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Sensitivity of microbial inhibition assay for screening antibiotic residues in retailed meats collected from the public market of Kabacan, Cotabato, Philippines

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ABSTRACT

The threat of antibiotic residues imposes a great concern in public health and at the same time in food safety. This study was conducted to screen antibiotic residues in retailed meats and evaluate the antibiotic sensitivity of indicator test organisms (*Bacillus subtilis* & *Staphylococcus aureus*). A total of 125 samples from three different types of meats (beef, chicken, and pork) were collected from the Public Market of Kabacan, Cotabato. Microbial Inhibition Assay utilizing *B. subtilis* and *S. aureus* were used for screening antibiotic residues. The total percentage of positive samples was 14.4%. The highest percentage was detected in chicken (22%) followed by beef (16%) and pork (6%), respectively. In evaluating the antibiotic sensitivity of test organisms, *Bacillus subtilis* detects Amoxicillin, Enrofloxacin, Tetracycline, and Oxytetracycline up to its maximum residual limits. There was no significant difference in beef ($p > 0.05$) and pork samples ($p > 0.05$) for the detection of antibiotics by the indicator organisms, while in chicken, the analysis revealed that the antibiotic sensitivity of test organisms was significantly different ($p = 0.021$). Hence, *B. subtilis* was ideal test organism than *S. aureus* ($p<0.05$). The binomial Logistic regression model also further suggests that *B. subtilis* was likely to be sensitive in detecting antibiotic residues in all meat types ($\beta = -1.23$, $OR = 0.29$, $p = 0.013$).

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1. Introduction

Meat products such as pork, chicken, and beef are choices of every consumer because of their distinct taste, as a protein source, and usually served on different occasions and even on ordinary days. As to the latest Agricultural Outlook of OECD-FAO, the world meat consumption per capita is expected to increase to 34.9 kg retail weight equivalent by 2029 (1).

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Data show that Philippine meat consumption will increase due to the popularity of meat-based products. The per capita consumption in the Philippines in 2020 by type are pork (14.91 kg), poultry (13.71 kg), beef and veal (3.15 kg), and sheep (0.52 kg) (2).

Food safety is a primary demand of every consumer (3). When Ahmadi et al, screened and investigated the microbial and chemical properties of meat products in Iran, they found that the level of contaminants was higher than the national standards which might be considered a major risk to consumers' health (4). Nevertheless, meats being sold in our local markets are usually ensured to come from animals, whose physiological and health conditions are maintained by giving antimicrobials. Manyi-Loh et al, pointed out in their review that because of the protein demand the practice of using antibiotics in farm management is increasing and antibiotic resistance is now a public health concern (5). Different types of antibiotics are usually given to these animals to combat certain types of diseases. In the country, a qualitative pilot study by Barroga et al, on antimicrobials used in backyard and commercial poultry and swine farms found that amoxicillin, Colistin, Norfloxacin, Oxytetracycline, Florfenicol, Streptomycin, and Enrofloxacin were the most frequently used antibiotics in poultry and swine farm (6).

The usual method for primary screening of antibiotic residues is microbiological inhibition assay because of its affordability, comfortability to use, and ability to detect a wide range of antibiotics. However, these tests don't give sufficient information about the quantity and type of antibiotic (3).

The purpose of this research is to identify the desired analyte (antibiotic residues) present in food samples. *Geobacillus stearothermophilus*, *Bacillus subtilis*, and *Escherichia coli* were the most frequently used indicator organisms in screening antibiotic residues. Notably, their sensitivity was dependent on the type of antibiotic residues present in the matrix (7).

In this study, we aimed to determine the sensitivity of microbial inhibition assay for screening antibiotic residues in retailed meats collected from the Public Market of Kabacan, Cotabato, Philippines. Specifically, we aimed to screen antibiotic residues in retailed meats and evaluate the antibiotic sensitivity of two indicator bacterial strains. Three (3) meat types: pork, chicken, and beef were sampled and tested in this study.

2. Materials and Methods

2.1. Sample collection

A total of 125 meat samples from three meat types (beef, chicken, and pork) with a corresponding sample size (muscles) of 25, 50, and 50, respectively, were collected randomly from the Public Market of Kabacan, Cotabato. The samples were packed in different sterile bags which are sealed, labeled, and placed in an icebox with crushed ice. Samples were transported on the same day to the laboratory and were kept frozen until used.

2.2. Preparation of test plate

Nutrient Agar (HiMedia Laboratories) was prepared following the procedure from the manufacturer. 28.0 g of the media was mixed in 1 L of distilled water and then dissolved using a hotplate with a magnetic stirrer. The medium was sterilized at 121°C (15 psi) for 15 min.

