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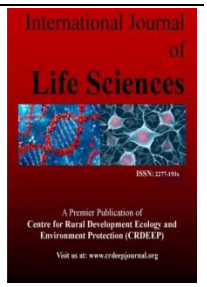

Full Length Research Paper

Isolates of *Echerichia. coli* and *Klebsella. pneumoniae* from microbiology laboratories, Hamad Medical Corporation (HMC) Qatar.

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ABSTRACT

Antimicrobial resistant pathogens increasingly pose a serious challenge to clinicians, infection control personnel and health care administrators. There is a large reservoir of resistance genes, in bacterial genomes and in extra-chromosomal pieces of DNA, that encode different mechanisms of drug resistance. A total of 134 consecutive nosocomial isolates of E. coli and K. pneumonia selected from in between 500 urine isolates collected from microbiology lab, Hamad Medical Corporation between September 2003 and July 2005, were tested for antibiotic resistance using Vitek1. All isolates were uniformly resistant to ampicillin in addition to at least two more ß-lactams. Plasmid DNA was isolated and screened by PCR for the presence of genes encoding beta-lactamases of SHV, TEM and CTX-M types. SHV-specific PCR revealed 25 blaSHV genes, 16 in E. coli and 9 in K. pneumoniae. In 71 strains, blaTEM genes were detected, 47 in E. coli and 24 in K. pneumoniae. To differentiate ESBL and non-ESBL among SHV genes, further analysis of PCR products was performed. Sixteen strains carried blaESBL-SHV genes. In a further five isolates a CTX-M gene was detected. The CTX-M genes could be affiliated to the CTX-M-8 cluster by RFLP analysis. Common plasmid restriction patterns among TEM producers provided evidence of horizontal spread. The importance of pre molecular detection is discussed.

Introduction

Antimicrobial resistant pathogens increasingly pose a challenge to clinicians, infection control personnel and health care administrators. The introduction of the third-generation cephalosporins into clinical practice in the early 1980s was heralded as a major breakthrough in the fight against ß-lactamase-mediated bacterial resistance to antibiotics. These cephalosporins had been developed in response to the increased prevalence of ß-lactamases in certain organisms, and the spread of these ß-lactamases into new hosts (Bradford P. A. 2001) Not only were the third-generation cephalosporins effective against most ß-lactamase-producing organisms but they had the major advantage of lessened nephrotoxic effects compared to aminoglycosides and polymyxins (Bisson et al, 2002).

The first report of plasmid-encoded ß-lactamases capable of hydrolyzing the extended-spectrum cephalosporins was published in 1983 (Knothe et al.1983). The gene encoding the ß-lactamase showed a mutation of a single nucleotide compared to the gene encoding SHV-1. Other ß-lactamases were soon discovered which were closely related to TEM-1 and TEM-2, but which had the ability to confer resistance to the extended-spectrum cephalosporins (Brun-Buisson, 1987, Sirot et al, 1997). Hence these new ß-lactamases were coined extended-spectrum ß-lactamases (ESBLs). These are detailed on the authorative website on the nomenclature of ESBLs hosted by Jacoby and Bush (http://www.lahey.org/studies/webt.htm). Published research on ESBLs has now originated from more than 30 different countries, reflecting the truly worldwide distribution of ESBL-producing organisms. In 100% of these studies, at least two patients were colonized or infected with genotypically similar strains, implying patient-to-patient transmission of the strain (Paterson and Bonomo, 2005). Transfer of genotypically related ESBLs from hospital to hospital within a single city (Bisson et al. 2002, Monnet et al. 1997, Sader et al. 1994,Yuan et al.1998), from city to city (Yuan et al.1998), and from country to country (Fiett et al. 2000, Gori et al. 1996, Shannon et al. 1990, Yuan et al.1998) has been documented. Intercontinental transfer has also been described (Shannon et al. 1990). The aim of this study was to investigate the presence of resistance genes among two gram negative pathogens; *Escherichia coli and Klebsiella pneumoniae,* causing urine tract infection at Hamad Medical Corporation, Qatar and to evaluate

molecular diagnosis methods for the detection of the common resistant genes.

