

WHOLE GENOME SEQUENCING OF ANTIBIOTIC RESISTANT GENES IN ISOLATES FROM SURFACES IN A SCIENCE LABORATORY

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INTRODUCTION

Microorganisms are ubiquitous. They have been detected in several areas of the environment. Air, water, soil and fluid from animals are carrier/vehicles of microorganisms. The quality of air is usually affected by the presence of microorganisms which include bacteria, fungi and viruses and people breathe in on average 14 m³ of air per day.¹ Poor air quality especially contaminated with microorganisms can lead to severe health challenges for humans. Microorganisms are transmitted through other routes which include contaminated food and food products, droplet contact by sneezing or coughing or contact with contaminated surfaces or soil. The mechanisms of how microorganisms attach to animate and inanimate things have been previously reported.² Biofilms may be found on a wide variety of surfaces. There are physical forces that determine how microorganisms are transmitted and attached to surfaces. Once bacteria get attached to surfaces they start to divide resulting in biofilms which cause the complex structure of natural sediments.³ The microorganisms may either be active reproducing immediately or remain inactive on surfaces for long periods making it difficult to identify the contamination source.⁴ Antibiotic resistance is a serious health challenge not only among human pathogens but also in isolates found in other habitats. Many resistant pathogenic bacteria and commensals are found in different hosts, or in the environment at large with the potential of causing infections that are usually difficult to treat.⁵ *Brucella* species, *Shigella* species, *Salmonella* species, *Mycobacterium tuberculosis*, and *Neisseria meningitidis* have been reported as the most common microorganisms causing laboratory-acquired infections (LAI). Infections as a result of the hepatitis virus, human immunodeficiency virus and fungal infections caused by dimorphic fungi have also been commonly reported.⁶ Laboratory-acquired and nosocomial infections poses an important challenge globally and characterization of microorganisms causing such infections is important as it provides possible therapeutic solutions for some laboratory-acquired infections

(LAI). It is important to characterize microorganisms causing laboratory-acquired infections in order to devise procedures to prevent subsequent outbreaks.⁷ This study aimed at identifying isolates, revealing resistance to antibiotics in isolates from surfaces in the Pharmaceutical microbiology laboratory, Igbinedion University Okada and characterizing the resistance mechanisms involved using whole genome sequencing.

Materials and methods

Materials

Collection of samples

Thirty samples were obtained aseptically from surfaces of work benches, tables, fridges, sinks, equipment, window and door in the Pharmaceutical microbiology laboratory using sterile cotton swab sticks. One sample was obtained per surface. Sterile cotton swab sticks were soaked in sterile peptone water before sampling was carried out.

Media preparation and sterilization

Four culture media were used in the study. These were eosine methylene blue agar (EMB), mannitol salt agar (MSA), Macconkey agar and Nutrient agar. All culture media were prepared and sterilized based on manufacturer's instructions.

Isolation and identification

Ten consecutive isolates were obtained in May 2021 from 30 sample surfaces of work benches, tables, fridges, sinks, equipment, window and door in the Pharmaceutical microbiology laboratory Igbinedion University Okada in Edo state Nigeria. Samples were aseptically taken and inoculated immediately on culture agar plates. Inoculated plates were incubated at 37°C for 24 hours. Distinct colonies formed were randomly obtained from culture plates. Pure cultures were obtained afterwards on agar slants maintained at 4°C in the refrigerator throughout the study. Identification of isolates was carried out using standard microbiological techniques.⁸ Identities of randomly selected four isolates were subsequently confirmed by MALDI-TOF mass spectrometry (Bruker Daltonik GmbH, Bremen, Germany) analysis.

Antimicrobial susceptibility testing

The Kirby-Bauer susceptibility testing technique, Bauer et al., 1966⁹ was performed and results were analyzed using European Committee on Antimicrobial Susceptibility Testing (EUCAST) criteria.¹⁰ The isolates were tested using 15 antibiotics: ampicillin, meropenem, ertapenem, ceftazidime, cefotaxime, amoxicillin/clavulanic acid, cefoxitin, cefepime, cefpodoxime, tigecycline, ciprofloxacin, amikacin, piperacillin/tazobactam, cefuroxime, gentamicin (Oxoid, Basingstoke Hampshire, UK).

