Genetic Profiling of *Raoultella* spp. in Pediatric Clinical Samples: Insights from Bauchi, North East Nigeria

ABSTRACT

Background: *Raoultella* species, emerging pathogens are increasingly linked to pediatric and neonatal infections in clinical settings.
Aim: isolation and accurate identification of *Raoultella* species from pediatric clinical samples to address challenges in identification and genetic characterization.
Study design: This is a prospective, cross-sectional study involving pediatrics aged 0-5 years.
Place and Duration of Study: Pioneering study in Bauchi, North-East Nigeria, from August 2021 to January 2022.
Methodology: Samples from 262 pediatric patients with septicemia symptoms were processed at Abubakar Tafawa Balewa University Teaching Hospital (ATBUTH). The VITEK 2 Compact system was used for initial identification, while PCR amplification of the 16S rRNA gene confirmed *Raoultella* species. The genomic DNA of identified isolates underwent sequence and bioinformatics analysis.
Results: The phylogenetic tree constructed using the 16S rRNA sequences of the isolates and reference strains visually represent the genetic relationships. The high homology observed in the sequence alignment is reflected in the phylogenetic clustering, with isolates RoP_2 (PQ213811), RoP_3 (PQ213812), and RoP_4 (PQ213813) grouping closely with known *Raoultella planticola* strains. This close genetic relationship further supports the species identification suggested by the gel electrophoresis and homology data. However, RoP_1 (PQ213810), identified as *Proteus mirabilis* through BLAST analysis of its 16S rRNA sequence, shows slightly lower homology with *Raoultella planticola* reference strains. In the phylogenetic tree, RoP_1 is positioned slightly apart from the other isolates, indicating some genetic variability. This discrepancy suggests that while RoP_1 shares characteristics with *Raoultella*, the distinct genetic makeup warrants further investigation to confirm its identification as *R. planticola* or otherwise.
Conclusion: The phylogenetic analysis supports the identification of isolates RoP_2, RoP_3, and RoP_4 as *Raoultella planticola*, showing close genetic relationships. However, RoP_1 exhibits genetic variability, clustering separately and aligning more closely with *Proteus mirabilis*, suggesting the need for further investigation to confirm its species identification.

Keywords: *Raoultella* spp., Pediatric infections, Molecular diagnostics, 16S rRNA gene, Vitek 2

1. INTRODUCTION

Raoultella spp. is Gram-negative, non-motile, encapsulated bacteria commonly found in aquatic environments and soil. However, the increasing rate of isolation in clinical settings raises concerns due to intrinsic resistance mechanisms, particularly to beta-lactam antibiotics. In recent years, the identification and study of these organisms in pediatrics have become crucial in understanding the clinical implications [1]. *Raoultella* spp. of the Enterobacteriaceae family, naturally occurring environmental organisms are recently been recognized as emerging pathogens in serious pediatric infections, potentially leading to multiple organ failure [2]. *Raoultella* spp. is frequently linked to severe infections and shows a tendency for multidrug resistance, complicating treatment strategies however, misidentification of these species using conventional methods remains a significant diagnostic challenge [3, 4].In recent years, there has been an increasing recognition of *Raoultella* spp. as significant contributors to pediatric infections, particularly in regions with limited

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healthcare resources. *Raoultella* species, previously classified under the *Klebsiella* genus, are emerging as significant nosocomial pathogens with the ability to cause a wide range of infections, including pneumonia, urinary tract infections and septicemia in neonates and young children [5, 6, and 7]. Accurate identification and differentiation of these species is crucial for effective management. Genetic profiling using 16S rRNA gene analysis offers a robust method for differentiating *Raoultella* spp. strains and understanding the epidemiological and resistance profiles [5, 8, and 9].

In Bauchi, North East Nigeria, the prevalence and genetic characteristics of *Raoultella* spp. in pediatric clinical samples remains underexplored. This study aims to isolate and adequately identify *Raoultella* species from pediatric clinical samples in the study population.

