Resistant bacteria a threat to antibiotics

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ABSTRACT
Objectives: The study was conducted to characterize the citywide epidemiology of ESBLs in Hail, to detect the frequency and types of enzymes by determining whether the responsible genes are spreading among different strains, or whether one or few strains are account for the spread of these enzymes among the local population. Methods: One hundred urine samples were collected and screened at local hospital during July- Aug 2011. The E.coli isolates resistant to third generation cephalosporins (ceftazidime). The CTX-M genes were detected by PCR and the RAPD typing done. E-Test was performed on 30 selected isolates and were reconfirmed by disc diffusion method for the confirmation of resistance. Results: Polymerase chain reaction (PCR) was used to detect the ESBLs genes. The collection of ESBLs positive isolate from the hospital and community for CTX-M were 78. Twenty two isolates did not contain blaCTX-M which may contain blaTEM, SHV or OXA genes (not tested). The overall prevalence of isolates of E.coli was high. ESBLs isolates were found in both the community and hospital, with the CTX-M type most common.

Keywords: ESBLs, polymerase chain reaction (PCR), E.coli, blaCTX-M.

INTRODUCTION
The cephalosporins are a class of β-lactam antibiotics. β-Lactam antibiotics are a broad class of antibiotics, consisting of all antibiotic agents that contains a β-lactam nucleus in its molecular structure. This includes penicillin derivatives (penams), cephalosporins (cephems), monobactams, and carbapenems. Together with cephamycins they belong to a sub-group called cephems.
Cephalosporin compounds were first isolated from cultures of cephalosporium acremonium from a sewer in Sardinia in 1948 by Italian scientist Giuseppe Brotzu. He noticed that these cultures produced substances that were effective against *Salmonella typhi*, the cause of typhoid fever. Researchers at the Sir William Dunn School of Pathology at the University of Oxford isolated cephalosporin C, which had resistance to β-lactamases but was not sufficiently potent for clinical use. The cephalosporin nucleus, 7-aminocephalosporanic acid (7-ACA), was derived from cephalosporin C and proved to be analogous to the penicillin nucleus 6-aminopenicillanic acid. Modification of the 7-ACA side-chains resulted in the development of useful antibiotic agents, and the first agent cephalothin (cefalotin) was launched by Eli Lilly in 1964. Cephalosporins are bactericidal and have the same mode of action as other β-lactam antibiotics (such as penicillins). Cephalosporins disrupt the synthesis of the peptidoglycan layer of bacterial cell walls. The peptidoglycan layer is important for cell wall structural integrity. The final transpeptidation step in the synthesis of the peptidoglycan is facilitated by transpeptidases known as penicillin binding proteins (PBPs). PBPs bind to the D-Ala-D-Ala at the end of muropeptides (peptidoglycan precursors) to crosslink the peptidoglycan. β-lactam antibiotics mimic this site and competitively inhibit PBP cross linking of peptidoglycan.

Cephalosporins are indicated for the prophylaxis and treatment of infections caused by bacteria susceptible to this particular form of antibiotic. First-generation cephalosporins are predominantly active against Gram-positive bacteria, and successive generations have increased activity against Gram-negative bacteria while reduced activity against Gram-positive organisms). Extensive studies have been made on effectiveness and the safety of the cephalosporin antibiotics.[1]

