EnvironmentAsia
The international journal by the Thai Society of Higher Education Institutes on Environment

EnvironmentAsia 15(3) 2022 133-142
DOI 10.14456/ea.2022.55
ISSN 1906-1714; ONLINE ISSN: 2586-8861

# Isolation, Characterization, and Antimicrobial Resistance of *Enterococcus* sp. from Irrigation Waters in Metro Manila, Philippines

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Received: June 17, 2022; Revised: July 18, 2022; Accepted: August 10, 2022

## Abstract

This study is the first in the Philippines to assess the presence of *Enterococcus* spp. in surface waters used for irrigation of agricultural produce. It aimed to assess enteric pathogen contamination of irrigation waters that can cause gastrointestinal illnesses. A total of 105 surface water samples from seven sampling sites in Metro Manila, Philippines were evaluated for the prevalence of *Enterococcus* using culture and molecular methods. Results showed that out of 105 surface-water samples, 70 (67%) were found to be contaminated by *Enterococcus*. Antimicrobial susceptibility testing by broth microdilution method showed low prevalence of antimicrobial resistant *Enterococcus* in the surface water samples. Out of 18 *Enterococcus* isolates, none was resistant to tetracycline, ampicillin, and chloramphenicol, while six (33%) exhibited intermediate resistance and one (6%) exhibited full resistance to ciprofloxacin. This study provides an initial estimation of the prevalence of *Enterococcus* in surface waters used for irrigation in urban farms in Metro Manila, Philippines and preliminary assessment of their antimicrobial resistance for disease detection and prevention.

***Keywords:*** Antimicrobial resistance; *Enterococcus* spp.; Prevalence, Produce, Surface-water

## 1. Introduction

*Enterococcus* sp. are group of non-motile, non-sporulating, facultatively-anaerobic, catalase-negative bacteria that usually occur singly, in pairs, or in short chains (Teixeira and Merquior 2013). They are ubiquitous and widely distributed in the environment, predominantly inhabiting the gastrointestinal tract of humans and animals, where they can be released to soil and water through human and animal feces. As a consequence, they are used as "indicator organisms" to evaluate fecal contamination of recreational waters worldwide (Kinzelman et al., 2003; Gilmore *et al.,* 2014). Further, they have been associated with a variety of infections such as bacteremia and endocarditis and have been shown to exhibit intrinsic and acquired resistance to antimicrobials (Gilmore *et al.,* 2014). This poses a significant health risk when potentially pathogenic and antimicrobial resistant *Enterococcus* are introduced to the human body directly through consumption of contaminated water and food, or indirectly through the recreational use of contaminated waters. Thus, investigating the presence of *Enterococcus* in water and food is of paramount importance to public health.

The presence of *Enterococcus* in surface waters used for irrigation has been reported in several countries (Ahmed *et al.,* 2006; Lata *et al.,* 2009; Luczkiewicz *et al.,* 2010; Larsen *et al.,* 2012; Carey *et al.,* 2016; Said *et al.,* 2016). The use of contaminated surface waters for irrigation is alarming, as this practice may transfer potentially pathogenic and antimicrobial resistant

bacteria from water to agricultural produce such as vegetables, that are typically consumed raw or processed minimally prior to consumption (Uyttendaele *et al.,* 2015). In Metro Manila, Philippines, high bacterial loads were reported in fresh produce and in surface waters used for irrigation (Vital *et al.,* 2014; Garcia *et al.,* 2015; Vital *et al.,* 2017; Vital *et al.,* 2018). However, little is known about the prevalence of *Enterococcus* in surface waters used for irrigation in the Philippines.

Thus, the study explored the presence of *Enterococcus* in surface waters used for irrigation in selected urban farms in Metro Manila, Philippines. Specifically, this study aimed to (1) isolate and characterize *Enterococcus* from surface waters using culture and molecular methods; (2) determine the prevalence of *Enterococcus* in surface waters; and (3) evaluate the antimicrobial resistance of *Enterococcus*. The results of this study will serve as an initial assessment of the presence of *Enterococcus* in surface waters used for irrigation in Metro Manila as no such study has been conducted in the Philippines thus far. This will provide information that could aid in the prediction, prevention, and management of the potential health risks associated with the presence of *Enterococcus* in irrigation waters.

