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Original Article

Pseudomonas aeruginosa: Prevalence of Pathogenic Genes, *OprL* and *ToxA* in Human and Veterinary Clinical Samples in Ardabil, Iran, 2020

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Abstract

Background & Objective: An opportunistic pathogen *Pseudomonas aeruginosa* can cause frequent hospital-acquired infections as well as one microorganism in the food spoilage. Also, the emergence of multidrug-resistant *P. aeruginosa* has become a serious threat to public health.This pathogen has many virulence factors which aid in bacterial invasion as well as toxicity, during infections. Out of different virulence genes in *Pseudomonas* aeruginosa, *oprL* (Encoding membrane lipoprotein L) and *toxA* (encoding exotoxin A i.e. ETA), are predominantly involved in, *P. aeruginosa*-related infections.
Materials & Methods: A total of 120 specimens of the bacteria *Pseudomonas aeruginosa* were collected from Veterinary microbiology and various hospital laboratories. The isolates were initially identified by culturing on MacConkey agar and Eosin Methylene blue (EMB) agar and were further characterized by morphological and biochemical tests. An antibiotic sensitivity test was carried out on 13 antibiotics using the disc diffusion method. Genotypic detection of *oprL* and *toxA* genes was performed using a specific PCR test.
Results: The results revealed that the *toxA* gene was detected by 84.62% in isolates belonging to human samples and 75% in the isolates of animal samples, whereas the *oprL* gene was detected by 80.77% and only 16.67 % in the isolates were derived from human and animal samples, respectively.
Conclusion: The PCR analysis can help in the fast and specific detection of *oprL* and *toxA* genes in *P. aeruginosa.* Monitoring of these pathogenic genes could prevent the risk of transmission of multi-drug resistant *P. aeruginosa*, from animals to humans.

Keywords: *Pseudomonas aeruginosa*, antibiotics, multi-drug resistance, lipoprotein, exotoxin, PCR

Introduction

An opportunistic pathogen, *Pseudomonas aeruginosa* is capable of causing infection throughout the body, especially in immunologically compromised patients that are underintensive care, catheterised or suffering from a chronic illness (1). These are slightly curved or straight rods that are uniformly stained, motile due to polar flagella and non-spore-forming in nature (2). They use a fermentative pathway for the carbohydrate metabolism with no gas production during the process. Their growth occurs between temperatures of 40-45^{0}C and is species-specific. Also, they can grow in the presence of arginine or nitrate (3). The infection caused by *P. aeruginosa* is dependent on many factors such as the action of

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virulence genes, strains, immune status of the host, and initial load of the pathogen. Under favourable conditions, and in a suitable host, some virulence gene factors in the bacteria get activated to make *P. aeruginosa* an opportunistic pathogen by invading the host's cells, where the proteins transcribed by the virulence gene factors trigger the infection process (3). The presence of virulence factors like exotoxin A, exoenzyme S, elastase and sialidase contribute cell to cell signalling, to confer infectious properties to *P. aeruginosa*. The two lipoproteins, which are present on the outer membrane of the *P. aeruginosa*, aid bacteria to become resistant to most antibiotics and antiseptics and are used for the identification using PCR (1). Additionally, it has been observed that biofilms protect these bacteria from external environmental damage and the attacks of phagocytes and antibodies (2). Along with being a causative agent in nosocomial infections, *P. aeruginosa* also has significance as a foodborne pathogen in food microbiology and is also considered a marker to assess the hygiene quality of the foodstuffs for human consumption. In the case of dairy products like milk, *P. aeruginosa* is found to be a milk-spoilage microorganism which causes alterations in milk's constituents and affects its shelf-life as well (4, 5). *P. aeruginosa* has multiple pathogenic factors which aid in its pathogenicity. *OprL* is the second most abundantly found outer membrane lipoprotein in *P. aeruginosa* and plays an important role in maintaining the cellular integrity of the bacteria and protects bacterial cells from oxidative stress (6, 7). Of the two important lipoproteins, *OprI* is used to detect the genus of *Pseudomonas* while, *OprL* can be used to detect *P. aeruginosa* species (8, 9). *Pseudomonas aeruginosa* exotoxin A (ETA), is one of the most potent virulence factors, which is an NAD^+-diphthamide ADP-ribosyl transferase (EC 2.4.2.36). It catalyses the transfer of ADP-ribose moiety from NAD^+ to the diphthamide residue on the eukaryotic elongation factor-2 (eEF-2) to disrupt the protein synthesis process, leading to host cell death. This bacterial toxin is heat-labile and is extremely toxic to mammalian cells (10).

