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Detection of veterinary antibiotic residues in fresh fecal samples collected from selected pig farms in the City of Baybay, Leyte, Philippines

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ABSTRACT

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Investigations on the presence of antibiotic residues are becoming an integral part on the quest against antimicrobial resistance (AMR). Moreover, as a potential hazard to human health and the environment, the threat of antibiotic residues cannot be taken lightly. As the biggest pig producer in Eastern Visayas, the Province of Leyte, Philippines is presented with the challenge to sustain the pork requirements of the region despite its limited overall production. This study aims to determine the prevalence of antibiotic residues present in the swine manure among selected commercial piggery farms in Baybay City, Leyte. Using 147 fresh fecal samples collected from different classes of pigs and following standard microbial inhibition plate technique, this study consistently found inhibitory action against the growth of *Bacillus subtilis* across three pH levels from fecal samples collected from grower-finishers, and breeders unlike samples collected from the lactating and farrowing sows. These results may provide insights and baseline information useful for similar studies on antibiotic residues in meat and meat products that may have implications on human health.

Keywords: pig manure, antibiotic residues, City of Baybay, Philippines

INTRODUCTION

Antibiotic use in farm animal production is almost a standard practice to treat sick animals, minimize mortality and promote faster growth. As food animal production becomes more complex, swine feeding has resulted in veterinary products becoming a common part of feed formulations. Antibiotics and growth

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could significantly improve the efficiency of livestock production thus helping to meet the demand for high-quality meat and poultry products at a reasonable price (Donoghue 2003). The use of antibiotics in livestock production serves three primary purposes: 1) therapeutic, 2) prophylactic, and 3) growth promotion (Schwarz et al 2001, Landers et al 2012). At therapeutic levels, antibiotics are administered at normal or higher doses for the treatment of specific diseases while at prophylactic levels, moderate doses are given to animals for relatively longer durations for disease prevention. While the mechanisms, reviewed by Mund et al (2017), on how antibiotics promote growth in animals may need further investigation, this could be attributed primarily to the inhibitory action against harmful pathogens. Antimicrobial agents could prevent overgrowth of intestinal bacteria which can help protect nutrients to be utilized by the animal and facilitate intestinal absorption (Butaye et al 2003), thus promoting growth. Antimicrobial growth promoters may improve the animal's efficiency to utilize feed by as much as 17% in beef cattle, 15% in swine and poultry, and 10% in lambs (Nisha 2008).

However, the risks posed by antibiotics both as residues in meat and meat products and as potential pollutants cannot be taken lightly (Shahani & Whalen 1986). Reports on residues in meat from food-producing animals suggest that at least 67% of pigs, 21% of cattle, 6% of sheep and goats, and 5% of poultry from a considerable number of samples tested were found to be non-compliant (EFSA 2010). In one study, at least 40% of chickens sampled confirmed positive of one or more antibiotics (see review by Vishnuraj et al 2016). This appears to support reports of a number of broiler and layer farms administering non-specific levels of antibiotics above therapeutic purposes for a prolonged period of time (Kabir et al 2004, Rokka et al 2012). Apparently, with many farmers having easy access to veterinary drugs compounded by poor restrictions and local regulations, non-specific medication along with indiscriminate and inappropriate use of antimicrobials have become a common problem in livestock production.

There is increasing concern relating to the rise of antimicrobial resistance (AMR) which has recently become a clear and urgent problem to human health and development (Bhatia & Narain 2010, Singh 2017). In India, for example, AMR could be linked to unregulated large-scale use of antibiotics in poultry farming (Vishnuraj et al 2016). Apparently, similar situations have been reported in Vietnam (Van Nhiem et al 2006), Africa (Darwish et al 2013, Lawal et al 2015), Egypt (Odwar et al 2014), and other places (Van Boeckel et al 2015). Antibiotic residues present a potential hazard to both human health and the environment (Ben et al 2019). Veterinary antibiotics are potential environmental pollutants when a significant number have been used in animal farms where a sizable fraction were used for non-therapeutic purposes. If not utilized by the body properly, these antibiotics are excreted in feces (Li et al 2013) and often introduced into the environment when animal manures are applied as fertilizers for crop production thereby putting human health at risk (Kumar et al 2005). These antibiotics also have the potential for migrating to bodies of water via surface runoff and/or leaching (Zheng et al 2019) and further escalate environmental contamination. A recent study showed that antibiotics belonging to tetracyclines, quinolones, macrolides, lincosamides, and pleuromutilins are highly persistent and could last for many months depending on the manure type (Berendsen et al 2018).

