RESEARCH ARTICLE

# Battling Resistance: Understanding Antibiotic Resistance in *Pseudomonas* sp. from Kinniya and Wahawa Hot Springs

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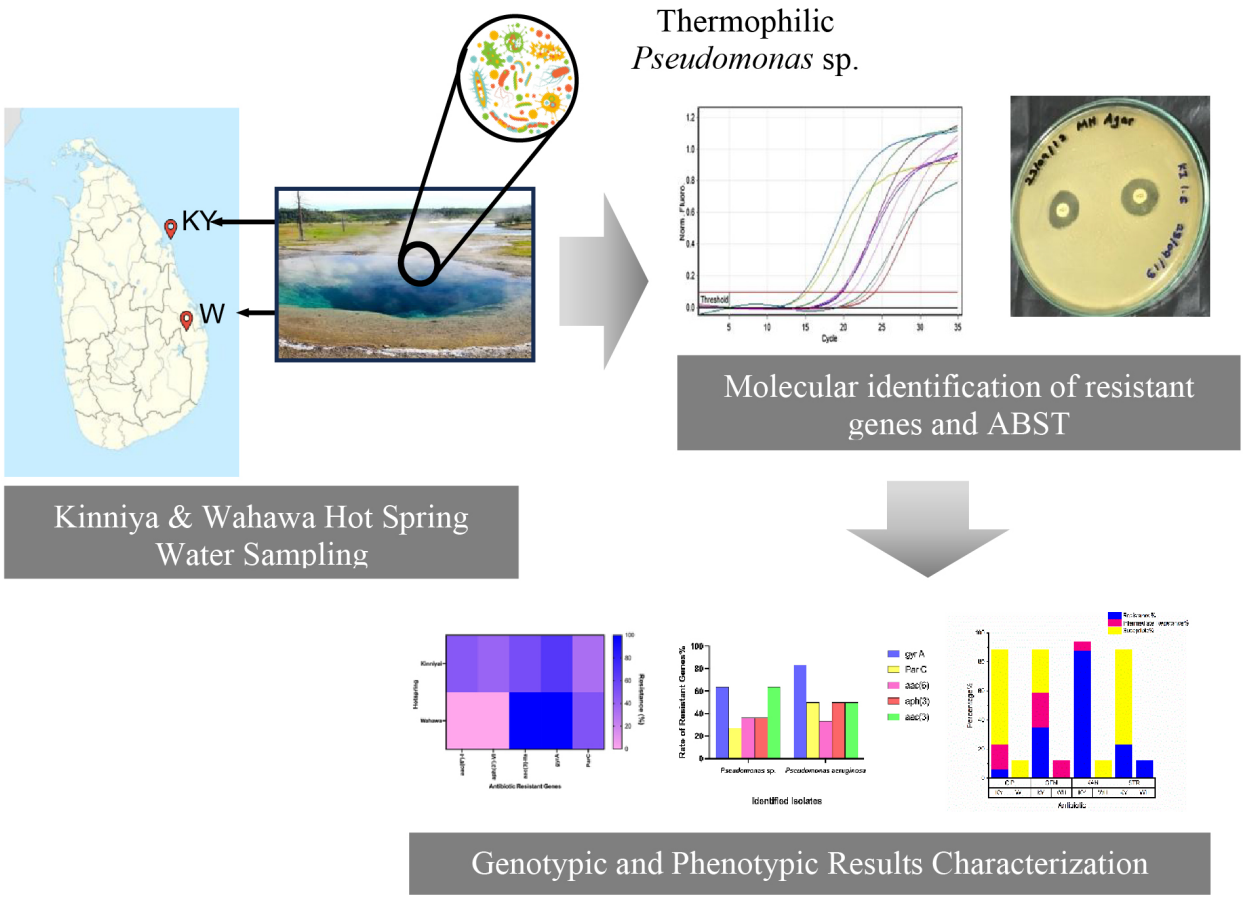


## Highlights

- Antibiotic-resistant *Pseudomonas* spp. were found in both hot springs.
- The *gyrA* gene is frequently linked to resistance against quinolones.
- The *aac(3)-II* gene is frequently associated with resistance to aminoglycosides.
- The *gyrA* and *aac(3)-II* (23.52%) were the most frequent partners of coexistence.

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# Battling Resistance: Understanding Antibiotic Resistance in *Pseudomonas* sp. from Kinniya and Wahawa Hot Springs

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**Abstract:** Certain *Pseudomonas* species can cause human diseases. This study focused on identifying antibiotic-resistant genes (ARG) in *Pseudomonas* species isolated from geothermal hot springs in Sri Lanka, specifically Kinniya (KY)(n=15) and Wahawa (WH)(n=2). Antibiotic sensitivity tests (ABST) were conducted using the Kirby-Bauer disk diffusion method for kanamycin, gentamycin, streptomycin, and ciprofloxacin. Real-time PCR with gene-specific primers was employed to detect aminoglycoside and quinolone-resistant genes (*aph (3')-VI*, *aac (6')-I*, *aac (3)-II, gyrA, ParC*). Out of the 17 examined isolates (*Pseudomonas* sp., n=11; *Pseudomonas aeruginosa*, n=6), *Pseudomonas* sp. from KY displayed resistance to all antibiotics, while WH showed resistance only to gentamycin and streptomycin. Resistance to two or more antibiotics was higher in the genus *Pseudomonas* sp. (75.54%) than in *Pseudomonas aeruginosa* (25.33%). The most frequently identified ARG was *gyrA* (66.66%), particularly in KY isolates. Notably, *aac(3)-II* and *gyrA* were detected in both WH isolates. The *gyrA* is the commonly found quinolone-resistance gene, and *aac(3)-II* is the aminoglycoside resistance gene. The *gyrA* and *aac (3)-II* were the most common co-existing resistance gene pattern (23.52%). *Pseudomonas* sp. exhibited co-existing resistance genes in 58.88% of cases, while 29.41% of *P. aeruginosa* isolates harbored them. Antibiotic-resistant *Pseudomonas* spp. were found in both hot springs, but fewer isolates from Wahawa (n=2) limit clear comparison with Kinniya. KY is more urbanized or populated by tourists than WH; there may be higher exposure to antibiotics from human activities. This study highlights the complexity of antibiotic resistance in *Pseudomonas* species from geothermal hot springs, emphasizing the importance of comprehensive genetic analysis for understanding their resistance patterns.

***keywords*:** *Aminoglycosides; Antibiotic resistance; Hot Spring; Pseudomonas sp.; Quinolone-resistance*

## INTRODUCTION

Extreme environments are hotspots for biodiversity, hosting various microorganisms such as bacteria, archaea, and fungi. Bacteria employ multiple survival strategies when exposed to challenging environmental conditions, often encountering multiple stressors simultaneously (Haruta & Kanno, 2015; Munita et al., 2015; Pednekar et al., 2011). Hot springs represent abundant sources of thermophilic microorganisms, comprising bacteria, archaea, and fungi that have adapted to grow and reproduce in natural geothermal settings (Bumrungthai et al., 2020; Chandrajith et al., 2013; Narsing Rao et al., 2021; Rajapaksha et al., 2014). The extreme conditions engaged by such environments help to trigger the microbial community to develop mechanisms of bacterial tolerance and persistence for survival (Amin et al., 2017; Tang et al., 2018). The ability of thermophilic bacteria to grow in harsh environmental conditions, characterized by high temperatures and minimal human impact, contributes to establishing a robust bacterial population. Sri Lanka has an impressive range of biological diversity, with a varied distribution of geothermal springs. These geothermal springs are situated in two geologically distinct regions in Sri Lanka, along a narrow east lowland belt extending from Hambantota to Trincomalee (Dissanayake & Jayasena, 1988; Rajapaksha et al., 2014).

