PREVALENCE OF ESBL GENES OF *PSEUDOMONAS AERUGINOSA* STRAINS ISOLATED FROM MAKKAH HOSPITALS, SAUDI ARABIA.

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ABSTRACT*: Due to annually recurring mass gatherings in Makkah, Saudi Arabia could be a hot spot for the collection of multidrug-resistant strains such as Pseudomonas aeruginosa (P. aeruginosa). The various genotypes of extended spectrum beta-lactamases (ESBLs) are SHV, TEM, CTX-M types VEB, PER, BEL-1, BES-1, ESBL enzymes in P. aeruginosa. The present study aimed to identify the prevalence these genes in P. aeruginosa isolates in Makkah hospitals. A total of 108 non-duplicated P. aeruginosa clinical isolates were identified in Makkah hospitals. ESBL production was confirmed using double disc synergy methods. All of ESBLs producer's isolates were submitted to PCR technique for detection for various ESBLs genes. Selected strains were subjected to whole genome sequencing. About 28 (25.9%) of P. aeruginosa isolates were confirmed as ESBL producers. Also, 78.6% of ESBLs producer carried blaGES gene while blaPER, blaCTX-M, bla VEB, blaOXA-10, blaOXA-4 genes appeared in 22.4%, 10.7%, 10.7%, 7.1% and 3.6% respectively. It was concluded that the incidence of ESBLs encoding genes among P. aeruginosa isolates in Makkah is near to the global prevalence. Continuous surveillance is essential to monitor the ESBLs producing P. aeruginosa. Also introduction of whole genome sequencing was found to be useful for both species and resistance genes identification.*

KEYWORDS: P.Aeruginosa, ESBL, Genes, blaGES, blaPER, Imipenem, Colistin.

INTRODUCTION

P.aeruginosa is an opportunistic pathogen that persists in both community and hospital settings due to its ability to survive on minimal nutritional requirements and to tolerate a variety of physical conditions (Lister et al., 2009). It is capable of infecting virtually all tissues and becoming a major cause of morbidity and mortality. The infections in hospitals mainly affect the patients in intensive care units and those having catheterization, burn, and/or chronic illnesses (Yetkin et al., 2006). *P. aeruginosa* is naturally susceptible to carboxypenicillins, ceftazidime and aztreonam; however, it can acquire resistance to third generation cephalosporins. *P. aeruginosa* is intrinsically resistant to several antimicrobial drug classes and can rapidly develop resistance to other drugs during chemotherapy, making medical treatment difficult and ineffective. The most frequent mechanism by which this occurs is through the constitutive hyperproduction of AmpC b-lactamase (so called stable derepression)(Bagge et al., 2002). The development of Beta -lactam resistance in *P. aeruginosa* can be caused by several mechanisms: (a) genetic mutations that lead to stable overexpression of AmpC, a chromosome-mediated cephalosporinase; (b) acquisition of transferable genes that code for a variety of Beta-lactamases; (c) overproduction of efflux systems; and (d) reduced permeability (Livermore, 2002). ESBL are Plasmid mediated enzymes that hydrolyze the oxyimino β monobactams (aztreonam) but have no **effect** on the cephamycins (cefoxitin, cefotitan) and the

carbapenems (Imipenem) but they are inhibited by clavulanic acid and tazobactam (Livermore, 1995). The various genotypes of ESBLs are the SHV, TEM, CTX-M types VEB, PER, BEL-1, BES-1, SFO-1, TLA, and IBC (Olowe and Adefioye, 2014). ESBL enzyme-encoding genes SHV-2a and TEM-42 have been found in *P. aeruginosa*, (Falagas and Karageorgopoulos, 2009, Jacoby and Munoz-Price, 2005)). In *P. aeruginosa* other enzymes that were identified like PER (mostly in clinical isolates from Turkey), VEB (from South-East Asia, France and Bulgaria), GES/IBC (France, Greece and South Africa) and BEL types. The annual Hajj to Makkah in Saudi Arabia is considered one of the largest annually recurring religious mass gatherings in the world. Saudi Arabia could be a hot spot for the collection of multidrug-resistant strains such as *P. Aeruginosa* (Yezli et al., 2014). Previous data from Saudi Arabia suggest that VEB and OXA-10-like enzymes are also frequent ESBLs in *P. aeruginosa* from Saudi Arabia, in accordance with reports from the Middle East, South East Asia, and parts of Europe (Strateva and Yordanov, 2009; Mirsalehian et al., 2010;Yezli et al., 2015). The present study aimed to identify the prevalence of genes responsible for ESBLs production in *P. aeruginosa* isolates in Makkah hospitals.

