Infection and Drug Resistance

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ORIGINAL RESEARCH

Monitoring Multi-Drug Resistant *Klebsiella pneumoniae* in Kitagata Hot Spring, Southwestern Uganda: A Public Health Implication

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Background: The concerning frequency of *K. pneumoniae* in various recreational settings, is noteworthy, especially regarding multi-drug resistant (MDR) strains. This superbug is linked to the rapid spread of plasmids carrying these resistance genes. The objective of this study was to evaluate the spatiotemporal prevalence of MDR-*K. pneumoniae* in the Kitagata hot spring, Southwestern Uganda.

Methods: A laboratory-based descriptive longitudinal study was conducted between May and July 2023. During rainy and dry seasons, we collected eighty water samples in the morning and evening from the hot spring. The temperature at each point was measured prior to sample collection, and two samples were obtained at varying depths. 5 mL of each homogenized sample were pre-enriched in brain heart infusion broth, and subsequently in both blood and violet red bile agar. The Kirby-Bauer disk diffusion method was performed, followed by the detection of carbapenemase (CR) and extended-spectrum β-lactamase (ESBL) production. Polymerase chain reaction showed resistance genes *viz.* bla_{TEM}, $bla_{\text{CTX-M}}$ and bla_{KPC}. Data were analyzed using SPSS-20 to obtain chi-square tests and regression analysis.

Results: *K. pneumoniae* accounted for 30.0% of isolates obtained from Kitagata hot springs, with all isolates classified as multi-drug resistant. All isolates were resistant to ampicillin, rifampicin, ceftazidime, and azithromycin (79.2%). Additionally, 95.8% of isolates harbored bla_{TEM} gene alone and both bla_{TEM} and bla_{CTX} genes, followed by bla_{KPC} alone (33.3%), with 25% harboring all three resistance genes. During the dry season, *K. pneumoniae* had a higher prevalence (35.0%) compared to the wet season (25.0%). The prevalence of MDR-*K. pneumoniae* significantly increased over the course of the study. The presence of the three studied resistance genes in the isolates showed a positive correlation with the second phase of sample collection and the dry season but exhibited a negative correlation with temperature, except for isolates harboring either bla_{TEM} alone or $bla_{\text{TEM+KPC+CTX}}$ genes.

Conclusion: Kitagata hot spring serves as a hotspot for continuous dissemination and acquisition of MDR-*K. pneumoniae* harboring resistance genes that encode for ESBL and CR production. The healthcare sector ought to implement an ongoing monitoring and surveillance system as well as robust antimicrobial resistance stewardship programs aimed at delivering health education to the community.

Keywords: antimicrobial resistomes, one health, first generation genomics, Kitagata healing hot spring, MDR-*K. pneumonia*

Introduction

The rising emergence of AMR bacteria has placed a substantial burden on patients and healthcare systems, particularly in low- and middle-income countries.[1] Among gram-negative bacteria, MDR-*K. pneumoniae* remains classified as one of

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the leading priority superbugs accountable for clinical infections.[2] *K. pneumonia*, a Gram-negative rod-shaped bacterium, has dimensions of approximately 2 µm by 0.5 µm. It possesses a capsule, lacks motility, and can ferment lactose. This organism is a facultative anaerobe and belongs to the *Enterobacteriaceae* family.[3]

Klebsiella pneumoniae has been rated among the major causes of several opportunistic and fatal hospital-acquired infections (HAIs) and community-acquired infections, including neonatal septicemia, wound infections, nasopharyngeal infections, pneumonia, meningitis, gastrointestinal tract infections, and urinary tract infections.[4,5] Recently, it has surpassed *Escherichia coli* as the leading cause of liver abscesses.[6] According to a systematic review conducted in Africa by the World Health Organization (WHO) in 2021, *K. pneumoniae* has been responsible for 29% of bloodstream infections.[7]

Globally, 84% of *K. pneumoniae* infections have been reported, while 55.9% in low-income countries have been registered.[8] In Africa, the frequency of recurring outbreaks of HAIs caused by *K. pneumoniae* has risen to an estimated rate ranging from 3% to 15%, in contrast to 7.1% among European countries.[9] Findings from studies conducted in Uganda have revealed a prevalence of 18.2% in the Eastern region,[10] while in Southwestern Uganda, rates of 36%, 12.57%, and 11.6% have been documented in Kabale District,[11] Mbarara District,[3] and Bushenyi Districts,[12] respectively.

The World Health Organization has identified *K. pneumoniae* as the most significant global health threat, with a prevalence rate exceeding 80%. Worldwide, mortality rates from MDR infections are projected to surpass 9 million deaths annually, with estimated costs exceeding US$100 trillion by 2050.[13] The increasing concern is particularly focused on CR and ESBL producing *K. pneumoniae*, which are now acknowledged as "infections of concern" for low-income countries.[7] Similarly, studies done in East Africa have reported a rising prevalence of CR (18.2%) and ESBL (13.1%) in *K. pneumoniae* isolates obtained from humans, livestock, wildlife, and the environment.[14] In Uganda, a nationwide prevalence of 23.3% (CR) and 46.3% pathogenic *K. pneumoniae*, identified through capsular typing was documented, with 44.1% reported in Bushenyi district.[7] Likewise, Baguma et al[11] reported an 89% prevalence of ESBL-producing *K. pneumoniae* in Southwestern Uganda. The concerning levels of AMR in Uganda are linked to the rapid increase in unregulated drug use and informal drug dispensing practices.[15]

Other studies done in Southwestern Uganda have showed highest levels of distribution of ESBL genes, mainly bla_{SHV}, $bla_{TEM,}$ and $bla_{CTX\text{-}M,}$[14] and CR genes, including bla_{IMP}-type (19.4%), bla_{KPC} (14.0%), bla_{VIM} (17.1%), $bla_{NDM\text{-}type}$ (13.2%) and $bla_{OXA\text{-}48}$ genes (36.4%), among *Klebsiella* sp. isolates obtained from both clinical and environmental samples.[7] These genes are carried by mobile genetic elements and plasmids, facilitating their transfer to other bacteria and promoting dissemination into the environment.[14] Furthermore, this study highlighted the high rate of intercontinental, cross-border, and local transmission of these resistance genes by visitors or tourists, primarily at recreational sites.

At present, Kitagata hot springs stand as tourist destinations in Southwestern Uganda, drawing both local and international visitors in search of adventure and the therapeutic benefits of the water post-steam bath (with spiritual benefits attributed to the hot springs). However, previous studies conducted in Ihimbo and Kitagata hot springs in Southwestern Uganda reported *Klebsiella* species as the predominant thermophilic pathogenic bacteria[16] and fecal coliforms.[17] Therefore, Kitagata hot springs continue to be significant locations for the potential spread and acquisition of *K. pneumoniae* superbugs among tourists and individuals with severe illnesses who partake in bathing in the geothermally heated groundwater.

Additionally, the increasing prevalence of AMR bacteria in hot springs located in developing nations may stem from poor sewage management, unsanitary environments, and insufficient hygiene practices.[18] This ultimately leads to heightened morbidity and extended hospital stays, potentially accompanied by elevated mortality rates within the human population.[19]

It should be noted that travelers serve as significant vectors for resistant bacteria and play a pivotal role in shaping the geographical patterns of AMR across regions, in contrast to national levels of antimicrobial usage.[20] Furthermore, both human and animal health serve as significant drivers for AMR, with the enduring impact of environmental factors also playing a crucial role. This interconnectedness is widely recognized as a "One Health" approach.[13] Nonetheless, Uganda grapples with a shortage of published studies adopting a One Health approach to address antimicrobial resistance (AMR). This is exacerbated by a deficiency in dependable and timely information, potentially hindering epidemiological surveillance and effective stewardship efforts.[15] Currently, there is insufficient information available regarding the occurrence and spatiotemporal prevalence of MDR-*K. pneumoniae* in Kitagata hot springs, Bushenyi district. Therefore, this study is necessary to address this knowledge gap.