After sterilizing, the medium was allowed to cool up to 40°C and poured at 20 mL/plate sterile petri dishes under the biological safety cabinet until it solidified. Petri plates were wrapped in sterile paper and placed in a sterile polyethylene bag as a secondary container and then refrigerated until used.

2.3. Bacterial strains

Bacillus subtilis and *Staphylococcus aureus* were utilized as indicator organisms in the study. Test organisms were procured from the Microbiology Laboratory, Department of Biological Sciences, College of Science and Mathematics, University of Southern Mindanao, Kabacan, Cotabato.

2.4. Preparation of bacterial culture

Nutrient agar (HiMedia Laboratories) was prepared according to the manufacturer's procedure. Briefly, 28.0 g of the medium was weighed, dissolved in 1 L distilled water, and boiled for 2-3 min. It was then sterilized at 121°C, 15 psi for 15 min. The sterile medium was dispensed on a sterile petri dish under aseptic conditions. The same procedure with some modifications was adopted from Hudzicki, for the preparation of bacterial strains (8). Strains of *Bacillus subtilis* and *Staphylococcus aureus* were streaked respectively on different plates. Plates were incubated at 37°C for 18-24 h. After incubation, colonies were fished-out using a flamed-sterilized inoculating loop and suspended on sterile distilled water. Turbidity was then compared to 0.5 McFarland solution for a viable amount of cells (1.0×10^8 cfu/mL) (9).

2.5. Preparation of antibiotic solutions

Antibiotic solutions were prepared for the antibiotic susceptibility screening of the indicator organisms. Amoxicillin, Enrofloxacin, Tetracycline, and Oxytetracycline were procured from a local pharmaceutical drugstore. Stock solutions were prepared using sterile distilled water as a diluent. Each concentration was stored in a glass vial for two weeks before being used.

2.6. Antibiotic sensitivity of indicator organisms

Briefly, conventional Kirby-Bauer Susceptibility testing was used in evaluating the antibiotic sensitivity of the indicator organisms according to the procedures of Sophila et al, with modifications (3). Nutrient agar was used as the medium and prepared according to the manufacturer's procedure (HiMedia Laboratories). Strains of *B. subtilis* and *S. aureus* were streaked in different plates using a sterile cotton swab. Sterile paper discs were soaked in varying concentrations of prepared antibiotic solutions. The discs were placed in plates with the bacterial lawn in triplicate. Moreover, the plates are incubated at 37°C for 18-24 h. Finally, the plates were evaluated using a caliper and noted as sensitive (>2mm in diameter) or resistant when no inhibition was observed (10).

2.7. Microbial inhibition assay

a. Sample preparation

Frozen meat samples (beef, chicken, and pork) were thawed under the biosafety cabinet. After thawing, a flamed sterilized steel spatula, scalpel, and forceps were used to remove the meat tissues (11), and samples were homogenized using a commercial blender.

Homogenized samples are transferred into respective sterile Petri dishes. Finally, a sterile paper disc was placed on homogenized samples to absorb the meat juices for 2-5 min.

b. Inoculation of indicator organisms on plates

Plates that were prepared and refrigerated were allowed to thaw at room temperature under the biosafety cabinet. Appropriate aliquots of *B. subtilis* and *S. aureus* were swabbed into plates using a sterile cotton swab. The plates were covered with a lid and allowed to stand under the biosafety cabinet for 20-30 min before sampling.

c. Screening of antibiotic residues

Paper discs soaked in samples were placed in prepared test plates using flame-sterilized forceps (11). Then plates are incubated at 37°C for 18-24 h. After incubation, the plates were evaluated. Those samples that do not have inhibition were reported as negative. A visible opaque layer or zone of inhibition surrounding the paper discs indicates positive for containing antibiotic residues.

2.8. Statistical analyses

Frequency count and percentage distribution were done for the number of positive and negative antibiotic residues of the different types of meats. On the other hand, we used the Chi-square test to study the statistical relationships between the different test organisms (10) to carry out the second objective. Further, we also used Binomial Logistic Regression Model in this study for comparison of identified antibiotic residues of test organisms across meat samples. We conducted all statistical tests using open-freeware JAMOVI version 2.00 (12). All statistical significance was set at $p<0.05$.

3. Results

Using Microbial Inhibition Assay, we collected a total of 125 meat samples which are tested for screening antibiotic residue. Out of the 125 samples, 14.4% (N = 18) tested positive. The chicken had the highest case of samples containing antibiotic residues, wherein 22% (N = 11) of the samples collected tested positive, followed by beef (16%; N = 4) and pork (6%; N = 3). In contrast, 85.6% (N = 107) of these samples were screened negative for antibiotic residues (Fig. 1).

