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Proteomic characteristics of resistant *Proteus* species in women with urinary tract infections in Nasarawa State, Nigeria

M. H. Muhammad[a], O. O. Orole[b,*], J. F. Nfongeh[b], E. S. Audu[c], F. Gbadeyan[b]

[a] *Department of Microbiology, University Teaching Hospital, Lafia, Nigeria.*
[b] *Department of Microbiology, Federal University of Lafia, Nasarawa State, Nigeria.*
[c] *Department of Medical Microbiology, Federal University of Lafia, Nasarawa State, Nigeria.*

Abstract

Urinary tract infections (UTIs) represent a recurring clinical challenge, primarily due to escalating pathogen resistance. Although *Proteus* species are not the most commonly isolated pathogens in UTIs, they typically exhibit high levels of resistance when present. This study investigated the proteomic profiles of resistance and virulence determinants in *Proteus* species isolated from women with UTIs in Nasarawa State, Nigeria. Urine samples were collected from 368 patients, from which *Proteus* isolates were identified and evaluated for resistance and virulence factors. Proteomic characteristics were determined following growth in Luria–Bertani (LB) broth and artificial urine (AU). Ten *Proteus* isolates, comprising four *P. vulgaris* and six *P. mirabilis*, were obtained from 21 urine samples exhibiting significant bacteriuria. Both species were found to harbor adhesion (20 kb) and hemolysin (30 kb and 50 kb) plasmids. Identified resistance elements included an ESBL plasmid (40 kb and 80 kb), a conjugative R-plasmid (50 kb), and a multidrug conjugative plasmid (200 kb). The outer membrane vesicle (OMV) yield ranged from 89.5–137μg/mL for *P. vulgaris* and 97.6–140μg/mL for *P. mirabilis*. The OMVs exhibited a unimodal size distribution, with average diameters ranging from 70 nm to 180 nm. Recovered outer membrane-associated proteins included outer membrane protein A (OmpA), porins (OmpC and OmpF), flagellin structural proteins (FliC and FlgE), lipoproteins (Lpp and Pal), and proteases (DegP and HtrA). Notably, *P. mirabilis* cultured in AU was enriched with virulence-related proteins, such as urease subunits (UreA, UreB, and UreC) and fimbrial adhesion proteins. This study confirms the complex nature of virulence and resistance factors in *Proteus* species, which significantly contribute to treatment failure in patients. 0.45μm

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1. Introduction

Urinary tract infections (UTIs) are more common in women than in men. These bacterial infections remain a major cause of illness across all age groups [1]. More than 50 percent of women will experience an episode of the infection in their lifetime. The anatomy of the female reproductive system, hormonal fluctuations, pregnancy, and sexual activity are responsible for the high rate of UTIs among women [2]. In Nigeria, the burden of UTIs is high due to limited access to healthcare, poverty, overcrowding, and widespread misuse of antibiotics [3]. Urinary tract infections are one of the leading reasons women of reproductive age visit

*Corresponding author Tel. No.: +234-806-786-2119.
Email address: orolekayode@gmail.com (O. O. Orole)

hospitals and labs for testing. The condition causes discomfort and can lead to complications such as pyelonephritis, kidney damage, miscarriage in pregnant women, and sometimes sepsis if not properly treated. Management in many healthcare facilities is inadequate due to limited diagnostic capacity and financial constraints. This leads to inappropriate antibiotic use and the development of resistant uropathogens.

Among the microorganisms involved in UTIs are *Proteus* species, alongside *Escherichia coli*, *Klebsiella* species, *Staphylococcus saprophyticus*, and *Enterococcus* species. These Gram-negative bacilli are opportunistic and are commonly found in complicated UTIs, catheter-associated UTIs, and recurrent infections. *Proteus* species produce urease, an enzyme that hydrolyzes urea into ammonia, which encourages the formation of struvite and apatite stones. The survival of *Proteus* species in the urinary tract is supported by swarming motility, fimbriae, hemolysin production, and biofilm formation. These traits contribute to their pathogenic potential and ability to evade host immune defenses. Recently, increasing resistance among *Proteus* species to commonly used antibiotics has raised significant clinical concerns [4].

