



Urban Cockroaches as Reservoirs of Multidrug-Resistant Bacteria: Insights from Morphological and Molecular Characterization

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ABSTRACT

Cockroaches are recognized as important mechanical vectors of antimicrobial-resistant bacteria in urban environments. This study examined the diversity of cockroach species, seasonal abundance, and associated bacteria across four urban biotopes, i.e., households, warehouses, hospitals, and canteens. We hypothesized that cockroach diversity and bacterial resistance profiles would differ significantly by sub-division, season, and habitat type, reflecting environmental and anthropogenic influences. Sampling was conducted by handpicking and using baited traps. Bacteria from the gut and surface were characterized using morphological, biochemical, and 16S rRNA sequencing. Resistance was tested using the Kirby–Bauer disk diffusion method against eight antibiotics, including azithromycin, ciprofloxacin, levofloxacin, cefixime, ceftriaxone, amoxicillin, tetracycline, and cefaclor. Four cockroach species were recorded; *Blattella germanica* and *Periplaneta americana* were the most widespread, while *Pycnoscelus surinamensis* occurred only in Wazirabad. Households showed the highest diversity (Simpson's 1–D = 0.573; Shannon's H = 0.939), and summer had the greatest richness and diversity (1–D = 0.565; H = 0.919), whereas winter had the lowest. Cefixime resistance was the highest (90%), while ciprofloxacin and levofloxacin had the lowest (5%). Multidrug resistance occurred in 35% of strains, 60% were resistant to at least one antibiotic class, and one strain was pan-susceptible. Seventeen of twenty strains (85%) had a Multiple Antibiotic Resistance (MAR) index >0.2, indicating a significant public health risk. The seasonal and habitat-related variations in both cockroach abundance and bacterial resistance patterns underscore their role in the spread of multidrug resistant bacteria. Integrated pest management and targeted antibiotic stewardship are essential to mitigate this emerging urban health threat.

Introduction

Cockroaches, belonging to the order Blattodea, comprise more than 4,600 described species worldwide (Memona et al., 2017; Guzman and Vilcinskis, 2020). While most species are harmless, a few, such as *Periplaneta americana*, *Blatta orientalis*, *Blattella germanica*, and *Supella longipalpa*, have become notorious household pests (Chompoosri et al., 2004; Memona et al., 2017). These pests thrive in human dwellings owing to their omnivorous feeding habits, tolerance for unsanitary conditions, and preference for warm, humid environments (Mullins, 2015). Commonly found in kitchens, hospitals, garbage areas, and sewers, pose considerable public health risks by contaminating food and disseminating pathogenic microorganisms (Graczyk et al., 2005; Bouamama et al., 2010; Nasirian, 2017).

In addition to being nuisance pests, cockroaches are recognized carriers of pathogenic microorganisms, with their occurrence linked to allergies, foodborne illnesses, and hospital-acquired infections (Peterson and Shurdut, 1999; Turner et al., 2022). Both the gut and body surfaces can harbor multidrug-resistant (MDR) bacteria such as *Escherichia coli*, *Salmonella* spp., *Klebsiella* spp., *Staphylococcus aureus*, and *Pseudomonas aeruginosa* (Naher et al., 2018; Kabantiyok et al., 2023). Reports suggest that up to 90% of gut-associated bacteria in cockroaches are MDR, with Gram-positive strains showing particularly high resistance (Solomon et al., 2018).

Their presence in human-inhabited environments represents a substantial health risk due to the increased likelihood of transmitting MDR bacteria (Soureshjani and Doosti, 2013). They may contribute to the spread of foodborne diseases and allergic reactions (Patel and Meher, 2016; Nasirian, 2019; Wang et al., 2019), and are known vectors of agents causing diseases such as plague, dysentery, abscesses, and leprosy (Adedara et al., 2022).

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Over 100 bacterial species have been documented in symbiotic association with cockroaches (Jalil et al., 2012). Evidence also suggests their potential to mechanically transfer viruses, including SARS-CoV-2, through contact with contaminated fecal matter (Sharawi, 2021).

Being nocturnal and regularly shedding their cuticle, cockroaches frequently encounter contaminated materials that enhance their efficiency as disease transmitters (Fischer et al., 2003; Mpuchane et al., 2006). Particularly concerning is their role in harboring and spreading antimicrobial-resistant bacteria, especially in healthcare settings. Studies have reported resistance to widely used antibiotics, including amoxicillin, vancomycin, and chloramphenicol, among bacteria isolated from cockroaches (Zarchi and Vatani, 2009; Kundera et al., 2020), though some strains remain susceptible to agents such as ciprofloxacin and imipenem (Ghasemi-Dehkordi et al., 2016).

Despite global recognition of this issue, a notable research gap exists in Pakistan. While some national studies have examined AMR in insects (Bilal et al., 2021; Kamran et al., 2021), few have specifically investigated cockroach-associated bacteria in urban environments, and no comprehensive, habitat-wide survey has been conducted in District Gujranwala. This densely populated and industrialized city in Punjab, Pakistan, offers favorable conditions for cockroach proliferation, including summer temperatures ranging from 36 to 42 °C, poor sanitation, and abundant food waste (Baig and Ehrenreich, 1997). We hypothesized that cockroach populations in Gujranwala exhibit species-specific seasonal abundance patterns influenced by local habitat conditions, and that these populations harbor diverse cultivable bacterial communities with varying levels of antimicrobial resistance.

Accordingly, this study aimed to identify the predominant cockroach species present in households, warehouses, hospitals, and canteens, isolate and characterize bacteria from both their gut and external surfaces, and assess the antimicrobial resistance of these isolates to eight commonly used antibiotics using the Kirby–Bauer disk diffusion method (Malinga et al., 2022). To confirm bacterial identity and explore their evolutionary relationships, 16S rRNA gene sequencing was performed. By integrating data on cockroach species diversity, seasonal abundance, and resistance profiles, this research provides valuable insights into their role as reservoirs and vectors of multidrug-resistant pathogens, with important implications for public health surveillance and control strategies.

Materials and methods

Study area

Gujranwala (32.1877°N, 74.1945°E; 231 meters above sea level), is an industrial city in Punjab, Pakistan, characterized by a hot semi-arid climate (BSh) with summer temperatures averaging 36°C to 42°C and winter temperatures averaging around 7°C (Baig and Ehrenreich, 1997). The district consists of five administrative subdivisions and has an area of 3,198 km², and lies at an elevation of 744 feet above sea level (Mahmood et al., 2013). The monsoon season peaks in July and August, which are also the rainiest months (Faheem et al., 2007; Mahmood et al., 2013).

