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Contamination of Sudanese Banknotes with *Acinetobacter radioresistens*

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ABSTRACT

Acinetobacter radioresistens is an environmental gram-negative species, ubiquitous in nature and resistant to radiation, desiccation and several antimicrobial agents. The bacterium is a potential human pathogen and has been detected in plants, soil, healthy individuals and patients. This study was carried out to detect the presence of *A. radioresistens* strains in the circulating Sudanese banknotes and to determine their sensitivity towards commonly used antimicrobial agents. The possible presence of *Acinetobacter spp.* in 130 used Sudanese banknotes, randomly collected from currency handlers in Khartoum state, in addition to 3 new control banknotes was investigated using various cultural techniques. Blood agar and MacConkey agar plates were used to recover and isolate bacteria from the banknotes. Biochemical tests such as oxidase, catalase, motility and fermentation tests were used to identify the *A. radioresistens* isolates and the multiple drug resistant isolates were further identified using a genotypic detection method, namely 16S rRNA gene amplification and sequencing. Isolates recovered from different 10 banknotes were identified as *Acinetobacter* spp., and there was no contamination detected in the controls. Antibiotic sensitivity test revealed that some isolates were resistant to multiple drugs. The 16S rRNA gene sequence of the most resistant isolate of *A. radioresistens* was deposited into NCBI GenBank with accession number MG203880. The results of the current study revealed that nearly 8% of the tested banknotes were contaminated with *A. radioresistens*. Antimicrobial sensitivity testing indicated the existence of multiple drug resistant isolates. Thus, appropriate measures should be taken to protect Sudanese currency holders.

Keywords: 16S rRNA, Drug resistance, *Acinetobacter radioresistens*, Contamination, Banknotes.

Introduction

Bacteria can be found in different ecological habitats with various roles ranging from carbon or nitrogen fixation, organic waste digestion, immune modulation up to cause of serious diseases and death. Environmental bacteria can easily be transmitted and contribute to host health/disease situation.[1] *A. radioresistens* is one of these environmental bacteria that is resistant to radiation and desiccation. It can be identified conventionally with biochemical tests such as oxidase, catalase motility and fermentation test in addition to genotypic methods.[2-4] Since it was originally detected in environmental samples and plants, and later detected in both healthy people and patients, *A. radioresistens* can be transmitted theoretically via various means, including person-to-person contact and contaminated objects.[4]

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Although, *A. radioresistens* is regarded as commensal, it has been associated with both topical and systemic infections including bacteremia and life threatening diseases.[5-7] Moreover, *A. radioresistens* has been reported to be associated with patients who did not respond to antimicrobial therapy resulting in death.[8]

A. radioresistens has been implicated with rapid development of antimicrobial resistance, possibly due to the fact that it is harboring genome or plasmid encoded bla_{OXA-23} oxacillinase gene, with ability of horizontal gene transfer to nearby bacteria. The ability of *A. radioresistens* to quickly exchange information, mainly by conjugation or transformation, combined with surviving desiccation and harsh environment conditions for longtime has attracted the clinicians attention.[9-11] The infections caused by this genus are difficult to be controlled due to rapid mutations, various virulence factors, and rapid development of antibiotic resistance to various antimicrobial agents.[12] The shortage of reports regarding information related to the identification of *A. radioresistens*, occurrence and virulence factors in developing countries like Sudan, as well as developed countries, might increase the fear of perceived misdiagnosis and treatment errors.[8,10,13] The shortage of control strategies appeared in several hospitalized cases associated with multiple drug resistant bacterial strains, identified later as carbapenem-resistant *Acinetobacter* strain, poses a significant challenge to healthcare providers.[11,14-16]

The traditional methods of microbial identification might not be suitable for accurate identification of *A. radioresistens*. Nonetheless, genotypic method such as 16S rRNA gene sequencing and the matrix-

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assisted laser desorption ionization-time of flight (MALDI-TOF) technique are more specific and sometimes highly recommended for identification of closely related strains that often misidentified.[8,13,17-20] The current study aimed to detect the occurrence of *A. radioresistens* in Sudanese banknotes and to assess their sensitivity pattern towards commonly used antibiotics.