Materials and Methods

Study Design

This study was a descriptive longitudinal laboratory investigation that collected quantitative data to evaluate the spatiotemporal prevalence of MDR-*K. pneumoniae* in the Kitagata hot spring, Sheema district. The study was conducted between May and July 2023.

Study Area

Water samples were collected from Kitagata hot spring (Figure 1), located along Ishaka-Kagamba road in Sheema South County, Sheema district, Southwestern Uganda (0°40'42.0" S, 30°09'38.0" E). The spring is situated approximately 2 km

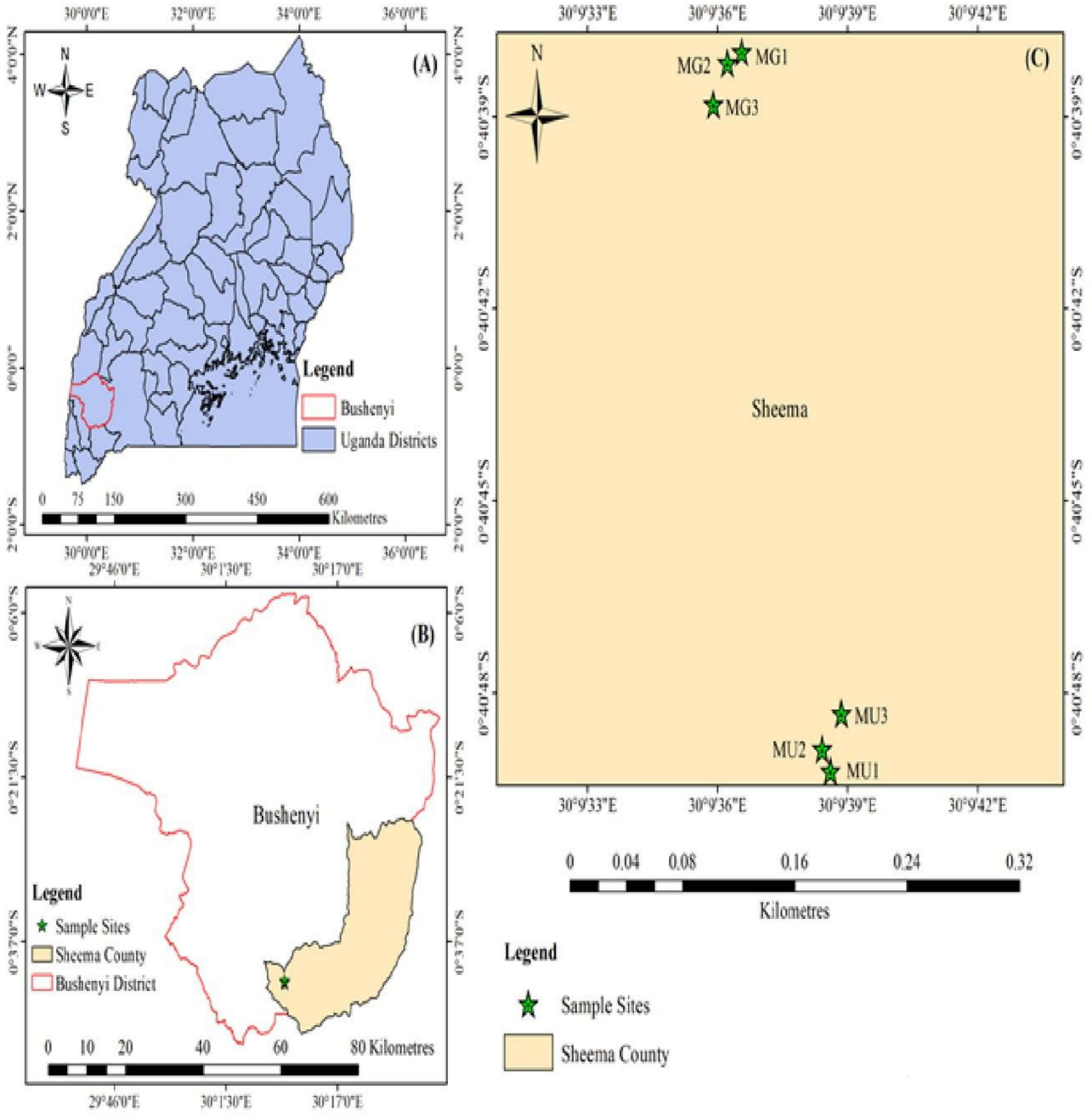

Figure 1 Map of Sheema district showing the study area (Kitagata hot spring).

southeast of Kitagata town, bordering Bushenyi district to the west, around 62 km west of Mbarara district by road, and 350 km from Kampala, the capital city. The hot spring, referred to locally as "Mulago" after Uganda's largest National Referral Hospital, is believed to possess healing properties and attracts over 200 daily visitors, both local and international. It stands as one of the region's primary tourist attractions. The Kitagata Mulago hot spring emits geothermally heated water reaching temperatures of 80 °C (176 °F) and also receives water from the Ngaromwenda River, which occasionally floods it during the rainy season. Situated close to a swamp, the hot spring boasts breathtaking scenery, with conical hills and inselbergs adorning its surroundings.[21]

Study Site

The water samples were analyzed at the Microbiology Laboratory of the Kampala International University campus and teaching hospital at KIU, western campus, Uganda.

Sample Size Determination

In total, 80 water samples were collected for analysis from five specifically chosen sampling points (inlet, outlet, and three spots within the bathing pool: middle and two border sides of the pool) during two periods, encompassing both the rainy and dry seasons in the months of May and June/July.[17] The current study adopted the aforementioned sampling strategy to ensure comprehensive representation and accurate assessment of the prevalence for both the spread and acquisition of *K. pneumoniae* across various points of the hot spring during different climatic seasons. At every sampling time, two samples (surface and ground/sediment water) were collected in the early morning (0600 hrs) and evening (1900 hrs), twice within each climatic period (dry and rainy seasons), with a one-week interval between collections.

Sample Collection

As described above, approximately 300 mL of water was collected in sterile bottles (Nalgene, New York, NY, USA) from each of the designated sampling sites. After collection, samples were placed in a cooler box (4–8°C) and then transported to the laboratory within 1 h.[22] The samples were processed for analysis upon arrival in the laboratory.

Determination of Temperature

The water temperature at the designated sampling points was assessed in triplicates just before sample collection using a digital thermometer.

Microbiological Analysis

Approximately 5 mL of each homogenized sample was pre-enriched in brain–heart infusion broth and incubated at 37 °C for 48 h. Subsequently, the broth cultures were inoculated on violet red bile agar using the streak plate technique and then incubated at 37 °C for 24 hr.[7] After incubation, the suspected colonies were subsequently sub-cultured on violet red bile agar and then incubated over night at 37 °C. Subsequently, presumptive *K. pneumonia* isolates with characteristic colony morphologies, such as mucoid and golden- or dull-yellow-colored colonies, were selected for further identification. Conventional microbiological identification methods, such as Gram staining and biochemical tests, have been used to identify *K. pneumonia* species.[8,23]

Biochemical Identification of K. Pneumonia

The presumptive colonies of *K. pneumonia* were subjected to preliminary conventional biochemical tests, such as negative tests for indole, methyl red, and oxidase and positive tests for Voges-Proskauer, Citrate, catalase, triple sugar iron fermentation, and delayed urease positivity, as presented in Table S1.[3,24]

Antibiotic Susceptibility Testing

The antibiotic resistance pattern of the *K. pneumoniae* isolates was determined using the Kirby-Bauer disk diffusion method, following the guidelines set by the Clinical Laboratory Standards Institute (CLSI).[25] Briefly, 2–3 colonies were emulsified in 5 mL of 0.85% w/v normal saline, achieving a turbidity matching the 0.5 McFarland standard.[26] This was

followed by immersing a sterile cotton swab into the bacterial suspension and then spreading it uniformly across the surface of Mueller–Hinton agar (HIMEDIA, India). The inoculated plates were allowed to air-dry at room temperature for 15 minutes. Following this, sterile forceps were used to place the chosen antibiotic discs (Oxoid, UK) onto the surface of the inoculated agar plates. The antibiotics included: Amoxycillin-clavulanic acid (AMC, 30 µg), Chloramphenicol (C 30 µg), Ciprofloxacin (CIP 5 µg), Ampicillin (AMP 10 µg), Tetracycline (TE 30 µg), Gentamicin (CN 10 µg), Sulphamethoxazole-trimethoprim (SXT 30 µg), Cefotaxime (CTX, 30 µg), Cefuroxime (CXM 30 µg), Ceftriaxone (CRO, 30 µg), Cefoxitin (FOX, 30 µg), Ertapenem (ERT, 10 µg), Ceftazidime (CAZ 30 µg), Cefipime (FEP, 30 µg), Nalidixic acid (NA 30 µg), Meropenem (MRP10 µg) and Erythromycin (E 15 µg). Subsequently, the plates were aerobically incubated overnight at 37 °C.