Sampling methodology

Sampling was conducted across five sub divisions of District Gujranwala: Gujranwala City, Gujranwala Saddar, Kamoke, Wazirabad, and Nowshera Virkan. Four urban biotopes were selected: households, warehouses, hospitals, and canteens. Cockroach collection occurred fortnightly from August 2020 to July 2021. Active sampling was performed manually for

approximately three hours per day during early mornings and at night to coincide with peak cockroach activity (Mehainaoui et al., 2021). Passive sampling involved placing 10 baited traps in each biotope per subdivision every two weeks and leaving them overnight. Trap spacing ranged from 10 to 50 ft, depending on site layout and accessibility.

Traps were placed in known cockroach-harboring areas, including kitchen cupboards, washrooms, and behind sinks in households; trash areas, drains, sinks, and food preparation zones in canteens; cafeterias, storage areas, and plumbing zones in hospitals; and corridors and damp, concealed areas in warehouses.

Identification of cockroach specimens

The collected specimens were identified to species level using published taxonomic keys and entomological literature specific to cockroaches (Hagenbuch et al., 1988; Choate et al., 2008).

Bacterial isolation from the Surface and gut

To investigate bacterial communities, four adult cockroaches were collected from each habitat type within each subdivision, except *Pycnoscelus surinamensis*, which was only found in household settings in Wazirabad. The live specimens were immobilized by placing them at 0 °C for 15 minutes. To isolate surface-associated bacteria, each cockroach was vigorously shaken in 200 µl of 2% saline solution, and the resulting suspension was subjected to two serial dilutions using autoclaved distilled water (Khan et al., 2018). For gut-associated bacteria, cockroaches were surface-sterilized by immersion in 70% ethanol for 5 min, rinsed with 2% saline, and air-dried under a laminar hood (Liu et al., 2016). Under sterile conditions, the alimentary canal was dissected and macerated, and six serial dilutions were prepared. Aliquots (0.02 ml) from both surface and gut samples were inoculated onto nutrient agar plates and incubated at 37 °C overnight (Ofoedu et al., 2021).

Morphological and biochemical characterization

Bacterial isolates were initially characterized by colony color, shape, and margin. Biochemical tests included catalase, citrate utilization, and indole, following established protocols (Garrity and Holt, 2001). Bubble formation indicated catalase positivity; green-to-blue color change indicated citrate utilization; and a red ring indicated an indole-positive reaction. Isolates were stored in 15% glycerol at –20 °C.

Bacterial DNA extraction

Genomic DNA was extracted from isolates using the boiling lysis method. A 200 µl aliquot of bacterial broth was centrifuged at 10,000 rpm for 2 minutes. The pellet was washed twice with distilled water. The cell pellet was then boiled for 12–15 minutes to lyse the cells, and the supernatant containing DNA was collected post-centrifugation for use in PCR.

16S rRNA gene amplification, sequencing, and phylogenetic analysis

The primer pair used in this study included the 16F (5'-GAGTTTGATCCTGGCTCAG-3') and 1510R (5'-GGCTACCTGTACGA-3'), which were utilized to get an amplification product of 1500 bp (Weisburg et al., 1991). PCR reactions contained 1× DreamTaq™ Green PCR Master Mix (12.5 µl), 10 µM of each primer, 5 µl DNA template, and nuclease-free water to 25 µl total volume (Máca et al., 2021).

Cycling conditions: initial denaturation at 94 °C for 5 min; 30 cycles of 95 °C for 45s, 50 °C for 30 s, and 72 °C for 10 min; final extension at 72 °C for 5 min (Christopher et al., 2021). Products were visualized by agarose gel electrophoresis. Sanger sequencing was performed commercially (Lab Genetics, Lahore). Sequences were edited using CHROMAS 2 software and aligned via BLAST against the NCBI database. Phylogenetic relationships were analyzed using MEGA 6.0 and the Neighbor-Joining method with bootstrap values based on 1000 replicates (Tamura and Nei, 1993; Kumar et al., 2018; Javaid et al., 2021).

Phylogenetic relationships of bacterial strains isolated from the gut and body surface of urban cockroaches based on 16S rRNA sequences were analysed by decoding the strain codes. Strain codes indicate the source, cockroach number, and colony morphology of each bacterial isolate.

Antibiotic Susceptibility Testing and AMR analysis

The antimicrobial resistance (AMR) profiles of the bacterial isolates were evaluated using the Kirby-Bauer disk diffusion method (Varaldo, 2002). The antibiotics tested included: azithromycin (AZM, 15 µg), ciprofloxacin (CIP, 5 µg), levofloxacin (LEV, 5 µg), cefixime (CFM, 5 µg), ceftriaxone (CRO, 30 µg), amoxicillin (AML, 10 µg), tetracycline (TE, 30 µg), and cefaclor (CEC, 30 µg), all from Oxoid UK.

Bacterial suspensions were adjusted to a 0.5 McFarland standard to standardize inoculum density (Jacobs and Chenia, 2007). Nutrient agar plates were poured to a depth of 4 mm as recommended by CLSI (2020). Plates were dried at room temperature for 3–5 minutes before placing antibiotic discs approximately 24 mm apart using sterile forceps.

Inhibition zones were measured in millimeters and interpreted as resistant, intermediate, or susceptible based on CLSI guidelines (Weinstein and Lewis 2nd, 2020). The Multiple Antibiotic Resistance (MAR) index was calculated as the ratio of the number of antibiotics to which an isolate was resistant over the total number tested. Strains were categorized as multidrug-resistant (MDR) if they were resistant to at least one agent in three or more antibiotic classes (Magiorakos et al., 2012).

Statistical analysis

Data were compiled based on monthly sampling conducted across five sub divisions of Gujranwala District, Pakistan. The seasons were categorized as Summer (May–July), Spring (March–April), Autumn (August–October), and Winter (November–February). Species diversity within each season, habitat, and biotype was assessed using the Shannon-Wiener diversity index and Simpson's diversity index. These indices were computed using PAST. To examine how ecological factors influence cockroach community composition, two separate two-way PERMANOVA tests were performed using PAST Software (v4.03). Analyses were based on a Bray-Curtis similarity matrix derived from species abundance data, with 9,999 permutations for statistical robustness (Dallarés et al., 2017). The first test assessed the effects of season (summer, autumn, winter, spring) and location (five sub divisions), while the second evaluated biotype (household, warehouse, hospital, canteen) and location. A significance threshold of $p < 0.05$ was applied.

