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Prevalence and Antibiotic Susceptibility Patterns of *Haemophilus* species Isolated from Clinical Samples in Port Harcourt, Nigeria

Everestus Adieme*, Owhonka Aleruchi, and Nedie Patience Akani
Department of Microbiology, Rivers State University, P. M. B. 5080,
Nkpolu-Oroworukwo, Port Harcourt, Rivers State, Nigeria.
***Corresponding Author:** *adiemeeverestus@yahoo.com*

ABSTRACT

Haemophilus species are important pathogens associated with respiratory infections, and their rising antibiotic resistance poses a serious treatment challenge. This study investigated the prevalence and antibiotic susceptibility patterns of *Haemophilus* species isolated from clinical samples obtained from patients with renal complications, respiratory infections, and underlying medical conditions at Rivers State University Teaching Hospital, Port Harcourt. A total of 128 clinical samples were collected across three age groups (0–18 years, 19–45 years, and ≥46 years), including blood (n = 20), sputum (n = 70), and nasopharyngeal swabs (n = 38). Samples were inoculated directly onto prepared agar plates using the swab technique and processed using standard microbiological, virulence, and molecular methods. Thirty-four bacterial isolates were recovered, of which sixteen were confirmed as *Haemophilus* species, giving an overall prevalence of 12.5%. Male patients accounted for ten positive cases, while females had six. Antibiotic susceptibility testing revealed resistance to ciprofloxacin (50%), cefotaxime (44%), and gentamicin (25%), while higher susceptibility was observed with Augmentin (81%), amoxicillin (75%), and sparfloxacin (69%). Haemolytic activity results showed that 68.8% of isolates were beta-haemolytic, 25% were alpha-haemolytic, and 6.25% were gamma-haemolytic. Additionally, 93.8% exhibited capsule formation. Molecular identification of five multidrug-resistant isolates showed 100% similarity with *Haemophilus influenzae* strains H1375, H54, and Rd KW20, *Haemophilus haemolyticus* CIP 103290, and *Streptococcus pneumoniae* strain K31. Gene profiling revealed the presence of the gyrA gene in all isolates and the blaTEM-1 β-lactamase gene in H54 and Rd KW20. The detection of multidrug-resistant *H. influenzae* highlights the urgent need for continuous local antimicrobial surveillance.

Keywords: *Haemophilus* Species, Antibiotic Resistance, Multidrug Resistance, Resistance Genes, Clinical Isolates

Introduction

Haemophilus species are Gram-negative coccobacilli, characterized by their pleomorphic shape, and belong to the family Pasteurellaceae (Kuhnert *et al*., 2008). *Haemophilus* species have a thin peptidoglycan layer surrounded by an outer membrane containing lipopolysaccharide. Some types contain a polysaccharide capsule around the outer membrane to aid in protection and colonization (Su et al., 2023).

The bacteria are pleomorphic, meaning the shape of the bacterium is variable, however it is typically coccobacillus or rod-shaped that contains pili, which are specialized to adhere to the human nasopharynx (Bjarnason *et. al.,* 2017).

Haemophilus spp are opportunistic pathogens capable of causing *a range of* infections such as otitis media, epiglottitis, and sinusitis, and pneumonia, particularly in children, the elderly and immunocompromised individuals. Transmission occurs through direct contact with respiratory droplets from pharyngeal carriers, and it can colonize the nasopharynx of healthy individuals, particularly in children under five years old, without causing disease. A newborn may contract the infection by contact with genital tract secretions containing living bacteria (Maddi *et al.,* 2017). In the absence of prompt and effective treatments, *Haemophilus* infection may result in life-threatening complications, such as bacteremia and meningitis. Bacteremia may result in limb amputation.

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Furthermore, up to 30% of adult patients who survive meningitis experience permanent hearing loss or other long-term neurological complications. Approximately 5% of invasive *Haemophilus* infections in children are fatal (Harabuchi *et al*., 1994).

However, data on the prevalence of *Haemophilus* infections in this region remains scarce. There is a need to understand the rates at which *Haemophilus* is isolated from clinical sources (e.g., sputum, blood) to determine the current antimicrobial susceptibility pattern. This knowledge is essential for informing treatment guidelines and improving patient outcomes (Saha, 2016).

