Preliminary Study of Emergence MDR of Providencia spp. Isolate Producing ESBL, AmpC and MBL among Patients Cases with RTI and in Wastewater in Al-Diwaniya City , Iraq.

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Abstract

Although there are a global spread of studies on Providencia genus, it has been studied very little or rare in Middle East countries especially in Iraq. Species of these bacteria are verified presence which are an uncommon as opportunistic pathogens in immunocompromised people or common in environment. It's gram negative bacilli belong to the family of Enterobacteriaceae . Due to wide and indiscriminate use of antibiotics, major problem has emerged the multi-drug resistance (MDR) of these bacteria.

The samples were collected from different sources (n=1209) which included clinical, environmental and food specimens from Al-Diwaniya city in September 2016 to December 2017. Patients profile included gender and age. The strains were identified, as well as, screened for antibiotic susceptibility pattern and for the presence of β-Lactamases. Moreover, the bacteria were stratified into MDR, extensively drug-resistant (XDR), and pandrug-resistant (PDR) depending on specific international standardized.

The results of 1209 specimens were examined as 684 (63.39%) positive clinical and environmental cultures, 18 (2.63%) strains were identified as *Providencia* species. Of these, 3 (0.44%) strains from respiratory tract infection (RTI) patients were found to be *Providencia rettgeri* , the mean age of these male patients 36 years range from 21-62 years, and 15 (2.19%) strains from wastewater were identified as *Providencia* spp. (*P. alcalifaciens n*= 10, and *P. rettgeri* , *n*=5). Finally, There are not identified any strains belong to *Providencia* genus in the food samples. Moreover, the antibiotic susceptibility pattern of 31 antibiotics tested showed all resistance for *Providencia* genus isolates. The high frequent of resistance was nitrofurantoin (*n*=18, 100%), followed by ampicillin (*n*=17, 94.4), azithromycin (*n* = 16, 88.9%), amoxicillin/clavulanic acid (*n* = 13, 72.2%), cephalexin (*n*=8, 44.4%), ampicillin/sulbactam (*n*=7, 38.8%), tetracycline and tigecycline (*n*=6, 33.3%). Resistance to nalidixic acid was found at rate of 16.7%, and at rate of 11.1% for each of the cefuroxime, trimethoprim , and trimethoprim/sulfamethoxazole. However, the percentages of resistance to imipenem and cefotaxime were 5.6% each one. The most effective of antibiotics were meropenem, ceftriaxone, cefixime , aztreonam, ciprofloxacin, norfloxacin, chloramphenicol and aminogycosides ; all the strains were found to be susceptible to this antibiotics. MDR occurred in 11 strains (61.1%) and 9 strains (50%) of *Providencia* species. Furthermore, *P. rettgeri* exhibits a MDR phenotype were 25% and 12.5%, while *P. alcalifaciens* were 90% and 80% according to the EUCAST and CLSI for year 2017 respectively. Phenotypic tests of β-Lactamases production showed that all *Providencia* spp. strains (100%) gave positive results with this tests. Phenotypic tests revealed that ESBL , AmpC and MBL production were recognized in 66.7 % , 37.5% and 80 % of the *Providencia* spp. respectively. Coexistence of a ESBL with other types of β-Lactamases (MBL) were documented in 4(22.2%) strains. Moreover, the vast majority of *Providencia* strains producing β-Lactamases and exhibits a MDR phenotypic were observed in 8(100%) strains in wastewater.

Conclusions : First papers have been provided baseline dissemination information on the presence of *Providencia* species and their antibiotic resistance profile in the environmental and clinical samples in AL-Diwaniya governorate of Iraq. In addition to, this study was considered first report on emerging *Providencia* species harboring of MDR which may be investigating potential reservoirs of ESBL, AmpC and MBL. The water sources in Iraq contains of resistant bacteria which classified as critical priority level according to latest report of WHO.