For phylogenetic analysis of bacterial isolates, 16S rRNA gene sequences (~1500 bp) were aligned, and evolutionary relationships were inferred using the Neighbor-Joining (NJ) method in MEGA version 6.0. Bootstrap analysis with 1000 replicates was performed to assess the robustness of branching nodes. The scale bar in the phylogenetic tree indicates the number of base substitutions per site. Sequence-based clustering patterns were used to infer relatedness among isolates and to validate taxonomic identification obtained through BLAST analysis.

Results

Diversity and relative abundance of cockroaches

A total of 2895 cockroach specimens were collected from various urban biotopes in District Gujranwala. These specimens represented four species: *Blattella germanica* (Family: Ectobiidae, formerly Blattellidae), *Periplaneta americana* (Family: Blattidae), *Blatta lateralis* (Family: Blattidae), and *Pycnoscelus surinamensis* (Family: Blaberidae). Among the collected specimens, *Blattella germanica* was the most dominant species, followed by *Periplaneta americana*. The relative abundance of cockroach species varied across biotypes and sub divisions. *Blattella germanica* and *Periplaneta americana* were present across all biotypes, with the highest abundances observed in households. *Blatta lateralis* was also most common in households, particularly in Kamonke (77.78%) and Gujranwala Saddar (57.78%). *Pycnoscelus surinamensis* was exclusively recorded in Wazirabad, comprising 14.29% of canteen and 85.71% of household collections. Overall, households consistently exhibited the highest species abundances across all sub divisions (Table 1).

Overall, diversity and dominance indices showed minor variation, suggesting a broadly similar cockroach community structure across locations. Summer displayed the highest diversity and abundance, while winter showed the lowest diversity and greatest dominance. Shannon diversity index (H') was highest in summer (0.928) and lowest in winter (0.686), indicating more even distribution and richness in summer. Simpson's diversity (1-D) ranged from 0.565 (summer) to 0.4668 (winter), supporting the same trend. Cockroach diversity varied across biotypes. Households had the highest individual abundance ($n = 1230$) and highest diversity, reflected by the Shannon index (0.939) and Simpson's 1-D (0.573). Canteens and hospitals showed moderate diversity, with Taxa_S = 4 and 3, respectively (Table 2). A two-way PERMANOVA (9,999 permutations) revealed a significant effect of season on cockroach community composition ($F = 28.07$, $p = 0.0001$), and a weaker but significant effect of location ($F = 2.22$, $p = 0.0388$). The season $\times$ location interaction was not significant ($F = 1.02$, $p = 0.4362$), indicating consistent seasonal patterns across sites.

In contrast, a second two-way PERMANOVA, assessing biotype and location, showed no significant effects of biotype ($F = 2.09$, $p = 0.0669$), location ($F = 0.98$, $p = 0.4488$), or their interaction ($F = 0.22$, $p = 1.000$), suggesting similar species composition across these categories.

Colonial morphology and biochemical characterization

Twenty bacterial strains isolated from the gut and surface of cockroaches were characterized based on colony morphology, specifically color, shape, and margin, and were assigned strain codes accordingly (Table 3). Most isolates were catalase positive, with only a few exceptions that showed no or weak effervescence. Similarly, the majority of strains exhibited positive citrate utilization, indicated by a color shift from green to blue, while a limited number remained negative. Indole production was observed in five strains, which turned red upon testing, whereas indole-negative strains showed no color change or turned yellow (Figure 1). These biochemical traits aided in a preliminary differentiation of the bacterial isolates before molecular identification.

Antimicrobial susceptibility

Antibiotic susceptibility testing revealed notable resistance trends among the twenty bacterial strains tested against eight antibiotics (Figure 2). Seventeen out of twenty strains exhibited a Multiple Antibiotic Resistance (MAR) index greater than 0.2, indicating exposure

Table 1
Relative abundance (%) of four cockroach species across different biotypes (household, warehouse, hospital, canteen) within five sub divisions of Gujranwala District.

Subdivisions	Biotypes	<i>B. lateralis</i>	<i>B. germanica</i>	<i>P. americana</i>	<i>P. surinamensis</i>
Gujranwala City	Canteen	13.89	22.26	19.56	--
	Hospital	8.33	16.61	10.67	--
	Household	44.44	33.89	43.11	--
	Warehouse	33.33	27.24	26.67	--
Gujranwala Saddar	Canteen	8.89	22.06	18.87	--
	Hospital	20.00	23.06	21.13	--
	Household	57.78	33.08	38.03	--
	Warehouse	13.33	21.80	21.97	--
Kamonke	Canteen	5.56	18.23	12.03	--
	Hospital	--	14.29	18.99	--
	Household	77.78	43.84	47.47	--
	Warehouse	16.67	23.65	21.52	--
Nowshera Virkaan	Canteen	6.25	19.57	17.32	--
	Hospital	18.75	16.09	14.53	--
	Household	56.25	43.91	47.49	--
	Warehouse	18.75	20.43	20.67	--
Wazirabad	Canteen	28.21	27.04	29.39	14.29
	Hospital	10.26	12.68	13.42	--
	Household	56.41	46.48	46.96	85.71
	Warehouse	5.13	13.80	10.22	--
Overall	Gujranwala City	21.18	20.23	18.29	--
	Gujranwala Saddar	26.47	26.81	28.86	--
	Kamonke	10.59	13.64	12.85	--
	Nowshera Virkaan	18.82	15.46	14.55	--
	Wazirabad	22.94	23.86	25.45	100.00

