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Phenotypic and Molecular Study of Pantoea spp. Isolated from Urinary Tract Infections among Pediatric Patients in Iraq

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ABSTRACT

Pantoea spp. are Gram negative opportunistic pathogen capable of causing disease when the hosts immune system is weak or disrupted as a result of pathogens, accidents, etc. It is also closely related to the worldwide spread of infections. Out of 100 urine specimens collected from UTI pediatric patients, 53 (53%) were Gram-negative bacteria, and out of Gram negative bacteria 14 (14%) samples showed a positive result as Pantoea spp, 27 (27%) Gram-positive bacteria were observed. The remaining 20 (20%) showed no growth on the medium of MacConkey and the Blood Agar. The ability of Pantoea to removes the dark blue color of the iodine starch complex for detection of β-lactamase resistance phenotypes, only 12 isolates (85%) gave positive results. The results were as follows that out of the 14 Pantoea spp. isolates β-lactamase producers, 11/14 (78.5%) Pantoea spp. exhibited zones enhancement with clavulanic acid, confirming their ESBL production, by using disk combination method. The results of screening test revealed that 9/14 (64.3%) Pantoea spp. isolates gave positive ESBLs production test, since the inhibition zone of synergism has been recognized clearly, using disk approximation method. Genotypic detection for β-lactamase resistances used blaTEM, blaSHV genes for detection. The results revealed that out of 14 Pantoea spp were gave 4 (28.6%) for the blaSHV gene. The results of blaTEM indicated for positive amplification and it has been found that blaTEM gene is found in 12 (78.5%) Pantoea spp.

Keywords: *Pantoea spp., β-lactamase, blaTemR, blaSHV, MDR*

INTRODUCTION

The Enterobacterales order contains the pigmented, Gram-negative rods known as Pantoea. Straight rods that are gram-negative non-capsulated facultative anaerobic, non-sporing and motile by peritrichous flagella make up the Pantoea genus (Morin, 2014). They are regarded as uncommon, opportunistic diseases, and they are often linked to nosocomial epidemics that mostly afflict newborns and patients with impaired immune systems (Gajdács, 2019) organisms that result in wounds, bleeding, acute infections in the urinary tract in immune compromised humans include plant pathogens and opportunistic pathogens (Morin, 2014).

Over the last 20 years, infections brought on by these bacteria have posed substantial challenges, especially in poor nations where they are linked to high rates of morbidity and death as well as prolonged hospital stays (Cunningham and Marcon, 2012). Hauben et al., (1998) documented a septicemia example caused by an Erwinia herbicola strains which are responsible for various antibiotics typically used to treat gram-negative bacilli but ampicillin resistant, carbenicillin, as well as cephalothin (cefalotin). Cruz et al., (2007) reported equivalent outcomes. The Pantoea species did not contain any β-lactamase. In 2000, it was discovered that clinical isolates of Pantoea agglomerans from a person who had septic arthritis had a high level of fosfomycin resistance (De Champs et al., 2000). Although inherent to microorganisms, clinically relevant to the resistance of antimicrobial (AMR) incidence has grown due to improper use of antimicrobial drugs, which is in turn a result of several reasons (Guevarra et al., 2021). When a bacterium loses its sensitivity to the antibiotics it formerly reacted to due to changes in its genetic composition, acquired resistance occurs. These types of resistance are often brought on by chromosomal or extra chromosomal structures (plasmid, transposon, etc.) (Hoffman, 2016). The genetic material from bacteria is transported by resistance genes and plasmids through the transduction, transformation, conjugation, and transposition mechanisms, respectively (Peterson et al., 2018). This syndrome is often seen in antibiotics with similar chemical structures, such as erythromycin, neomycin-kanamycin, or cephalosporin and penicillin. It sometimes appears in entirely different pharmacological categories, however. A case of cross-resistance between erythromycin and lincomycin exists. This might have extra chromosomal or chromosomal origins. (Reygaert, 2018). The second form of resistance, known as multidrug resistance, may also be brought on by altered target shape, enzymatic inactivation, or increasing expressions of the gene which encodes to multi-drug efflux pump (Ventola, 2015). The aim of the study was to determine the antimicrobial susceptibility patterns and frequency of β-lactamase resistance genes of Pantoea spp. isolated from pediatric patients with UTI.

METHODS

At the Biology Department, Science Faculty, Kufa University, Bacteriology Iraq, and Molecular Labs, the study was conducted.