Zones of inhibition were measured (in millimeters) and thereafter interpreted using CLSI standards. The standard MDR strain of *K. pneumoniae* ATCC BAA-1705 was adopted as the positive control.[7] All isolates that exhibited multi-drug resistance were considered for genotypic characterization of resistance genes ($bla_{\text{CTX-M}}$, bla_{TEM} and bla_{KPC}), as discussed below.

Genotypic Screening for Antibiotic Resistance K. Pneumoniae

DNA Extraction

DNA extraction was performed using the heat shock (boiling) method as previously described by Ibrahim et al[27] with slight modifications. Briefly, 3–6 discrete colonies of *K. pneumoniae* were picked from an overnight culture on nutrient agar and placed in 100 µL of 1 × TE buffer (10 mM Tris-Cl, 1 mM EDTA buffer, pH 7.9). The bacterial suspension in an Eppendorf tube (Thermomixer comfort, Germany) was heated to 100 °C for 10 min and centrifuged at 13,000× *g* for 15 min after cooling. The crude DNA extract contained in the supernatant was transferred into sterile microcentrifuge tubes and stored at −20 °C until use. A NanoDrop® ND-1000 Spectrophotometer (Thermo Fisher, Loughborough, UK) was used to estimate DNA concentration and purity. The purity of the extracted DNA was checked at an absorbance of 260/280 nm and a ratio of approximately 1.8–2 was considered to be of good purity.[27]

PCR Amplification of Resistance Genes

Resistance genes were amplified from the extracted DNA of all isolates displaying either resistance or intermediate resistance to ceftazidime and/or cefotaxime. This was accomplished using conventional uniplex PCR methods with minor adjustments.[27] The amplification of bla_{TEM}, $bla_{\text{CTX-M}}$, and bla_{KPC} genes was performed using the primer sets shown in Table 1, as previously documented in related studies by Gröbner et al.[28] Ibrahim et al[29] and Tuhamize et al[30] respectively.

The PCR master mix was prepared as follows: 12.5µL Hot Start Taq2x master mix (M0496S)-New England Bio-labs, 1.0µL forward primer (10µM), 1.0µL reverse primer (10µM) for $bla_{\text{CTX-M}}$ and bla_{TEM} genes while for bla_{KPC} gene, 75uM of forward and reverse primers each, 5.0µL DNA template and 5.5µL RNAase-Free-H_2O, making up to 25.0 a final reaction volume.

A conventional PCR Thermocycler (CLASSIC K960 Thermal Cycler) was used for PCR amplification. The program was set with initial denaturation at 95 °C for 3 min, followed by 40 cycles (denaturation at 95 °C for 30s, annealing at (59°C for bla_{TEM}, 55 °C for $bla_{\text{CTX-M}}$ and bla_{KPC} genes) for 30s, and elongation at 72 °C for 1 min and a final extension cycle at 72 °C for 5 min.[28–30]

Table 1 Genes and Their Respective Primers

Gene	Primer Sequence (5'-3')	Amplicon Size (bp)	References
CTX-Mu-F	CGCTTTGCGATGTGCAG	551	[28]
CTX-Mu-R	ACCGCGATATCGTTGGT		
TEM-F	GCGGAACCCCTATTTG	800	[29]
TEM-R	ACCAATGCTTAATCAGTGAG		
KPC-2-F	GCTCAGGCGCAACTGTAAG	150	[30]
KPC-2-R	AGCACAGCGGCAGCAAGAAAG		

Gel Electrophoresis of PCR Products

The gel electrophoresis of the PCR amplicons was conducted using the methods described by Tuhamize et al.[30] DNA Amplicon was electrophoresed using 1.5% agarose gel, in 1x Tris-Borate EDTA buffer (TBE), 5µL Safe View Classic™ DNA stain (cat # G108), 6x loading dye (Thermo Scientific #R0611), and DNA ladder/marker 1kb (NEB-Biolabs #N3231L). Electrophoresis was run at 200V and 80mA for 1 h. Bands were visualized using a Gene-Flash Trans-illuminator. The PCR products for the reference strains *E. coli* NCTC 13352, *K. pneumoniae* ATCC 700603, and *K. pneumoniae* NCTC 13368 were used as positive controls to detect $bla_{\text{CTX-M}}$, bla_{TEM}, and bla_{KPC} genes, respectively, whereas *K. pneumoniae* ATCC® 700603™ was used as a negative control. The formation of bands at a specific bp in reference to the DNA ladder and positive control was considered positive for a specific gene, whereas those without bands were regarded as negative.

Data Analysis

The data was inputted into Microsoft Excel and then transferred to the Statistical Package for Social Sciences (SPSS) Version 21 for analysis. Descriptive statistics for the occurrence of *K. pneumoniae* during the study period were obtained using cross-tabulation, and a chi-square test comparison was conducted. Regression analysis was utilized to calculate Pearson's product-moment correlation coefficient to determine the relationship between study variables. A significant difference of $p \leq 0.05$ was applied for all compared variables. The findings from this study are depicted using graphs, pie charts, tables, and images (especially for gel electrophoresis).

Results

Prevalence of Klebsiella Pneumoniae in Samples

Out of the 80 samples examined, 24 (30.0%) presumptive *K. pneumoniae* isolates were identified, as illustrated in Figure 2. The likely identities of these isolates were established through various biochemical tests, as detailed in Table S1. Other isolates identified from the samples were *Staphylococcus aureus, Enterobacter aerogenes, Micrococcus* sp, *Aeromonas* sp, *Enterococcus faecalis, Pseudomonas aeruginosa, Vibrio cholerae, Escherichia coli, Shigella* sp, *Bacillus* sp, *Salmonella* sp, *Proteus mirabilis, Serratia marcescens, Citrobacter freundii*.

Antibiotic Susceptibility Pattern of Klebsiella Pneumoniae Isolated in Kitagata Hot Spring

Most of the isolates showed high sensitivity to cefixime (79.2%), ceftriaxone (54.2%), imipenem (54.2%), and kanamycin (54.2%). Some isolates (29.2%) exhibited intermediate susceptibilities to cefotaxime and ceftriaxone. Moreover, all *K. pneumoniae* isolates (100%) were resistant to ampicillin, rifampicin, and ceftazidime, followed by azithromycin (79.2%) (Table 2).

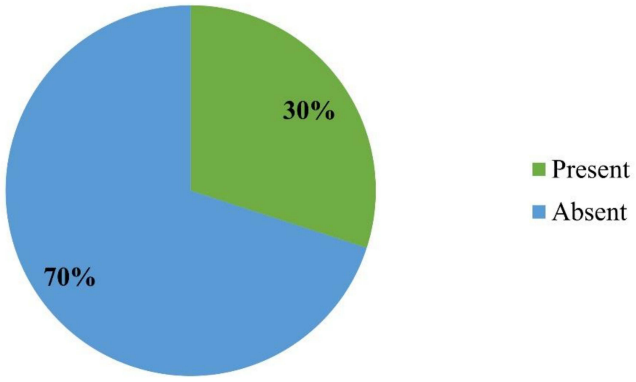

Figure 2 Isolation rate of *K. pneumoniae* in Kitagata hot spring, Sheema district, Southwestern Uganda.