Rivers State in southern Nigeria has a large population and numerous healthcare facilities where cases of *Haemophilus* infections could be prevalent.

This study aims to determine the prevalence and antibiogram of *Haemophilus* species isolated from patients attending a government hospital in Port Harcourt, Rivers State, to provide data that will inform clinical management and policy decisions in the region.

Materials and Methods

Description of Study Area

The study was conducted at a tertiary health facility; the Rivers State University Teaching Hospital (RSUTH) (Latitude 4, 7804246 and Longitude 7, 0135091), which was established in 1925 and is located at old GRA, Port Harcourt Rivers State.

Ethical Consideration

Ethical approval was obtained from the Ethical Committee at Rivers State University Teaching Hospital with approval number RSUTH/REC/2025/690.

Study Population

The study focused on patients from different demographic backgrounds starting from 0-18years; 19-45years; and 46years and above, who visited or were admitted to Rivers State University Teaching Hospital.

Inclusion Criteria

The study involved all age groups, from neonates to elderly individuals, to understand the epidemiology of *Haemophilus* species across different age ranges.

Clinical Presentation: Patients with respiratory tract infections (such as otitis media pneumonia, or bronchitis), meningitis, or other related conditions where *Haemophilus* species is a known pathogen were included.

Exclusion

Patients that are already on antibiotic therapy for more than 72 hours prior to sample collection and patients who refuse to provide informed consent or participate in the study.

Study Design

A cross-sectional design was employed to assess the presence and antibiotic resistance of *Haemophilus* species in patients attending a government hospital in Port Harcourt, Rivers State.

Sampling Period

The study was carried out between April, 2025 to July, 2025, and clinical specimens were collected bi-monthly to determine the prevalence and antibiogram of *Haemophilus* sp.

Sample Collection and Processing

A total of 38 nasopharyngeal swabs, 70 sputum samples, and 20 blood specimens were collected from patients presenting with respiratory and renal conditions. Standard aseptic techniques were employed to ensure specimen integrity, with efforts made to minimize contamination, particularly in sputum samples.

Microbiological Analysis

The streak plate method was used to isolate pure colonies of *Haemophilus* species from clinical specimens and to study colony morphology. A sterile inoculating loop was used to spread the sample over the surface of the chocolate agar. The specimen was streaked in quadrants to dilute bacteria progressively, allowing individual colonies to develop.

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The culture plate was labeled with the specimen ID, date, and medium. The sterilized inoculation loop was used to pick a portion of the clinical specimen and it was inoculated using a streak pattern. The inoculum was streaked to obtain isolated colonies and incubated at 37°C, and the plate was inspected for growth (typically 24-48 hours). The colonies formed on the plates were counted and described morphologically.

Biochemical Characterization

The catalase test was performed by mixing bacterial colonies with 3% hydrogen peroxide; immediate bubble formation indicated a positive reaction (Cheesbrough, 2006). The oxidase test involved smearing colonies on oxidase reagent-impregnated filter paper, with a color change to dark purple within 10–30 seconds indicating positivity (Forbes et al., 2007). Indole production was assessed by inoculating bacteria in tryptone broth, followed by the addition of Kovac's reagent post-incubation; a red layer formation denoted a positive result (Holt et al., 1994). Citrate utilization was determined using Simmons' citrate agar slants, where a color shift from green to blue signified positive utilization (Cheesbrough, 2006). Nitrate reduction was tested by inoculating bacteria in nitrate broth and adding reagents A and B after incubation; red coloration confirmed nitrate reduction, while zinc addition ruled out false negatives (Forbes et al., 2007). Hydrogen sulfide production was detected by black precipitate formation in SIM medium (Holt et al., 1994). The Voges-Proskauer test employed MR-VP broth inoculation, with reagents A and B added post-incubation; development of a red color indicated a positive result (Cheesbrough, 2006).

