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Detection of Different Types of Class 1, 2 and 3 Integrons among *Pseudomonas aeruginosa* Isolates from Raw Milks

Parisa Mobasseri [1], Mahdi Jamali Harsini [1], Sedigeh Mehrabian [1], Kumarss Amini [2]*

[1] *Department of Microbiology, Faculty of Biology Sciences, North Tehran Branch, Islamic Azad University, Tehran, Iran.*

[2] *Department of Microbiology, Faculty of Basic Sciences, Saveh Branch, Islamic Azad University, Saveh, Iran.*

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ABSTRACT

Background: *Pseudomonas aeruginosa* is a leading cause of hospital-acquired infections that causes severe diseases in immuno-compromised individuals. integrons have a major role in multidrug resistanct, diversity, evolution and recombination strains. Various animals may act as the reservoir for bacterial humans pathogens. This study is aimed to evaluate the frequency of class 1, 2 and 3 integrons in *P. aeruginosa* isolates detected in raw milks.

Methods: Identification of isolates were confirmed with morphology, Gram staining and biochemical tests. Drug resistance to various antibiotics was investigated using agar disk diffusion method. After DNA extraction of the isolates, they were subjected to a polymerase chain reaction for detection of class 1, 2 and 3 integrons.

Results: In this study, 60 *P. aeruginosa* isolates were isolated from raw milk samples. The isolates showed resistance to amikacin (100%), ampicillin (100%), gentamicin (86.6%), cefotaxime (10%), ciprofloxacin (6.6%) and ceftazidime (3.3%). PCR analysis revealed the presence of *intI-1* in 49(81.6%), *intI-2* in 9(15%), and *intI-3* in 31(51.6%) isolates. Furthermore, class 1 and class 2 integrons were detected in 8(13.3%). In place, class 1 and class 3 integrons were observed in 26(43.3%) and class 2 and class 3 integrons in 6(10%) isolates.

Conclusion: Ciprofloxacin and ceftazidime were the most effective antibiotics against *P. aeruginosai* isolates in this study. The distribution of different classes of integrons in this study was high and it sheds light on the importance of regulations on the antibiotic uses.

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*Corresponding Authors: Kumarss Amini, Department of Microbiology, Faculty of Basic Science, Saveh Branch, Islamic Azad University, Saveh, Iran.
Tel: +98-21-44850954, Email: Dr_kumarss_amini@yahoo

Introduction

Pseudomonas aeruginosa is a gram-negative, aerobic, non-fermentative, flagellated, non-fermentative, nosocomial and opportunistic organism (1). *P. aeruginosa* causes severe invasive infections in iatrogenic immuno-compromised, cystic fibrosis, AIDS, cancer and neutropenia patients. It has been long responsible for pneumonias, bloodstream infections, surgical site infections and urinary tract infections (2, 3). Mutation of genes or change of action mechanisms in chromosomal or mobile genetic elements such as transposons, integrons, miniature inverted-repeat transposable elements and plasmids are the resistance mechanisms in *P. aeruginosa*. They have a role in acquiring and spreading resistance in gram-negative bacteria (4, 5).

Class I integron has been observed in gram-negative organisms such as *Pseudomonas, Acinetobacter, Burkholderia, Campylobacter, Citrobacter, Alcaligenes, Salmonella , Aeromonas* and *Klebsiella.* Class II integron is most commonly found in *Burkholderia, Salmonella enterica, Acinetobacter baumannii* and *Escherichia coli.* Class III integron has been identified within *Pseudomonas aeruginosa, Pseudomonas putida, Acinetobacter* spp., *Citrobacter freundii, Klebsiella pneumoniae, Serratia marcescens, Salmonella* spp., *Alcaligenes* and *Escherichia coli* (6). Integrons can cause resistance to β –lactams, aminoglycoside, chloramphenicol, rifampicin, trimethoprim, quinolone, macrolides and quaternary ammonium compounds (7).