In this study, *Bacillus subtilis* and *Staphylococcus aureus* were used as test organisms to detect antibiotic residues in meat samples. A positive result is exhibited by a zone of inhibition of any size. Table 1 reveals the number of meat samples that tested positive for antibiotic residue using these test organisms. When beef samples were screened using *Bacillus subtilis*, four (16%) out of 25 samples were found positive, and 21 (84%) out of 25 samples were negative. When *Staphylococcus aureus* was used as a test organism, only one (4%) out of 25 samples was screened positive for antibiotic residues. When *Bacillus subtilis* was used as a test organism in screening chicken samples, eleven (22%) out of 50 samples tested positive. Meanwhile, 39 (78%) out of 50 samples tested negative. With *Staphylococcus aureus* as the test organism, four (6%) out of 50 samples were positive, and 46 (94%) were negative. Prevalence of antibiotic residues was also found in pork, wherein three (6%) out of 50 pork samples were positive for antibiotic residues using *Bacillus subtilis*.

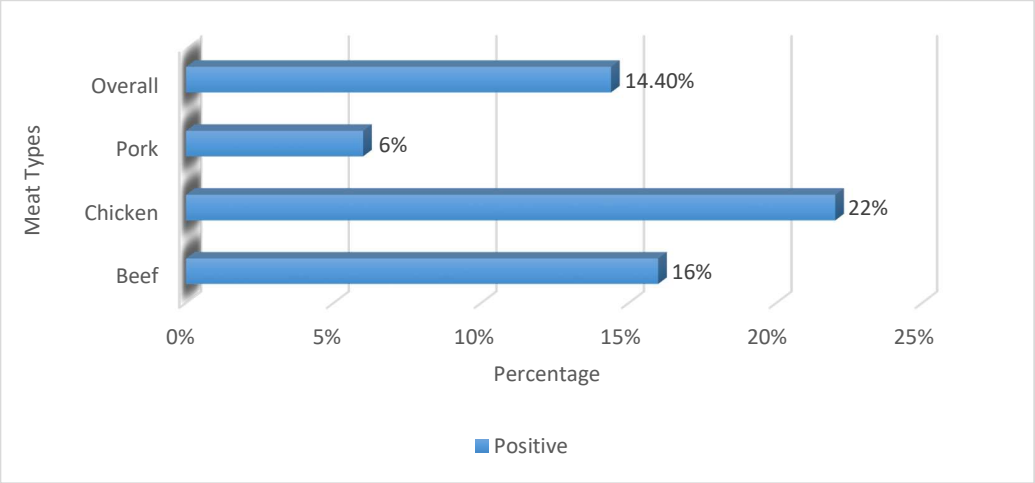

Figure 1. Total positive antibiotic residues in meat samples.

Table 1. Screening of antibiotic residues in all meat types (Frequency & Percentage).

Meat Types	Bacterial Group	Positive	Negative	Total
Beef	*B. subtilis*	4	21	25
		16%	84%	100%
	S. aureus	1	24	25
		4%	96%	100%
Chicken	*B. subtilis*	11	39	50
		22%	78%	100%
	S. aureus	4	47	50
		6%	94%	100%
Pork	*B. subtilis*	3	47	50
		6%	94%	100%
	S. aureus	2	48	50
		4%	96%	100%

Table 2. Antibiotic susceptibility testing of *B. subtilis* and *S. aureus*.

Antibiotic	MRL (µg/L)	Concentration (µg/L)	*Bacillus subtilis*	*Staphylococcus aureus*
Amoxicillin	4	16	+	+
		8	+	+
		4	+	-
Enrofloxacin	10	20	+	+
		10	+	-
		5	-	-
Tetracycline	100	120	+	+
		100	+	+
		90	+	-
Oxytetracycline	100	120	+	+
		100	+	+
		90	-	-

(+) sensitive; (-) resistant
Maximum residue limits (MRLs) of the European Union (13)

Table 3. Overall comparison of bacterial groups on screening antibiotic residues in retailed meats.

Bacterial Group		Frequency	Percentage
B. subtilis	Positive	18	14%
	Negative	107	86%
S. aureus	Positive	6	5%
	Negative	119	95%

Table 4. Chi-squared test of Independence comparison across meat groups.