Virulence Assessment:

The pathogenic potential of *Haemophilus* species was evaluated through hemolysis, capsule detection, motility, and coagulase testing, following established protocols (Quinn et al., 2011). Haemolytic activity was assessed on blood agar, while capsule presence was confirmed microscopically using capsule staining techniques. Motility was determined via stab inoculation in semi-solid agar. These assays collectively contributed to the characterization of virulence attributes among the isolates.

Antibiogram

The preparation of a standard bacterial inoculum involved using the McFarland standards, which provide a reference turbidity correlating to bacterial concentration. Specifically, a 0.5 McFarland standard represents approximately 1.5×10^8 CFU/mL. A fresh bacterial colony was inoculated into broth and incubated to reach the exponential growth phase.

The optical density of the suspension was measured with a spectrophotometer and adjusted by dilution with sterile saline to match the 0.5 McFarland standards. Visual comparison against a Clinical and Laboratory Standards Institute (CLSI) turbidity reference card was performed to ensure accurate bacterial density, following the Clinical and Laboratory Standards Institute (CLSI, 2020) guidelines.

Molecular Identification

DNA Extraction

Genomic DNA was extracted from 24-hour-old pure cultures using the boiling method as described by Forbes et al. (2007). Briefly, isolates grown in Luria-Bertani (LB) broth were centrifuged at 14,000 rpm for 3 min, washed thrice with normal saline, and resuspended in 500 µL of saline. The suspension was heated at 95°C for 20 min, cooled on ice, and centrifuged. The supernatant containing DNA was stored at −20°C for downstream analysis.

16S rRNA Gene Amplification

Amplification of the 16S rRNA gene was performed using an ABI 9700 Thermal Cycler following Srinivasan et al. (2015). Primers used were 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-CGGTTACCTTGTTACGACTT-3′). PCR was conducted in a 30 µL reaction volume containing Taq polymerase mix, 0.5 µM primers, and template DNA. Cycling conditions were: 95°C for 5 min; 35 cycles of 95°C for 30 s, 52°C for 30 s, and 72°C for 30 s; with a final extension at 72°C for 5 min. Amplicons were resolved on 1% agarose gel at 130 V for 30 min and visualized under blue light.

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DNA Sequencing

PCR products were sequenced using the BigDye Terminator v3.1 kit on an ABI 3510 sequencer (Inqaba Biotechnologicals, Pretoria, South Africa). Sequencing reactions (10 µL) contained 0.25 µL BigDye mix, 2.25 µL 5× buffer, 10 µM primer, and 2–10 ng template DNA. Thermal cycling involved 32 cycles of 96°C for 10 s, 55°C for 5 s, and 60°C for 4 min (Srinivasan et al., 2015).

Phylogenetic Analysis

Sequences were edited using TraceEdit, aligned with MAFFT, and compared with NCBI BLASTN database entries. Phylogenetic trees were constructed using the Neighbor-Joining method in MEGA 6.0 with 500 bootstrap replications (Saitou and Nei, 1987; Forbes et al., 2007).

Amplification of Resistance Genes (gyrA and blaTEM-1)

The gyrA and blaTEM-1 genes were amplified using primers gyrA-F (5′-CAGTCAGGAAATGCGTACG TCCTT-3′), gyrA-R (5′-CAAGGTAATGCTCCAGG CATTGCT-3′), blaTEM-1F (5′-ATAAAATTCTTGA AGACGAAA-3′), and blaTEM-1R (5′-GACAGTTA CCAATGCTTAATCA-3′). Reactions (50 µL) contained DreamTaq Master Mix (Inqaba, South Africa), 0.4 µM primers, and 50 ng DNA. PCR cycling was: 95°C for 5 min; 35 cycles of 95°C for 30 s, 58°C for 30 s, and 72°C for 30 s; final extension at 72°C for 5 min. Amplicons were visualized on 1% agarose gel at 120 V for 25 min, yielding products of 1484 bp (gyrA) and 372 bp (blaTEM-1) (Forbes et al., 2007).