In general, bacteria have the genetic ability to transmit and acquire resistance to drugs, which are utilized as therapeutic agents[2]. Resistant bacteria are emerging worldwide as a threat to the favourable outcome of common infections in community and hospital settings. Nearly half century ago the antibiotic era began with the discovery of penicillin. With in few years of its discovery and use in medicine, some bacteria e.g *Staphlococcus aureus* showed resistant due to the production of penicillinase, with the ability to hydrolysing penicillin. Then there was an introduction of broad spectrum penicillins and first generation cephalosporins. In the past it was believed that cephalosporins were relatively immune to attack by β-lactamases, but it was surprising to find cephalosporin resistant *Klebsiella spp.* among clinical isolates. Increasing resistance to third-generation cephalosporins amongst *E. coli* and *Klebsiella spp* is predominantly due to the production of extended-spectrum β-lactamases (ESBLs). ESBLs are defined as beta-lactamases capable of hydrolyzing oxyimino cephalosporins[3]. These β-lactamases are resistant to inactivation by β-lactamase inhibitors(clavulanic acid, sulbactum, tazobactam). These plasmid mediated enzymes mostly evolved via point mutations of the classical TEM-1 and SHV-1 β-lactamases but other groups are increasingly prominent, notably the CTX-M types, which evolved via the escape and mutation of chromosomal β-lactamases from *Kluyvera* spp.[4] These plasmids are easily transmissible in and between bacterial species[5]. T ESBL producers are associated with increased morbidity and mortality, especially amongst patients on intensive care and high-dependency units. Plasmids responsible for ESBLs production frequently carry genes encoding resistance to other drug classes. Therefore antibiotic options in the treatment of choice for serious infections due to ESBLs producing organisms are
extremely limited [6]. The abuse of broad spectrum chemo-therapeutants has resulted in an increased number of antibiotic-resistant bacteria.[7]. Wide spread use of third generation cephalosporins and aztreonem is believed to be the major cause of mutations in these enzymes that has led to the emergence of ESBLs. Other risk factors for colonization or infection with ESBL producing organisms are long term antibiotic exposure, prolonged ICU stay, nursing home residency, severe illness, instrumentation or catheterisation. Accurate laboratory detection is important to avoid clinical failure due to inappropriate antimicrobial therapy. Treatment of infections in non-hospitalized patients caused by these multi drug resistant ESBL-producers is difficult or impossible due to lack of available antimicrobials. In hospitalized patients adequate empiric treatment may be delayed which may increase both morbidity and mortality. Endemic ESBLs lead to increase use of carbapenems with emerging new resistance problems. The strains of ESBLs remain difficult to isolate. Although epidemiology has been found to vary between hospitals, in one institution there were 16 different stains in 18 different cases. such variations has made ESBLs difficult to detect.[8]

The production of extended-spectrum β-lactamases (ESBLs) by Enterobacteriaceae has been documented since the introduction of third-generation cephalosporins (3GCs) into clinical usage.[9] Whereas plasmid-mediated or hyperproduction of AmpC-type and other β-lactamases have been the most common cause of resistance to 3GCs, ESBL-producing Enterobacteriaceae have recently become more frequent in the UK. Currently, the ESBLs associated with 3GC-resistant Enterobacteriaceae can be divided into the ‘big three’ families of TEM-, SHV- and CTXM-type β-lactamases. [9] TEM and SHV variants are reliant on key amino acid substitutions to increase their substrate profile to include the 3GCs, whereas the CTX-Ms have an intrinsic extended-spectrum profile. A national survey on the prevalence and mechanisms of resistance to 3GCs in clinically relevant Enterobacteriaceae in the UK was last conducted over a decade ago, where only TEM- and SHV-type ESBLs were screened for at a molecular level. The findings of this report gave an ESBL phenotype frequency of 1% of unselected isolates of Enterobacteriaceae from a wide range of locations. Since this time, we have seen the emergence and global dissemination of the CTXM-type β-lactamases, which have become the predominant ESBL type [10,11] in a number of Asian and South American countries. During the past 15 years, CTX-M-type ESBLs have undergone a rapid and global spread. Enterobacterial strains producing these enzymes have now been reported almost everywhere and, in some settings, CTX-M-type enzymes outnumber the classic TEM- and SHV-type ESBLs [12, 13]. This massive worldwide dissemination, which could be referred to as the ‘CTX-M pandemic’, is one of the most striking examples of rapid and global dissemination of plasmid-mediated resistance determinants among bacterial pathogens, and has been compared to the dissemination of the broad-spectrum TEM type β-lactamases observed since the 1960s. The reason(s) for such an explosive dissemination of CTX-M-type ESBLs in Enterobacteriaceae remain(s) to be clarified. Carriage on plasmids that are highly efficient at conjugal transfer, and/or a lower fitness cost imposed by these enzymes and cognate genetic elements upon the bacterial hosts (as compared with other types of ESBLs), could be included among the possible explanations for the remarkable success of CTX-M-type enzymes, as compared with other types of ESBLs.