Milk and milk products are among the most commonly consumed food by humans. Along with the increasing prevalence of multi-drug resistant *P. aeruginosa* in humans, the presence of MDR strains of this opportunistic pathogen in foodstuffs like milk poses a serious threat to public health (11). To avoid the transmission of MDR strains of *P. aeruginosa* from such foodstuffs to humans, maintaining hygiene and quality of milk is essential (12). Due to its remarkably developed antibiotic resistance, *P. aeruginosa* has become an increasingly difficult pathogen. *P. aeruginosa* had shown antibiotic resistance through various mechanisms which involve restricting outer membrane permeability, using an efflux system and producing different antibiotic-inactivating enzymes (13). According to World Health Organisation (WHO), such globally increasing incidences of MDR *P. aeruginosa* are worrisome and hence considered microorganisms with the critical necessity for the development of novel therapeutic modalities (14, 15).

Therefore, to assess the prevalence of *oprL* and *toxA* genes and multi-drug resistance, in humans and animal-related foodstuff, this study selected human clinical and raw cow milk samples. After culturing, morphological and biochemical tests were used to recognize *P. aeruginosa* which is further confirmed by species-specific polymerase chain reaction (PCR) to detect virulence genes like *OprL* (which is the most commonly observed in outer membrane lipoprotein in *P. aeruginosa*) and toxicity related virulence gene *ToxA* (1, 8, 9). In this study, we carried out the identification of *P. aeruginosa* from the clinical samples of humans and animals using PCR amplification of the *oprL* and *toxA* genes and the isolates were further characterized by biochemical tests. Also, antibiotic sensitivity was tested to evaluate the potentially developed antibiotic resistance within the *P. aeruginosa* isolates obtained from two different sources, i.e. humans and animals.

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Materials & Methodes

Sample collection, bacterial strains growth and identification

For this cross-sectional study, 120 specimens for the *Pseudomonas aeruginosa* were studied, which were collected in Ardabil in 2020 of which, 60 specimens were collected from the Veterinary microbiology laboratory from the cold-stored raw cow milk sources obtained from industrial livestock farming and the remaining 60 human samples were collected from the hospital and pathobiological laboratories from blood and urine sources. Only *P. aeruginosa* positive samples were selected from both human and veterinary sources. Samples which were negative for *P. aeruginosa* were excluded from this study. All collected samples were cultured in specific media like MacConkey agar [Merck, Germany]. From the 60 animal samples, 24 isolates were obtained and from the remaining 60 human clinical samples, 52 isolates of *P. aeruginosa* were obtained. The growth and colony characterization was done by culturing the samples on MacConkey media [Merck, Germany] and eosin methylene blue (EMB) agar [HiMedia, India] at 37°C overnight (16). These isolates were further characterized based on gram staining and biochemical tests of oxidase, catalase, motion, SIM (Sulfur, Indole, Motility), TSI (triple sugar iron), Agara agar, VP (VegsProsquier) broth, MR (methyl red), OD (ornithine decarboxylase), SiMc (Simon citrate) agar, AD (arginine dehydrogenase), LD (lysine decarboxylation), OF (Oxidation – Fermentation medium test) [Merck, Germany] (17-19). The cultured isolates of *Pseudomonas aeruginosa* were further used for the subsequent tests.

Antibiotic sensitivity test

Antibiotic sensitivity test (AST) was performed for different antibiotics, such as Ceftriaxone (30µg), Co-trimoxazole (1.25/23.75 µg), Ampicillin (10 µg), Cefixime (30µg), Colistin (10 µg), Coloxacilin (30µg), Enrofloxacin (30µg), Meropenem (10µg), Vancomycin (5µg) (20, 21), Nitrofurantoin (30µg), Doxycycline (30µg) and Sulphametoxazole+trimethoprim (75/25µg) by diffusion discs method (Kirby–Bauer method) (22) in Miller-Hinton agar medium. *Pseudomonas aeruginosa* ATCC27853 was used as a strain control in all AST performed. The results were compared with the Clinical Laboratory Standards Institute (CLSI) and decided as susceptible (S), resistant (R), and intermediate (I) accordingly (20, 23).

