Molecular detection of virulence genes and Extended Spectrum Beta-lactam producing enzymes of Proteus mirabilis isolates from fishes in Federal Capital Territory (FCT) Abuja, Nigeria

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ORIGINAL ARTICLE

Molecular Detection of Virulence Genes and Extended Spectrum Beta-lactam Producing Enzymes of *Proteus mirabilis* Isolates from Fishes in Federal Capital Territory Abuja, Nigeria

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Abstract

OBJECTIVE: To study the molecular characterization of three virulence genes and phenotypic detection of extended spectrum beta-lactam (ESBL) producing enzymes in *Proteus mirabilis* isolates from fish in the Federal Capital Territory.
DESIGN: Cross-sectional study.
ANIMAL: A total of 400 fish were purchased from various markets and sites in the Federal Capital Territory. Thirteen (3.25%) *P. mirabilis* isolates were obtained. This study used amoxicillin with clavulanate and ceftazidime, a third-generation cephalosporin antibiotics, to treat all phenotypically typed isolates utilizing ESBL. PCR was used for molecular characterization to identify the pathogenic genes of interest, including *ure*C, *rsb*A, and *lux*S.
RESULTS: The prevalence of *P. mirabilis* by area councils shows that 4.81% for AMAC, 2.99% for Bwari and 2.5% for Gwagwalada. The isolates screened for the detection of ESBL enzymes showed that three (23%) were positive for ESBL. The *ure*C (0%) gene was not detected in any of the 13 isolates, *rsb*A (100%) and *lux*S (100%) genes were detected in all 13 isolates *P. mirabilis* obtained in our study.
CONCLUSION: and clinical relevance: In conclusion, the present study showed that *P. mirabilis* occurs in this environment, which contains ESBL enzymes, and may create virulence factors that contribute to the pathogenicity of the organism. Public health awareness and education initiatives on the risks associated with *P. mirabilis* infection are therefore necessary, and should be intensified.

Keywords: Antibiotic susceptibility, Extended spectrum beta-lactam, Fish, PCR, *Proteus mirabilis*, Virulence genes

1. Introduction

Proteus mirabilis is an important gram-negative rod-shaped pathogen commonly found in the soil, water, and gastrointestinal tracts of many animals, including humans. The morphology and physiological traits of this dimorphic *Enterobacteriaceae* can change in response to its environment and growth conditions. These modifications causes the organism to exhibit its most peculiar activity, called swarming behavior, in which short, swimming vegetative cells convert into long, highly flagellated forms called swarmer cells [1]. *P. mirabilis* is known to pose a serious public health concern because of its association with urinary tract infections (UTIs), biofilm formation, antimicrobial resistance (AMR), and other healthcare-associated infections [1]. The bacterium is not widely known as *Escherichia coli* (*E. coli*) or *Klebsiella pneumoniae* in terms of AMR, *P. mirabilis* poses

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significant public health concerns, especially because of its ability to develop resistance to multiple antibiotics. *P. mirabilis* is responsible for UTIs, especially in those with underlying medical problems, such as urinary catheterization, structural abnormalities of the urinary tract, or weakened immune systems. If left untreated, *P. mirabilis*-caused UTIs can result in complications, such as bacteremia and pyelonephritis [2].

As the beta-lactam antibiotic families of cephamycin (cefotetan, cefoxitin) and carbapenem (imipenem, ertapenem, and meropenem) are known to be ineffective against extended spectrum beta-lactam (ESBL) enzymes, carbapenems are typically used for the treatment of ESBL-producing bacteria [2]. Beta-lactam resistance, which results from the presence of these enzymes in an organism, is a significant clinical and therapeutic issue [3]. A major risk to public health arises from the potential for amicroorganisms to generate different ESBL variations, which can impact treatment options and results. In the current study, we sought to carry out phenotypic detection of ESBL enzymes in *P. mirabilis* isolates from fish sold in Federal Capital Territory (FCT), Abuja [2].