Introduction

Organism of the genus *Providencia* is non-fermenting gram negative bacterium belong to Enterobacteriaceae family (Jneid *et al*., 2016; Perween *et al*., 2016; Kamga *et al*., 2012). *Providencia* genus is contains 5 species are *P. rettgeri* , *P. stuartii* , *P. alcalifaciens*, *P. heimbachae* and *P. rustigianii* (O'Hara *et al*., 2000; Manos and Belas, 2006;Sharma *et al*., 2017). This species are opportunistic pathogens in immunocompromised people (Jneid *et al*., 2016), which has been found in sputum (Feyzioğlu, *et al*., 2013), urine (Marquez-Ortiz, *et al*., 2017 ; Liu, *et al*., 2016), burns (Perween *et al*., 2016 ; Arpin *et al*., 2012), Wounds (Washington *et al*., 2015) , and stool specimens (Shima *et al*., 2016). Additionally, it is found in fishes (Akinyemi *et al*., 2017 ; Oyelakin *et al*., 2016; Ololade *et al*., 2016) , beef meat and chicken meat, (Shima *et al*., 2016). As well as soil(Sharma and Gupta, 2016), water river (Kuczynski, 2016) , cows water (Bento Rodrigues, *et al*., 2017), chicken and bird feces (Nahar *et al*., 2016; Foti *et al*., 2017), and wastewater (Xia *et al*., 2013). On the other hand, the enormous evolution and increase of MDR in many bacterial pathogens, including *Providencia* is a significant public health challenging (Tshisevhe, *et al*., 2017). The terms MDR, XDR and PDR are increasingly frequently used in the biomedical literature to describe various degrees of antimicrobial resistance among bacteria. Unfortunately, there are currently no internationally accepted definitions for these terms for bacteria other than *Mycobacterium tuberculosis*. As a result, these terms are used arbitrarily creating great confusion among researchers, health care professionals and the public (Falagas and Karageorgopoulos, 2008). So over all, the scientists agree on standardized definitions for MDR, XDR, and PDR, depending on antimicrobial susceptibility pattern of organisms.They have been creating a standardized international terminology in order to describe resistance pattern in bacteria which is usually be responsible for healthcare-associated infections and prone to one a previous terms (Magiorakos *et al*., 2012). Furthermore, other researchers found the pathogens according to the species and the type of resistance and then classifying the results in three priority levels: critical, high and medium.The critical level contains Enterobacteriaceae including *Providencia* spp. which are resistance to carbapenems and third generation cephalosporin's (WHO, 2017). Several surveillance studies have revealed a relatively high incidence of MDR in *Providencia* spp. and β-Lactamases production (Ahasan *et al*., 2017 ; Marquez-Ortiz, *et al*., 2017; Sharma *et al*., 2017 ; Voukeng *et al*., 2017; Foti *et al*., 2017; Perween *et al*., 2016; Jamal *et al*., 2016; Saavedra-Rojas *et al*., 2015; Feyzioğlu *et al*., 2014). However, in the Middle East countries including Iraq have a limited studies focused on phenotypic characterization of Providencia spp. Therefore, Present study seeks to detection the prevalence of Providencia spp. in, clinical, environmental and food specimens and their antibiotic susceptibility profiles as well as phenotypic β-Lactamases production.

Materials and Methods

Collection and Handling Sampling

The samples (n= 1209) was carried out in the laboratories of Biology Department/ Science College, University of Al-Qadisiya, Iraq in September 2016 to December 2017, from different sources such as environmental and food specimens in Al-Diwaniya city. The total of 791 specimens were obtained from patients whom they suffering from different infections and admitted to the Al-Diwaniya city hospitals: RTI (n=305), burns (n=245), UTI (n=174), GIT (n= 37), and wounds (n=30). The total of environmental samples were investigated in chicken, and bird feces (n=116), the wastewater (n=116), water of cows (n=32), and soil (n=32). Finally, the total of food samples were examined in fishes (n= 53), beef meat (n= 42), and chicken meat (n= 35). All specimens or swabs were transported in refrigeration conditions to the previous laboratories and streaked on the blood agar (Himedia, India) and MacConkey agar (Oxoid, UK) using sterile standard loop method. The media were incubated at 37˚C for 24 hours (Collee *et al*., 1996 ; Brown, 2005).

Identification of Bacterial Strains

The strains were identified depending on their cultural characteristics, Gram's stain, and reaction to standard biochemical tests (MacFaddin, 2000). All strains were further confirmed as *Providencia* species by API 20E system (BioMerieux, Marcy L'E toile, France) and Vitek-2 identification system (BioMerieux, Marcy L'E toile, France).

Antimicrobial susceptibility testing (AST)

Antibiotic sensitivity profiles of the bacterial strains performed identification to susceptibility testing by modified disc-diffusion method (Kirby-Bauer) (Bauer *et al.,* 1966) on Mueller-Hinton agar (MHA) (Oxoid, UK) plates were inoculated by sterile swab dipped into the inoculums (0.5 McFarland). The selection of antibiotic disc was performed according to (CLSI, 2017), while the strains were considered resistant or susceptible according to (CLSI, 2017; EUCAST, 2017). All strains tested for susceptibility of 12 antibiotic classes divided into 31 antimicrobial agents which are : Ampicillin (AMP,10µg), amoxicillin/clavulanic acid (AMC, 30 µg), Ticarcillin/clavulanic acid(TIM, 85 µg), Ampicillin/sulbactam (SAM,20µg), Piperacillin/tazobactam (PTZ, 110 µg), Cephalexin (CL, 30 µg), Cefoxitin (FOX, 30 µg), Cefuroxime (CXM, 30 µg), Ceftazidime (CAZ, 30 µg), Cefotaxime (CTX, 30 µg), Ceftriaxone (CTR, 30 µg), Cefixime (CFM, 5 µg) , Cefepime (CFP, 30 µg), Aztreonam (ATM, 30µg), Imipenem (IMP,10µg), Meropenem (MEM,10µg), Amikacin (AK, 30µg), Gentamicin (GM,10µg), Tobramycin (TOB,10µg), Ciprofloxacin (CIP, 5µg), Levofloxacin (LEV, 5 µg), Norfloxacin (NOR, 10µg), Ofloxacin (OFX, 5 µg) Nalidixic acid (NA, 30µg),Tetracycline (T, 30µg), Tigecycline (TGC, 15 µg), Trimethoprim (TM, 5µg), Trimethoprim/sulfamethoxazole (TS, 25µg), Chloramphenicol (C, 30µg), Nitrofurantoin (NIT, 300 µg), Azithromycin (ATH,15 µg) (Himedia, India and Mast Diagnotics, UK). For purpose of this study, the isolates were considered a MDR, if an isolate is resistant to representatives of three or more classes of antibiotics above. While the definition of XDR is an isolate that is resistant to all but one or two classes. A PDR in Gram-negative strains was defined if resistance of the isolates was observed to all available classes of antibiotics (Magiorakos *et al*., 2012).The control positive was used *E. coli* ATCC 25922.