Preparation of bacterial DNA

Shortlisted *Pseudomonas* aeruginosa bacterial isolates were used for DNA extraction. DNA extraction was done using CinnaGen commercial kit called CinnaPure® commercial kit (CinnaGen Co) following the manufacture's protocol.

Primer selection and preparation of dilution

The sequence of the oligonucleotide with forward and reverse primers, which were used to detect *oprL* and *toxA genes* are listed in Table 1 (24). 10mL of 100 pmol/mL of the primer solution, was added to 90mL of nuclease-free water to make the final concentration of primer 10 pmol/mL.

Table 1. List of primers

Primer target	Primers sequence 5' to 3'	Product
OprL-F	ATGGAAATGCTGAAATTCGGC	504
OprL-R	CTTCTTCAGCTCGACGCGACG	504
ToxA-F	GACAACGCCCTCAGCATCACCA	397
ToxA-R	CGCTGGCCCATTCGCTCCAGCG	397

Detection of virulence genes by PCR

PCR amplification of the *OprL* and *ToxA* genes, was performed in a 25µL reaction mixture containing 0.5µL of dNTPs (10 mM), 0.5µl of each primer (10 pmol), 1.5µL $MgCl_2$ (25 mM), 0.2µL Taq DNA polymerase (5 U/µL). Each gene was amplified separately. *Pseudomonas aeruginosa* ATCC27853 was used as a positive control. PCR products were visualized by electrophoresis, using a 1% agarose gel stained with Ethidium bromide.

Analysis of the Data

Data obtained in this study were analysed using descriptive statistics and expressed in percentages. It was further used for the comparative analysis for the presence of *oprL* and *toxA* genes in different specimens.

Results

From the total 120 samples which were used in the study, we found that the presence of *ToxA* and *OprL* genes differed in both clinical samples of humans and animals as shown in Table 2.

Table 2. Numbers and % of the isolates from the animals and human clinical samples

Sample types	Total samples	Isolates obtained	% of isolates of the total samples
Animals (Cow Milk)	60	24	40%
Humans (Blood/urine)	60	52	86.67%

Our outcomes showed that all cultured isolates possess harbouring genes. The occurrence of the *toxA* gene in the obtained isolates of the human specimens was found to be 84.62% and for the animal specimens it was 75%. The presence of susceptible gene *OprL* in human samples was found to be 80.77% and in animal isolates, it was found to be 16.67%. Similarly, the presence of both genes in animal isolates was found to be 62.50% and 73.1% in human isolates, as shown in Table 3.

Table 3. Occurrence of the virulence genes in the cultured isolates of *P. aeruginosa*

Name of the gene/species	Number (n) of isolates in which respective genes are found / total number of isolates in respective clinical samples	Percentagewise occurrence of the gene in each animal and human specimen
ToxA in animal isolates	18/24	75%
ToxA in human isolates	44/52	84.62%
OprL in animal isolates	4/24	16.67 %
OprL in human isolates	42/52	80.77%
ToxA+OprL in animal isolates	15/24	62.50%
ToxA+OprL in human isolates	38/52	73.1%

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In the case of the antibiotic susceptibility test, two types of properties were examined which include resistance and susceptibility factors. The highest percentage of resistance was displayed against antibiotics such as Ceftriaxone, Co-trimoxazole, Ampicillin, Cefixime, and Sulphametoxazole+trimethoprim ranging from 95-99% by the resistant isolates of *P. aeruginosa*. In the case of susceptibility factors, Colistin, Vancomycin, and Nitrofurantoin show the highest percentage. Results showed that bacteria cannot grow in presence of these drugs, as depicted in Table 4.