Medical Samples and Patients

In general, there were 110 urine obtained from patients suffering from urinary tract infections during the period from1st October 2020 to 27th January 2021. The specimens contained information about the patients' age, sex. Patients who had not gotten an antibiotic for a full week before the accumulation of sample collecting had their specimens taken. The specimens were incubated as well as inoculated in aerobically form for time 24- 48 hours at 37°C on the culture media (MacConkey agar, blood agar) for biochemical testing to diagnose bacteria (McFadden, 2000).

Isolates and Identification

All urine specimens had collected in sterile container by midstream, urine bag, and catheter methods had based on age and physical status of patients. Then each urine specimen has labeled and transported directly to a microbiology laboratory for processing and were divided into two portions. One portion was for the microscopically examination, for pus cells, casts, red blood cells, and others. The second portion has streaked on the blood, MacConkey and CHROM agars by using a calibrated wire loop (0.001 mL). Then after incubation at 37°C for 24 hour, if the growth of colonies more than 105 CFU/mL it was considered as significant growth (bacteriuria) (Batabyal and Himanshu, 2018). The identifications of colonies bacterial growth after 24 hour in 37°C can be depended on morphological characteristics on the CHROMAgar TM and MacConkey agar, Conventional biochemical test, and Vitec2 compact system has used for final identification.

Detection of β-Lactamase Generating Isolates Phenotypically

The identification of β-Lactamase production was carried out using a direct capillary tubes technique (Koneman et al., 1997). This approach entails:

1- Penicillin G solution was mixed with two ml of a 0.5% phenol red indicator solution.
2- Until the color turned violet, a few drops of Sodium hydroxide solution (1N) have been added to the mixture of solutions.
3- After submerging the capillary tubes one end in the prescribed solution till it arrived to the height of 1-2 cm, this end then was placed in a culture of bacterial colonies. Trying to make a bacterial plug takes 24 hours (avoid the production of bubbles between the colonies and indeed the liquid).
4- Within fifteen min of the capillary tubes being incubated vertically at 37 ℃, the findings were obtained., with the tops of the colonies changing color to yellow to imply a successful outcome.

Conclusive extended-spectrum β-lactamase Production Test

All β-lactamase-resulting isolates have too been examined for confirmation of ESBL synthesis utilizing two different techniques;

a. Test of Disk Combination

The phenotypic verification of potential Extended - spectrum beta isolates has been done using the disk diffusion approach. We assess the effectiveness of tazobactam as well as clavulanic acid alone and in combination with ceftriaxone and ceftazidime. When an antibiotic is tested in conjunction with Tazobactam as well as clavulanic acid as opposed to when it is tested alone, an ESBL-producing strain is identified by a 5-mm increase in the antibiotic's inhibitory zone. (CLSI, 2020).

b. Disk Approximation Test

All isolates that generate -lactamase were examined, claim Batchoun et al., (2009). Cefotaxime (30 gram), ceftazidime (30 gram), ceftriaxione (30 gram), and azetreonam (30 gram) antibiotic disks have been positioned 15 millimeter (edge to edge) around a center disk of amoxi-clav on Muller-Hinton agar plates with the organism being tested for ESBL formation (twenty gram amoxicillin plus 10-gram clavulanate). Plates were then incubated aerobically at 37°C for 24 hours. The organism has been regarded as an ESBL producer if there had been any increase (increment in diameter, of inhibition zone) between the center amoxiclav disk or some of the β-lactam antibiotic disks rendering resistance or transitional sensitivity.

Molecular Methods

DNA Extraction and Isolation

Pantoea spp. isolate DNA was told to prepare utilizing the boiling process. Colonies were placed in sterile TE buffer at 100 microliters, boiled for 15 minutes at 100 ℃ in a water bath, quickly cooled to -20 ℃ for 60 minutes, The supernatant from the centrifugation was retained to be utilized during amplification procedures (Shah et al., 2017).

PCR Test Polymerase Chain Reaction

In the monoplex PCR technique used in this research, two genes responsible for the β- lactam resistant have been chosen to be amplified separately. The gene sequences showed in table 1 and the program in table 2:

TABLE 1: Specific primers that are used in the present study.

Gene name	Sequences (5 to 3)	Primer size(bp)	References
blaTEM	F: ATCAGCAATAAACCAGC	766	Svärd, (2007)
	R: CCCCGAAGAACGTTTTC		
blaSHV	F:GGCCGCGTAGGCATGATAGA	714	Ensor et al., (2009)
	R:CCCGGCGATTTGCTGATTTC		

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TABLE 2: The program settings of PCR.

