Original Article

Detection of Extended-spectrum Beta-lactamase Genes in Members of the *Proteeae* Tribe Isolated from a Tertiary Hospital in Southeast, Nigeria

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Abstract

Background: Tribe *Proteeae* consisting of *Proteus, Providencia*, and *Morganella species* are critical opportunistic pathogens causing various health care-associated infections. Widespread extended-spectrum β-lactamase (ESBL) activities have been reported among members of these groups of organisms. **Aim:** This study aimed to detect ESBL genes in clinical isolates of these bacteria from Nnamdi Azikiwe University Teaching Hospital (NAUTH). **Materials and Methods:** Two hundred and sixteen Gram-negative bacilli isolated from patients receiving care in NAUTH were identified using the Microbact 12A Gram-negative bacilli identification system. Antimicrobial susceptibility tests were performed on the isolates using antibiotics of various classes. The isolates resistant to any third-generation cephalosporins were screened for ESBL activity using the combination disc test. The presence of cefotaximase (CTX), oxacillinases (OXA), and transmission electron microscopy (TEM) genes were determined using polymerase chain reaction. **Results:** Out of the 216 Gram-negative isolates, 12 (5.6%) were *Proteus spp*., 3 (1.4%) were *Morganella spp.,* and 8 (3.7%) were *Providencia spp*. The prevalence of the *Proteeae* bacteria was 23/216 (10.6%). Of the 23 *Proteeae* bacteria, 5 (21.7%) were confirmed phenotypically as ESBL producers, while 3 (13%) expressed the actual ESBL genes. CTX, OXA, and TEM genes were detected in all three isolates. Furthermore, 13% of the isolates exhibited carbapenem resistance. **Conclusion:** The results confirmed that ESBL-producing *Proteeae* bacteria existed in NAUTH. The incidental detection of carbapenem resistance among the *Proteeae* isolates whispers terror in waiting because carbapenems are last-resort antibiotics for managing life-threatening ESBL infections. This worrisome development highlights the need to improve infection control practices in Nigerian tertiary hospitals drastically.

Keywords: Antimicrobial resistance, beta-lactamase, carbapenem-resistant, cephalosporin, *proteeae*

Introduction

The tribe *Proteeae* consists of *Proteus*, *Providencia*, and *Morganella* genera. They are Gram-negative, nonlactose fermenting, and urease-producing bacilli and are members of the *Enterobacteriaceae* family of bacteria.[1] They are widespread in the environment and makeup part of the normal flora of the human gastrointestinal tract. Tribe *Proteeae species* are important opportunistic pathogens that cause various health care-associated infections.[2] They are among the most common causes of urinary tract infections in both community and hospital settings.[3] *Proteus mirabilis* infection is of utmost concern due to its ability to form crystalline biofilms that block the catheter.[4] The routine use of antimicrobial agents has resulted in widespread antibiotic resistance and antibiotic resistance genes, especially among Gram-negative bacteria.[5] The worldwide dissemination of extended-spectrum β-lactamase (ESBL) producing *Enterobacteriaceae* is of great concern due to the limited treatment options available to tackle infections caused by them.[6] These enzymes can hydrolyse penicillins, aztreonam,

Access this article online

Quick Response Code:

Website: www.njmonline.org

DOI: 10.4103/NJM.NJM_39_22

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How to cite this article: Akujobi CN, Okwesilieze CV, Aghanya IN, Ukibe SN, Okoro AE, Ushie SN, *et al*. Detection of extended-spectrum beta-lactamase genes in members of the *Proteeae* tribe isolated from a tertiary hospital in Southeast, Nigeria. Niger J Med 2022;31:429-34.

Submitted: 14-Mar-2022 **Revised:** 02-Jun-2022
Accepted: 24-Jun-2022 **Published:** 27-Aug-2022

and first-, second-, and third-generation cephalosporins, thus limiting treatment options.[7] In such cases, there is a resultant increase in carbapenems use (the last-resort antibiotics for the treatment of ESBL-producing *Enterobacteriaceae* infections) and subsequent emergence and dissemination of carbapenem-resistant bacteria worldwide.[8]

The increase in ESBL production among hospital clinical isolates is a growing public health concern. Consequently, there is a need for regular screening of clinical isolates to determine their antimicrobial susceptibility patterns, thus preventing treatment failure and uncontrolled spread of multidrug-resistant pathogens. Although a similar study has been conducted in Nnamdi Azikiwe University Teaching Hospital (NAUTH), Nnewi by Akujobi and Ewuru,[9] this study aims to review their findings with specific interests in ESBL production in the *Proteeae* tribe of bacteria.

