antimicrobial therapy and inhibiting effect of the normal flora [17]. Understanding the characteristics and antimicrobial resistance/susceptibilities of the organisms responsible for chronic sinusitis is crucial to the success of antimicrobial therapy and must be considered when selecting the antimicrobial regimen [2].

Having established the significant role of gram-negative bacteria in sinus infections, and the important role of extended-spectrum beta-lactam resistance in the pathogenesis of gram-negative bacteria, we aimed this study to investigate the beta-lactam resistance profile of bacterial strains isolated from patients with chronic sinusitis, by determining the prevalence and characterizing two important ESBL genes, namely bla_{TEM} and $bla_{CTX\text{-}M}$ [11, 18].

Materials and methods

This prospective study was conducted during the period 2018–2019, on samples collected from 90 individuals, out of which 70 were admitted to a large public hospital in Tehran, Iran, suffering from chronic sinusitis. The Ethical Code for this study is IR.IUMS.REC.1395.28467, and all participants were aware of and consented to entering this study and publication of its data. Sinusitis was diagnosed by specialist doctors, based on clinical findings, radiographic imaging (Rx) and CT scan. The other 20 participants had no sinusitis symptoms.

Samples were collected during endoscopy, and the specimens were sealed and immediately transferred to the laboratory under prescribed conditions [19]. Individuals who had received antibiotics in the three weeks preceding sample collection were excluded from the study. Specimens were cultured on blood agar, on enriched Schaedler agar and in thioglycolate broth (enriched with yeast extract, vitamin K and hemin) media. After incubation, we carried out gram staining, standard identification tests and antimicrobial susceptibility tests on isolates as previously described [2]. The antibiotics used in the antimicrobial susceptibility tests were obtained from Mast, England (MASTDISCS™ AST), and included the following disks as previously described [20]: *ciprofloxacin* (5ug), *gentamicin* (10ug), *penicillin* (10U), *ampicillin* (10ug), *clindamycin* (2ug), *vancomycin* (30ug), *tetracycline* (30ug), *erythromycin* (15ug), *cotrimoxazole* (1.25/23.75ug), *amikacin* (30ug), *amoxicillin* (20ug), *oxacillin* (1ug), *imipenem* (10ug), *ceftazidime* (30ug), *meropenem* (10ug), *cefotaxime* (30ug), *piperacillin–tazobactam* (30/6ug), *colistin*

Table 1 Primers sets used for detection of bla_{TEM} and $bla_{CTX\text{-}M}$ genes

Gene	Primers	Product length (bp)	References
bla_{TEM}	F- GAGTATTCAACATTTCCGTGTC R- TAATCAGTGAGGCACCTATCTC	861	[22]
$bla_{CTX\text{-}M}$	F- CGCTTTGCGATGTGCAG R- ACCGCGATATCGTTGGT	550	[22]

(10ug) and *ampicillin–sulbactam* (10/10ug). Antibiotic susceptibility test results were interpreted and reported using CSLI guidelines (CSLI M021, 2020).

DNA was extracted using the boiling method from all the gram-negative bacterial strains isolated in the previous steps, then stored in double distilled water at − 4 °C. To check the quality of the extracted DNA, the 260/280 nm absorbance ratio was determined (using a Thermo Scientific™ NanoDrop™ 2000 spectrophotometer) prior to storage [21].

All 26 gram-negative isolates were checked for the presence of bla_{TEM} and $bla_{CTX\text{-}M}$ genes using the PCR technique. *Staphylococcus aureus* ATCC 29213 was used as the blank control strain. Primer sets used in this study as described previously are provided in Table 1. PCR procedures and mixtures were used as previously described [22, 23]. PCR products were separated using a standard 1.5% agarose gel electrophoresis procedure.