Types of genes	Temperature (c) / time					Number of cycles
	Cycling of conditions					
	Initial desaturations	Denaturation	Annealing	Extensions	Final extensions	
blaTEM	94/5min	94/1 min	50/1min	72/1min	72/7 min	30
blaSHV	94/5 min	94/30 sec	55/30sec	72/90 sec	72/5 min	

Electrophoresis on Agarose Gel

According to the Bartlett and Stirling technique, all technical, preparation and criteria for DNA agarose-gel electrophoresis analysis as well as detection were carried out (2003).

Preparation of Agarose Gel and DNA Loading

Agarose-gel electrophoresis, according to Mainiatis et al. was carried out (1982). This method has been using adequate electrophoresis buffer (TBE) as the running buffer. In order to make agarose gel, a gram of agarose has been dissolved in 100 ml of TBE 1X in a glass flask, which was then continuously mixed while being melted in a pot of boiling water. The mixture was then token to cool to between 50 and 60 degrees Celsius, 3 liters of ethidium bromide dye has been mixed in, and agarose has been poured into a gel before being fixed with a comb to inhibit bubble formation. The mixture then was left to solidify. The jar has been placed in the electrophoresis tank after the bubbles had been successfully removed. Six microliters of the 100 bp DNA ladder were injected into the initial left well of the agarose electrophoresis gel. Then a well of the agarose electrophoresis gel was carefully filled with 5 L of the PCR product that had been amplified. The special lid of the electrophoresis tank has been closed, and the electric current has been matched (70 volts for 1.5-2 hrs).

Detection of DNA by Agarose Gel Electrophoresis

Agar-gel electrophoresis has been utilized to identify the PCR Amplification products, which were then seen after being stained with ethidium bromide. The electrophoresis result was discovered using the gel documentation system. When the sample's DNA band's base pairs matched the intended resulting size, the results have been deemed positive (Bartlett and Stirling, 2003). The gel has finally been imaged using the Biometra gel documentation system (Mishra et al., 2009).

RESULTS AND DISCUSSION

Isolates and Designation

Bacterial isolation and identification during the study period from November 2021 to January 2022, 100 urine were collected from pediatric patients suffering UTI. Out of 100 clinical samples, 53 (53%) were Gram-negative bacteria, and out of Gram negative bacteria 14 (14%) samples showed a positive result as Pantoea spp, 27 (27%) Gram-positive bacteria were observed. The remaining 20 (20%) showed no growth on the medium of MacConkey and the Blood Agar.

The biochemical outcomes of the isolates of Pantoea spp. were revealed by the TSI, Sugars, Oxidase, Indole, Ureases, and simmone citrate assays. Other assays on Pantoea spp. bacteria produced negative findings for oxidase, indole negative, and urease production, as indicated in Table (3). Many biochemical assays have been utilized to identify Pantoea spp. isolates utilizing the VITEK2 GN ID Cards System. The findings suggested Pantoea spp. with cards identifying a variety of high-quality isolates.

TABLE 3: Conventional Biochemical Tests for Pantoea spp.

Test	P. agglomerans	P. ananatis	P. calida
Urease	-	-	-
Citrate	+	/-+	+
Indole	-	-	-/+
Motility	+	+	+
Oxidase	-	-	-
Catalase	+	+	+
Hemolysis	-	-	-
MR	+	-/+	+
VP	+	-/+	+
KIA	K/A	K/A, A/A	K/A, A/A
Gas	-/+	-/+	-/+
H2S	-	-	-
Lactose	-	+	+

Results show that 100% of Pantoea spp which have been isolated are urease test positive, 88.88% are standard test positive, 96% are indole test negative, 88% are motility test negative, and all of it is oxidase and hemolysis test negative. When these findings have been contrasted with those from Abdalhussen et al., (2018). The diagnosis was made by the Vitek-2 system (bioMerieux Inc. USA) which takes less time to achieve microbial identification and antibiotic susceptibility results.

Primary detection of β-lactsamase resistance phenotypes

After culturing 14 isolates of Pantoea bacteria on a MHA medium equipped with amoxicillin and ampicillin (Each of them alone). The result was that all isolates of these bacteria can grow in the medium of MHA in the presence of amoxicillin 100%, and ampicillin 100% and therefore they are considered resistant isolates to β-lactam antibiotics (Figure 1). This study does not agree with the findings of Rumyana et al., (2014).