Materials and Methods

Materials

Used Sudanese banknotes were randomly collected from currency handlers of Khartoum state, while the new banknotes were obtained from central bank of Sudan. Hydrogen peroxide was obtained from BELL SONS & Co. Ltd. England. The Immersion oil was obtained from The British Drug houses Ltd. England. The blood was obtained from Khartoum teaching hospital blood bank. Nutrient broth, Nutrient agar, Nitrate Agar, H_2S Test medium and MacConkey agar in addition to other various reagents including oxidase disc, Urea agar base, phenol red, DNase test agar base, Simmon's citrate agar, Methyl Red, and Voges Proskauer were obtained from HiMedia Laboratories Pvt. Ltd. India. All antibiotic discs (mentioned below) were obtained from HiMedia Laboratories Pvt. Ltd. India. Chelex (InstaGene, 6% w/v Chelex resin) was obtained from Bio Rad, England. Primers were from iNtRON, Korea. Maxime™ PCR PreMix Kit (i-Taq™) was obtained from Boca Scientific, Inc.

Detection of Acinetobacter radioresistens in Sudanese banknotes

The possible presence of *Acinetobacter* spp. in 130 used Sudanese banknotes that were randomly collected in the summer of 2018, May to July, from currency handlers of Khartoum state and 3 new control notes was carried out by collecting the paper currency into sterile disposable Petri dishes and shortly transferred to the laboratory for swabbing and cultivation. All currency notes, including new ones, were swabbed by sterile moisten cotton-tipped swab and inoculated on 5% blood agar and MacConkey agar (MAC) plates. Some colonies with different characteristics appeared in the plates after aerobic incubation at 37˚C for 24hrs. The cultural characteristic of the isolates closely related to enterobacteria on inoculated agar plates were isolated and subjected to various biochemical and gram staining techniques. The gram-negative bacilli were further identified using biochemical tests such as catalase, Methyl Red, Voges Proskauer, oxidase, nitrate reduction, motility, H_2S production test in addition to glucose, mannose, lactose and mannitol fermentation tests.[21,22]

Antibiotic Susceptibility Testing

Kirby-Bauer disc diffusion technique was performed to examine the susceptibilities of the isolates towards the commonly used anti-infective agents in the study area. Ten antimicrobial discs (Hi-Media laboratories) namely, Ampicillin (AMP) 10 µg, Ceftriaxone (CTR) 30 µg, Ceftazidime (CAZ) 30 µg, Gentamicin (GEN) 10 µg, Amikacin (AK) 30 µg, Co-trimoxazole (COT) 25 µg, Ciprofloxacin (CIP) 30 µg, Levofloxacin (LE) 5 µg, Chloramphenicol (C) 30 µg, and Meropenem (MEM) 10 µg were used. The active/resistant agents were determined according to EUCAST standard (the European Committee on antimicrobial susceptibility).[23]

Genotypic analysis

The chromosomal DNA of the biochemically identified *A. radioresistens* was extracted as described previously.[24] Briefly, 3 well isolated colonies of the sub-cultured *A. radioresistens* were added to 300 µL (1x) phosphate buffer solution, vortexed for ten seconds and centrifuged at 10,000 RPM for five minutes. The pellet was re-suspended in 200 µL of 6% Chelex and incubated at 56℃ for thirty minutes, boiled at 100℃ for fifteen minutes, vortexed for ten seconds, transferred to water bath (30℃) for 5 min, and then centrifuged (10,000 RPM) for one minute. The supernatant which expected to contain the genomic DNA was transferred into clean 0.5 mL Eppendorf tube and stored at 4℃ for Polymerase chain reaction (PCR).

The extracted DNA was used as a template to amplify the 16S rRNA with aid of the universal primers (27F; 5′ AGAGTTTGATCCTGGCTCAG-3′, 1495R; 5′-CTACGGCTACCTTGTTACGA-3′) (iNtRON, Korea). The PCR conditions were set as shown in table 1.