*Pseudomonas* species are a group of bacteria that are ecologically important. They are characterised by their rod-shaped, aerobic, non-sporulating nature and are classified as gram-negative bacilli. Most *Pseudomonas* species are known to be opportunistic pathogens that can cause diseases in humans. Notably, *P. aeruginosa* and *P. maltophilia* significantly contribute to opportunistic human infections (Muggeo et al., 2023; Reynolds & Kollef, 2021; Ruffin & Brochiero, 2019). The development of multiple resistances in *P. aeruginosa* is due to the production of various virulent factors, such as toxins, enzymes, and extracellular metabolites. The bacterium also utilizes several mechanisms to transfer resistance genes from generation to generation (Arabameri et al., 2021; Sader et al., 2017; Vaziri et al., 2011; Von Wintersdorff et al., 2016). *Pseudomonas aeruginosa* develops antibiotic resistance

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by acquiring resistance genes or undergoing modifications in antibiotic target sites resulting from gene mutations (Mandsberg et al., 2009; Martinez & Baquero, 2000).

Aminoglycosides disrupt bacterial protein synthesis by binding to the site 'A' of 16S rRNA in the bacterial 30S ribosomal subunits (Tsai et al., 2013). Recently, specific genes have emerged as a significant resistance mechanism against clinically important aminoglycosides. These 16S ribosomal RNA methyltransferase genes include *armA, rmtA, rmtB, rmtC*, and *rmtD*.Amikacin is an aminoglycoside that is commonly used to treat Pseudomonal infections (Wachino et al., 2020). However, *P. aeruginosa* is more resistant to amikacin than other aminoglycosides like gentamicin, netilmicin, tobramycin and isepamicin (Tsai et al., 2013). One of the primary mechanisms of aminoglycoside resistance is the inactivation of drugs by aminoglycoside-modifying enzymes that are either plasmid or chromosomally encoded (Kim et al., 2008). In *P. aeruginosa*, the most common gentamicin resistance acetyltransferase gene is *aac(3)-II*, associated with a transposon (Tn801) located on the chromosome (Ahmed et al., 2004; Díaz et al., 2006; Holmes et al., 1974). Mutations in the quinolone-resistance-determining regions (QRDRs) of *gyrA* and *ParC* genes can cause fluoroquinolone (FQ) resistance by altering DNA gyrase or topoisomerase IV in *Pseudomonas* sp. (Xu et al., 2021; Zhao et al., 2020).

Hot springs are essential natural habitats that provide extreme conditions for the growth of thermophiles. These also act as reservoirs of environmental resistors. The emergence of drug-resistant bacterial strains is linked to the increasing global concern over antibiotic resistance in the environment. It is posing a significant threat to public health (Kolář et al., 2001). Globally, antibiotic resistance is becoming more prevalent, decreasing the effectiveness of antibiotics. This highlights the urgent need to develop new antibiotics with innovative mechanisms to combat resistant pathogens. Exploring environmental resistors, especially in less-explored niches like hot springs, holds promise for understanding antibiotic resistance evolution and developing effective infection treatment strategies (Czatzkowska et al., 2022; Fletcher, 2015). While previous research has focused on microbial diversity and distribution in natural geothermal hot springs in Sri Lanka (Rupasinghe et al., 2022; Sadeepa et al., 2022), there needs to be more research on environmental resistomes, particularly antibiotic-resistant genes, and their potential transfer to clinical pathogens. This study aims to fill this gap by expanding our knowledge of environmental resistomes and gaining insights into the mechanisms underlying antibiotic-resistant development in bacteria within the Kinniya (KY) and Wahawa (WH) hot springs of Sri Lanka.

## MATERIALS AND METHODS

The two geothermal hot springs, namely Kinniya (Trincomalee district) and Wahawa (Ampara district), were selected as the study sites. The study analysed 17 preserved bacterial isolates, biochemically identified as belonging to *Pseudomonas*.

### Sample Collection

In January 2019, subsurface water samples were collected from seven wells in Kinniya and the tube well in Wahawa, Sri Lanka. After collection, all water samples were transported to the research laboratory within 12 hours, and bacterial samples were isolated immediately.

### Physicochemical parameters of surface waters in hot springs

The temperature, pH, conductivity, total dissolved solids (TDS), salinity, and dissolved oxygen (DO) of the surface water were measured in situ using a multi-parameter (SyberScan PCD 650, Eutech instrument) (Table 2).

### Confirmation of bacterial isolates as *Pseudomonas* sp.

Thermophilic bacteria isolation and identification were carried out according to the method outlined in Samarasinghe et al. (2021). The stored bacterial isolates belonging to *Pseudomonas* sp. were used for this study. Bacterial DNA was extracted from the cultured isolates in LB (Luria-Bertani) broth by the modified cetyltrimethylammonium bromide (CTAB) method (Somerville et al., 2005).

The tentatively identified isolates were confirmed using polymerase chain reaction (real-time qPCR) with *Pseudomonas* genus-specific and *Pseudomonas aeruginosa* species-specific primers. Positive controls of *P. aeruginosa* (ATCC 27853) were used for all reactions. Additionally, sequencing was performed for three biochemically confirmed isolates to validate the molecular identification. The remaining 14 isolates were confirmed through qPCR assays. All PCRs were conducted using a Rotor-Gene Q PCR cycler (Qiagen) under the following conditions. The PCR reaction mixture was performed in 25 µL total volume consisting of 1 × PCR buffer, 1.5 µL of 25 mM $MgCl_2$, 2.5µL of 1 mM of each dNTP, and 400 nM of each primer, 2x SYBR green in the presence of 1 unit of Taq polymerase enzyme and ~100 ng of the DNA template. As a negative control, 5µL of Milli-Q water was used (Table 1).

### Antimicrobial susceptibility testing

*Pseudomonas* spp. isolates from Kinniya and Wahawa geothermal springs were subjected to antimicrobial susceptibility testing as per the reference guidelines against the following antibiotic classes: aminoglycoside and fluoroquinolone (Weinstein, 2019). Quality control strains, including *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853, were tested alongside the isolates to ensure accuracy of zone diameter measurements. All media, antibiotic disks, and reagents were stored according to the manufacturer's instructions. Tests were conducted in triplicate on separate days to verify reproducibility, and any ambiguous or inconsistent results were retested. Antibiotic sensitivity was tested by disk diffusion on Mueller-Hinton agar plates. The Kirby-Bauer disk diffusion assay (Bauer et al., 1966) was conducted according to Clinical and Laboratory Standards Institute (CLSI) standards using the following antibiotics: kanamycin (30 mg), gentamycin (10 mg), streptomycin (30mg), and ciprofloxacin (30 mg). After

overnight incubation, the inhibition zone diameters were measured. Inhibition zone breakpoints were interpreted according to CLSI standards. Bacterial isolates resistant to more than one antibiotic were considered Multiple antibiotic resistance (MAR) strains, and the MAR index was calculated. The MAR index is calculated as the ratio of the number of antibiotics to which an isolate shows resistance (a) to the total number of antibiotics tested against it (b), expressed as MAR = a/b.