From the PCR products positive for either bla_{TEM} or $bla_{CTX\text{-}M}$, 25–50 µl was sent to Microsynth Company, Switzerland, for DNA sequencing (via Topaz Gene Kavosh Company, Karaj, Alborz Province, Iran). DNA sequencing data was checked for the most similar sequences in online databases, using the online NCBI Blast tool. All data were analyzed by SPSS 23.

Sequences obtained were submitted to GenBank, with the following accession numbers: OP374105, OP374106, OP374107, OP374108, OP374109, OP374110, OP374111, OP424993, OP424994 and OP424995.

Results

Bacteriology

Of all the samples collected in this study from 90 people (70 patients with sinusitis and 20 healthy individuals), 74 yielded positive cultures. Fifty-seven (77.03%) of the cultures had a single organism, while 17 (22.97%) contained multiple organisms. A total of 97 different isolates were obtained from these cultures.

Samples collected from individuals with no sinusitis symptoms were mostly sterile (80%), and in 4 cases, *Staphylococcus saprophyticus* and *Staphylococcus aureus* were isolated. The most significant bacteria isolated from sinusitis patients are provided in Table 2.

Antimicrobial susceptibility test

The highlights of antibiotic susceptibility tests results are as follows:

All group D *Streptococci* isolates were resistant to clindamycin, and all were susceptible to gentamicin. Penicillin and vancomycin were observed to be equally effective against these isolates, with 42.8% of the isolates showing resistance to each of these antibiotics.

Corynebacteium isolates demonstrated the most resistance to ciprofloxacin (69.2% were resistant) and the most susceptibility to clindamycin and tetracycline (0% were resistant).

Staphylococcus aureus isolates were observed to be 100% resistant to penicillin. The most effective antibiotic against these isolates was ciprofloxacin, with 16.6% of the isolates showing resistance.

Escherichia coli isolates demonstrated mild resistance to some beta-lactam antibiotics (18.1% were resistant to ceftazidime and 27.3% to meropenem), but no resistance to other carbapenems or aminoglycosides.

In case of *Staphylococcus saprophyticus,* 100% of the isolates were resistant to penicillin and 90.9% were resistant to erythromycin. Ciprofloxacin, ampicillin and cotrimoxazole were observed to be fully (100%) effective against these isolates.

Klebsiella isolates showed the most resistance to beta-lactam antibiotics among all the isolates (87.5% resistant to ampicillin, 75% resistant to cephalosporins and 50% resistant to both imipenem and meropenem), but no resistance to beta-lactams in combination with

Table 2 Most significant bacteria isolated from sinusitis patients and the number of isolates in 70 patients with sinusitis

Bacteria	Number of isolates	Percentage (%)
Group D Streptococci	14	20
Corynebacteium spp.	13	18.6
Staphylococcus aureus	12	17.1
Escherichia coli	11	15.7
Staphylococcus saprophyticus	11	15.7
Kebsiella spp.	8	11.4
Pseudomonas	7	10
Enterococcus spp.	5	7.1
Staphylococcus epidermitise	4	5.7
Nonhemolytic Streptococci	4	5.7
Other bacteria	8	11.4

beta-lactamase inhibitors, such as piperacillin–tazobactam and ampicillin–sulbactam (0% resistant).

Pseudomonas isolates demonstrated some resistance to gentamicin**,** ceftazidime, colistin and meropenem in equal measure (all 42.8%), but they were all susceptible to other beta-lactam antibiotics.

Enterococcus isolates were observed to be most resistant to erythromycin and clindamycin (both 100%) and least resistant to penicillin and gentamicin (both 0%). Isolates from other bacteria showed varied resistance patterns (results not reported).

Overall, the most drug resistance was observed in *Pseudomonas* isolates (with 100% of the isolates showing multi-drug resistance), and the most beta-lactam resistance was observed in *Klebsiella* isolates.