Table 4. Antibiotic susceptibility test results of *P. aeruginosa* from all samples

Antibiotic name	Quantity	Both Specimen		Resistant strains	
		Resistance strain%	Susceptible strain%	Animal%	Human%
Ceftriaxone	30ug	96.66	0.83	94.50	98.82
Co-trimoxazole	1.25/23.75 µg	95.83	1.66	94.80	96.86
Ampicillin	10ug	97.5	2.5	95.60	99.40
Cefixime	30ug	98.33	0.83	97.10	99.56
Colistin	10ug	89.16	5.80	87.92	90.40
Coloxacilin	30ug	95	2.5	93.70	96.30
Enrofloxacin	30ug	94.16	4.1	92.80	95.52
Meropenem	10ug	84.16	3.47	76.82	91.50
Vancomycin	5ug	94.46	8.1	92.30	96.62
Nitrofurantoin	30ug	91.66	5	90.50	92.80
Doxycycline	30ug	82.5	4.1	80.30	84.70
Sulphametoxazole + trimethoprim	75/25ug	95.15	1.6	94.10	96.20

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P. aeruginosa isolates isolated from both types of clinical samples carried both *ToxA* and *oprL* genes. The distribution of virulence genes with respect to isolate origin was compared using the chi-square test. Statistically significant was considered to be P value below 0.05.

The difference between the prevalence of the genes in the *P. aeruginosa* isolates obtained from the human and animal samples was significant on a statistical level. The prevalence of *ToxA* and *OprL* genes was significantly higher in human specimens as compared to isolates obtained from animal samples. Independent determination of the 76 isolates suggests that they are associated with different levels of intrinsic virulence with 23.68% and 57.89% for *toxA gene* while, 5.26% and 55.26% for oprL gene in animals and humans, respectively. Data are depicted by the graph represented in Chart 1 and the number of genes found in the respective specimen is shown in Table 3.