**Antibiotic-Resistant Gene (ARG) screening**

The selected ARGs were further investigated using PCR: aminoglycoside-modifying enzymes (*aac(6')-II, aph(3')-VI, aac(3)-II*); enzymes responsible for fluoroquinolone resistance (*gyrA, ParC*). PCR amplification was performed under the following conditions using gene-specific primers. All PCR assays were conducted using validated primers from peer-reviewed studies, with detailed primer sequences and references provided in Table 1. Each PCR run included positive controls (*Pseudomonas aeruginosa* ATCC 27853 and *Escherichia coli* ATCC 25922) and negative (no template) controls to monitor assay performance and prevent contamination. DNA was extracted following a standardized protocol (Somerville et al., 2005), and concentrations were accurately measured using a fluorometer with a double-stranded DNA-specific assay. PCR conditions, such as annealing temperatures and cycle numbers, were optimized for each primer set to maximize specificity and sensitivity, consistent with published methodologies.

The PCR reaction mixture for each gene was performed in 25 µL total volume consisting of 1 × PCR buffer, 1.5 µL of 25 mM $MgCl_2$, 2.5µL of 1 mM of each dNTP, and 400 nM of each primer, 2X SYBR green in the presence of 1 unit of Taq polymerase enzyme and ~100 ng of the DNA template. As a negative control, 5µL of Milli-Q water was added to the PCR mixture. Positive controls of *P. aeruginosa* (ATCC 27853) and *E. coli* ATCC 25922 were used for all reactions.

**Table 1:** List of Primers used for PCR amplification

| Primer | Nucleotide Sequence (5'-3') | PCR Condition | Reference |
|---|---|---|---|
| **Genus *Pseudomonas*** | $F_W$-TACCTGGCCTTGACATGCTG | 95°C 5 min, [95°C 15 s, 59°C 30 s, 72°C 20 s] 35 times, 72°C 10 min | (Khaksar et al., 2016) |
| | $R_V$-CAGACTGCGATCCGGACTAC | | |
| ***Pseudomonas aeruginosa*** | $F_W$ -CAGGCCGGGCAGTTGCTGTC | 95°C 10 min, [95°C 15 s, 60°C 15 s, 72°C 15 s] 40 times, 72°C 5 min | (Savli et al., 2003) |
| | $R_V$ -GGTCAGGCGCGAGGCTGTCT | | |
| **aac(6´)-I-F** | CGCGCGGATCCCACACTGCGCCTCATGA | 94°C 15 min, [94°C 45 s, 55°C 45 s, 72°C 45 s] 34 times, 72°C 10 min | (Kim et al., 2008) |
| **aac(6´)-I-R** | GACGGGTCGTTTGAATTCTGGTG | | |
| **aph(3´)-VI-F** | GACGACGACAAGGATATGGAATTGCCCAATATTATT | 94°C 15 min, [94°C 1 min, 60°C 1 min, 72°C 1 min] 30 times, 72°C 10 min | (Kim et al., 2008) |
| **aph(3´)-VI-R** | GGAACAAGACCCGTTCAATTCAATTCATCAAGTTT | | |
| **aac (3)-IIaF** | GGGAATTCAGAGGAGATATCGCGATGCATACG | 96°C for 5 min, [ 96°C 30 s, 58°C 30 s] 35 times, 70°C 60 s | (Díaz et al., 2006) |
| **acc (3)-IIaR** | CATTGTCGACGGCCTCTAACC | | |
| **gyrA-F (P)** | AGTCCTATCTCGACTACGCGAT | 94 °C for 5 min, [94 °C 30 s, 58 °C 30 s 72 °C 30 s] 36 times, 72 °C 7 min | (Zhao et al., 2020a) |
| **gyrA-R (P)** | AGTCGACGGTTTCCTTTTCCAG | | |
| **parC-F (P)** | CGAGCAGGCCTATCTGAACTAT | 94 °C for 5 min, [94 °C 30 s, 60 °C 30 s , 72 °C 30 s] 40 times, 72 °C 7 min | (Zhao et al., 2020a) |
| **parC-R (P)** | GAAGGACTTGGGATCGTCCGGA | | |

**Statistical analysis**

Statistical analysis was performed using the SPSS program (Version 11.5, SPSS Inc., Chicago, IL, USA). A *P* value of 0.05 was considered significant. Descriptive statistics were used to calculate the mean and standard error of the mean for antibiotic resistance. Principal component analysis (PCA) implemented with GraphPad Prism (Version 11) was carried out to represent the clustering among *Pseudomonas* species based on the most critical components of antimicrobial resistance patterns of ARG.

**RESULTS AND DISCUSSION**

The study analysed culturable bacterial isolates (n=17) from selected hot springs, with 15 *Pseudomonas* sp. (n=15) from KY and 02 from WH. Table 2 presents the physiological parameters of the selected hot springs. Some isolates were identified at the genus *Pseudomonas* level using genus-specific PCR assays targeting conserved regions of the 16S rRNA gene. That does not always provide sufficient resolution for species-level discrimination. In contrast, other isolates were explicitly identified as *Pseudomonas aeruginosa* using PCR assays targeting species-specific genes using PCR approaches designed for *P. aeruginosa* detection. Therefore, results are reported separately

for *Pseudomonas* spp. (genus-level identification) and *P. aeruginosa* (species-level identification) to accurately reflect the taxonomic resolution achieved with each method. This approach is consistent with prior studies that employ hierarchical PCR identification strategies (Spilker et al., 2004), allowing distinction between broadly detected genera and specifically confirmed species within complex environmental samples. Isolates positive only by genus-specific PCR but negative for *P. aeruginosa* species-specific primers are reported as *Pseudomonas* sp., indicating they likely belong to other *Pseudomonas* species. The three isolates were identified (Isolation code: PAS) as *Pseudomonas aeruginosa* based on sequencing methods, which provide species-level resolution. Three isolates (Isolation code: PAP) were identified as *P. aeruginosa* using species-specific PCR assays. The remaining isolates (Isolation code: PS) were confirmed only to the genus level (*Pseudomonas* sp.) through PCR targeting conserved gene regions insufficient for species-level discrimination (Table 3)

**Table 2:** Description of the sampling sites, hot spring samples, and physicochemical parameters of hot spring water

| Sampling site | GPS data | Sample Code | Temperature(°C) | pH | Electrical conductivity (mS/m) | TDS (ppt) | Salinity (ppt) |
|---|---|---|---|---|---|---|---|
| Wahawa | 7°21.42′N 81°19.33′E | WT | 42.8 | 7.17 | 1.245 | 1.263 | 1.298 |
| Kinniya | 8°36.27′N 81°10.28′E | KI | 39.4 | 6.20 | 0.267 | 0.276 | 0.271 |

