JBS
Research Article

Journal of Biological Studies

eISSN: 2209-2560

V. 7, N. 4:169-183, 2024

https://doi.org/10.62400/jbs.v7i4.11525

Dominant bacterial pathogens in a river receiving swine farm effluent

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Received 08 July 2024|Accepted 15 September 2024|Published 15 December 2024

Abstract

Swine production dominates the livestock sector in the Philippines, providing 60% of the nation's meat supply. Some of the swine industry's challenges include waste management, resulting in environmental issues such as air pollution, water contamination, and the spread of pathogens that can affect human health. Severe impacts of swine waste on water bodies can lead to eutrophication and render water systems unproductive. This study aimed to isolate and characterize the dominant bacterial pathogens in swine waste effluent in a municipality in southern Iloilo, Philippines. Water samples from the receiving river were collected and cultured to isolate bacteria, followed by genetic identification and phylogenetic analysis to understand the microbial contamination in rivers impacted by the swine farm. Four dominant bacterial strains were isolated and identified using 16S rRNA gene sequencing. All strains, confirmed as Gram-negative, belong to the Enterobacteriaceae family, specifically *Klebsiella pneumoniae* and *Enterobacter cloacae*. *K. pneumoniae*, highly resistant and associated with significant health risks, is found in diverse environments and causes severe infections. *E. cloacae*, an opportunistic pathogen, contributes to water contamination and antibiotic resistance. The study underscores the environmental and public health concerns linked to these bacteria, emphasizing the need for stringent waste management and infection control strategies.

Keywords: Effluent-associated pathogens, *Enterobacter cloacae*, environment, *Klebsiella pneumoniae*, wastewater disposal

1. Introduction

The Philippines, an archipelagic Southeast Asian country, has diverse and prosperous natural resources that sustain its thriving agriculture industry. The agriculture sector is integral to the country's economy, imparting 25-30% to the labor force. Its major sub-sectors, comprising livestock, crops, fish, and poultry, directed a 10% contribution to the gross national product (FAO, 2024). A significant contributor to the agricultural sector is crops, preceded by livestock and fisheries. Primarily, rice and corn dominate crop production, accounting for 28%

of agricultural produce. A significant portion of 66% is imparted by horticultural crops such as rubber, sugarcane, coconut, abaca, fruits, root crops, and vegetables (Philippine Statistics Authority, 2015).

Conversely, the livestock industry is also pivotal in the country's agricultural domain. With an approximate 18.23% gross output value contribution, the livestock industry plays a significant role in the agricultural sector (Chen et al., 2022). Additionally, this sector not only plays an integral part in the economy but also substantially provides livelihood opportunities to the people in rural communities (Ortega et al., 2021).

The Philippines' livestock agriculture predominantly comprises swine, goats, cattle, and water buffalo, including rabbits, sheep, and horses, gradually gaining popularity and importance because of their significance (Ortega et al., 2021). In 2019, the livestock industry had a high-value gross output of 328. One billion pesos contribute to the total agricultural output value of 1.8 trillion or 74.16 billion euros (Sanchez, 2020). Among the entire livestock and poultry industry, swine production is appraised to be the largest. With a 191-billion-peso value industry and 60% meat provision to the Filipino people, swine production is valuable in shaping the country's economy and food security. The Philippines' swine industry is top-notch, ranking eighth in the volume yield of pork production and breeding sows' population (DOST-PCAARRD, 2016).

The swine industry in the Philippines is of two forms, as differentiated by the Philippine Statistics Authority (2021). Backyard farms usually consist of 1-20 finisher or adult hogs, typically without piglets, or either 1-40 individual piglets or 1-10 count of sows with the inclusion of 1-21 piglets. In contrast, commercial farms house more than 21 finishers or 41 piglets and up or over ten sows with 22 piglets. The swine industry in the Philippines is dominated by smallholder producers or backyard farms that constitute 70.6 % of the national swine industry (PSA, 2021).