2. MATERIAL AND METHODS

2.1 Study design

Pediatric patients exhibiting suspected symptoms of scepticaemia within Bauchi metropolis, North-east Nigeria were selected for this study following a simple random sampling method. A total of two hundred and sixty two (262) were sampled between August and December 2021. The study was conducted in Tertiary and State owned hospitals chosen based on representation of diverse paediatric populations and geographical distribution. Samples were collected from four hospitals to include: Abubakar Tafawa Balewa University Teaching Hospital, Bauchi (ATBUTH); Bauchi State Specialist Hospital, Bauchi; Women and Children Hospital, Railway, Bauchi and Yelwa Domiciliary Hospital, Bauchi. All samples were conveyed to ATBUTH, Bacteriological Laboratory tests were carried out in the Microbiology laboratory after which isolated bacteria were taken to Molecular Genetics and Infectious Disease Research Laboratory (MOGID), ATBUTH for the molecular analysis. Febrile patients and suspected cases of septicemia who sought medical attention during the research period were included.

2.2 Blood Sample Processing

From each patient, 2 ml of intravenous blood was collected using a sterile syringe. The blood samples were immediately inoculated into BactAlert culture bottles and incubated using the BactAlert system for 24-48 hours. Samples that tested positive for bacterial growth were subsequently sub-cultured on blood agar plates to isolate pure colonies.

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2.3 Processing urine samples

The culture media used for isolation was Cystein-Lactose Electrolyte-Deficient (CLED) (Difco Co, USA) and chocolate agar plates. Each urine sample was inoculated using heat-flamed standard wire loop which was dipped directly to the bottom and streaked on to the agar plates. The plates were incubated aerobically at 37°C for 24hrs and then examined. The cultural and morphological characteristics of distinct and isolated colonies were studied. Distinct and isolated colonies from each significant growth were Gram stained and viewed under a light microscope to ascertain the gram status of isolates.

2.4 Rectal swab Sample Processing

Rectal swab samples were cultured on Salmonella-Shigella (SS) agar for the isolation of enteric pathogens. Following incubation, bacterial colonies were examined based on their phenotypic characteristics.

2.5 Bacterial Identification

Isolated bacterial colonies from all samples were identified with gram stain and *Klebsiella* like isolates were subjected to VITEK 2 Compact system (bioMérieux, France) for precise identification based on biochemical profiles.

2.6 Molecular Analysis

The genomic DNA of isolates identified as *Raoultella* species on VITEK 2 was extracted using the Quick-DNA™ Bacterial Miniprep Kit according to the manufacturer's protocol and used to confirm the organism identification on PCR. The species-specific primers for PCR were used for the accurate identification of *Raoultella* spp. in the clinical isolates, ensuring that the organism was correctly characterized.

2.7 Species-Specific Primer Design

Primers were specifically designed to target unique genetic sequences within the *Raoultella* genome. These sequences are distinct from other related genera, allowing for precise identification of *Raoultella* spp. The primers used are as in Table 1.

Table 1: Primers used for Polymerase chain reaction in identification of *Raoultella* spp.

Gene Target	Primer sequence (5'-3')	Amplicon Size (bp)	Reference
R. ornithinolytica	F- TCG GTC TCC ATG CCT TCA TAG	2135	This study

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	R- CGA TCT GAT TCC GGA CGT CAT		
R. planticola	F- TAG CTG GTC TGA GAG GAT GAC C	1058	This study
	R- GCG ATT CCG ACT TCA TGG AGT C		
R. terrigena	F- GAC TCC ATC TAT ATG CGC GGT	1666	This study
	R-GAT ACT GGC TCC AGA CGG TAA		

2.8 PCR Amplification

PCR amplification was performed using a PCRMax thermal cycler with the cycling conditions optimized for *Raoultella* spp. the PCR conditions were as shown on the table 2.

Table 2: optimized conditions for Polymerase chain reaction used in amplification of *Raoultella* spp.

Step	Temperature (^{0}C)	Time (Sec)	Step
Activation	94	30	Activation
Denaturation	94	15	Denaturation
Anealing	57	30	Anealing
Extension	68	30	Extension
Final Extension	68	180	Final Extension

2.9 Gel Electrophoresis

The PCR products were resolved using gel electrophoresis, 0.8% agarose gel stained with ethidium bromide to visualize the amplified DNA fragments. The expected product size was compared against a 1kb DNA ladder to confirm the presence of *Raoultella* spp.