Table 3: Antibiotic Resistance Profiles and MAR Index of *Pseudomonas* spp. isolated from Hot Springs, Kinniya (KY) and Wahawa (WH).

| Sample code | Organism | Hot spring | Ciprofloxacin Resistance | | Aminoglycoside Resistance | | | Multidrug Resistance | | | | MAR Index |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | *gyrA* | *par C* | *aac-(6')-I* | *aph-(3') VI* | *aac-(3)-II* | CIP | STR | KAN | GEN | |
| KI1.6(PS) | *Pseudomonas* sp. | KY | + | - | + | + | + | - | - | + | - | 0.25 |
| KI1.7(PS) | *Pseudomonas* sp. | KY | + | - | - | - | + | + | - | + | + | 0.75 |
| KI1.8(PS) | *Pseudomonas* sp. | KY | + | - | - | - | + | - | - | + | - | 0.25 |
| KI2.3(PAP) | *Pseudomonas aeruginosa* | KY | + | + | + | - | - | + | + | + | + | 1 |
| KI2.4(PAP) | *Pseudomonas aeruginoas* | KY | + | + | - | - | + | - | - | + | + | 0.5 |
| KI2.6 (PAS) | *Pseudomonas aeruginosa* (MT113094.1) | KY | - | - | - | + | + | - | - | + | - | 0.25 |
| KI2.7(PAS) | *Pseudomonas aeruginosa* (MT113093.1) | KY | + | + | + | + | - | - | + | + | + | 0.75 |
| KI2.9(PS) | *Pseudomonas* sp. | KY | + | - | - | + | - | - | - | + | + | 0.5 |
| KI2.11(PS) | *Pseudomonas* sp. | KY | + | - | + | + | + | - | - | + | + | 0.5 |
| KI3.2(PS) | *Pseudomonas* sp. | KY | + | - | - | - | + | - | - | + | - | 0.25 |
| KI4.2(PAS) | *Pseudomonas aeruginosa* (MT113099.1) | KY | + | - | - | + | - | - | - | + | + | 0.5 |
| KI5.3(PS) | *Pseudomonas* sp. | KY | - | - | - | - | - | - | - | + | - | 0.25 |
| KI6.3(PS) | *Pseudomonas* sp. | KY | - | + | + | - | - | - | - | + | - | 0.25 |
| KI7.2(PS) | *Pseudomonas* sp. | KY | - | + | - | + | + | + | + | + | + | 1 |
| KI7.3(PS) | *Pseudomonas* sp. | KY | - | - | + | - | - | + | + | + | + | 1 |
| W1(PS) | *Pseudomonas* sp. | WH | + | + | - | - | + | - | + | - | + | 0.5 |
| W2(PAP) | *Pseudomonas aeruginosa* | WH | + | - | - | - | + | - | + | - | + | 0.5 |

**Antimicrobial susceptibility of selected isolates**

*Pseudomonas* spp. Isolates included in this study were widely resistant to kanamycin (~88.23%) and gentamycin (35.29%), as shown in Figure 1(a). The percentages were calculated for the total of isolates. According to the ABST, the selected isolates exhibited higher susceptibility to ciprofloxacin (76.47%) and streptomycin (64.70%). Some isolates exhibited intermediate resistance to kanamycin, gentamycin, and ciprofloxacin.

Although the Wahawa hot spring area comprises approximately 18 natural springs and tubewells, sampling in this study was conducted exclusively from a single tubewell chosen for its minimal human contamination. This approach aimed to ensure the collection of water samples with reduced risk of external microbial contamination. Consequently, fewer *Pseudomonas* isolates were obtained from Wahawa than from Kinniya, which may affect the observed lower antibiotic resistance prevalence in Wahawa isolates (Figure 1(b)). This limitation should be considered when interpreting comparative resistance patterns.

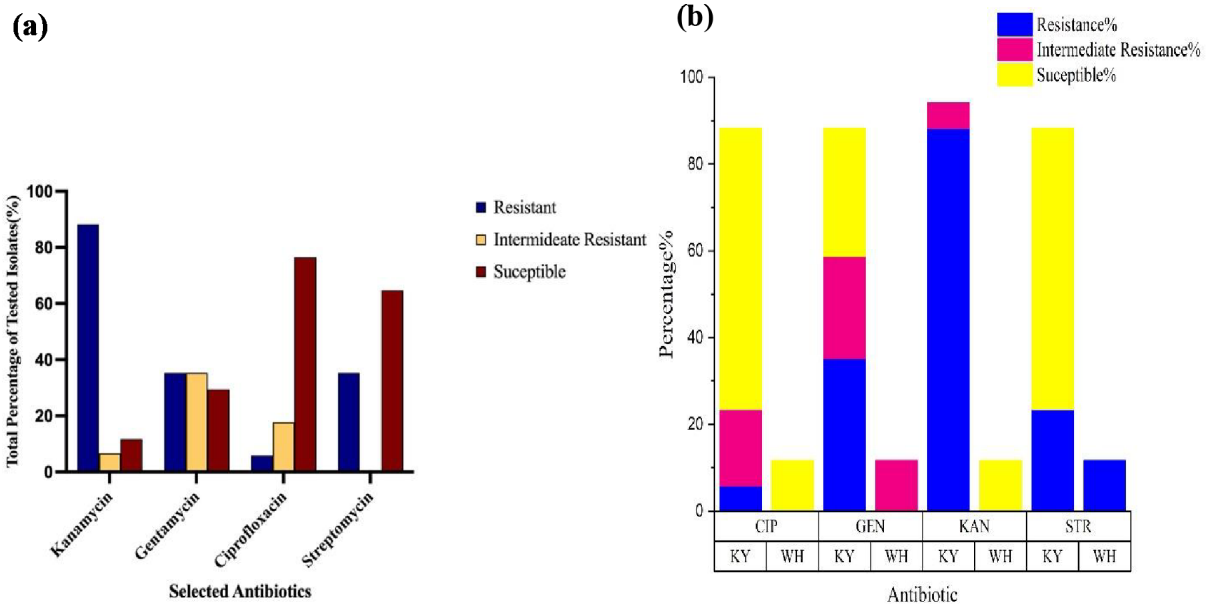


**Figure 1: (a)** Distribution of resistant patterns of all selected isolates in different antibiotics. **(b)** Phenotypic resistance of *Pseudomonas* spp. isolated from each hot spring; KY-Kinniya Hot Spring (n=15); WH- Wahawa Hot spring (n=02); KAN-Kanamycin; CIP- Ciprofloxacin; GEN-Gentamycin; STR-Streptomycin

*Pseudomonas* species, including *Pseudomonas aeruginosa*, are known for their ability to develop resistance to antibiotics through various mechanisms. They possess inherent mechanisms that make them less susceptible to certain antibiotics, known as intrinsic resistance (Poole, 2011). The resistance could be due to the permeability of their outer membrane, efflux pump systems, or antibiotic-modifying enzymes. *Pseudomonas* species possess efflux pump systems that actively exclude antibiotics from the bacterial cell, reducing drug concentration and efficacy (Lorusso et al., 2022). Species can form biofilms, complex communities of bacteria encased in a protective matrix. Biofilms provide a physical barrier that shields bacteria from antibiotics and immune system attacks. It makes it challenging for drugs to reach and eliminate the bacterial cells (Thi et al., 2020). *Pseudomonas* species can produce enzymes that modify or degrade antibiotics, making them ineffective. For example, *P. aeruginosa* can break down beta-lactam antibiotics like penicillin by producing beta-lactamase enzymes (Ahmadian et al., 2021).