A recorded growth in swine production was registered in the subsequent years of 2011-2020 (PSA, 2021). Across the regions of the Philippines, Western Visayas had the highest swine inventory, accounting for 12.1% in 2021. The regions of Central Visayas are ranked next in swine inventory production with 11.6%, Northern Mindanao (11%), CALABARZON, and Davao with 10.2% and 8.5% respectively. High-volume swine production in Western Visayas is accounted for by the provinces of Negros Occidental, Antique, and Iloilo (PSA, 2024).

With the continuous increase in the swine population comes a need for better waste management practices, in a study by Castelo et al. (2019) on a community where swine raising is identified as an essential economic activity. Several inferences were made that were deduced from hog-raising occupations in the community. Within the community, the perceived negative impact of swine-raising activity includes air pollution resulting from foul odors and greenhouse gas emissions. Another severe environmental impact is the contamination of groundwater and nearby bodies of water caused by waste spills. The data obtained in the study that mitigates the negative impacts includes the lack of wastewater treatment facilities and indiscriminate discharging of hog waste to the bodies of water. Overall, the study results implicate hog-raising activity complications as a severe threat to the environment and the community's health.

Significant environmental risks are associated with introducing diverse microbial communities in river systems. A manifold of microorganisms such as protozoa, viruses, fungi, and bacteria play critical roles in the ecological health of aquatic ecosystems. Animal wastes are a reservoir of a wide array of zoonotic pathogens that have become a significant public health concern in recent years. The fecal wastes, as well as other biological component materials like respiratory secretions, sloughed feathers, fur, or skin, and urine from agricultural livestock, can harbor elevated concentrations of pathogens capable of causing diseases in humans (Strauch and Ballarini 1994; Sobsey et al., 2006). Animal excreta encompasses diverse path-

ogens such as *Salmonella, Campylobacter, E. coli, cryptosporidium, Giardia, Cholera, Streptococcus,* and *Chlamydia*. Other pathogenic strains, such as *Cryptosporidium* and *Giardia,* are resilient to standard chlorination processes, leading to a heightened risk of waterborne contamination if hog manure lagoons with high concentrations of these pathogens experience leakage and spills (Carpenter et al., 1998; Steinfeld et al., 2006).

In the Philippines, several studies have been done to gauge the environmental impact of swine farm waste discharge on different bodies of water. Such a study reveals that eutrophication impacts aquatic systems because of high levels of phosphorus and nitrogen compounds from these effluents (Castelo et al., 2001). Untreated swine farm wastewater that is directly released into creeks, rivers, and other bodies of water has rendered aquatic systems unproductive and unfit for several significant recreational and economic activities. The Department of Environmental and Natural Resources (DENR) Administrative Order No. 2016-08 states that discharges from any point source shall at all times meet the effluent standards in order to maintain the required water quality per water body classification, and the General Effluent Standards (GES) shall be used regardless of the industry category (DENR, 2016). This policy mandates all industries, including swine farming, to adhere to the required effluent standards to ensure that their wastewater effluent does not degrade the receiving body of water. Currently there are no specific policies mandated by the DENR regarding management of wastewater effluent from backyard swine farming and some of the farmers have limited knowledge about the possible eutrophication of nearby bodies of water due to untreated wastewater effluents. Pollution in the aquatic system due to massive swine waste dumping has been documented in various parts of the country, including Northern Mindanao, Central Luzon, and Southern Luzon (Catelo et al., 2001; Rola et al., 2003).

Despite these extensive explorations, more comprehensive studies on the microbial profiles of the river ecosystems utilized as discharge points of numerous commercial and backyard hog farms still need to be conducted. Thus, this study aimed to extensively profile the dominant bacterial species in swine waste effluent from a river in the Southern part of Iloilo, Philippines. Specifically, this study isolated dominant bacterial strains present in wastewater effluent samples. Additionally, this study aimed to morphologically describe and assess the biochemical properties of the isolated strains, as well as to determine the accurate identification of the bacterial species through molecular characterization. The results of this study will eventually lead to better understanding of the environmental and health implications of untreated wastewater discharge and develop strategies that will mitigate the risks related to microbial contamination.

