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Carbapenem-resistant *Pseudomonas aeruginosa* originating from farm animals and people in Egypt

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Abstract

Introduction: Carbapenem-resistant *Pseudomonas aeruginosa* (CRPA) has become the leading cause of health care-associated infections. Treatment is difficult due to the lack of an effective antimicrobial therapy, and mortality is high. This study investigated the occurrence of CRPA in farm animals (buffaloes and cattle), livestock drinking water, and humans in Egypt. **Material and Methods:** A total of 180 samples were examined: 50 faecal each from buffaloes and cattle, 30 of livestock drinking water, and 50 stool from humans. The samples were cultured on cetrimide agar and the plates were incubated aerobically at 37°C for 24 h. The isolates were examined for the presence of the bla_{KPC}, bla_{OXA-48}, and bla_{NDM} carbapenemase-encoding genes using PCR and investigated for the exotoxin A (*toxA*) gene. The *toxA* gene from carbapenem-group resistant isolates was phylogenetically analysed. **Results:** *P. aeruginosa* was isolated from buffaloes, cattle, drinking water, and humans, with occurrences of 40%, 34%, 10%, and 20%, respectively. Carbapenem resistance genes were found in 60%, 59%, 67%, and 70% in buffalo, cattle, water and human samples, respectively. The *toxA* gene was detected in 80% of samples. The phylogenetic analysis showed that cattle and water sequences were in one cluster and more related to each other than to human isolates. **Conclusion:** Occurrence of CRPA among farm animals, drinking water, and humans was high, reflecting the environmental origin of *P. aeruginosa* and highlighting contaminated water as a potential transmitter of CRPA to livestock and next to humans.

Keywords: farm animals, humans, water, antibiotic-resistant *Pseudomonas aeruginosa*, carbapenem, Egypt.

Introduction

Pseudomonas aeruginosa is Gram-negative bacterium which exhibits a wide distribution range in nature and colonises diverse ecological niches in both soil and water. The host range of *P. aeruginosa* is very broad, spanning aquatic and terrestrial plants, animals, and humans (25). *P. aeruginosa* is an opportunistic human pathogen and can pose serious health risks to elderly and immunocompromised individuals. Aquatic reservoirs of *P. aeruginosa* are rivers (22), open oceans (12), recreational waters (2), and wastewater (15), making these bacteria a growing public health concern (24). Several factors, including biofilm formation, contribute to the survival of *P. aeruginosa* in a variety of environmental conditions (13, 23) and aid bacterial proliferation and distribution. *P. aeruginosa*, like many other pseudomonads, harbours intrinsic resistance to many classes of drugs, and to make matters worse, the bacteria can expand their antibiotic resistance by mutations or by acquiring resistance through horizontal gene transfer, causing therapeutic failures in humans (6). On the other hand, the dissemination of these drug-resistant bacteria from hospitals to the natural environment may increase the occurrence of community-acquired infections. In fact, although the source has yet to be clearly established, wastewater treatment plants have been found to harbour antibiotic-resistant *P. aeruginosa* strains in their effluent and in the surface water downstream (21).

Carbapenems have recently begun to be utilised for treatment of serious *P. aeruginosa* infections. They are regarded as the last-line treatment against multidrug-resistant (MDR) Gram-negative bacterial diseases (16). Carbapenem resistance in *P. aeruginosa* is particularly troublesome because this class of β-lactams represents the last therapeutic resource for the control of bacterial infections. Authorisation for use in veterinary pharmaceuticals around the world (27) has not been given to carbapenems. In fact, the precise origins of carbapenemase genes in animals remain undefined, and this indicates that these genes may have been mobilised

from environmental bacteria into animals due to the close contact animals have with the environment.

Since the carbapenemases in *P. aeruginosa* from non-human sources are not yet being systematically investigated, the objective of this study was to examine carbapenem-resistant *P. aeruginosa* in livestock animals and water and its implication for human health.

Material and Methods

Study samples. A total of 180 samples were examined. Samples of faeces totalling 100 were collected using sterile swabs from apparently healthy animals (50 buffaloes and 50 cattle) on three farms in the Giza Governorate. Thirty samples of livestock drinking water (1 L each) were collected from the same farms. Water samples were collected in sterile glass bottles containing sodium thiosulphate as a dechlorinating agent. In addition, 50 human faecal samples were also collected from people working on these farms. All samples were stored on ice and immediately sent to the laboratory for further testing.