It was found that 64.70% of the isolates had a Multiple Antibiotic Resistance (MAR) index greater than 0.25, while 35.29% had a MAR index of $\leq 0.25$. This suggests that more than half of the isolates were likely to be affected by high-risk sources of antibiotics (Table 3). Among the isolates, a significant proportion of *Pseudomonas aeruginosa* (80%) and *Pseudomonas* species (41.17%) exhibited a MAR index value greater than 0.25, with the highest mean value of MAR index (0.677±0.06) observed for these isolates. *Pseudomonas* isolates in Kinniya hot spring were mostly resistant to a single antibiotic, but resistance to up to four antibiotics was also recorded (Figure 2(a)). In the Wahwa hot spring, resistance was observed for only two antibiotics, gentamycin and streptomycin, which are aminoglycosides. The overall frequency of MAR strains was 60%, with the highest occurrence in the Kinniya hot spring.

The percentage of resistance for each bacterial species was assessed to evaluate the extent of isolates resistant to multiple antibiotics. Approximately 23.52% of isolates showed resistance to two or more antibiotics. Figure 2(b) illustrates that the *Pseudomonas* sp. (75.54%) category was the most prevalent among these drug-resistant organisms.

Bacteria can develop resistance to multiple antibiotics through various mechanisms. One such mechanism involves acquiring specific genes that encode proteins capable of transporting or deactivating drugs, thereby reducing the effective drug concentration within the bacterial cell. Additionally, mutations in bacterial genetic material can alter antibiotic target sites, decreasing drug effectiveness and contributing to resistance (Afunwa et al. 2020).

**Resistance gene profiles among the tested isolates**

Table 4 shows the genotypic resistance of *Pseudomonas* spp. to different antibiotic-resistance genes. The highest frequency of genetic determinants for ciprofloxacin resistance was detected in Kinniya samples, and in Wahwa samples, it was detected for both ciprofloxacin and gentamycin. Despite the low abundance of phenotypically resistant isolates, ARGs in *gyrA* (66.66%) and *ParC* (33.33%) displayed resistance to ciprofloxacin in the Kinniya hot spring. ARGs for gentamicin resistance were found in 53.33% of KY and 100% of WH strains, but phenotypically in 35.29% of all strains in both hot springs. PCR testing revealed that 40% of KY strains carried *aac(6')-I* and 46.66% had *aph(3')-VI* genes for amikacin resistance, while all WH strains were susceptible to these two genes. Phenotypically, 93.33% showed resistance to kanamycin, which was used as an alternative to amikacin (Figure 3(a)).

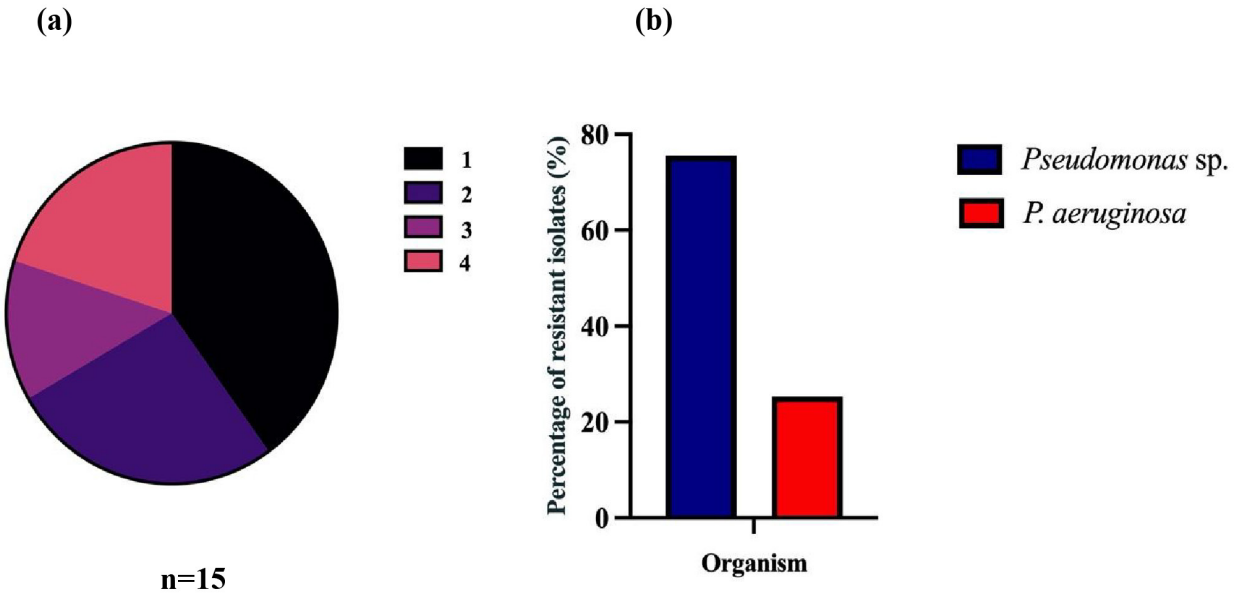


**Figure 2: (a)** Degree of antimicrobial resistance was expressed by the number of antibiotics (ranging from 1 to 4) to which *Pseudomonas* sp. isolates from the Kinniya Hot Spring showed resistance. "n" represents the total number of isolates for each sampling site. **(b)** Frequency of resistance to two or more antibiotics among the two identified groups of *Pseudomonas* organisms

**Table 4:** Genotypic resistance of *Pseudomonas* sp.

| Antibiotic | ARG | Genotypic resistant isolates, number (%) | |
|---|---|---|---|
| | | Kinniya | Wahawa |
| Amikacin | *aac (6')-I* | 6(40) | 0(0) |
| | *aph (3')-VI* | 7(46.66) | 0(0) |
| Gentamycin | *aac (3)-II* | 8(53.33) | 2(100) |
| Ciprofloxacin | *gyrA* | 10(66.66) | 2(100) |
| | *ParC* | 5(33.33) | 1(50) |

The percentages of antibiotic resistance genes were assessed to observe the resistance gene spectrum among different bacterial isolates. As of Figure 3(b), *Pseudomonas aeruginosa* strains harbored all of the resistance genes analyzed to a greater extent than the *Pseudomonas* sp. Some *P.aeruginosa* isolates carried four selected genes except *aac(3)-II.* Figure 3(c) shows the inconsistencies among the isolated organisms for the ARG and antibiotic disk analyses. Of the 17 isolates tested, 58.88% harbor any of the genes tested for ciprofloxacin resistance, even

though they were phenotypically resistant by disk diffusion. Nevertheless, 29.41 % of the total isolates were observed to be phenotypically inconsistent, although these isolates harbored resistance genes for gentamycin resistance. Of the isolates, 64.70% had matching consistency regarding genotypes and phenotypes for amikacin resistance. Co-presence of multiple resistance genes is a big concern in the spread of antibiotic resistance.