The Philippine Statistics Office (PSA) recorded a very low swine inventory in Region VIII at 3.2%, indicating the need to increase the level of pig production (PSA 2019). Based on recent data, the Province of Leyte, accounts for more than half (52.4%) of the pig population (PSA 2019) and provides about 60% of slaughtered pigs within the region (PSA 2018). This study attempts to identify the prevalence of and to characterize antibiotic residues present in the swine manure from selected commercial piggery farms in Baybay City, Leyte. Results of this study could provide insights and baseline information before extensive studies on antibiotic residues in meat and meat products are initiated, including specialized identification and subsequent quantification (Gondová & Kožárová 2012). It is also envisaged that the results of this study could direct pig farm owners, animal technicians and pig enthusiasts about the risks associated with antibiotic residues in manure as a potential source of pollution within the region.

MATERIALS AND METHODS

Pig Farms and Collection of Fecal Samples

Selected commercial pig farms strategically located in Baybay City, Leyte, Philippines (10°39'50.3"N 124°50'55.3"E) were included in the study. A total of 147 fresh fecal samples were collected from different age groups and/or classes either as individual samples from lactating sows (4), farrowing sows (12), breeders (45), or as pooled samples from grower-finisher pigs (86). All fecal samples about 10g each were collected in the morning from freshly evacuated manure and placed inside clean resealable plastic bags and labeled accordingly. The samples were transported in a polystyrene box with ice to the Microbiology Laboratory of the College of Veterinary Medicine, Visayas State University for processing and the identification of antibiotic residues. The study was conducted following approval of the Student Research Committee of the College.

Preparation of Fecal Residue Assay

About two grams of each fecal sample were weighed and added into a clean 15mL tube with 3mL of McIlvaine buffer solution. The McIlvaine buffer (pH 3.0) consisted of 41.1mL of 0.2M Na_2HPO_4 and 158.9mL of 0.1M citric acid prepared previously as a stock solution. The mixture was homogenized gently using a spatula followed by centrifugation at 5000rpm for 5min and then the supernatant was transferred to another 15mL tube. This procedure was repeated thrice to maximize extraction of potential antibiotic residues from the fecal sample and all supernatants were mixed together.

Preparation Test Organisms and Reference Antibiotics

Batches of Mueller Hinton Agar (MHA) media each weighing 38g were prepared in a beaker with 1000mL of distilled water and adjusted to either pH of 6, 7.2 and 8, accordingly. The MHA medium was used as it generally favors the growth of bacteria due to its non-selective characteristics while allowing better diffusion of antibiotics resulting in clearer zones of inhibition (Deka et al 2022, Ruangpan & Tendencia 2004, Tendencia 2004). The prepared MHA with adjusted pH was homogenized then autoclaved at 121°C (10psi) for 15mins. Thereafter, the beaker containing the MHA was maintained in a hot water bath to keep the medium from solidifying until it was used.

The reference organisms used in this study were pure culture of *Escherichia coli* (BIOTECH 1634) and *Bacillus subtilis* (BIOTECH 1679) purchased earlier from the University of the Philippines, Los Baños, Laguna. Each of these test microorganisms were cultured accordingly using standard microbiology laboratory protocols and served as the source suspensions. The suspensions contained an approximate bacterial density of 300 million cells/mL as indicated by the turbidity standard set using McFarland Nephalometer barium sulfate preparation (Coles 1984).

The reference antibiotics used in this study were standard antibiotic discs of penicillin (10µg), trimethoprim-sulfamethoxazole (25µg), tetracycline (30µg), and ciprofloxacin (5µg). The same antibiotic discs were used in different procedures conducted in the laboratory for relevant research or diagnostic activities, thus ensuring that such protocols using these antibiotics had already been established. The test protocol used media seeded with *Bacillus subtilis* at different pH values (6.0, 7.2 and 8.0) with one medium seeded with *Escherichia coli* at pH 8.0. On agar at pH 6.0, 7.2 and 8.0, *Bacillus subtilis* is sensitive to tetracyclines, sulfonamides and aminoglycoside antibiotics, respectively, while *E. coli* is sensitive to quinolones on agar at (Kožárová 2012).