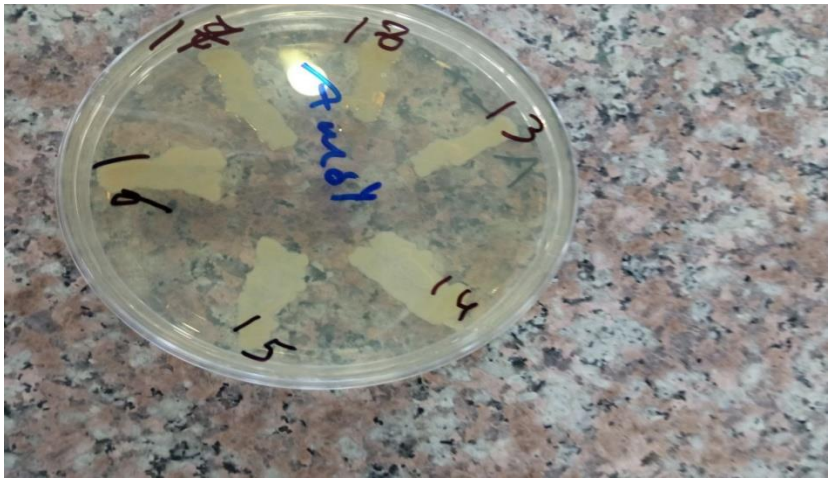

FIGURE 1: Pantoea Species Susceptibility to Ampicillin Supplemented on Muller-Hinton Agar at 37°C for 24hrs.

Perhaps, Gram-negative bacteria are intrinsically resistant to penicillin-G by virtue of their double membrane structure, which prevents the antibiotic from accessing the cell wall target. Moreover, the acquired resistance to β-lactams operates through different mechanisms;

production of β-lactamases, changes in the outer membrane permeability or alterations in the PBPs (Wilke et al., 2005). Reduced permeability through porin losing may reduce the steady state of periplasmic drug concentrations and thereby reduces PBP inactivation. Therefore, decreased permeability may act synergistically with the expression of β-lactamases or active efflux to confer higher levels of β-lactam resistance (Livermore and Woodford, 2006). However, β-lactam resistance mostly associated with transmissible plasmids can be transferred between different bacterial species among hospital isolates (Carattoli, 2008).

Detection of β-lactamase Producing

Direct iodine method was used for the detection of β-lactamase production in β-lactam resistance Pantoea isolates. The result revealed that out of 14 β-lactam resistance Pantoea spp. isolates only 12 isolates (85%) gave positive results with direct iodine method. This result indicated that the enzymatic resistance was not prevalent among isolates.

Different mechanisms of resistance may be associated with non-susceptibility to βlactam antibiotics in Gram negative bacteria; however the enzymatic, for intrinsic and acquired β-lactamases, is the main mechanism (Bush and Jacoby, 2010). Therefore, the diversity of mechanisms, such as porin loss, efflux pumps or lack of expression of acquired genes and the presence of other unsearched β-lactamases may be associated with these inconsistencies between genes found and expressed phenotype (DavinRegli and Pages, 2015).

Phenotypic Detection of Extended Spectrum β–Lactamases (ESBLs)

Pantoea spp. isolates resistant to β-lactam antibiotic are suspected to be highly producers of ESBLs; therefore, all were subjected to ESBLs production test. Performance of the test isolates in the ESBL initial screen disk test was assessed using ceftazidime disks. According to the CLSI, (2021) the isolate is considered to be a potential ESBL producers, if the inhibition zone of ceftazidime disks (30 μg) was ≤ 22 mm. The study found that 11/14 (78.5%) isolates of Pantoea spp. were ESBL positive during the initial screening using ceftazidime disk, which considered as suspected of ESBL producing studied isolates. The detection of ESBL producing in studied isolates was performed using disk combination method. In this method ceftazidime and ceftriaxone disks compared to cefotaxime and aztroname disks each alone. The isolate was considered ESBL producer, when the inhibition zone of combined disks was more than or equal to 5 mm increased than the inhibition zone of disk alone. The results were as follows that out of the 14 Pantoea spp. isolates β-lactamase producers, 11/14 (78.5%) Pantoea spp. exhibited zones enhancement with clavulanic acid, confirming their ESBL production. Also all isolates were further confirmed by disk approximation method. In this method augmentation of the inhibition zone between a 30μg antibiotic disks (ceftazidime, ceftriaxone, cefotaxime and aztreonam) toward amoxicillin-clavulanate disk (20/10μg), was interpreted as synergy, indicating the presence of an ESBL (Table 4). The results of screening test revealed that 9/14 (64.3%) Pantoea spp. isolates gave positive ESBLs production test, since the inhibition zone of synergism has been recognized clearly (Table 4).