Isolation and identification of *Pseudomonas aeruginosa*

Faecal samples. Faecal samples from animals and humans were cultured on cetrimide agar aerobically at 37°C for 24 h.

Water samples. Bacteria were trapped by filtration of 1 L of water through 0.45 µm sterile nitrocellulose filters (Sartorius, Germany). The filters were then vortexed in peptone broth and bacteria were allowed to grow in this broth at 37°C for 20 h according to Hikal *et al*. (11), then they were transferred to cetrimide agar plates and incubated aerobically at 37°C for 24 h.

The suspected colonies from faecal and water samples were identified according to Quinn *et al.* (20), and the pure isolates of *P. aeruginosa* were confirmed using API 20NE identification kit (bioMérieux, France).

Antibiotic susceptibility test. The standard disc diffusion method (Kirby–Bauer) on Mueller–Hinton agar plates) was performed for the antibiotic susceptibility test according to Clinical and Laboratory Standards Institute guidelines (7). *P. aeruginosa* isolates were tested using three carbapenem derivatives: imipenem, meropenem, and ertapenem.

Genomic DNA extraction. All *P. aeruginosa* isolates were extracted using a DNA Mini Kit (Qiagen, Germany) according to the manufacturer's protocol.

PCR amplification of the resistant genes. All isolates were examined for the carbapenemase-encoding genes bla_{KPC}, bla_{OXA-48}, and bla_{NDM} (9). These gene fragments were amplified using the gene-specific PCR primers listed in Table 1. PCR mixtures consisted of 1 µL of genomic DNA template, 12.5 µL of DNA polymerase master mix (Takara Bio Inc., Japan), and 0.4 µM of each primer, for a total reaction volume of 25 µL. PCR amplifications of the bla_{KPC}, bla_{OXA-48}, and bla_{NDM} fragments were carried out with the following cycling parameters: 30 cycles with a 1-min denaturation step at 94°C, a 1-min annealing step at 55°C, and a 2-min extension step at 72°C, and a final single 10-min extension step at 72°C.

PCR amplification of the toxin gene. All *P. aeruginosa* isolates were examined for *toxA* gene (28). The gene-specific primers are listed in Table 1.

PCR mixtures consisted of 3 µL of genomic DNA template, 25 µL of DNA polymerase master mix (Takara Bio Inc., Japan), and 0.4 µM of each primer, for a total reaction volume of 50 µL. The cycling parameters for the *toxA* PCR were as follows: 30 cycles with a 1-min denaturation step at 94°C, a 1-min annealing step at 68°C, and a 1-min extension step at 72°C, and a final single 7-min extension step at 72°C.

PCR product in a 5 µL volume was electrophoresed on 1.5% agarose gel to determine the size of the product.

Sequence analysis of *toxA* gene. After selecting one isolate each from animal, water, and human samples that showed resistance to carbepenem genes, the amplified *toxA* fragments were purified using the QIAquick gel extraction kit (Qiagen, Germany) according to the manufacturer's instructions and sequenced at Promega Lab Technology (Germany) by using the forward and reverse primers of *toxA* listed in Table 1. The sequences of the *toxA* gene were deposited in the National Center for Biotechnology Information (NCBI) GenBank database under the accession numbers KY407567, KY407566, and KY454872 from animal, water, and human, respectively.

The three *toxA* genes sequenced in this study were compared with the sequences available in the public domain using the NCBI BLAST server. Publicly available *toxA* gene sequences were downloaded from the NCBI GenBank and aligned using ClustalW in BioEdit version 7.0.1.4. Phylogenetic analysis was performed with MEGA version 7, using the neighbour-joining approach. The bootstrap consensus tree was inferred from 950 replicates (Fig. 1).