β-Lactamases production

*** ESBL production**

All isolates tested for confirmatory ESBL production according to Batchoun *et al*. (2009). Antibiotic disks of cefotaxime, ceftazidime, ceftriaxione, and azetreonam (30µg each) were placed 15 mm (edge to edge) around a central disk of amoxi-clav (20µg amoxicillin plus 10 µg clavulanate) on MHA plates seeded with organism being tested for ESBL production. Plates were incubated aerobically at 37°C for 24 hours. Any augmentation (increase in diameter of inhibition zone) between the central amoxi-clav disk and any of the β-lactam antibiotic disks showing resistance or intermediate susceptibility was recorded, and the organism was thus considered as an ESBL producer. Cefotaxime alone and in combination with clavulanic acid were tested. Inhibition zone of ≥ 5 mm increase in diameter for antibiotic tested in combination with clavulanic acid versus its zone when tested alone confirms an ESBL producing isolate (Cantarelli *et al*., 2007; CLSI, 2017).

***Detection of AmpC β-lactamase**

The strains would be considered potential AmpC β-lactamase producers, if the inhibition zone of cefoxitin (30µg) disk was ≤ 18mm (Coudron *et al.*, 2003). Cetazidime-Imipenem Antagonism Test (CIAT) was carried out for detection of inducible AmpC β-lctamases according to Cantarelli *et al*. (2007). An imipenem disk (10µg) placed 20 mm a part (edge-to-edge) from a ceftazidime disk (30µg) on a MHA plate previously inoculated with a 0.5 McFarland bacterial suspension, and incubate for 24 hours at 35ºC. For comparison, a cefoxitin disk was also placed 20 mm a part from the ceftazidime disk. Antagonism indicated by a visible reduction in the inhibition zone around the ceftazidime disk adjacent to the imipenem or cefoxitin disks, regarded as positive for the inducible AmpC β-lactamase production.

***Detection of Carbapenemases**

Three methods were performed to determine of Metallo-β-lactamase by Imipenem-EDTA double disk synergy test(DDST) according to Lee *et al*. (2003). A 10µg imipenem disk was placed in the center of a MHA plate inoculated with a 0.5 McFarland dilution of the test isolate. An EDTA disk (1900 µg) was placed at a distance of 15 mm center to center from the imipenem disk. The plate was incubated at 37ºC overnight. The zone around the imipenem disk would be extended on the side nearest the EDTA disk for a metallo-β-lactamase producer. Disc potentiation test to organism was inoculated onto plates of Mueller-Hinton agar plate (opacity adjusted to 0.5 McFarland opacity standards). A 0.5-m EDTA solution was prepared by dissolving 186.1 g of disodium EDTA 2H 2 O in 1000 ml of distilled water and adjusting it to pH 8.0 by using NaOH. The mixture was sterilized by autoclaving. Two 10-µg imipenem discs and meropenem discs were placed on the plate; 5 µl of EDTA solution was added to one of the disc each. The inhibition zones of the imipenem and imipenem-EDTA discs and meropenem and meropenem-EDTA discs were compared after 16-18 h of incubation at 35°C. An increase in the zone size of at least 7 mm around the imipenem-EDTA disc and meropenem-EDTA discs was recorded as an MBL-positive strain (Hemalatha *et al*., 2005). A Modified Hodge Test (MHT) was carried out by Prepare a 0.5 McFarland dilution of *E. coli* ATCC 25922 (using either direct colony suspension or growth method) in broth or saline, and dilute 1:10 in saline or broth, a inoculate an MHA plate and allow to dry (3-10) min, meropenem or ertapenem disk (10µg) was placed in the center of the test area. In a straight line the test organism streaked from the edge of the disk to the edge of the plate, the streak should be at least 20–25 mm in length, the plates were incubated 16-20hours at ±35ºC. Four organisms were tested on the same plate with one disk. MHT positive test has a clover leaflike indentation of *E. coli* ATCC 25922 growing along the test organism growth streak within the disk inhibition zone. MHT negative test has no growth of *E. coli* ATCC 25922 along the test organism (CLSI, 2017).

Statistical Analysis

The χ^2 (Chi-square) tests were applied to determine the statistical significance of the data. *P* value of < 0.01 or < 0.05 was considered significant, Prism 5 (Graph Pad Software Inc., San Diego, CA, USA).

Results

Isolation and identification of *Providencia* species

The strains have showed significant differences in the frequencies of sources specimens ($P < 0.01$). In this study, were received and examined 1209 specimens from different sources during year and six months. Among these, 791 (65.4%), 288(23.8%) and 130(10.8%) obtained from clinical, environmental and food specimens respectively. However, a total of 396 (50.0%) positive clinical culture were implicated in community- acquired and nosocomial infections including; 152(49.8%) from sputum, 123 (50.2%) from burns, 76 (43.6%) from urine, 37 (100%) from stool and 8 (26.6%) from wounds. Additionally, 100% positive environment and food cultures. From the total 814 (67.3 %) positive clinical, food and environmental cultures, 18 (2.2%) strains were identified as *Providencia* species. Of these, 3 (0.7%) strains from sputum patients were found to be *Providencia rettgeri* , the mean age of these male patients 36 years range from 21-62 years, and 15(2.19%) strains from wastewater were identified as *Providencia* spp. (*P. alcalifaciens n*= 10, , and *P. rettgeri* , *n*=5). Moreover, don't identified any isolates belong to *Providencia* genus in food samples (Table 1).