Conventional culturing of microorganisms and susceptibility testing against antimicrobials offer limited insight into antimicrobial resistance. While these methods can identify bacteria and effective antibiotics, they cannot explain how or why resistance develops at the molecular level. An approach that reveals the mechanisms of bacterial resistance and virulence is crucial for addressing the challenge of antibiotic resistance. Proteomics involves identifying and characterizing proteins expressed by an organism under specific environmental or physiological conditions. Since proteins are functional molecules responsible for cellular processes, proteomic analysis provides insights into metabolism, virulence, stress responses, and resistance mechanisms in bacterial cells.

Studies focusing on the proteomic characteristics of uropathogens in Nigeria are scarce. In Nasarawa State, research on UTIs has focused primarily on prevalence, risk factors, and antibiotic susceptibility patterns [3, 5, 6]. This study aimed to determine the proteomic characteristics of resistant *Proteus* species among women with UTIs in Nasarawa State. Knowledge of protein expression in bacteria could inform the development of therapies targeting proteins involved in resistance, aligning with global and national efforts to combat pathogen resistance.

2. Materials and methods

2.1. Study location

The study was carried out at the Federal University Teaching Hospital in Lafia, Nasarawa State, Nigeria. The state is situated at latitude 8.53895 and longitude 7.7082112, in Nigeria's North Central region. As of 2016, Nasarawa State had an estimated population of approximately 2.5 million.

2.2. Study Population and Participation Criteria

Participants were women diagnosed with urinary tract infections, aged 18–65 years. Only those who provided informed consent participated in the study. Exclusion criteria included individuals who had used antibiotics within the past two weeks and patients with underlying chronic conditions. Ethical approval (NHREC Protocol No. 18/06/2017) was obtained from the Nasarawa State Ministry of Health.

2.3. Sample Collection

Midstream clean-catch urine samples were collected from 368 women. The sample size was determined using Naing's [7] formula, $Z^2P(1-P)/d^2$, based on the prevalence (39.8%) reported by Nwankwo *et al.* [8].

2.4. Isolation of Bacteria

Bacterial isolation was performed using Cystine Lactose Electrolyte Deficient (CLED) and Chocolate agar plates. Inoculated plates were incubated at 37 °C for 24 h; chocolate agar plates were incubated at the same temperature under anaerobic conditions in a candle jar. Samples exhibiting significant bacteriuria ($\geq 1 \times 10^5$ CFU/mL) were selected for further analysis [9].

2.5. Identification of Isolates

Colonial characteristics and morphology were recorded, followed by Gram staining of the isolates. Biochemical characterization, including citrate utilization, urease production, indole test, hydrogen sulphide production, and motility testing, was performed to identify the isolates.

2.6. Molecular identification of Proteus species

The DNA was extracted from an overnight broth culture following the methods described by Hiett *et al.* [10]. Approximately 1 mL of the isolate in Luria-Bertani (LB) broth was centrifuged at 14,000 rpm for 3 min. The supernatant was decanted, and the pellet was suspended in 1 mL of distilled water in a 1.5 mL centrifuge tube and centrifuged again at 14,000 rpm for 10 min. This process was repeated after suspending the pellet in 100μL of sterile distilled water. The pellet was subsequently suspended in 500μL of normal saline and heated at 95 °C for 20 min. The suspension was cooled on ice for 10 min and centrifuged at 14,000 rpm for 3 min. The supernatant containing DNA fragments was transferred to a 1.5 mL tube and stored at −20 °C. The PCR mixture consisted of 4μL Master mix, 2μL forward primer, 2μL reverse primer, 2μL DNA template, and 15μL DNase-free water (Zymo Research, USA).

For the identification of *Proteus*, universal 16S rRNA primers were utilized, while *ureR*, *ureaseC*, and *pureR* genes were targeted for identification to the species level. The primer sequences used were: 16S rRNA (27F: AGAGTTTGATCCTGGCTCAG; 1492R: GGTTACCTTGTTACGACTT); *ureR* (F: GCAAATTGAGTGACTTTGGCTGGACC; R: GGTGAGATTTGTATTAATGG); *ureaseC* (F: CGCTTTGCGATGGCAAGTACAAGTAAG; R: GCAAATTGAGTGACTTTGGCTGGACC); and *pureR* (F: GCGTG-GAGTGATTTGTGGTTA; R: TTGAGGAGCCGTAGAGTGAA). Amplification was performed with an initial denaturation at 95 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 52 °C for 30 s, and extension at 72 °C for 85 s, with a final extension at 72 °C for 10 min.