Table 2 Antibiotic Susceptibility Pattern of K. Pneumoniae Isolated in Kitagata Hot Spring, Sheema District, Southwestern Uganda

Antibiotics	Number of Isolates, n (%)		
	Sensitive	Intermediate	Resistant
Cefixime	19 (79.2)	2 (8.3)	3 (12.5)
Cefotaxime	10 (41.7)	7 (29.2)	7 (29.2)
Ceftriaxone	13 (54.2)	7 (29.2)	4 (16.7)
Imipenem	13 (54.2)	2 (8.3)	9 (37.5)
Kanamycin	13 (54.2)	1 (4.1)	10 (41.7)
Ampicillin	0	0	24 (100.0)
Rifampicin	0	0	24 (100.0)
Ceftazidime	0	0	24 (100.0)
Azithromycin	1 (4.2)	4	19 (79.2)

Genotypic Characterization of Selected Resistance Genes Among K. Pneumoniae Isolated from Kitagata Hot Spring

PCR amplification of DNA from all *K. pneumoniae* isolates showed the presence of ESBL genes $Bla_{CTX\text{-}M}$ genes (551bp), Bla_{TEM} (800 bp), and carbapenem bla_{KPC} (1150 bp), as shown in Figures 3–5, respectively. Out of the 24 *K. pneumoniae* isolates obtained from the samples, the majority 23 (95.8%) harbored bla_{TEM} alone and both bla_{TEM} and bla_{CTX} genes, followed by bla_{KPC} 8(33.3%). Meanwhile, six (25%) isolates harbored a combination of three resistance genes (bla_{TEM}, bla_{CTX} and bla_{KPC}), five (20.8%) had bla_{CTX} genes only, while the remaining three (12.5%) isolates harbored both bla_{TEM} and bla_{KPC} (Figure 6).

Spatial Temporal Prevalence of Klebsiella Pneumoniae in the Sampling Site

Overall, a higher recovery of *K. pneumoniae* was recorded during the dry season 10/80 (17.5%) than during the wet season 10/80 (12.5%). However, a higher prevalence of *K. pneumoniae* (17.5%) was noted during the second phase of sample collection compared to the first phase within the same season. Similarly, *K. pneumoniae* (17.5%) was isolated more frequently during the dry season than the wet season. The study documented an equal count of *K. pneumoniae* (12, 15.0%) during both morning and evening or nighttime hours. The temperatures at the sample collection points within the hot spring ranged from 29.0 to 65.5°C, with a mean temperature of 36.5 ± 9.2°C. The study indicated a higher incidence of *K. pneumoniae* in water samples with temperatures of ≤45°C (26.3%) compared to those with temperatures >45°C

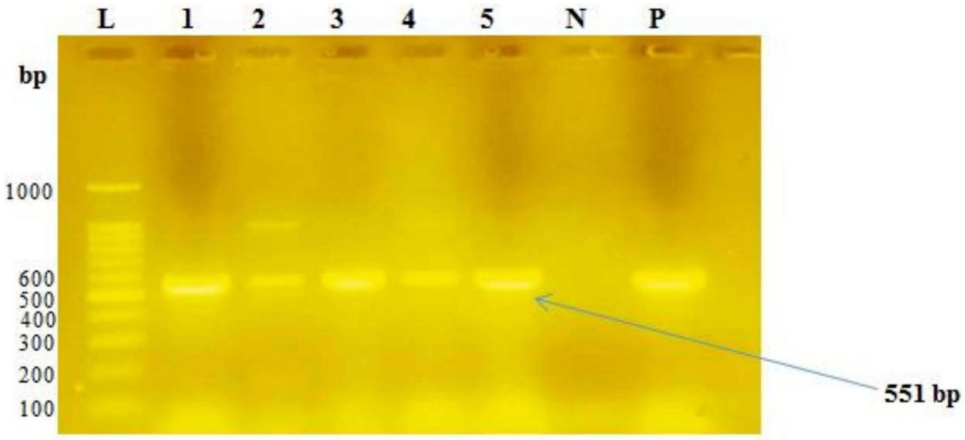

Figure 3 Gel electrophoresis of PCR products for $Bla_{CTX\text{-}M}$ gene from selected *K. pneumoniae* isolates. L- DNA ladder (100 bp to 1000 bp); 1–5 are positive isolates for $Bla_{CTX\text{-}M}$ gene (551 bp); N- Non template control; P- Positive control (*K. pneumoniae* ATCC 700603).

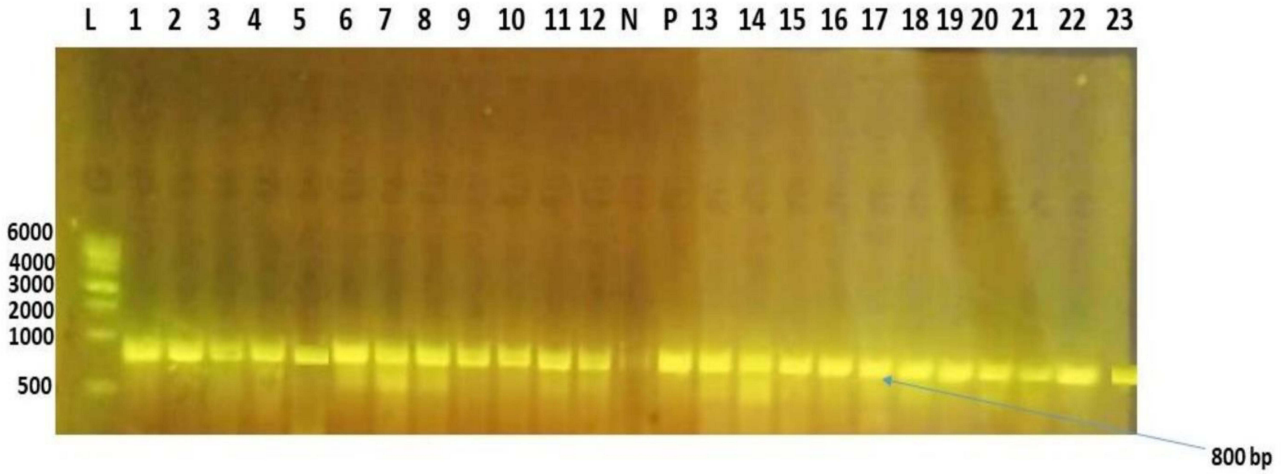

Figure 4 Gel electrophoresis of PCR products for Bla_{TEM} gene from selected *K. pneumoniae* isolates. L- DNA ladder, 1–23 are isolates positive for Bla_{TEM} gene (800 bp); N- Non template control; P- Positive control (*E. coli* NCTC 13352).

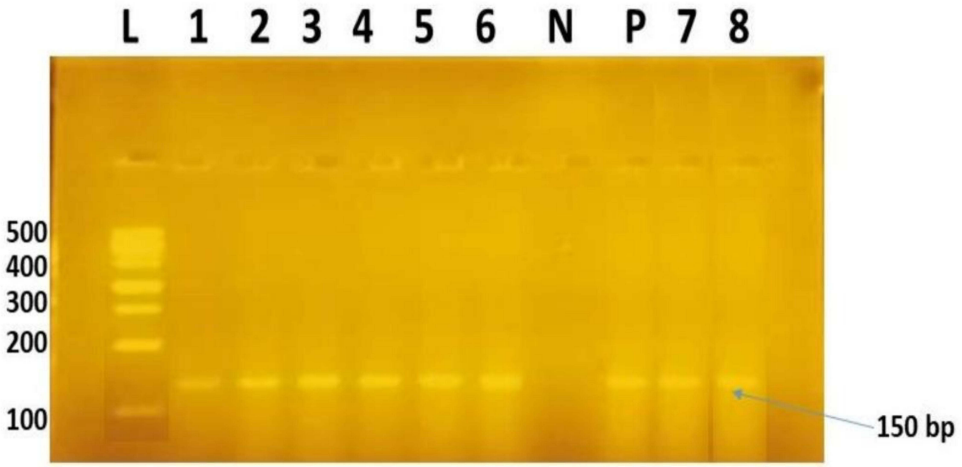

Figure 5 Gel electrophoresis of PCR products for Bla_{KPC} gene from selected *K. pneumoniae* isolates. L- DNA ladder (100 bp to 500 bp); 1–8 are positive isolates for Bla_{KPC} gene (150 bp); N- Non-template control; P- Positive control (*K. pneumoniae* NCTC 13368).