Table 1: Number and percentage of *Providencia* species isolated from different sources of specimens.

Source of specimens			**Positive culture No.(%)**	***Providencia* species**	**No. (%)**
Pathogenic	sputum	305	152(49.8)	*P. rettgeri*	3 (0.7)
	burns	245	123(50.2)		
	urine	174	76(43.6)		
	stool	37	37(100)		
	wounds	30	8(26.6)		
Total No. (%)		791 (65.4)	396(50.0)		
Environment	wastewater	107	107(100)	*P. alcalifaciens* *P. rettgeri*	10 (3.5) 5(1.7)
	chicken and bird feces	116	116(100)		
	water of cows	33	33(100)		
	soil	32	32(100)		
Total No. (%)		288 (23.8)	288(100)		15(5.2)
Foods	fishes	53	53(100)		
	beef meat	42	42(100)		
	chicken meat	35	35(100)		
Total No. (%)		130 (10.8)	130(100)		
Total		1209	814(67.3)		18 (2.2)

The present investigation showed that 18 strains belong to two *Providencia* species isolated from sputum and wastewater sources. The majority 10 (55.6%) strains of isolated species which were found in wastewater belong to *P. alcalifaciens* , and 8 (44.4%) strains which were

found in sputum and wastewater belong to *P. rettgeri* . The strains were identified by their cultural characteristics, Gram staining, reactions to standard biochemical tests (Table 2) and confirmed as *Providencia* species by API 20E system (Figure 1, and 2) and Vitek-2 identification system.

Table 2 : Standard biochemical tests of *Providencia* species.

Tests / Type of bacteria	Gram-negative bacilli	Lactose fermentation	Indole	Methyl red	Vogas-Proskaur	Citrate utilization	Motility	Urease	TSI (K/A+ G)	H_2S production	Oxidase	Catalase	Gelatin hydrolysis	Mannitol fermentation	Inositol fermentation
P. rettgeri	+	–	+	+	–	+	+	+	+	–	–	+	–	+	+
P. alcalifaciens	+	–	+	+	–	+	+	–	+	–	–	+	–	–	–

Figure 1:Picture of *P. rettgeri* by API 20E system.

Figure 2:Picture of *P. alcalifaciens* by API 20E system.

Antibiotics susceptibility testing of *Providencia* species

All the *Providencia* species obtained from patients and wastewater were tested for their antibiotic susceptibility against the selected 31 antibiotics. Strains have significant differences in the frequencies of antibiotics resistance ($P < 0.01$). Table (3) showed comparative between CLSI and EUCAST in year 2017 on the basis of the resistance, intermediate resistance and susceptibility of the isolated *Providencia* to different antibiotics as represented by the diameter in mm. The highlight indicates cases considered to be resistant to the respective antibiotics. The high frequent of resistance (*n*=18, 100%) was to nitrofurantoin, followed by ampicillin (*n*=17, 94.4), azithromycin (*n* = 16, 88.9%), amoxicillin/clavulanic acid (*n* = 13, 72.2%), cephalexin (*n*=8, 44.4%), ampicillin/sulbactam (*n*=7, 38.8%), tetracycline and tigecycline (*n*=6, 33.3%) for each one. Resistance to nalidixic acid was found at rate of 16.7%, and at rate of 11.1% for each of the cefuroxime, trimethoprim, and trimethoprim/ sulfamethoxazole. However, the percentages of resistance to imipenem and cefotaxime were 5.6% each one. The most effective of antibiotics were meropenem, ceftriaxone, cefixime , aztreonam, ciprofloxacin, norfloxacin, chloramphenicol and aminogycosides ; all the strains were found to be susceptible to this antibiotics (Table 3).

Table 3: Comparative between susceptibility profile of CLSI and EUCAST for year 2017 of *Providencia* species for different antibiotics.

Antibiotic	Susceptibility profile % CLSI- 2017			Susceptibility profile % EUCAST- 2017		
	R	I	S	R	I	S
Ampicillin	94.4	5.6	0	-	-	-
Amoxicillin/clavulanic acid	72.2	11.1	16.7	83.3	0	16.7
Ticarcillin/clavulanic acid	0	88.9	11.1	-	-	-
Ampicillin/sulbactam	38.8	5.6	55.6	-	-	-
Piperacillin/tazobactam	22.2	16.7	61.1			
Cephalexin	44.4	22.2	33.3	-	-	-
Cefoxitin	0	38.9	61.1	44.4	0	55.6
Cefuroxime	11.1	5.6	83.3	16.7	0	83.3
Ceftazidime	0	5.6	94.4	-	-	-
Ceftriaxone	0	0	100	-	-	-
Cefotaxime	5.6	0	94.4	-	-	-
Cefixime	0	0	100	-	-	-
Cefepime	0	5.6	94.4	5.6	11.1	83.3
Aztreonam	0	0	100	-	-	-
Imipenem	5.6	50	44.4	-	-	-
Meropenem	0	0	100	-	-	-
Amikacin	0	0	100	-	-	-
Gentamicin	0	0	100	0	5.6	94.4
Tobramycin	0	0	100	0	5.6	94.4
Nalidixic acid	16.7	11.1	72.2	-	-	-
Ciprofloxacin	0	0	100	16.7	0	83.3
Norfloxacin	0	0	100	-	-	-
Ofloxacin	0	11.1	88.9	-	-	-
Levofloxacin	0	5.6	94.4	11.1	5.6	83.3
Tetracycline	33.3	0	66.7	-	-	-
Tigecycline	33.3	11.1	55.6	-	-	-
Trimethoprim	11.1	0	88.9	11.1	5.6	83.3
Trimethoprim/sulfamethoxazole	11.1	11.1	88.8	11.1	0	88.9
Chloramphenicol	0	0	100	-	-	-
Nitrofurantoin	100	0	0	-	-	-
Azithromycin	94.4	0	5.6	-	-	-