In Europe, (ESBLs were discovered in Germany [14])where the TEM- and SHV-type ESBLs were first described and have played a major role as ESBL determinants, the CTX-M-type ESBLs have recently achieved a remarkable diffusion in several countries [15]. A remarkable
diffusion of CTX-M-type ESBLs has been observed in Spain [16] with a rate of CTX-M enzymes were found to be 52.3% among ESBL producing isolates of \textit{E.Coli}, with a predominance of group 9. The spread of CTX-M-producing strains was first reported in eastern Europe [17,18], but has subsequently involved also western and southern European countries. One of the most striking examples of rapid dissemination of these ESBLs has been reported in the UK, where CTX-M enzymes were first reported in 2000 and have subsequently undergone an explosive spread involving \textit{E. coli} and also \textit{K. pneumoniae}, with a predominance of group 1 enzymes (mostly CTX-M-15) [19].

Recent European studies have confirmed the persistence of TEM and SHV ESBLs and the increase in CTX-M producers among \textit{Enterobacteriaceae}. The identification of other ESBLs remains rare, and when detected they have been mostly found in \textit{P. aeruginosa} and \textit{Acinetobacter} [21]. Pooling different European studies, it seems that certain isolates expressing specific enzymes are better adapted to specific environments and geographical areas. Moreover, in the last few years, new ESBL variants from different families, including CTX-M, have emerged. In addition, it has been suggested that co-resistance might have played a relevant role in the current endemic situation, allowing the maintenance of ESBL-producing organisms. Other ESBLs are linked to specific clones and/or plasmid incompatibility groups, and have been shown to be widespread in some European countries [21].

\textit{CTX-M family}

CTX-M beta-lactamases are commonly found in \textit{K. pneumoniae}, \textit{E. coli}, thypoidal, and non thypoidal \textit{Salmonella}, \textit{Shigella}, \textit{Citrobacter freundii}, \textit{Enterobacter} spp., and \textit{Serratia marcescens}. Within the CTX-M family, the CTX-M-9 cluster has been shown to be highly represented in Spain. In particular, CTX-M-14 is frequently detected in \textit{E. coli} isolates recovered from non-hospitalised patients [21]. These enzymes have also been reported in Portugal, France and the UK, but are infrequent in other European countries. Within the CTX-M-1 cluster, CTX-M-1 and CTX-M-32 were originally found to be prevalent within the Mediterranean area, but nowadays they are prevalent in nearly all European countries.

CTXM-15 has risen to prominence all over Europe, whereas CTX-M-3 is mainly described in eastern European countries [21]. CTX-M-15 was initially detected in an isolate recovered in India in 2001 [21]. It is distinguished from CTX-M-3 by a point mutation that increases hydrolytic activity against ceftazidime. CTX-M-15-producing isolates have been increasingly recognised in community isolates, particularly in healthcare-associated patients, and more recently in the nosocomial setting [21]. In the UK, there were many reports of TEM and SHV ESBLs during the 1990s but despite rare large outbreaks producers did not become common. The successful dispersion of CTX-M-15 has been associated with specific clones, particularly in the UK, and the transfer of specific epidemic plasmids harbouring the \textit{blaCTXM-15} gene. These
epidemic plasmids have been identified in Europe (Spain, France, Portugal, Austria, UK), Africa (Tunisia and Central Africa Republic) and North America (Canada) [21]. It is of note that some of the epidemic E. coli clones expressing CTX-M-15 in Europe, the CTX-M-8 cluster is only represented in the UK, by the CTX-M-40 variant CTX-M-26 from the CTX-M-25 cluster has also been detected in the UK.

