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Identification and 16s rRNA Gene Sequence Analysis of Multidrug-Resistant *Pseudomonas Aeruginosa* in Paper Currency Notes

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Abstract

Pseudomonas aeruginosa is one of the most feared opportunistic human pathogens due to its capability to colonize a broad range of ecological sites, infect various hosts and organs and capable of acquiring resistance to multiple antimicrobial agents.

This study aimed to investigate the likelihood of contamination of currency notes with multidrug-resistant Pseudomonas aeruginosa.

One hundred and twenty used Sudanese currency notes were collected from people working in different sites such as public transportations, shops, restaurants and hospitals, moistened using sterile water, swabbed, inoculated in cetrimide agar plates and incubated for 24 hours. The motile gram-negative bacilli grown in cetrimide agar were subcultured in nutrient agar plates and prepared for further identification with phenotypical, molecular and antimicrobial sensitivity tests. Bioinformatics analyses for 16S rRNA gene sequence of the most resistant Pseudomonas aeruginosa isolate was also performed.

Out of 120 collected samples of paper currency notes, 17.5% was found to be contaminated with Pseudomonas aeruginosa strains. All isolates were resistant to 6 out of 13 tested antimicrobial agents including Kanamycin, Cefuroxime and Amoxyclav. All isolates were sensitive to Ciprofloxacin, Levofloxacin and Azithromycin, however, drugs used for treatment of Pseudomonal infections such as ceftazidime and piperacillin showed different rates of resistance. One nucleotide substitution mutation, T550C, was detected in the most resistant isolate's 16S rRNA gene, however, its exact effect is unclear.

Forthcoming studies should further explore the impact of mutation detected in the 16S rDNA, the gene that known to be stable over many generations..

Keywords: *Pseudomonas aeruginosa, currency notes, antibiotics resistance, 16S rDNA*

Introduction

Pseudomonas aeruginosa is an opportunistic gram-negative bacterium with ability to grow in different conditions and infect various hosts (1). It is a non-fermentative aerobic, motile and normally lives in moist environment but can adapt easily to various conditions. Due to its staggering versatility and metabolic flexibility in using wide types of organic compounds for its growth and development, in addition to its ability to form biofilm (2), it has superior capability to colonize a broad range of ecological sites including detergents and disinfectants and infect most eukaryotes (3-6). Infections due to *Pseudomonas aeruginosa* are difficult to be eradicated as a result of its intrinsic resistance as well as acquired resistance to different available antibiotics (3, 7). This opportunistic pathogen readily acquires resistance to multiple drugs and infects mainly immunocompromised individuals leading to severe nosocomial infections (8), chronic respiratory infections in cystic fibrosis patients (1, 9-13), and chronic infections in patients with bronchiectasis or chronic obstructive pulmonary disease (COPD) (14). In addition to environmental sites, *Pseudomonas aeruginosa* was also detected in pharmaceutical products, surgical environments, materials and equipment, and implicated in urinary tract infections (UTI) that mainly acquired by catheterization (8, 15, 16), and keratitis in contact lens users (17). *Pseudomonas aeruginosa isolates*, which considered as a critical health hazard, are quite susceptible to microenvironmental pressure and as a result, each population of *Pseudomonas aeruginosa* carries a large amount of variations in antimicrobial susceptibilities (18). This might lead to difficulties in choosing a representative isolate and basing the therapeutic policies accordingly. Therefore, it is necessary to investigate clinical, environmental and fomites for possible presence of multidrug-resistant *Pseudomonas aeruginosa* and other problematic microorganisms. In this study, both phenotypical and the more advance 16S rRNA gene sequencing methods were applied for detection of *Pseudomonas aeruginosa* in Sudanese paper currency notes, which considered to be one of the most circulating items among communities and as a potential source of infections.

Methods

Sample collection

One hundred and twenty used denominations of Sudanese paper currency were randomly collected from public transportations, shops, restaurants and hospitals in sterile petri dishes and kept at room temperature for bacterial isolation and identification. New currency notes were collected from bank and used as control.

Bacterial isolation and identification

Paper notes were swabbed with sterile wet swab, streaked over cetrimide selective media and the plates were incubated at 37°C for 18~20 hours. The suspected *Pseudomonas aeruginosa* grown in the selective media were confirmed using colony morphology, motility test, gram staining technique and various biochemical tests including indole test, glucose and lactose fermentation, catalase and oxidase tests, urease test, and citrate utilization test as described previously (19).

Antimicrobial Susceptibility test (AST)

Well isolated colonies of identified *Pseudomonas aeruginosa* culture were used to prepare standardized inoculum. Mueller-Hinton agar and antibiotic susceptibility disks (Hi-Media laboratories) were used to study the susceptibilities of isolates towards commonly used antibacterial agents. Kirby-bauer disc diffusion technique was performed as described previously (20). Results of the AST were interpreted according to EUCAST (the European committee on antimicrobial susceptibility) standard (21).