2.10 Bioinformatics Analysis

The PCR product was further sequenced and data subjected to bioinformatics analyses using Geneious 9.50 software for multiple sequence alignments and phylogenetic tree construction to explore the genetic relationship among *Raoultella spp* isolates as well as percentage homology of isolates with existing reference strains.

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3. RESULTS AND DISCUSSION

The integrated analysis of agarose gel electrophoresis, homology data and phylogenetic analysis provides a comprehensive understanding of the identification and genetic characterization of *Raoultella* species in this study.

The data on table 3 presents *Raoultella ornithinolytica* strains isolated from pediatric patients, detailing the sources of isolation, patient demographics and identification probabilities using the VITEK 2 automated system. The isolates were obtained from urine, rectal swab, and blood samples, indicating the presence organism in different clinical specimens commonly associated with infections [9]. The patients range in age from neonates (≤28 days) to toddlers (13-24 months), demonstrating ability of the organism to affect a wide range of young children, with both male and female patients represented. The probability of identification by the VITEK 2 system ranges from 78% to 90%, reflecting a relatively high level of confidence in the identification of *R. ornithinolytica*, though the variation suggests potential challenges in phenotypic identification, possibly due to atypical strains or the complex nature of the bacterium. The identification of *R. ornithinolytica* in pediatric patients, particularly in neonates, is significant due to the organism potentials to cause serious infections like urinary tract infections and bacteremia, especially in vulnerable populations agreeing with reports of Chen *et al*, 2020 that R. planticola are considered a potential pathogen. The data emphasizes the importance of accurate identification and suggests that while VITEK 2 is a valuable tool, supplementary methods may be necessary to confirm the presence of this pathogen, especially in critical cases like neonatal sepsis. The in-efficiency of current phenotypic and conventional methods to detect *Raoultella species* suggests that the incidence of these organisms may be underestimated. More so, the difficulties in identification may have led to an underestimation of its incidence world over. Several Scholars have reported that conventional phenotypic identification methods hardly distinguished *Raoultella spp* from *Klebsiella* spp. [11, 12 and 13]. El-Shannat, *et al.* (2020) emphasized that the phenotypic identification methods are not reliable in an attempt to identify *Raoultella ornithinolytica*. In this study however, the conventional phenotypic methods identified the *Raoultella* isolates as *Klebsiella* spp due to the high morphological and phenotypic characteristics shared by organisms of these genera. Subsequently, Vitek 2 Compact system identified the four isolates as *Raoultella ornithinolytica* as shown on table 3 this agrees with reports of Salimiyanrizi and Farsiani. (2022) that the misidentification is thought to result from the genetic similarity between *Raoultella spp.* and *Klebsiella* spp.

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Table 3: Properties of *Raoultella* species Isolated from Paediatric Patients and Identified on Vitek 2 Automated System

Organism	Source	Age Group	Gender	VITEK 2 Prob. of Id. (%)	Designation
R. ornithinolytica	Urine	29D-12M	M	78	RoP_1
R. ornithinolytica	Rectal swab	<=28D	F	90	RoP_2
R. ornithinolytica	Blood	13- 24M	M	87	RoP_3
R. ornithinolytica	Blood	<=28D	M	81	RoP_4

Legend: M: Male; F: Female; D: days; M: months.

The monoplex PCR results, as demonstrated in the agarose gel electrophoresis, revealed clear bands at approximately 1,779 bp (Fig.1) for isolates identified as bacteria. These results align with the expected size of the 16S rRNA gene (Table 1), a universal bacterial marker, confirming the successful amplification and suggesting that the isolates are indeed bacteria as the 16S rRNA gene is highly conserved among bacterial species.The absence of bands in the negative control further validates the specificity of the primers used in the PCR process, indicating no contamination which supports the reliability of the results. This result validates the presence of bacterial DNA in the samples and supports the identification of the isolates as bacterial, providing a crucial step towards further characterization.