Materials and methods

500 urine specimens from hospitalized patients with Urine Tract Infections (UTIs) at Hamad Medical Corporation (HMC), Doha, Qatar were inoculated on Cysto-Pyrin Electrolyte Deficient (CED) medium with standard loop and incubated in an incubator at 37ºC for 24 hr. Viable bacterial counts revealed significant bacterial number. Colonies of each type were sub-cultured in peptone water for purity, and identified to species level using a series of standard biochemical tests according to Murray et al. (1999). Confirmation was done using the AP120 STEP (Biomeriex Vitec, USA). A total of 134 specimens (87 *Escherichia coli* and 47 *Klebsella pneumonia*) were identified, serially numbered and kept frozen at -20˚C under specific code till used for further biochemical and molecular investigations.

Bacterial strains. A total of 134 specimens of *Escherichia coli and Klebsella pneumonia* strains included in this study were selected from in between 500 urine isolates collected and isolated between September 2003 and July 2005 from urine specimens of patients hospitalized at Hamad Medical Corporation (HMC), Doha, Qatar. The isolates were identified using a series of standard biochemical tests according to Murray et al. (1999) and were phenotypically tested for production of β-lactams by the Vitec 1 test.

Preparation of plasmid DNA. Plasmid DNA was obtained using a modified alkaline-lysis method (Sambrook *et al.*, 1989), with slight modifications. Briefly, a further purification step with phenol/chloroform was added before DNA precipitation in 2-propanol. Plasmids were separated on a 0.8 % (w/v) agarose gel in Tris/borate/EDTA buffer for 1.5 h at 120 V

PCR. In order to detect genes encoding Ambler class A beta-lactamases (*bla*), a standard PCR was performed with plasmid DNA as a template. For amplification of genes encoding SHV beta-lactamases (bla_{SHV}), primers MN I (5'-CGC CGG GTT ATT CTT ATT TGT CGC-3') and MN II (5'-TCT TTC CGA TGC CGC CGC CAG TCA-3') were used (Nüesch-Inderbinen *et al.*, 1996). The thermal cycling conditions used for the PCR with these primers included 30 cycles at 94 °C for 30 s, 68 °C for 60 s, and 72 for 60 s, with a final extension of 72 °C for 300 s. The 1016 bp PCR product contained the entire ORF. For amplification of genes encoding TEM beta-lactamases (bla_{TEM}), primers TEM-F (5'-ATA AAA TTC TTG AAG ACG AAA-3') and TEM-R (5'-GAC AGT TAC CAA TGC TTA ATC A-3') were used with thermal cycling conditions as described elsewhere (Wu *et al.*, 2001). The 1080 bp amplicon reached from 214 bp upstream of the start codon to the stop codon. In order to detect genes for CTX-M beta-lactamases (bla_{CTX-M}), the following primers were used: CTX-M (5'-CCG CGC TAC ACT TTG TGG C-3') and primer CTX-M (5'-TTA CAA ACC GTT GGT GAC G-3'). The cycling conditions used for the PCR with these primers were 35 cycles of 94°C for 45 s, 56°C for 45 s, and 72 for 45 s, with a final extension of 72°C for 10 min (Oliver et al, 2001). CTX-M primers were provided and certified by The Midland Certified Reagent Company Inc, Midland, Texas. The amplicon is a 544bp intragenic fragment. Ten micro liters of PCR product was electrophoresed in a 1 % (w/v) agarose gel for 1 h at 140 V together with a Smart ladder DNA standard (Eurogentec).

Differentiation between $bla_{ESBL-SHV}$ and $bla_{non-ESBL-SHV}$ in a PCR product.