Production of AmpC beta lactamase, aminoglycoside modifying enzymes, decrease permeability of outer membrane, presence of several efflux pumps, DNA gyrase and topoisomerase are resistance mechanism to antibiotics in *P. aeruginosa* (3, 8). To date, five classes of integrons have been described in gram-negative bacterial isolates (9). The structure of class 1 integrons consists two conserved regions, including 3′ conserved segment (3′ CS) and 5′ conserved segment (5′ CS). While class 1 integrase gene encoding a site-specific integrase (intI), the adjacent recombination site, (attI) and a promoter region (Pant), sulfonamide resistance gene (sulI) and antiseptic resistance gene (qacEΔ1) (9). Class I integron has been found to code for resistance to several antimicrobial agents in bacteria, including cassettes for resistance to fluoroquinolones, β-lactams, aminoglycosides, trimethoprim and chloramphenicol (10). In recent years, treatment of infections caused by *P. aeruginosa* has encountered a number of difficulties due to the emergence of multiple drug resistance *P. aeruginosa* and leads to increased mortality. *Pseudomonas* has been identified as predominant milk-associated psychrotrophic bacteria groups in dairy industry and their presence is indicator for fecal contamination. During cold storage after milk collection they dominate the flora, and their extracellular enzymes, mainly proteases and lipases, contribute to the spoilage of dairy products. The extra cellular enzymes can resist pasteurization and even ultrahigh temperature processing.

This study is aimed to evaluate the frequency of class 1, 2 and 3 integrons in *P. aeruginosa* isolated from raw milks.

Materials and Methods

Bacterial isolates

P. aeruginosa isolates were obtained from raw milks and transported to the laboratory for further

analysis. Samples were collected from milk via test tubes and containers carefully to minimize contamination and processed adequately until transportation to work lab by ice box, then refrigerated at 4 °C as critical control point in isolation and identification procedure of *P. aeruginos*. samples were diluted and inoculated in buffered tryptone soya yeast extract broth and incubated at 37 °Cfor 24 hours, then inoculated in Blood agar, MacConkey agar and in Cetirmide agar, then incubated at 37 °C. Bacterial isolates were identified as *P. aeruginosa* using standard microbiological methods including gram staining, oxidase, oxidative-fermentative, catalase, urea, methyl red and Voges-Proskauer test, production of the pyocyanin pigment, nitrate reduction, motility, growth at 42◦C and growth on Cetrimide agar.

Antibiotic susceptibility testing

Antimicrobial susceptibility testing was carried out at 37°C on Mueller-Hinton agar by using the Kirby-Bauer disk diffusion method on Mueller-Hinton agar plates according to the Clinical and Laboratory Standards Institute guidelines (CLSI). The antimicrobial disks included cefotaxime (30 µg), ceftazidime (30 µg), ciprofloxacin (5 µg), gentamicin (10 µg), amikacin (30 µg), ampicillin (10 µg) provided by a commercial company (Padtan Teb, Iran). For antimicrobial susceptibility testing *P. aeruginosa* ATCC 27853 was used as a control.

DNA Extraction

For DNA extraction, 1.5 ml of fresh overnight cultures in LB broth were centrifuged and the pellete was used as a template for DNA extraction using Gene Transfer Pioneers kit (Cat No DM04050).

Detection of class 1, 2 and 3 integrons

The presence of class 1, 2 and 3 integrons in *P. aeruginosa* was investigated by amplification of integrase genes including *intI1, intI2,* and *intI3* specific primers (Table 1). The PCR reactions were prepared in a total volume of 25 µL and amplification PCR mixture contained DNA template 2 µL, Mastermix 12.5 µL, distilled water 8.5 µL and 1 µL of forward and reverse primers. PCR reactions were performed in a Eppendorf master cycler, thermaocycler. *P. aeruginosa* ATCC 27853 was used as positive control. After performing PCR reactions, the PCR products with DNA Ladder 100 bp were visualized using DNA electrophoresis on 1% agarose gels. The condition for PCR amplification of different genetic regions in this study are shown in Table 2. After staining with ethidium bromide the bands were visualized by a UV-gel documentation system.