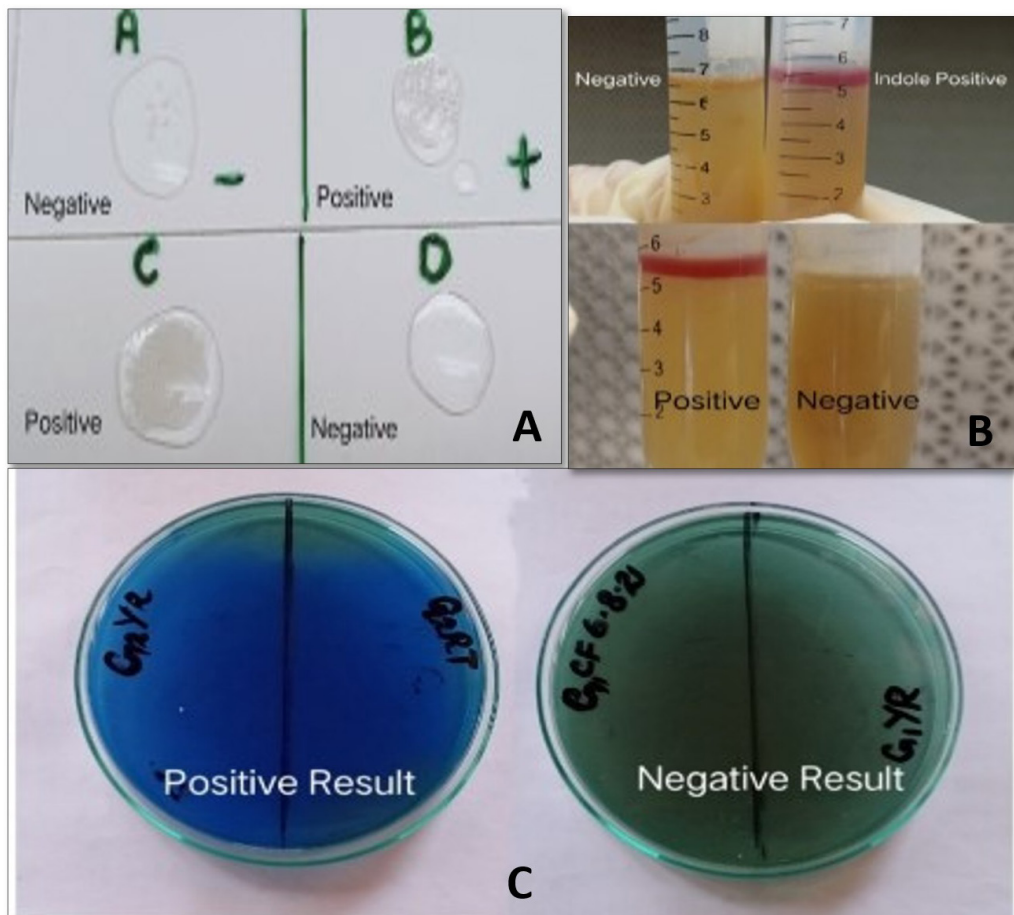
Table 2
Diversity indices of cockroach communities across different sub divisions, biotypes, and seasons in Gujranwala District.

Category Type	Description	Taxa_S	Individuals	Simpson (1-D)	Shannon_H	Evenness (e^H/S)
Subdivisions	Gujranwala City	3	562	0.549	0.877	0.801
	Gujranwala Saddar	3	799	0.550	0.869	0.795
	Kamonke	3	379	0.537	0.844	0.775
	Nowshera Virkaan	3	441	0.558	0.896	0.817
Biotype	Canteen	4	610	0.529	0.830	0.573
	Hospital	3	472	0.536	0.842	0.774
	Household	4	1230	0.573	0.939	0.640
	Warehouse	3	583	0.538	0.848	0.779
Season	Autumn	3	691	0.539	0.848	0.778
	Spring	3	324	0.537	0.847	0.777
	Summer	4	1712	0.565	0.919	0.627
	Winter	3	168	0.467	0.686	0.662

Table 3

MAR Index calculation for all tested bacterial strains against eight antibiotics used.

Strain ID	Description	Number of Resistant Antibiotics	MAR Index
G1CF	Gut 1 – Creamy Filamentous	6	0.75
G1YR	Gut 1 – Yellow Round	4	0.50
G2RT	Gut 2 – Round Transparent	3	0.38
G2CR	Gut 2 – Creamy Round	3	0.38
G2WR	Gut 2 – White Round	0	0.00
G4OC	Gut 4 – Off-white Curled	3	0.38
G4CF	Gut 4 – Creamy Filamentous	1	0.13
G4WC	Gut 4 – White Curled	5	0.63
G5YCF	Gut 5 – Yellowish Creamy sc Filamentous	3	0.38
G5YRW	Gut 5 – Yellowish Round Whitish	6	0.75
G5WLI	Gut 5 – White Large Irregular	4	0.50
G6CR	Gut 6 – Creamy Round	1	0.13
G6PYF	Gut 6 – Pale Yellow Filamentous	3	0.38
S1WF	Surface 1 – White Filamentous	6	0.75
S1CF	Surface 1 – Creamy Filamentous	4	0.50
S2WF	Surface 2 – White Filamentous	5	0.63
S2CC	Surface 2 – Creamy Curled	2	0.25
S2CF	Surface 2 – Creamy Filamentous	2	0.25
S3WR	Surface 3 – Whitish Round	5	0.63
S4WIC	Surface 4 – White Irregular Curled	2	0.25

**Figure 1** Biochemical characterization of bacterial isolates: (A) Catalase test, (B) Citrate Utilization test, (C) Indole Test.