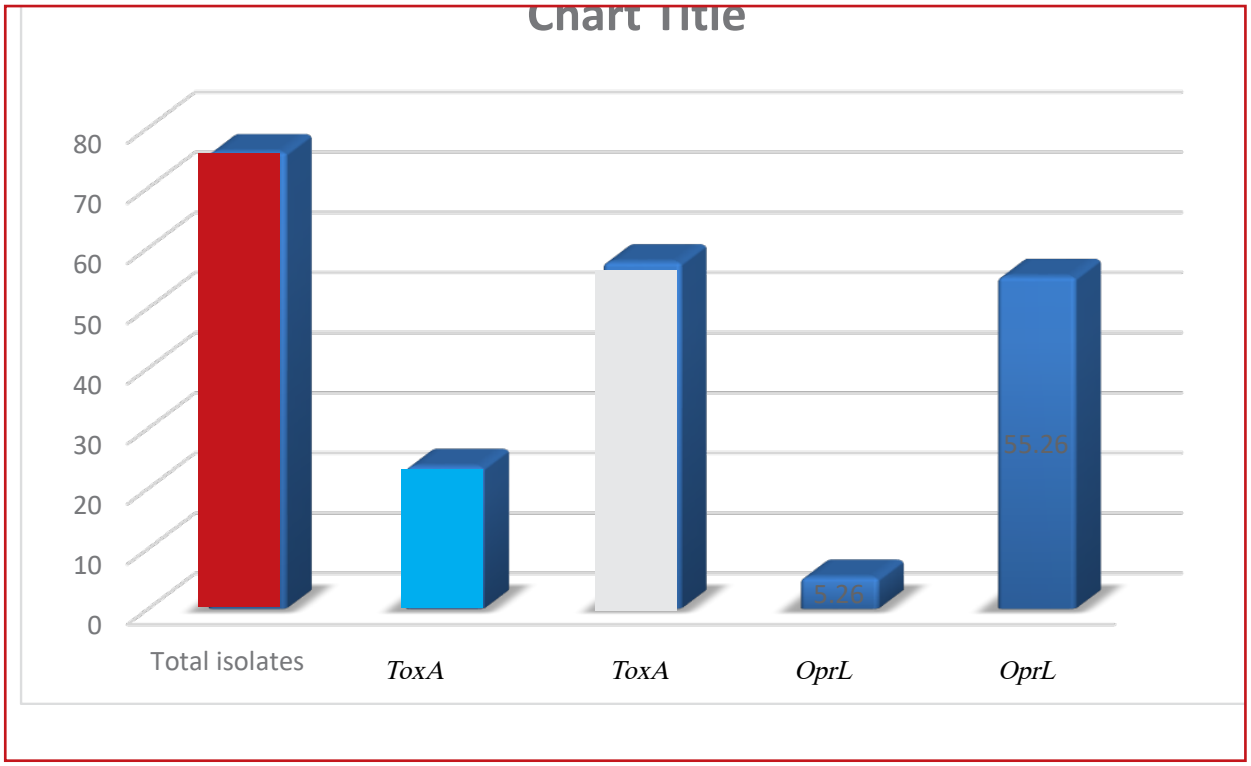

Chart 1. The percentagewise occurrence of both *ToxA* and *OprL* genes independently in humans and animals out the total 76 isolates of the *P. aeruginosa* obtained from both human and animal clinical samples

Discussion

P. aeruginosa is a worldwide health problem, especially with their increasing resistance towards generally used antibiotics. These antibiotic-resistant strains are difficult to handle with traditionally used antibiotic treatment due to their characteristic biofilm formation and the presence of different types of virulent genes. This emergence is linked with the abuse of antibiotic therapy (25). Phenotypic methods have been mostly used for the identification of *P. aeruginosa* and are still used as the most accurate standard when dealing with the typical isolates. Biochemical tests are reliable yet common procedures. However, a superior method based on molecular biology can be used, rather than phenotypic or biochemical methods for the identification of *P. aeruginosa* (26). These bacteria have a high genotypic diversity due to their ability to adapt to the environment, which leads to unusual phenotypic characteristics.

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Hence, molecular methods such as PCR can be more effective in identifying pathogenic isolates (27). Many studies in different parts of the world favour the use of molecular methods for the diagnosis of *Pseudomonas aeruginosa* and it has been done by using different genes to evaluate the results (28, 1).

Nikbin *et al.*, (2012) studied the presence of various pathogenic genes like; *oprL, oprI, toxA, lasB, exoS*, and *nan1*. They reported that the prevalence of *nan1* and *toxA* genes was significantly higher in pulmonary tract and burn isolates than that of *oprL*, *oprI* and *lasB* genes. Hence, they have suggested that there are different levels of intrinsic virulence and pathogenicity associated with different pathogenic genes in *P. aeruginosa* (1). Khan and Cerniglia, (1994) proposed the use of PCR amplification of the *toxA* gene. It can help to detect *P. aeruginosa* in environmental and clinical samples even at a low level without using selective media or additional biochemical tests (2).

Amini et al. (2010) examined the presence of the *toxA* gene on 70 strains of *P. aeruginosa* from patients with type 2 and 3 burns. Out of 70 strains isolated from burn patients, 66 strains had this gene in PCR reaction. The sensitivity of this experiment was determined to be 94.3% (29). Khosravi et al.'s (2016) study evaluated the prevalence of two exotoxin genes in *Pseudomonas* aeruginosa strains obtained from samples of burn patients. Their study found MDR *P. aeruginosa* strains in clinical samples with an equal frequency of both exotoxin genes, indicating the significance of these genes in the development of antibiotic resistance to this pathogen (30). In Bogiel et al.'s (2021) study, MDR *P. aeruginosa* showed the presence of the *toxA* gene in 96.3% of strains in human clinical samples (31). While in another study by Goncalves et al. (2017), the prevalence of the *toxA* gene was found to be 87.5% in Carbapeneme-resistant *P. aeruginosa* (CRPA) strains and this prevalence value is close to the prevalence value of the *toxA* gene (84.62%) observed in the human clinical samples of this study (32). Hence, the results of this study support that the prevalence of the *toxA* gene is commonly observed in MDR strains of P. aeruginosa and might be contributing to the increased virulence of this strain, as suggested by the previous studies (31, 32).

Some researchers in different animal- and human-related clinical studies have found that genes such as *oprL* and *toxA* are associated with the multidrug-resistance in *P. aeruginosa* related infections (33, 34). Chand *et al.*, (2021) study observed that out of 87 isolates of *P. aeruginosa* obtained from the clinical samples collected from a Tertiary Hospital of Nepal, 38 (43.68%) isolates showed multidrug resistance (MDR). They found that the *oprL* gene was present in all isolates (100%), while 95.4% of isolates were positive for the *toxA* gene. Hence, they explained that identifying virulence genes such as *oprL* and *toxA* genes in *P. aeruginosa* isolates along with the multidrug resistance, becomes essential. They proposed that it can assure the need of planning strategic treatment options to prevent the development and spreading of antimicrobial resistance (AMR) (34).