Data Analysis

Data obtained from the study were analyzed using descriptive statistical methods**,** including frequency distribution and percentage analysis**,** to determine the prevalence of *Haemophilus* species among different patient groups, age categories, and genders. The data was processed using the Statistical Package for Social Sciences (SPSS) version 27**.** Results were presented in tables to show the distribution patterns of isolates, antibiotic susceptibility profiles, virulence properties, and molecular identification outcomes.

Results

A total of 128 clinical specimens collected from patients presenting various health conditions including respiratory infections, renal disorders, and other underlying medical conditions were examined for the presence of *Haemophilus* species. The overall prevalence was 12.5% (16 out of 128 samples). When stratified by age, the highest prevalence occurred among children and adolescents (0–18 years) at 19.4%, followed by young adults (19–45 years) at 12.3%, while the lowest was recorded in older adults (≥45 years) at 5.7%.

Based on clinical conditions, *Haemophilus* spp. was detected in 12.3% of respiratory infection cases, 19.4% of patients with underlying medical conditions, and 5.7% of those with renal-related illnesses. The gender distribution was nearly equal, with infection rates of 12.7% in males and 12.2% in females, as presented in Tables 1, 2, & 3.

Table 1: Prevalence of *Haemophilus* species among patients groups

Patients Group	Total Samples	Positive cases	Prevalence (%)
Patients with respiratory infections	57	7	12.3
Patients with renal issues	35	2	5.7
Patients with underlying medical conditions	36	7	19.4

Table 2: Prevalence of *Haemophilus* species among age groups

Age Group	Total Samples	Positive cases	Prevalence (%)
0—18years	36	7	19.4
19—45years	57	7	8.8
46 and above	35	2	5.7

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Table 3: Prevalence of *Haemophilus* species among males and females

Gendre	Total Samples	Positive cases	Prevalence (%)
Male	**79**	**10**	**12.7**
Female	**49**	**6**	**12.2**
Overall	**128**	**16**	**12.5**

Antimicrobial susceptibility testing of the 16 isolates (Table 4) revealed notable resistance trends. Resistance to ciprofloxacin was recorded in 50% of isolates, while 44% showed resistance to cefotaxime and ofloxacin. Amoxicillin and gentamicin each exhibited a resistance rate of 25%, whereas Augmentin demonstrated the lowest resistance at 19%. Resistance to trimethoprim-sulfamethoxazole and other fluoroquinolones, including pefloxacin and sparfloxacin, was approximately 31%. Virulence profiling (Table 5) showed that 68.8% of isolates were beta-hemolytic, 25% were alpha-hemolytic, and 6.25% were gamma-hemolytic. Capsule formation was detected in 93.8% of isolates, indicating a strong virulence potential. Molecular identification based on 16S rRNA sequencing confirmed the isolates as *Haemophilus* spp. and closely related species, showing 100% sequence similarity with *Haemophilus influenzae* strains and *Streptococcus pneumoniae*. Phylogenetic analysis (Figure 1) using the Jukes–Cantor method (Table 6) supported these relationships.

Table 4: Antibiotic sensitivity pattern of *Haemophilus* spp. isolated from clinical sources

Antibiotics/Conc.	*Haemophilus* (n=16)		
	Resistant n (%)	Intermediate n (%)	Susceptible n (%)
Ciprofloxacin (30 µg)	8(50)	0(0.0)	8(50)
Amoxicillin (30 µg)	4(25)	0(0.0)	12(75)
Augmentin (10 µg)	3(12)	0(0.0)	13(81)
Gentamicin (30 µg)	4(25)	0(0.0)	12(75)
Peflaxacin (30 µg)	5(31)	0(0.0)	11(69)
Tarivid (10 µg)	7(44)	0(0.0)	9(56)
Septrin (10 µg)	5(31)	0(0.0)	11(69)
Cefotaxim (10 µg)	7(44)	0(0.0)	9(56)
Streptomycin (10 µg)	5(31)	0(0.0)	11(69)
Sparfloxacin (10 µg)	5(31)	0(0.0)	11(69)

Table 5: Hemolysis and capsule formation of *Haemophilus* spp

Virulence property	Number of isolates	Percentage (%)
Alpha Hemolysis	1	6.25
Beta Hemolysis	11	68.8
Gamma Hemolysis	4	25
Capsule	15	93.8