Results

From a total of 100 samples that were collected from raw cows' milk, 60% were found as *P. aeruginosa*-positive. In this study the frequecy of resistance to antibiotics was as: amikacin (100%), ampicillin (100%), gentamicin (86.6%), cefotaxime (10%), ciprofloxacin (6.6%) and ceftazidime (3.3%). PCR analysis showed that

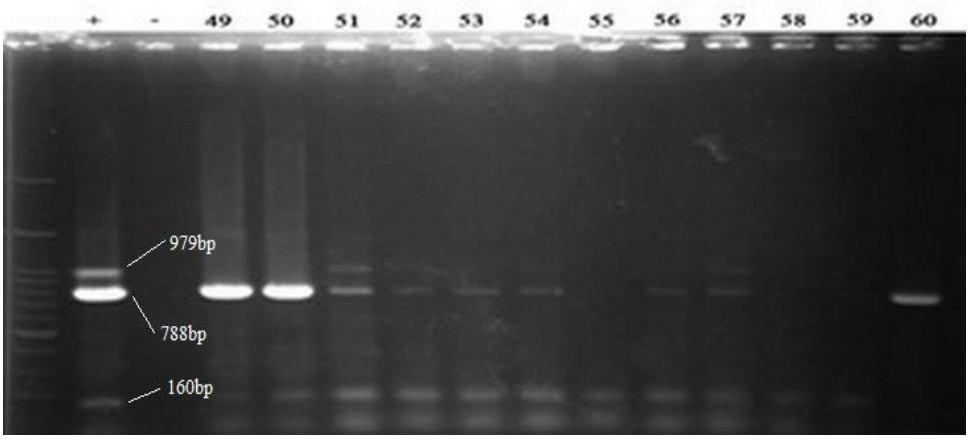

Figure 1. Gel electrophoresis of PCR products of intI-1 gene (160 bp), intI-2 gene (788 bp), intI-3 gene (979 bp) Lane: +, positive control; Lane: -, negative control; 51-60, raw milk isolates.

Table 1. List of primers, sequences and sizes used for the PCR amplification**.**

Target gene	Primers sequence(5′-3′)	Size of product	References
intI-1F *intI*-1R	CAGTGGACATAAGCCTGTTC CCCGAGGCATAGACTGTA	160bp	11
intI-2F *intI*-2R	GTAGCAAACGAGTGACGAAATG CACGGATATGCGACAAAAAGGT	789bp	11
intI-3F *intI*-3R	GCCTCCGGCAGCGACTTTCAG ACGGATCTGCCAAACCTGACT	980bp	11

class 1 integron in 49 (81.6%) class 2 integron in 9 (15%) and class 3 integron in 31(51.6%) isolates. Class 1 and class 2 integrons in 8 (13.3%) class 1 and class 3 integrons in 26 (43.3%) and class 2 and class 3 integrons in 6 (10%) isolates simultaneously were detected.

Discussion

Pseudomonas can survive in various terrestrial and aquatic niches. It possesses several virulence factors (12). Psychrotrophic bacteria are defined as those that grow at 7°C, although their optimal growth temperature is higher. *Pseudomonas* is the most frequently reported psychrotroph in raw milk. During cold storage after milk collection they dominate the flora, and their extracellular enzymes, mainly proteases and lipases, contribute to the spoilage of dairy products. Food-associated pseudomonads might transfer antibiotic resistance genes to human pathogenic bacteria during food processing or after ingestion, raising possible risks for human health.

Proliferation of antibiotic resistance in microbes is the result of extraordinary abuse of antibiotics in both clinical and veterinary treatments. The global public health concerns are

the risk of antibiotic resistance in various microorganisms (13). Plasmids, transposons, integrons, phages and multi-drug resistance genomic islands are responsible for the rapid emergence of antibiotic resistance among clinical isolates of bacteria. These elements play an important role in evolution, bacterial adaptation and diversity of multi-drug resistant strains (14, 15). Integrons can capture external drug resistance gene cassettes and integrate gene cassettes by site-specific recombination and express gene cassettes. Obtaining gene cassettes and expression of them can facilitate via integrons (12, 15, 16).