Several factors contribute to the virulence of *Proteus* species, and virulence genes encoded in operons control and express these factors [4]. These virulence genes make *Proteus* species more pathogenic, including urease, the key enzyme in the development of kidney and bladder stones [5], and allows it to create a habitat where it can survive [6]. The *lux*S gene contributes to the production of autoinducer 2, which is released by bacteria and is used to convey the metabolic potential and cell density of the environment [7]. The *rsb*A gene-mediated swarming behavior of *P. mirabilis* has been associated with the development of biofilms and extracellular polysaccharides [8].

There has been very little data within Nigeria that elucidate its pathogenicity and expose the virulence genes of *P. mirabilis* in fish. In Nigeria, bacterial fish infections pose a major threat to aquaculture systems, resulting in significant damage and mortality [9]. *P. mirabilis* has been identified as an opportunistic pathogen and is thought to be an indicator of sewage pollution in fish [9]. Pathogenic strains of *P. mirabilis* may cause diarrhea in fish [10]. In addition to being present in human, animal, and poultry excrements, *P. mirabilis* is omnipresent in nature. *P. mirabilis* is a common water-borne bacterium found in the tissues of normal fish [11]. Our research will be useful to provide firsthand information on the molecular characteristics of virulent genes of *P. mirabilis* strains in fishes, which could affect humans, as well as to elucidate the presence of the ESBL enzyme in the organism.

2. Materials and methods

2.1. Sample collection and bacterial isolation

From August 2021 to February 2022, a purposive sampling technique was employed in the FCT, based on the availability and convenience of fish from the markets. To purchase fish from markets in a few local government areas within the FCT, a basic random sample technique was used.

P. mirabilis was isolated in accordance with the guidelines provided by the International Organization for Standardization (ISO 6579, 2017) for the identification and isolation of *Enterobactereaca*. For nonselective pre-enrichment, 1 g of tissue sample was aseptically extracted. It was then homogenized in 9 ml buffered peptone water (LabM, UK) in a test tube to obtain a dilution of 1 : 10 test tubes were labeled, corked properly, and overnight stored at 37 °C. For selective enrichment, a little less than 0.1 ml of the pre-enrichment was incubated overnight at 37 °C on Rappaport-Vassiliadis (RV) (Oxoid, England). Selective agar plating was performed by plating 10 µl onto Xylose Lysine Deoxycholate agar (Himedia, India) and incubating it at 37 °C for the entire night. Suspect colonies were sub-cultivated on Xylose Lysine Deoxycholate (Himedia, India) and incubated overnight (18–24 h) at 37 °C for sub-cultivation/purification. The colonies mostly had black surfaces and tiny transparent black centers. After an overnight incubation period at 37 °C, pure cultures were maintained on nutrient agar slants and refrigerated at 2–8 °C. All pure *P. mirabilis* were stored in nutrient agar slants and transported to the biotechnology laboratory of the National Veterinary Research Institute Vom (NVRI) for molecular analysis [12,13].

2.2. Phenotypic detection of ESBL gene

The combination disc test method was used to screen ESBL-producing organisms. Disc combinations containing cephalosporins (Cefotaxime, Ceftazidime, and Cefpodoxime) with and without clavulanic acid were used in each combination disc test [14]. A standardized test organism (0.5 McFarland turbidity standard) was inoculated onto Muller–Hinton agar using a sterile cotton-tipped applicator to achieve semi-confluent growth. The inoculated plates were lined up with paired discs separated by at least 25 mm. After overnight incubation at 35 ± 20 °C for the entire night, the zones of inhibition were

assessed. The formation of ESBL was indicated by an inhibition zone greater than or equal to 5 mm or an extension of 50% surrounding the combination disc (Cephalosporin with Clavulanic acid) in comparison to the cephalosporin disc alone [15].

2.3. DNA extraction

Boiling was used to extract genomic DNA. Briefly, 5 ml of bacterial isolates was centrifuged at 14 000 rpm for 3 min after being cultured in Laura Bertani broth at 37 °C for 8 h. The cells were heated at 95 °C for 20 min in a heating chamber after being resuspended in 500 µl of normal saline. After cooling on ice, the heated bacterial suspension was centrifuged at 14 000 rpm for 3 min. For later use, the DNA-containing supernatant was transferred to 1.5 ml microcentrifuge tubes and kept at −20 °C. The extracted genomic DNA was quantified using the NanoDrop 1000 spectrophotometer by placing a drop (~2 nmol/pmol) in the sample space and analyzed using NanoDrop 1000 software.