The results of the antibiotic résistance gene are shown in Plate 1 and 2. Plate 1 shows Agarose gel electrophoresis of the *gyrA* gene bands. Lane 1-5 represent the *gyrA* gene bands at 345bp of the five (5) bacterial isolates while M stands for 100bp molecular ladder while plate 3 shows Agarose gel electrophoresis of the bla_{TEM-1} gene bands. Lane 1-5 represents the bla_{TEM-1} gene bands at 500bp of the five (5) bacterial isolates while M represents the 100bp molecular ladder. The *gyrA* resistance gene was positive for

Haemophilus influenzae strain Hi375, *Haemophilus influenzae* strain H54, *Haemophilus influenzae* strain Rd KW20, *Streptococcus pneumoniae* strain K31, *Haemophilus haemolyticus* CIP 103290. The bla_{TEM-1} was present in *Haemophilus influenzae* strain H54, *Haemophilus influenzae* strain Rd KW20 *Haemophilus haemolyticus* CIP 103290 but absent in *Haemophilus influenzae* strain Hi375 and *Streptococcus pneumoniae* strain K31.

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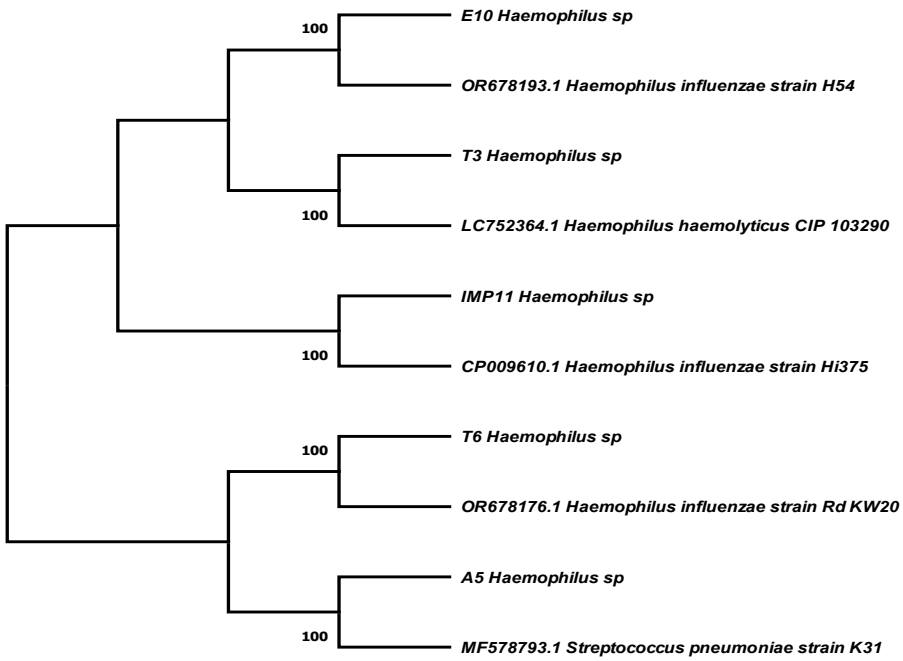

Figure 1: Phylogenetic tree showing the evolutionary relationships between the bacterial isolates

Table 6: Identified bacterial 16Sr RNA sequence relatedness and assigned GeneBank accession numbers

Isolates Code	Tentative Identity	Genotypic	NCBI GeneBank Accession Number	Similarity Index (%)
E10	*Haemophilus* sp.	*Haemophilus influenzae*	OR678193.1	100
T3	*Haemophilus* sp.	*Haemophilus Haemolyticus*	LC752364.1	100
IMP11	*Haemophilus* sp.	*Haemophilus influenzae*	CP009610.1	100
T6	*Haemophilus* sp.	*Haemophilus influenzae*	OR768176.1	100
A5	*Haemophilus* sp.	*Streptococcus pneumonia*	MF578793.1	100

