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Use of Polymerase Chain Reaction (PCR) Technique to Detect of Extended-spectrum β-lactamases in Klebsiella granulomatis Isolated from Vagina of Al-Diwaniya City (Iraq) Wom

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ABSTRACT

100 vaginal swabs were collected from women in Al-Diwaniya city (Iraq). It had been isolated Klebsiella granulomatis from vaginal swabs then subjected all isolates under study to test the sensitivity (disc diffusion method) towards some antimicrobial and follow the PCR technique were identified extended-spectrum of genes responsible for the effectiveness of the β-lactamases antibiotics (bla TEM, bla SHV, bla CTX-M and bla AMPC) and the results showed fully resistance isolates K. granulomatis to penicillin, ampicillin and cephalosporin antibiotics (100%), while resisted to ceftazidime and cloxacillin by (88 and 80)%, respectively, compared to other antibiotics under study which showed K. granulomatis isolates different sensitivity towards it. Also identified the extended-spectrum of isolates toward the β-lactamases antibiotics by using Polymerase Chain Reaction technique which recorded the percentage of genes bla CTX-M (30/ 93.75%), bla SHV (25/ 78.12%), bla TEM (18/ 56.25%) and bla AMPC (22/ 68.15%), respectively.

Keywords: K. granulomatis, Antibiotics, β-lactamases,pcr technique

1. INTRODUCTION

Extended-spectrum beta-lactamases (ESBL) are enzymes that confer resistance to most beta-lactam antibiotics, including penicillins, cephalosporins, and the monobactam aztreonam. Infections with ESBL-producing organisms have been associated with poor outcomes. Community and hospital-acquired ESBL-producing Enterobacteriaceae are prevalent worldwide [1]. Reliable identification of ESBL-producing organisms in clinical laboratories can be challenging, so their prevalence is likely underestimated. Carbapenems are the best antimicrobial agent for infections caused by such organisms. The types and detection of extended-spectrum beta-lactamases as well as the epidemiology and treatment of organisms that produce them are discussed in this topic. The clinical features and diagnosis of the infections that ESBL-producing organisms often cause are discussed elsewhere [2].

β-Lactamases are most commonly classified according to two general schemes: the Ambler molecular classification scheme and the Bush-Jacoby-Medieros functional classification system. The Ambler scheme divides β-lactamases into four major classes (A to D). The basis of this classification scheme rests upon protein homology (amino acid similarity), and not phenotypic characteristics. In the Ambler classification scheme, β-lactamases of classes (A, C, and D) are serine β-lactamases. In contrast, the class B enzymes are metallo-β-lactamases [3, 4, 5].

The Bush-Jacoby-Medeiros classification scheme groups β-lactamases according to functional similarities (substrate and inhibitor profile). There are four main groups and multiple subgroups in this system [4]. This classification scheme is of much more immediate relevance to the physician or microbiologist in a diagnostic laboratory because it considers β-lactamase inhibitors and β-lactam substrates that are clinically relevant [5].

There is no consensus of the precise definition of ESBLs. A commonly used working definition is that the ESBLs are β-lactamases capable of conferring bacterial resistance to the penicillins, first-, second-, and third-generation cephalosporins, and aztreonam (but not the cephamycins or carbapenems) by hydrolysis of these antibiotics, and which are inhibited by β-lactamase inhibitors such as clavulanic acid. For the purpose of this review, the term ESBL will be taken to mean those β-lactamases of Bush-Jacoby-Medeiros group 2be and those of group 2d which share most of the fundamental properties of group 2be enzymes [3].

Group 2b enzymes hydrolyze penicillin and ampicillin, and to a lesser degree carbenicillin or cephalothin [3, 5]. They are not able to hydrolyze extended-spectrum cephalosporins or aztreonam to any significant degree. TEM-1 is the most common plasmid-mediated β-lactamase of ampicillin resistant enteric gram-negative bacilli (for example, Escherichia coli), while SHV-1 is produced by the vast majority of Klebsiella [6]. TEM-2 is a less common member of the same

group with identical biochemical properties to TEM-1. The ESBLs derived from TEM-1, TEM-2, or SHV-1 differ from their progenitors by as few as one amino acid [7].