Table 1: PCR conditions for 16S rRNA gene amplification

Process	Temp.	Time	PRC cycle
Initial denaturation	94 ℃	5 mins	1 cycle
Denaturation	94 ℃	1 min	35 cycles
Annealing	58 ℃	1 min	
Extension	72 ℃	1 min	
Final Extension	72 ℃	10 mins	1 cycle

The dsDNA and protein concentration (µg/mL) were determined by spectrophotometer (GeneQuant), while the approximate length, purity and quality of the amplified gene were detected by gel electrophoresis and visualized under UV transilluminator (Uvite –UK).[25]

GeneBank accession number

The amplified PCR product, 16S rRNA gene, was further purified and sequenced (Macrogen Company), while the received nucleotides sequence was submitted to NCBI to get new accession number.[26]

Bioinformatics analysis

The obtained 16S rRNA nucleotide sequence was submitted to nucleotide BLASTn (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch) to search for sequence similarity, and https://www.arb-silva.de/ to check its quality.[27,28] Highly similar sequences (more than 99% identity) of lowest E-value were retrieved, compared and analyzed using Clustal Omega multiple sequence alignment tool (EMBL-EBI, https://www.ebi.ac.uk/Tools/msa/); the same tool was used to construct the Phylogenetic Tree for evolutionary analysis. All data retrieved from Clustal Omega multiple sequence alignment tool were visualized using BioEdit 7.2 (https://bioedit.software.informer.com/7.2/).[29]

Statistical analysis

The descriptive statistics of the data were conducted in form of frequencies, percentages and graphs using GraphPad® Prism software Version 5.03, CA, USA. IBM SPSS v20 was used for inferential statistics. The Chi-square test was used to investigate the association between *A. radioresistens* contamination and the source of banknotes or denominations with confidence interval of 95% ($p \leq 0.05$).

Results and Discussion

Most of the *Acinetobacter* spp. are similar in their culture characteristics. The currency notes swabbing and inoculation on MacConkey agar plate resulted in a few colonies. This was attributed to either the banknotes were not too much contaminated, or the media used was inappropriate since the bacteria might be injured and thus high nutritional content media was needed. To this end, blood agar was used and much more colonies were grown compared to the MacConkey agar plates. The use of blood agar was also useful for the identification process where colonies showed no blood hemolysis only were selected for further identification techniques as *A. radioresistens* is not a hemolytic bacterium. With the aid of gram staining technique, the initial phenotypic identification of the isolates recovered from banknotes (130 used plus 3 new notes) revealed 74.6% of the notes (N= 97) were contaminated with gram-negative bacteria. All strains expected to be *Acinetobacter spp*. were found to be catalase positive, oxidase negative, nonmotile, urease negative, nitrate reduction negative, and non-blood hemolytic (Table 2). This have supported our postulation about the identity of the isolate as *A. radioresistens* which was also found to be citrate negative, sugar fermentation tests negative and the most tolerable to desiccation.

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A. radioresistens recovered from 10 examined banknotes (7.7%) showed susceptibility to different antibiotics, e.g. Meropenem (Table 3) and they were resistant to Ampicillin, Ceftriaxone and ceftazidime. Furthermore, the isolates showed resistance to Amoxiclav, Penicillin, Azithromycin, Erythromycin, Cefuroxime and Nitrofurantoin.

Although all analyzed denominations (2, 5, 10, 20 and 50 SDG (26 notes of each) which collected in the summer of 2018 were contaminated with various bacteria, only 10 denominations were revealed to be contaminated with *A. radioresistens*. The isolates were mostly found in the lower denomination that collected from vendor, food seller and traveler (Figures 1 and 2). Statistical analysis revealed no significance association between *A. radioresistens* contamination and the source of banknotes or denominations ($p > 0.05$).

The nucleotides sequence of the amplified 16S rRNA gene from the most resistant isolate submitted to NCBI was given an accession number [MG203880]. The BlASTn database search revealed many similar sequences of *A. radioresistens'* 16S rRNA genes. The top sequences from different countries, including two reference strains NCBI: NR_114074.1 and NBRC102413: AB681769, were aligned with MG203880 (Figure 3) and the results showed three insertion mutations at positions 730, 763, and 777 in the isolate MG203880's 16S RNA gene.

Using the isolate's 16S rRNA gene sequences shown in figure 3, phylogenetic analysis was carried out and evolutionary relationship [30] between *A. radioresistens* [MG203880] isolate and *A. radioresistens* **[**MK780049.1] Indian strain and *A. radioresistens* [LM994721.1] France strain was detected, as shown in figure 4.