2. Materials and methods

Collection of water samples

The water samples were collected in a riverside municipality in southern Iloilo, Philippines (Figure 1) in April 2024, which coincided with the dry season. The water effluent was collected approximately 500 meters from a local hog farm. Two hundred (200) mL of water, from composite samples, was collected in a sterile plastic bottle and stored at 4°C in a styrofoam box during transport from the sampling site to the laboratory. The collected water sample was transferred to the Central Science laboratory of West Visayas State University in La Paz, Iloilo City, where most bacterial isolation and PCR amplification were conducted.

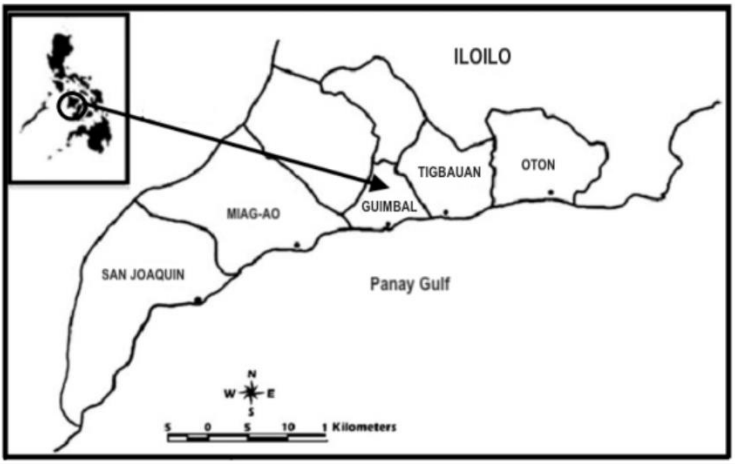

Figure 1. Map of the Philippines showing the municipality of Guimbal in Iloilo Province (Image Source: Espectato et al., 2012)

Preparation of culture media

Three culture media were utilized in this study, with a focus on precision and thoroughness. The Eosin-Methylene Blue (EMB) agar and MacConkey agar, both selective culture media, were chosen for their ability to isolate specific types of bacteria. The Nutrient agar, a general-purpose medium, was also used. The Eosin-Methylene Blue (EMB) Agar was prepared following the manufacturer's instructions (Lal and Cheeptham, 2007). The MacConkey Agar was also prepared following the manufacturer's protocol (Allen, 2005; Jung and Hoilat, 2022). The Nutrient Agar was also prepared following the manufacturer's protocol (Sapkota, 2022).

Incubation of water samples

The collected water samples were prepared in a ten-fold serial dilution using 10 mL before plating (Reynolds, 2005). The spread plate technique was done following the procedures of Buck and Cleverdon (1960) and Sanders (2012). The agar plates were incubated at 27°C for 24 hours and observed for microbial growth.

Isolation of dominant bacteria from collected water samples

The incubated plates were observed for any microbial growth and the most frequently occurring bacteria, based on distinct morphological characteristics of the colonies, were selected. The isolation of the pure culture of bacteria was carried out using the 10^{-3} dilution and the quadrant streak plating method (Katz 2008; Jensen and Støy, 2022). The plates were then incubated at 27°C for 24 hours. All the activities for the isolation of bacteria were done following the aseptic technique (Sanders, 2012). Isolation was performed three consecutive times, a critical step to ensure the purity of the cultures. The pure cultures were then transferred to Nutrient Agar plates and subjected to characterization and observation. A stock of the pure cultures was prepared in a 15 mL Nutrient Broth, which was used for DNA extraction and sequencing.

Identification of bacterial isolates

Bacterial isolates were observed based on their cultural and microscopic characteristics. Biochemical tests were also performed to determine and differentiate the biochemical and enzymatic activities of the isolated bacteria. Moreover, conducting biochemical tests further sup-

ported the identification of bacteria (Moore, 2021). Stock cultures of bacterial isolates underwent Genomic DNA extraction following the manufacturer's protocol (PureLink™ Genomic DNA Mini Kit, Invitrogen). Forty (40) µL of purified gDNA was transferred to a microcentrifuge tube for sequencing, and the remaining was transferred to another microcentrifuge tube and was stored at -20°C for future assays.