(3.8%). Furthermore, more *K. pneumoniae* were isolated from ground/sediment water (32.5%) than from surface water (27.5%), as shown in Table 3.

The factors affecting the spatiotemporal prevalence of *K. pneumoniae* in the sampling sites were also determined (Table 3). During the dry season, a higher proportion of *K. pneumoniae* was isolated (35.0%) compared to the wet season (25.0%), with no statistically significant difference in bacterial load ($p = 0.329$). A weak positive correlation was observed between the season of sample collection and the occurrence of *K. pneumoniae* ($r = 0.109$, $p = 0.335$). The prevalence of *K. pneumoniae* increased during the sampling period ($r = 0.109$, $p = 0.335$), with 35.0% of isolates collected during the initial phase compared to 25.0% during the subsequent phase. However, there was no observed correlation between the timing of sample collection and the prevalence of *K. pneumoniae*. The temperature at the sampling point exhibited a significant negative correlation ($r = -0.242$, $p = 0.03$) with the presence of *K. pneumoniae*, with temperatures ≤45°C showing higher contamination rates (26.3%) compared to temperatures >45°C (3.8%). The occurrence of *K. pneumoniae* demonstrated a weak correlation with sediment and groundwater samples, with a *p*-value >0.05 ($r = 0.05$, $p = 0.631$) (Table 3).

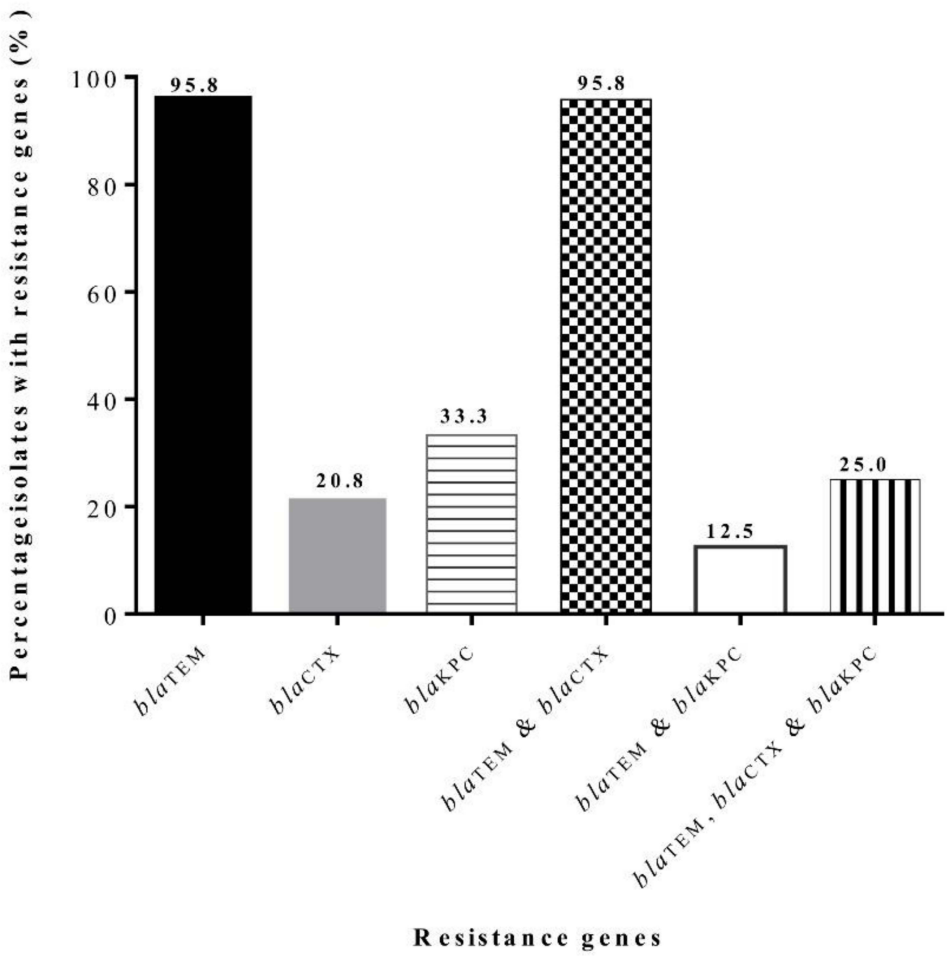

Figure 6 Prevalence of resistance genes in *K. pneumoniae* isolated from the sampling site. Each block represent one or more resistome(s) harbored by the test organisms.

Spatiotemporal Prevalence of Resistance Genes Associated with K. Pneumoniae in the Sampling Site

There was no significant difference ($p > 0.05$) in the prevalence of *K. pneumoniae* that harbored the resistance genes bla_{TEM}, bla_{CTX}, bla_{TEM}, bla_{CTX}, both bla_{TEM} and bla_{KPC} during the two phases of sample collection. However, there were significantly ($p = 0.003$) higher occurrences of *K. pneumoniae* that harbored bla_{KPC} (33.3%) during the second sampling phase compared to none isolated during the first phase. Additionally, there were significantly ($p = 0.017$) higher occurrences of *K. pneumoniae* with bla_{TEM}, bla_{CTX} and bla_{KPC} (25.0%) during the second sampling phase compared to none isolated during the first phase. The presence of these three genes in the isolates was positively correlated with the second phase of sample collection ($r = 0.488$, $p = 0.016$). The study revealed a negative correlation between the stage of sample collection and the occurrence of bla_{TEM} gene ($r = -0.176$, $p = 0.410$), in which the majority (54.2%) of *K. pneumoniae* isolates were isolated during the second phase of resistance genes of bla_{TEM} gene as compared to those isolated in the first phase (41.7%), as shown in Figure 7a.

The current study showed that during the wet season, 41.7% of *K. pneumoniae* isolates harbored both bla_{TEM} and bla_{CTX}, and the same percentage (41.7%) carried only bla_{TEM}, while 4.2% of isolates carried only bla_{KPC}. In contrast, during the dry season, a majority (58.3%) of the isolates carried both bla_{TEM} and bla_{CTX}, and 54.2% of isolates carried only bla_{TEM}. However, during this season, none of the isolates harbored either bla_{TEM} or bla_{KPC}. The presence of bla_{KPC} was significantly associated with the season ($p = 0.025$), as depicted in Figure 7b.

Table 3 Factors Influencing the Spatiotemporal Prevalence of Klebsiella Pneumoniae in the Sampling Sites

Parameter during sample collection	Prevalence of *K. pneumoniae*, N=80, n (%)		Pearson correlation, r	*p*-value
	Absent, n (%)	Present, n (%)		
Season			0.109	0.335
Wet	30 (75.0)	10 (25.0)		
Dry	26 (65.0)	14 (35.0)		
Stage of sample collection			0.109	0.335
First phase	30 (75.0)	10 (25.0)		
Second phase	26 (65.0)	14 (35.0)		
Time of sample collection			0.000	1.000
Morning hours	28 (70.0)	12 (30.0)		
Evening/night hours	28 (70.0)	12 (30.0)		
Temperature at sample collection point (n=80)			−0.242	**0.031***
≤ 45 °C	38 (47.5)	21 (26.3)		
>45 °C	18 (22.5)	3 (3.8)		
Depth of sampling collection			0.055	0.631
Surface water	29 (72.5)	11 (27.5)		
Sediment/ ground water	27 (67.6)	13 (32.5)		

Note: *statistically significant at $p \leq 0.05$.

