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Emergence of an Antimicrobial Resistant *Pseudomonas aeruginosa* from Human and Animal Clinical Samples: A zoonotic and Public Health Hazard

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Abstract: There is very little factual evidence to clarify the nature and extent of the multi-drug resistance (MDR) producing PER-1 Extended-spectrum β-lactamase (PER-1 ESBL) *Pseudomonas aeruginosa* problem In Egypt. The obtained results revealed that all isolates from human origin were totally resistant to ampicillin (100%) while the same isolates were totally sensitive to imipenem (100%). Most isolates were resistant to amoxicillin and less resistant to amoxicillin/clavulanic acid, but most isolates were sensitive to piperacillin while they were less sensitive to aztreonam. Results of detection of antimicrobial resistance of *P. aeruginosa* producing PER-1 ESBL revealed positive amplification of the 966 bp of PER-1 ESBL from the extracted DNA (only 3 PER-1 ESBL were positive isolates and 16 PER-1 ESBL were negative isolates). The results of detection of MDR to *P. aeruginosa* producing PER-1 ESBL revealed that, the gene appeared to be chromosomally located and present in isolates from human burns and from the internal organs of diseased chicken. The prevalence of resistance gene reported here suggests that *P. aeruginosa* from cattle operations, poultry farms and veterinary or/and human clinics may be important contributors to environmental reservoirs of resistance genes. It also highlights the importance of spreading of the beta-lactamase-mediated resistance mechanisms between countries and continents.

Key words: *Pseudomonas aeruginosa* · *bla*PER-1 · Antipseudomonal agents · Chicken · Milk · Calves · Burns · Urine · Sputum

INTRODUCTION

Pseudomonas aeruginosa, a Gram-negative non-fermenting bacillus, is a much feared pathogen. *Pseudomonas* spp. are ubiquitous organisms which occur in water, soil and decaying organic matter even contaminating distilled water [1]; it is also the cause of infections associated with hot tubs and contaminated contact lens solutions [1, 2]. They are able to colonize many clinical environments and components, including disinfectant solutions. They tend to persist in hospitals where an exchange can occur between patients and environmental habitats. Treatment with antibiotics to which the pseudomonads are resistant promotes infection with resistant strains of *Pseudomonas* [3, 4, 5] that are more virulent and may become established in hospital or farm premises. *P. aeruginosa* has also been identified as an animal pathogen. *Pseudomonas aeruginosa* is a bacterium capable of causing mastitis in dairy cows [6]. Intramammary infection by *Pseudomonas aeruginosa* remains one of the most refractory to antibiotic therapy [7]. The Eurosurveillance special issue on 20th November, 2008 comprised the emerging antimicrobial resistance threats in Europe such as extended-spectrum beta-lactamase (ESBL)-producing drug-resistant Gram-negative bacilli [8].

PER (*Pseudomonas* extended resistant) ß-lactamases are one of the rarer ESBL families; however, their prevalence may be increasing. The identification of PER-1 producers in America, Europe, in the Middle and Far East and Latin America suggests their proceeding dissemination [9-12]. The production of plasmid-mediated extended-spectrum ß-lactamases (ESBLs) has emerged as an important mechanism of resistance to ß-lactams, drugs

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that account for approximately 50% of antibiotic consumption [13]. Since ESBL distribution has been shown to differ among countries [14] monitoring of the prevalences and the types of extended-spectrum enzymes may contribute to defining the breadth of the problem in the geographic area of interest.

In this study, we report for the first time the detection of multidrug-resistant *P. aeruginosa* isolates producing the PER-1 ESBL that have been involved in nosocomial infections (human and animal) in various geographical locations in Cairo, further supporting the global spread of such strains. A molecular characterization of these isolates was performed to investigate their relationships and the spreading patterns of the bla_{PER-1} determinant. The importance of this study becomes evident and crucial when we review the study that was carried out in Hungary by Szabó *et al*. [15] in which he reported the first detection of PER-producing *P. aeruginosa*, in Hungary from a patient who was hospitalized in Egypt and transferred to Hungary suggesting the proceeding dissemination of bacteria carrying the PER-type ESBL enzyme outside the geographic area of origin. This case highlights the importance of spreading of the beta-lactamase-mediated resistance mechanisms between countries and continents illustrating the possibility of the inter-country and the inter-continent spread of the beta-lactamase-mediated resistance mechanisms. Their study featured the intercontinental spread of antimicrobial resistance, showing the importance of careful screening of human or animal patients arriving from a different country.

