Nosocomial infections: Importance of rapid and early detection of ESBL Enterobacteriaceae by molecular biology

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Abstract. Nosocomial infections are a major public health problem. They are mainly caused by bacteria that often present antibiotic resistance profiles, which complicates their management. The diagnosis of these infections is based on clinical and biological criteria that lack sensitivity and specificity, and on microbiological examinations that are lengthy. Unfortunately, the inadequate empirical antibiotic therapy in many cases, and the late implementation of an effective treatment, are associated with the emergence and spread of bacteria that are multi-resistant to antibiotics, i.e. strains producing extended-spectrum beta-lactamases (ESBL). Rapid diagnostic methods, identifying the pathogen and its resistance profile, are therefore necessary. Our laboratory is committed to develop PCR techniques to genotypically identify the most frequent ESBLs from four hospital facilities. Among all the enterobacteria detected, we observed the predominance of OXA type ESBL (38%), followed by CTX-M type ESBL (33%). TEM and SHV genes represent respectively 19% and 10%.The optimization of these technologies could allow the identification of all known resistance mechanisms in only a few hours and find new preventive or curative strategies in the fight against these nosocomial infections, to better respond to this health threat. **Key words:** nosocomial infections, ESBL, PCR, resistance genes, surveillance.

Introduction

The production of beta-lactamases is the main cause of resistance to beta-lactamase antibiotics in gram-negative bacteria. These enzymes cut the amide bond in the beta-lactamase cycle, rendering beta-lactamase antibiotics harmless to bacteria [1].

There are many types of ESBLs such as TEM, VHS, CTX-M, OXA, AmpC, etc., but TEM and sulphydryl-variable VHS are the major types of ESBLs and these enzymes are most commonly found in E. coli and K. pneumoniae [2].

Determination of TEM and VHS genes by molecular techniques in ESBL-producing bacteria and their antimicrobial resistance profile can provide useful data on their epidemiology and risk factors associated with these infections [4].

The genes encoding these enzymes are located on transferable plasmids [5].

CTX-M type betalactamases are a new group of enzymes encoded by transferable plasmids [6]. The name CTX-M type Betalactamases is due to their high activity against cefotaxime. Unlike TEM and SHV ESBLs, most CTX-M enzymes preferentially hydrolyze and confer resistance to cefotaxime and ceftriaxone rather than ceftazidime. In recent years, a new family of plasmid-mediated CTX-M extended-spectrum b-lactamases (ESBLs), called CTX-M, has emerged and has been reported in the literature with increasing frequency in Europe, Africa, Asia, South America and North America [1].

OXA-like enzymes are another growing family of ESBLs [7].

1 Material and methods

1.1 Bacterial strains

37 strains of enterobacteria stored since the year 2014 were tested for genotypic identification, these strains isolated in four Moroccan regions are distributed as follows: including 16 Escherichia coli, 10 Klebsiella pn, 7 Enterobacter Cloacae, 2 Morganella Morganii, 1 Proteus Mirabilis and 1 Citrobacter Freundi.

1.2 Preparation of the reaction mixture

For the PCR technique, the reaction mixture is composed of one unit of Taq polymerase, 0.4 mM of each primer (1 µl) (Table 1), 100 mM of each deoxynucleoside triphosphate (1 µl), 2.5 mM of MgCl2 (5 µl), PCR Enzyme Buffer (10 µl) Complete with PCR water (ultra pure) to a volume of 50 µl.

Table 1. Primer sequences used in the PCR technique

OXA	OXA-1/F OXA-1/R	5'– ACACAATACATATCAACTTCGC – 3' 5'– AGTGTGTTTAGAATGGTGATC – 3'
CTX-M	CTX-MF	5'- ATGTGCAGYACCAGTAARGT - 3'
	CTX-MR	5'- ACCGCRATRTCRTTGGTKGT - 3'
TEM	TEML	5'- ATGAGTATTCAACATTT - 3'
	TEMR	5'- TTACCAATGCTTAATCA - 3'
SHV	OS5	5'- TTATCTCCCTGTTAGCCACC - 3'
	OS6R	5'- GATTTGCTGATTTCGCTCGG - 3'

1.3 Amplification

The amplifications were performed under a volume of 52 µl (including 50 µl of reaction mixture and 2 µl of DNA extract) using the thermocycler (PROGEN), programmed. The different steps of the amplification, the temperature and the time to perform each step are mentioned in the (Table 2).

Table 2. PCR amplification conditions for SHV, TEM, CTX- M and OXA genes [8].

gene	Initial stage	Denaturation, hybridization, elongation	Number of cycles	Final stage
OXA	96°C 5 min	96 °C 1 min/60 °C 1 min/72 °C 2 min	35	72 °C 10 min
CTX-M	94°C 5 min	94°C 30s/56C 30s/ 72°C 45s	30	72°C 7min
SHV	94°C 5 min	94°C 30s/55°C 30s/ 72°C 30s	25	72°C 7min
TEM	94°C 5 min	94°C 30s/42°C 30s/ 72°C 30s	25	72°C 7min

2 Results

In 2014, 37 strains of enterobacteria were isolated from four Moroccan regions to study their resistance profile. Among these 37 isolates, 22 indicate the profile of ESBL enterobacteria.