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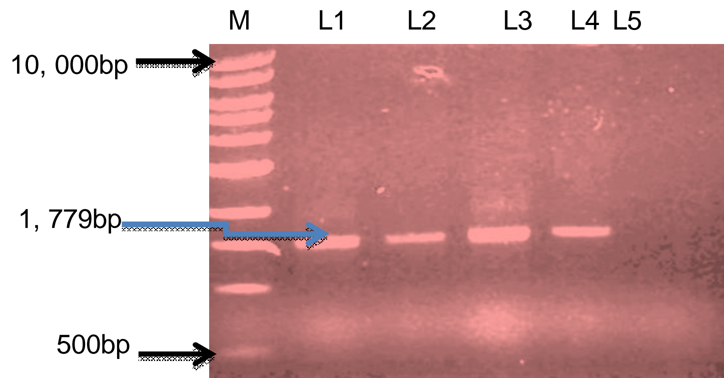

Fig.1: Agarose gel electrophoresis of monoplex PCR amplified products from extracted total DNA bacteria initially identified on VITEK 2 as *R. ornithinolytica*.

M- Marker (1kb); Lane: 1- 4 (isolates) amplified with 16S ribosomal RNA of universal bacteria giving positive results at 1779 bp. L5: Negative control

The electrophoresis was performed at 48 volt for 60 min.

The multiplex PCR conducted to identify *Raoultella* spp showed bands at approximately 1058 bp in the gel (Fig.2), corresponding to the expected size for *R. planticola* 16SrRNA gene. This consistency with the predicted band size indicates that the isolates are *R. planticola*. The high specificity of the assay is reinforced by the negative control, which displayed no bands, confirming that the results are free from non-specific amplification.

M L1 L2 L3 L4 L5

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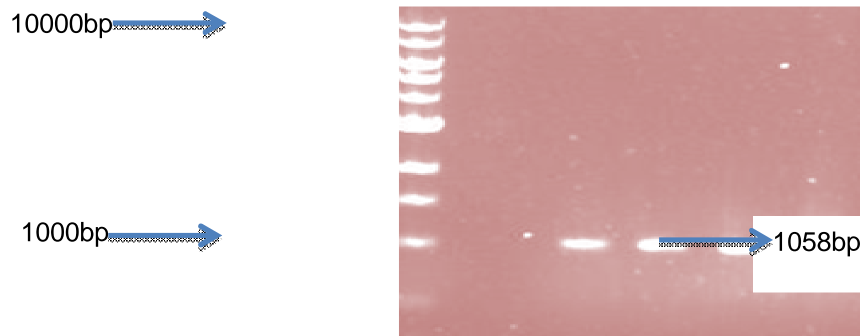

Fig.2: Agarose gel electrophoresis of multiplex PCR amplified products from extracted DNA of Isolates.

M- Marker (1kb); L1: Negative control; Lane: 2- 5 (isolates) amplified with 16S ribosomal RNA of *Raoultella* spp. giving positive results at 1058 bp. for *Raoultella planticola*

The electrophoresis was performed at 48 volt for 60 min.

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Although discrepancy between VITEK 2 and multiplex PCR results highlights the challenges and limitations inherent in bacterial identification methods, Monteiro*et al*, 2016 recommended VITEK 2 to be used for rapid phenotypic identification due to the high probability of identification of >90%.

The homology analysis further corroborates these findings, isolates RoP_2, RoP_3, and RoP_4 exhibited high sequence similarity (95-97%) with multiple reference strains of *Raoultella planticola*, such as strains R6, FYF_35, DD30, and CEMTC_3606. This high level of homology supports the identification of these isolates as *R. planticola*, as initially suggested by the gel electrophoresis results, RoP_1 however, displayed slightly lower homology (90-95%), which indicates some genetic variability or potential sequencing errors which supports Demiray and others reported where Vitek 2® automated system identified the isolates as R. planticola but 16S rRNA sequencing and blast analysis of the same isolate showed that the it was R. terrigena with 92% homology [17]. Similarly, Salimiyan and Farsiani reported that as this method may not efficiently differentiate *Klebsiella* from *Raoultella* genera. The comparison with *Klebsiella planticola*, showed lower homology percentages, emphasizing the genetic distinction between *Raoultella* and *Klebsiella* species, despite the historical taxonomic overlap.