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Extended spectrum β-lactamases are often plasmid mediated and derived from mutations in classic TEM, SHV, CTX-M, and AMPC genes by one or more amino acid substitution around the active site [3]. ESBLs are most commonly detected in Klebsiella, which is an opportunistic pathogen associated with severe infections in hospitalized patients, including immunocompromised hosts with severe underlying diseases [8].
Klebsiella granulomatis is gram-negative, rod-shaped bacterium of the genus Klebsiella [9]. known to cause the sexually transmitted disease granuloma inguinale (or donovanosis). It was called Calymmatobacterium granulomatis [10].
Extended-spectrum β-lactamases such as SHV and TEM are the classical B-lactamase had resistance to penicillin and narrospectrum cephalosporins, the CTX-M β-lactamases are more active against cefotoxim and cefriaxon than ceftazidime, the AMPC β-lactamases has cephalosporin activity in K. granulomatis [11]. .In addition, outbreak of multidrug resistant Klebsiella spp. Especially extended-spectrum B-lactamase has lead the treatment to limited option in recent year [12].

Aim of Study

Determination of Extended-spectrum β-lactamases (ESBLs) (*blaTEM*, *blaSHV*, *blaCTX-M* and *blaAMPC* genes) founded in *K. granulomatis* that isolated from vagina of women in Al-Diwaniya city/ Iraq, by using polymerase chain reaction (PCR) technique.

Materials and Methods

- **Bacterial isolates:** 100 *K. granulomatis* that isolated from vagina provided from Microbiology laboratory of Al-Diwanyia Hospital. After that *K. granulomatis* isolates were inoculated on molar Hinton agar media and incubation at 37°C overnight. Then, the antimicrobial susceptibility test was doing by using of penicillin (10µg), ampicillin (10µg), cephalosporin (10µg), cefotaxime (30µg), cloxacillin (10µg), ceftriaxone (10µg), ceftazidime (10µg) and cefoxitin (10µg) processed from Bioanalyse company/Turkey, and it's tested by disk diffusion methods.

- **Bacterial genomic DNA extraction:** Bacterial genomic DNA was extracted from *K. granulomatis* isolates by using (PrestoTM Mini gDNA Bacteria Kit. Geneaid. USA). 1ml of bacterial overnight growth on BHI broth was placed in 1.5ml micro-centrifuge tubes and then transferred in centrifuge at 10000 rpm for 1 minute. After that, the supernatant discarded and the bacterial cells pellets used in genomic DNA extraction and the extraction done according to company instruction. After that, the extracted gDNA checked by Nanodrop spectrophotometer, then store at -20ºC in refrigerator until perform PCR assay.
- **Polymerase chain reaction:** PCR assay was performed for detection of Extended-spectrum β-lactamases (ESBLs), (*blaTEM, blaSHV, blaCTX-M* and *blaAMPC* genes) according to method described by [13], using of specific ESBLs primers that designed by using NCBI-GenBank and primer plus-3 design online (table 1).

Table 1: DNA primers processed from Bioneer/ South Korea company

Primer	DNA Sequence (5`-3`)		Amplicon (bp)	GenBank
blaCTX-M	F	5`-AGCGATAACGTGGCGATGAA-3`	247	JN411912.1
	R	5`-TCATCCATGTCACCAGCTGC-3`		
blaSHV	F	5`-CCGCCATTACCATGAGCGAT-3`	410	FJ668798.1
	R	5`-AATCACCACAATGCGCTCTG-3`		
blaTEM	F	5`-GGTGCACGAGTGGGTTACAT-3`	531	JN037848.1
	R	5`-TGCAACTTTATCCGCCTCCA-3`		
blaAMPC	F	5`AAACGACGCTCTGCACCTTA-3`	670	AY533245.1
	R	5`-TGTACTGCCTTACCTTCGCG-3`		