This study showed no significant difference in the type of resistance genes carried by the isolates obtained during the morning and evening hours of sample collection. The majority of the samples (50.0%) collected during the morning and evening carried both bla_{TEM} and bla_{CTX} genes. However, 50.0% of the isolates collected in the morning harbored bla_{TEM} only compared to those collected in the evening (4.2%) at *p*-value >0.05 (Figure 7c).

Out of the 24 *K. pneumoniae* isolates obtained, the majority of MDR strains 20/24 (83.3%) were mesophiles. There was no correlation between *K. pneumonia* identified resistance genes and the variation in the temperature of the sampling points. Nonetheless, there were higher occurrences of bla_{TEM} 20 (83.3%) and both bla_{TEM} and bla_{CTX} 21 (87.5%) at temperatures ≤ 45 °C than in isolates recovered at >45 °C. Of the isolates screened from water at temperatures above 45 °C, none harbored bla_{CTX} ($r = -0.169$, $p = 0.430$) and both bla_{CTX} and bla_{KPC} ($r = -0.143$, $p = 0.508$) as compared to isolates from temperatures ≤ 45 °C with occurrences of 16.7% and 12.5%, respectively. Only 5/24 (20.8%) isolates at temperatures below 45 °C harbored all three resistance genes studied compared to those at temperatures above 45 °C (12.5%). There was a weak positive correlation was observed between the temperature and the presence of bla_{TEM}
($r = 0.097$) and $bla_{TEM+KPC+CTX}$ ($r = 0.073$), as shown in Figure 7d.

Discussion

The increasing incidence of multi-drug resistance in *K. pneumoniae* has posed a significant challenge for the healthcare sector in developing nations such as Uganda, leading to both nosocomial and community-acquired infections.[7] Consequently, the World Health Organization (WHO) has enlisted this superbug as a top-priority pathogen, emphasizing the importance of surveillance and the urgency for research into novel treatments.[2] Yet, the lack of dependable and timely epidemiological surveillance data on antimicrobial resistance at recreational sites could trigger an outbreak of multi-drug resistant infections.[9,15]

The present study reported a contamination prevalence of 24.0% for *K. pneumoniae* in the bathing pools of Kitagata hot springs. Located in the Sheema district of Southwestern Uganda, Kitagata hot spring serves as a tourist attraction and has been identified as an ecological habitat for various *Klebsiella* species and fecal coliforms.[16,17] Reporting such a high prevalence rate is not unexpected, given that the hot spring primarily attracts patients from neighboring districts such as Bushenyi, Fort Portal, and Mbarara, who seek healing for various ailments by dipping the affected body part in the water.

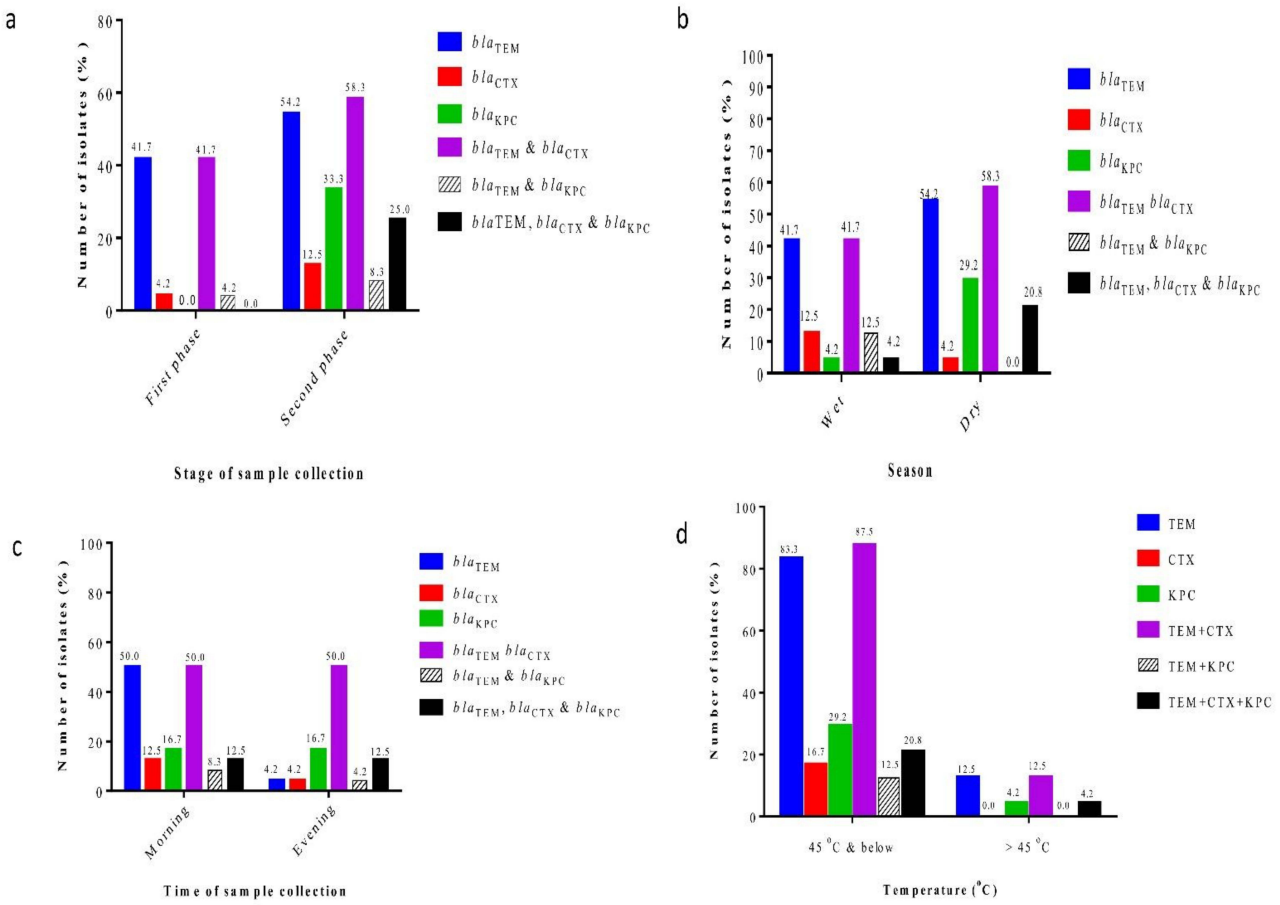

Figure 7 Spatiotemporal prevalence of resistance genes associated with *K. pneumonia* under conditions such as (**a**) sampling phases, (**b**) Season, (**c**) Time of sample collection and (**d**) Temperature.

Furthermore, numerous studies conducted in southwestern Uganda have consistently documented a high prevalence of *Klebsiella* spp. among these patient populations.[3] The high occurrence of *K. pneumoniae* in the bathing pool of the hot spring aligns with the findings of Lubega et al.[31] Lubega and colleagues reported a high prevalence of this pathogen among patients with surgical site infections in the region. However, these patients believed that the water helped heal their wounds, inadvertently contributing to the spread of these superbugs.