Table4: Homology of Identified *Raoultella planticola* Isolates with Five (5) Reference Strains.

Reference Strain	Homology of Isolate (%)

	RoP_1	RoP_2	RoP_3	RoP_4
Raoultella planticola strain R6 16SrRNA gene, partial sequence	93	96	97	96
Raoultella planticola strain FYF_35 16SrRNA gene, partial sequence	90	96	96	96
Raoultella planticola strain DD30 16SrRNA gene, partial sequence	95	95	95	95
Raoultella planticola strain CEMTC_3606 16SrRNA gene, partial sequence	95	95	95	95
Klebsiella planticola 16SrRNA gene, strain 7444, partial sequence	91	95	95	95

The phylogenetic tree constructed using the 16S rRNA sequences of the isolates and reference strains visually represent the genetic relationships. The high homology observed in the sequence alignment is reflected in the phylogenetic clustering, with isolates RoP_2, RoP_3, and RoP_4 grouping closely with known *Raoultella planticola* strains (Fig.3). This close genetic relationship further supports the species identification suggested by the gel electrophoresis and homology data. However, RoP_1, identified as *Proteus mirabilis* through National Centre for Biotechnology Information (NCBI) using the basic local alignment search tool (BLAST) analysis of 16S rRNA sequence shows slightly lower homology with *Raoultella planticola* reference strains. In the phylogenetic tree, RoP_1 is positioned slightly apart from the other isolates, indicating some genetic variability. This discrepancy suggests that while RoP_1 shares characteristics with *Raoultella*, its distinct genetic makeup warrants further investigation to confirm its identification as *R. planticola*. This highlights the importance of using a combination of molecular and bioinformatics tools to accurately identify bacterial species in clinical settings.

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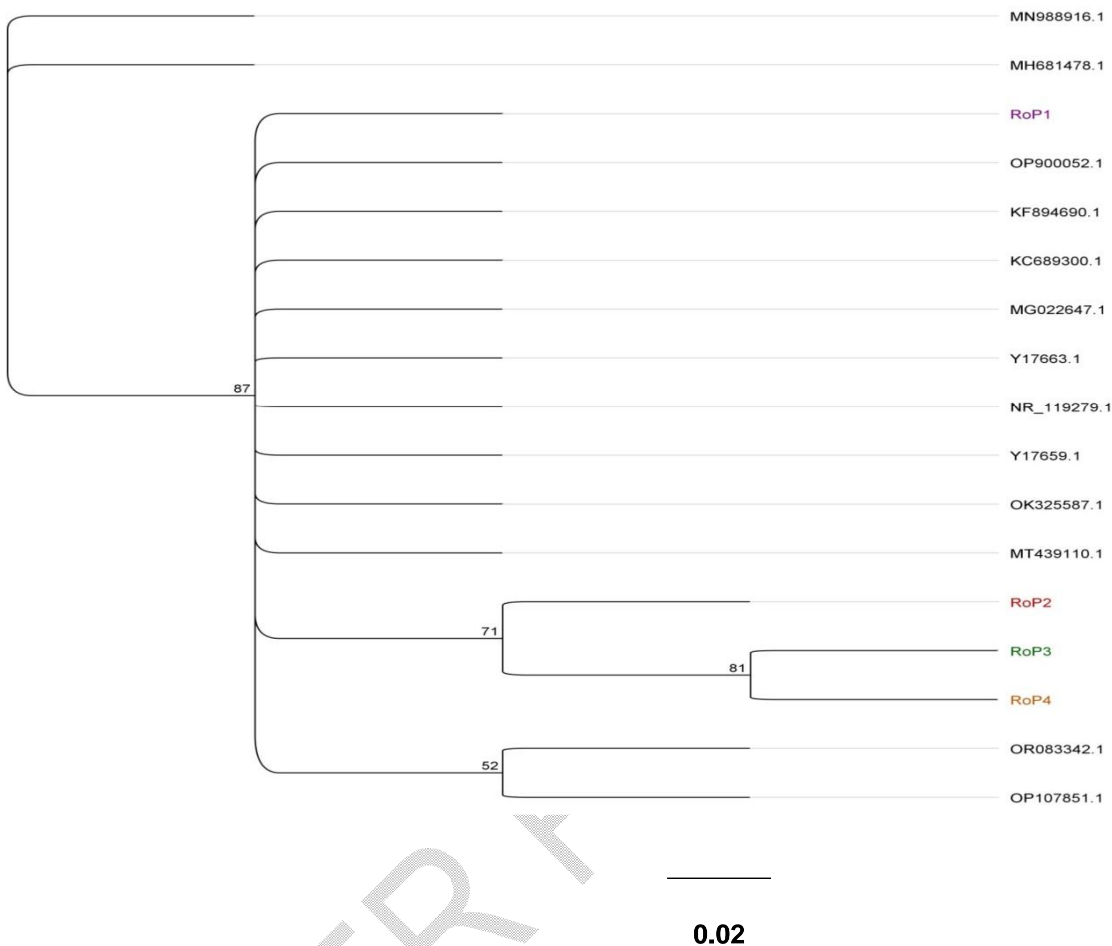