(R):resistance, (I): intermediate resistance , (S): susceptibility, (-): Same CLSI

The resistant effect of strains to antibiotics including; amoxicillin/clavulanic acid, cefoxitin , cefuroxime, ciprofloxacin, levofloxacin and cefepime are comparable; the rates of resistance were 72.2%, 0%, 11.1,0%, 0% and 0% respectively, on the basic CLSI-2017. Whereas, 83.3%, 44.4%, 16.7%, 16.7%, 11.1% and 5.6% strains respectively were resistant to same antibiotics on the basic EUCAST- 2017. Differences between the rates of resistance of CLSI and EUCAST for year 2017 of *Providencia* species were significant ($P < 0.01$) (Figure 3).

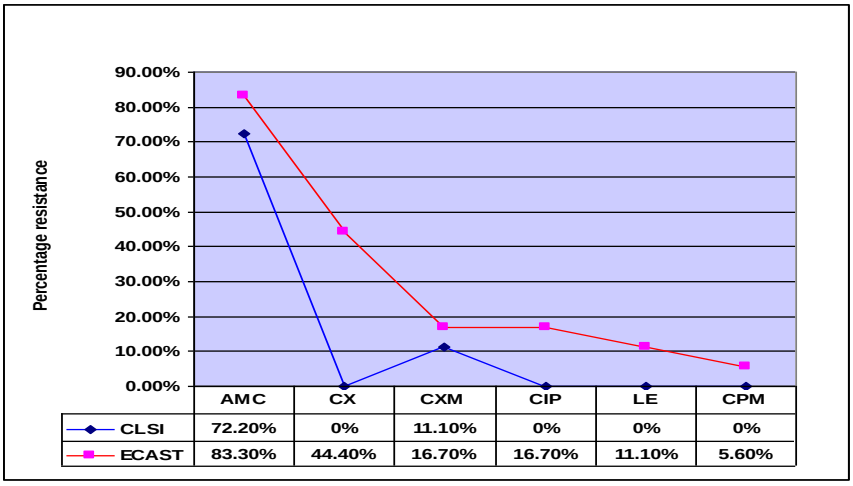


Figure 3: Difference between the rates of resistance of CLSI and EUCAST for year 2017 of *Providencia* species.

Interestingly, the strains of *P. rettgeri* showed resistance to 14 antibiotics (45.1 %), and *P. alcalifaciens* to 12 antibiotics (38.7%). Strains have significant differences in the frequencies of single species of *Providencia* ($P < 0.01$). Antibiotics susceptibility of the single species of *Providencia* (*P. alcalifaciens* and *P. rettgeri*) showed low percent resistance to most antibiotic tested except for nitrofurantoin (100%, 100%), ampicillin (100%, 87.5%), azithromycin (90%, 100%), amoxicillin/clavulanic acid (80%, 62.5%), ampicillin/sulbactam (50%, 25%), and cephalexin (40%, 50%) respectively. The *P. rettgeri* isolates showed that high resistance to tetracycline (75%) (Figure 4).

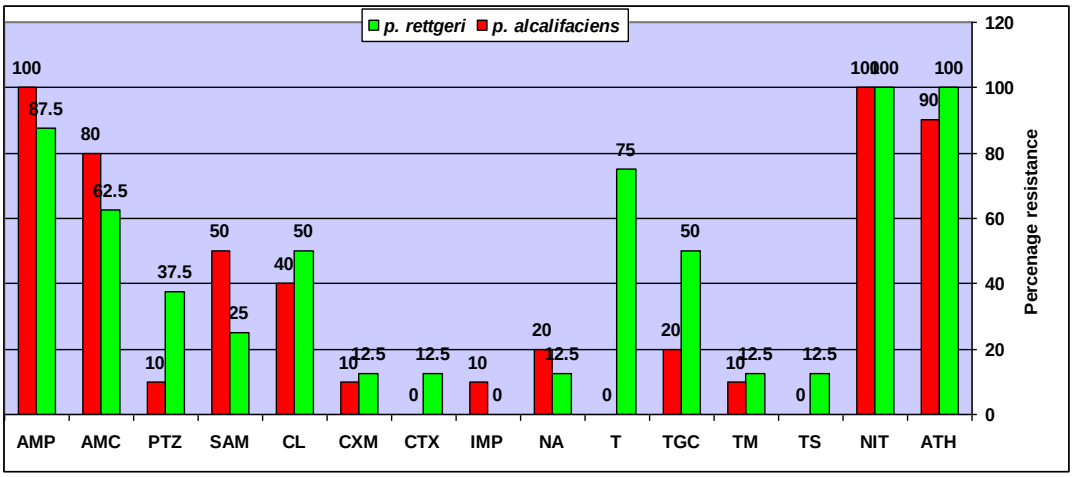


Figure 4: Comparative between the rates of resistance for single species *Providencia*.

