First Detection of *Proteus cibarius* sp. of clinical significance

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ABSTRACT

The rapidly changing resistance pattern of multidrug resistant Gram negative bacilli over the last decade is challenging. Their ability to develop resistance to β-lactam antibiotics and produce extended spectrum β-lactamases is most current clinical issue in antibiotic resistance, globally. This study was carried out to identify Gram negative bacterial responsible for β-lactam antibiotics treatment failure in an 88-year-old patient not responding to cephalosporin treatment. Wound sample was obtained from patient with long term infected diabetic wound and cultured on MacConkey agar. Antimicrobial susceptibility and double disk synergistic test were carried out to ascertain antibiotics resistance profile of isolate and production of extended spectrum beta-lactamases, respectively. Isolate was presumptively identified using standard biochemical tests. Genomic DNA extraction, amplification of the 16S rRNA gene with 27F (f5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (r5′-GGTTACCCTTGTTACGACTT-3′) primers and sequencing analysis were carried out to confirm presumptive identification. Nucleotides sequences were aligned with bioedit 7.2.5 and aligned sequences were compared with similar sequences on Gene bank. Construction of phylogenetic tree and evolutionary analysis of related sequences was conducted in MEGA7, using the Neighbor-Joining method. Isolate was identified to be *Proteus cibarius* strain ADE 1 with ascension number MH037129. *Proteus cibarius* strain ADE 1 was resistant to all β-lactam antibiotics and positive for ESBL production. This is the first report on *Proteus cibarius* associated with human pathogenicity. It is also the first detection of antibiotics resistance and ESBL production in *Proteus cibarius*. *Proteus cibarius* has a pathogenic potential and is an emerging opportunistic pathogen capable of spreading multiple antibiotics resistance.

Keywords: *Proteus cibarius*, Extended spectrum β-lactamases, Antibiotics resistance, β-lactam antibiotics.

1. INTRODUCTION

The spread of extended spectrum β-lactam resistant Gram negative bacilli is the most current clinical issue in antibiotic resistance globally (Thaden, Pogue & Kaye., 2016; Logan & Weinstein., 2017; Senchyna et al., 2018). The β-lactam antibiotics are the most clinically useful antibiotics in the treatment of several bacterial infections (Abdallah et al., 2015). They have a broad use because of their wide spectrum of activity and well-established safety profile (Mitić et al., 2014; Naas et al., 2017). These antibiotics are commonly used for empirical and prophylactic therapy against bacterial infections in Nigeria (Onalo, Adeleke & Onalo, 2013; Raji et al., 2013; Eyo et al., 2015). Not only are they used as first line antibiotics in Nigeria, they are also commonly prescribed for therapeutic use (oral and intravenous) for several bacterial infections in the clinical setting and are readily available over the counter antibiotic drugs in community settings (Oli et al., 2017; Iroha et al., 2017; Ibadin et al., 2018). However, their widespread usage has created a selective pressure leading to resistance and many treatment failures.

A recent study by Bernabbe et al., (2017) on failure of β-lactam antibiotics as first line antibiotics in empirical therapy has reported failure rate of 86.2% (ampicillin), 34.6% (amoxicillin), 77% (ceftriaxone) and 82% (ceftazidime). Resistance to extended spectrum beta-lactam antibiotics commonly used, have been reported across all geopolitical zones in Nigeria to be between 5.1% in Ogun and 83.3% in Sokoto (Akinduti et al., 2015; Oghenevo et al., 2016; Horsefall et al., 2017; Ogbolu et al., 2017; Braide et al., 2018; Mohammed et al., 2016 & Zubair & Iregbu, 2018). Resistance to extended spectrum β-lactam antibiotics results more often from production of extended spectrum β-lactamases; an enzyme encoded by resistance genes and often borne on plasmids.

Many Gram-negative bacilli carry plasmid mediated ESBLs and have been successful in proliferating and spreading ESBL resistance through horizontal gene transfer (Raji et al., 2013; Lukac, 2015). This growing threat poses a significant challenge to clinical microbiologists, particularly in Africa (Muhammad, et al., 2014; Khan, Maryam & Zarrili, 2017). Several antibiotic drug combinations initially used for treatment have become ineffective, resulting in a more complex drug resistance and a serious limitation to therapeutic options (Nathwani, Raman, Sulham, Gavaghan, & Menon, 2014).

Centre for Disease and Control classifies Gram negative bacilli resistant to extended spectrum β-lactam as a serious threat. These organisms require consistent monitoring They pose several limitations to antibiotics therapy and are global threats of public health concern (Pokhrel et al., 2014; Duin & Doi, 2017; Founou, 2018). and the development of prevention strategies, including their prompt detection, within the middle to low income countries (Nasir, Babyo, Emeribe, & Sani, 2015).