2.4. Molecular detection of virulence genes of P. mirabilis by polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) was used to characterize the virulence genes of *P. mirabilis*. The following primer sets were used: (5′ − TTGAAGGACGCGATCAGACC − 3′) and (3′-ACTCTGCTGTCCTGTGGGTA-5′), which amplify a 467 bp sequence of *rsbA* gene [16]; (5′-GTATGTCTGCACCTGCGGTA-3′) and (3′−TTTGAGTTTGTCTTCTGGTAGTGC−5′), which amplifies the 464 bp sequence of *luxS* gene [16]; and (5′-ACTCTGCTGTCCTGTGGGTA-3′) and (3′-GTTATTCGTGATGGTATGGG-5′), which amplify the 317 bp sequence of *ureC* gene [17]. Gene amplification was performed in a thermal cycler (AB Biosystem, USA) with a final volume of 25 l for 35 cycles. Taq polymerase, dinucleotide triphostes (dNTPs), and MgCl were included in the X2 Dream Taq Master Mix provided by Inqaba, South Africa, along with the primers at a concentration of 0.2M and 0.5 l DNA as the template for the PCR [13].

The DNA amplification (PCR) was carried out in a final volume of 20 nmol/pmol containing 2 nmol/pmol of the template DNA, 0.2 nmol/pmol of each primer (forward and reverse), 2 nmol/pmol PCR buffer (including 15 mM $MgCl_2$), 2.0 nmol/pmol of 2 mM dNTPs, 0.2 nmol/pmol of Taq DNA polymerase and 13.40 nmol/pmol Nuclease-free water (Qiagen) to complete the total volume of the reactions. The tubes were placed in an Applied BiosystemsVeriti 96 well thermocycler (Applied Biosystems, Germany). The PCR cycling conditions was initial denaturation at 94 °C for 5 min, 40 cycles of denaturation at 95 °C for 30 s, annealing at 59 °C for 30 s, and extension at 72 °C for 30 s. The final extension was performed at 72 °C for 10 min. The PCR products were stored at 4 °C for electrophoresis [18].

3. Results

The frequency of *P. mirabilis* isolated from different area councils of the FCT, Abuja, is shown in Table 1. The highest frequency of *P. mirabilis* was observed in fish bought in Abuja Municipal Area Council (AMAC) (4.81%), with fish bought in Bwari (2.99%) and Gwagwalada (2.57%) having the next highest prevalence of *P. mirabilis*. Of the 400 fish samples purchased in the FCT, *P. mirabilis* accounted for a total of 3.235%.

The combination disc test method for phenotypic detection of ESBL enzymes in *P. mirabilis* isolates is displayed in Fig. 1, where the inoculated plates containing Cephalosporin with Clavulanic acid in comparison to the cephalosporin disc alone were placed at least 25 mm apart from each other, and zones of inhibition were observed.

Table 1. Prevalence of Proteus mirabilis *in different area councils of Abuja.*

Area councils	Number of fishes used	Number of isolate	% prevalence
AMAC	104	5	4.81
Bwari	134	4	2.99
Gwagwalada	162	4	2.57
Total	400	13	3.25