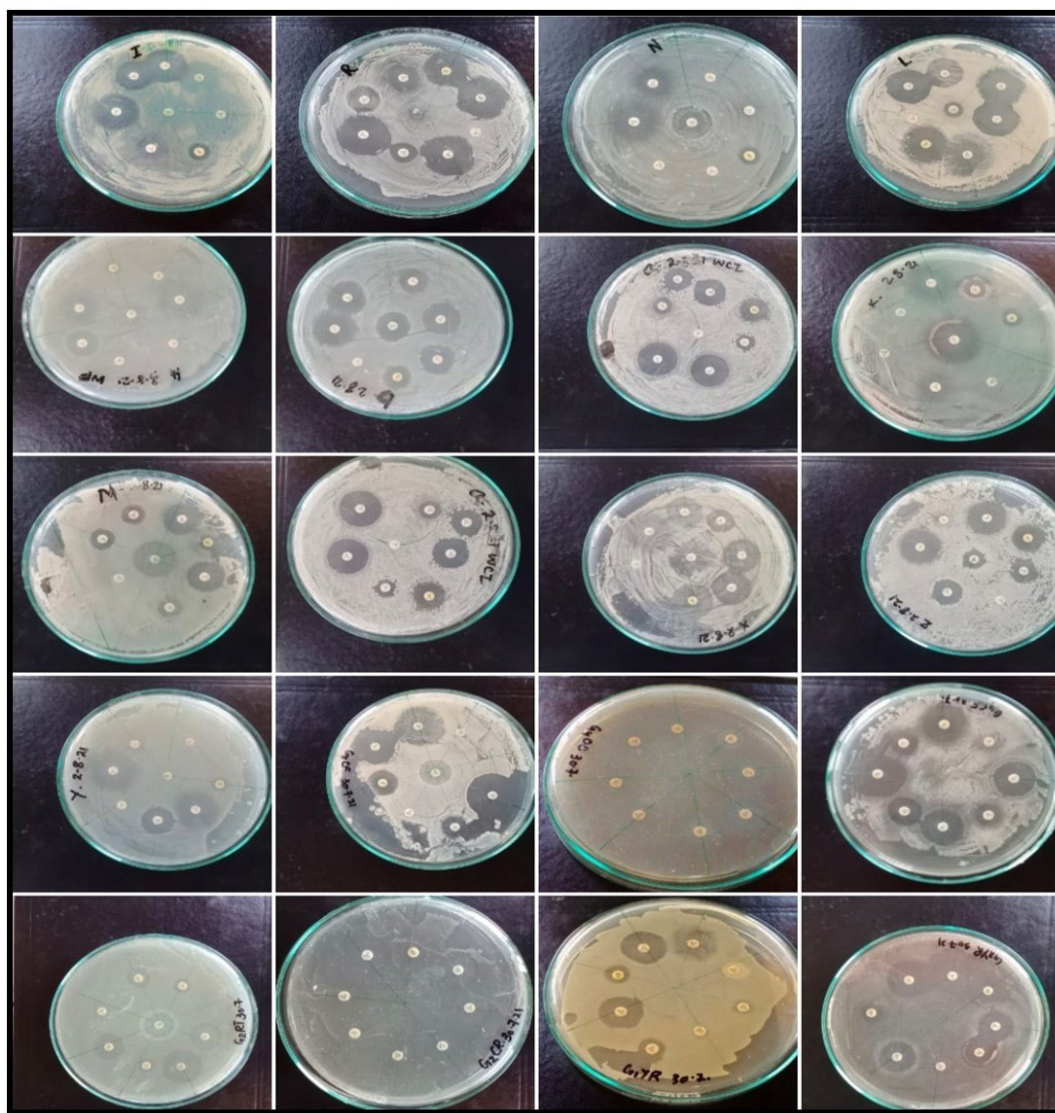


Figure 2 Antimicrobial resistance (AMR) of isolated bacterial strains using the Kirby-Bauer disk diffusion method.

to high-risk contamination sources (Table 3). In total, 35% (7/20) of the strains were classified as multidrug-resistant (MDR), showing resistance to at least three different antibiotic classes. An additional 60% (12/20) of the strains were resistant to at least one antibiotic class, while only one strain (G2WR) was pan-susceptible, exhibiting no resistance to any of the antibiotics tested.

The highest resistance was observed against cefixime (90%), followed by amoxicillin (70%) and ceftriaxone (60%). In contrast, the greatest susceptibility was recorded for levofloxacin (75%), ciprofloxacin (65%), azithromycin (65%), and tetracycline (60%) (Table 4).

Phylogenetic analysis

Molecular identification using 16S rRNA gene sequencing with 1,000 bootstrap replicates revealed close homology between the isolated strains and 20 distinct bacterial species (Table 5). Of the total isolates, 13 originated from the gut and seven from the external surface of freshly collected cockroach specimens. Gut-associated strains showed close genetic similarity to a range of clinically relevant bacteria, including *Staphylococcus aureus*, *Stenotrophomonas maltophilia*,

Bacillus paramycoides, *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella quasipneumoniae*, *Bacillus subtilis*, *Klebsiella pneumoniae*, *Cronobacter sakazakii*, *Paenibacillus thiaminolyticus*, *Paenibacillus dendritiformis*, *Klebsiella variicola*, and *Citrobacter freundii*. In contrast, surface-associated isolates showed the highest sequence similarity to species such as *B. atrophaeus*, *B. safensis*, *S. haemolyticus*, *S. gallinarum*, *B. pumilus*, *B. cereus*, and *B. paranthracis* (Figure 3). An evolutionary tree was constructed for all 20 bacterial strains using the Neighbor-Joining method, with bootstrap values (e.g., 52%, 54%, 65%, 79%) indicating statistical support at branch nodes, higher values reflecting greater confidence. A major bifurcation in the tree, supported by a bootstrap value of 79%, revealed two primary clades stemming from a common ancestor. Notably, both gut- and surface-derived isolates were distributed across these clades, indicating no strict phylogenetic separation between internal and external microbiota. For instance, strains G6CR, S3WR, G4WC, G6PYF, G5YCF, G1YR, G1CF, G2CR, and G2WR clustered into a large clade, suggesting a shared evolutionary lineage. The second major clade included S2WF, S2CF, G2RT, G4CF, S4WIC, S1WF, S2CC, and S1CF. Interestingly, three gut isolates (G4OC, G5WLI, and G5YRW) formed a distinct minor clade at the end of the tree, suggesting a unique evolutionary trajectory (Figure 4).

Table 4

Antimicrobial resistance (AMR) measurement (mm) of the tested strains against eight antibiotics according to CLSI, 2020 and multidrug resistance (MDR).