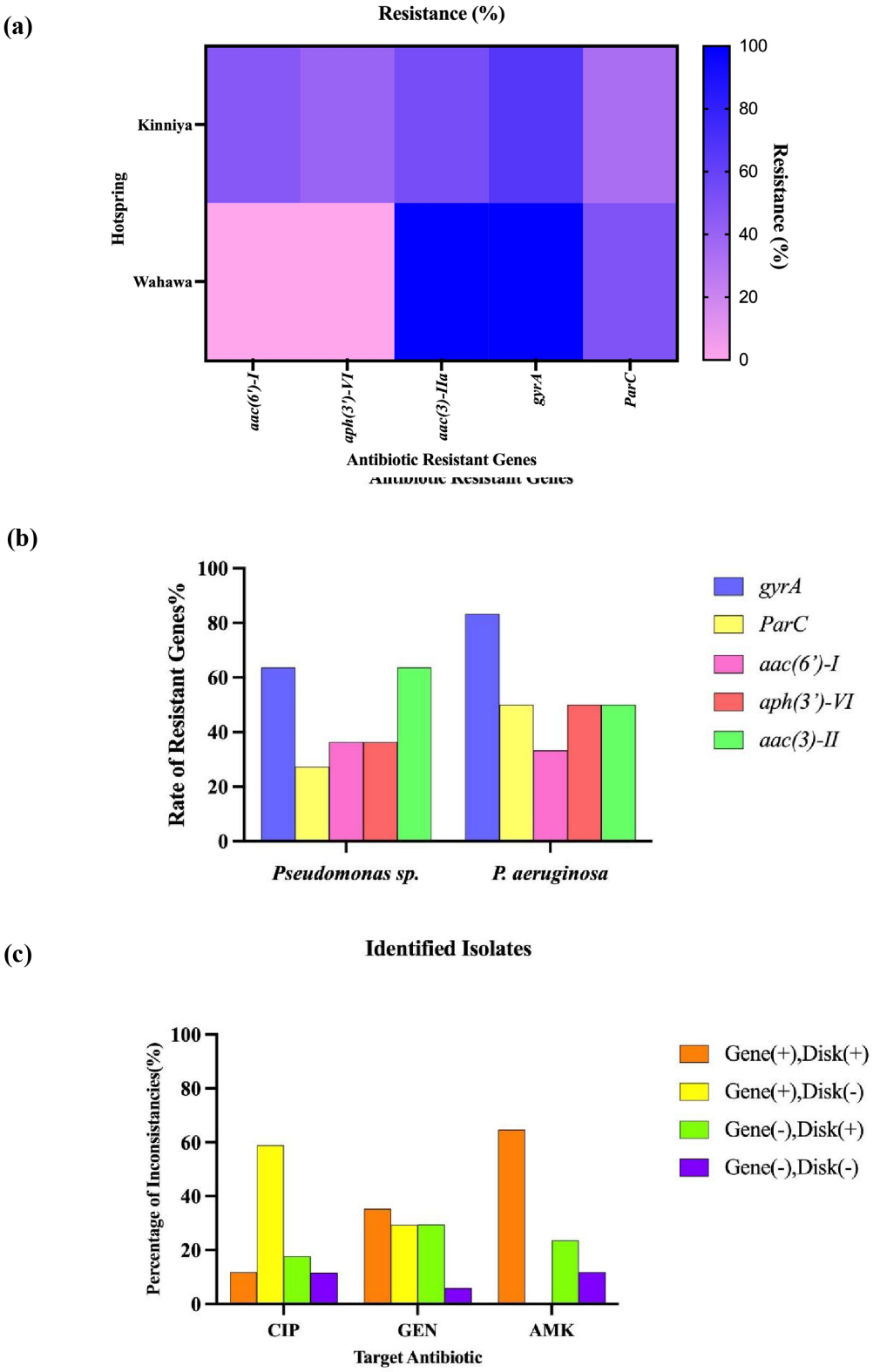


**Figure 3: (a)** Heatmap analysis showing the clusters of antibiotic resistance profiles and the resistant genes in Kinniya versus Wahawa geothermal springs. **(b)** Distribution of *aac(6')-I, aac(3)-II, aph(3')-VI, gyrA,* and *ParC* genes among the selected isolates.**(c)** Existing inconsistencies among the isolated organisms (CIP) Ciprofloxacin;(GEN) Gentamycin;(AMK) Amikacin

The combinations of resistance genes can vary widely, and new combinations may emerge due to selective pressures, horizontal gene transfer, and the adaptation of bacteria to different environments (Begum & Shamsuzzaman, 2016). PCR detects specific resistance genes in bacterial genomes, but a resistance gene does not always indicate phenotypic resistance, as other factors can influence gene expression (Bajpai et al., 2019). This could be one reason for the difference in phenotypical and genotypical resistance patterns among the selected KY and WH hot springs isolates. *Pseudomonas* species are known for their genetic diversity. Different strains within the same species may carry unique sets of resistance genes or mutations, leading to variations in antibiotic resistance profiles. PCR may detect the presence of specific resistance genes, while ABST measures the actual phenotypic resistance expressed by the bacteria. ABST assesses the phenotypic resistance of bacteria by exposing them to antibiotics and observing their growth response.

*Pseudomonas* species are capable of horizontal gene transfer, meaning they can acquire resistance genes from other bacteria. This transfer may not be immediately reflected in phenotypic resistance but can be detected through PCR. As a result, a strain may carry resistance genes without expressing resistance in ABST (Von Wintersdorff et al., 2016). *Pseudomonas* species have relatively high mutation rates, allowing them to adapt rapidly to changing environments. Mutations in genes associated with antibiotic resistance may lead to phenotypic resistance that is not detectable by PCR targeting specific resistance genes (Mandsberg et al., 2009). The regulation of gene expression plays a crucial role in antibiotic resistance. PCR typically detects the presence of genes but may not provide information on their expression levels. Differences in gene regulation can lead to variations in phenotypic resistance (Cabot et al., 2016). *Pseudomonas* species can exhibit adaptive resistance, where exposure to sublethal concentrations of antibiotics can induce temporary resistance. Such adaptive responses may not be captured by PCR targeting specific resistance genes (Kim et al. 2008). These reasons may help to show the phenotypical and genotypical antibiotic resistance differences in the results of this study.