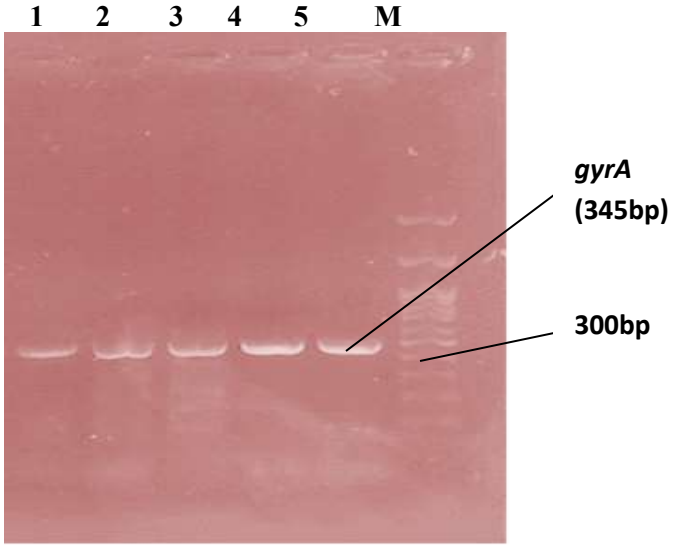

Plate 1: *gyrA* gene bands of *Haemophilus* spp. Lane 1-5 represent the *gyrA* gene bands at 345bp of the five (5) bacterial isolates. M = 100bp mol ladder.

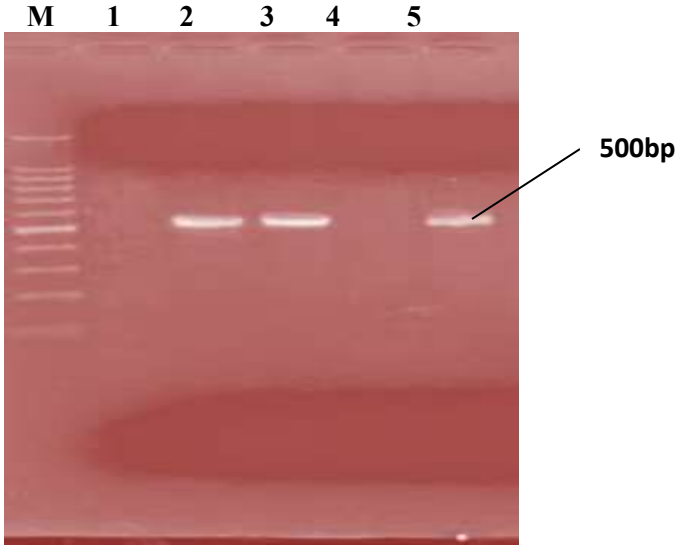

Plate 2: bla_{TEM-1} gene bands. Lane 1-5 represents the bla_{TEM-1} gene bands at 500bp of the five (5) bacterial isolates. M represents the 100bp molecular ladder.

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Discussion

This study investigated the prevalence, antibiotic susceptibility, virulence traits, and molecular characteristics of *Haemophilus* species isolated from clinical samples at Rivers State University Teaching Hospital, Port Harcourt. Out of 128 samples examined, 16 isolates (12.5%) were confirmed as *Haemophilus* species. This prevalence indicates that *Haemophilus* remains a relevant etiological agent of respiratory and opportunistic infections in the studied population. Similar prevalence rates have been reported elsewhere, where *Haemophilus influenzae* accounted for 10–15% of bacterial isolates from respiratory samples (Agrawal et al., 2015).

Age-specific distribution showed that children and adolescents (0–18 years) recorded the highest infection rate (19.4%), followed by young adults (19–45 years) at 12.3%, and the lowest rate (5.7%) among older adults (≥45 years). The higher rate in younger individuals reflects their greater susceptibility to respiratory pathogens due to immature immune responses and closer contact in communal environments such as schools (Barenkamp et al., 2004; Murphy et al., 2009). Conversely, lower prevalence among adults may be linked to acquired immunity, vaccination, or prior microbial exposure that limits colonization. Analysis based on clinical conditions revealed that patients with respiratory infections and those with underlying medical conditions each recorded 12.3% infection rates, whereas renal patients had a lower prevalence (5.7%). This trend supports the organism's established association with respiratory infections and its opportunistic nature in immunocompromised individuals (van Eldere et al., 2014). Gender-based analysis showed minimal variation — 12.7% in males and 12.2% in females — indicating no gender bias in infection risk, consistent with previous epidemiological findings (Agrawal et al., 2015). Antibiotic susceptibility testing revealed variable resistance profiles. Resistance was highest to ciprofloxacin (50%) and cefotaxime (44%), while moderate resistance was observed for gentamicin (25%).