Strain ID	Azithromycin (AZM) 15 µg	Ciprofloxacin (CIP) 5 µg	Levofloxacin (LEV) 5 µg	Cefixime (CFM) 5 µg	Ceftriaxone (CRO) 30 µg	Amoxicillin (AML) 10 µg	Tetracycline (TE) 30 µg	Cefaclor (CEC) 30 µg	Number of Resistant classes	MDR
G1CF	0 (R)	30 (S)	15 (R)	0 (R)	16 (R)	9 (R)	20 (S)	14 (R)	3	Yes
G1YR	30 (S)	22 (I)	26 (S)	0 (R)	15 (R)	9 (R)	15 (S)	0 (R)	2	No
G2RT	13 (S)	23 (I)	24 (S)	0 (R)	10 (R)	11 (R)	21 (S)	28 (S)	2	No
G2CR	20 (S)	28 (S)	22 (S)	0 (R)	25 (S)	0 (R)	14 (I)	0 (R)	2	No
G2WR	21 (S)	28 (S)	30 (S)	23 (S)	25 (S)	27 (S)	15 (S)	35 (S)	0	No
G4OC	15 (S)	25 (I)	20 (I)	0 (R)	23 (S)	0 (R)	12 (I)	0 (R)	2	No
G4CF	21 (S)	30 (S)	27 (S)	10 (R)	20 (I)	20 (S)	31 (S)	30 (S)	1	No
G4WC	0 (R)	30 (S)	20 (I)	0 (R)	15 (R)	0 (R)	15 (S)	0 (R)	3	Yes
G5YCF	14 (S)	35 (S)	26 (S)	0 (R)	21 (I)	0 (R)	13 (I)	0 (R)	2	No
G5YRW	0 (R)	30 (S)	24 (S)	0 (R)	0 (R)	0 (R)	11 (R)	0 (R)	4	Yes
G5WLI	22 (S)	31 (S)	34 (S)	0 (R)	0 (R)	17 (S)	10 (R)	0 (R)	2	No
G6CR	20 (S)	27 (S)	25 (S)	25 (S)	25 (S)	0 (R)	20 (S)	33 (S)	1	No
G6PYF	20 (S)	28 (S)	28 (S)	0 (R)	15 (R)	0 (R)	22 (S)	20 (S)	2	No
S1WF	0 (R)	20 (R)	19 (I)	0 (R)	21 (I)	0 (R)	9 (R)	0 (R)	3	Yes
S1CF	20 (S)	32 (S)	20 (I)	0 (R)	23 (S)	0 (R)	11 (R)	0 (R)	3	Yes
S2WF	7 (R)	22 (I)	38 (S)	0 (R)	2 (R)	8 (R)	26 (S)	4 (R)	3	Yes
S2CC	35 (S)	38 (S)	29 (S)	0 (R)	13 (R)	21 (S)	28 (S)	30 (S)	1	No
S2CF	23 (I)	24 (I)	32 (S)	0 (R)	16 (R)	22 (S)	30 (S)	34 (S)	2	No
S3WR	0 (R)	30 (S)	30 (S)	0 (R)	13 (R)	0 (R)	11 (R)	25 (S)	4	Yes
S4WIC	20 (S)	25 (I)	24 (S)	0 (R)	15 (R)	20 (S)	22 (S)	30 (S)	1	No

Periplaneta americana: G1CF, G1YR, G2RT, G2CR, S1WF, S1CF, S2WF, S2CC; *Blatta lateralis*: G4OC, G4CF, G4WC; *Blattella germanica*: S3WR, S4WIC, G5YCF, G5YRW, G5WLI; *Pycnoscelus surinamensis*: G6CR, G6PYF

Table 5

Closely related bacterial species identified by the Maximum Likelihood Method (MEGA X) for each strain.

Strain ID	Cockroach species	Biotypes	Closest bacterial species
G1CF	<i>Periplaneta americana</i>	Hospital, Gujranwala City	<i>Staphylococcus aureus</i>
G1YR	<i>Periplaneta americana</i>	Canteen, Wazirabad	<i>Stenotrophomonas maltophilia</i>
G2RT	<i>Periplaneta americana</i>	Households, Kamonke	<i>Bacillus paramycoides</i>
G2CR	<i>Periplaneta americana</i>	Households, Gujranwala Saddar	<i>Escherichia coli</i>
G2WR	<i>Periplaneta americana</i>	Hospital, Gujranwala City	<i>Pseudomonas aeruginosa</i>
G4OC	<i>Blatta lateralis</i>	Households, Nowshera Virkan	<i>Klebsiella quasipneumoniae</i>
G4CF	<i>Blatta lateralis</i>	Canteen, Gujranwala city	<i>Bacillus subtilis</i>
G4WC	<i>Blatta lateralis</i>	Households, Gujranwala Saddar	<i>Klebsiella pneumoniae</i>
G5YCF	<i>Blattella germanica</i>	Hospital, Kamonke	<i>Cronobacter sakazakii</i>
G5YRW	<i>Blattella germanica</i>	Households, Wazirabad	<i>Paenibacillus thiaminolyticus</i>
G5WLI	<i>Blattella germanica</i>	Canteen, Gujranwala Saddar	<i>Paenibacillus dendritiformis</i>
G6CR	<i>Pycnoscelus surinamensis</i>	Households, Wazirabad	<i>Klebsiella variicola</i>
G6PYF	<i>Pycnoscelus surinamensis</i>	Households, Wazirabad	<i>Citrobacter freundii</i>
S1WF	<i>Periplaneta americana</i>	Hospital, Gujranwala City	<i>Bacillus atrophaeus</i>
S1CF	<i>Periplaneta americana</i>	Canteen, Wazirabad	<i>Bacillus safensis</i>
S2WF	<i>Periplaneta americana</i>	Households, Kamonke	<i>Staphylococcus haemolyticus</i>
S2CC	<i>Periplaneta americana</i>	Households, Gujranwala Saddar	<i>Bacillus pumilus</i>
S2CF	<i>Periplaneta americana</i>	Hospital, Gujranwala City	<i>Staphylococcus gallinarum</i>
S3WR	<i>Blattella germanica</i>	Hospital, Kamonke	<i>Bacillus cereus</i>
S4WIC	<i>Blattella germanica</i>	Canteen, Gujranwala Saddar	<i>Bacillus paranthracis</i>

G = Gut isolate, S = Surface isolate; The number (1–6) represents the individual cockroach from which the strain was isolated. The final letters represent colony morphology as observed on agar plates: CF = Creamy Filamentous, YR = Yellow Round, WR = Whitish Round, WC = White Curled, PYF = Pale Yellow Filamentous, WF = White Filamentous, YF = Yellowish Filamentous, CC = Creamy Curled, CRT = Creamy Round Threadlike, WIC = White Irregular Curled, RT = Round Transparent, OC = Off-white Curled, CR = Creamy Round, YCF = Yellowish Creamy Filamentous, WLI = White Large Irregular, YRW = Yellowish Round Whitish