Whole genome sequencing

Whole genome sequencing (WGS) was carried out for four randomly selected isolates which identities were confirmed by MALDI-TOF mass spectrometry (Bruker Daltonik GmbH, Bremen, Germany) analysis. Genomic DNA (gDNA) extraction was carried out using the MagAttract HMW DNA extraction kit (Qiagen, Hilden, Germany). Quantification of Genomic DNA was performed on a Qubit® 2.0 Fluorometer using the dsDNA BR Assay kit (Invitrogen by Thermo Fisher Scientific, Waltham, MA, USA) and diluted to 0.2ng/µl based on the manufacturer recommendations (Illumina sample preparation guide, Illumina Inc, San Diego, CA, USA). Preparation of fragment libraries of the bacterial genomes was carried out using the Illumina Nextera XT DNA library preparation kit (Illumina Inc, San Diego, CA, USA). DNA fragment library preparation was carried out using 1ng of gDNA (Illumina sample preparation guide). Paired end sequencing using a read length of 2x300bp on an Illumina Miseq (Miseq v3.0, Illumina Inc) was performed using Miseq reagent kit v3 containing the reagent cartridge and

flow cell. Pooled libraries were loaded on the reagent cartridge. Samples were sequenced to obtain a minimum average coverage of 100-fold based on Illumina's recommended standard protocols.

Raw reads (FASTQ files) were trimmed at their 5' and 3' ends until an average base quality of 30 was reached in a window of 20 bases, and assembly was performed using Velvet version 1.1.04 Zerbino, 2010¹¹ using optimized k-mer size and coverage cutoff values based on the average length of contigs with >1,000 bp. Species identification via MALDI-TOF MS was confirmed using ribosomal multilocus sequence typing (rMLST) (<https://pubmlst.org/species-id>). Identification of Antimicrobial resistance genes (ARGs) was carried out using the Comprehensive Antibiotic Resistance Database-Resistance Gene Identifier (CARD-RGI).¹² ARGs were identified based on a minimum cutoff of 98% nucleotide identity for perfect or strict hits predicted by RGI. Sequences were analyzed for their plasmid replicon types using Plasmidfinder and their multi locus sequence types using MLST 1.8 software both available from the Center for Genomic Epidemiology.^{13, 14}

Nucleotide Sequence Accession Numbers

This Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under the accession numbers JAJNEH000000000-JAJNEK000000000. The version described in this paper is version JAJNEH010000000-JAJNEK010000000. Table 4 shows the genome assembly statistics of the recovered isolates.

Results and Discussion

Species identification using MALDI-TOF-MS and ribosomal MLST assigned the 4 isolates to 4 different species (Table 1). Identified laboratory isolates include *Atlantibacter hermannii*, *Stenotrophomonas maltophilia*, *Enterobacter hormaechei* and *Leclercia adecarboxylata* (Table 1). Out of 4 randomly selected identified isolates, 3 isolates revealed antibiotic resistance and were further analyzed by whole genome sequencing (WGS) (Table 2). No information about antibiotics was obtained in the EUCAST table for *Stenotrophomonas maltophilia*. The laboratory isolates were resistant to ampicillin (100%), cefotaxime (67%), amoxiclav (100%), cefepime (67%), ceftazidime (67%), cefpodoxime (67%), cefuroxime (67%), ciprofloxacin (67%), gentamicin (67%), tigecycline (67%). Two isolates were positive in the phenotypic testing of ESBLs and had ESBL gene *bla_{CTX-M-15}* detected (Table 2).

Whole genome sequencing revealed that 3 out of the 4 identified isolates harbored more than one resistance gene. Resistance genes were detected in *Stenotrophomonas maltophilia* but didn't pass the minimum cutoff of 98% nucleotide identity for perfect or strict hits predicted by RGI. The resistance determinants in the isolates included β -lactamase genes, *bla_{TEM-1}*, *bla_{ACT-24}*, *bla_{CTX-M-15}*, *bla_{OXA-1}*; aminoglycoside modifying enzymes, *aac(3)-IId*, *aph(6)-Id*, *aph(3'')-Ib*, *aac(6')-Ib-cr4*; qnr gene, qnrB1; sulphonamide resistance gene, *sul2*; tetracycline resistance gene, tet(D); phenicol resistance gene, catII and trimethoprim resistance gene, *dfrA14*. Other resistance determinants which included the regulatory systems modulating antibiotic efflux CRP, antibiotic target alteration gene EF-Tu were also detected in the antibiotic resistant isolates. Table 3 shows the characteristics of the antibiotic resistant laboratory isolates. The *Enterobacter hormaechei* isolated was of the sequence type ST 78. *Atlantibacter hermannii*, *Stenotrophomonas maltophilia*, and *Leclercia adecarboxylata* had previously unknown sequence type (Table 3). Plasmids of the incompatibility group detected among the isolates were predominantly of Inc F and Col family types (Table 3).