Strains have significant differences in the frequencies of MDR according to the EUCAST-2017 and CLSI-2017($P < 0.01$). All *Providencia* genus strains (100%) were resistant to a minimum of three classes of antibiotics. MDR occurred in 11 strains (61.1%) and 9 strains (50%) of *Providencia* species. Furthermore, *P. rettgeri* exhibits a MDR phenotype were 25% and 12.5%, while *P. alcalifaciens* were 90% and 80% according to the EUCAST-2017 and CLSI-2017 respectively (Table 4). Unexpectedly, 10/15 (66.7%) and 9/15 (60%) of the wastewater strains were characterized as MDR, while one (33.4)% and 0% of the sputum strains respectively were characterized as MDR according to the EUCAST-2017 and CLSI-2017 respectively.

Table 4: Multi-drug resistance of *Providencia* single species

Bacterial species	CLSI-2017		EUCAST-2017	
	No. (%) of Bacterial strains	No. of resistance to antibiotic classes	No. (%) of Bacterial strains	No. of resistance to antibiotic classes
P. rettgeri	1(12.5)	5	2(25)	3,6
P. alcalifaciens	1(10) 1(10) 2(20) 4(40)	5 6 3 4	2(20) 2(20) 2(20) 3(30)	3 5 6 4
Total	9(50)		11(61.1)	

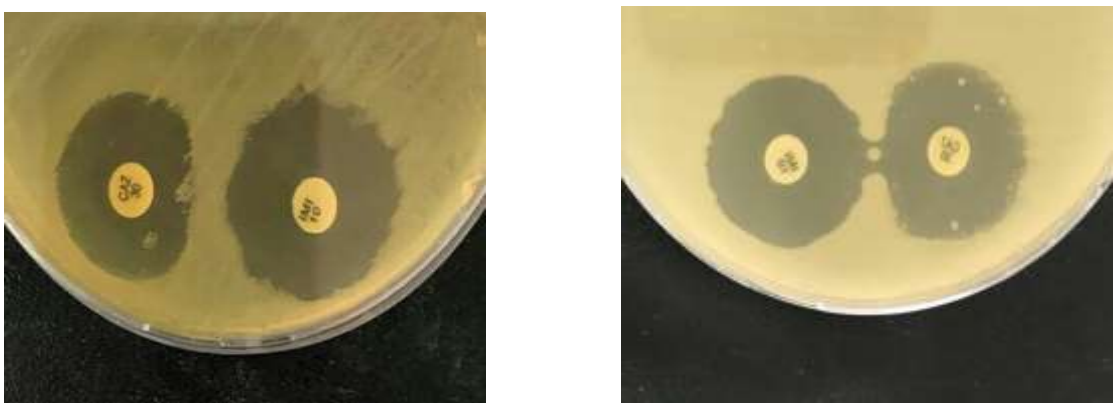
β-Lactamases production

In generally, phenotypic tests of β-Lactamases production showed that all *Providencia* strains (100%) gave positive results with these tests including initial screening test. The frequency of ESBL, AmpC, MBL producing strains by disk diffusion assay are summarized in table (5). A ESBL production was detected in 2/18 (66.7%) strains (Figure 5), AmpC production was observed in 3/8(37.5%) strains (Figure 6) and increase production to MBL was documented in 8/10(80%) strains (Figure 7). Strains have significant differences in the frequencies of β-Lactamases production (ESBL, AmpC and MBL) of *Providencia* strains , so there are significant differences between β-Lactamases production and sources specimens ($P < 0.05$). Based on this phenotypic detection, the study revealed a high rate of ESBL, AmpC and MBL producing from *Providencia* spp. had been identified as resistant or intermediate resistance to β-Lactam antibiotics and recovered from patients with RTI and in wastewater in AL-Diwaniya city / Iraq. However, the study indicated that there are correlation was found between the results obtained with IST and CIAT.

Table 5: Frequency of phenotypic ESBL, AmpC and MBL producing *Providencia* spp. strains by different methods.

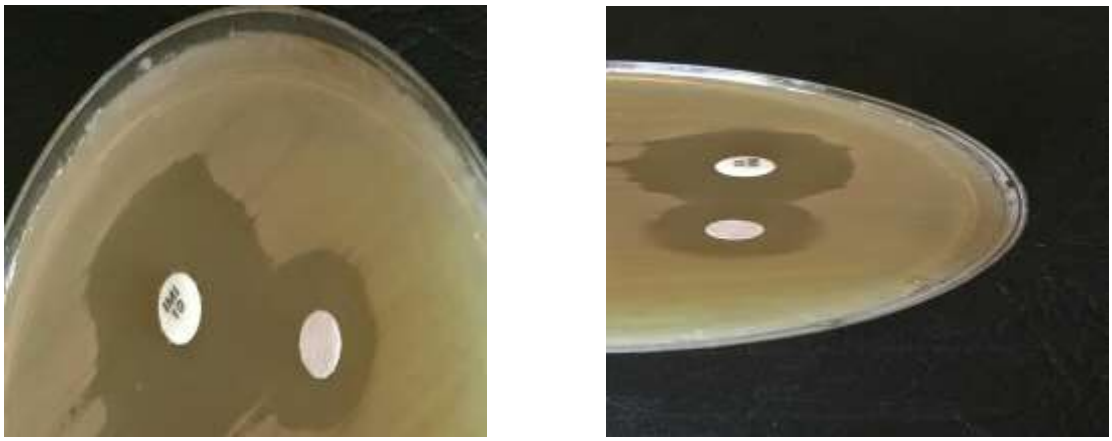
Source of sample	No. (%) *Providencia* spp. strains						
	ESBL		AmpC		MBL		
	Disk approximation n=18	Disk combination n=18	IST n=18	CIAT n=8	Imipenem-EDTA (DDST) n=10	Disc potentiation test n=10	MHT n=10
Sputum	3(16.7)	0(0)	0(0)	0(0)	1(10)	0(0)	0(0)
Wastewater	9(50)	0(0)	8(44.4)	3(37.5)	7(70)	0(0)	0(0)
Total	12(66.7)	0(0)	8(44.4)	3(37.5)	8(80)	0(0)	0(0)

Figure 5: Disk approximation test exhibiting ESBL in *Providencia* sp.