Materials and Methods

This prospective hospital-based study was carried out on the *Proteeae* bacteria isolated from clinical specimens of patients receiving care at the NAUTH, Nnewi from August 2019 to May 2020.

Study location

This study was conducted in Anambra State, Nigeria, at the Medical Microbiology and Parasitology Department of NAUTH, Nnewi. The hospital is a tertiary institution and a major referral center serving individuals from most parts of Southeast Nigeria.

Study design

This was a prospective hospital-based cross-sectional study.

Study population

The study involved Gram-negative bacilli isolated from clinical specimens of patients in the study duration.

Sampling method

A convenience sampling technique was employed in this study.

Bacteria identification

In total, 216 nonduplicate Gram-negative bacilli obtained from patients' clinical samples, including urine, blood, sputum, and wounds, were identified using the Microbact 12A semi-automated Gram-negative bacilli identification system. Out of these, 23 were confirmed to be members of the *Proteeae* tribe of *Enterobacteriaceae*.

Antimicrobial susceptibility testing

The modified Kirby–Bauer susceptibility testing technique as described by the Clinical and Laboratory Standard Institute (CLSI) was carried out.[10] The isolates were tested with the following antibiotics: amoxicillin-clavulanate (20/10 µg), piperacillin-tazobactam (TZP: 100/10 µg), cefuroxime (30 µg), ceftriaxone (30 µg), ceftazidime (30 µg), cefepime (30 µg), meropenem (10 µg), ertapenem (10 µg), imipenem (10 µg), gentamicin (30 µg), amikacin (30 µg) and ciprofloxacin (5ug) (Oxoid Ltd., UK). Incubation was performed at 35–37°C for 18–24 h. After incubation, the inhibition zone diameter of each antibiotic produced by the *Proteeae* bacteria was measured in millimeters (mm). This was considered sensitive, intermediate, or resistant to the test antibiotics based on the documented break-point guidelines of the CLSI standard interpretive criteria.[10] *Escherichia coli* American Type Culture Collection (ATCC) 25922 was used for quality control.[10]

Screening for suspected extended-spectrum beta-lactamase production

The isolates were screened for resistance to third-generation cephalosporins and possible ESBL producers according to the 2020 CLSI guidelines.[10] In this method, third-generation cephalosporins (cefotaxime [30 µg] and ceftazidime [30 µg]) discs (Oxoid, UK) were placed on the surface of Mueller–Hinton Agar (Oxoid, UK) plates inoculated with each isolate and then incubated for 16–18 h at 35–37°C. Following incubation, the zones of inhibition were read off. Isolates that showed a zone of inhibition ≤27 mm in diameter for cefotaxime or ≤22 mm for ceftazidime were considered suspected ESBL producers and were subjected to phenotypic confirmation by the combined disc method for detecting ESBLs. *Klebsiella pneumoniae* ATCC 700603 and *E. coli* ATCC 25922 were used to quality control the screening test.[10]

Phenotypic confirmation of extended-spectrum beta-lactamase production (Combination disc test)

All isolates suspected of producing ESBLs were then subjected to confirmatory ESBL testing using the combination disc technique.[10] Ceftazidime (30 µg) and cefotaxime (30 µg) were used alongside their combinations with clavulanic acid. The standard disc diffusion procedure was followed as described earlier, and plates were incubated at 35–37°C for 16–18 h. An increase in the diameter of the zone of inhibition of ≥5mm when the disc containing a single antibiotic is compared to the disc containing a combination of the same antibiotic and clavulanate confirms the production of ESBL in the test organism.[10] *K. pneumoniae* ATCC 700603 and *E. coli* ATCC 25922 were used to quality control the test.[10]

Molecular detection of extended-spectrum beta-lactamase genes (cefotaximase, transmission electron microscopy, and oxacillinases)

Bacteria DNA from the confirmed *Proteeae* bacteria isolates was extracted using the boiling method for DNA extraction previously described by De Medici *et al.*,[11] with slight modifications. Genes encoding transmission electron microscopy (TEM), cefotaximase (CTX)-M, and oxacillinases (OXA) group beta-lactamases (bla) were detected using conventional polymerase chain reaction (PCR) that were based on the protocols published previously by Iroha *et al.*[12] and Dallenne *et al.*[13] The primers used were designed and supplied by Inqaba, SA,

and are listed in Table 1. The PCR conditions were as follows: initial denaturation at 94°C for 5 min, followed by 32 cycles of denaturation at 95°C for 30 s, annealing at 54°C for 1 min, extension at 72°C for 2 min, then final extension at 72°C for 5 min.

The products were resolved on a 2.0% agarose gel at 100V for 30 min and visualized on a blue light transilluminator at 280 nm.