Fig.3: Phylogenetic Analysis of Isolated *Raoultella planticola* Strains for Assessing Genetic Relatedness and Evolutionary Relationships with Reference Strains Retreived from the NCBI GeneBank Database

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Enterobacteriaceae family and provides reliable results in clinical and environmental samples [13, 21]. Moreover, the specificity of PCR assays targeting the 16S rRNA gene has been validated in studies involving both clinical and environmental isolates, reinforcing the accuracy of this molecular approach [5]. In particular, the phylogenetic tree that includes *Raoultella planticola* strain SA2 as a reference strain (Fig.4) shows that the isolates cluster near this strain, confirming identity as *R. planticola*. The evolutionary proximity to strain SA2 suggests that these isolates share a common ancestor, consistent with the high sequence similarity reported in table 4.

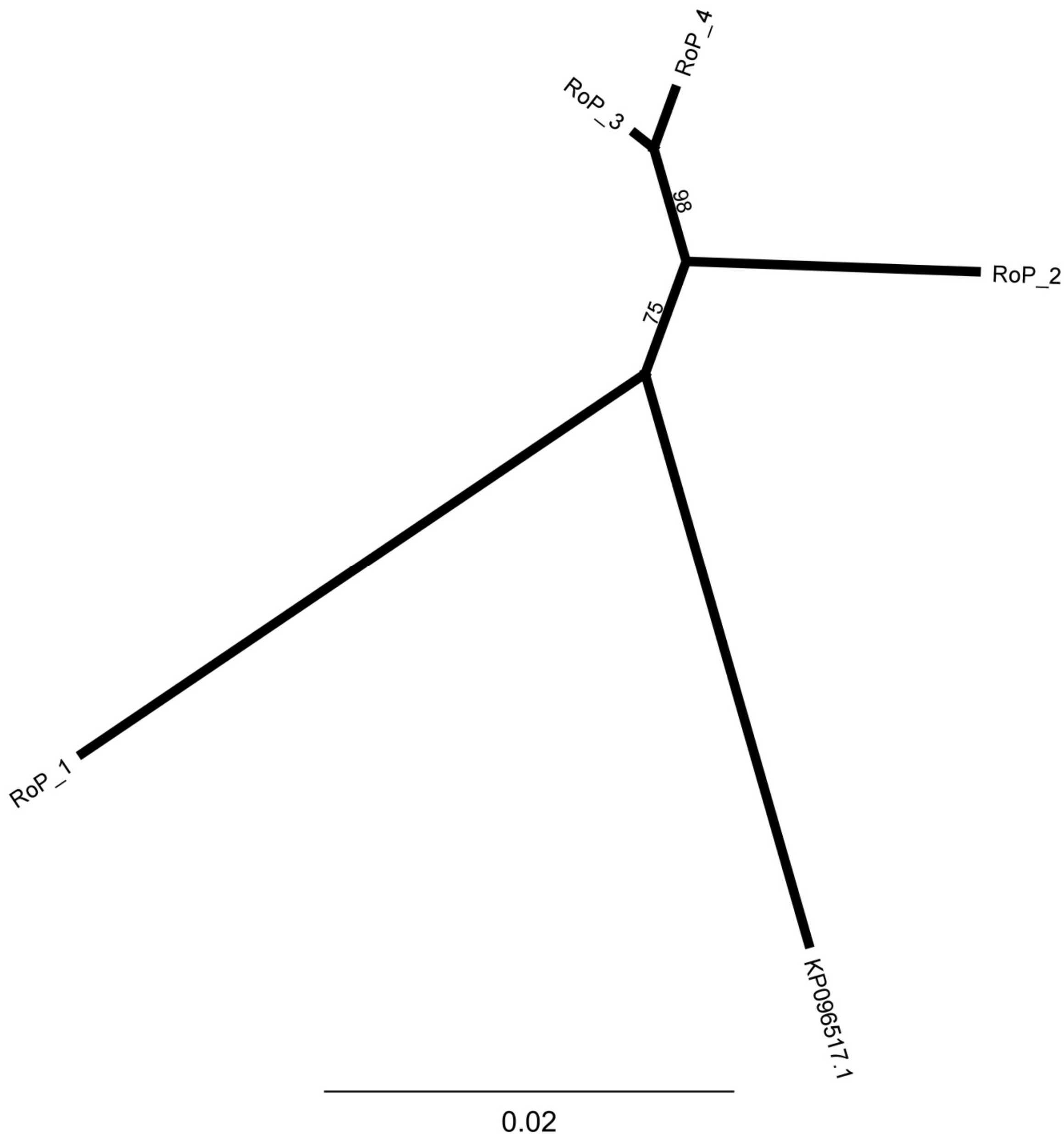

Fig.4: Phylogenetic Tree of *Raoultella planticola* Strains with Reference Strain: *Raoultella planticola* strain SA2 16S ribosomal RNA gene, partial sequence (KP096517.1) retrieved from NCBI database

The findings in this study, where isolate RoP_1 was identified differently by VITEK 2 (*R. ornithinolytica*), PCR (*R. planticola*) and 16S rRNA sequencing (*Proteus mirabilis*), highlight the challenges associated with bacterial identification in clinical settings. Although PCR-based identification is often reliable in research laboratories, the clinical environment introduces complexities that can influence the accuracy of results this is in line with review reports of Franco-Duarte*et al.*, 2019 that clinical samples typically contain low bacterial load, necessitating several pre-processing steps to remove PCR inhibitors and maximize bacterial extraction without contamination [18]. These factors can lead to discrepancies in PCR results, as observed in this study. Despite these challenges, PCR remains a widely used and successful method for