A B

Figure 6: Inducible AmpC β-lactamase production in *Providencia* sp. by CIAT(A+B)



A B

Figure 7: MBL production in *Providencia* sp. by imipenem - EDTA DDST (A+B)

Phenotypic tests of *P. rettgeri* and *P. alcalifaciens* revealed that ESBL production were recognized in (38.8% and 27.9%) strains respectively, AmpC production were detected in (12.5% and 25%) strains respectively and MBL 40% strains for each one (Figure 8).

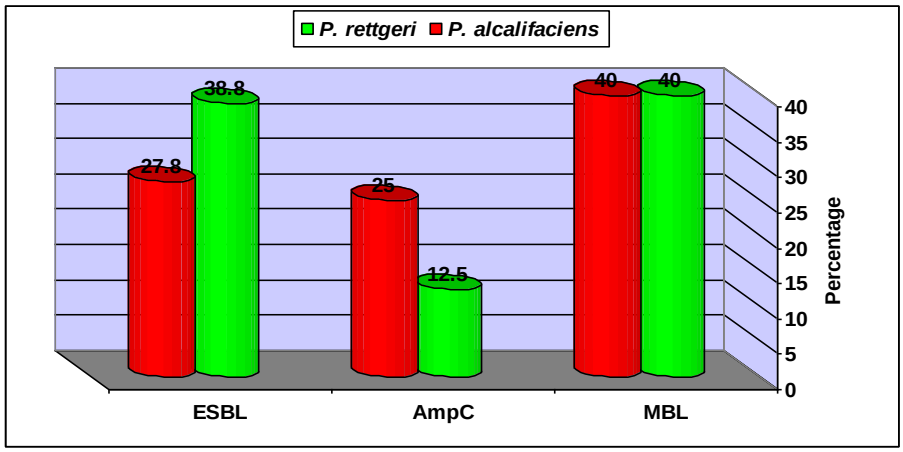


Figure 8: Comparative between the β-Lactamases production for single species *Providencia*.

Coexistence of a ESBL with other types of β-Lactamases producing *Providencia* strains was as follows: ESBL+ AmpC (5.6%), ESBL+ MBL (22.2%) and AmpC + MBL (5.6%) (Table 6).

Table 6: Relatedness among phenotypic ESBL, AmpC and MBL producing *Providencia* strains.

Source of Sample	*Providencia* speceis	No. (%) *Providencia* spp. strains			Total	
		ESBL+ AmpC	ESBL+ MBL	AmpC + MBL		
Sputum	*P. rettgeri*		1(5.6)		1(5.6)	
Wastewater	*P. rettgeri*	1(5.6)	2(11.1)		3(16.7)	5(27.8)
	P. alcalifaciens		1(5.6)	1(5.6)	2(11.1)	
Total		1(5.6)	4(22.2)	1(5.6)	6(33.4)	

Alarmingly, prevalence of β-Lactamases producing MDR *Providencia* strains were the highest (88.9% to 90.9%) strains depending on CLSI and EUCAST respectively for year 2017 (Table 7).

Table 7: Relatedness regarding between *Providencia* strains producing β-Lactamases and exhibits a MDR phenotypic depending on CLSI and EUCAST for year 2017.

β-Lactamase types	No. (%) *Providencia* spp. strains			
	CLSI-2017		EUCAST-2017	
	β-Lactamases exhibits a MDR n=9	β-Lactamases non exhibits a MDR	β-Lactamases exhibits a MDR n=11	β-Lactamases non exhibits a MDR
ESBL	4(44.5)	5(55.5)	6(54.6)	5(45.4)
AmpC	2(22.2)	7(77.8)	2(18.2)	9(81.8)
MBL	5(55.5)	4(44.5)	5(45.4)	6(54.6)
Total	8(88.9)	1(11.1)	10(90.9)	1(9.1)

Moreover, the vast majority of relatedness regarding between *Providencia* strains producing β-Lactamases and exhibits a MDR phenotypic were 8(100%) and 9(90%) depending on CLSI and EUCAST respectively for year 2017 in wastewater table (8). Strains have significant differences in the frequencies between β-Lactamases production and exhibits a MDR phenotypic and *Providencia* spices depending on sources specimens ($P < 0.01$).

Table 8: Relatedness between *Providencia* single spices producing β-Lactamases and exhibits a MDR phenotypic depending on CLSI and EUCAST for year 2017.