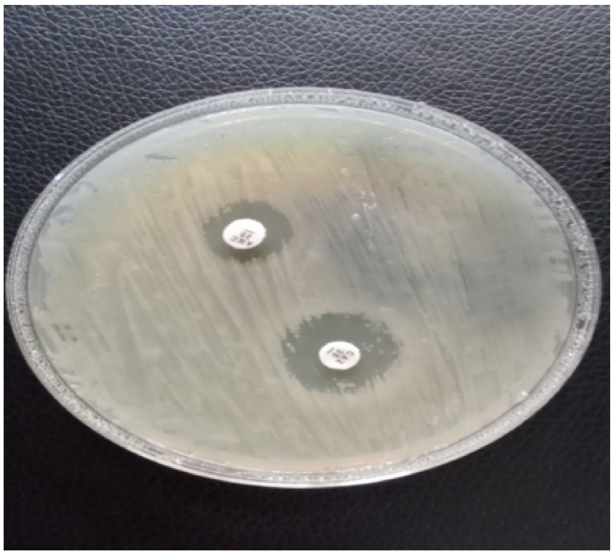

Fig. 1. Extended spectrum beta-lactam culture plate of Proteus mirabilis *isolates showing the zones of inhibition of Cephalosporin with Clavulanic acid in comparison to the Cephalosporin disc alone.*

As observed, the PCR patterns of *ure*C gene of 13 genomic DNA isolates of *P. mirabilis* from FCTand Abuja are presented in Fig. 2. The agarose wells were ladder (L), 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, and −C (negative control). The *P. mirabilis* isolates were loaded in to the wells with the ladder, and nothing was loaded into the negative control well. The ladder was labeled in 1000 bp. None of the 13 isolates showed visible bands for *ure*C, which amplifies at 317 bp after charging it through gel electrophoeresis. The 13 isolates of *P. mirabilis* from fish in FCT did not carry the *ure*C gene. Meanwhile, the results of the PCR patterns of *lux*S gene of 13 genomic DNA isolates of *P. mirabilis* from FCT, Abuja is presented in Fig. 3, where the agarose wells were ladder (L), 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, and −C (negative control). The *P. mirabilis* isolates were loaded in to the wells with the ladder, and nothing was loaded into the negative control well. The ladder was labeled in 1000 bp. All 13 isolates of *P. mirabilis* showed visible bands of *lux*S gene, which were amplifies at 464 pb after passing through gel electrophoresis. On the other hands, Fig. 4 shows

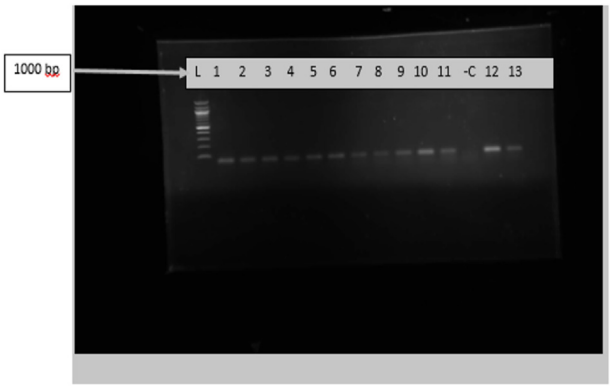

*Fig. 2. A representative gel of 13 polymerase chain reaction products for ureC gene (*Proteus mirabilis*) with no visible band at 317 bp form fishes in Federal Capital Territory. L, ladder; −C, negative control.*

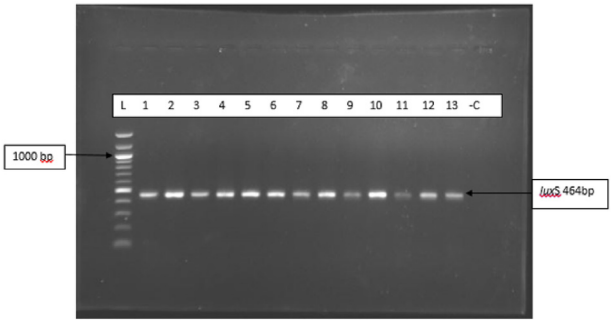

*Fig. 3. A representative gel of 13 polymerase chain reaction products for luxS gene (*Proteus mirabilis*) located at 464 bp from fishes in Federal Capital Territory. L-, ladder; −C, negative control.*