PCR

Out of the 26 isolates selected for molecular assessment of beta-lactamase genes, 9 contained the $bla_{CTX\text{-}M}$ gene, 10 carried the bla_{TEM} resistance gene, and 3 harbored both the $bla_{CTX\text{-}M}$ and the bla_{TEM} genes. The results are illustrated in Table 3. PCR products were separated using 1.5% agarose gel electrophoresis, as depicted in Fig. 1.

DNA sequencing

Nineteen isolates containing at least one of the two beta-lactam resistance genes were selected for DNA sequencing. These included 10 isolates with the bla_{TEM} gene and 9 isolates with the $bla_{CTX\text{-}M}$ gene.

Our bla_{TEM} gene sequencing data analysis demonstrated a similarity range of 93% to 99% with sequences reported from India, China, Japan, Switzerland and the USA. The bla_{TEM} gene obtained from *Escherichia coli* showed the most variation from bla_{TEM} gene sequences present in GenBank database (93% similarity), while sequences from other isolates all showed the same 99% similarity. For the $bla_{CTX\text{-}M}$, the similarity range was observed to be between 69 and 100% with sequences reported from Spain, the USA, China, the UK, Germany, Colombia, Brazil, Singapore, Norway, Sweden, South Korea, Australia, Switzerland, Canada and France.

The $bla_{CTX\text{-}M}$ gene sequence obtained from our *Klebsiella* isolates showed the least similarity (69%) with sequences reported from other countries, while the sequences from *Escherichia coli* had 100% similarity with sequences reported from the UK.

Table 3 Prevalence of the bla_{TEM} and $bla_{CTX\text{-}M}$ resistance genes in gram-negative bacterial isolates from 70 patients with sinusitis

Resistance gene	bla_{TEM}		$bla_{CTX\text{-}M}$		bla_{TEM} & $bla_{CTX\text{-}M}$	
Bacterial isolates	**Number**	**Percentage**	**Number**	**Percentage**	**Number**	**Percentage**
Escherichia coli	4 out of 11	(36.36%)	4 out of 11	(36.36%)	1	(9.09%)
Klebsiella spp.	5 out of 8	(62.50%)	4 out of 8	(50%)	2	(25%)
Pseudomonas spp.	1 out of 7	(14.29%)	1 out of 7	(14.29%)	0	(0%)
Total	10 out of 26	(38.46%)	9 out of 26	(34.62%)	3 out of 26	(11.54%)

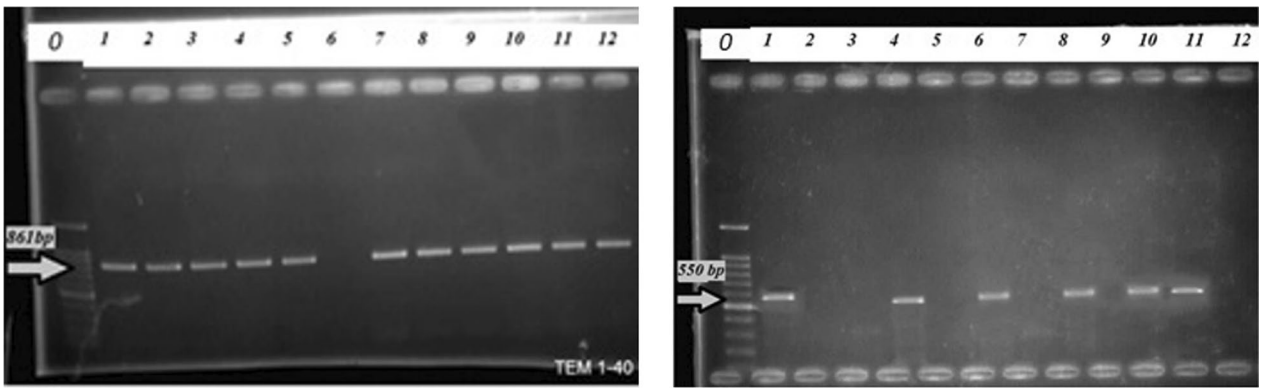

Fig. 1 bla_{TEM} (left image) and $bla_{CTX\text{-}M}$ (right image) genes PCR products. Image on the left: bla_{TEM} gene PCR products; lane 0 is the ladder; lane 1 is the positive controls; lane 6 is the negative control; lanes 2–12 (except lane 6) are sample results. Image on the right: $bla_{CTX\text{-}M}$ gene PCR products; lane 0 is the ladder; lane 1 is the positive control; lane 2 is the negative control; lanes 3–12 are sample results