MATERIALS AND METHODS

Bacterial Strains and Susceptibility Testing: Source of Bacterial Isolates: *Pseudomonas* strains isolated from human (23 isolates), cattle (14 isolates) and chicken (12 isolates) sources in Egypt were selected in this retrospective study. Isolates were obtained from human origin in the form of sputum samples from patients suffering from lung manifestations (n = 123) and burn samples from patients exposed to third degree burns (n = 68) specimens and urine samples. In addition, mastitic milk samples (n= 195), lung tissues (n= 43) and internal organs of diseased chickens (n= 140) were also included in the study. Primary isolation was performed on Columbia agar containing 5% sheep blood. The isolates were identified with ID32 GN strips (bioMérieux, Marcy l'Etoile, France) and stored at -70°C in tryptone soya broth (Oxoid) supplemented with 15% (v/v) glycerol.

Antimicrobial Assay: Antibiotic susceptibility was determined using Mueller-Hinton agar by a disc diffusion method. The Antimicrobial agents used were: Amoxicillin (10 µg), amoxicillin/ clavulanic acid (20/10 µg), ampicillin (10 µg), aztreonam (30 µg), piperacillin (10 µg) and imipenem (100 µg). The discs were obtained from Himedia Laboratories. After the 18-24 hour incubation periods, the visible and clear zone of growth inhibition was measured and the interpretations were based on CLSI guidelines [16].

Plasmid DNA Extraction: The plasmids were extracted by the alkaline lysis method [17, 18]. The plasmid DNA pellet was resuspended in 20 µl DDW containing DNase free pancreatic Rnase (20 µg/ml) and incubated at 37°C for 30 minutes. The extracted DNA plasmid was mixed with the loading buffer and loaded directly to a well of a 0.7% agarose gel (Life Technologies, Bethesda, MD) containing 0.5 µg/ml of ethidium bromide (Life Technologies). The samples were electrophoresed in 1× TAE buffer (Tris-acetate, 0.5 M EDTA, pH 8.0) at 1.5 V/cm for 1-3 h parallely with a supercoiled DNA ladder marker (2 to 16 kb) (Initrogen). The plasmids and ladder were visualized over a UV transilluminator (model VWR M- 20E, VWR Scientific, Plainfield, NJ) and photographed using type 55 Polaroid film (Polaroid Corporation, Cambridge, MA).

PCR Detection and Sequencing of ß-lactamase (bla_{PER-1}) Genes: Bacterial cells were grown in 2 ml of Luria-Bertani (LB) broth overnight at 37°C and the DNA was extracted using the GenElute ™Genomic kit protocol (Sigma, USA). The amplified reactions were performed on the plasmid extraction material and DNA extraction material to detect *P. aeruginosa* isolates producing (PER1- ESBL) according to Pereira *et al.* [19]. In 0.5 ml thin walled microcentrifuge tubes. All reactions were performed in a 100-µl volume and the mixture consisted of 1µl (50ng) of extracted DNA and extracted plasmid materials 5 µl 10 x PCR buffer provided by the manufacturer containing 75 mM Tris-HCl, 2 mM $MgCl_2$, 50 mM KCl, 20mM $(NH_4)_2SO_4$, 1 µl (2 mM) deoxynucleoside triphosphates, 80 pmol of each primer and 200 ng of plasmid DNA as a template, 1 µl dNTPs (10mM), 25 pmol of each primer, 1 µl of Taq DNA polymerase (5 units/µl) and the volume (50 µl) of the reaction mixture was completed using DDW. The primers used for amplification of the bla_{PER-1} allele were Oligo Name: BLA-PER/F (5'-GGGACARTCSKATGAATGTCA) and BLA-PER/R (5'-GGYSGCTTAGATAGTGCTGAT).