PCR Amplification and sequencing of 16S rRNA gene

PCR amplification and gel electrophoresis of the extracted DNA samples were done at the Biotechnology Laboratory of West Visayas State University, Iloilo City. The amplification of 16S rRNA utilized the 16S forward primer GAGTTTGATCCTGGCTCAG and the 16S reverse primer AAGGAGGTGATCCAGCC (Srinivasan et al., 2015) in a 25 µL PCR reaction. This PCR reaction consisted of: 2 µL (10-15 ng) of DNA as the template, 2 µL of each primer (5 pmol), 2.5 µL of 10 PCR buffer, 1.5 µL of 2 mM dNTP, 1µL of 50 mM MgCl2 and scaled up to the desired volume using distilled water. PCR amplification was carried out following the protocol described by Caipang et al. (2010). The amplicons were then visualized by gel electrophoresis using the Gel Doc system. The PCR products were cleaned and sent for sequencing (Macrogen, Korea). Using publicly available data from NCBI GenBank (blast.ncbi.nlm.nih.gov), sequenced data were aligned and analyzed to construct the phylogenetic trees of the bacterial isolates.

Construction of phylogenetic tree of bacterial isolates

The generated sequences of the bacterial isolates were subjected to a BLASTn search for their related taxa in the GenBank database (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The taxa table was assembled based on the closest matches from the BLASTn search results. Finally, the phylogenetic trees were constructed using the MEGA11 software. Sequence data of isolates were loaded in FASTA format and aligned prior to phylogenetic tree construction. Aligned sequence data were then utilized in generating the phylogenetic tree by employing neighbor-joining with 1,000 bootstrap replications (Tamura et al., 2021).

3. Results

Isolation of Dominant Bacterial Colonies from Hog Waste Discharge Effluent

The water samples were collected in a river that served as a waste discharge point for several commercial and backyard swine farms in southern Iloilo, Philippines. Figure 2 shows the collection site of water samples. The collected water samples were serially diluted and plated on Eosin Methylene Blue (EMB) agar and MacConkey Agar. All agar plates were incubated at 27°C for 24 hours. After incubation, the total bacterial count was 9.93 x 10^5 colony forming units (CFU) ml^{-1}. From the agar plates that were spread with water samples at the highest dilution factor, four dominant bacterial colonies designated as: S1, S2, S3 and S4, were isolated and characterized.

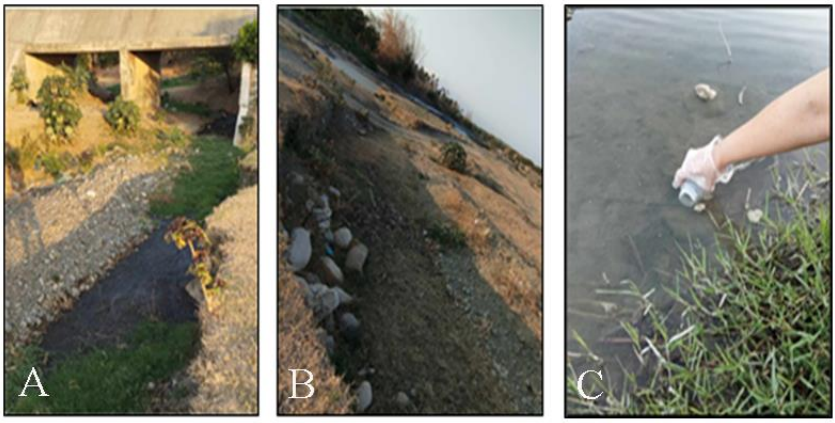

Figure 2. Swine waste discharge area in a river situated in Southern Iloilo. (A) shows the wastewater effluent from the local hog farm, (B) presents the area where the wastewater effluent reaches the river, (C) shows the actual collection of water samples.