Previous studies have documented the prevalence of *K. pneumonia*e among patients attending regional referral hospitals, such as the Mbarara Hospital (52.9%) and Kampala International University Teaching Hospital (44.1%).[7] In contrast, a study by Odoki et al[1] in the Bushenyi district reported a slightly lower prevalence of K. pneumoniae (11.6%) compared to the present study, likely due to differences in sample types.[12] Notably, similar research has shown that hot springs harbor thermophilic bacteria known for producing bioactive compounds and thermostable enzymes such as DNA polymerases, lipases, proteases, xylanases, pectinases, chitinases, cellulases, and amylases, many of which are utilized in biotechnological processes. The inherent functional attributes of the sampling site might contribute to the perceived healing potential of the hot spring.[32]

Klebsiella pneumoniae naturally inhabits the gastrointestinal tract, making fecal contamination a likely contributor to its prevalence at the sampling site as individual bathe.[31] Some et al[33] and Stec et al[34] have noted that the presence of fecal coliforms, such as *Klebsiella* spp, in water indicates pollution from fecal matter from warm-blooded animals. Additionally, environmental contamination from sewage and hospital effluents, particularly from the nearby Kitagata Referral Hospital, could be polluting the hot springs due to their proximity to the surrounding swampy area. Some et al[33] showed that surface runoff water can potentially harbor pathogenic bacteria after coming into contact with sewage and

human waste from surrounding areas, making hot spring water extremely hazardous for recreational activities such as swimming, bathing, and drinking. Additionally, the spread of pathogens and their resistance genes into recreational areas and water sources aligns with the findings of Apecu et al[35] who reported contamination of environmental waters and drinking water sources by sewage effluents from nearby communities and hospitals in the same region.[33,35] Likewise, using an amplicon dependent sequencing, Janssen et al[36] identified new sequence type ST5236 – *K. pneumoniae* from Urban lake with recreational and domestic use. The isolated strains were similar to those isolated from a nearby hospital, indicating a putative gene flow from the hospital network into Paranoa Lake.[36]

The present study showed a lower occurrence of thermophilic strains (3.8%) compared to mesophiles propagated from water at temperatures below 45 °C. This lower isolation rate of thermophilic *K. pneumoniae* aligns with a study conducted in Fujian province, China, which reported the *K. oxytoca* HP1 strain from a hot spring sampling site at 65 °C.[37] Similarly, two thermophilic *Klebsiella* spp. were isolated from the Mahapellessa hot spring in Sri Lanka, consistent with the reported low recovery rate.[38] It should be noted that among all enteric pathogens, *K. pneumoniae* is the most heat-tolerant, with a specific growth rate at maximal temperatures approaching 36.9 °C.[39] Studies have indicated that the prevalence of bacteria at high temperatures (>45 °C) largely depends on microbial phenotypes, which can be influenced by various physicochemical conditions, as well as the geological and biogeographical history of the hot spring.[40]

Furthermore, the findings of this study align with those of Collignon et al[41] who documented a positive correlation between the aggregate AMR index and environmental temperature. Similarly, a European study highlighted a positive association between the resistance patterns of *K. pneumoniae* and temperature.[42] Additionally, research conducted across 31 provinces in China from 2014 to 2020 reported a significant positive correlation, noting increased detection rates of CR and third-generation CR *K. pneumoniae* in areas with higher ambient temperatures.[43] These studies ascertained that temperatures between 55°C and 60°C promote bacterial proliferation, accelerate antibiotic selection, and facilitate the acquisition and transmission of resistance genes within and between hosts, through both food and environmental sources. Likewise, Zeng and colleagues[43] revealed that the spread of thermal resistance genes, such as *clpK*, promotes the adaptability of *K. pneumoniae* in its environment.

During the dry season, there was a notable increase in the number of people visiting the hot spring compared to the wet season, which often experiences heavy rainfall that may restrict human activities around the recreation center. The study reveals a higher prevalence of *K. pneumoniae* and its resistance genes during the dry season. Similarly, studies have reported seasonal fluctuations in the susceptibility pattern of *K. pneumoniae* to selected antibiotics such as imipenem, ertapenem, and amikacin, which is in agreement with our findings. Interestingly, a study conducted in China documented the influence of climatic seasons on the abundance of antibiotic resistance genes harbored by *K. pneumoniae* in the environmental samples.[44]

Nonetheless, Anderson et al[39] showed increased incidence rates of *K. pneumoniae* bloodstream infections during the warm season of the year, possibly elucidating the heightened prevalence of this superbug and its resistance genes noted in the dry season. Despite this, studies have ascertained a greater risk of cross-infection with environmental K. pneumoniae during the warmer seasons compared to the dry season. However, the correlation between the dissemination of *K. pneumoniae* and environmental conditions during summer remains ambiguous.

The present study demonstrated the sensitivity of *K. pneumoniae* isolates to cefixime, imipenem, cefotaxime, and ceftriaxone. The sensitivity of *K. pneumoniae* to ceftriaxone was reported in a similar study by Lubega et al.[31] Also, Turugurwa et al[3] reported high sensitivity rate of the isolates to imipenem, which is in accordance with this study. Studies by Atta et al.[45] Falgenhauer et al[46] and Montezzi et al[47] on hospital wastewater, German surface water, and coastal recreational waters, respectively, also revealed the same susceptibility trend for *K. pneumoniae* as reported in this study. It is noteworthy that carbapenem antibiotics, such as imipenem, are currently considered among the drugs of last resort in the treatment of most ESBL producers despite the current emerging resistance.[7]

This study showed that all *K. pneumoniae* isolates produce CR and ESBL. The high prevalence of *K. pneumoniae* harboring these resistomes was consistent with several studies that documented the rapid dissemination of MDR *K. pneumoniae* into the environment and high rates of person-to-person transmission.[3,31,45,48] The resistance of *K. pneumoniae* to antibiotic classes, such as β-lactams and phenicols. Penicillins, cephalosporins, fluoroquinolones, and aminoglycosides have been reported by studies, such as Ferreira et al,[8] Katale et al[14] Osei & Reta,[18] Janssen et al[36]

and Montezzi et al[47] on both environmental and clinical samples. Falgenhauer et al[46] reported high prevalence level of Carbapenemase/ESBL producing *K. pneumoniae* in sediment and surface water in Germany. Odoki et al[1] reported 20.0% fluoroquinolone-resistant *K. pneumoniae* among UTI patients who attended hospitals in Bushenyi District, Uganda. Our findings were contrary to the study conducted at the (MRRH), which reported a higher prevalence of imipenem resistance (96.2%) and ESBL producers (29.0%).[49] Additionally, Turugurwa et al[3] reported a high resistance rate to cefotaxime and ceftazidime antibiotics among *K. pneumoniae* clinical isolates in the same hospital.

Lubega et al[31] reported 100% MDR-*K. pneumonia* in surgical site infections, which is in agreement with our findings. The above findings indicate a possible vehicle for the dissemination of resistance genes from patients who attend these hospitals and visit the hot spring for spiritual bathes because of their proximity. Likewise, Iramiot et al[19] reported high prevalence level of MDR *K. pneumoniae* among humans and cattle, respectively, at the human–animal interface in Kasese district, Southwestern Uganda. This could explain the possible transmission of resistance genes from animals, as they tend to access unprotected hot springs during the night hours.

This study showed a high occurrence of carbapenemase and ESBL genes $bla_{\text{CTX-M}}$, and bla_{KPC} which is in agreement with related studies conducted in southwestern Uganda on clinical isolates.[3] Studies conducted on recreational waters have identified several ESBL and carbapenemase genes associated with *K. pneumoniae*. For instance, a draft genome analysis of *K. pneumoniae* from an urban river in Brazil revealed a high-risk hospital-associated clonal lineage containing $bla_{\text{KPC-2}}$, isolated from the sample.[50] Atta et al[45] detected ESBL genes (bla_{TEM}, bla_{SHV}, and bla_{CTX-M}) in strains of *E. coli* and *K. pneumoniae* isolated from recreation waters and tertiary hospital wastewater in Zaria, Nigeria. In addition, Montezzi et al[47] isolated Carbapenemase producing *K. pneumoniae* from coastal recreational water. The isolate produced Guyana extended-spectrum (GES)-type Carbapenemase and Carbapenemase (KPC) resistomes. The use of conventional PCR to detect resistance genes associated with cephalosporins, β-lactams, and carbapenem antibiotics has been widely recommended; thus, the present study adopted a similar method to detect the above resistance genes.[51] These resistance genes detected in the isolates were similarly reported in ESBL-producing bacteria in patients who had a high degree of diversity regarding hospitalization.[7,14] Additionally, a review study conducted in Nigeria reported resistance genes bla_{TEM} and $bla_{\text{CTX-M}}$ among *K. pneumoniae*.[13] Still, a high prevalence of bla_{TEM} gene (95.8%) was reported in the present study as compared to 47% prevalence detected among clinical isolates in a study performed at MRRH by Turugurwa et al.[3] Moreover, $Bla_{\text{CTX-M}}$ has been widely detected among the studied isolates, and its resistance mechanism has been associated with porin mutations or drug efflux pumps.[3]