Table 1. List of primers used in this study

Gene	Sequence (5'–3')	Size (bp)	References
bla_{KPC}	F: ATGTCACTGTATCGCCGTCT R: TTTTCAGAGCCT TACTGCCC	882	Hamza *et al.* (9).
bla_{OXA-48}	F: TTGGTGGCATCGATTATCGG R: GAGCACTTCTTTTGTGATGGC	743	
bla_{NDM}	F: GGTTTGGCGATCTGGTTTTC R: CGGAATGGCTCATCACGATC	621	
toxA	F: GACAACGCCCTCAGCATCACCAGC R: CGCTGGCCCATTCGCTCCAGCGCT	396	Xu *et al.* (28).

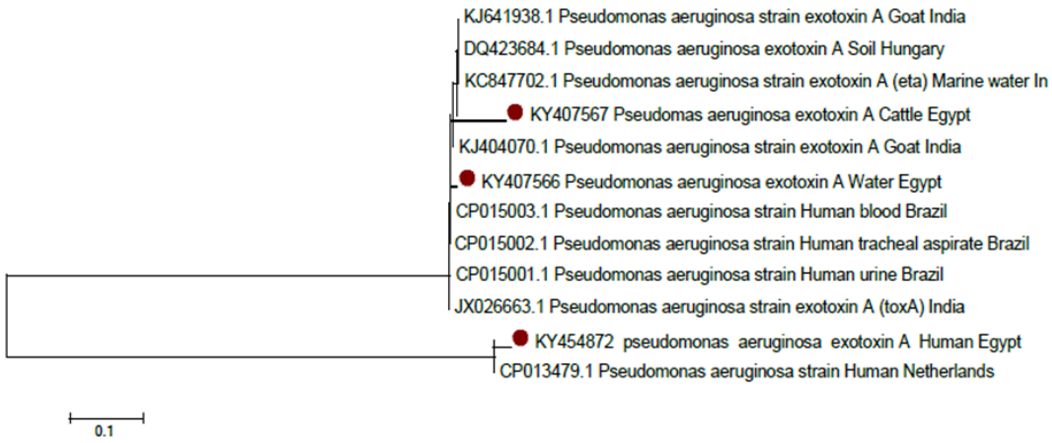

Fig. 1. Phylogenetic analysis of the *toxA* gene. The *toxA* genes sequenced in this study are marked with red solid dots. All other sequences are derived from the NCBI GenBank database

Table 2. Occurrence among different sources of *P. aeruginosa*, carbapenem-resistant *P. aeruginosa* (CRPA), and exotoxin A in the tested samples

Origin of sample	Number of samples	Number of samples positive for *P. aeruginosa*	Occurrence of *P. aeruginosa* (%)	Number of samples positive for CRPA	Occurrence of CRPA (%)	Exotoxin A
Buffalo	50	20	40	12	60	40 strains out of 50 (80%)
Cattle	50	17	34	10	59	
Livestock drinking water	30	3	10	2	67	
Human	50	10	20	7	70	

Results

Samples from buffaloes, cattle, livestock drinking water, and humans were examined for the presence of *P. aeruginosa*. The incidence of *P. aeruginosa* in faecal samples of buffaloes was 40% (20 out of 50 samples) whereas 34% of samples from cattle (17 out of 50 samples) were positive for this bacterium. Its incidence in drinking water was 10% (3 out of 30 samples). In human stool samples, the occurrence was 20% (10 out of 50 samples). Therefore, out of 180 samples collected, we identified 50 isolates of *P. aeruginosa* (Table 2).

All *P. aeruginosa* isolates were subjected to an antibiotic disc diffusion assay with carbapenem-group antibiotics (Table 2). We further characterised these isolates for the presence of the β-lactam resistance genes bla_{KPC}, bla_{OXA-48}, and bla_{NDM}, and the virulence of the *toxA* gene (Table 2). We found that 60% and 59% of animal isolates (buffalo and cattle), 67% of drinking water isolates, and 70% of human isolates were carbepenem resistant by both the disc diffusion method and testing for resistant genes. Furthermore, we found that 40 out of 50 isolates (80% prevalence) were positive for *toxA* as they showed amplification of a 396 bp *toxA*-specific fragment. Sequence analysis of the *toxA* gene from isolates derived from cattle, water, and humans fell in two clusters, with sequences from cattle isolates (KY407567) and water isolates (KY407566) being more related to each other than to the sequence from bacteria isolated from human samples (KY454872).