Genotypic analysis

Multiple antimicrobial resistant isolate recovered from the examined paper currency notes was subjected to genotypic analysis to confirm the phenotypic results. Three colonies from pure sub-cultured isolate were suspended in 200µl 1x Phosphate Buffer Saline (PBS). Genomic DNA was extracted using chelex extraction protocol as described previously (22), detected using 1% agarose gel electrophoresis, visualized using U.V. transilluminater (Cleaver scientific, UK) and quantified using GeneQuant, (Amersham Biosciences), according to manufacture protocol.

16S rRNA gene was amplified using the extracted DNA as templates, forward 27F; 5′-AGAGTTTGATCCTGGCTCAG-3′ and reverse 1495R; 5′-CTACGGCTACCTTGTTACGA-3′ universal primers (iNtRON, Korea) (23). The Polymerase chain reaction (PCR) conditions adopted at 94◦C for 5 mins as starting denaturation temperature, followed by 35 cycles (94◦C for 1 min, 58◦C for 1 min, 72◦C for 1min) and final extension at 72◦C for 10 mins.

Sequencing and bioinformatics analysis

One amplified 16S rRNA gene of multiple drug resistant isolate of *Pseudomonas aeruginosa* was selected for sequencing (Macrogen). The received nucleotide sequence was submitted to NCBI to get an accession number. The obtained nucleotide sequence of the 16S rRNA gene was searched using SILVA (24) database, and Basic Local Alignment Search Tool (BLAST) (25). Highly similar sequences, 21 different strains of different countries, were retrieved and aligned to identify regions of similarity and evolutionary relationship using BioEdit 7.2 (https://bioedit.software.informer.com/7.2/) and Clustal Omega of EMBL-EBI database (https://www.ebi.ac.uk/Tools/msa/).

Results

Bacterial identification

Phenotypic analysis such as gram stain and biochemical reactions were used for identification. Gram negative bacilli strains that were motile, catalase and oxidase-positive capable of growing over selective cetrimide agar plates were preliminary identified as *Pseudomonas aeruginosa*. Out of 120 collected samples of paper currency notes, 21 notes were contaminated with *Pseudomonas aeruginosa strains* (17.5%).

Antimicrobial sensitivity

Antimicrobial susceptibility testing using disc diffusion method showed that: six antimicrobial agents including Amoxyclav, and Kanamycin were inactive against all identified *Pseudomonas aeruginosa isolates* (100%), 2 antimicrobial agents were inactive against more than 90% of isolates, however, all isolates were sensitive to 6 antimicrobial agents including Polymyxin B and Meropenem (Table1).

16S rRNA gene sequence and Bioinformatics

The search output of BlAST database revealed high similarity between the obtained isolate's 16S rRNA gene sequence and some *Pseudomonas aeruginosa* 16S rRNA gene sequences from different countries. The top 21 sequences which showed 99% similarity to the isolate's 16S RNA gene, including China-EU931548.1, Algeria-KR349493.1, India-KJ819583.1 and others, were subjected to multiple sequences alignment and the results showed only one substitution mutation, thymine to cytosine at position 550 of the isolate's 16S RNA gene sequence, corresponding to nucleotide number 604 of Morocco strain's sequence (Figure 1).

The constructed phylogenetic tree using 16S rRNA gene showed evolutionary relationship between the studied isolate and the other related 21 *Pseudomonas aeruginosa* strains from different countries. The identified isolate sequence was closely related to China (EU931548.1) strain's sequence and clustered together in the same genomovar. The common ancestors of different strains were Japan (AB037545.1) and USA(NR_117678.1) 16S RNA genes' sequences (Figure 2).

Discussion

Paper currency notes as one of the most circulated items between many people in all communities could be exposed to microorganisms, however, contamination with pathogenic bacteria is a matter of great concern especially to vulnerable groups such as immunocompromised populations. In this study, 120 paper currency notes were examined for possible presence of *Pseudomonas aeruginosa* and detected on 21(17.5%) of the tested paper notes. Similar studies were carried out in some countries and different percentages of contamination on their banknotes were recorded. Three denominations of Indian currency notes, Rs. 10, Rs. 50, and Rs. 100, were tested for bacterial contamination and *Pseudomonas aeruginosa* were detected in Rs. 10 and Rs. 50 (19%) (26). In Pakistan, 13.7 % of their 100 studied banknotes, 4 different denominations, exhibited contamination with *Pseudomonas* spp. (27). The reasons beyond contamination of this very important item under study with dangerous opportunistic pathogens are

unclear, however, lifestyle and personal hygiene might be among them. It looks that people in study area and some neighbouring countries share the same behaviour of storing paper currency notes in areas like drawer with different items, that prone them to contamination with pathogenic bacteria (28).