Further statistical analysis was performed to compare their ARG patterns. In the PCA (Figure 4), the first two components account for 74% variability. Table 5 details the corresponding values of these components(PC1 and PC2) for each gene. The *aac-(3)-II*(0.916), *gyrA*(0.815) and *ParC*(0.923) have a strong influence on PC1, and *aac-(6')-I* (0.419), *aph(3')-VI*(0.849) and *aac-(3)-II*(0.298) on PC2, respectively.The biplot shows a higher presence of *aac(3)-II*, *ParC*, and *gyrA* genes in the WH hot spring compared to *aac(6')-I* and *aph(3')-VI* genes. Similarly, *aac(3)-II, aac(6')-I*, *aph(3')-VI*, and *ParC* genes are commonly present in KY hotspring isolates compared to the *gyrA* gene**. Fluoroquinolone** resistance

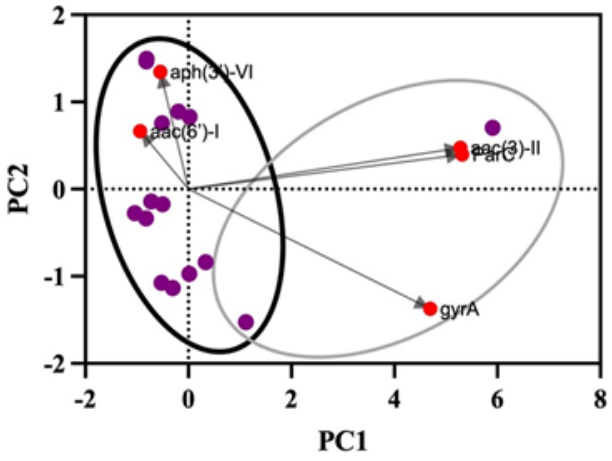


**Figure 4:** Principal component analysis (PCA) of antimicrobial resistance genes for Kinniya hot spring (black circle) and Wahawa hot spring (grey circle)

The ABST results showed high susceptibility to ciprofloxacin (76.47%). However, when considering the genotypic analysis, with the two genes considered, *gyrA* and *ParC*, the *gyrA* gene showed the highest resistance (66.66%) compared to *ParC*. Resistance to ciprofloxacin in *Pseudomonas aeruginosa* is often associated with mutations in the genes *gyrA* and *ParC*, which encode DNA gyrase and topoisomerase IV subunits, respectively. These enzymes are essential for bacterial DNA replication and repair. Mutations in these genes can alter the target site for ciprofloxacin, leading to reduced binding and efficacy of the antibiotic.

Ciprofloxacin primarily targets DNA gyrase, which is encoded by the *gyrA* gene. It works by inhibiting the activity of DNA gyrase, which prevents the supercoiling of bacterial DNA. However, mutations in the *gyrA* gene can directly affect the binding site of ciprofloxacin, reducing its effectiveness. DNA gyrase plays a crucial role in the unwinding and supercoiling of DNA during replication. Mutations in the *gyrA* gene can alter the enzyme's structure, affecting its interaction with ciprofloxacin and promoting resistance (Xu et al., 2021; Zhao et al., 2020).

**Table 5:** Loadings of individual antibiotic resistance genes (ARGs) on the first two principal components (PC1 and PC2)

| Gene | PC1 | PC2 |
|---|---|---|
| *aac(6')-I* | -0.423 | 0.419 |
| *aph(3')-VI* | -0.247 | 0.849 |
| *aac(3)-II* | 0.916 | 0.298 |
| *gyrA* | 0.815 | -0.144 |

Mutations in *gyrA* are known to impact ciprofloxacin resistance significantly (Zhao et al., 2020). Even a single point mutation in *gyrA* can substantially reduce the binding affinity of ciprofloxacin to DNA gyrase, leading to higher resistance levels. Resistance often develops sequentially, with *gyrA* mutations occurring before *ParC* mutations. The initial mutations in *gyrA* may confer a certain level of resistance, and later mutations in *ParC* may further contribute to resistance. Therefore, when evaluating resistance, mutations in *gyrA* may be observed more frequently (Arabameri et al., 2021). Specific amino acid residues in the *gyrA* gene are crucial for binding ciprofloxacin. Mutations at these residues, such as Ser83 or Asp87, can significantly impact ciprofloxacin susceptibility (Mahapatra et al., 2022). DNA gyrase is the primary target of fluoroquinolone resistance mechanisms in *Pseudomonas aeruginosa*. Mutations in *gyrA* are frequently linked to high-level resistance, making it a key determinant. According to the study results, although all isolates showed high susceptibility (76.47%) to ciprofloxacin in ABST, most isolates presented the *gyrA* gene (66.66%) in their genome, compared to the *ParC* gene (33.33%) for fluoroquinolone resistance.

**Aminoglycoside resistance**

Amikacin and gentamycin belong to the aminoglycoside class of antibiotics and are commonly used to treat serious infections caused by gram-negative bacteria, including *Pseudomonas.* Aminoglycoside-modifying enzymes, such as acetyltransferases, phosphotransferases, and adenyltransferases, can chemically modify the structure of aminoglycoside antibiotics. This modification can make the drugs inactive and contribute to resistance (Poole, 2005). The *aac(3)-II* genes typically encode an aminoglycoside acetyltransferase, which adds acetyl groups to the aminoglycoside molecule. This modification alters the antibiotic's structure and reduces its binding affinity to the bacterial ribosome (Riccio et al., 2003). On the other hand, the *aph(3')-VI* gene typically encodes a phosphotransferase that adds a phosphate group to the antibiotic (Zeng & Jin, 2003). Acetylation (*aac(3)-II*) can have a more significant impact on gentamicin resistance compared to phosphorylation (*aph(3')-VI*). Aminoglycoside-modifying enzymes may exhibit substrate specificity, meaning they prefer certain aminoglycosides. The acetyltransferase encoded by *aac(3)-II* has higher activity against gentamicin than the phosphotransferase encoded by *aph(3')-VI* (Ahmed et al., 2004). Based on the results, it has been found that most isolates displayed a higher resistance to the *aac(3)-II* gene (53.33%) compared to the amikacin resistance genes. Even the WH isolates showed complete resistance (100%) to the *aac(3)-II* gene despite being completely susceptible to amikacin.

Some modifying enzymes may have a broader range of substrates, while others may be more specific. If *aac(3)-II* is more efficient at modifying gentamicin, it could result in higher resistance to gentamicin than amikacin (Holmes et al., 1974). Aminoglycoside-modifying enzymes may have varying affinities for different aminoglycoside substrates. If the acetyltransferase encoded by *aac(3)-II* has a higher affinity for gentamicin than the phosphotransferase encoded by *aph(3')-VI*, it could lead to more effective modification and higher resistance (El-Far & Abukhatwah, 2023). *Pseudomonas* strains may possess multiple resistance mechanisms simultaneously.

The presence of *aac(3)-II* in combination with other resistance mechanisms may result in a cumulative effect on gentamicin resistance, influencing the overall resistance level (Díaz et al., 2006; Holmes et al., 1974). These isolates show higher gentamycin resistance than amikacin resistance, possibly due to these genotypic factors.