During this period, the most ESBL-producing bacterial species were *Escherichia coli* (n= 11), *Klebsiella pneumoniae* (n= 8), *Enterobacter cloacae* (n= 2) and *Morganella Morganii* (n= 1) (Fig. 1).

These 22 ESBL-producing isolates were selected for detection of CTX-M, SHV, TEM and OXA genes by conventional PCR.

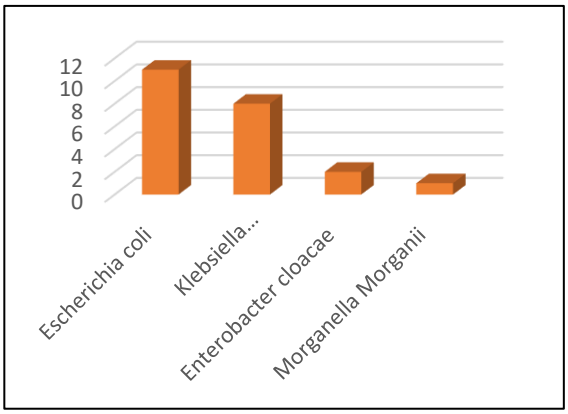

Fig. 1. ESBL-producing bacterial species isolated in 2014.

The search for resistance genes was done by simplex PCR, i.e. each gene independently of the others. After this search, the results obtained are shown in Fig.2.

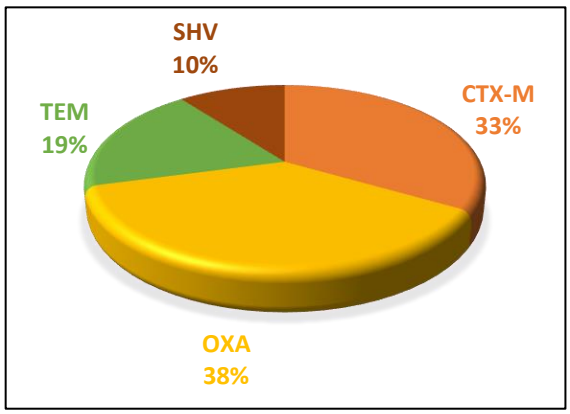

Fig. 2. Distribution of CTX-M, OXA, TEM and SHV genes in the tested strains.

When we studied the different profiles existing in our strains, we had 8 different profiles presented in (fig 3).

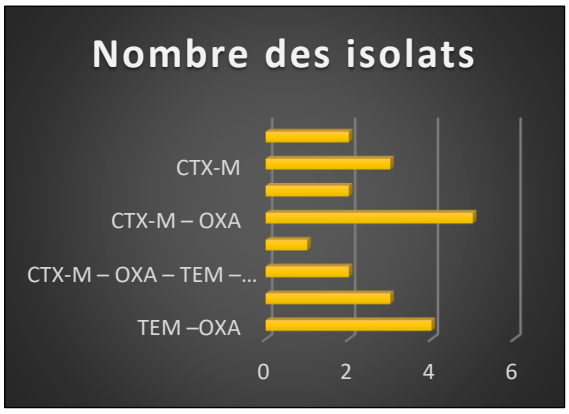

Fig. 3. Different gene profiles existing for the strains studied.

Discussion

The results of our study confirm the high prevalence of the OXA and CTX-M genes, while the prevalence of the other TEM and SHV genes was moderately low. The majority of strains had two or more ESBL genes, and only two strains carried all four ESBL gene types. Our isolates came from a variety of Moroccan hospitals, suggesting that these beta-lactamases are widely distributed.
ESBL production is much lower in Europe than in Latin America and Asia, and even lower in the Pacific than in North America.
CTX-M ESBLs are easily recognized in antibiotic susceptibility testing, as CTX is the most affected molecule with very good inhibition around the tazobactam-containing disc. These primers have been used to amplify all strains with this profile [8].

In our study, 16 ESBL strains among the 22 expressed the CTX-M gene; Escherichia coli being the most common (7 strains), followed by Klebsiella pneumoniae (6 strains) and finally Enterobacter cloacae (3 strains). It appears clearly that the CTX-M gene predominates in our ESBL strains. The same result was found in Europe, while in other countries the ESBL genes are more diversified [9].
ESBLs were inhibited by clavulanic acid and tazobactam. Importantly, the isolates were more resistant to cefotaxime and aztreonam than to ceftazidime, suggesting that they were CTX-M producers. Some CTX-M ESBLs confer high resistance to ceftazidime [10].