2.7. Proteomics analysis

2.8. Bacterial culture conditions

The confirmed *Proteus vulgaris* (P1, P8, and P10) and *P. mirabilis* (P3, P5, P6, P7, P8, and P9) were stored in glycerol at -80 °C. They were grown on Luria–Bertani (LB) agar at 37 °C before proteomic analysis [11].

2.9. Isolation and purification of the bacterial outer membrane vesicles (OMVs)

To isolate and purify outer membrane vesicles (OMVs), the isolates were cultured separately in LB broth and artificial urine (AU), with the pH adjusted to 6.2. The AU medium was sterilized by filtration through a 0.45μm Millipore membrane. Cultures (100 mL) were centrifuged at 6,000 $\times g$ for 15 min at 4 °C, and the resulting supernatants were filtered through 0.45μm and 0.20μm membranes to remove remaining cellular debris.

The filtrate was subsequently ultracentrifuged at 100,000$\times g$ for 2 h at 4 °C (90 Ti rotor, Beckman). The OMV-containing pellets were resuspended in 300μL of HEPES–NaCl buffer (50 mM HEPES, 0.85% NaCl). For purification, crude OMVs were adjusted to 1 mL of 45% (w/v) iodixanol (OptiPrep™, Sigma) in HEPES–NaCl and placed at the bottom of ultracentrifuge tubes. Density gradients were established by layering 2 mL each of 40%, 35%, 30%, 25%, and 20% iodixanol solutions.

2.10. Characterization of OMVs

The OMVs for each bacterium were characterized using a Zetasizer (ZS) instrument (Malvern Instruments) equipped with a He–Ne laser (633 nm) at a fixed scattering angle of 175° [12]. The instrument measured the hydrodynamic radius, polydispersity index (PdI), and zeta potential of the OMVs. The OMV samples were diluted in Milli-Q water and analyzed in DTS1070 cuvettes.

OMV protein content was analyzed using SDS–PAGE. Protein concentrations were determined through densitometric analysis of Coomassie-stained gels. Samples were loaded onto 4–12% Bis–Tris precast gels (NuPAGE™, Invitrogen) with an LMW-SDS molecular weight marker (Cytiva) and run at 150 V. After electrophoresis, gels were fixed in 50% ethanol and 10% acetic acid, stained overnight with colloidal Coomassie blue, and destained with ultrapure water. Gel images were captured with a UMAX Power-Look 1120 scanner and LabScan 5.0 software (GE Healthcare). Protein band intensities were quantified using ImageQuant TL software (v8.1).

2.11. Preparation of OMV proteins for mass spectrometry

Analysis of the OMVs obtained from the nine *P. mirabilis* and *P. vulgaris* isolates cultured in LB and AU media was performed using three biological replicates. For each sample, 25μL of OMVs was resolved on 12.5% acrylamide gels until the proteins migrated approximately 1 cm into the resolving gel. The corresponding gel region was excised, destained, and processed for in-gel digestion.

The protein samples were reduced with 10 mM dithiothreitol (DTT) at 56 °C for 1 h, alkylated with 50 mM iodoacetamide at room temperature for 45 min in the dark, and digested overnight at 37 °C with sequencing-grade trypsin (Promega). Peptides were extracted with 60% acetonitrile containing 0.1% trifluoroacetic acid (TFA), vacuum dried, and resuspended in 0.1% TFA. The peptides were desalted using C18 OMIX pipette tips (Agilent) and eluted with acetonitrile containing 0.1% formic acid. Eluates were vacuum dried, resuspended in 0.1% formic acid, and normalized based on peptide concentration determined by absorbance at 215 nm using a Denovix DS-11 FX+ spectrophotometer/fluorometer.