Data collection methods

Data were collected by the observation method and then manually entered into a structured Microsoft Excel spreadsheet (Microsoft Office 2013™), pending analysis using the STATA software.

Ethical considerations

Ethical approval was obtained from the Research and Ethics Committee of NAUTH, Nnewi, with reference number NAUTH/CS/66B/VOL. 2/068.

Data analysis

Statistical analysis was done using STATA version 13 (Stata Corp LP, Texas, USA). Frequency distribution tables were used to determine rates.

Results

Out of 216 Gram-negative clinical isolates collected from NAUTH Medical Microbiology Laboratory, 3 (1.4%) were *Morganella spp.,* 8 (3.7%) were *Providencia spp.,* and 12 (5.6%) were *Proteus spp*. The individual species and their prevalence were *M. morganii* 3/216 (1.4%), *Providencia rettgeri* 4/216 (1.85%), *Providencia stuartii* 4/216 (1.85%), *P. mirabilis* 5/216 (2.31%), and *Proteus vulgaris* 7/216 (3.24%). The overall prevalence of the *Proteeae* bacteria was 10.6%.

The antibiogram showed that the *Proteeae* isolates were markedly resistant to amoxicillin-clavulanic (91.3%) and cefixime (60.9%). In contrast, none of the isolates showed resistance to piperacillin-tazobactam, meropenem, and amikacin. Resistance to the carbapenems was detected against ertapenem (8.7%) and imipenem (8.7%) [Table 2].

Out of the 23 *Proteeae* isolates, 15 (65.2%) showed resistance to at least 1 of the third-generation cephalosporins, and of these 15 isolates, 5 (33.3%) were confirmed phenotypically as ESBL producers using the combination of disc test.

Table 1: Primer sequences used in the study

Primer	Sequence (5′→3′)	Location	Product size
TEM (F)	TCAACATTT CCGTGTCG	bla_{TEM}	900 bp
TEM (R)	CTGACAGTTACCAATGC TTA	$bla_{TEM,}$ R	
CTX-M (F)	CGCTTTGCG ATGTGCAG	bla_{CTX-M}	517 bp
CTX-M (R)	ACCGCGATA TCGTTGGT	$bla_{CTX-M,}$ R	
OXA 1 (F)	TCTTTCGAGTACGGCAT TAGC	bla_{OXA-1}	800 bp
OXA 1 (R)	TTGCAGCTTTTCA AGAATGCGC	$bla_{OXA-1,}$ R	

Bla: Beta-lactamase, bp: Base pair, F: Forward primer, R: Reverse primer

PCR analysis revealed that the 3 (60%) out of five phenotypically confirmed extended bla producers harboured either TEM, OXA, or CTX [Table 3]. Isolates 3, 18, and 28 expressed the CTX gene [Figure 1]; isolate 18 harboured the OXA gene [Figure 2], while isolates 18 and 28 expressed the TEM gene [Figure 3]. The remaining two isolates were negative for the ESBL genes tested. The prevalence of ESBL producers among the *Proteeae* species was 21.7%.

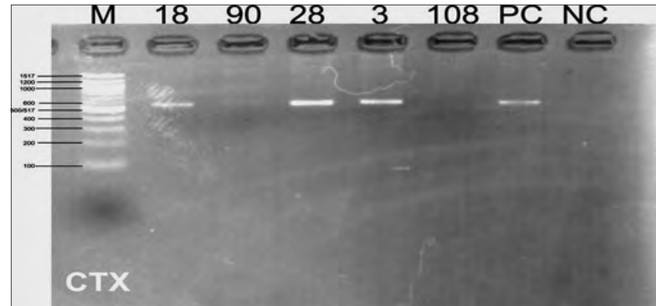

Figure 1: Agarose gel electrophoresis showing the CTX bands. Lanes 3, 18, and 28 represent the CTX bands at 600bp, while lane M represents the 100bp molecular ladder. Lane PC represents blaCTX -positive control. Lane NC represents negative control. CTX: Cefotaximase, NC: Negative control, PC: Positive control

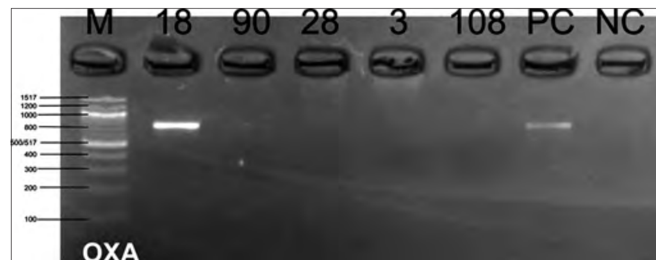

Figure 2: Agarose gel electrophoresis showing the OXA bands. Lanes 18 represents the OXA bands at 800bp, while lane M represents the 100bp molecular ladder. Lane PC represents bla_{OXA}-positive control. Lane NC represents negative control. OXA: Oxacillinases, PC: Positive control, NC: Negative control