TABLE 4: ESBLs Producing in Pantoea spp. Isolates by Confirmation Methods.

Species of Pantoea	No. of Isolates	No. (%) of Positive	
		Disk combination	Disk approximation
Pantoea spp.	14	11(78.5)	9(64.3)

The dramatic escalation of antimicrobial resistance among Enterobacteriaceae worldwide over the past two decades reflects the emergence and widespread dissemination of novel extended spectrum β-lactamases (ESBLs) (Chong and Kamimura, 2011; Salabi et al., 2013). Among Gram negative bacteria, hydrolysis of the β-lactam ring by β-lactamase(s) is the most common mechanism of resistance to β-lactam antibiotics (Bush and Jacoby, 2010), but additional mechanisms (e.g., alterations in porin channels, efflux pumps) may contribute to or amplify resistance (Canton et al., 2012).

Genetic Method

Detection blaSHV gene

The results revealed that out of 14 Pantoea spp in this study were gave 4 (28.6%) for the blaSHV gene (figure 2), the greatest incidence was recorded for blactx-m (3.9%) followed by blaTEM (26.1%) and blaSHV (14.2%) in a previous study by the researchers Ejaz et al., (2021) just some few temporary reports available on the destinations of blaSHV gene among Enterobacteria of healthy domestic and farm animals in Pakistan.

FIGURE 2: Electrophoresis of PCR amplification products of Pantoea isolates amplified with blaSHV gene primers with product 714 bp for 1 hrs at 80 volt on an ethidium bromide-stained agarose gel. Lane M: DNA Ladder (10000bp). Lanes 1,3,4,13,14,15: PCR product (positive case band 714 bp).

The multiple β-lactamases within a single isolate may confer complex phenotypic expression, like SHV and AmpC enzymes production which exhibit resistance to third, fourth generation cephalosporins and aztereonam (Hammond, 2004). Initially, the ESBLs among these species were typical TEM or SHV enzymes (Dhillon and Clark, 2012).

Detection of blaTEM Gene

The molecular detection of blaTEM gene by using specific PCR primer is the most common plasmid-encoded β-lactamase in Gram-negative bacteria. The results of this experiment indicated for positive amplification and it has been found that blaTEM gene is found in 12 (78.5%) Pantoea spp as shown in figure (3).

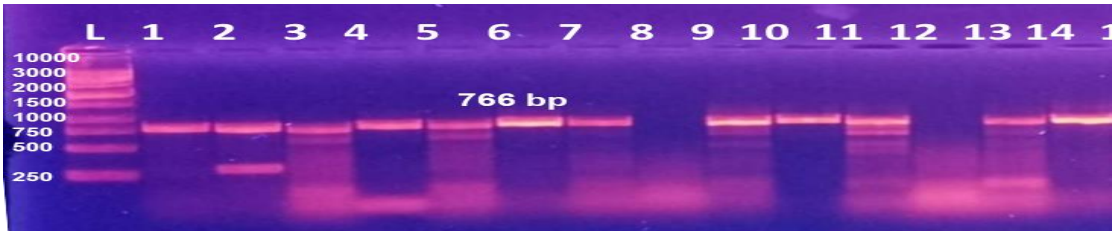

FIGURE 3: Electrophoresis of PCR amplification products of Pantoea isolates amplified with blaTEM gene primers with product 766bp for 1 hrs at 80 volt on an ethidium bromide-stained agarose gel. Lane M: DNA Ladder (10000bp). Lanes 1-24: PCR product (positive case band 766 bp).

Almost all species of the Enterobacteriaceae family were found to produce extended-spectrum beta-lactamases (ESBLs). A small number of mutations led to the development of the enzymes, which have been primarily plasmid-mediated as well as developed from the beta-lactamases broad-spectrum TEM-1 and TEM-2. This investigation was performed to identify ESBL producers within this species using PCR-RFLP after phenotypic screening (Jyoti et al., 2010). While PCR for both plasmid and chromosomal DNA revealed 60percent positivity for SHV and 56% positivity for Transmission electron microscopy, Polymerase chain reaction on the plasmid DNA alone only discovered at 30% ESBL positive isolates utilizing Transmission electron microscopy primer and 38% utilizing SHV primer.

CONCLUSION

In conclusion, regarding the important role of the Pantoea spp. as a causative agent in nosocomial infections and the widespread presence of ESBL. blaTEM was highly prevalent among Pantoea spp isolates compared with other BlaSHV group I. The study revealed that the PCR technique was more accurate than phenotypic methods for detecting β-lactamase production in Pantoea isolates.

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