This study aims to isolate ESBL producing Gram negative bacilli from an 88-year-old patient with relapsing chronic diabetic wound infection following initial amputation of lower-extremity.

2. MATERIALS AND METHODS

2.1 Sample site and collection

Sample was obtained from an 88-year-old patient not responding to cephalosporin treatment for 3 months at Lagos island General Hospital (Odan), Nigeria. Patient was administered third generation cephalosporin antibiotics for over two months with no clinical sign of improvement. Samples were aseptically obtained during wound dressing, using sterile swab sticks enclosed in a tube, as described by Godebo, Kibru & Tassew (2013).

2.2 Identification of Isolates

Sample was cultured aseptically on MacConkey (Rapid Lab), Salmonella Shigella (Rapid Lab) and Eosine Methyelene Blue (Rapid Lab) culture media and identification was carried out using phenotypic method. Wound exudates on swab sticks were dissolved in 0.5 ml of normal saline solution and inoculated on MacConkey agar plates using an inoculating loop with a 10 µl volume calibration and aerobically incubated at 37^0 C for 24 hours. Pure cultures were obtained by inoculating distinct colonies on fresh sterile petri dishes containing MacConkey agar and incubated at 37^0 C for 24h. Isolates obtained were identified using routine biochemical tests such as sulphur reduction, indole, motility, methyl red, voges-proskauer, urease, oxidase, sugar fermentation and citrate were carried out, following Gram stain reaction (Prescott et al., 2008).

2.3 Antibiogram Studies for Multidrug Resistance Detection of isolates obtained from wound sites

Antibiotics such as 2^{nd} and 3^{rd} generation cephalosporins (ceftriaxone, ceftazidime, cefuroxime, cefotaxime), penicillins (ampicillin, augmentin), fluoroquinolones (ciprofloxacin, ofloxacin), aminoglycosides (gentamicin) and nitofurantoin, commonly prescribed by clinicians in Nigeria for the treatment of wound infection were obtained from Oxoid^{TM} and used to determine multidrug resistance patterns of all identified isolates. Kirby Bauer disk diffusion method was employed by seeding isolate suspension of 0.5 McFarland turbidity standards on sterile Mueller-Hinton agar plates. Antibiotic discs were placed on seeded agar plates and incubated aerobically for 18h at 37^0 C. Results were interpreted based on their inhibition zone diameter breakpoints, based on Clinical and Laboratory Standards Institute (CLSI, 2016).

2.4 Phenotypic detection of ESBLs

Double disc synergy test was carried out using cefrtriazone (CRX, 30 µg), ceftazidime (CAZ, 30 µg) and cefotaxime (CTX, 30 µg) disks alone and in combination with amoxicillin-clavulanate (AMX, 20/10 µg), purchased from Oxoid^{TM}, were used to detect ESBL activity in isolates resistant to third generation cephalosporins. Mueller-Hinton agar plates were inoculated with isolate suspension of 0.5 McFarland turbidity standards. Antibiotic discs were placed 24 mm distances at the center and plates were aerobically incubated for 18h at 37^0 C. An increase of more than 5 mm in the inhibition zone diameter of plates with amoxicillin-clavulanate combination, in comparison with the diameters of those with cephalosporins alone, indicated ESBL activity (CLSI, 2016).

2.5 Genetic identification of isolates

16S rRNA gene sequence analysis was carried out to confirm the identity of carbapenem resistant ESBL producing isolates.

2.5.1. Genomic DNA extraction

Overnight Luria-Bertani broth bacterial culture of ESBL producing isolate was harvested by centrifugation at 10,000 rpm for 1 min. Genomic DNA was extracted using AidLab (China) genomic DNA extraction kit as instructed by manufacturer.

2.5.2. PCR amplification and sequencing

16S rRNA genes amplification for ESBL producing isolates was carried out by a thermal cycler using simplex PCR, as described by El Allaoui, Rhazi, Essahale, Bouchrif, Karraouan & Ameur (2013). The 27F (f5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (r5′-GGTTACCCTTGTTACGACTT-3′) primer pairs specific for bacteria domain was used. PCR product along with 1kb DNA ladder (Norgen, Canada) were visualized with 4 µl ethidium bromide fluorescence after 45 min electrophoresis on 2% TBE agarose gel, under the UV-transilluminator (Bio- Rad, UK). PCR products were purified using PCR clean up kit (Zymo Research, U.S.A.) and sequenced using automated sequencer (Applied Biosystems SeqStudio Genetic Analyzer) and the dye-deoxy termination procedure.

2.5.3. Phylogenetic analysis

16SrRNA gene sequences was edited using Bioedit (version 7.2.5) software and aligned with CLUSTAL W. Aligned sequence was compared with those on GenBank, using the NCBI BLAST-n program. Neighbor joining method in MEGA version 7.2.5 was used to statistically determine evolutionary relationship and construct phylogenetic tree (Adelowo and Fagade, 2012).