Furthermore, Turugurwa et al[3] reported the co-existence of multiple ESBL genes within a single isolate, aligning with our current findings that indicate a combination of either two or three genes: $bla_{\text{CTX-M}}$, bla_{KPC} and bla_{TEM}. Janssen et al[36] also identified multiple ESBL genes (bla_{TEM} and $bla_{CTX-M-15}$) in *K. pneumoniae* isolated from urban lakes for drinking and recreational water reuse. The combination of bla_{OXA-1}, $bla_{\text{CTX-M-15}}$, $bla_{\text{SHV-28}}$, and $bla_{\text{TEM-1}}$ was also found in *K. pneumoniae* identified from sediments/surface waters in Germany, as reported by Falgenhauer et al.[46] This finding clearly indicated the occurrence of MDR-*K. pneumoniae* in the hot spring, which makes the waters of Kitagata hot spring unhygienic for bathing and recreation purposes. In addition, a systematic review showed a high abundance of similar genes mediating beta-lactam resistance among *K. pneumoniae* isolates of animal and human origin.[18]

Notably, the resistance genes in *K. pneumoniae* are linked to mobile genetic elements such as insertion sequences (ISs), integrons, transposon and plasmids, mainly in environmental, animal, and human isolates.[18,36,48] *IncF*-type plasmids are reportedly linked to genes encoding ESBLs and carbapenemase resistance, while IntI1 has been documented in both non-lactamases and β-lactamases genes, such as *qnrA/B/D/S, qepA, floR, catA/B, dfrA*, and *sul1/2/3* while *IS*EcP1 is frequently linked with $bla_{\text{CTX-M-15}}$ in environmental isolates.[18]

The wide array of antibiotic resistance exhibited by MDR *K. pneumoniae* isolates could be attributed to several mechanisms, like efflux pumps, production of β-lactamases, and alterations in the expression of porins and penicillin-binding proteins (PBPs) as a result of mutations.[8,36] *K. pneumoniae* contains over 77 different cellular capsules (K antigens) around its cell wall and is responsible for its pathogenicity, acting as a shield from bacterial phagocytosis.[31]

Currently, there are possibilities of dissemination of resistant bacteria by travelers both local and international tourists who visit Kitagata hot springs in search of adventure and the purported healing properties of its waters during steam baths. This dissemination could lead to the widespread distribution of antimicrobial resistance (AMR) across various

regions of Uganda and beyond continents. The hot spring serves as a consistent breeding ground for recurrent seasonal outbreaks, contributing to significant but unquantified rates of illness and mortality.[20] Studies have shown that medical tourists and patients in the communities move between hospitals and health facilities with different resistant pathogens which potentially enhances the transmission of superbugs. It should be noted that human travelers are major live carriers for resistant bacteria.[20] Still, effluents from nearby hospitals such as Kitagata Hospital and sewage treatment plants that end up in the swampy area surrounding the study site could also eventually leak into the hot spring, causing dissemination of superbugs.[18]

Decano et al[52] documented that 100% *K. pneumoniae* strains containing genes associated with MDR were disseminated across the East African region via clonal and horizontal gene transfer mechanisms. Studies have reported bla_{KPC} gene is carried on a transposon known asTn440 a mobile piece of genetic material that elevates the risk of dissemination.[3] Moreover, International clones of *K. pneumoniae* ST208 and ST101 have been associated with several clonal disseminations of carbapenemases in addition to colistin and MDR; however, ST208 was absent in Africa as compared to ST101, which is predominantly isolated in several countries.[18]

The result of this study also align with related reports that showed the continued spread of a superbug-*Klebsiella pneumoniae* ST101 through hospital wastewaters that are known to harbor a wide range of micro- and macro-pollutants, such as antibiotic compounds and resistance gene.[53] Additionally, Stec et al[34] reported the high survivability of MDR *Enterobacteriaceae* in various recreational water bodies (brackish water, coastal water, and swimming pool water), either as planktonic cells or in biofilm form. Moreover, a study conducted in Saudi Arabia reported that 63.63% *K. pneumoniae* isolates from private recreational facilities exhibited resistance to 56% of antibiotics.[53]

The high rate of resistance observed among the isolates may be attributed to the misuse or overuse of antibiotics, self-medication, weak infection surveillance and control measures, and insufficient drug usage monitoring and regulatory policies.[3,15] The current study's reported rates of carbapenem resistance and ESBL-producing *Enterobacteriaceae* are now regarded as "priority infections of concern" for East African countries, necessitating an urgent response.[14]

Notably, *K. pneumoniae* is the most frequently isolated clinical bacterial pathogen worldwide, causing numerous fatal and multi-drug resistant infections. According to a study conducted at Mbarara Regional Referral Hospital, *K. pneumoniae* infections were linked to conditions such as chronic obstructive pulmonary disease, renal failure, liver disease, and impaired respiratory host defenses, which are common illnesses among the patient population in the region.[3,7]

The presence of antibiotic resistance genes in human, animal, and environmental samples is well-documented both globally and across Africa. This calls for the need to strengthen "One Health" surveillance systems and establish antimicrobial resistance stewardship, including periodic microbiological monitoring and assessment of antibiotic resistance in humans, animals, and environmental samples.[9,13,18] However, the "One Health" perspective on the spread of resistomes is lacking in developing countries, particularly in Uganda, which exacerbates the spread of resistance genes in recreational areas.[15] Therefore, if no significant measures are taken, Kitagata hot spring will become a hotspot for the dissemination of infections, posing a risk to all individuals who bathe in the geothermally heated water pool.

Conclusions and Recommendation

Our study reported the prevalence of MDR-*K. pneumoniae* in Kitagata hot spring, with higher occurrences observed during the dry season compared to the wet season. The dissemination of the pathogen continued to increase over the duration of the study. Additionally, the prevalence of *K. pneumoniae* in hot springs exhibited a significant negative correlation with the temperature at the sampling point.

All isolated *K. pneumoniae* strains were multi-drug resistance, showing particularly high resistance levels to ampicillin, rifampicin, ceftazidime, and azithromycin. Moreover, all isolates were identified as ESBL producers, with the majority carrying the bla_{TEM} gene alone or the combination of both bla_{TEM} and bla_{CTX} genes, followed by bla_{KPC} alone, and 25% harboring all the three resistance genes (bla_{TEM}, bla_{CTX} and bla_{KPC}). The presence of these three studied resistance genes in the isolates was positively associated with the second phase of sample collection and the dry season, but negatively correlated with temperature, except for isolates harboring either bla_{TEM} alone or $bla_{TEM+KPC+CTX}$ together. Therefore, without intervention, Kitagata hot springs could become a significant source of infection for individuals

bathing in the geothermally heated water pool. Meanwhile, this study recommends maintaining cleanliness and implementing basic interventions, such as disinfection, to significantly enhance the quality and sustainability of the spring, ensuring clean and safe water for both the community and tourists.

Data Sharing Statement

The datasets generated and/or analyzed during the current study are not publicly available due [because the isolates were not sequenced; rather, a traditional PCR method was adopted] but are available from the corresponding author upon reasonable request.

Ethics Approval and Consent to Participate

Ethical clearance for this research was obtained from the Mbarara University of Science and Technology Institutional Research and Ethics Committee and the study was registered with the National Council for Science and Technology (MUST-REC-2023).

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Author Contributions

All authors significantly contributed to the reported work, whether in the conception, study design, execution, data acquisition, analysis, interpretation, or all these areas. They participated in drafting, revising, or critically reviewing the article; approved the final version for publication; agreed on the journal for submission; and accepted responsibility for all aspects of the work.

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All authors declare that there are no conflicts of interest in this work.

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