It is thought that multidrug-resistant *Pseudomonas aeruginosa* is normally found in clinical specimens, however, all identified strains in this study were multidrug-resistant since they were resistant to 6 different drugs, and one isolate was resistant to 13 antimicrobial agents tested (Table 1). We detected 4(19%) and 3(14%) isolates resistant to Gentamicin and Amikacin, respectively, which commonly used as part of empirical therapy for treatment of infections in critically ill patients. As expected, antipseudomonal agents are still active against most isolates and there were 2 isolates resistant to the 3rd generation cephalosporin ceftazidime (9.5%), and only 1 strain resistant to the extended spectrum penicillin piperacillin (4.8%). It stands to reason that all isolates were sensitive to Polymyxin B and Meropenem since they are not routinely prescribed and reserved for severe cases in the study area, however, the sensitivity of multidrug-resistant isolates towards drugs that used extensively in Sudan, with or without prescriptions, such as Azithromycin, ciprofloxacin and levofloxacin is unclear. Irrational use of antimicrobial drugs is one of the reasons behind drug resistance, nonetheless, further studies considering the complete map of resistant genes, physiological and environmental factors might be required. Surprisingly, all isolates were found to be sensitive to co-trimoxazole, a drug that showed high rate of resistance in Sudan for over 2 decades ago (29). This might be attributed to implementation of antibiotic policy and standard treatment guidelines that might help in limiting the emergence of resistant strains (30).

Contamination of banknotes with resistant isolates is of great concern since the vast majority of population used paper currencies and cash services, that might account in cross transmission and spread of infections. We think that the irrational and miss use of antibiotics in Sudan, and most developing countries, at the forefront of reasons behind the presence of antibiotic resistant strains in paper notes, however, the open boundaries in Sudan could be among reasons since the notes and goods are exchanged in a daily bases. So generally, and based on this finding, contamination of currency notes with multidrug-resistant *Pseudomonas aeruginosa* is an alarming thread to be highlighted by public and governmental efforts to stop the progression of resistance and protect the people especially the immunocompromised individuals from infections by resistant isolates.

16S rRNA gene amplification and sequencing confirmatory test was used due to it is sensitivity and suitability for identification of different species, and in some cases, to the strain level (31, 32). Here we used this method to confirm the phenotypic identification results of the most resistant isolate. The 16S rRNA gene sequence analysis revealed that the isolate of this study is 99% identical to *Pseudomonas aeruginosa* from different countries. We detected only one nucleotide substitution mutation, T550C, in the isolate's 16S rRNA gene (Figure 1), which is probably inconsequential. We believe that the resistance manifestation of the current strain due to this single mutation is unlikely and further investigations such as restricted permeability and the activity of efflux system, antibiotic-inactivating enzymes, mutational changes in essential proteins or other mechanisms (33) might be required.

Paper currency notes are normally released from banks clean, and sometimes sterile, however, poor handling or storage subjects them to microbial contaminated. In conclusion, Sudanese banknotes, the most exchanging item between people, were found to be contaminated by multiple-drug resistant *Pseudomonas aeruginosa*, an organism that known to cause various infections especially to immunocompromised individuals. The 16S rRNA gene sequence of the most resistant strain was found to be identical to that of *Pseudomonas aeruginosa* from different countries, however, one nucleotide substitution mutation was detected in the isolate's gene. All identified *Pseudomonas aeruginosa* isolates were resistant to 6 of the routinely used antimicrobial agents in study area including Cefuroxime, Amoxyclav and Kanamycin. This finding highlights the importance of performing antibiotic sensitivity testing before prescriptions

since we detected several environmental isolates that were resistant to routinely prescribed drugs, as well as studying the impact of the detected mutation in the marker gene, 16S rDNA, that preferred over functional genes in molecular identification and taxonomic purposes.