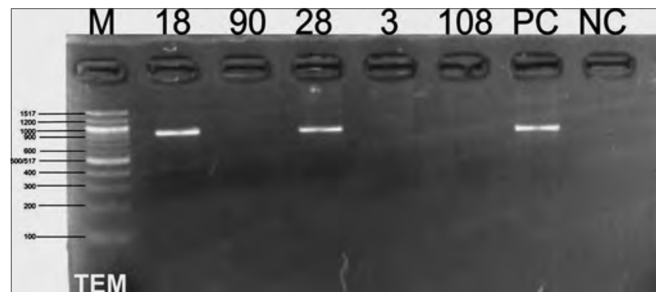

Figure 3: Agarose gel electrophoresis showing the TEM bands. Lanes 18 and 28 represent the TEM bands at 900bp, while lane M represents the 100bp molecular ladder. Lane PC represents bla_{TEM}-positive control. Lane NC represents negative control. TEM: Transmission electron microscopy, PC: Positive control, NC: Negative control

Table 2: Antibiogram of the proteeae bacteria

Antibiotic class	Antibiotic	Disc content (μg)	Sensitive		Resistant	
			Frequency	Rate (%)	Frequency	Rate (%)
B-lactam/B-lactam inhibitor	Amoxicillin-clavulanate	20/10	2	8.7	21	91.3
Cephalosporins	Third-generation: Ceftriaxone	30	15	65.2	8	34.8
	Third-generation: Cefixime	30	9	39.1	14	60.9
	Third-generation: Ceftazidime	30	16	69.6	7	30.4
	Fourth-generation: Cefepime	30	18	78.3	5	21.7
Aminoglycosides	Gentamicin	30	16	69.6	7	30.4
	Amikacin	30	23	100	0	0
Carbapenems	Ertapenem	10	21	91.3	2	8.7
	Meropenem	10	23	100	0	0
	Imipenem	10	21	91.3	2	8.7
Quinolones	Ciprofloxacin	30	14	60.9	9	39.1
Penicillin	Piperacillin-tazobactam	100/10	23	100	0	0

Table 3: Distribution of isolates in relation to their specimen sources, resistance to third-generation cephalosporins and extended-spectrum β-lactamase activity

Isolate ID	Organism	Specimen source	Third-generation cephalosporin resistance	ESBL activity	blaCTX	blaOXA	blaTEM
3	*M. morgani*	Urine	+	+	+	−	−
13	*P. vulgaris*	Urine	+	−	−	−	−
18	*P. rettgeri*	Wound	+	+	+	+	+
20	*P. rettgeri*	Wound	+	−	−	−	−
22	*P. stuartii*	Urine	+	−	−	−	−
28	*P. mirabilis*	Wound	+	+	+	−	+
34	*P. mirabilis*	Urine	−	−	−	−	−
36	*P. vulgaris*	Wound	+	−	−	−	−
41	*P. vulgaris*	Urine	−	−	−	−	−
67	*P. mirabilis*	Blood	−	−	−	−	−
75	*M. morgani*	Urine	+	−	−	−	−
90	*P. vulgaris*	Urine	+	+	−	−	−
101	*P. rettegeri*	Urine	−	−	−	−	−
106	*P. vulgaris*	Sputum	−	−	−	−	−
107	*P. rettegeri*	Sputum	−	−	−	−	−
108	*P. stuartii*	Sputum	+	+	−	−	−
144	*M. morgani*	Wound	+	−	−	−	−
157	*P. vulgaris*	Wound	−	−	−	−	−
215	*P. stuartii*	Wound	+	−	−	−	−
223	*P. mirabilis*	Wound	−	−	−	−	−
226	*P. mirabilis*	Wound	+	−	−	−	−
230	*P. stuartii*	Urine	+	−	−	−	−
244	*P. vulgaris*	Urine	+	−	−	−	−

M. morgana: Morganella morgana, P. vulgaris: Proteus vulgaris, P. rettgeri: Providencia rettgeri, P. stuartii: Providencia stuartii, P. mirabilis: Proteus mirabilis, P. rettgeri: Providencia rettgeri, ESBL: Extended-spectrum β-lactamase, + means detected, - means undetected