## 2. Materials and Methods

### *2.1 Sampling sites*

The sampling locations were small scale urban farms in Metro Manila, Philippines, which include: five sites in Quezon City, one site in Marikina City, and one site in Taguig City (Figures 1A-1F). The sampling sites were situated near residential areas and cultivate different fresh produce, which were sold in nearby markets. Several animals such as cats, dogs, and birds were also observed among the sites. The sampling sites were chosen because the nearby surface waters such as rivers, ponds, creeks, brooks, puddles, and seeps were used for the irrigation of fresh produce in these farms.

### *2.2 Sample collection and processing*

Sample collection was performed during the wet season of 2014, which covers the months of June to November. Samples (100-mL volume) were collected from surface waters by drawing water into sterile sampling bottles. A total of 15 samples were collected in each site. The samples were placed in an ice box kept at 10 °C and were transported to the laboratory for processing within 6 hr after collection. Sample processing was performed following the method of Litsky *et al.* (1953). Briefly, tenfold serial dilutions of the samples were performed three to four times in azide dextrose enrichment broth consisting of 7.5 g/L enzymatic digest of casein, 7.5 g/L protease peptone no. 3, 4.5 g/L beef extract, 7.5 g/L dextrose, 7.5 g/L sodium chloride, and 0.2 g/L sodium azide (HiMedia, India). Then, the dilution series were incubated at 37 °C for 4 hr.

### *2.3 Isolation of Enterococcus sp.*

A 100-µL aliquot from the dilution series was spread plated into bile esculin (BE) agar (Scharlau™, Scharlab, Sentmenat, Spain) plates and incubated at 35 °C for 24 hr. Dark brown to black colonies were considered as culture-positive *Enterococcus*. Three random colonies from BE agar plates were isolated and purified into bile esculin azide (BEA) agar (Scharlau™, Scharlab, Sentmenat, Spain) which were incubated at 35 °C for 18 to 24 hr (Bae and Hou 2013). The purified isolates were subcultured into trypticase soy broth (BBL™, Becton, Dickinson, and Company, Maryland, USA) and incubated for 24 hr. Then, 20% glycerol were added and stored at -20 °C until use for analysis.

### *2.4 DNA extraction*

Extraction of DNA was performed using the boil lysis method previously described by De Medici *et al.* (2003). In its procedure, overnight cultures of the isolates were centrifuged (Eppendorf, Germany) at 10,000 × g for 10 min. The resulting pellet was dissolved in 1 mL sterile distilled water and mixed by vortexing for 10 min. The solution was then centrifuged for 10,000×g for 10 min


**Figure 1.** Representative sampling locations for the collection of irrigation water to detect *Enterococcus* spp. in urban farms in Metro Manila, Philippines. (A) River in Marikina City; (B) Brook site in Quezon City; (C) Pond with vegetable farm in Quezon City; (D) Seep with vegetable farm in Quezon City; (E) Natural puddle with vegetable farm in Quezon City; (F) Seep site with vegetable farm in Quezon City

and the resulting pellet was added with 100 µL sterile distilled water. Then, the solution was placed in a digital dry bath (Bio-Rad, California, USA) set at 100 °C for 15 min. The supernatant was collected and stored -20 °C until use for PCR amplification.

*2.5 Polymerase chain reaction*

The extracted DNA was subjected to a PCR assay previously described by Ke *et al.* (1999). The primer sequences were: (Ent-1) 5'-TAC TGA CAA ACC ATT CAT GAT G-3,' and (Ent-2) 5'-AAC TTC GTC ACC AAC GCG AAC -3,' which amplify the 112-bp *tuf* gene that encodes the elongation factor EF-Tu of *Enterococcus*. The reaction was carried out using a 20-µL mixture consisting of 10 µL GoTaq® Master Mix (Promega Corporation, Wisconsin, USA), 0.5 µL forward primer (Macrogen, Korea), 0.5 µL reverse primer (Macrogen, Korea), 8 µL nuclease-free water (Vivantis, Malaysia), and the extracted 1 µL DNA template.

The cycling conditions were as follows: initial denaturation at 95 °C for 3 min, followed by 34 cycles of denaturation at 95 oC for 30 sec, annealing in the range of 45 to 60 °C for 30 s, elongation at 72 °C for 30 sec, and termination at 72 °C for 5 min.