Discussion

The present study was undertaken to examine the prevalence and origin of *P. aeruginosa* in farm animals, livestock drinking water, and humans in Egypt. A large number of investigations have been carried out on *P. aeruginosa* isolated from human infections while fewer are available on strains isolated from animals. In veterinary medicine, *P. aeruginosa* may be responsible for difficult-to-treat infections (8). The bacterium was detected in enzootic or epizootic flare-ups of mastitis in bovine species (18). In the present study, *P. aeruginosa* isolates were found in 40% and 34% of faecal samples of buffaloes and cattle, indicating the dissemination of *P. aeruginosa* in farm animals.

The occurrence of *P. aeruginosa* in the surface water used for drinking by the livestock was 10%. It is apt to note that the cells of *P. aeruginosa* are able to proliferate in different environmental conditions or survive in a "viable but nonculturable state" (19). This observation can partially explain the low recovery of *P. aeruginosa* from water.

The use of antimicrobials in farming and livestock management has contributed to the spread of antibiotic resistance genes in the environment (14). Carbapenems are not licensed for use in veterinary medicine or livestock production anywhere around the world (27). However, in this study CRPA was recovered from 60% and 58% of animal isolates, 66% of water isolates, and 70% of human isolates. Screening for carbapenemase-encoding genes (bla_{KPC}, bla_{OXA-48}, and bla_{NDM}) by PCR showed that all phenotypically CRPA isolates harboured

three resistant genes. The presence of such resistant genes in non-human sources including animals and water indicates that these carbapenemase genes may originate from environmental bacteria and mobilise into animals due to the close contact between the two. Another scenario was highlighted by Walsh *et al.* (26), who reported that chromosomal carbapenemase is naturally co-transcribed with other chromosomally located β-lactamases; moreover, the over-expression of such genes is induced by the administration of β-lactam or carbapenem antibiotics. Hence, the use of penicillins or penicillin-β-lactamase inhibitor combinations in veterinary medicine can create selective pressure for naturally occurring chromosomal carbapenemases, which, in theory, can eventually be mobilised. These findings are of great concern because carbapenemases in bacteria from non-human sources are not yet being systematically investigated. In addition, the close contact between animal and human populations may be a high risk factor for acquiring such bacteria.

P. aeruginosa produces two different ADP-ribosyltransferase toxins: exotoxin A (ETA) and exoenzyme S (4, 5). The majority of *P. aeruginosa* strains produce the highly toxic ETA which inhibits eukaryotic protein biosynthesis at the level of polypeptide chain elongation factor 2. The high percentage of *toxA* gene (80%) among the *P. aeruginosa* isolates may seriously threaten public health, as ETA is cytotoxic to numerous mammalian cells (17). Importantly, in our study all carbapenem-resistant *P. aeruginosa* isolates harboured *toxA* gene.

To explain the possible genetic link between the activity of exotoxin A gene and antibiotic resistance inherited in *P. aeruginosa*, a phylogenetic tree was constructed based on the three *toxA* sequences recovered from carbapenem-resistant *P. aeruginosa* isolates from cattle (KY407567), water (KY407566), and humans (KY454872) and aligned with the other related *toxA* gene sequences obtained from GenBank (Fig. 1). Analysis of these sequences demonstrated that cattle and water isolates were found in the same cluster and are more related to each other than human isolates. This finding reflects the environmental origin of *P. aeruginosa* and highlights the potential role of contaminated water in the transmission of highly pathogenic CRPA to livestock and subsequently to humans. Epidemiologically, hospitals and healthcare settings are regarded as reservoirs for large numbers of pathogenic *Pseudomonas* strains. Wastewater from hospitals may contain a large number of these bacteria, some of which can also be multidrug resistant (3, 24). Therefore, we cannot eliminate the possibility of water contamination by hospital wastewater.

In conclusion, the presence and persistence of CRPA in non-human sources may pose a great risk to public health. Environmental water may play a pivotal role in transmission of CRPA between humans and animals and this requires further work to fully characterise and quantify the input of CRPA strains specifically from hospitals compared with those originating from the general community or other wastewater-related sources.

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Animal Rights Statement: Collection of samples was conducted according to the guidelines of the Ethical Committee of the Faculty of Veterinary Medicine, Cairo University, Egypt.

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