Morphological and Biochemical Characterization of Bacterial Isolates

Figure 3 presents a visual presentation of the dominant bacterial isolates on two selective culture media. The colonies of the bacterial isolates vary in color from pink and deep purple to a green metallic sheen.

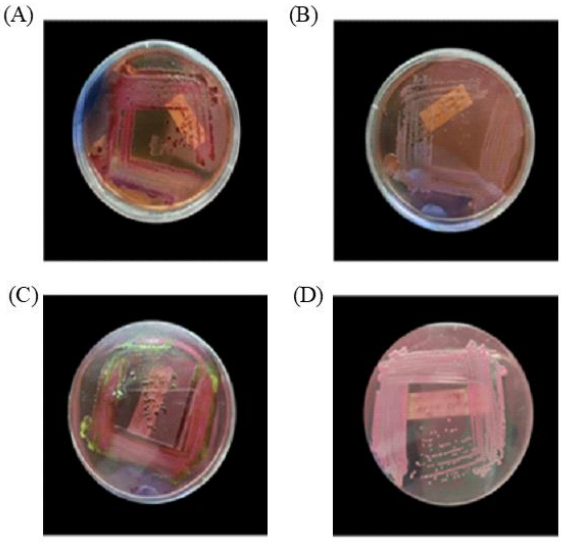

Figure 3. The four bacterial isolates on EMB and MacConkey Agar. (A) presents isolate S1, (B) shows isolate S2, (C) represents isolate S3, and (D) visualizes isolate S4. Isolates S1-S3 on EMB agar and isolate S4 on MacConkey Agar.

Table 1 presents the morphological characterization of bacterial isolates, and Table 2 presents their biochemical attributes. Bacterial isolates were subjected to Gram staining to differentiate their structural characteristics. The four bacterial isolates from the swine wastewater sample were all shown to be Gram-negative. The bacterial cell shape of the isolates was bacillus,

specified as a thin and elongated rod in cocci form. The biochemical test results showed that all bacterial isolates were positive in catalase activity, methyl red test, citrate utilization, methyl red-Voges Proskauer (MR-VP), and triple sugar iron test. Isolate S4 tested negative on the citrate utilization test.

Table 1. Morphological characteristics of the isolated bacterial strains.

Characteristics	Isolate 1	Isolate 2	Isolate 3	Isolate 4
Colony Form	Circular	Circular	Circular	Circular
Colony Color	Dark purple to pink	Creamy purple to pink with a mucoid appearance	Green metallic sheen	Purple to pink
Colony Margin	Entire	Undulate	Entire	Entire
Cell Shape	Thin rods (bacillus)	Rods (bacillus)	Elongated rods (bacillus)	Coccobacillus
Gram reaction	Negative	Negative	Negative	Negative
Cell arrangement	Singly present	Singly present	Singly present	Singly present

Table 2. Biochemical attributes of isolated bacterial strains

Characteristics	Isolate 1	Isolate 2	Isolate 3	Isolate 4
Catalase test	+	+	+	+
Methyl red (MR) test	+	+	+	+
Methyl red – Voges Proskauer (MRVP test	+	+	+	+
Triple sugar iron test	+	+	+	+

(+) indicates positive reaction; (-) indicates negative reaction

Molecular Identification of Bacterial Isolates

Bacterial isolates were subjected to 16S rRNA gene sequencing. The DNA of isolated bacterial strains was analyzed using gel electrophoresis, and the bands were visualized using a Gel Doc system. The results of the amplification are presented in Figure 4. The amplified 16S rRNA of the isolated bacterial strains was subjected to purification and sequencing analysis. UV absorbance ratio A260/A280 was used to evaluate DNA extract purity.