These findings are consistent with global trends showing increasing resistance of *Haemophilus* species to fluoroquinolones and third-generation cephalosporins (Tristram et al., 2007; Gilsdorf, 2008). In contrast, high susceptibility was observed to Augmentin (81%), amoxicillin (75%), and sparfloxacin (69%), suggesting that β-lactam/β-lactamase inhibitor combinations remain effective treatment options. The molecular identification and resistance gene profiling provided deeper insight into the mechanisms behind these patterns. The detection of the gyrA gene in all isolates is significant because mutations within this gene are known to confer resistance to quinolones by altering the DNA gyrase enzyme, which is the primary target of these antibiotics. This likely explains the high ciprofloxacin resistance (50%) observed in this study. Similarly, the blaTEM-1 β-lactamase gene, identified in isolates H54 and Rd KW20, encodes a β-lactamase enzyme capable of hydrolyzing penicillins and cephalosporins, which accounts for the resistance to cefotaxime (44%) and reduced sensitivity to other β-lactam antibiotics. These findings are in line with reports that *Haemophilus influenzae* frequently carries both chromosomal mutations and plasmid-borne resistance genes that enhance its survival under antibiotic pressure (Tristram et al., 2007).

Virulence profiling revealed that 68.8% of isolates were beta-haemolytic, 25% were alpha-haemolytic, and 6.25% were gamma-haemolytic, while 93.8% exhibited capsule formation. The capsule is a major virulence determinant, protecting the organism from phagocytosis and complement-mediated killing, thus facilitating persistent colonization and invasion (Moxon et al., 1990). The predominance of beta-haemolytic activity suggests active secretion of haemolysins that damage host tissues and erythrocytes, supporting the invasive potential of the isolates. Interestingly, molecular characterization using 16S rRNA sequencing revealed that five multidrug-resistant isolates showed 100% similarity not only with *Haemophilus influenzae* strains (H1375, H54, and Rd KW20) but also with *Haemophilus haemolyticus* CIP 103290 and *Streptococcus pneumoniae* strain K31. The presence of *S. pneumoniae* sequences among the isolates indicates that not all isolates belonged exclusively to *Haemophilus* species. This could result from either co-infection, sample contamination, or genetic exchange between respiratory tract bacteria sharing similar ecological niches. The close phylogenetic relationship between *Haemophilus* and *Streptococcus* species has been documented, as both inhabit the nasopharyngeal mucosa and can exchange genetic material through horizontal gene transfer (Redfield *et al.,* 2005).

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Such interactions may contribute to the acquisition and dissemination of resistance genes, including blaTEM-1 and gyrA, within the respiratory microbiome. Overall, the detection of antibiotic-resistance genes that directly correlate with observed phenotypic resistance confirms that these genetic determinants were indeed responsible for the high resistance levels seen in isolates. Moreover, the coexistence or close relationship of *Haemophilus* and *Streptococcus* species in clinical specimens highlights the complex dynamics of polymicrobial infections in the respiratory tract.

Conclusion

This study confirms that *Haemophilus* species remain important pathogens in Port Harcourt, with 12.5% prevalence, mainly affecting children and young adults. High resistance to ciprofloxacin and cefotaxime was linked to the presence of gyrA and blaTEM-1 genes, confirming their role in mediating antibiotic resistance. The predominance of beta-haemolytic and encapsulated isolates indicates strong virulent potential. The detection of *S. pneumoniae* sequences suggests possible co-infection or gene exchange within the respiratory microbiome. Overall, antimicrobial surveillance and prudent antibiotic use are essential to curb resistance and guide effective treatment.

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