The Thermal Cycler Was Adjusted as Follows: Denaturation, 95°C for 40 s; annealing, 53°C for 1 min; extension, 72°C for 1 min. The first cycle was repeated for 35 cycles; final extension, 72°C for 2 min. After preparation of the gel (0.7% in 1x TAE buffer) containing 0.5 µg/ml ethidium bromide, 10 µl of each of the PCR product samples and 5µl molecular weight marker were applied to the gel after mixing each with 1µl loading buffer on a piece of parafilm. Each mixture was applied to the well using 10 µl micropipette. The electrophoresis cell was covered and the power supply was adjusted at 10 volt/cm. Supercoiled DNA ladder marker (Fermentas) was used as DNA Molecular weight markers. The gel was taken out from the cell and examined on U.V. transilluminator and photographed with a polaroid camera. Calculation of the fragment size was performed at the National Center of Biotechnology Information website (http://www.ncbi.nlm.nih.gov/) using NCBI BLAST software.

RESULTS

The obtained results presented in Table 1 revealed that all *P. aeruginosa* isolates from human origin (sputum, burns and urine) were totally resistant to ampicillin (100%). The *P. aeruginosa* isolated from human sputum resistance dropped to 70% (amoxacillin and amoxicillin/clavulanic acid), 50% (aztreonam) and 30% (pipracillin), while the same *P. aeruginosa* isolates were totally sensitive to imipenem (100%). The *P. aeruginosa* isolated from human burns were resistant to amoxacillin and amoxicillin/clavulanic acid by 90% and 80% respectively to drop sharply to 40% and 10% to aztreonam and pipracillin respectively. The resistance of *P. aeruginosa* isolated from urine to amoxacillin and amoxicillin/clavulanic acid was 100% and dropped slightly to 66.7% aztreonam and pipracillin. Sensitivity of all of the *P. aeruginosa* isolates from human origin (sputum, burns and urine) was 100% to impenem.

As shown in Table 2 the isolates from milk, chickens and lung of calves were variably resistant to amoxicillin (80%, 100% and 75%, respectively). Resistance of the *P. aeruginosa* isolates to ampicillin from the same locations was higher (80%, 100% and 100% respectively). Resistance of the *P. aeruginosa* isolates to amoxicillin/clavulanic acid slightly dropped (70%, 100% and 75%, respectively). Resistance of the *P. aeruginosa* isolates from milk and chicken to aztreonam dropped significantly to 60% and 58.3% The *P. aeruginosa* isolates from milk, chickens and lung of calves were totally sensitive to imipenem (100%) and less to piperacillin (80%, 100% and 100%, respectively). Peculiarly, the *P. aeruginosa* isolates from milk were extremely non-sensitive to amoxicillin (20%), amoxicillin/clavulanic acid (30%), ampicillin (20%) and aztreonam (30%). Imipenem and pipracillin were the most active among the tested antibiotics against human and animal isolates.

Table 1: Results of the antibiogram sensitivity test of the collected isolates from human origin

	Isolates from sputum							Isolates from burns							Isolates from urine						
		Sensitive		Intermediate		Resistant			Sensitive		Intermediate		Resistant			Sensitive		Intermediate		Resistant	
Antibiotic Discs	No. of tested isolates	No.	%	No.	%	No.	%	No. of tested isolates	No.	%	No.	%	No.	%	No. of tested isolates	No	%	No	%	No	%
Amoxicillin	10	2	20	1	10	7	70	10	-	0	1	10	9	90	3	-	0	-	0	3	100
Amoxicillin + Clavulanic Acid	10	2	20	1	10	7	70	10	2	20	-	0	8	80	3	-	0	-	0	3	100
Ampicillin	10	-	0	-	0	10	100	10	-	0	-	0	10	100	3	-	0	-	0	3	100
Aztreonam	10	3	30	2	20	5	50	10	-	0	6	60	4	40	3	-	0	1	33.33	2	66.67
Imipenem	10	10	100	-	0	-	0	10	10	100	-	0	-	0	3	3	100	-	0	-	0
Piperacillin	10	7	70	-	0	3	30	10	9	90	-	0	1	10	3	1	33.33	-	0	2	66.67