A. radioresistens can be routinely misidentified in environmental as well as clinical samples due to closely related characteristics with other *Acinetobacter* species, thus, inappropriate management might occur. In this study, the currency notes were collected from areas and people that are exposed to direct sunlight and dryness. In such an environment it is expected that only *Acinetobacter* species, namely *A. radioresistens*, that survive more harsh conditions such as irradiation, hydrogen peroxide, presence of some antimicrobial agents and desiccation could be isolated. The biochemical-based methods applied in this study revealed that 97 of the tested used notes (74.6%) were contaminated with gram-negative bacteria and 10 notes (7.7%) contaminated with *A. radioresistens*. The true epidemiology of the current isolate is questionable and there are some authors doubt about the previously reported infections of *Acinetobacter* species.[8]

Due to possible misidentification of *A. radioresistens*, it is expected that most environmental samples might harbor this organism since it can survive desiccation and radiation. The studied Sudanese currency notes were 100% contaminated and gram-negative species constitute 74.6%. Therefore, this report in addition to others[31,32] must raise concerns and get the attention of policymakers that currencies are an important potential source of highly resistant and nosocomial bacteria.

In previous studies, different types of bacteria in different currency notes were detected,[31-40] and, to best of our knowledge, the current study is the first to report the detection of *A. radioresistens* in Sudanese banknotes. Therefore, strategies and measures necessary to protect the currency handlers such as washable plastic currency and other preventive measures might be required.

In this study, 3 multiple drug resistant *A. radioresistens* out of 10 isolates were identified in the examined currency notes (Table 3). The previous thought that antimicrobial resistance could be observed with clinical samples only is now changed due to numerous reports, including the present study, that identified resistant environmental bacteria. In fact, the rate of antimicrobial resistance is normally higher in the developing countries due to irrational use of antimicrobial agents. Some previous reports showed that *A. radioresistens* is resistant to chloramphenicol.[21] It is worth noting that all the current isolates were found sensitive to Chloramphenicol indicating the success of the antibiotic policy such as shifting the treatment towards other new agents in the study area and the application of national action plan.[41] Albeit the revealed resistance to some marketed drugs, the isolates showed susceptibility to many drugs available in Sudanese market such as Amikacin, Gentamycin, Ciprofloxacin, Levofloxacin, Cotrimoxazole, and Meropenem.

The significance of antibiotic resistance showed by the current isolates in addition to that of previously identified *A. radioresistens* is alarming since the bacterium could transfer the resistant genes to other species both vertically and horizontally.[21,42,43]

Multiple sequence alignment of 16S rRNA of the identified *A. radioresistens* with the top similar sequences from different countries, retrieved from BLASTn search output, detected insertion mutations. The impact of the frame shift mutation by insertion at positions 730, 763, and 777 in the current MG203880 gene sequence (Figure 3) is unclear, however, the function of the gene and antimicrobials targeting this gene might be affected leading, therefore, to functional consequences or development of drug resistance.

The phylogenetic analysis showed evolutionary relationship (Figure 4) between the current strain MG203880 and *A. radioresistens* Indian strain [MK780049.1 isolated from traditionally preserved fish products of Sikkim] as they are clustered together in the same genomovar, and both are closely related to *A. radioresistens* France strain [LM994721.1, from grapes berries].

Harsh environmental conditions can favor the abundance of some pathogens, that can easily be transmitted to people. Therefore, similar studies on the possible microbial contamination and large-scale identification of problematic microorganism in currencies and other objects are highly recommended to be routinely undertaken globally for suitable prevention or treatment options towards better health and wellbeing.

Table 2: Cultural and phenotypic characterization of *A. radioresistens* isolates

Test	Characteristic
Nutrient broth	Uniform turbidity
Nutrient agar	Small, circular, convex, smooth and slightly opaque colonies with entire margins
MacConkey agar	Non- lactose-fermenter
Blood agar	No blood hemolysis
Catalase	+
Oxidase	-
Motility	Nonmotile
Nitrate reduction	-
Urease	-
Citrate	-
Methyl Red	-
Voges Proskauer	-
H_2S production	-
Sugar (glucose, mannose, lactose and mannitol) fermentation tests	-

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Table 3: Antibiotic sensitivity pattern of *Acinetobacter radioresistens* isolates: SN: strain number, R = resistant, S = sensitive