3. RESULTS AND DISCUSSION

Phenotypic identification revealed bacterial isolate recovered from the chronic wound sample to be a *Proteus* specie (Table 1) and was genotypically confirmed to be *P. cibarius* ADE1 with ascension number MH037129 (Figure 1). *Proteus cibarius* showed resistance to ceftriaxone, ceftazidime, cefuroxime, cefotaxime, ampicillin, augmentin and nitrofurantoin but sensitive to ciprofloxacin, ofloxacin and gentamicin. *Proteus cibarius* was also positive for extended spectrum β-lactamase production.

Table 1: Morphological characteristics and biochemical test result of isolate

Bacterium	Form	Margin	Elevation	Optical property	Appearance	Consistency	Colour on NA	Colour on MacC	Gram stain
Proteus sp.	circular	Entire	Flat	opaque	Glistening	Moist	White	Colourless	Negative

Bacterium	Indole production	Methyl-red	Voges-proskauer	Citrate	Uurease	Hydrogen sulphide	Motility	Lactose (A/G)	Maltose (A/G)	Sucrose (A/G)	Glucose (A/G)	Manose (A/G)	Xylose (A/G)	Mannitol	Oxidase

Proteus sp.	-	+	+	+	+	+	+	-/-	-/-	-/-	+/+	-/-	+\+	-\-	-

KEYS: -=Negative, +=Positive, MacC= MacConkey, NA= Nutrient Agar.

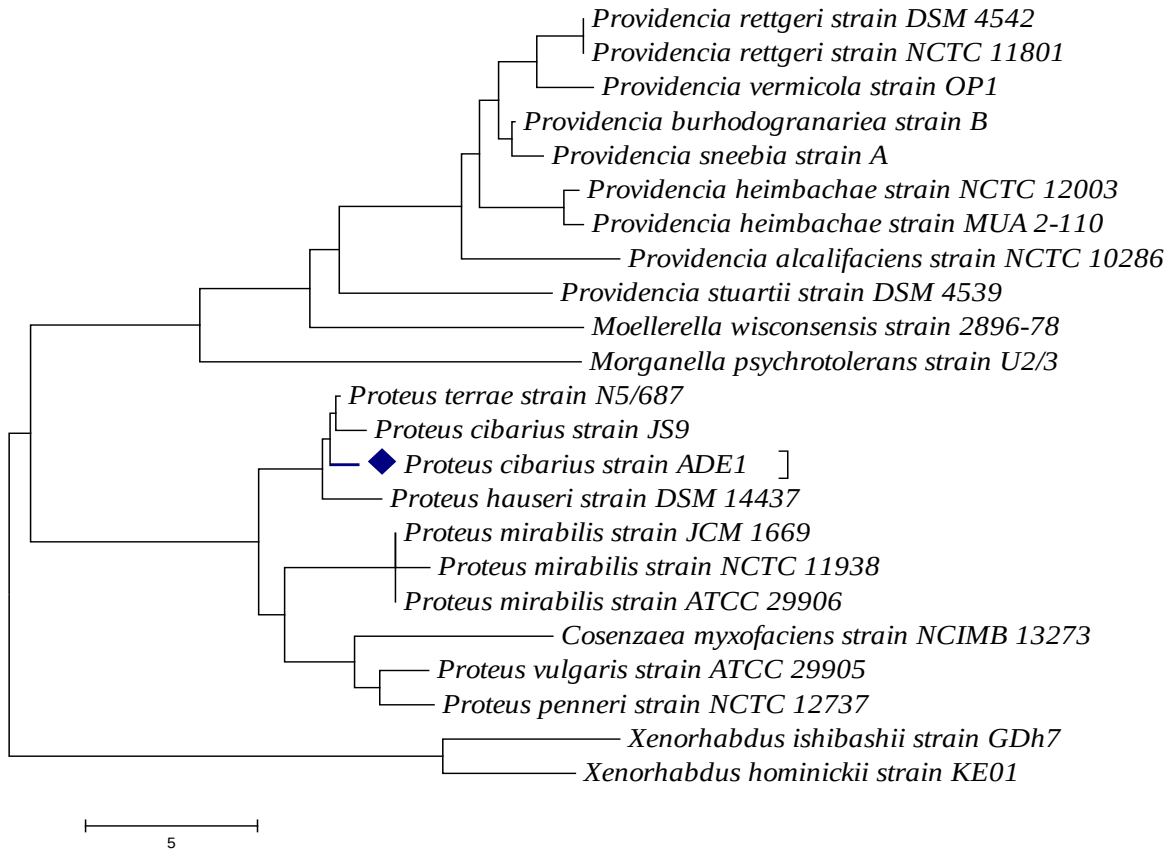

Figure 1: Phylogenetic tree showing evolutionary relationship of *Proteus cibarius* with other members of the genus *Proteus*. *P. cibarius*. Twenty-two nucleotide sequences pulled from Genbank was compared with 16SrRNA nucleotide sequence of *Proteus cibarius* strain ADE 1 and used to infer evolutionary relationship, using the neighbour-joining method.