Discussion

The study of the molecular mechanisms of antimicrobial resistance in bacterial rhinosinusitis has not received due attention in recent years, even though it is of significant clinical importance. To our knowledge, this study is one of the very few of its kind in recent years. However, findings from various phenotypical studies suggest great diversity in bacterial causative agents of sinusitis, in terms of both genus and species variation, and frequency of isolation. Bacterial agents most commonly reported in association with sinusitis include *Haemophilus influenzae, Streptococcus pneumonia*, *Staphylococcus species, Streptococcus species* and *Corynebacterium species* [3, 6, 8, 15, 24].

Other reported pathogens include Moraxella catarrhalis, Escherichia coli, Klebsiella Pneumonia and anaerobic bacteria, to a lesser extent [3, 9, 16]. In our study, the most commonly isolated bacteria were Group D Streptococci, *Corynebacterium* spp., *Staphylococcus aureus*, *Escherichia coli*, *Staphylococcus saprophyticus*, *Kebsiella* spp. and *Pseudomonas* genus. These results are in line with some previous studies [2, 3, 8], but not so with a number of other studies [16, 25]. This could be largely attributed to varied bacterial populations in different geographical areas, as well as differences in sample sizes, and sample collection and culturing methods [2].

Resistance to beta-lactam antibiotics was observed to be very significant, with 58.7% of all the isolates showing resistance to at least one beta-lactam antibiotic in the disk diffusion test. Similar results have been reported by other research groups in Iran [16, 25]. While phenotypic resistance to beta-lactam antibiotics in gram-negative bacteria was also similar to the above figure (57.69% resistant to at least one beta-lactam antibiotic), the molecular assessment (i.e., beta-lactamase genes PCR test) indicated that beta-lactam resistance genes are slightly more prevalent than detectable by phenotypic methods (61.54% of the isolates harboring at least one beta-lactamase gene). Other factors might also affect the expression of resistance genes, which bear further investigation [18]. No resistance was observed to amikacin, piperacillin–tazobactam and ampicillin–sulbactam, which confirms the accepted practice of prescribing beta-lactam antibiotics in conjunction with beta-lactamase inhibitors as the first-line therapy for bacterial sinusitis [15].

Of the two beta-lactamase genes investigated in this study, the bla_{TEM} gene had a slightly higher prevalence (38.46%) than the $bla_{CTX\text{-}M}$ gene (34.62%). This may help explain the poor resistance of our isolates to cefotaxime [26]. The relative prevalence of these two genes is very important from an epidemiological view point; Reports from different parts of the world indicate that the prevalence of the $bla_{CTX\text{-}M}$ gene, which was once markedly less than the bla_{TEM} gene, is now on the rise [27–29]. If a gradual increase be observed in the relative prevalence of the more recent ESBL gene (i.e., the $bla_{CTX\text{-}M}$ gene) in future studies, we could conclude that the bacterial infections might be originating from source outside our region. As mentioned earlier, however, although many research teams have investigated the bacteriology and antimicrobial resistance patterns in chronic and acute sinusitis, their studies have mainly been concerned with the phenotypic manifestation of beta-lactam resistance, and up-to-date literature on the prevalence of beta-lactamase genes among bacterial isolates from sinusitis patients is very limited.