CTX-M β-lactamases, of which there are now more than 40 types, can be divided into five major groups based on their amino-acid identities. In the UK, an epidemic clone producing CTX-M-15 is thought to be widely distributed.

Control of infection classically involves hand and healthcare hygiene, reduction of selective and ineffective chemotherapy, reduction of invasive procedures and achlorhydria and adequate staffing, along with appropriate containment and concentration of patients. Investigation and control of any continuing sources of infection in food and water supplies is important also, as is recognition of individuals carrying high-risk strains and species. The onset of infection may be distant from the time of acquisition and may critically affect epidemiological assessment of control points. Carriage may be prolonged, increasing the likelihood of recurrent infection and exacerbating the difficulty of control. Mortality associated with resistance is difficult to assess retrospectively and may not be high, complicating analysis of the success or failure of control measures.

The aim of the present study is to characterize the citywide epidemiology of ESBLs, to detect the frequency and types of these enzymes by determining whether the responsible genes are spreading among different strains, or whether one or a few strains account for the spread of these enzymes in the local population. The material consists of about 100 case-patients collected during July -Aug 2011.

EXPERIMENTAL SECTION

Bacterial strains

100 resistant (3rd generation cephalosporin-ceftazidime) E.coli isolates were collected randomly from urine samples during the period July 2011 to August 2011 were accessed for CTX-M screening.

Susceptibility testing by E-Test

ESBLs E Tests were used in order to reconfirm the resistance of the selected isolates. The E Test was performed using E test strips were purchased from (AB Biodisk). E Test strips were stored at -20°C to +8°C, as recommended by the manufacturer. The E test called TZ/TZL is two sided strips containing gradients of ceftazidime (TZ) at one end and, combined with (TZL) clavunate on the other end.

Single colonies were picked into nutrient broth. These broths were incubated overnight at 37°C in hot room. Using cotton swab colonies were plated out on isosensitive agar plate. The strips were applied in the centre of the plates and were incubated overnight.

Readings were noted at the point of intersection of the inhibition zone with the strip.
The isolates were considered true ESBLs when the MIC value of tested isolates is reduced by one fourth in the presence of clavunate.

E Test susceptibility was determined after 24 hours of incubation at 37°C.

**Susceptibility testing by Disc diffusion method**

8 isolates which failed to show any resistance by E test were tested for ampicillin sensitivity by disc diffusion method. Iso sensitive agar plates were supplied by Oxoid Pharma, Limited, UK and ampicillin discs were obtained from same company.

Plates were streaked in two directions at 90° using standard microbiology laboratory techniques. Ampicillin discs were placed in the centre of the discs. Plates was incubated upside down. Disc diffusion susceptibility was determined after 24 hours of incubation at 37°C.

**Control Strains used for PCR**

As positive controls for amplicons of \( \text{bla}_{\text{CTX-M}}, \text{bla}_{\text{TEM}}, \text{bla}_{\text{SHV}}, \text{bla}_{\text{OXA}} \) genes, the strains of \( E.coli \) CTX-M 1 (ED 499), CTX-M 8, CTX-M (E 395), \( E.coli \) KT 1118, \( E.coli \) 1059, \( E.coli \) KT 1114 were used respectively.