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Table 1. Antimicrobial Sensitivity Patterns of *Pseudomonas Aeruginosa* Isolates from Paper Currency Notes

Antimicrobial disc	No. of resistant isolates	Percentage of resistance
Kanamycin (K) 30µg	21	100%
Cephalexin (CN) 30µg	21	100%
Cefuroxime (CXM) 30µg	21	100%
Amoxicillin (AMX) 25µg	21	100%
Amoxyclav (AMC) 30µg	21	100%
Penicillin-G (P) 10IU	21	100%
Erythromycin (E) 5µg	20	95%
Nitrofurantoin (NIT) 200µg	19	90.5%
Gentamicin (GEN) 10µg	4	19%
Amikacin (AK) 30µg	3	14%
Chloramphenicol (C) 30µg	2	9.5%
Ceftazidime (CAZ) 30µg	2	9.5%
Piperacillin (PI) 100µg	1	4.8%
Ciprofloxacin (CIP) 30µg	0	0%
Levofloxacin (LE) 5µg	0	0%
Azithromycin (AZM) 15µg	0	0%
Co-trimoxazole (COT) 25µg	0	0%
Meropenem (MEM) 10µg	0	0%
Polymyxin B (PB) 300 Units	0	0%

Figure 1. Multiple alignment of 16S rRNA gene sequences of different countries strains: A substitution mutation (T550C) appeared only in the isolate's 16S RNA gene sequence. The numbers showing above the sequences is according to the Morocco strain's gene sequence.

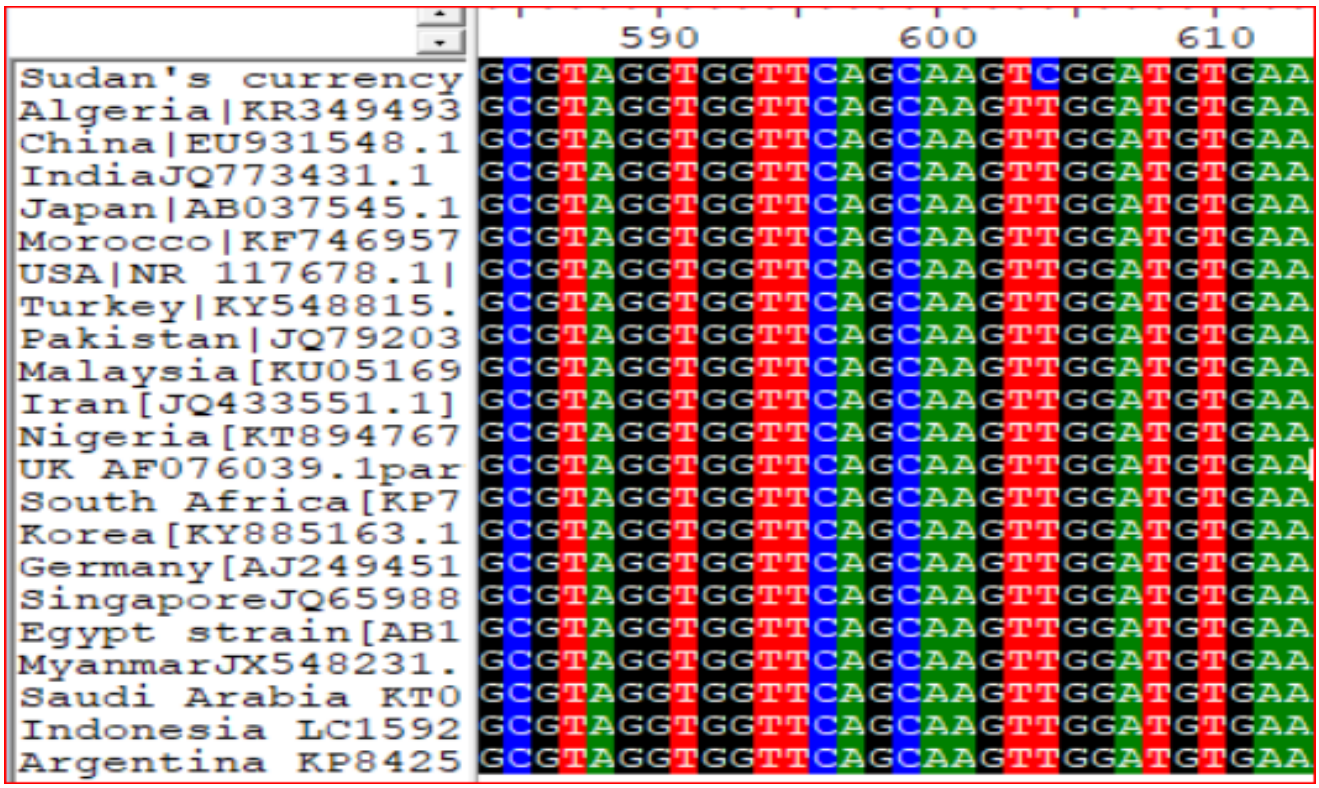

Figure 2. Phylogenetic tree of the identified isolate and closely related *Pseudomonas aeruginosa* strains: The identified isolate sequence is clustered together with China (EU931548.1) strain's sequence in the same genomovar. The common ancestors of different strains were Japan (AB037545.1) and USA(NR_117678.1) 16S RNA genes' sequences.