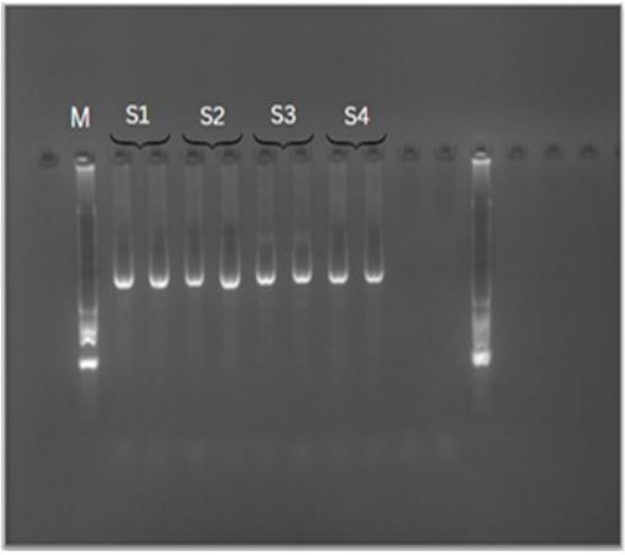

Figure 4. PCR products of the extracted genomic DNA on a 2% agarose gel. M, Marker; S1, Isolate 1; S2, Isolate 2; S3, Isolate 3; S4, Isolate 4.

Sequencing

The DNA samples of all four bacterial isolates were run on the agarose gel, and the bands were visualized when observed under the Gel Doc system. To understand the evolutionary relationships between the isolates, phylogenetic trees were constructed based on the results obtained from 16S rRNA sequencing. Table 3 reflects the identified bacterial strains with their corresponding accession codes.

Table 3. Results of the 16S rRNA analysis of the bacterial isolates.

Bacterial Isolate Code	Highest Identity	Strain Code	Percent Identity (%)	Accession Number
S1	*Klebsiella pneumoniae*	FM8348	99.91	KJ803905.1
S2	*Enterobacter cloacae*	PW 113	99.34	KP969041.1
S3	*Enterobacter cloacae*	CP22	99.34	MN244519.1
S4	*Klebsiella pneumoniae*	MST3	92.92	KY550379.1

Isolates 1 and 4 (S1 and S4) had high sequence identity with *Klebsiella pneumoniae* while isolates 2 and 3 (S3 and S4) had high sequence identity with *Enterobacter cloacae*. Figure 5 shows the phylogenetic tree of the isolated bacterial species based on the 16S rRNA sequencing results.

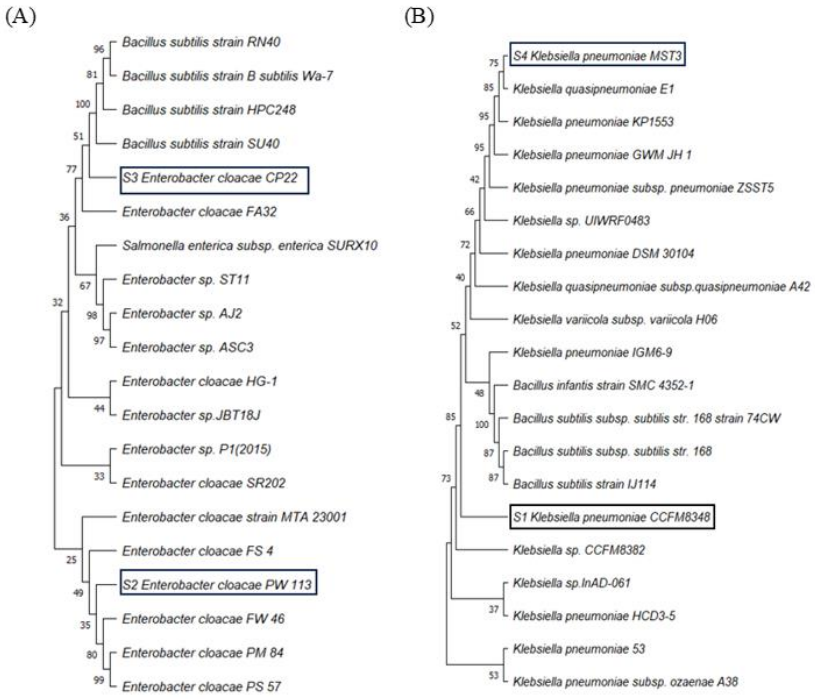

Figure 5. Phylogenetic trees of *Klebsiella* and *Enterobacter* isolate from water effluent in Southern Iloilo. The phylogenetic trees of *Klebsiella* species are shown in 5A, while *Enterobacter* species are presented in 5B. Identified bacterial isolates are highlighted in the box.