**PCR**

Presence of \( \beta \)-lactamases genes was investigated by PCR amplification using the primers listed in Table No 1 [22]

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>size of product(bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \text{bla}_{\text{CTX-M}} )</td>
<td>CTX-M-fMA3</td>
<td>5'-CAAACCGTTGGGTACGAT</td>
<td>693</td>
</tr>
<tr>
<td>CTX-M-cxr1</td>
<td>5'-CGTGGGTTACGATTTCGC</td>
<td>684</td>
<td>22</td>
</tr>
<tr>
<td>CTX-M-cxr2</td>
<td>5'-AACCAGTGGGTACGACGAT</td>
<td>684</td>
<td>22</td>
</tr>
<tr>
<td>CTX-M-cxr9</td>
<td>5'-AACCAGTGGGTACGACGAT</td>
<td>683</td>
<td>22</td>
</tr>
<tr>
<td>ERIC2</td>
<td>5'-AAGTAAGTGACGGGAGGCACGAG</td>
<td>683</td>
<td>22</td>
</tr>
</tbody>
</table>

Sequence of primers used to identify CTX-M PCR products

**Preparation of DNA extracts**

Single colonies were picked from each isolate with the help of inoculation loop, into 100 µl of molecular graded water in 200 µl labeled tubes.

Omn E thermal cycler was used for DNA lysis. Heat treatment (98°C for 15 min) was done to prepare the template DNA from the control and clinical isolates.

The suspension recovered from the extraction was used as a DNA template for amplification by PCR.
**Gel preparation**

The amplification products were run on 1.5% agarose gel with 1 X Tris borate EDTA buffer to confirm the presence and size. The gel was stained with ethidium bromide (5 \( \mu g / ml \)) and photographed under UV light. [23]

**CTX-M**

Multiplex PCR amplifications was performed in 200 \( \mu l \) small reaction tube (illust puReTaq Ready-To-Go PCR Beads) as previously described.25 \( \mu l \) final volume containing 0.5 \( \mu l \) of the universal forward primer fMA3 (bla\(_{\text{CTX-M-fMA3}}\), 0.5 \( \mu l \) bla\(_{\text{CTX-M cxr1}}\), 0.5 \( \mu l \) bla\(_{\text{CTX-M cxr2}}\), 0.5 \( \mu l \) bla\(_{\text{CTX-M cxr8}}\), 0.5 \( \mu l \) bla\(_{\text{CTX-M cxr9}}\), 20.5 \( \mu l \) of molecular graded water and 2 \( \mu l \) of DNA. The primers listed above are designed to give product size of 693 bp (CTX-M-1 group), 684 bp (CTX-M-2 group), 684 bp (CTX-M-8 group) and 683 bp (CTX-M-9 group).[24].

A negative (no DNA template) control and a positive control containing control strain of CTX-M were used. Every effort was made to avoid nucleic acid contamination as described above.

DNA was amplified with Omn E thermal cycler using the following cyclic parameters: 1 cycle consisting denaturation at 94°C for 1 min, and then 30 cycles with 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min, followed by extension at 72°C for 10 min.

The amplification products were run on 1.5% agarose gel with 1 X Tris borate EDTA buffer to confirm the presence and size. The gel was stained with ethidium bromide (5\( \mu g / ml \)) and photographed under UV light.

**RAPD**

To obtain more insight into wider distribution of ESBL producing isolates of *E.Coli*, it was decided to type the *E.coli* by RAPD.

PCR amplifications was performed in 200 \( \mu l \) small reaction tube (illust puReTaq Ready-To-Go PCR Beads). 25 \( \mu l \) final volume containing 1 \( \mu l \) ERIC-2, 22 \( \mu l \) of molecular graded water and 2 \( \mu l \) of DNA.

DNA was amplified with Omn E thermal cycler using the following cyclic parameters: 1 cycle consisting denaturation at 94°C for 2 min, and then 36 cycles with 94°C for 1 min, 36°C for 1 min, and 72°C for 3 min.

The amplification products were run on 1.5% agarose gel with 1 X Tris borate EDTA buffer to confirm the presence and size. The gel was stained with ethidium bromide (5\( \mu g / ml \)) and photographed under UV light. A molecular size (DNA) ladder was used on all gels. Banding patterns were evaluated visually.
RESULTS

Fig.1 Agarose gel electrophoresis of products obtained by CTX PCR. Lanes: L, DNA Ladder:55-69, E.coli isolates numbering from 55 to 70.