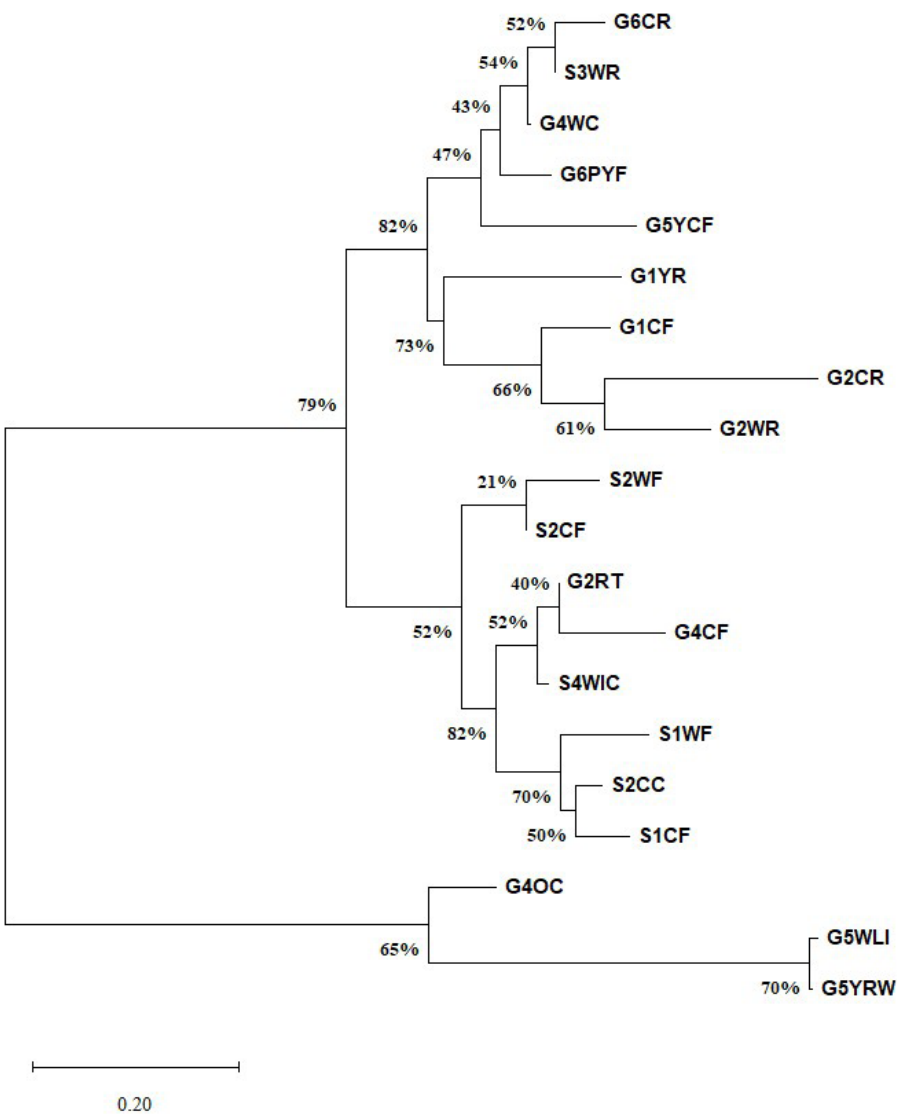


Figure 3 Phylogenetic relationship among tested bacterial strains based on 16S rRNA gene sequence through the Neighbor Joining method using 1000 bootstrap replicates.

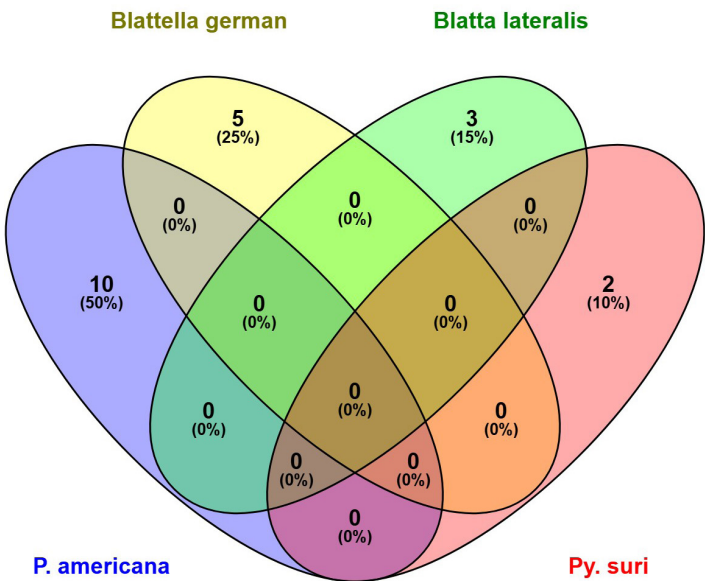


Figure 4 Venn diagram illustrating the distribution of closely related bacterial species isolated from the gut and surface of four cockroach species.

Table 6

Distribution of bacterial isolates by cockroach species, site, and ecological/pathogenic category.

Subdivisions	Cockroach Species	Human Pathogens	Environmental Bacteria	Opportunistic / Other	Entomophilic
Gujranwala City	<i>P. americana</i>	<i>P. aeruginosa</i> , <i>S. aureus</i>	<i>B. atrophaeus</i>	--	<i>S. gallinarum</i>
	<i>B. lateralis</i>	--	<i>B. subtilis</i>	--	--
Gujranwala Saddar	<i>B. germanica</i>	--	<i>P. dendritiformis</i>	<i>B. paranthracis</i>	--
	<i>B. lateralis</i>	<i>K. pneumoniae</i>	--	--	--
	<i>P. americana</i>	<i>E. coli</i>	<i>B. pumilus</i>	--	--
Kamonke	<i>P. americana</i>	<i>S. haemolyticus</i>	<i>B. paramycoides</i>	--	--
	<i>B. germanica</i>	<i>B. cereus</i>	--	<i>C. sakazakii</i>	--
Nowshera Virkan	<i>B. lateralis</i>	--	--	<i>K. quasipneumoniae</i>	--
Wazirabad	<i>B. germanica</i>	--	<i>P. thiaminolyticus</i>	--	--
	<i>P. americana</i>	<i>S. maltophilia</i>	<i>B. safensis</i>	--	--
	<i>P. surinamensis</i>	--	--	<i>C. freundii</i>	<i>K. variicola</i>

Ecological and pathogenic diversity of cockroach-associated bacteria

Bacterial isolates obtained across five sub divisions were grouped into human pathogens, environmental bacteria, opportunistic pathogens, and entomophilic species (Table 6). *Periplaneta americana* carried the most diverse bacterial community (ten species), which included important human pathogens such as *P. aeruginosa*, *S. aureus*, and *E. coli*, particularly from hospital and household settings. In contrast, *B. germanica* mainly was associated with environmental and opportunistic bacteria like *B. cereus* and *C. sakazakii*. *B. lateralis* and *P. surinamensis* yielded fewer isolates, which included some opportunistic and potentially insect-associated bacteria, such as *K. variicola* and *S. gallinarum* (Figure 4).