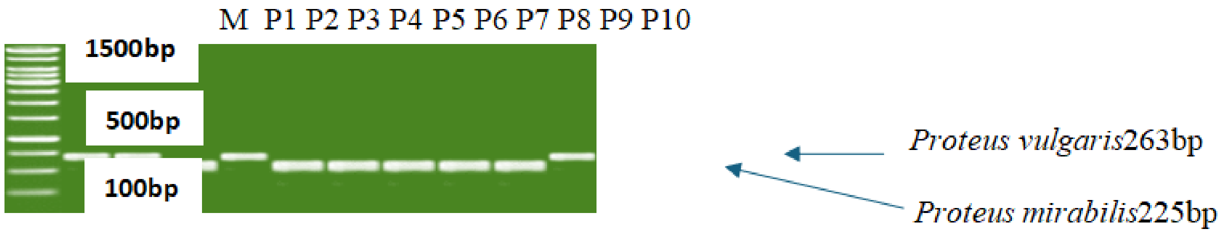

Figure 1: Plate 1: Agarose gel electrophoresis of the amplified *Proteusmirabilis* and P. *vulgaris*. Lane M - 1500bp DNA molecular ladder; Lanes P1, P2, P4, and P10 - expression urease C for P. *vulgaris* (263bp)gene; Lanes P3, P5, P6, P7, P8 and P9 - expression ureR for P. *mirabilis* (225bp) gene.

3. Results

3.1. Significant bacteriuria and isolated bacterial species

A total of 21 women had significant bacteriuria in their urine samples. Eight of the 368 women had urine colony-forming units > 20.0×10^5 CFU/mL. From the 21 women with significant bacteriuria, nine bacterial species were isolated. *Escherichia coli* was the most prevalent species, accounting for 66.7% of isolates. Other isolated species were *Klebsiella* sp. (57.1%), *Proteus* sp. (42.9%), *Enterobacter* sp. (28.6%), *Pseudomonas aeruginosa* (38.1%), *Enterococcus faecalis* (23.8%), *Staphylococcus aureus* (23.8%), *Corynebacterium* sp. (4.8%), and *Streptococcus agalactiae* (14.3%). Only ten (10) patients had *Proteus* species isolated in their mid-stream urine samples in the study.

The *Proteus* species isolated were identified as *P. mirabilis* (Isolates P3, P5, P6, P7, P8, and P9), while isolates P1, P2, P4, and P10 were identified as *P. vulgaris* (Plate 1).

3.2. Outer membrane vesicle isolation and yield

OMVs were isolated from nine *Proteus* isolates cultured in LB broth and AU. Isolate P2 was not productive and was discarded. Each isolate produced a visible, translucent, light-brown pellet after ultracentrifugation, confirming the recovery of OMVs. Bradford protein assay showed variable OMV yields among isolates (Table 1).

Comparison of OMV yields within isolates showed that LB medium resulted in significantly higher protein concentrations than AU medium (mean difference = 29.9μg/mL, 95% CI: 27.9–31.9μg/mL; $p < 0.0001$). This pattern was consistent across all nine isolates examined. Between species, *P. mirabilis* produced slightly more OMVs than *P. vulgaris* under both conditions, with differences of approximately 8–9μg/mL; however, this difference was not statistically significant ($p > 0.05$). The standard deviations across measurements were low (±2.9–5.6μg/mL), indicating high reproducibility and minimal experimental variability. The isolates included *P. mirabilis* (Isolates P3, P5, P6, P7, P8, and P9) and *P. vulgaris* (Isolates P1, P4, and P10).

3.3. Dynamic light scattering (DLS) and zeta potential

The OMVs exhibit a unimodal size distribution, as determined by DLS analysis. The average diameters ranged from 70 nm to 180 nm (Figure 2). OMVs from AU cultures exhibited a lower mean value of 105 ± 12 nm compared to the LB cultures at 121 ± 15 nm. The polydispersity index (PdI) values (0.21–0.28) showed moderate sample uniformity. The zeta potential ranged between −25 mV and −35 mV, reflecting the negatively charged vesicle surfaces characteristic of Gram-negative bacterial OMVs, as shown in Figure 3.

3.4. Nanoparticle tracking analysis

Nanoparticle Tracking Analysis confirmed the presence of abundant nanoparticles consistent with OMVs, with concentrations between 1.8×10^9 and 4.2×10^9 particles/mL. The average particle diameter was consistent with DLS results, which showed a population between 80 nm and 150 nm. Isolates P3 and P8 of *P. mirabilis* produced the highest vesicle concentrations under both growth conditions.