4. Discussion

This study is of significant importance as it delves into the microbial composition of hog waste discharges in a river located in an area with numerous backyard and commercial farms. The research involved the isolation of bacterial strains, with a focus on four dominant strains. These selected bacterial strains underwent rigorous testing to evaluate their morphological characteristics and biochemical properties. The identification of these bacterial strains was carried out through 16S rRNA gene sequencing, a crucial step in understanding their potential impact on the environment and human health.

All bacterial isolates were confirmed to be Gram-negative and performed similar positive responses to catalase activity, methyl red test, citrate utilization, methyl red-Voges Proskauer (MRVP), and triple sugar iron test that is by the standard (Ng et al., 2010; Oliveira & Reygaert, 2023).

The results of the 16S rRNA gene sequencing confirmed the presence of bacterial strains under *the Enterobacteriaceae family* in hog waste effluent. These bacterial strains, commonly found in the intestinal tracts of both humans and animals, were identified as *Klebsiella pneumoniae* strain CCFM8348, *K. pneumoniae* strain MST3, *Enterobacter cloacae* strain PW 113, and *Enterobacter cloacae* strain CP22. The potential impact of these strains on human health and the environment is a matter of urgent concern, given their prevalence in hog waste discharges.

Klebsiella pneumoniae is classified within the *Enterobacteriaceae* family and is characterized as a Gram-negative bacterium that possesses a capsule and is non-motile (Ashurst &

Dawson, 2023). This bacterium is commonly encountered in water, sewage, soil, and on the surfaces of plants (Martin & Bachman, 2018). This bacterium usually inhabits the mucosal lining of the human throat and digestive system. Once inside the body, it can exhibit significant aggressiveness and antibiotic resistance (Jondle et al., 2018; Aghamohammad et al., 2020). Further, they are responsible for infections in various body parts, including the lungs, urinary tract, bloodstream, wounds, surgical sites, and brain. These infections are more prevalent among individuals with existing health issues. This bacterium, in particular, has become a significant global concern due to the rising occurrences of highly virulent and carbapenem-resistant strains (Chang et al., 2021).

Klebsiella pneumoniae was found to be the most common pathogen, second to *E. coli,* responsible for deaths attributed to antimicrobial resistance (AMR), accounting for >600,000 AMR-associated deaths globally in 2019 (Kohler et al., 2017). This species is further classified into two subtypes: classical *Klebsiella pneumoniae* (cKp) and non-classical *Klebsiella pneumoniae* (ncKp). It is also important to note that many clones of ncKp are capable of mutation and acquisition of plasmids and transposons, which carry resistant and virulent genes, leading to severe and difficult-to-treat infections, leading to the emergence of strains such as hypervirulent *Klebsiella pneumoniae* (hvKp) or hypermucoviscous *Klebsiella pneumoniae* (HMKP) (Effah et al., 2020). According to Chang et al. (2021), hypervirulent *K. pneumoniae* (hvKp), designated as a *K. pneumoniae* strain, can cause infections in relatively healthy subjects, often in community settings. This species is ubiquitous in the environment, and sources, such as soil, vegetation, feces-contaminated surfaces, and water preceding gut-colonization are yet to be known. However, once the strain colonizes the gut, community person-to-person spread through fecal-oral entry in hvKp-endemic regions is one mechanism for its presence in the community causing infections.

Enterobacter cloacae is an opportunistic and multi-resistant human bacterial pathogen that has been the cause of several outbreaks of hospital-acquired infections in Europe and France (Davin-Regli & Pagès, 2015). It is commonly found in terrestrial and aquatic environments and has been shown to have genomic heterogeneity (Mezzatesta et al., 2012). Belonging to the family *Enterobacteriaceae*, this Gram-negative, facultatively anaerobic, rod-shaped, and non-spore-forming bacterium is the most frequently isolated species from humans and animals (Guglielmetti & Bartoloni, 2003; Jin et al., 2022).