*2.6 Agarose gel electrophoresis*

The PCR products were loaded into a 1.5% (w/v) agarose gel stained with SYBR® Safe DNA gel stain (Thermo Fisher Scientific, California, USA) in 1X TAE buffer (40 mM Tris, 20 mM acetic acid, and 1 mM EDTA at pH 8.0) (Vivantis, Malaysia) set at 180 V for 30 min. A positive control, *Enterococcus* faecium, and no template control were included in the electrophoresis. A 2 µL 1.0 - kbp DNA ladder (KAPA Biosystems, Massachusetts, USA) was also included to estimate the molecular weight of the PCR products. The agarose gel was viewed and photographed using Bio-Print ST4 and Vision-Capt ver. 16.08 gel documentation system (Vilber Lourmat, Marne-La Vallee, France).

*2.7 Antimicrobial susceptibility testing*

A total of 18 *Enterococcus* isolates were randomly selected and subjected to antimicrobial susceptibility testing following the broth microdilution method described by the American Society for Microbiology (Coyle 2005) and Clinical and Laboratory Standards Institute (2012). The minimum inhibitory concentrations (MICs) were determined for the following antimicrobials: tetracycline (Oxiod, Australia), ampicillin (Sigma Aldrich, Germany), chloramphenicol (Mast Diagnostics, United Kingdom), and ciprofloxacin (Becton, Dickinson and Company, Maryland, USA). *Enterococcus* isolates were subcultured in tryptic soy agar (Difco™, Becton, Dickinson, and Company, Maryland, USA) and incubated at 35 °C for 24 hr. Then, three random colonies were subcultured into Mueller-Hinton (MH) broth (Difco™, Becton, Dickinson, and Company, Maryland, USA) and the turbidity of the suspension was adjusted to that of 0.5% MacFarland standard (Hardy Diagnostics, USA). A 0.05-mL aliquot from the standardized suspension was inoculated into MH broth. Within 15 min, a 0.05-mL aliquot from the broth cultures was distributed into 96-well plate containing 0.05 mL antimicrobial broth microdilution series (Coyle 2005). The antimicrobial concentrations range from the highest susceptibility concentration for *Enterococcus*: 8 µg/mL for chloramphenicol and ampicillin, 4 µg/mL for tetracycline, and 1 µg/mL for ciprofloxacin, up to 5,120 µg/mL antimicrobial (CLSI 2012). The panels were then incubated at 35 °C for 16 to 20 hr. MICs were determined by visual inspection and were defined as the lowest antimicrobial concentration that showed absence of growth or complete growth inhibition.

## 3. Results and Discussion

*3.1 Results*

*3.1.1 Prevalence of Enterococcus sp. in irrigation waters*

From a total of 105 surface water samples analyzed in this study, 70 (67%) samples were found to be contaminated by *Enterococcus*, as shown in Table 1. Highest prevalence of *Enterococcus* was observed in the sampling site (river) in Marikina City (100%) and sampling sites 1 (brook) and 2 (pond) in Quezon City (both 100%). This was followed by sampling site 5 (seep) in Quezon City (60%), followed by sampling sites 3 (seep) and 4 (natural puddle) in Quezon City (both 53%). Inversely, no *Enterococcus* was recovered from the sampling site (seep) in Taguig City.

*3.1.2 Culture and molecular identification of Enterococcus sp.*

Colonies appearing as dark brown to black in BEA agar plates were considered as culture-positive *Enterococcus* isolates (Figure 2). The identity of the culture-positive *Enterococcus* isolates was confirmed using a PCR assay that amplifies the 112-bp *tuf* gene which encodes the elongation factor EF-Tu of *Enterococcus* (Figure 3).

**Table 1.** Prevalence of *Enterococcus* sp. in irrigation waters in urban farms in Metro Manila, Philippines

| Sampling location | Sampling sites | No. of samples analyzed | No. of positive samples (%) |
|---|---|---|---|
| Quezon City | 1 – brook | 15 | 15 (100%) |
| | 2 – pond | 15 | 15 (100%) |
| | 3 – seep | 15 | 8 (53%) |
| | 4 – puddle | 15 | 8 (53%) |
| | 5 – seep | 15 | 9 (60%) |
| Marikina City | 1 – river | 15 | 15 (100%) |
| Taguig City | 1 – seep | 15 | 0 (0%) |
| **Total** | | 105 | 70 (67%) |