MATERIAL AND METHODS

A total of 108 non-duplicated *P. aeruginosa* clinical isolates were identified in Makkah hospitals during March-November 2014. ESBL production was confirmed among 28 strains by double disc synergy methods. DNA was prepared by guanidium thiocyanate extraction as previously described (Pitcher et al., 1989). All of ESBLs producers isolates were submitted to PCR technique to detection for some genes; *bla* TEM, *bla* SHV, *bla* CTX-M , *bla* OXA-1 *bla* OXA-4, *bla* OXA-10 *bla* VEB, *bla* GES, *bla* PER (encoded for some extended spectrum β-lactamases) (Table 1). The primers used in this study were obtained from IDT Integrated DNA technologies (IDT, Belgium). Amplification of DNA was performed using Master cycler Personal Thermal Cycler (Eppendorhoff, Germany) PCR was carried in 50μl PCR reaction volumes containing 4 μl of template DNA, 1μl (100 pmol) of each primer and a 25μl of Taq PCR Master Mix (promega company). Two multiplex reactions, the conditions of the first one include *bla* TEM, *bla* SHV, *bla* OXA-4, *bla* OXA-10 and *bla* VEB conditions were as following: pre-denaturation at 94°C for 4 minutes, followed by 35 amplification cycles of 94°C for 1 minute, 55°C for 1 minute and 72°C for 1.5 minutes, with a final extension step of 72°C for 5 minutes. The conditions of the second reaction include *bla*GES, *bla*CTX-M and *bla*PER conditions were as following: pre-denaturation at 94°C for 4 minutes, followed by 35 amplification cycles of 94°C for 1 minute, 50°C for 1 minute and 72°C for 1.5 minutes, with a final extension step of 72°C for 5 minutes. Amplified PCR products were detected by agarose gel electrophoresis. A DNA marker (Promega/USA) was run with each gel.

DNA Sequencing

Bacterial cells of 4 selected *P. aeruginosa* strains which were positive four ESBLs genes (2 *bla*GES and 2 *bla*OXA-10) for whole genemoe sequencing. Fresh cultures of these strains were used for DNA extraction. DNA quantity and quality were determined using Qubit® (Invitrogen, Applied Bio systems, USA), and Agilent Bio analyzer 2100 using 1000 DNA Chip (Agilent Inc., USA). Libraries for whole genome DNA sequencing were prepared using Illumina NexteraXT Library Preparation Kit and samples were barcoded using NexteraXT Index Kit (Illumina Inc., USA). *P. aeruginosa* genomes were sequenced in Illumina MiSeq

using pair ends protocol and version-2 500 cycles nano kit. The quality of pair ends sequence reads were checked by FastQC (BaseSpace Labs, Illumine Inc., USA).

De novo assembly of *P. aeruginosa* genomes were done using SPAdes Genome Assembler 3.0 (Algorithmic Biology Lab, St. Petersburg, Russia). Assembled contigs were used for 16s rRNA based species identification using Species Finder 1.0 Server from The Center for Genomics Epidemiology (http://www.genomicepidemiology.org/). Antibiotics resistance mechanisms of *P. aeruginosa* were predicted by mapping of assembled contigs and pair ends sequence reads against The Comprehensive Antibiotic Resistance Database (CARD), which was downloaded from Michael G. DeGroote Institute for Infectious Disease Research, McMaster University.