Microbial Inhibition Assay

Standard procedures, run in three replicates per fecal sample, following the microbial inhibition plate test were used to screen for the presence of antibiotic residues depending upon the response of the test organisms at different pH levels (Gondová & Kožárová 2012). Using a sterile pipette, one mL of each of the reference organisms was dispensed in individually labeled sterile petri plates containing 15mL MHA. The petri plate was swirled gently to homogenize the mixture and was allowed to harden for 15min at room temperature. Thereafter, holes were made in the agar using a 5mm metal cork borer.

The supernatants of two fecal samples, 0.05mL each in three replicates were then infused into individual holes including distilled water as the negative control, and antibiotic discs were overlaid on the agar at the center of the plate to serve as positive controls. Each of the test and control samples were laid out at about 15-20mm from each other to avoid overlapping of the zones of inhibition. The plates were allowed to stand for 10-15min at room temperature and then incubated at 37°C for 24h. Following incubation, samples that exhibited zones of inhibition (ZI) ≥9mm diameter (2mm from the edge of the disc to the edge of the clear zone) were considered the presence of antibiotic residue.

Data Management and Statistical Analysis

All data were encoded using Microsoft Excel and descriptive statistics were generated using XLSTAT (Addinsoft 2020). Observations were calculated as percentage positive over total number of samples tested while zones of inhibition were calculated as means (±SEM) presented per class of pigs examined.

RESULTS AND DISCUSSION

A total of 147 fecal samples were collected according to the different classes of pigs that were available when the study was conducted. Consistently, fecal samples collected from growers-finishers and breeders demonstrated inhibitory action against the growth of *Bacillus subtilis* across the three pH levels. On the other hand, none of the samples collected from both the lactating and farrowing sows had any inhibitory action (Tables 1, 2, and 3). At pH of 6.0, fecal samples collected from both the grower-finishers (10.5%) and the breeders (13.3%) demonstrated inhibitory action against the growth of *Bacillus subtilis* at 8.4±0.4mm and 7.9±0.3mm, respectively (Table 1). Interestingly, both had zones of inhibitions that were relatively greater than the positive control. Similar results can be seen at pH 7.2 except for the marked increase in the zones of inhibition in the positive control using the Trimethoprim-sulfamethoxazole (Table 2) while a relatively lower number of positive samples at pH 8.0 (Table 3). None of the fecal samples across all classes were found to inhibit the growth of *E. coli*.

Table 1. Fecal samples from different classes of pigs that exhibited inhibition of bacterial growth and mean (±SEM) zone of inhibition against *Bacillus subtilis* at pH 6.0

Classes of Pigs	Observations (%)	Zone of inhibition (mm)	Control-Penicillin (mm)
Grower-finishers	10.5	8.4±0.4	4.4±0.4
Breeders	13.3	7.9±0.3	2.1±0.5
Lactating	0.0	0.0±0.0	0.7±0.7
Farrowing	0.0	0.0±0.0	0.0±0.0

Samples (n) = grower-finisher (86), breeders (45), lactating (12), farrowing (4)

Table 2. Fecal samples from different classes of pigs that exhibited inhibition of bacterial growth and mean (±SEM) zone of inhibition against *Bacillus subtilis* at pH 7.2

Classes of Pigs	Observations (%)	Zone of Inhibition (mm)	Control – Trimethoprim – sulfamethoxazole (mm)
Grower-finishers	8.1	7.9±0.4	14.9±0.7
Breeders	4.4	8.8±0.8	14.4±1.4
Lactating	0	0.0±0.0	16.1±0.8
Farrowing	0	0.0±0.0	17.1±2.6

Samples (n) = grower-finisher (86), breeders (45), lactating (12), farrowing (4)

Table 3. Fecal samples from different classes of pigs that exhibited inhibition of bacterial growth and mean (±SEM) zone of inhibition against *Bacillus subtilis* at pH 8.0

Classes of Pigs	Observations (%)	Zone of Inhibition (mm)	Control - Tetracycline (mm)
Grower-finishers	1.2	8.0±0.0	2.5±0.4
Breeders	4.4	7.0±0.5	2.0±0.5
Lactating	0.0	0.0±0.0	0.0±0.0
Farrowing	0.0	0.0±0.0	0.0±0.0

Samples (n) = grower-finisher (86), breeders (45), lactating (12), farrowing (4)