Microbial contamination in a laboratory varies in different laboratories based on its geographical location and measures used to control infection which poses an important challenge. There are

few reports that have been made on this issue especially in developing countries like Nigeria which is really a drawback. Most of the previous studies were based on phenotypic characterization of microbial isolates from surfaces in the laboratory. In a previous Nigerian study by Isola and Olatunji, 2016¹⁵ bacterial isolates obtained from laboratory surfaces were characterized and identified. The result showed that the most frequent microorganisms from laboratory surfaces were *Bacilli*. Others included *Salmonella typhae* and *Staphylococcus aureus*. Another previous study identified *Staphylococcus epidermis* and aerobic spore bearers, i.e. *Bacillus subtilis* as common microorganisms contaminating working areas in a Microbiology laboratory.¹⁶ Strains identified were possible pathogens and could cause Laboratory acquired infections. In another study by Veena et al., 2012¹⁷ laboratory surface samples were assessed for microbial contaminants. Out of the 60 surface samples assessed, Coagulase- negative staphylococci (CNS) were the most frequent contaminant, followed by Gram positive bacilli (*Corynebacterium spp*). In our study, isolates obtained were on a relatively small scale compared to previous studies. Significantly, no Gram positive isolate was identified among the isolates obtained in this study. All isolates were Gram negative bacteria. More than one resistance determinant was found on the draft genome sequences of the resistant isolates which showed that they are potential pathogens that may cause laboratory acquired infections. Resistance mediated by *bla*_{CTX-M-15} have been reported globally including in Nigeria.^{18, 19, 20} Recently, a first report on *bla*_{CTX-M-15} in clinical isolates of *Providencia spp.*, *Citrobacter freundii* and *Atlantibacter hermannii* isolated from humans in Nigeria was published.²¹ To the best of our knowledge we report the first genomic characterization of resistance to antibiotics in isolates obtained from surfaces of a University science laboratory for Nigeria. This suggests the circulation of the gene *bla*_{CTX-M-15} in a different setting. Significantly, resistance genes were detected in *Leclercia adecarboxylata* observed to be sensitive to most of the antibiotics in the susceptibility testing. This shows the importance of studying antibiotic resistance not using phenotypic methods only but further genotypic and molecular characterization techniques like whole genome sequencing. *E. cloacae* ST66, ST78, ST108 and ST114 strains known to be extended spectrum cephalosporin-resistant have been reported to spread internationally as high-risk clones.²² *E. hormaechei* ST78 isolate detected in this study belonged to the high-risk clone known especially for the nosocomial spread carbapenemases and ESBLs²². Jesumirhewe et al., 2020²¹ in a recent report detected *E. hormaechei* ST78 isolates in a Nigerian hospital. *E. hormaechei* ST78 identified in this study suggests the circulation of this high-risk lineage in a different setting. The detection of the predominant plasmid replicon types (Inc F and Col) among the resistant isolates in this study show their importance in the transmission of antibiotic resistance. A recent report showed IncF plasmid type as the most prevalent among human ESBL *Enterobacteriaceae* isolates in Nigeria.²¹

Conclusion

Microorganisms are ubiquitous including the laboratory environment. Techniques for reducing contamination should be employed which includes the use of soaps to clean laboratory surfaces, Pre and Post- treatment of hands in disinfectant before carrying out any experiment in the laboratory, the use of protective clothing when working in the laboratory. Laboratory coats must be strictly used in the laboratory and not worn outside the laboratory. Measures should be aimed at eliminating/ significantly reducing these microorganisms from laboratory using proper procedures. Frequent assessments of surfaces of the laboratory should be carried out not using phenotypic methods only but molecular methods to identify and explore the genetic mechanisms of resistance to antibiotics in isolates which is important to understand the dissemination of

resistant isolates. Further molecular studies are required to characterize the prevailing clonal lineages and plasmids that harbor resistance mediating genes in the isolates. Frequent assessments would assist laboratories to either avoid or eliminate most microbial contaminants found in the laboratory.