Discussion

This study reports the first record of *Pycnoscelus surinamensis* in Pakistan, expanding its known distribution and contributing to the growing documentation of urban cockroach fauna in tropical and subtropical regions. The detection of four cockroach species across diverse habitats demonstrates their ecological adaptability and capacity to act as mechanical and/or biological vectors of pathogens. The widespread occurrence of *B. germanica* across all habitats and seasons underscores its high adaptation to human-associated environments, consistent with previous findings (Latorre et al., 2022). Its high reproductive rate, ecological plasticity, and resilient gut microbiome, enabling survival in urban settings, likely explain its co-dominance with *P. americana* (Wang et al., 2000). The association of all four species with at least one bacterial pathogen reinforces their potential role in disease transmission, in line with earlier reports from residential, healthcare, and food-handling environments (Correia et al., 2018; Gaire and Romero, 2020; Mehainoui et al., 2021). These findings confirm the growing belief that urban cockroaches act as both mechanical and biological carriers of pathogens (Tatfeng et al., 2005).

Biochemical characterization using catalase, citrate, and indole tests generally aligned with expected metabolic profiles of the respective genera. For example, indole-positive reactions in *E. coli*, *Klebsiella* spp., and *C. freundii* are well-documented, as is citrate utilization in *Pseudomonas*, *Paenibacillus*, and *Bacillus* spp. (Fang et al., 2025). Unexpected catalase-negative reactions in *B. paramycoides* and *S. aureus* may reflect strain-level variation or environmental modulation of gene expression (Burford-Gorst and Kidd, 2024). While useful for preliminary screening, phenotypic assays require molecular confirmation, particularly for environmental isolates with variable traits (Church et al., 2020).

Molecular identification via 16S rRNA sequencing revealed *S. aureus*, *P. aeruginosa*, *K. pneumoniae*, and *E. coli*, pathogens implicated in both hospital- and community-acquired infections due to their virulence and antibiotic resistance (Hailemariam et al., 2021). Their presence in densely populated, unsanitary urban environments highlights significant public health concerns. Notably, *P. americana* harbored the most diverse and resistant strains, supporting prior observations of its importance as a carrier of antibiotic-resistant bacteria (Pai et al., 2005; Islam et al., 2016). The isolation of *K. quasipneumoniae*, *K. pneumoniae*, *B. subtilis*, and *E. coli* in the guts of *B. lateralis* and *P. americana* confirms earlier reports that cockroach guts harbor members of the Firmicutes and Bacteroidetes phyla, often linked to fecal contamination and gastrointestinal infections (Shiota et al., 2010; Makvana and Krilov, 2015).

The isolation of *P. aeruginosa* from *P. americana* and *C. sakazakii* from *B. germanica* underscores the heightened risk of cockroach infestations in healthcare settings (Neuhauser et al., 2003; Jain et al., 2021; Adeyemi et al., 2022). Other opportunistic pathogens, such as *P. thiaminolyticus* and *P. dendritiformis*, further exemplify the microbial diversity in cockroach microbiota that may affect vulnerable populations.

Interestingly, *P. surinamensis*, though less abundant, contained *Klebsiella variicola* and *C. freundii*, both known to cause respiratory, urinary, and intestinal infections (Wang et al., 2000; Garza-Ramos et al., 2018). Environmental isolates such as *Bacillus atrophaeus*, *B. safensis*, and *B. pumilus* were recovered from *P. americana*, and *B. cereus* and *B. paranthracis* from *B. germanica*, indicating exposure to soil- or dust-borne bacteria that can be pathogenic or toxin-producing (Benardini et al., 2003; Malik et al., 2013; Chu et al., 2019). Of particular concern is *B. cereus*, a known foodborne pathogen with notable resistance traits and stress tolerance (Bottone, 2010; Isaac et al., 2014; Abdolmaleki et al., 2019).

Antimicrobial susceptibility testing revealed widespread β -lactam resistance, particularly to cephalosporins (cefexime, ceftriaxone, cefaclor) and amoxicillin, consistent with global trends linked to overuse in human and veterinary medicine (Alraey et al., 2025). Tetracycline resistance, although moderate, is concerning due to its frequent linkage to mobile genetic elements that facilitate horizontal gene transfer (Grossman, 2016). The high proportion of isolates (85%) with a MAR index > 0.2 suggests origin from environments under strong antibiotic pressure such as hospitals, canteens, and wastewater-contaminated areas (Krumperman, 1983; Muwonge et al., 2025).

Widespread β -lactam resistance (e.g., cefexime, amoxicillin) aligns with global trends, while relatively higher susceptibility to fluoroquinolones and tetracyclines may reflect limited local use. While spontaneous mutations can confer resistance, horizontal gene transfer (HGT) is a faster, more influential driver of resistance dissemination in microbial communities.

Overall, these findings demonstrate strong links between cockroach behavior, habitat type, and microbial acquisition, with gut microbiota likely reflecting long-term colonization and surface microbiota indicating recent environmental contact. The potential for HGT amplifies the risk of pathogen spread, particularly in overcrowded, unsanitary urban settings. Integrating cockroach surveillance into vector control and antimicrobial resistance monitoring programs, especially in healthcare and food-handling environments, should be prioritized to mitigate public health risks.

Conclusion

This study identified four cockroach species across five sub divisions of District Gujranwala, with *B. germanica* emerging as the most abundant and *P. surinamensis* reported for the first time in Pakistan. Overall, bacterial isolates exhibited high resistance to cephalosporins and penicillins, while fluoroquinolones showed the lowest resistance rates. The notable prevalence of multidrug-resistant (MDR) strains (35%), most with a Multiple Antibiotic Resistance (MAR) index greater than 0.2, underscores the significant public health risk posed by cockroaches as reservoirs of MDR bacteria. Phylogenetic analysis revealed that twenty strains from the four cockroach species were closely related to pathogenic bacteria, except *B. pumilus*, which is generally considered non-pathogenic or beneficial. Given the higher infestation rates in human dwellings and the associated health hazards, implementation of Integrated Pest Management (IPM) strategies is strongly recommended to reduce cockroach populations and curb the dissemination of pathogenic and MDR bacteria in urban environments.

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Research permit

This study was approved by the Ethical Committee of the University of Gujrat.

Conflicts of interest

The authors have declared no conflict of interest.

Compliance with ethical standards

All efforts were taken to minimize pain and discomfort to the animal while conducting this research according to the ethical protocol of the University of Gujrat.

Author contribution statement

JR and MH presented the idea, conducted the research, and wrote the manuscript. MH, JR, KA, AI, critically reviewed the manuscript. AZ, SS, AN, SHS performed analysis and revised the manuscript.

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