Contamination by *E. cloacae* can occur when the said bacterium enters water systems through agricultural runoff, sewage discharge, and industrial effluents, leading to waterborne diseases and the overall decline in water quality. Contamination and increased levels of nutrients can lead to eutrophication, resulting in excessive algal blooms, depleting oxygen levels in the water, and destroying aquatic life (Kim et al., 2015; Zagui et al., 2020).

Furthermore, this bacterium is also known for its ability to acquire and disseminate antibiotic-resistance genes (Davies & Davies, 2010; Naidoo & Olaniran, 2014), particularly the chromosomal AMpC-type cephalosporinase, which can induce resistance from first- and second-generation cephalosporins and penicillins (Haenni et al., 2016). Studies have shown that *E. cloacae* can be a reservoir of multidrug-resistant genes, specifically in aquatic environments contaminated by agricultural and hospital wastes. A study conducted by Harada et al. (2017) demonstrated how this bacterium became resistant to several antibiotics, including ampicillin, amoxicillin-clavulanic acid, cefmetazole, chloramphenicol, ciprofloxacin, and tetracycline. Moreover, *E. cloacae* is also known to cause human infections, especially in immunocompromised individuals. *E.* cloacae can serve as a vector for transmitting diseases through contaminated water or food supplies (Davin-Regli & Pages, 2015). *E. cloacae* trains that produce OXA-48 were recently reported in the hospital environment in Algeria and were suggested to have been infecting humans through horizontal transfer and have been present in vegetables and river water of the country (Yousfi et al., 2018).

Overall, the detection of *K. pneumoniae* and *E. cloacae* in environmental settings, specifically in this study in swine waste discharges, is critical in advancing our knowledge regarding

their mechanisms, environmental impacts, influence on human health, and notable characteristics of global health problems. Their detection raises a pressing concern, particularly in public health and environmental safety. Immense attention is attributed to their varying pathogenic profiles and resistance mechanisms. Appropriate strategies to mitigate their impact include stringent infection control practices instituted if every swine farm has an established waste management treatment system. Addressing these challenges in recent times requires a multiple approach that involves intervention and management of the local government unit (LGU).

4. Conclusions

In this study, the researchers isolated, identified and characterized four dominant bacterial colonies from a swine farm waste effluent in a river in southern Iloilo, Philippines. The results showed that two distinct bacterial strains belong to putative *Klebsiella pneumoniae*, and the other two belong to *Enterobacter cloacae*. These species are long known to have detrimental impacts on human health, inflicting common diseases and infections that could be life-threatening without proper intervention. Therefore, microbial profiling of dominant bacterial species of swine waste effluent is crucial in mitigating public health concerns, environmental problems, wastewater treatment, and resource management. Further studies are needed to maximize the results and data gathered and explore its impacts on various fields, including pathogen surveillance, antimicrobial resistance (AMR) monitoring, and ecological assessments of various strains isolated from this study. The conduct of this study is limited only to culturable bacteria and future studies shall also identify non-cultural bacteria using the metagenomics approach. Nevertheless, the information acquired from this study will contribute to the advancement of making informed decisions for the policymakers, stakeholders, business owners, swine farmers, and the general public.

Acknowledgments

The authors would like to thank the Central Science Laboratory and the Biotechnology Laboratory at West Visayas State University, and the Microbiology Laboratory of the Division of Biological Sciences at the University of the Philippines Visayas, Miagao for providing the laboratory materials and equipment for the conduct of the study; the University of the Philippines Visayas – Graduate School for provision of logistics in the study; and Mr. John Kelly Padernilla for technical assistance. KKDC and MAG would also like to acknowledge the scholarship support of the Department of Science and Technology - Accelerated S&T Human Resource Development Program (DOST-ASTHRDP). We would like to thank the reviewers for their valuable comments and suggestions to improve paper quality.

Conflict of interests

The authors declare that they have no competing interests.

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