bacterial identification in clinical samples [19]. The discrepancy between the PCR results and 16S rRNA sequencing highlights the need for a comprehensive approach that integrates multiple identification techniques to for accurate diagnosis and treatment.

The genetic variability observed in isolate RoP_1, as indicated by being more distinctly positioned on the phylogenetic tree, suggests some degree of intra-species diversity, which is consistent with findings in other studies of *Raoultella* species [20]. Recent studies emphasize the importance of 16S rRNA gene sequencing in the accurate identification of *Raoultella planticola*, especially in distinguishing it from closely related species. The use of 16S rRNA gene amplification has been a cornerstone in differentiating *R. planticola* from other members of Enterobacteriacae.

4. CONCLUSION

This study, the first to isolate *Raoultella planticola* in pediatric clinical samples in Bauchi, North-East Nigeria, highlights the challenges of accurately identifying *Raoultella* species, often misidentified as *Klebsiella or different species* due to phenotypic similarities. The integration of molecular techniques such as 16S rRNA sequencing, PCR assays and phylogenetic analysis proved essential for precise identification. This study brings out the need for advanced diagnostic methods to ensure accurate diagnosis and effective treatment, particularly in critical cases like neonatal sepsis.

ETHICAL APPROVAL

Ethical clearance was sought and obtained from the Research and ethics committee of ATBUTH and Bauchi state hospital management board, also informed consent of the care givers was obtained before commencement.

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