3.5. SDS–PAGE protein profiling analysis

SDS–PAGE analysis of OMV preparations revealed distinct protein banding patterns among isolates under the culture conditions used. Protein bands were observed at 35–40 kDa, 60 kDa, and 90 kDa, respectively (Figure 4). OMVs derived from the AU medium had fewer high molecular weight proteins than those from the LB broth. Densitometric quantification revealed higher overall protein intensity in P. *mirabilis* isolates than in P. *vulgaris*. Nano-LC–MS/MS analysis identified a total of 1,328 non-redundant proteins in all nine isolates. Of these, 742 proteins were common to both P. *vulgaris* and P. *mirabilis* species, 298 were unique to P. mirabilis,

Table 1: Proteus isolates *Protein* yield of OMVs under LB and AU conditions.

Isolate ID	Species	Culture Medium	OMV Yield (μg/mL ± SD)	*t*-value	*p*-value	Significance
P1	*P. vulgaris*	LB AU	125.3 ± 4.2 94.7 ± 5.6	7.54	0.0016	**
P4	*P. vulgaris*	LB AU	137.8 ± 3.8 110.2 ± 4.1	9.18	0.0008	***
P10	*P. vulgaris*	LB AU	120.1 ± 5.0 89.5 ± 3.7	8.27	0.0012	**
P3	*P. mirabilis*	LB AU	142.5 ± 2.9 115.8 ± 4.3	9.12	0.0008	***
P5	*P. mirabilis*	LB AU	131.4 ± 4.8 103.2 ± 5.0	7.22	0.0019	**
P6	*P. mirabilis*	LB AU	128.9 ± 3.5 97.6 ± 4.4	9.64	0.0007	***
P7	*P. mirabilis*	LB AU	138.1 ± 4.1 108.7 ± 3.8	10.11	0.0006	***
P8	*P. mirabilis*	LB AU	140.7 ± 4.6 111.3 ± 4.2	9.49	0.0007	***
P9	*P. mirabilis*	LB AU	135.9 ± 3.9 100.5 ± 5.1	8.01	0.0013	**

$Key : *** \longrightarrow p < 0.001 (LB vs AU within isolate)$

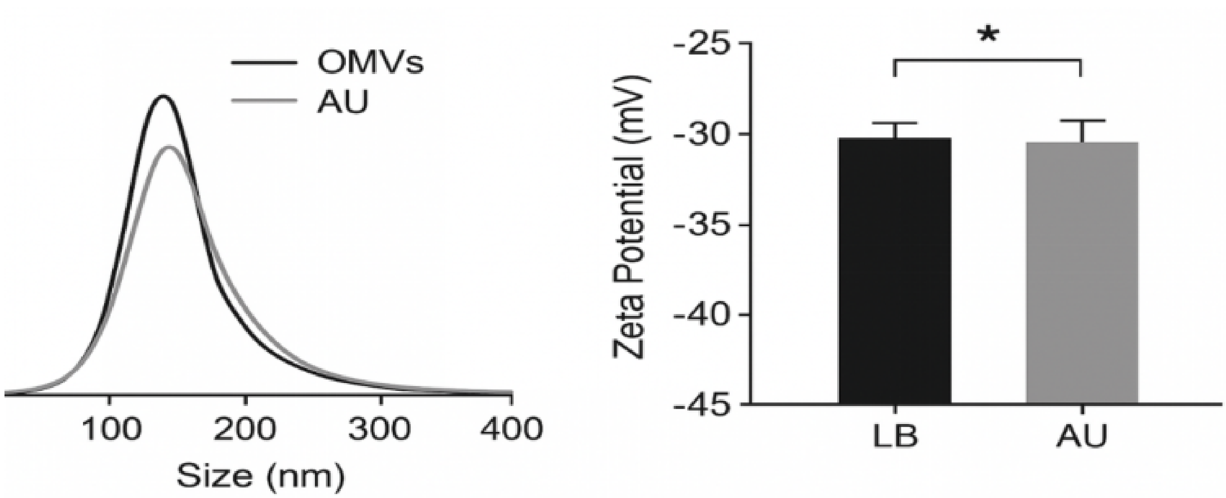

Figure 2: Average zeta potential comparison of OMV from both media.

and 288 were unique to P. *vulgaris*. Based on PSORTb subcellular localization predictions, approximately 42% of the proteins were of outer membrane origin, 24% cytoplasmic, 18% periplasmic, and 16% predicted to be extracellular, as shown in Figure 5.