PCR master mix was prepared by using (AccuPower® multiplex PCR PreMix kit. Bioneer/ South Korea). The PCR premix tube contains freeze-dried pellet of (Taq DNA polymerase 5U, dNTPs 250μM, Tris-HCl (pH 9.0) 10mM, KCl 30mM, $MgCl_2$ 1.5mM, stabilizer and tracking dye) and the PCR master mix reaction was prepared according to kit instructions in 20μl total volume by added 5μl of purified genomic DNA and 1.5μl of 10pmole of forward primer and 1.5μl of 10pmole of reverse primer, then complete the PCR premix tube by deionizer PCR water into 20μl and briefly mixed by Exispin vortex centrifuge (Bioneer/ South Korea). The reaction performed in a thermocycler (Mygene Bioneer/ South Korea) by set up the following thermocycler conditions; initial denaturation temperature at 95°C for 5 min; followed by 30 cycles at denaturation at 95°C for 30s, annealing at 58°C for 30s, extension at 72°C for 1min and then final extension at 72°C for 10 min. The PCR products examined by electrophoresis in 1.5% agarose gel, stained with ethidium bromide and visualized under UV transilluminator.

Results and Discussion

The antimicrobial susceptibility tests were doing as phenotypic antibiotics resistance profile of all *K. granulomatis* isolates (100 isolates). The test was conducted to examine the sensitivity of all samples to a group of antibiotics and resistance to it by measuring the diameter of growth inhibition zone around the antibiotics used discs and compare with the provisions of [14].

Figure (1) show that *K. granulomatis* isolates did not show any sensitivity to antibiotics (penicillin, ampicillin and cephalosporin) resisted as it fully (100%) in addition to resistance to ceftazidime (88%) and cloxacillin (80%). While the sensitivity to ceftriaxone, ceftazidime and cefoxitin were higher than compared with resistance, reaching percentage (62, 67 and 74)%, respectively. Which indicates that the proportion of resistant isolates *K. granulomatis* of the total antibiotics used in the study have a higher resistance in terms of sensitivity for more than 60% of these antibiotics. Bacterial isolates showed a marked variation in resistance to B-lactam antibiotics and the reason for this may be due to the diversity of mechanisms of resistance to isolates of *K. granulomatis* for B-lactam antibiotics by producing enzymes B-lactam antibiotics inhibitory to these antibiotics, or by changing the correlation of protein sites association of penicillin (PBPs) [15]. The other reason depends on the virulence of the bacteria, the same factors; it isolates show some amount of resistance than others and that the difference in testing conditions and the type of techniques used in the study, all of this society there is a difference in resistance levels [16].

Klebsiella spp. consider as an important cause of hospital acquired infections, especially among patients in the neonatal intensive care unit and can be causes mortality rates as high (70%) over the last two decades, the incidence of infections caused by multidrug-resistant *Klebsiella* strains has increased [10]. Extended-spectrum β-lactamases (ESBLs) were

first described in *Serratia marcescens* and *K. pneumoniae* isolates in 1983 in Europe country [17]. In United States at 1989 were described *K. pneumoniae* and *Escherichia coli* isolates that marked increase in the incidence of bacteria that produce ESBL enzymes and show about 20% of strains were resistant to ceftazidime in some teaching hospitals [12]. Epidemiological studies proposed that the increasingly extensive use of third-generation cephalosporin is a major risk factor that has contributed to the emergence of Extended-spectrum β-lactamases producing from K. granulomatis [18].