The general and growing concern associated with antimicrobial resistance (AMR) is becoming a serious problem worldwide. Aside from the risks associated with the development of drug resistance, antibiotic resistant pathogens, as well as environmental pollutants coming from antibiotic residues (Van Epps & Blaney 2016), there is also the danger of antibiotic residues in meat and meat products. One question to consider is the extent of antibiotics being excreted as residues in the feces of animals particularly in commercial pig operations. It has to be noted that there are a number of factors that could influence drug metabolism and thus the occurrence of antibiotic residues in the animal manure. These include, but may not be limited to, species, age, size, tissue storage, nutritional and health status, and spatial variation (Wei et al 2019) including animal health practices and husbandry. Moreover, while detection of antibiotic residues in feces is not new, our study primarily aims to demonstrate the presence of antibiotic residues in fresh fecal samples collected from commercial pigs in the City of Baybay, Leyte, Philippines. Results of this study may guide future research in the region to 1) identify and quantify the relative levels of antibiotic residues over time, and 2) explore mitigation measures either to control its prevalence, and/or treatment procedures to minimize or prevent environmental contamination. We note however that the procedures used in this study are not the gold standard and therefore interpretation of the results should be taken with caution. There could be other chemical substances coming from various sources that are excreted in the feces which could also have antibiotic properties (Terzi et al 2016, Prabhurajeshwar & Chandrakanth 2019). Moreover, it was not possible during the extraction process to exclude those substances with potential antibiotic activities that might confound the actual results.

The application of livestock manure to plants is a common practice in rural agriculture particularly crop production. This practice provides an easy, readily available, and relatively cheaper fertilizer for plants and is being promoted for healthy vegetable production. Unfortunately, the presence of antibiotic residues in manure as well as animal wastewater and surface water around livestock and poultry farms (Wei et al 2019) has been the subject of interest between animal health professionals and health-conscious crop growers because of its impact on the environment and the potential risk to human health. Several studies highlight potential effects of antibiotic residues against the structure and function of microbial contaminants in the soil, water contamination and the spread of antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs) among others (Wei et al 2019). A recent study for example has found that antibiotic residues coming from doxycycline, sulfadiazine, and lincomycin were among those most frequently detected; doxycycline being the highest (mean of 1476μg kg^{-1} pig manure) while a mean concentration of less than 100μg kg^{-1} manure was detected in at least 18 antibiotic residues tested (Rasschaert et al 2020). Moreover, antibiotic residues from sulfadiazine, tetracycline, and trimethoprim have been found to persist up to 100 days after the manure application signifying the risks involved in such events (Menz et al 2019). Research and regulatory efforts to mitigate and control the presence of antibiotic residues in livestock manure should be given attention. These efforts may include production of education materials to inform livestock farmers, adoption of good animal health practices, and proactive participation of animal health professionals particularly livestock veterinarians. Composting techniques that employ controlling composting temperature and pH may also be explored which could potentially reduce the load of microbial ARGs (Liu et al 2020). Similarly Zhang et al (2019) demonstrated possible removal of veterinary antibiotic residues by almost 65% after 171 days of composting. The same authors further showed higher removal rates for lincomycin, trimethoprim and the macrolides (>89.7%) when compared to sulfonamides, tetracyclines and fluoroquinolones (<63.7%). There are also other manure management strategies that include the biological removal of nitrogen from pig manure. This manure treatment was found to reduce not only the levels of certain antibiotic residues but also zoonotic bacteria present in the manure thus lessening contamination of the environment (Van den Meersche et al 2019).

CONCLUSION

In conclusion, this study confirms the prevalence of antibiotic residues in the fecal samples collected from grower-finisher and breeder pigs as demonstrated by the inhibitory action against the growth of *Bacillus subtilis* across three pH levels. The simplicity of the method presented in this research should encourage small laboratories to conduct similar studies while taking into consideration the number of samples to be examined. The zone of inhibition assay is relatively cheap and a quick method which could be used to broadly evaluate the prevalence of antibiotic residues. The results of which could then be used as a basis for further research using more sophisticated laboratory techniques to identify and quantify the levels of antibiotic residues. Notwithstanding, the use of more sophisticated techniques should not preclude other considerations or factors which could provide more information that would lead to a better understanding of the occurrence of antibiotic residues in meat and/or in manure. These factors may include the individual animal, animal health programs, history of prolonged drug treatment and other risk factors.

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