Discussion

Infections caused by the *Proteeae* bacteria are common and were previously treated easily using available antibiotics, but with the increasing rate of antibiotic resistance in this group of organisms, morbidity and mortality have significantly increased, especially in healthcare settings.[14] The development of antimicrobial resistance in pathogens that were previously easy to eradicate has become a major issue confronting the global health community with the acquisition of ESBL genes resulting in a drastic reduction in available treatment options.[15]

The prevalence of the *Proteeae* bacteria in this study was 10.6%. This finding was similar to that observed in a Hungarian study where 9.56% of the isolates were ESBL producers.[3] Slightly higher rates of 14% and 18.1% were recorded in Kano and Benin, Nigeria, respectively.[16,17] The somewhat different rates may result from varying infection control practices in the various institutions.

The prevalence of ESBL production in the *Proteeae* bacteria was 21%. A higher rate was observed in a study by Meharaj *et al.*, where 36% of the isolates were ESBL producing.[18] ESBL production was confirmed in 9.1%, 12.5%, and 25% of the *Proteus species, Providencia species,* and *Morganella species,* respectively. This finding contrasted with a similar study done in the same institution in 2010, where no ESBL production was detected among the *Proteus species*.[9] This observation may be due to the increased use of antibiotics that were not readily available for use during the period of the older study and as a result of an increase in the population of society, which will also translate to increased hospital admissions and hence increased risk of transfer of resistance genes.

The most prevalent gene detected among the 23 *Proteeae* bacteria was the *bla*CTX gene (13%), followed by the *bla*TEM gene (9%), while the *bla*OXA gene was the least prevalent (4%). Findings corresponded with those of Olowe *et al.*, Jin *et al.,* and Alabi *et al.*, in which the CTX gene was found to be the most prevalent ESBL gene.[19-21]

One of the isolates, *Providencia rettgeri,* harboured CTX, TEM, and OXA genes, while *P. mirabilis* harboured CTX and TEM genes. coharbouring of genes has also been reported by Olowo-Okere *et al.,* in 2020.[22] An explanation for this trend is that some plasmids can carry more than one resistance gene, which allows bacteria cells to acquire multidrug resistance during a single genetic transfer step, thus resulting in coselection for resistance.[23]

Out of the five isolates that showed a positive result for the phenotypic ESBL test, only 3 (60%) expressed the ESBL genes tested. The remaining two may have been harbouring one of the ESBL genes not tested in this study (e.g. SHV).

Urine harboured the highest population of the *Proteeae* bacteria 9 (39%), similar to the studies done by Olowo-Okere *et al.,* where urine bore most of the *Proteeae* bacteria (49%).[22] These findings agree with claims by Umar *et al.* that urine is the most implicated specimen in tribe *Proteeae* infection.[5] Only one isolate was obtained from a blood specimen. This low *Proteeae* isolation rate in blood corresponds with scientific claims that members of the *Proteeae* tribe are not common causes of bloodstream infections as it accounts for just 1%–3% of all episodes of bacteremia.[20]

Some of the *Proteeae* bacteria 6/23 (26.1%), resistant to at least one of the third-generation cephalosporins but failed to produce ESBLs, were susceptible to cefepime. This finding could result from a sole production of AmpC β-lactamase, which also causes resistance to third-generation cephalosporins. It has been reported that the fourth-generation cephalosporin, cefepime, is clinically useful against organisms producing AmpC-type β-lactamases but may be less useful in treating ESBL-producing organisms.[24]

Carbapenem resistance was exhibited by 3 (13%) of the isolates in this study. This was a worrisome observation because the carbapenems are considered the antibiotics of last resort in treating infections caused by ESBL-producing organisms. If not checked, it may result in a global health emergency.

Conclusion

Extended-spectrum β-lactamase-producing *Proteeae* bacteria (*Proteus spp., Providencia spp.,* and *Morganella spp.* were prevalent in clinical specimens obtained from NAUTH. The incidental detection of carbapenem resistance whispers terror in waiting since they are the last-resort antibiotics for managing most drug-resistant life-threatening infections.

Recommendations

This worrisome observation necessitates the need to re-enforce antimicrobial stewardship in hospital settings to curb the spread of these resistant pathogens. Since 13% of the *Proteeae* bacteria also exhibited carbapenem resistance, it is recommended that further molecular studies to look for the production of carbapenemase genes in these groups of organisms should be explored.

Limitations

This study was limited due to the fact that not all the possible ESBL genes were detected. For example, the blaSHV gene was not tested.

Acknowledgment

The authors of this work will like to appreciate the staff of the Medical Microbiology department at Nnamdi Azikiwe University for their contributions toward the specimen processing and isolation processes of this work.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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