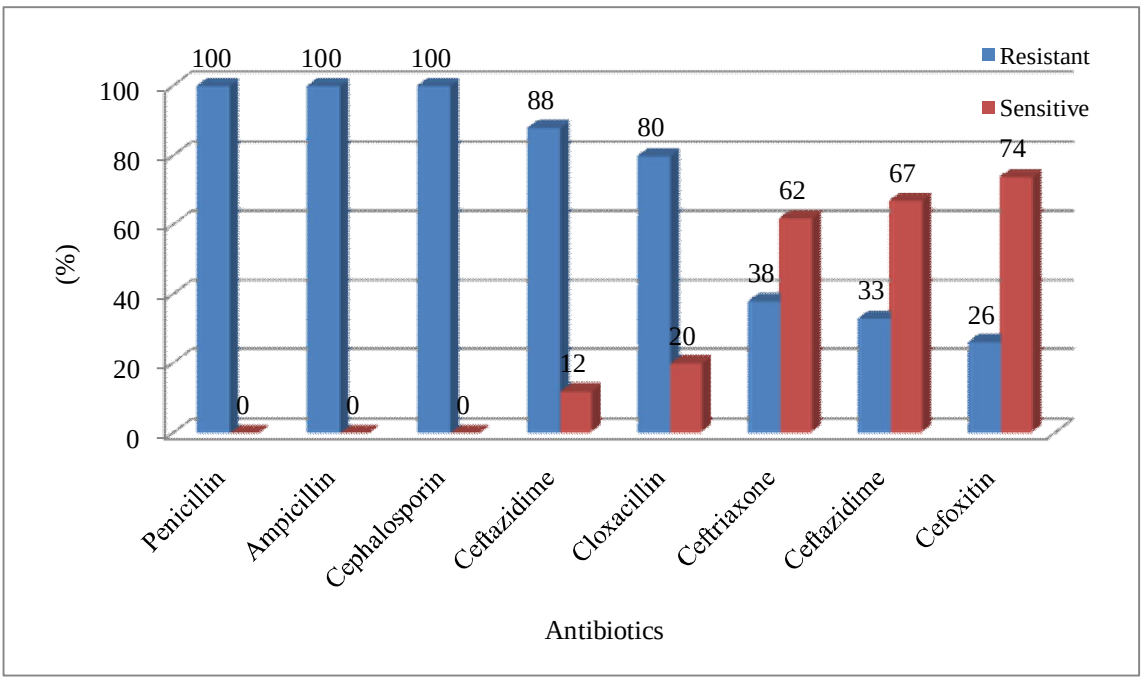

Figure 1: The antimicrobial susceptibility *K. granulomatis* isolates by using disc diffusion method.

Polymerase chain reaction (PCR) results were show that Extended-spectrum β-lactamases (ESBLs) to (*blaCTX-M*, *blaSHV*, *blaTEM* and *blaAMPC*) genes by PCR technique were given *blaCTX-M* (30/ 93.75), *blaSHV* (25/ 78.12%), *blaTEM* (18/ 56.25%) and *blaAMPC* (22/ 68.75%), respectively. These results agreement with [19, 20], which explained CTX-M-type ESBLs have become more prevalent worldwide.

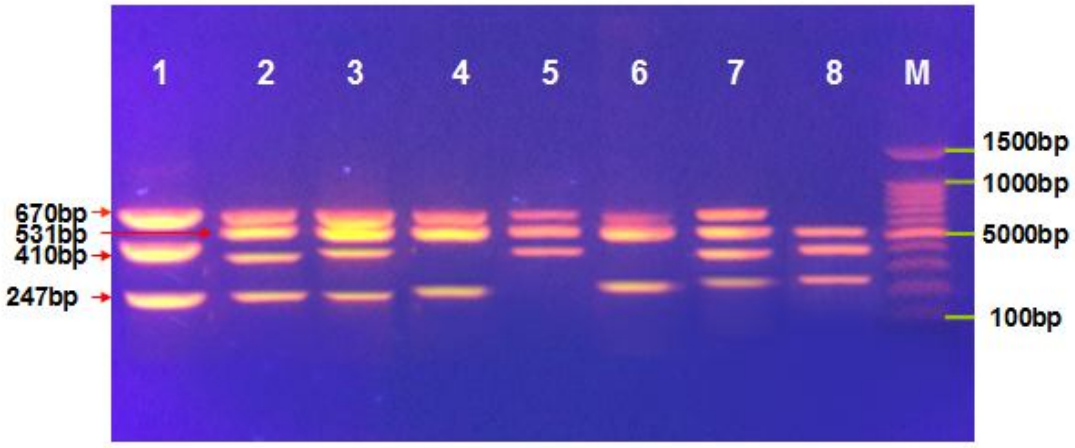

Figure 2: Agarose gel electrophoresis of PCR assay show that some positive *K. granulomatis* isolates results of Extended-spectrum β-lactamases gene. Where: Lane (M) DNA marker (1500-100bp), Lane (1-8) show positive (*blaTEM*, *blaSHV*, *blaCTX*-M and *blaAMPC* genes) at 670bp, 531bp, 410bp and 247bp PCR product respectively.

Conclusion

This study emphasizes the major role that Extended-spectrum β-lactamases CTX-M plays in facilitating ESBL-mediated antimicrobial resistance in *K. granulomatis* of Vagina infection that association with multiple antibiotics.

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