Detection and identification of CTX-M genes
A multiplex PCR was designed to detect $bla_{CTX-M}$ in ESBLs E.coli isolates. Each PCR amplification reaction produced a specific DNA fragment of the expected size; see Table 1. 78 out of 100 isolates were positive for CTX-M genes. However, as the CTX-M reaction was performed as a multiplex reaction, and the amplification products were very similar in size (Table 1), it was not possible to differentiate between the different CTX-M enzyme families.

RAPD-typing
In order to illustrate the epidemiology of the ESBL producers in the area of Nottingham, some examples of RAPD are presented in Fig 2. The 100 ESBL producing E.Coli isolates collected from Queen’s Medical Centre, City Hospital and community displayed various distinct profiles. Several comprising single isolates and 2 corresponding to clusters: cluster 1 with 30 isolates and cluster 2 with twenty five isolates (Table 2). All remaining isolates showed different banding patterns by RAPD typing and were considered different strains. Some of them being very close to one another, but clearly distinguishable from each other. On the basis of global analysis it was observed that clonal strains were more common than unique strain in the area.

Table 2

<table>
<thead>
<tr>
<th>Clone</th>
<th>Number of isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>30</td>
</tr>
<tr>
<td>B</td>
<td>25</td>
</tr>
<tr>
<td>C</td>
<td>6</td>
</tr>
<tr>
<td>D</td>
<td>4</td>
</tr>
<tr>
<td>E</td>
<td>3</td>
</tr>
<tr>
<td>Sporadic</td>
<td>22</td>
</tr>
</tbody>
</table>

DISCUSSION

Out of 100 selected E.coli isolates, 78 were positive for CTX-M. Fifteen isolates did not contain $bla_{CTX-M}$. Absence of or $bla_{CTX-M}$ in fifteen isolates may contain $bla_{TEM}, blaoXA$ or $bla_{SHV}$, cannot
be excluded which were beyond this study. These results are shown in Fig.3. The results are in accordance with production of CTX-M as the most common ESBL.

This study gives an idea of the current prevalence and molecular types of ESBLs producing organisms in both community and hospital settings.

A similar study done in 1990-1991 regarding prevalence and types of ESBLs produced by Enterobacteriaceae in UK hospitals showed a frequency of 1% ESBLs, and most common was found to be SHV-type.

That previous survey indirectly reflects the possibility of $\text{bla}_{\text{CTX-M}}$ at that time also.

This comparison of previous study shows that there is sudden increase in number and patterns of ESBLs producer is in accordance to world wide spread of ESBLs in a very short time. A survey conducted in public hospitals of Argentina consider -CTX-M as 66% of ESBLs found.[24] Studies from China [25], Japan [26]and Taiwan[27] and Spain[28], also shows predominant prevalence of CTX-M[29]. On the other hand studies done in early 1990s demonstrated absence or very few occurrences of CTX-M-type $\beta$-lactamases.

Several limitations should be kept in mind in the interpretation of this study. First no information was available on the resistant pattern of collected $E.\text{coli}$ samples. The isolates of $E.\text{coli}$ proved to be positive for CTX-M type ESBLs were not further investigated for sub types .We did not study the two other common ESBLs genes including TEM, OXA and SHV genes. Additional analyses needs to be performed for further characterization and sub typing.

In conclusion our data suggests: that CTX-M type ESBLs are most common in both hospital and community settings in the area. Results shows that the responsible genes (ESBLs) are spreading among different strains, and at the same time, few of these strains contribute a major role for the spread of these enzymes in the local population. Hence, both processes are involved in the spread of resistance. Therefore, it is required to take every possible step to restrict the further dissemination of the resistant strains. It may include continuous monitoring of data by regular surveys and studies, reviewing antibiotic policy and its effective implementation, improving personal hygiene by health education campaigns and public awareness.

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