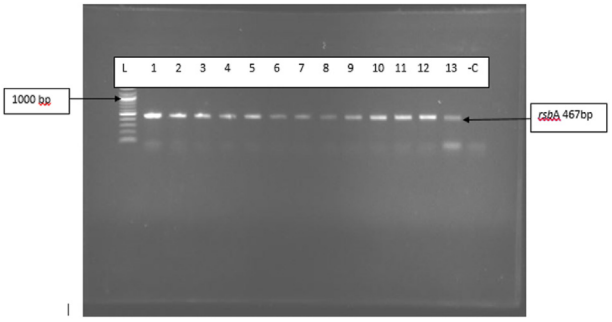

*Fig. 4. A representative gel of 13 polymerase chain reaction products for rsbA gene (*Proteus mirabilis*) located at 467 bp from fishes in Federal Capital Territory. L-, ladder; −C, negative control.*

the results of the PCR patterns of *rsb*A gene of 13 genomic DNA isolates of *P. mirabilis* from FCT, Abuja. The agarose wells were ladder (L), 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, and −C (negative control). The *P. mirabilis* isolates were loaded in to the wells with the ladder, and nothing was loaded into the negative control well. The ladder was labeled in 1000 bp. All 13 isolates of *P. mirabilis* showed visible bands of *rsb*A gene, which were amplifies at 467 pb after passing through gel electrophoresis.

4. Discussion

P. mirabilis is becoming prevalent as an opportunistic pathogen, especially in Nigerian hospitals [19]. *P. mirabilis* is frequently linked to urinary tract infections, especially in patients who use indwelling catheters for an extended period or who receive regular antibiotic therapy [20,21]. Bacteria can be native to aquatic habitats or exogenous, sporadic, and occasionally found in water due to shedding from soil, vegetation, or animals. Because of the use of this heavy compound in aquaculture, resistant strains of antibiotics are present, some of which are non-biodegradable. This increases the selective pressure for antibiotics in water, facilitates the transfer of antibiotic: determinants between aquatic bacteria and human pathogens, and permits the presence of residual antibiotics in commercial fish and shellfish products [20,22].

The β-lactam ring of penicillins and cephalosporins is opened by beta-lactamases, which eliminate the ability of the antibiotic to fight microorganisms. Bete-lactamases have been identified in numerous gram-positive and gram-negative bacterial species, beta-lactamases have been identified [2]. In this study, it was discovered that beta-lactam hydrolyzing enzymes are probably one of the factors responsible for *P. mirabilis* isolate resistance. In our study, three (23%) out of 13 isolates of *P. mirabilis* were detected to have beta-lactamase enzymes

using the disc combination method. This method measures the zone of inhibition around the disc of cephalosporin plus clavulanate. This result is in agreement with the findings of Kwiecinska-Pirog [23], in Poland, who reported that 11 (22.0%) *P. mirabilis* strains produced ESBL. The research conducted determined that three, out 13 *P. mirabilis* strains, were resistant either to Ciprofloxacin or Ceftazidime. The suspected presence of ESBL: producing genes in *P. mirabilis* isolates from fish could be the result of microbial interactions in the transfer of resistance genes within their habitat. Treatment has become more difficult because of *P. mirabilis*'s global development of antibiotic resistance to multiple classes of *P. mirabilis*. *P. mirabilis* has been reported of being resistant to β-lactams (cephalosporin and penicillin), fluoroquinolones, nitrification, fosfomycin, aminoglycosides, tetracyclines, and sulfonamides [24,25]. Most isolates were resistant to tetracycline [25]. The tendency of this organism to implant itself as crystalline biofilms on urinary catheters, which can serve as a bacterial barrier or urine stones may lead to treatment failure. Additionally, *P. mirabilis* has a high level of resistance to antimicrobial peptides such as defensin, Polymyxin B, Protegrin, and LL-37 [26].