El-Ghany WA (2021) discussed that there is a possibility of passing down the *P. aeruginosa* associated infections, from animals to humans during the consumption of animal-related food products. Hence, we have studied if there is a similar prevalence of pathogenic genes like *oprL* and *toxA* in animals (as from cow milk samples) and humans, which are positive for P. *aeruginosa* bacteria (35). Similarly, Abdullahi et al., (2013) studied the presence of the *oprL* gene for the detection of the *P. aeruginosa* isolates obtained from aquaculture environment and their study found that the *oprL* gene was present in *P. aeruginosa* isolates and almost all isolates showed antibiotic resistance against different antibiotics used in that study. Hence, they suggested the potential risks of transmitting such multidrug-resistant *P. aeruginosa* bacteria, from aquaculture to humans, which are exposed to such environment (36).

Therefore in this study, we have evaluated

the presence of *oprL* and *toxA* genes in the isolates of *P. aeruginosa* isolated from the animal and human clinical samples. Out of 120 specimens (60 in animals and 60 in humans) of *P. aeruginosa*, 76 isolates (24 and 52 from animals and humans) were isolated and used for the subsequent analysis. The antibiotic susceptibility testing of the bacteria on Müller-Hinton agar medium was done, according to CLSI guidelines using antibiotic discs and it showed that most of the isolates were resistant to the different antibiotics. In this test, the highest value of resistance was shown by the Cefixime antibiotic and highest susceptibility of the isolated *P. aeruginosa* strains was shown for the antibiotic Colistin. Aslani et al., (2009) identified genus-specific genes *oprI* and *oprL* on *P. aeruginosa* specimens isolated from respiratory infections. 100% of the samples were positive for *oprI* and *oprL* specific genes and 83% of samples were found to be positive for *toxA* gene in human samples by PCR method (28). Similar to the Aslani et al.'s (2009) study, in this study, too, we examined the presence of *toxA* gene by 84.62% in *P. aeruginosa* isolates obtained from human clinical samples. Later, these isolates were grown on the specific media to harvest the bacterial DNA and were further processed to check the presence of *oprL* and *toxA* genes by the PCR method. The results obtained in this study suggest that the prevalence of the *toxA* gene in both *P. aeruginosa* isolates of animals and humans clinical samples were 75-85%. However, the presence of the *oprL* gene was found higher in humans (80.77%) than animals (16.67%). Out of 13 antibiotics or combinations of antibiotics used, *P. aeruginosa* showed antibiotic resistance against most of the antibiotics except polymyxin class antibiotics such as colistin and glycopeptide antibiotics such as vancomycin.

Conclusion

In conclusion, the prevalence of pathogenic genes such as *oprL* and *toxA* genes differ in both animals and humans. Determination of different virulence genes of *P. aeruginosa* isolates from two different species suggests that they are associated with different levels of intrinsic virulence, pathogenicity and host specificity. These factors might be causing varied consequences on the outcome of infections. Observed multidrug resistance in both samples is worrisome. Since animal sources used here are cow's milk samples, there is a need to study potential risks of transmitting such MDR *P. aeruginosa* from animals to humans due to consumption of milk or milk-related products. Therefore, there is a need to find out the significant correlations between some virulence genes and sources of infections. Also, the presence of these two genes and antibiotic resistance observed in the isolates indicate that further studies are required for finding out the actual role of these genes in different clinical infections caused by *P. aeruginosa* and they are also related to the multidrug resistance shown by *P. aeruginosa*. Along with it, the isolates which did not show the presence of either of the genes of interest need to be further investigated for the presence of other virulence genes from *P. aeruginosa* and their possible role in the infections. More research on virulence genes can also help to assess whether these genes can be used as potential targets while developing new therapeutic strategies for treating multi-drug resistant *P. aeruginosa* related infections. Also, further study is required to properly plan the handling and processing the animal food products, to avoid the risks of transmitting such pathogens and their infections from animals to humans.

Conflict of interest

There is no conflict of interest.

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