Regardless of the species of bacteria harboring beta-lactamase genes, resistance to beta-lactam antibiotics could occur in multi-bacterial infections through the shielding phenomenon, if even only one species carries a beta-lactamase gene [2]. Therefore, it is prudent to consider using beta-lactamase inhibition in all chronic bacterial sinus infections.

Although a number of research groups have investigated the prevalence of bla_{TEM} beta-lactamase genes in *H. influenzae* and other sinusitis-related bacteria isolated from various infections, no recent studies have been conducted to determine the prevalence of this gene in other causative agents of chronic sinusitis at a molecular level [30–34]. These studies reported the prevalence of bla_{TEM} genes in beta-lactamase-producing isolates from various sources to be from 53.7 to 93.7% in different countries, which is higher than our results (38.46%). Hara et al. suggested that beta-lactamase genes could be transferred from one sinusitis agent to another [30], therefore, it is imperative to investigate the presence of these genes in all bacterial agents of sinusitis, especially in multi-bacterial chronic sinusitis.

Similarly, contemporary research on the prevalence of $bla_{CTX\text{-}M}$ gene in bacterial agents of sinusitis is even more limited than the bla_{TEM} gene. This could be attributed to the fewer number of sinusitis-related isolates reported to harbor this beta-lactamase gene. Bajpai et al. reported the prevalence of $bla_{CTX\text{-}M}$ beta-lactamase gene in isolates from urinary tract infections to be 7.6% [13]. In contrast, another study by Rajivgandhi et al. reported $bla_{CTX\text{-}M}$ to be the predominant beta-lactamase gene (53%) in gram-negative isolates from urinary tract samples in India [22], while in our study, 34.62% of the beta-lactamase-producing bacterial isolates carried the $bla_{CTX\text{-}M}$ gene. These varied results could be due to different geographical distribution of beta-lactamase genes, as well as different infection sources.

Our sequencing data analysis indicates that the $bla_{CTX\text{-}M}$ is the beta-lactamase gene with the most variation (69% to 100% similarity with other reported sequences), and

it is most similar to $bla_{CTX\text{-}M}$ sequences reported from the UK. This heterogeneity may suggest a greater rate of horizontal transfer as compared to the more conserved bla_{TEM} beta-lactamase gene (93–99% similarity with sequences reported from other countries). As a growing global concern in terms of extended-spectrum beta-lactamase, prevalence and variations of $bla_{CTX\text{-}M}$ genes need to be constantly monitored [13, 35]. The heterogeneity that we have observed in this gene's sequence could be a point of concern, as it might lead to more potent mutant beta-lactamases, and emerging new variations of the gene may escape detection by existing molecular methods.

Conclusion

The presence of antibiotic resistant bacteria in sinuses of patients with chronic sinusitis is indicative of the importance of effective antimicrobial therapy to overcome this major health problem. Our findings indicate that beta-lactam resistance patterns in our samples are in line with existing resistance patterns in other countries, and indeed have the same origin. Although the accepted combination of a beta-lactam antibiotic and a beta-lactamase inhibitor (such as amoxicillin–clavulanic acid) is evidently still effective as the first-line therapy, vigilance programs need to be put in place in all Iranian hospitals to monitor its continued effectiveness, as new resistance mechanisms are emerging in other parts of the world, which will inevitably affect us.

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Author contributions
All authors contributed to the study conception and design. Material preparation, data collection and laboratory procedures were performed by MAN, AA, DAP, KMY and SM. Data analysis was performed by MS and DAP. The first draft of the manuscript was written by MS, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
The datasets generated during and/or analyzed during the current study are in the care of the corresponding author (Dr. Sara Minaeian) and will be provided upon reasonable request.

Declarations

Ethics approval and consent to participate
All participants were aware of and consented to entering this study (Ethical Code for this study is IR.IUMS.REC.1395.28467).

Consent for publication
All participants were aware of and consented to entering this study and publication of the data (ethical code for this study is IR.IUMS.REC.1395.28467).

Competing interests
The authors declare that they have no competing interests.

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