References

1. Wemedo SA, Ede PN, Chuku A. Interaction between building design and indoor airborne microbial load in Nigeria. *Asian Journal Biol Sci*, 2012; 5:183–191.
2. Costerton JW, Geesey GG, Cheng K-J. How bacteria stick. *Sci Am*. 1978; 238: 86–95.
3. Donlan RM. Microbial Life on Surfaces. *J. emerg. Infect. Dis.* 2002; 8; 9.
4. Mendoza J, Caso W, Valdez C, Pons M, Valle L, Ore V, Michelena D, Mayra J, Gavidea V, Vargas M, Ruiz, J. Diagnosis of carriage of disease by direct blood PCR in thin blood smear negative samples. *J. Clin. Microbiol.* 2014; 9: 92-183.
5. Taylor LH, Latham SM, Woolhouse MEJ. Risk factors for human disease emergence. *Phil. Trans. R. Soc. Lond.* 2001; B 356: 983– 989. (doi:10.1098/rstb.2001.0888)
6. Singh K. Laboratory-Acquired Infections. *Clin. Infect. Dis.* 2009; 49: 142–147
7. Porte L, Soto A, Andrighetti D, Dabanch J, Braun S, Saldivia A, Flores J, Wozniak A, Garcia P, Weitzel T. Catheter-associated bloodstream infection caused by *Leifsonia aquatica* in a haemodialysis patient. A case report. *J. Med. Microbiol.* 2012; 61: 868–873
8. Cheesbrough M. *District Laboratory Practice in Tropical Countries*. 2nd ed. Cambridge University Press, UK; 2006: 178-187.
9. Bauer AW, Kirby MM, Sherris JC, Truck M. Antibiotic susceptibility testing by a standardized single disk method. *Am J Clin Pathol.* 1966; 45: 493-496.
10. European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MICs and zone diameters. 2021 version 11.0.
11. Zerbino DR. Using the Velvet de novo assembler for short-read sequencing technologies. *Curr Protoc Bioinformatics* 2010; Sep; Chapter 11: Unit 11.5. doi: 10.1002/0471250953.bi1105s31.
12. Jia B, Raphenya AR, Alcock B, Wagglechner N, Guo P, Tsang KK et al. CARD 2017: expansion and model-centric curation of the comprehensive antibiotic resistance database. *Nucleic Acids Res.* 2017; 45(D1): D566–573. <http://dx.doi.org/10.1093/nar/gkw1004>
13. Larsen MV, Cosentino S, Rasmussen S, Friis C, Hasman H, Marvig RL., Jelsbak L, Sicheritz-Ponténa T, Usserya DW, Aarestrup FM, Lunda O (2012) Multilocus sequence typing of total-genome-sequenced bacteria. *J. Clin. Microbiol.* 2012; 50: 1355–1361.
14. Carattoli A, Zankari E, García-Fernández A, Voldby Larsen M, Lund O, Villa L, Aarestrup M, Hasman H. In silico detection and typing of plasmids using Plasmid Finder and plasmid multilocus sequence typing. *Antimicrob. Agents Chemother.* 2014; 58: 3895–3903. doi:10.1128/AAC.02412-14.
15. Isola OB, Olatunji EO. Isolation and identification of microorganisms from surfaces in Science laboratories. *SAU Sci-Tech. J.* 2016; 1(1): 80-87.
16. Ghayoor M, Qadoos A, Bahadar S, Hayat A, Daud M, Hassan A, Ali F, Zeb A, Rahman KU, Wahab A, Khattak ZF, Khattak B. Isolation and Identification of Common Contaminants Bacteria from Working Area in Microbiology Laboratory. *J Biomol. Sci.* 2015; 3(2): 74-78.
17. Veena KHB, Nagarathna S, Reddemma K, Lalitha K, Mary B, Sateesh VL. Containment of case-file contamination-- infection control. *JEMDS.* 2012; 1(6): 1166-1171