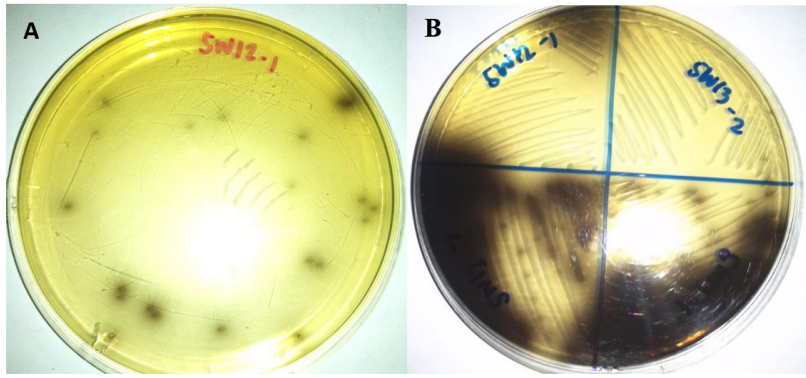


**Figure 2.** Representative culture-positive (A) spread plate; and (A) streak plate in BEAA of *Enterococcus* taken from water sample in a seep site in Quezon City as indicated by colonies exhibiting dark brown or black color

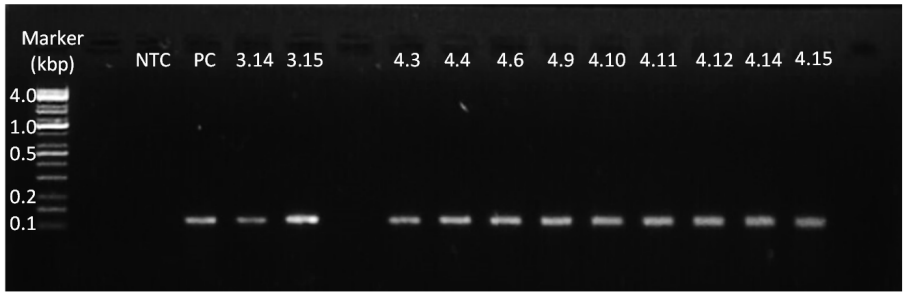


**Figure 3.** Representative agarose gel showing the PCR amplification of the 112-bp *tuf* gene of *Enterococcus* spp. where NTC = non-template control; PC = positive control; 3.14-3.15 = *Enterococcus* spp. isolated from Marikina City; 4.3-4.4; 4.6; 4.9-4.12; 4.14-4.15 = *Enterococcus* sp. isolated from Quezon City

*3.1.3 Antimicrobial susceptibility of Enterococcus sp.*

The MICs of each antimicrobial against *Enterococcus* are presented in Table 2. Highest MIC was observed in tetracycline (4.00 ± 0.00 µg/mL), followed by ampicillin (8.15 ± 0.61 µg/mL), chloramphenicol (8.00 ± 0.00 µg/mL), and ciprofloxacin (1.52 ± 0.80 µg/mL). All of the 18 *Enterococcus* isolates (100%) were susceptible to tetracycline, ampicillin, and chloramphenicol. Meanwhile, 11 isolates (61%) were susceptible, six isolates (33%) were intermediate, and one (6%) isolate was resistant to ciprofloxacin, as shown in Table 3.

**Table 2.** Minimum inhibitory concentrations (MICs) of antimicrobials against *Enterococcus* sp.

| Antimicrobial | Antimicrobial class | MICs[a] (µg/mL) |
|---|---|---|
| tetracycline | tetracyclines | 4.00 ± 0.00 |
| ampicillin | penicillins | 8.15 ± 0.61 |
| chloramphenicol | phenicols | 8.00 ± 0.00 |
| ciprofloxacin | fluoroquinolones | 1.52 ± 0.80 |

[a] values are reported as mean ± standard deviation

**Table 3.** Antimicrobial resistance of *Enterococcus* sp. from irrigation waters in Metro Manila, Philippines

| Antimicrobial | Susceptible | Intermediate | Resistant |
|---|---|---|---|
| tetracycline | 18 (100) | 0 (0) | 0 (0) |
| ampicillin | 18 (100) | 0 (0) | 0 (0) |
| chloramphenicol | 18 (100) | 0 (0) | 0 (0) |
| ciprofloxacin | 11 (61) | 6 (33) | 1 (6) |