The proteins identified in the outer membrane included outer membrane protein A (OmpA), porins (OmpC, OmpF), structural flagellins (FliC, FlgE), lipoproteins (Lpp, Pal), and proteases (DegP, HtrA). In *P. mirabilis*, OMVs from AU cultures were enriched with virulence-related proteins such as urease subunits (UreA, UreB, UreC) and fimbrial adhesion proteins, suggesting adaptive modulation of vesicle composition in response to the urine environment.

4. Discussion

Bacterial counts in urine samples with significant bacteriuria ranged from 3.7×10^5 CFU/mL to 28.8×10^5 CFU/mL. The high counts indicated the severity of infection and the patients' susceptibility. Baimakhanova *et al.* [13] and Bhugra and Gachinmath [14] explained that the time of urine collection, pathogen virulence, and the onset of infection could determine the recorded counts. This points to the progression of infection in the women and possible late diagnosis. Plasmid profiling of the isolates revealed the co-occurrence of virulence and antimicrobial resistance plasmids on *Proteus* DNAs. The 20 kb adhesion plasmid was the most common

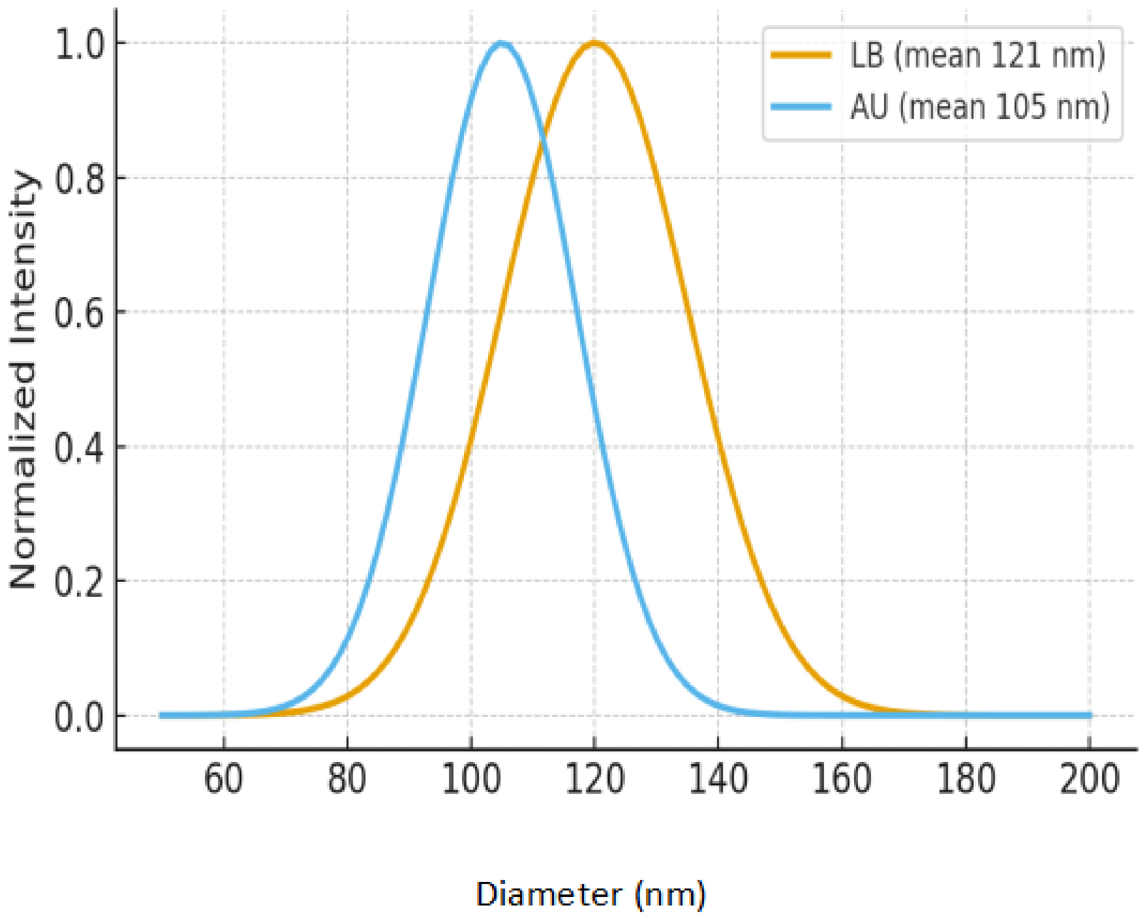

Figure 3: Size distribution of OMVs.

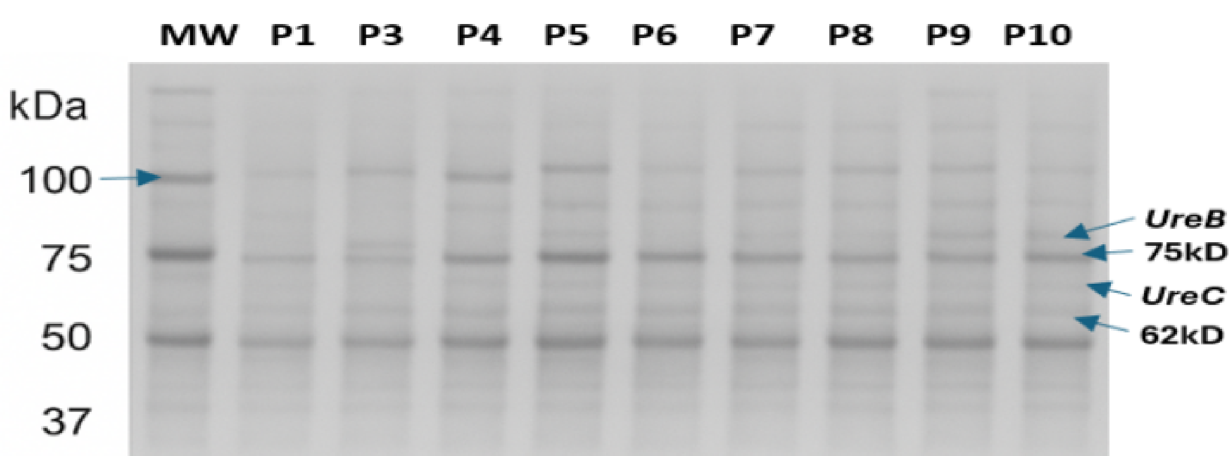

Figure 4: Putative protein profiles(UreB and UreC) of OMVs from P. *mirabilis* isolates compared with P. *vulgaris* in LB and AU run in 12% SDS-PAGE. Molecular weight standard with corresponding kDa indicated on the left.

virulence plasmid that aids adherence to host uroepithelial cell surfaces [15, 16]. Hemolysin plasmids of 30 kb and 50 kb [15] are associated with cytotoxic activity and promote host cell lysis, tissue damage, and the development of complicated or recurrent infections [1, 17]. Some plasmids, such as the 200 kb multidrug plasmid, are known to spread resistance horizontally across species

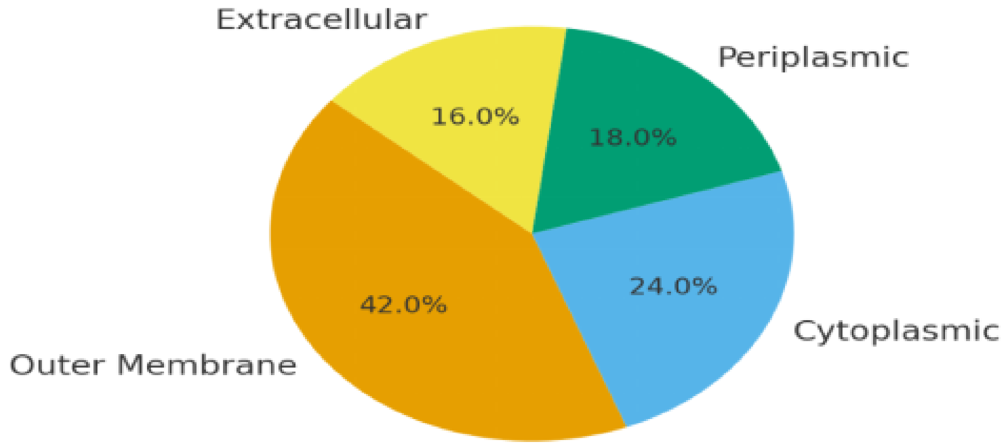

Figure 5: The predicted subcellular localization of OMV-associated proteins.

and to accelerate the transfer of antimicrobial resistance to multiple antibiotic classes in the environment [18, 19].