As described elsewhere (Nüesch-Inderbinen *et al.*, 1997), SHV-ESBL genes contain an *Nhe*I restriction site, in contrast to $SHV_{non-ESBL}$ genes. Thus, resulting in two fragments that could be separated from each other. A 40 µl aliquot of the corresponding PCR product was digested with 5 µl *Nhe*I (10 U μl^{-1}; Fermentas) and 5 µl 10x Buffer (Fermentas) with BSA for 3 h at 37 °C. Digested DNA was separated on 1.5 % (w/v) agarose gels for 2 h at 140 V. Staining, de-staining and documentation as described above.

RFLP analysis of bla_{CTX-M}.

A further subgrouping of CTX-M was performed according to Edelstein et al (2003). For this purpose, 10 µl of the products of CTX-M-specific PCR were digested with 0.9 µl *Pst*I (10 U μl^{-1}), 0.2 µl *Pvu*II (20 U μl^{-1}), 2 µl 10-fold NEBuffer 3 (enzymes and buffer, New England BioLabs) and 6.9 µl nuclease-free distilled water at 37 °C for 3 h. Restriction fragments were electrophoresed in a 3 % (w/v) agarose gel for 2 h at 140 V together with the DNA standard Smart ladder SF (Eurogentec). Restriction patterns were compared with the expected ones described in the original publication (Edelstein et al. 2003) and up-dated by Joachim et al, 2007 to include the recently discovered CTX-M subgroups.

Results

Plasmid DNA analysis: Plasmid DNA was obtained in all of the strains. In all cases, the predominant plasmids were large ones (above 20Kb). The plasmid profile typing criteria (Sahm F.D., 1996), is used to determine the plasmid relatedness among strains based on the similarities in the digested DNA electrophoresis patterns (table 1).

Polymerase chain reaction SHV-specific PCR revealed 25 blaSHV genes (fragment size is 930 Kb), 16 in *E. coli* and 9 in *K. pneumoniae*. Figures (1). Since the above-mentioned SHV and TEM primers are specific for all blaSHV and blaTEM, including the non-ESBL variants, further analysis of the PCR products was necessary.). In 71 strains, blaTEM genes were detected, 47 in *E. coli* and 24 in *K. pneumoniae* (Figure 2). Finally, PCR revealed only 5 CTX-M genes in E. coli (figure 3) and non in K. *pneumonia.*

Restriction Fragment Length Polymorphism (RFLP): The presence of an NheI site in the SHV-specific PCR product will give two bands, and its absence will result in no cleavage and, thus, in a full-length fragment of 930 bp (Nüesch- Inderbinen et al, 1996, Rasheed et al, 1997). According to this criteria we found 9 of the 16 E. coli isolates carrying SHV genes were of ESBL type, while 8 out of 10 of K. pneumoniae isolates carrying SHV genes were of ESBL type (Figure 4). RFLP analysis of PCR products was performed in order to obtain information about the cluster affiliation of the CTX-M genes found. Non of the two restriction enzymes cleaved the CTX-M genes we have got, Non of the two restriction enzymes cleaved the CTX-M genes we have got.

Table 1: Plasmid patterns shown by *E. coli & K.pneumoniae* isolates.

Plasmid pattern	Size in kb	E.coli	K.pneumoniae	total	%of total	
1	20,9,6	24	17	41	33.10%	
2	20,8,5	11	4	15	12.10%	68.60%
3	20,5	19	10	29	23.40%	
4	5	1	5	6	4.80%	
5	2,3	17	4	21	16.90%	31.40%
6	2	10	2	12	9.70%	
Total		82	42	124		

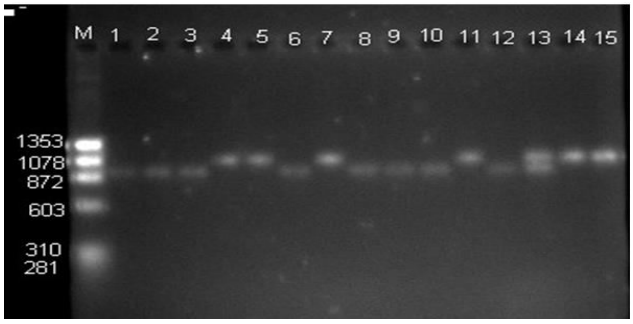

Fig 1. Agarose gel electrophoresis of a mixture of PCR products for amplification of TEM and SHV resistance genes from representative plasmids isolated from multi-drug resistant Escherichia coli isolates .M = DNA size marker, 13= positive control, 1-12 = co17, 18, 22,6,15 ,39,24,58,59,60,25,61. respectively, 14=27, 15=29.