SN	Ampicillin	Gentamicin	Amikacin	Ciprofloxacin	Levofloxacin	Ceftriaxone	Ceftazidime	Co-trimoxazole	Chloramphenicol	Meropenem
1	R	S	S	S	S	S	S	S	S	S
2	R	S	S	S	S	R	R	S	S	S
3	R	S	S	S	S	R	R	S	S	S
4	R	S	S	S	S	S	S	S	S	S
5	R	S	S	S	S	R	R	S	S	S
6	R	S	S	S	S	R	R	S	S	S
7	R	S	S	S	S	R	R	S	S	S
8	R	S	S	S	S	S	S	S	S	S
9	R	S	S	S	S	S	S	S	S	S
10	R	S	S	S	S	R	R	S	S	S

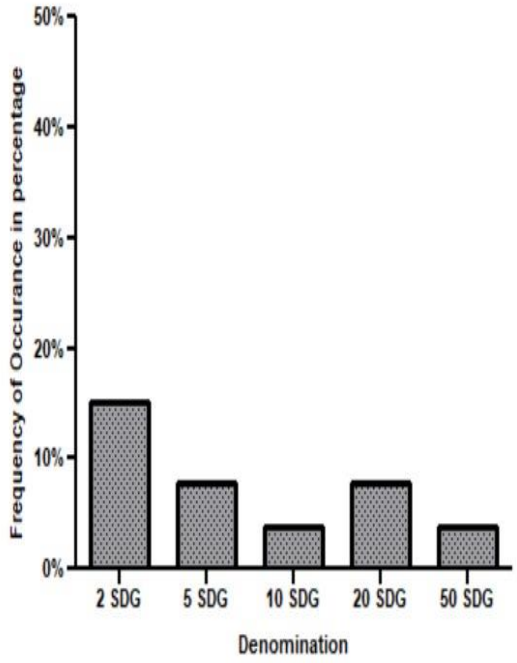

Figure 1: Occurrence of *A. radioresistens* (percentage) in different denominations (26 notes from each denomination): 4 isolates from 2 SDG (15%), 2 isolates from 5 SDG and 20 SDG, each (7.8%), and there was 1 isolate from 10 and 50 SDG, each (3.8%).

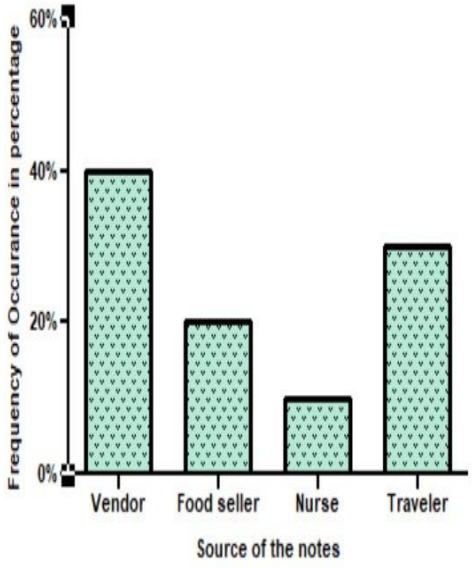

Figure 2: Occurrence of *A. radioresistens* (percentage) according to the source of notes: 4 isolates from vendor (40%), 3 isolates from traveler (30%), 2 isolates from food seller (20%) and 1 isolate (10%) from nurse.

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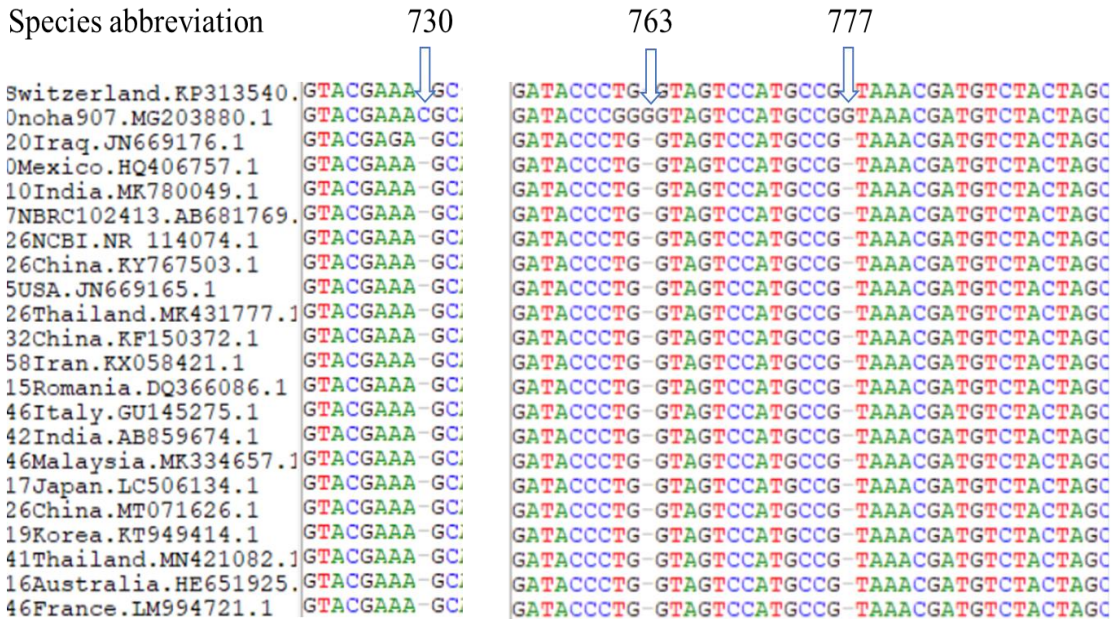