*3.2 Discussion*

Irrigation waters are considered as an important vehicle of microbial contamination of produce. Previous studies have shown that surface waters used for surface or gravity irrigation in selected urban farms in Metro Manila, Philippines were contaminated by thermotolerant *E. coli*, pathogenic *Salmonella* spp., and somatic coliphages (Garcia *et al.,* 2015). Both *E. coli* and coliphages are used as indicator organisms to monitor pollution in different resources as their presence correlates with fecal contamination (Odonkor and Ampofo 2013; Vital *et al.,* 2014). This suggests that surface waters used for irrigation in Metro Manila may be contaminated by other microorganisms that are fecal in origin.

In this study, high prevalence of *Enterococcus* was observed in majority of the sampling sites (six out of seven) (Table 1). The highest prevalence of *Enterococcus* was observed in water samples obtained from a brook and a pond in separate urban farms in Quezon City, and from a river used for irrigation in Marikina City. The high prevalence of *Enterococcus* in these sites may be due to contamination caused by the presence of fecal material from domesticated and stray animals, such as cats and dogs that were noted near the surface waters during sample collection. A relatively high abundance of water birds was also observed, particularly in the brook in an urban farm in Quezon City. Further, the sampling sites were situated near residential areas. These results concur with the findings of Larsen *et al.* (2012) where 78.8% of the surface water samples from Des Moines River in Iowa, USA were found to contain *Enterococcus* and 38.1% of the samples reached a colony count that is equal or greater than the number established as the level of concern by the European Union (100 - 400 CFU/100 mL). The sampling sites were located near confinement animal (e.g. chickens, turkeys, and cattle) feeding operations where fecal streams may combine with runoffs that are eventually introduced in the surface waters. Similar results were obtained by Kouadio *et al.* (2017) and Said *et al.* (2016) where 100% of surface waters samples in Cote d'Ivoire and 50% of surface water samples in Tunisia, that are used for irrigation, were found to be contaminated by *Enterococcus.*

The proximity of various animals to the sampling sites is a plausible reason for finding a large number of surface water samples contaminated with *Enterococcus* in this study. The gastrointestinal tract of humans and animals are considered as the primary habitat of *Enterococcus* where they exist as harmless commensals and opportunistic pathogens (Johnston and Jaykus 2004; Gilmore *et al.* 2014; Sidhu *et al.* 2014; Fisher and Phillips 2018). This habitat provides a warm, nutrient-rich environment that supports vigorous growth and rapid reproduction of *Enterococcus*.

From the gut, *Enterococcus* are released into its secondary environments through feces during animal excretion, where it can survive for extended period of time. This accounts for the abundance of *Enterococcus* in human and animal feces, making it an important vector of *Enterococcus* transmission and deposition to its secondary habitats (Byappanahalli *et al.,* 2012). The secondary habitats of *Enterococcus* are usually open and limiting environments characterized by nutrient deprivation, osmotic stress, fluctuating temperature and pH, and predation; and include water, soil, sediments, and plant surfaces. *Enterococcus* spp. overcomes these harsh conditions by exhibiting a remarkable metabolic versatility and physiological adaptability in their secondary environments which also provides opportunities for reintroduction to humans and animals through ingestion of contaminated water and food (Johnston and Jaykus 2004; Gilmore *et al.,* 2014; Sidhu *et al.,* 2014; Fisher and Phillips 2018).

The emergence of antimicrobial resistance among bacteria recovered from contaminated food has also raised substantial concerns about food safety and sparked interest over the potential sources of antimicrobial resistant bacteria in food. In Metro Manila, Philippines, fresh produce that are typically consumed raw or minimally processed were found to be contaminated by *E. coli* and *Salmonella* spp. that exhibit mono- or multi-resistance to commonly used antimicrobials such as tetracycline, chloramphenicol, ciprofloxacin, and nalidixic acid. Additionally, genes conferring resistance to tetracycline and chloramphenicol were also observed (Vital *et al.* 2017). While the exact source of antimicrobial resistance bacteria in fresh produce in Metro Manila is difficult to ascertain, several studies have recognized the increasingly important role of irrigation waters in the emergence and dissemination of antimicrobial resistant bacteria (Aijuka *et al.* 2015), especially as antimicrobial resistance determinants such as antimicrobial residues and antimicrobial resistance genes were detected among the bacterial community in surface waters in Metro Manila, Philippines (Suzuki *et al.* 2013).