The *aph(3')-VI* gene typically encodes a phosphotransferase enzyme, which adds a phosphate group to the amikacin molecule. This modification alters the antibiotic's structure and reduces its binding affinity to the bacterial ribosome. On the other hand, the *aac(6')-I* gene encodes an acetyltransferase enzyme, which adds acetyl groups to the antibiotic (Sader et al., 2017; Vaziri et al., 2011). Phosphorylation (*aph(3')-VI*) has a more significant impact on amikacin resistance compared to acetylation (*aac(6')-I*), potentially leading to higher levels of resistance. This could be why there is higher resistance to the *aph(3')-VI*, 46.66% gene, compared to the *aac(6')-I* gene in amikacin resistance in KY hot springs.

The results of the co-presence of resistance genes are displayed in Table 6. Out of 17 strains, a total of 15 strains were revealed to possess the co-presence of resistance gene combinations. Specifically, five strains of *P. aeruginosa* and ten strains of *Pseudomonas* sp. were identified with co-existence of resistance genes. In *Pseudomonas* isolates analysed, the co-existence of *gyrA* and *aac(3)-II* (n=3) was recognized as the most prevalent combination. *P. aeruginosa* isolates that had been analysed showed co-presence of resistance genes in different combinations. *P. aeruginosa* isolate was seen to contain multiple aminoglycoside resistance genes (*aph (3')-VI, aac (3)-II,* and *aac(6')-I*). The data demonstrate that *Pseudomonas* sp. has a common phenomenon (58.88%) of harbouring coexisting resistance genes, whereas only 29.41% of *P.aeruginosa* isolates carried co-existence of resistance genes.

Bacteria, such as *Pseudomonas*, can develop genetic mutations over time that alter the structure of target proteins involved in antibiotic binding, reducing the effectiveness

of antibiotics (Ahmadian et al., 2021). The horizontal gene transfer mechanisms of *Pseudomonas* species can acquire resistance genes. This includes the transfer of resistance genes via plasmids, transposons, or other mobile genetic elements from other bacteria. This facilitates the rapid spread of antibiotic resistance within bacterial populations (Michaelis & Grohmann 2023; Von Wintersdorff et al., 2016). The widespread and improper use of antibiotics in clinical settings, agriculture, and other sectors creates selective pressure on bacteria. Over time, this selective pressure favours the survival and proliferation of antibiotic-resistant strains, including resistant *Pseudomonas* species (Alonso et al., 1999; Boyer et al., 2011; Kolář et al., 2001). *Pseudomonas* species, particularly those found in environmental reservoirs like water sources, may encounter low levels of antibiotics due to human activities (e.g., discharge of pharmaceuticals and tourism). Exposure to sub-lethal antibiotic concentrations can contribute to the development of resistance over time (Panahi et al., 2020).

**Table 6:** Detection of the presence of co-resistance genes in the tested organisms

| Co-existence-resistant gene pattern | Number of isolates | | % of co-existence resistant gene pattern |
|---|---|---|---|
| | *Pseudomonas* sp. | *Pseudomonas aeruginosa* | |
| Only one gene | 1 | - | 5.88% |
| Without any gene | 1 | - | 5.88% |
| *gyrA,aac(3)-II* | 3 | 1 | 23.52% |
| *gyrA,aph(3')-VI* | 1 | 1 | 11.76% |
| *ParC, aac (6')-I* | 1 | - | 5.88% |
| *aac (3)-II, aph (3')-VI* | - | 1 | 5.88% |
| *gyrA,ParC, aac(3)-II* | 2 | - | 11.76% |
| *gyrA, ParC, aac (6')-I* | - | 1 | 5.88% |
| *ParC, aph (3')-VI, aac (3)-II* | 1 | - | 5.88% |
| *gyrA, aph (3')-VI, aac (3)-II, aac (6')-I* | 2 | - | 11.76% |
| *gyrA,ParC, aac(6')-I, aph(3')-VI* | - | 1 | 5.88% |
| All selected genes | - | - | 0 |


**Figure 5** **(a)** Map of Sri Lanka showing the distribution of hot water springs. **(b)** KY-Kanniya(41.7°C) **(c)** WH-Wahawa (43.4 °C)

This image (Figure 5) visually compares the Wahawa and Kinniya hot springs, highlighting the influence of human activities on these natural geothermal sites. According to the study results, the Kinniya hot spring shows high antibiotic resistance to kanamycin (88.23%), gentamycin (35.29%), streptomycin (23.52%), and ciprofloxacin (5.88%). Human activities near Kinniya Hot Spring may contribute to higher antibiotic resistance. Kinniya Hot Spring is more urbanized or populated by tourists than Wahawa Hot Spring; there may be higher exposure to antibiotics from human activities. Urban areas and tourism hubs can be sources of antibiotic residues, contributing to the selection of antibiotic-resistant strains. Different geographical regions may have unique antibiotic usage patterns in medical, agricultural, or industrial practices, influencing the prevalence of antibiotic resistance. The resistance patterns among the Wahawa isolates may be reflected in less urban development, industrial activities, or other anthropogenic sources of pollution.

## CONCLUSION

Data obtained from Kinniya and Wahawa, hot springs in Sri Lanka, showed phenotypical and genetic antibiotic resistance of *Pseudomonas* species. This study reports that antibiotic-resistant *Pseudomonas* isolates were present in both the Wahawa and Kinniya hot springs. Antibiotic-resistant *Pseudomonas* spp. were found in both hot springs, but fewer isolates from Wahawa (n=2) limit clear comparison with Kinniya. It is the first report on genetic resistance to antibiotics in *Pseudomonas* spp. found in both Wahawa and Kinniya hot springs. However, among selected isolates, *Pseudomonas* sp. showed resistance to two or more antibiotics than *Pseudomonas aeruginosa*. The *Pseudomonas* species found in KY carry antibiotic resistance genes, including aminoglycoside-modifying enzymes (*aac(6')-I, aph(3')-VI, aac(3)-II*) and enzymes responsible for fluoroquinolone resistance (*gyrA, ParC*). It appears that WH is carrying aminoglycoside-modifying enzymes *aac(3)-II*. Additionally, WH has enzymes responsible for fluoroquinolone (FQ) resistance: *gyrA* and *ParC*. The *gyrA* gene is commonly found among the selected two quinolone-resistance genes and *aac(3)-II* for aminoglycoside resistance. The *gyrA* and *aac(3)-II* (23.52%) were the most frequent partners of coexistence. In comparison to *P.aeruginosa* isolates, *Pseudomonas* sp. harbours more multiple resistance genes. Monitoring anthropogenic activities regularly, thoroughly, and aggressively is critical to preventing antibiotic resistance among environmental microbial communities.

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## *DECLARATION OF CONFLICT OF INTEREST*

*The authors confirm that they have no conflicts of interest to declare.*

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## AUTHOR CONTRIBUTION

Conceptualization: **D.N.M**., **R.P.W.**; Methodology: **H.M.S.A.T.G.**, **D.G.S.N.S., T.M.U.E.K.S.**; Statistical analysis: **H.M.S.A.T.G.;** Investigation**: D.N.M.**, **R.P.W.**; Writing - Original draft preparation: **H.M.S.A.T.G.;** Manuscript Review and editing: **D.N.M**.; Overall Supervision**: D.N.M.**

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