In our study 60% of our samples were *P. aeruginosa*-positive. In a study conducted by Meng et al., raw milk samples that were collected directly from 87 bulk tanks and a total 143 isolates were confirmed as *Pseudomona*s (17). In Bader et al. study, nine strains out of sixty milk samples (15%) were identified as *P. aeruginosa-positive* (18). In Bekci et al. study, from a total of 50 samples that were collected from raw milk, 15 *Pseudomona*s isolates were detected (19).

In the present study, ciprofloxacin and ceftazidime were the most effective antibiotics against *P. aeruginosa*. The selective pressures and uses of inappropriate antibiotics are factors that have roles in drug resistance in various bacterial species. In one study conducted by Arslan et al., *Pseudomonas* spp. from homemade white cheese samples had the highest resistance to penicillin G (20). In one study by Haenni et al., 68.2% of *P. aeruginosa* isolated from dairy cows and horses were resistant to fosfomycin (21). In study carried out by Fazlani et al., *P. aeruginosa* from clinical mastitic milk samples of camels, had highest resistance to chloramphenicol, amoxicillin and kanamycin (22).

Difference in antibiotic therapy regimens and difference in geographical regions, are responsible for differences between the results in this study and other studies. In a study by Khosravi et al., in 2016, 89 *P. aeruginosa* isolates isoalted from different clinical specimens at the hospital, 95.7% of them were contained *int*1 gene (23). In a study by Salimizadeh et al., the class 1 integrons were found in 90.5% *P. aeruginosa* isolates collected from hospitals in Tehran (24). In a study by Hosseini et al. around 90% prevalence of class 1 integrons has been reported for *P. aeruginosa* isolated from burn patients who were hospitalized (25). In a study by Shojapour et al., in 2019 class 2 integron was not found and class 1 integron was detected in 95% of *P. aeruginosa* isolates (26). These studies have almost similar results to our study.In one study on *P. aeruginosa* isolates collected from different clinical specimens, 95% of them were positive for *int*1, 54% for *int* 2 and10% for *int* 3 gene (27). It has been shown that 55.5% and 29% of *P. aeruginosa* isolates were obtained from clinical specimens such as urine, wounds, blood and other samples were contained class 1 and 2 integrons (28). In other studies carried out on different clinical specimens results revealed that 56.6% of *P. aeruginosa* contained class1 integron (29-30). The different results in these studies and our results maybe because of the geographical distribution, source of isolates and inappropriate use of antibiotics. In a study conducted by Ebrahimpour et al., 30% of *P. aeruginosa* isolates were positive for the presence of class 1 integrons (31). In one study the prevalence of class I integrons was reported at 57% but none of them carried class 2 and class 3 integrons (16). In study by Rajabnia et al. 39.4% of *P. aeruginosa* collected from different environments and equipments at a hospital have

intl gene (32). Another study on isolates of *P. aeruginosa* from sputum samples indicates that 33.3% of *P. aeruginosa* isolates carried this gene (33). In a study by Cicek et al , ten isolates (4.8%) of *P. aeruginosa* were identified as being positive for class 1 integrase and class 2 integrase was not detected (9). In a study by Xu et al. detected45.8% of *P. aeruginosa* were class 1 integron-positive strains, 19.5% class 2 integron-positive strains, and 2.5% class 1 and 2 integron-positive strains from total of 118 strains (34). This difference can be attributed to the geographical locations, the number of samples studied and the bacterial isolates.

Conclusion

Raw milk is a significant reservoir of antibiotic resistant pathogens. Dissemination and acquisition of resistance genes can be facilitated with integrons among pathogens. So, improvement of the infection control policies and the appropriate uses of antibiotics seems to be necessary for controling the antibiotics resistance.

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Ethics approval and consent to participate

Not needed.

Conflict of interest

The authors declare no competing financial interest.

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