18. Canton R, Coque TM. The CTX-M beta-lactamase pandemic. *Curr Opin Microbiol.* 2006; 9 (5): 466-475.
19. Aibinu I, Pfeifer Y, Peters F, Ogunsola F, Adenipekun E, Odugbemi T, Koenig W. Emergence of *bla* CTX-M-15, *qnrB1* and *aac(6′)-Ib-cr* resistance genes in *Pantoea agglomerans* and *Enterobacter cloacae* from Nigeria (sub-Saharan Africa). *J Med Microbiol.* 2012; 61:165–167.
20. Raji MA, Jamal W, Ojemeh O, Rotimi VO. Sequence analysis of genes mediating extended-spectrum beta-lactamase (ESBL) production in isolates of *Enterobacteriaceae* in a Lagos Teaching Hospital, Nigeria. *BMC Infect Dis.* 2015; 15:259. doi: 10.1186/s12879-015-1005-x.
21. Jesumirhewe C, Springer B, Allerberger F, Ruppitsch W. Whole genome sequencing of extended-spectrum β -lactamase genes in *Enterobacteriaceae* isolates from Nigeria. *PLoS ONE.* 2020; 15(4): e0231146. doi: 10.1371/journal.pone.0231146
22. Izdebski R, Baraniak A, Herda M, Fiett J, Bonten MJ, Carmeli Y, Goossens H, Hryniewicz. W, Brun-Buisson C, Gniadkowski M, on behalf of the MOSAR WP2, WP3 and WP5 study groups MLST reveals potentially high-risk international clones of *Enterobacter cloacae*. *J Antimicrob Chemother.* 2015; 70(1):48–56. doi: 10.1093/jac/dku359. Epub 2014 Sep 12.

Table 3 Details of the resistant isolates

Isolates	Sequence type	Plasmid replicon type	Antibiotic resistance genes/determinants detected
<i>Enterobacter hormaechei</i>	78	Col(pHAD28), Col3M	MD-regulatory system modulating antibiotic efflux CRP, antibiotic target alteration gene Ef-Tu ,BL- <i>bla</i> _{ACT-24} , <i>bla</i> _{CTX-M-15} ,
<i>Atlantibacter hermannii</i>	Unknown	IncFIB(pECLA), IncFII(pECLA)	<i>A-aac(3)-lld</i> , <i>aph(6)-ld</i> , <i>aac(6)-lb-cr4</i> , <i>P-catII</i> , <i>T-dfrA14</i> , MD-regulatory system modulating antibiotic efflux CRP,TE-tet(D), S-sul2, BL- <i>bla</i> _{TEM-1} , <i>bla</i> _{OXA-1} , <i>bla</i> _{CTX-M-15} FQ- <i>QnrB1</i> ,
<i>Leclercia adecarboxylata</i>	Unknown	Col(pHAD28), IncFII(pCTU2)	<i>A-aph(6)-ld</i> , <i>aph(3'')-lb</i> , <i>T-dfrA14</i> , MD-regulatory system modulating antibiotic efflux CRP,S- <i>sul2</i> , BL- <i>bla</i> _{TEM-1}
<i>Stenotrophomonas maltophilia</i>	Unknown	No plasmid replicon type	No resistance gene detected
			Key-A-Aminoglycosides, BL-Beta-lactams, MD-Multi-drug, P-Phenicol, T-Trimethoprim,TE-Tetracycline,S-sulphonamide,FQ-Fluoroquinolones

Table 4 Genome assembly statistics of the recovered isolates

Isolate	Isolation source	Genome size (GB)	Genome coverage	N ₅₀ (bp)	Number of contigs	Accession Number
<i>Atlantibacter hermannii</i>	Laboratory sink	4,7	28	175,641	114	JAJNEI000000000
<i>Stenotrophomonas maltophilia</i>	Laboratory bench	4,6	24	38,623	220	JAJNEH000000000
<i>Leclercia adecarboxylata</i>	Laboratory bench	4,9	51	142,618	158	JAJNEK000000000
<i>Enterobacter hormaechei</i>	Laboratory sink	4,7	85	244,270	88	JAJNEJ000000000