Table 2: Results of the antibiogram sensitivity test of the collected isolates from animal origin

	Isolates from milk							Isolates from chickens							Isolates from lung of calves						
		Sensitive		Intermediate		Resistant			Sensitive		Intermediate		Resistant			Sensitive		Intermediate		Resistant	
Antibiotic Discs	No. of tested isolates	No.	%	No.	%	No.	%	No. of tested isolates	No.	%	No.	%	No.	%	No. of tested isolates	No	%	No	%	No	%
Amoxicillin	10	2	20	-	0	8	80	12	-	0	-	0	12	100	4	1	25	-	0	3	75
Amoxicillin + Clavulanic Acid	10	3	30	-	0	7	70	12	-	0	-	0	12	100	4	1	25	-	0	3	75
Ampicillin	10	2	20	-	0	8	80	12	-	0	-	0	12	100	4	-	0	-	0	4	100
Aztreonam	10	3	30	1	10	6	60	12	-	0	5	41.67	7	58.33	4	4	100	-	0	-	0
Imipenem	10	10	100	-	0			12	12	100	-	0	-	0	4	4	100	-	0	-	0
Piperacillin	10	8	80	-	0	2	20	12	12	100	-	0	-	0	4	4	100	-	0	-	0

Results of assay of Resistant *P. aeruginosa* producing PER-1 Extended- Spectrum β-lactamase:

The results revealed positive amplification of the 966 bp of PER-1 ESBL from the extracted DNA (only 3 *P. aeruginosa* isolates were positive for bla_{PER-1} gene and 16 *P. aeruginosa* isolates were negative).

DISCUSSION

The present investigation was directed towards investigating for the presence of PER-like ESBL determinants in isolates of *P. aeruginosa* that exhibited a resistance phenotype that was suggestive of ESBL production and that had been associated with nosocomial outbreaks in humans and animals. A molecular characterization of these isolates was performed to investigate their relationships and the spreading patterns of the bla_{PER-1} determinant. In light of the uncertainty surrounding the issue of antibiotic resistance, the Animal Remedies Board Operational Policy in New Zealand [20] considered that the potential for resistance must become a hazard that is addressed when assessing applications for a new licence for antibiotic trade name products. The Animal Remedies Board and the OIE Ad hoc Group of experts on antimicrobial resistance created by the Office International des Epizooties [21] have also directed that the licences of all existing antibiotic trade name products be reviewed with regard to the potential to cause antibiotic resistance in order to limit the spread of resistant bacteria among animals and to protect the health of consumers [21].

Nosocomial outbreaks of multidrug-resistant *P. aeruginosa*, usually caused by clonal spread, have been described globally for various hospitals and may be an emerging problem in outpatient settings in various parts of the world [14, 22, 23, 24]. The particular interest and clinical relevance of PER-1 ESBL is in the management of severe infections through at least three reasons [25]: (i) it confers resistance to most β-lactams, including aztreonam and newer antipseudomonal cephalosporins (i.e. ceftazidime and cefepime); (ii) it may be carried on a plasmid that has been transferred *in vitro* from PER-1-positive *P. aeruginosa* to PER-1-negative strains of the same species; and (iii) unlike other class A β-lactamases of *P. aeruginosa*, PER-1 appeared to be transmissible among different species.

The on-going detection of organisms producing ESBLs in clinical microbiology laboratories remains a contentious issue and compliance varies widely. Proficiency-testing studies performed by the World Health Organization and Centers for Disease Control have raised concerns about the current ability of many clinical laboratories to detect organisms producing ESBLs [26]. In proficiency testing of laboratories outside the United States only 2 of 129 laboratories specifically identified a highly resistant ESBL-producing *K. pneumoniae* isolate. A study recently published showed that only 8% of clinical laboratories from rural hospitals in the USA routinely screened for ESBL-producing organisms [26].