The AU and LB culture media affected the yield of the outer membrane vesicle (OMV) protein obtained from isolates [20, 21]. LB produced higher yields compared to the AU medium. More than 20% of the OMV protein concentration was recovered in the LB medium, indicating higher bacterial growth. Higher growth rate results from increased metabolism, leading to more outer membrane components that drive OMV biogenesis [22]. AU medium yields limited protein synthesis in the bacteria, resulting in reduced OMV yield. The OMV protein yield was species-specific, with *P. mirabilis* isolates producing more protein than *P. vulgaris* [23, 24]. This might be due to their outer membrane structure, metabolic pathways, or vesiculation. Another reason is their virulence factors, which are associated with increased protein production [25]. The average zeta potential of the OMVs from the *Proteus* isolates was negative, indicating a net negative surface charge. The zeta potential is a measure of OMV stability, aggregation behavior, and interactions with the environment [26, 27]. Environmental stress is a known factor influencing phosphorylation and acylation of the lipopolysaccharide (LPS) layer of the cell wall. The state of the LPS affects its surface charge and electrostatic interactions [28]. This mechanism is protective, aiding bacterial survival under stress conditions.

The functional capacity of the cell is also influenced by OMV size. Larger vesicles are more stable because they carry greater protein loads; however, this is a disadvantage compared to smaller OMVs, which can easily enter host cells to deliver virulence factors or signaling molecules [29]. In our study, OMV proteins were derived from the outer membrane and the periplasm. This aligns with Schwechheimer and Kuehn [22], who reported that vesicles bud from the outer membrane and encapsulate periplasmic components during scission. Although OMVs originate from the outer membrane, cytoplasmic and inner membrane proteins may be incorporated during membrane disturbances, envelope stress, or cell lysis [30]. Finding these proteins in the cytoplasm shows that OMV selection is not entirely passive. Additionally, the outer membrane localization, central to ESBL-associated plasmids, was detected in nine isolates. OMV-mediated export of ESBLs has been shown to protect both producer cells and nearby bacteria by degrading β-lactam antibiotics outside the cell [22, 30].

The presence of periplasmic proteins in OMVs is particularly relevant to antimicrobial resistance. β-lactamases encoded on ESBL plasmids are localized to the periplasm, where they intercept antibiotics before they reach their targets [31]. Their incorporation into OMVs extends enzymatic activity beyond the bacterial envelope [32, 33]. This creates a community wall of resistance in the urinary tract, suggesting that stress may promote the selective export of resistance-associated periplasmic proteins. Their incorporation into OMVs suggests a potential role for vesicles in facilitating horizontal gene transfer and enhancing multidrug resistance [22]. Isolation of P4 (*P. vulgaris*) harboring the adhesion, hemolysin, and ESBL plasmids simultaneously represents a convergence of multiple virulence and resistance determinants. This mirrors the heterogeneous localization of OMV-associated proteins and supports the concept that OMVs function as multifunctional platforms that integrate virulence and resistance factors within a single secretory system.

5. Conclusion

Proteus species are distinctive UTI pathogens with advanced virulence and resistance. The OMV analyses confirmed that the bacterium possesses pathogenic attributes that enhance its colonization and survival on uroepithelial surfaces, as well as its resistance to commonly administered antibiotics. The study provides molecular evidence that the observed pathogen characteristics are innate and that UTIs caused by *Proteus* species are becoming increasingly complex and challenging to treat. These findings emphasize the need to improve diagnostic measures in hospitals and enhance public health hygiene to improve health outcomes among women.

Data availability

Data will be made available upon reasonable request from the corresponding author.

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