Shelenkov *et al*. [27] reported in their study that The ESBL gene and plasmid-mediated resistance to aminoglycosides, macrolides, and fourth-generation cephalosporins were present in Multi-drug Resistances (MDR) and virulent *P. mirabilis* isolates from Moscow, Russia. Treatment failure is a concern because integrons and transposons, which are plasmids, are the means by which resistance-encoding genes are acquired [28]. Our inability to use molecular methods, the gold standard for determining the existence of genes responsible for enzyme production, to validate the presence of ESBL genes in our isolates was a significant limitation of our work. The virulence genes of *P. mirabilis* detected in this study using PCR method including *ure*C, *lux*S, and *rsb*A, which are responsible for virulence factors in the organism. From all 13 isolates, there was no prevalence of *ure*C, which is in contrast with the report of Owoseni *et al*. [13], who reported *ure*C gene as the predominant gene detected in their study. *ure*C gene is responsible for the elevation of urinepH, resulting in stone formation, and it plays an important role in the virulence of *Proteus* species [5]. The result does not corroborate the report of Alsherees *et al*. [29], who reported that 33.3% of *ure*C gene was detected in isolates of *P. mirabilis* from urine and wounds in Iraq. *ure*C gene is responsible for the production of the urease enzyme, which is regarded as a diagnostic feature of *P. mirabilis*. The urease enzyme obtained from *P. mirabilis* exhibited higher activity than the urease enzymes derived from other bacterial species [17].

The *rsb*A gene amplified in the isolate was 100% for all 13% of isolates, which differs from the findings of Abbas *et al*. [16], who claimed that *P. mirabilis* was unable to amplify the *rsbA* gene. The *rsb*A gene may be responsible for swarming motility, which probably associated with *P. mirabilis*. It is well known to function as a protein sensor and is useful in enabling organisms to withstand harsh environmental conditions. Subsequently, *rsb*A facilitates biofilm and extracellular polysaccharide formation [16].

Additionally, 100% of the *P. mirabilis* isolates contained *luxS* genes present in them. The *luxS* gene generates a signal that is used to detect species interactions and cell density in a polymicrobial community, which is crucial for the regulation of virulence genes. The 100% prevalence rate in *lux*S and *rsb*A is similar to that reported by Hussein *et al*. [7] in Jordan, who recorded a100% prevalence rate for *lux*S and *rsb*A in *P. mirabilis* isolates. A study from Iran reported *lux*S and *rsb*A prevalence rates of 70% each [30]. The distribution of virulence genes varies among populations and geographical locations, which is most likely the cause of disparities and similarities in the prevalence rates. The synthesis of *lux*S has been linked to the formation of biofilms by *Proteus* species and other Enterobacteriaceae members [13].

5. Conclusion

The variance in sanitary methods observed in the current investigation may be the cause of the higher prevalence of *P. mirabilis* and virulence factors. In our study, three (23%) of the *P. mirabilis* 13 isolates were positive for ESBL, which can be implicated in the antibiotic resistance of the organism. In our study, *P. mirabilis* was found to carry *lux*S (100%) and *rsb*A (100%) genes using conventional PCR, whereas *ure*C was not detected in any of the isolates. All fish samples were collected from nearby markets within the FCT. Hence, using unsanitary methods when handling, transporting, storing, and cooking fish may be a significant factor in the increased prevalence of *Proteus* species in fish and the presence of bacteria in fish ambient water. *P. mirabilis* is associated with UTIs, biofilm formation, and antibiotic resistance, making it a serious public health concern. The fight against AMR in *P. mirabilis* requires a variety of approaches, including cautious use of antibiotics, infection prevention and control protocols, monitoring of resistant strains, and creation of novel antimicrobial agents. To reduce the

effects of AMR linked to *P. mirabilis*, it is also critical to raise awareness among the public, legislators, and healthcare professionals regarding the significance of prudent antibiotic usage and infection control measures. In addition, hygienic practices followed by retail fish shops were very poor, and the lack of knowledge among fish handlers may have been the primary cause for the increased occurrence of *Proteus* species in fish. Therefore, it is concluded that *P. mirabilis* in this environment produces virulence factors that could be involved in the pathogenicity of the organism.

Ethical statement

Ethical approval for this study was obtained from the Environmental Control Agency in collaboration with the Laboratory Animal Unit of the Department of Veterinary Microbiology, University of Abuja.

Availability of data and materials

All data are available in the present study.

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Author's contributions

This study is a rigorous effort and the contribution of all the authors by ensuring that the aim of the study was achieved.

Conflict of interest

There are no conflicts of interest.

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