Among the 95 CTX-M producing isolates, the CTX-M-1 gene was positive in 45 isolates (47.3%). Of these, 36% were E. coli isolates, 36.3% were Enterobacter spp. and 58.3% were Klebsiella spp [11]. In another study, 67 isolates were positive using PCR. CTX-M ESBLs were observed in 22.72% of E. coli isolates [4].
As elsewhere in Europe, the rate of CTX-M genes is increasing with the new century, especially in northern France [9].
In recent years, the appearance of new variants of ESBL producers, in particular CTX-M, has raised the implication of co-resistance to other drug classes in endemic situations. This co-resistance is due to the propagation of different types of resistance genes within the same clone.
Some works have reported that bla CTX-M genes are frequently located on large plasmids that often carry other genes attributing resistance to other antimicrobial agents, citing aminoglycosides, fluoroquinolones, chloramphenicols, tetracyclines, and others…
This may explain the high rate of spread of the CTX-M gene among E. coli strains through the acquisition of the R plasmid, and constantly the high prevalence of the CTX-M resistance gene is combined with another resistance gene in these strains [12].
Recent European studies on enterobacterial resistance suggest that, EMT and VHS now replace as the predominant ESBL isolate. The prevalence of ESBL production has shown remarkable geographical differences, ranging from 0% in (Iceland) to less than 1% for (Estonia) and from 41% for E. coli and 91% for K. pneumonia in (Romania).
A recent study by Lal et al describes the occurrence of genetic variants in K. pneumoniae from clinical specimens of diverse origins. This study reported that isolates with both TEM and SHV genes were more frequent than TEM and SHV alone [3].

In a study that was conducted in Iran, Amir Peymani et al. found that 58.5% of ESBL-producing Enterobacter cloacae isolates carried OXA-1 genes [13].
Unfortunately, very few epidemiological studies have been conducted to assess the spread of OXA ESBLs [14].
Several studies have been cited that address the resistance and frequency of ESBL in Asia and specifically in India. This may be due to a number of factors that may give rise to this problem; the lack of sewage (the "Delhi belly") and poor quality of drinking water, coupled with a lack of control over the prescription and sale of antibiotics, are probably major factors that have facilitated the spread of resistance [15].

In Brazil, Cristina et al. showed that (8/12) isolates carried bla TEM, bla CTX-M, bla OXA, and bla SHV genes, (1/12) strain has bla TEM gene and (3/12) having bla TEM, bla CTX-M, and bla OXA genes [16].

In another study, conducted by Elif Burcu Bali et al. from Turkey, based on a total of 94 isolates, they found 50% (n = 47), 14.89% (n = 14) and 11.70% (n = 11) ESBL

rates for TEM, SHV, and CTX-M beta-lactamases, respectively. While, there were no strains harboring OXA type beta-lactamase in their study [4].
From Portugal, Soraia Necho Amaral reported that the genes bla TEM and bla OXA were found in those isolates representing by a number of (8/12 and 9/12, respectively) [17].

Another study summarized that bla TEM was present in 63.3% (55/87) and bla SHV in 52% (45/87) isolates alone or in combination. OXA-type beta-lactamases were present in combination with other enzymes. It was present in 26.4% (23/87) isolates only in combination with other genes [18].

The genes encoding β-lactamase identified in addition were 14 SHV, 8 OXA, 6 TEM and 4 of CTX-M-IV types. This was revealed by J. kim et al. in their study [19].

Using Multiplex PCR analysis of 51 amoxicillin-clavulanic acid resistant E. coli isolates, Karmele Colom et al. detected bla TEM and bla SHV genes in 45 and two strains, respectively, and only one strain harbored a bla OXA-1 gene [20].

Given the epidemiological importance of β-lactamase genes, all families should be studied in Europe, especially the OXA and PER enzyme families that have been prevalent in other sites in Turkey [21].

Another study showed that transfer of genotypically related ESBLs from hospital to hospital within a single city, from city to city and from country to country. Intercontinental transfer has also been reported. VHS-type ESBLs may be more common in clinical isolates than any other ESBL type.

The evolution of OXA-type β-lactamases from related enzymes with narrower spectra has many parallels with the evolution of SHV- and TEM-type ESBLs [22].

The epidemiology of ESBL is quite complicated. Initially, there are certain factors that are involved: the wider geographical area, the country, the hospital, the community and the host.
In addition, there are bacteria and their mobile genetic elements, usually plasmids. In addition, there are several reservoirs, including the environment, wild animals, farm animals and pets [17].

Conclusion

Correct detection of ESBL-producing microorganisms is a challenge for laboratories, as it requires not only phenotypic tests, but also genotypic tests for all genes associated with beta-lactamase production. The prevalence of ESBL-producing bacteria is increasing every year, especially in tertiary hospitals.

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