Table 1. Primers used in the study

| Gene	Primer	Sequence (5′ to 3′)	size	Ref
Bla VEB	VEB-1(F) VEB-1(R)	CGACTTCCATTTCCCGATGC GGACTCTGCAACAAATAC GC	643	(Lee et al., 2005)
Bla GES	GES-1(F) GES-1(R)	ATGCGCTTCATTCACGCAC CTATTTGTCCGTGCTCAGG	860	(Poirel et al., 2001)
Bla CTX-M	CTX-M(F) CTX-M(R)	CGCTTTGCGATGTGCAG ACCGCGATATCGTTGGT	550	(Bonnet et al., 2000)
Bla OXA-4	OXA-4(F) OXA-4(R)	TCAACAGATATCTCTACTGTT TTTATCCCATTTGAATATGGT	216	(AL-Thwani, 2013)
Bla OXA-10	OXA-10(F) OXA-10(R)	TCAACAAATCGCCAGAGAAG TCCCACACCAGAAAAACCA	277	(Bert et al., 2002)
Bla PER	PER-1(F) PER-1(R)	AATTGGGCTTAGGGCAGAA ATGAATGTCATTATAAAAGC	925	(Lee et al., 2005)
Bla TEM	TEMF TEMR	ATGAGTATTCAACATTTCCGTG TTACCAATGCTTAATCAGTGAG	840	(Sidjabat et al., 2009)
Bla SHV	SHV S1 SHV S2	ATTTGTCGCTTCTTTACTCGC TTTATGGCGTTACCTTTGACC	1051	(Sidjabat et al., 2009)
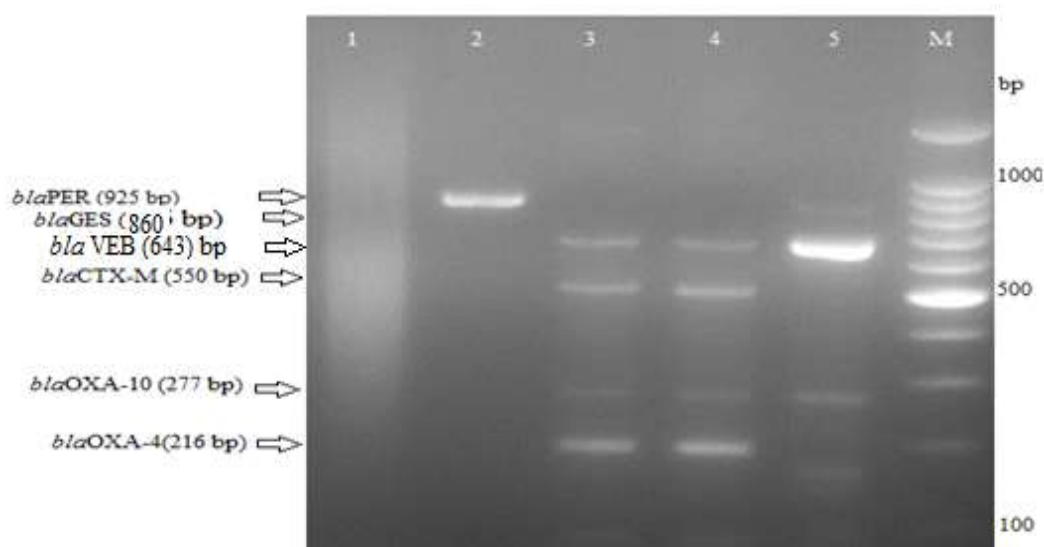
Figure 1: The ESBL genes after PCR on 2% agarose gel electrophoresis. Lane 1: Control negative. Lane 2; positive blaPER, Lanes 3 and 4; positive blaVEB, blaCTX-M, blaOXA-10 and blaOXA-4; Lane 5: positive blaGES, ; positive blaVEB and blaOXA-10; Lane M: 100-bp DNA ladder.