Source of Sample	*Providencia* speceis	No. (%) *Providencia* spp. strains producing β-Lactamases exhibits a MDR			Total
		CLSI-2017	Total	EUCAST-2017	
Sputum	*P. rettgeri*	0(0)	0(0)	1(10)	1(10)
Wastewater	*P. rettgeri*	1(12.5)	8(100)	1(10)	9(90)
	P. alcalifaciens	7(87.5)		8(80)	
Total		8(100)	8(100)	10(100)	10(100)

Discussion

Microbiological analysis led to the isolation and identification of two species from *Providencia* genus which they are *P. alcalifaciens* and *P. rettgeri*. These species are often found in wastewater , soil (Manos and Belas, 2006; Foti *et al*., 2009; Interaminense *et al*., 2010; Feyzioğlu, *et al*., 2013), and an un common cause of diseases humans and animals (O'Hara *et al*., 2000). This is similar with that reported by other studies in wastewater and RT (Urbanova *et al*., 2000; Shiroto *et al*., 2005; Xia *et al*., 2013; Abo-Amer *et al*., 2013; Tada *et al*., 2014; Shenoy *et al*., 2014). API technique had 99.9% accuracy in identify of *P. rettgeri* among *Providencia* spp. (Ahasan, *et al*., 2017). AST featured a high frequency of isolates resistant to some of antimicrobials studied. The rate of resistance to β-Lactams, co-trimoxazole and nitrofurantoin are consistent with other authors in their results (Laupland *et al*., 2007; Linhares *et al*., 2013). Thus, is similar results to resistance broad-spectrum penicillin's , co-trimoxazole and aminoglycosides with another study (Ahasan, *et al*., 2017). Strikingly, more than half (55.6%) of the strains were either resistance or intermediate resistance to imepenem compare with meropenem. This may be due to the strains have mechanisms resistance other than production of β-Lactamases against imipenem (CLSI, 2017). Tribe Proteeae strains have high rate of Tigecycline susceptibility for (EUCAST, 2017).

The present study classified resistance patterns in *Providencia* spp. according to (Magiorakos *et al.*, 2012). In Iraq, like other parts of the world, *Providencia* spp. have been shown to have low prevalence rate with they have multiple antibiotic resistance (Linhares *et al*., 2013; Wang *et al*., 2014; Olaitan *et al*., 2015; Baran and Aksu, 2016;

Perween *et al*., 2016; Marquez-Ortiz, *et al*., 2017; Sharma *et al*., 2017; Tshisevhe, *et al*., 2017; Cao *et al*., 2017). To the best of investigation knowledge, this is the first report to describe the MDR of these organisms . The alarming situation with global dissemination of MDR *Providencia* spp. strains highlights the need for their epidemiological monitoring and prudent use of antibiotics in AL-Diwaniya governorate /Iraq.

This study was the first reported signed to investigate the distribution and made a rough estimate of the status of β-lactamases produced *Providencia* spp. isolated from patients and environment in Iraq. Several surveillance studies revealed a relatively high incidence of ESBL, AmpC and MBL-producing organisms in world. This findings are in agreement with other results in Nigeria (Ibadin *et al*., 2017) and in India (Jena *et al*., 2014).

Although the coexistence of a ESBL with other types of β-Lactamases are no longer a rare event, the concomitant presence of 2 β-Lactamases in the same strain is reported (Jena *et al*., 2014; Ibadin *et al*., 2017). Moreover, the vast majority of *Providencia* strains producing β-Lactamases and exhibits a MDR phenotypic were observed in 8(88.9%) from total 8(100%) strains in wastewater . However, similar results with β-Lactamases producing MDR strains have been reported with other authors (Jena *et al*., 2014).

Epidemiologic surveillance of antimicrobial resistance is indispensable for empirically treating infections, implementing resistance control measures, and preventing the spread of antimicrobial-resistant microorganisms in AL-Diwaniya governorate /Iraq. Resistance of *Providencia* spp are among the most important resistance priority Where classify in critical level specifically carbapenem-resistant and third generation cephalosporin's- resistant (WHO, 2017).

Unfortunately, extensive use of antibiotics is the cause of resistance phenomena, and emergence of these resistance by β-Lactamases production mechanism especially carbapenemases in environment faces a very serious problem. Because the fact that carbapenems are the last choice used to treat infections in humans only. It is unknown how this resistance reached to the environment. Several explanations have been suggested to the phenomenon. One of the most important hypotheses it is overuse of antibiotics and their careless disposal through the ecosystem which may be assist in acquire genetic information's

through vertical transfer gene and horizontal transfer gene among different species. Many reports have reported that soil bacteria are an important reservoirs of resistance mechanisms to antibiotics, including carbapenemase enzymes production (Gudeta *et al*., 2015; Nesme and Simonet, 2015). The water polluted via faeces or human waste contains of pathogenic bacteria of human and animal origin , which is a source of their transport to wild birds via their food (Pindi *et al*., 2013; Pinto *et al*., 2015). It has been observed that antibiotic susceptibility of *Providencia* strains is not constant and varies with time and environment. This therefore demands the need for periodic screening of *Providencia* strains for their antibiotic susceptibility profiles in different environments and hospitals. Widespread occurrence of drug resistant *Providencia* in the environment and hospitals have necessitated the need for regular monitoring of antibiotics susceptibility trends to provide the basis for developing rational prescription programs and assessing theirs effectiveness.

Conclusions : The water sources and clinical specimens in AL-Diwaniya governorate / Iraq were contains of *Providencia* spp. harbored a diverse community of MDR producing of ESBL, AmpC and MBL, raising concerns about the overuse of antibiotics and their careless disposal through the ecosystem.

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