Interestingly, only a small fraction (6%) of *Enterococcus* showed full resistance to at least one antimicrobial (ciprofloxacin), which was somewhat unexpected, as antimicrobial selection pressure has been predicted based on the presence of antimicrobial resistance determinants in surface waters of Metro Manila as reported by Suzuki *et al.* (2013). Additionally, the results of this study are discordant with the findings of other studies which showed high prevalence of antimicrobial resistant *Enterococcus* in surface waters used for irrigation. For instance, *Enterococcus* recovered from surface waters near an area of intensive poultry production in British Columbia, Canada exhibited resistance to lincomycin (87.1%), tetracycline (24.1%), penicillin (7.6%), and ciprofloxacin (12.9%) (Furtula *et al.,* 2013). Similarly, Luczkiewicz *et al.* (2010) reported the prevalence of *Enterococcus* that are resistant to erythromycin (55%), ciprofloxacin (22%), and tetracycline (14%) in surface waters of Gdansk and Puck Bays in Poland. Meanwhile, surface waters used for irrigation in western Tehran, Iran were found to harbor *Enterococcus* spp. that are resistant to tetracycline (41.5%), erythromycin (27.1%), and ampicillin (12.7%) (Enayati *et al.* 2015).

The results of this study may have also been influenced by the time of sampling which took place during the wet season. In the eastern part of the Philippines, including Metro Manila, wet season is characterized by abundant rainfall throughout the year due to the southwest monsoon (Capistrano and Marten 1986). Seasonal variation was noted to affect the prevalence of indicator organisms such as *E. coli.* For instance, Garcia *et al.* (2015) reported that *E. coli* counts in surface waters in Metro Manila, Philippines were significantly higher during the dry season as compared to the wet season. The same results were obtained by Widmer *et al.* (2013) where the combined *E. coli* counts in surface waters of major cities in Vietnam, Thailand, Cambodia, and Indonesia were significantly higher during the dry season as opposed to the wet season. This disparity may be explained by the movement of surface waters during the dry season, which is characterized by less runoff feeding and reduced and more stagnant water flow, resulting to higher counts (Garcia *et al.,* 2015).

This study is limited by the following: (1) isolation of *Enterococcus* hinges on the use of conventional culture methods that may fail to take into account the presence of viable but non-culturable entities that may also display antimicrobial resistant phenotypes; (2) limited sample size encompassing a small geographic area does not provide absolute estimation of the prevalence of *Enterococcus* in surface waters; and (3) lack of data to accurately measure the extent to which surface waters in the sampling sites are used for irrigation makes it difficult to estimate the negative impacts of utilizing resources for irrigation of fresh produce. Nevertheless, this study showed the presence of pathogenic *Enterococcus* in surface waters in Metro Manila, Philippines, which may contaminate agricultural produce when these waters are utilized for irrigation purposes. Comparison of the prevalence of *Enterococcus* during the wet and dry seasons and tracking of the sources, distribution, and fate of *Enterococcus* are important follow up work that warrants attention in surface waters used for irrigation in Metro Manila, Philippines.

This manuscript is important in the Philippine setting as currently, the government's Department of Agriculture – Bureau of Fisheries and Aquatic Standards is creating and adopting standards for agricultural food safety. Particularly, there was no local data to support microbiological criteria that can be included in the standards. The results of this study will greatly contribute in the much needed data by the government.

## 4. Conclusion

*Enterococcus* sp. were recovered from surface waters used for irrigation in selected urban farms in Metro Manila, Philippines, suggesting that their use for irrigation may have a negative impact on the quality of vegetables. A factor that may significantly contribute to the presence of *Enterococcus* in surface waters is the presence of domesticated and stray animals on the farms and their proximity to residential areas. A sizable proportion of *Enterococcus* recovered was susceptible to antimicrobials. This study provides baseline information and initial estimation of the prevalence of *Enterococcus* in surface waters used for irrigation in Metro Manila, and preliminary assessment of their antimicrobial resistance, areas that remain relatively unexplored in the Philippines thus far.

## Acknowledgements

The authors would like to thank the Department of Agriculture – Bureau of Agricultural Research and the Scientific Career System of the Department of Science and Technology and Civil Service Commission for providing funding support.

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