Table 2 Resistance Gene Database of four selected samples after whole genome sequencing

Sample1 Genes	Sample2 Genes	Sample3 Genes	Sample4 Genes
aac(3)-Ic	aac(6')	aph(3')-IIb	aph(3')-IIb
aac(6')	aph(3')-IIb	blaOXA	blaOXA
aph(3')-IIb	aph(3')-XV	blaPAO	blaPAO
blaGES	blaGES	cat	cat
blaOXA	blaOXA	fosA	fosA
blaPAO	blaPAO		
cml	cat		
cmlA	fosA		
fosA	sul1		
sul1	tet(G)		

RESULTS AND DISCUSSION

About 28 (25.9%) of *P. aeruginosa* isolates were confirmed as ESBL producers. ESBLs enzymes are primarily produced by the *Enterobacteriaceae* family in particular *Klebsiella pneumonia* and *Escherichia coli* and can be carried on chromosomes or plamids, (Falagas and Karageorgopoulos, 2009). They could also be produced by *P. aeruginosa* (Jacoby and Munoz-Price, 2005). The results of the present study is near to Aggarwal *et al*. (2008), who reported that 20.27% of *P. aeruginosa* isolates that were ESBLs producers. Also that's very close to Sheikh et al. (2015) and Peshattiwar and Peerapur., (2011) who reported (25.13%) and (22.22%) respectively. In the present study, the results of ESBLs genes detection showed that 78.6% of ESBLs producer of *P. aeruginosa* strains carried *bla*GES gene (Figure 1) while *bla*PER, *bla*CTX-M, *bla* VEB *bla*OXA-10, *bla*OXA-4 genes appeared in 22.4%, 10.7%,

15

10.7%, 7.1% and 3.6% respectively (Figure 1). The *bla* TEM, *bla* SHV, didn't appeared in any isolate of ESBLs producer *P. aeruginosa* strains . GES is a known class-A ESBL in *P. aeruginosa*. A GES-1 producing *P. aeruginosa* isolate was detected in Brazil while GES-2, was described from South Africam (Sidjabat et al., 2009; Castanheira et al., 2004). In similar study, AL-Thwani et al., (2013) reported that OXA-10 gene in 100% of *P. aeruginosa* isolates while detected 39.2% for OXA-4 and VEB-1 was found in 71.4% of the ESBL positive isolates. Bert et al., (2002) reported that PCR detected only in 26.3% while Strateva et al. (2007) reported that *bla*VEB-1 was detected in 33.1%, *bla*PER-1 0%, *bla*OXA-I in 41.3% . Shacheraghi et al., (2010) reported that *bla*VEB-1 was detected in 100% while *bla*CTX-M, , *bla*PER-1, *bla*GES-1, *bla*OXA-1, *bla*OXA-4 and *bla*OXA-10 genes were detected in the 2.43%, 68.3%, 24.4%, 70.7%, 17.1% and 92.7% of the ESBL producing isolates respectively. Similarly, Picão et al., (2009) found GES-1 and CTX-M-2 were of 16.3% and 4.6% respectively. Whole genome sequencing of selected *P. aeruginosa* strains confirmed the presence of ESBL genes which were detected by PCR and revealed no mutation in the restriction sites of the above genes (Table 2). Additional genes responsible for resistance of antibiotics other than third generation of cephlosphoreins were detected by the whole genome sequencing such as Aminoglycoside, Fosfomycin, Phenicol and other Beta-lactam which are beyond the scope of the present study. We can conclude that *bla* GES gene was the most common ESBL genes and none of *bla* TEM, *bla* SHV genes appeared in any isolate. The incidence of ESBLs encoding genes among *P. aeruginosa* isolates in Makkah is near to the global prevalence. Therefore it seems that continuous surveillance is essential to monitor the ESBLs producing *P. aeruginosa* in Makkah hospitals. Introduction of whole genome Sequencing is useful to identify both species and resistance genes in in *P. aeruginosa* organism.

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