However, in our region, testing for ESBL production is not routinely done in any human or/and veterinary clinics. This may allow the dissemination of ESBL-producing strains within and between humans and animals to remain undetected for long periods. The consequence can be serious zoonotic outbreaks. The acquired resistance to the antipseudomonal ß-lactams agents poses an extensive threat to any anti-*Pseudomonas* chemotherapy, with special emphasis to the cases conjoined with drug resistance to other groups of drugs [27].

For this study, 100% of the PER-1 ESBL producers were resistant to amoxicillin and ampicillin (extended-spectrum penicillins) in contrast to the findings of Lim *et al*. [23] and Gad *et al.* [28]. The expression of PER-1 ESBL usually confers clear resistance to oxyimino-ß-lactams, especially aztreonam [29]. We found that our *P. aeruginosa* isolated strains harboured the bla_{PER-1} gene on the chromosome in agreement with Pagani *et al.* [29] not on the plasmids as recorded by Danel *et al.* [30].

The isolated *P. aeruginosa* producing PER-1 ESBL from hospitals in the Cairo area supports the hypothesis that PER-1 ESBL producing *P. aeruginosa* isolates have been endemic to this area for several years reflecting further evidence for an International Clonal Complex. On the other hand, the failure to detect PER-1 ESBL producers from mastitic cases and lungs of claves suggests that the resistance genes may be absent or less widespread in that area. Several studies on antimicrobial resistance and their relation to the presence of plasmids have been carried out in isolates of *P. aeruginosa* [31]. In the present study the strains of *Pseudomonas aeruginosa* isolated from a variety of human and animal sources were found to harbour plasmids. The findings of Padilla *et al.* [32] and Wolska and Bukowski [31] recorded that more than half of all *P. aeruginosa* strains examined had extrachromosomal DNA.

The results showed that *P. aeruginosa* isolates producing the PER-1 enzyme have been present in hospitals in the Cairo area and the surrounding region further supports the notion that PER-1-producing *P. aeruginosa* isolates have been endemic to this area for several years reflecting further evidence for an International Clonal Complex. In contrast, PER-1 producers were not detected in the isolates from mastitic cases and lungs of claves which is located in a different region suggesting that the resistance determinant may be absent or less widespread in that area. All of the PER-1 producers were not only resistant to extended-spectrum cephalosporins and monobactams, but also exhibited a multidrug-resistant phenotype that included most other antipseudomonal agents, leaving few therapeutic choices. Imipenem and pipracillin were the most active among the tested drugs against human and animal isolates.

In *P. aeruginosa*, the bla_{PER-1} gene has been found either in plasmids or on the chromosome [12]. The isolates investigated in this study, the bla_{PER-1} determinants were apparently located on the chromosome. The fact that *P. aeruginosa* isolates producing the PER-1 enzyme tended to exhibit a multidrug-resistant phenotype that usually included aminoglycosides [28, 29] could be related to a linkage between bla_{PER-1} and aminoglycoside resistance determinants within the same genetic element. In Europe, the bla_{PER-1} gene is found mostly in *P. aeruginosa*, where it resides within a specific transposon, named Tn*1213* by Poirel *et al.* [33] and Tn*4176* by Mantengoli and Rossolini [34]. The expression of PER-1 (and that of PER-2) usually confers clear resistance to oxyimino-ß-lactams, especially ceftazidime, ceftibuten and aztreonam [29].

CONCLUSION

In conclusion, the present results in conjunction with the results of Osman *et al.* [35] show that isolates of *P. aeruginosa* producing PER-1 ESBL indicate a considerable zoonotic potential for spread between human and animal patients. Therefore, we feel that it is extremely important to implement a revised strategy to monitor routinely for PER-1 ESBL production in the human and veterinary hospitals for devising suitable control strategies in Egypt.

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