In this study, *Proteus* specie isolated in the sample obtained from the patient with chronic diabetic wound was genetically identified to be *P. cibarius*; a recently described member of the genus *Proteus*. The 16S rRNA of *Proteus cibarius* strain ADE 1 isolated from chronic diabetic wound during this study had the closest similarity sequence of 97% with that of *P. cibarius* strain JS9. *Proteus cibarius* is a Gram negative bacilli recently discovered from a fermented sea food called "Jeoneo Jeotgal" (Hyun et al., 2016). Although Hyun et al., (2016) reported swarming motility similar to that of pathogenic *P. mirabilis* in P. *cibarius* strain JS9,

another study carried out by Yu et al., (2017) reported that *P. cibarius* had no pathogenicity for human. However, *Proteus cibarius* strain ADE 1 isolated from this study was responsible for relapsing chronic diabetic wound infection, revealing human pathogenicity. Resistance to all β-lactam antibiotics by *Proteus cibarius* strain ADE 1 substantiates the failure of treatment with cephalosporin antibiotics. *Proteus cibarius* strain ADE 1 isolated during this study was also positive for Extended spectrum β-lactamase production, an indication that *P. cibarius* is likely to encode β-lactamase genes which poses a serious challenge to antibiotics resistance globally. *Proteus* species are important opportunistic pathogens implicated in a number of infections (Chen et al., 2017). They are common hospital acquired pathogens also present within the community (Alabi et al., 2015). *Proteus mirabilis* have been reported in chronic diabetic foot ulcers to form a large community of highly persisting biofilm formation (Wasfi et al., 2012). Biofilm formation aided by swarming is believed to be a mechanism for multidrug resistance in *Proteus* species; it enhances cell-cell interaction and horizontal gene transfer of various plasmid mediated genes particularly those that code for extended spectrum β-lactamases (Banu et al., 2015). Studies have shown that *Proteus* species have over the years developed resistance to extended spectrum β-lactam antibiotics often used to treat the infections they cause (Alabi et al., 2015; Hoban et al., 2015). Yusuf et al., (2014) reported a prevalence of 19.8% ESBL producing *proteus* species, from various clinical samples, with *P. mirabilis* being the dominant producers. Ogefere, Aigbiremwen and Omoregie., (2015) reported a prevalence of ESBL producing *P. mirabilis* and *P. vulgaris* from wound infection to be 25% and 37.5% respectively. Another study conducted by Alabi et al., (2015) in Southwest Nigeria, reported 44% ESBL production in *P. mirabilis*, isolated from wound infection. *Proteus mirabilis* have been reported to acquire plasmid-mediated resistance gene that codes for β-lactamase production, through horizontal gene transfer. A recent study also reported ESBL production in *P. mirabilis* (34.6%) and *P. vulgaris* (40.0), (Ibadin et al., 2017). *Proteus mirabilis* have been reported to acquire plasmid-mediated resistance gene that codes for β-lactamase production, through horizontal gene transfer. A recent study in Nigeria reported *P. mirabilis* as an opportunistic pathogen with the potential to disseminate Extended spectrum β-lactamase (ESBL) resistance gene (Alabi et al., 2017). This study reported clonal CTX-M-15 gene in two *P. mirabilis* isolates obtained from a study carried out across five tertiary hospitals (Ibadan, Sagamu, Abeokuta, Ile-Ife and Lagos) in the South West region. The CTX-M-15 genes had an association with ISEcp1 and presumed to be chromosomally encoded following plasmid curing and transconjugation experiment (Alabi et al., 2017). A study in Taiwan also reported class 1 integron association with CTX-M-3 and CTX-M-14 genes in *P. mirabilis* (Chen et al., 2017). This first study to report P. cibarius in Nigeria and also the first to report *P. cibarius* with human pathogenicity.

4. CONCLUSION

Proteus cibarius has a pathogenic potential and is an emerging opportunistic pathogen capable of spreading multiple antibiotics resistance. Further studies will reveal detailed information on their potential threat to the clinical setting, mechanisms used for acquisition of resistance genes and possibility of undergoing horizontal gene transfer; a mechanism that has rendered most antibiotics ineffective.

5. ACKNOWLEDGEMENT

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COMPETING INTERESTS

None declared

ETHICAL PERMISSION

Ethical permit for this research was obtained from Lagos State Ministry of Health, Health Research and Ethics Committee LASUTH and Covenant University (Biological Sciences Research Ethics Committee (BIOSCREC).

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