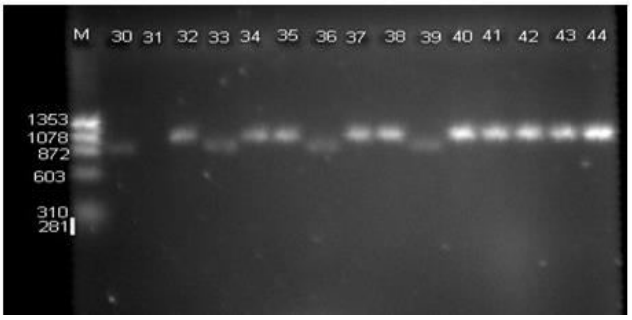

Fig 2. Agarose gel electrophoresis of a mixture of PCR products for amplification of TEM and SHV resistance genes from representative plasmids isolated from multi-drug resistant K. pneumani isolates . M = DNA size marker, 30=Eco64,31=negative control,32=Eco72,33=Eco65, 34 &35=Eco73&75,36=Eco66,37&38=Eco78&79,39=Eco71,40-44=Eco80-84.

Fig 3. Agarose gel electrophoresis of a mixture of PCR products for amplification of CTX-M resistance genes from representative plasmids isolated from multi-drug resistant Escherichia coli isolates. M = DNA size marker, 30=Eco64,-ve=negative control, 1-5=Eco5, 19,26, 68 & 69, respectively.

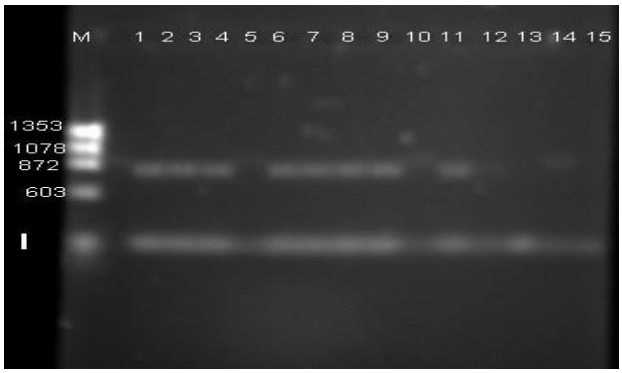

Fig 4. Nhe1 analysis of PCR products for amplification of SHV resistance genes from representative plasmids isolated from multi-drug resistant Escherichia coli & K. pneumani isolates. M = DNA size marker, 1-10= Eco17,22,58,59,61,62,Klp10,16,27&29.

Discussion

The determination of antimicrobial susceptibility of a clinical isolate is often crucial for the optimal antimicrobial therapy of infected patients. The need is increasing with increasing resistance and the emergence of multidrug-resistant microorganisms (Fluit et al, 2000, 1999). Testing is required not only for therapy but also to monitor the spread of resistant organisms or resistance genes throughout the hospital and community.

TEM-1 is the most commonly encountered β-lactamase in gram negative bacteria. Up to 90% of ampicillin resistance in *E. coli* is due to the production of TEM-1 (Livermore, 1995). TEM-1 is able to hydrolyze penicillins and early cephalosporins such as

cephalothin and cephaloridine. In this study TEM accounts for resistance in only 47 (54%) of *E. coli* isolates and 24 (51%) of *K. pneumonia* isolates.