Figure 3: Multiple sequence alignments of *Acinetobacter radioresistens* 16S rRNA [MG203880] from Sudan and related sequences obtained from BLAST. Insertion mutations shown at positions 730, 763, and 777

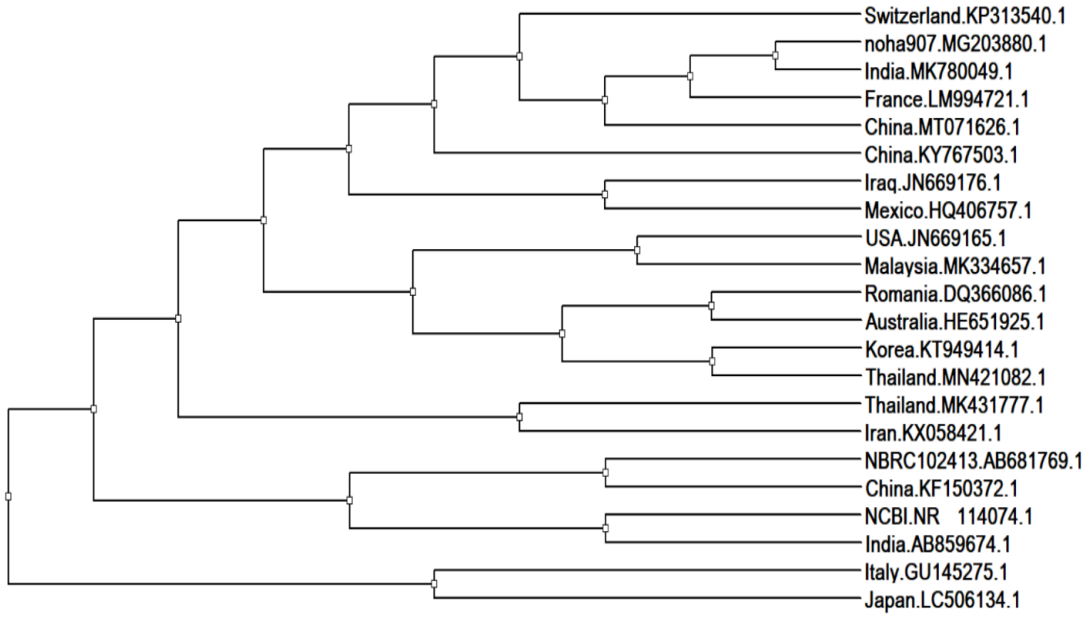

Figure 4: Phylogenetic tree of *Acinetobacter radioresistens* isolate's 16S rRNA from Sudan (noha907.MG203880.1) and closely related strains retrieved from NCBI database

Conclusion

A. radioresistens was detected in Sudanese banknotes using culture-based and 16S rRNA sequence analysis techniques. Some identified strains were resistant to multiple antimicrobial agents with possibility of donating their resistant genes horizontally to nearby microbial population. The findings from this study demonstrated the presence of multi-drug resistance *A. radioresistens* strains in tested currencies. Thus, calls should be raised to policy makers and health providers to develop and implement preventive measures to protect vulnerable people who are more likely to become in contact with contaminated banknotes.

Conflicts of Interests

The authors declare that they have no competing interests.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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