As shown by Bradford, 2001, the amino acid substitutions that occur within the TEM enzyme occur at a limited number of positions. The combinations of these amino acid changes result in various subtle alterations in the ESBL phenotypes, such as the ability to hydrolyze specific oxyimino-cephalosporins such as ceftazidime and cefotaxime. A number of amino acid residues are especially important for producing the ESBL phenotype when substitutions occur at that position. They include glutamate to lysine at position 104, arginine to either serine or histidine at position 164, glycine to serine at position 238, and glutamate to lysine at position 240 (Perilli, 1997). Primers are usually chosen to anneal to regions where various point mutations are not known to occur. However, PCR will not discriminate among different variants of TEM or SHV. Several molecular methods that will aid in the detection and differentiation of ESBLs without sequencing have been suggested.

The SHV-1 β-lactamase is most commonly found in *K. pneumoniae* and is responsible for up to 20% of the plasmid-mediated ampicillin resistance in this species (Tzouvelekis and Bonomo, 1999). Similar results were obtained in our study, 9 out of 47 of K. *pneumonia* isolates were found to bear plasmids with SHV resistance genes and 16 out of 87 of E. *coli* isolates showed plasmids with SHV resistance genes (Figures 3.4-3.7), In many strains of *K. pneumoniae, bla*$_{SHV-1}$ or a related gene is integrated into the bacterial chromosome (Livermore, 1995). This may account for the antibiotic resistance in the remaining 13 isolates of K. *pneumonia* that no resistance genes were detected in their plasmids. Unlike the TEM-type β-lactamases, there are relatively few derivatives of SHV-1. Furthermore, the changes that have been observed in *bla*$_{SHV}$ to give rise to the SHV variants occur in fewer positions within the structural gene.

Restriction Fragment Length Polymorphism (RFLP):

The presence of an *Nhe*I site in the SHV-specific PCR product will give two bands, and its absence will result in no cleavage and, thus, in a full-length fragment of 930 bp (Nüesch-Inderbinen et al, 1996, Rasheed et al, 1997). According to this criterion we found 9 of the 16 *E. coli isolates* carrying SHV genes were of ESBL type, while 8 out of 10 of K. *pneumonia* isolates carrying SHV genes were of ESBL type. In recent years a new family of plasmid-mediated ESBLs, called CTX-M, that preferentially hydrolyze cefotaxime has arisen. These enzymes are notclosely related to TEM or SHV β-lactamases in that they show only approximately 40% identity with these two commonly isolated β-lactamases in this study we have got only 5 E. *coli* isolates carrying CTX-M type of genes and none of the K. *pneumonia* isolates. RFLP analysis of PCR products was performed in order to obtain information about the cluster affiliation of the genes found. Restriction patterns were compared with the expected ones described Edelstein et al, 2003 (Tzouvelekis et al, 2000). None of the two restriction enzymes cleaved the CTX-M genes we have got, indicating that they are all of the CTX-M-8 type.

With respect to the Vitek 1 ESBL test, it is known that it cannot distinguish between a K1 hyperproducer and a 'classical' ESBL carrier (Leverstein-van Hall et al., 2002) Although K1 was assigned to group 2be (ESBL) within the functional beta-lactamase classification scheme of Bush–Jacoby–Medeiros (Bush et al.,1995), in fact, ESBL detection in K1 hyperproducers represents a false-positive result, because it is not necessary to regard such strains as resistant to all cephalosporins independently of their resistance patterns, as is the case with ESBL carriers. Moreover, Paterson et al. (2001) have described the higher risk of therapy failure in the treatment by oxyimino-cephalosporins of infections with ESBL-producing strains, even if the strains appear to be susceptible *in vitro*. In this context, the importance of molecular diagnostics will increase despite their cost, as they are more reliable.

Conclusion

In conclusion, it may be difficult to detect and interpret the presence of low-level resistance using phenotypic tests that provide general information about the involved resistance mechanisms only. Alternatively, molecular techniques could be the choice to show the possible resistance mechanism that can be involved. Molecular detection gives a definite answer for the presence or not of specific resistance determinants such a beta-lactamase within a study isolate. However, molecular assays may also show some limitations technically and costly.

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