Meat group	χ^2 (d.f.)	p-value	N
Beef	2 (1)	0.157	50
Chicken	5.32 (1)	0.021	100
Pork	0.21 (1)	0.646	100
Overall comparison of antibiotic sensitivity of test organisms across all meat types.			
All meat types	6.64 (1)	0.01	250

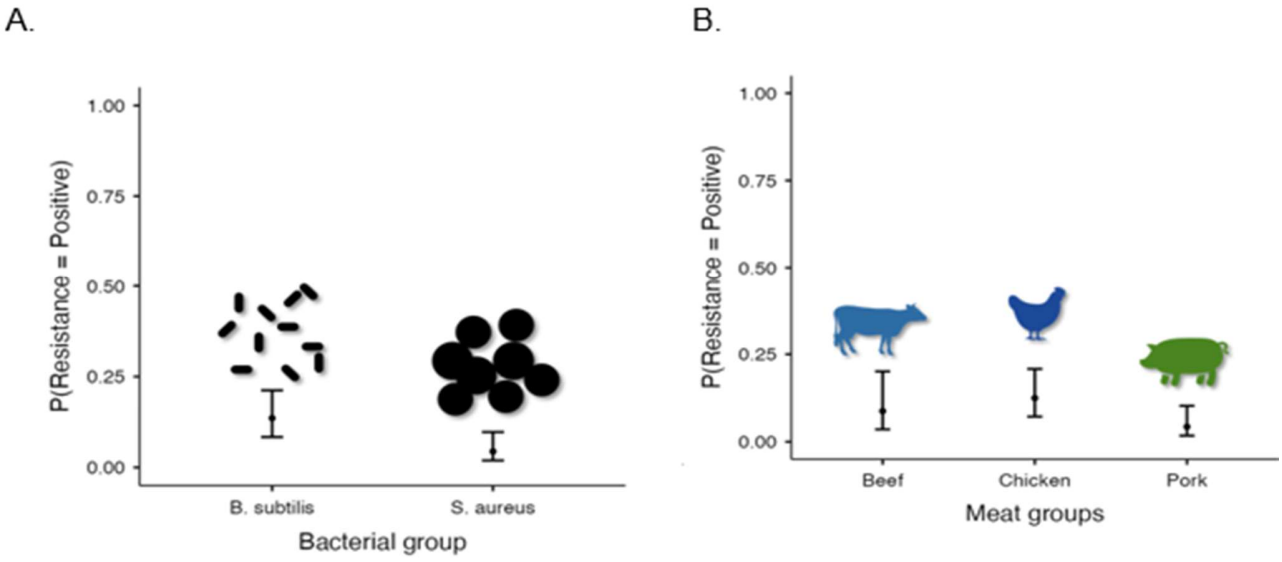

Figure 2. Comparison of detection of residues and likelihood on bacterial group (A) across meat samples (B). The error bars show that 95% confidence interval.

In contrast, 47 (94%) out of 50 samples tested negative. When the samples were screened using *Staphylococcus aureus,* two (4%) out of 50 samples showed a zone of inhibition. On the other hand, 48 (96%) of the samples were negative.

Table 2 presents the antibiotic sensitivity of the indicator organisms using varying concentrations of antibiotics. It was observed that *B. subtilis* detects higher than *Staphylococcus aureus. Bacillus subtilis* was sensitive up to its maximum residue limits on Amoxicillin, Enrofloxacin, Tetracycline and Oxytetracycline than *Staphylococcus aureus.*

Table 3 summarizes and compares the number of samples that tested positive per test organism. All meat samples were tested for antibiotic residues using the two test organisms. Out of 125 total meat samples, 14% (N = 18) of the samples were observed positive by inhibiting *B. subtilis*. Moreover, 5% (N = 6) of the samples tested positive by inhibiting *S. aureus*.

Table 4 presents the Chi-squared test of independence comparison across meat groups. *B. subtilis* and *S. aureus* were not significantly different ($p = 0.157$) in detecting antibiotic residues in beef samples. It appears that when evaluating the antibiotic sensitivity of test organisms on chicken meat, analysis revealed that test organisms were significantly different ($p<0.05$). Moreover, the Chi-square test revealed that there was no significant difference between the test organisms ($p>0.05$) for their antibiotic sensitivity when pork was used as a matrix. Furthermore, overall comparison of antibiotic sensitivity of test organisms across all meat types, revealed that there was a significant difference among test organisms ($p<0.05$).

The binomial Logistic regression model (Fig. 2) shows that the detection of antibiotic residues (i.e., positive or negative) in all meat types was dependent on the bacterial group. The model further suggests that *B. subtilis* was likely to be sensitive in detecting antibiotic residues in all meat types compared to *S. aureus* ($\beta = -1.23$, OR = 0.29, $p = 0.013$).

4. Discussion

There are a vast number of studies across the globe that were being carried out to test and investigate cases of cumulative toxicants such as antibiotic residues present in livestock. So far, no other studies for a decade have been published in the country for investigating antibiotic residues on livestock using Microbial Inhibition Assay. The main objective of this study was to determine the sensitivity of the Microbial Inhibition Assay for screening antibiotic residues in retailed meats. Specifically, it aimed to: screen antibiotic residues present in beef, chicken, and pork, and to evaluate the antibiotic sensitivity of the two test organisms. In the qualitative pilot study of Barroga et al, in the Philippines, Enrofloxacin was the most frequently used antibiotic in both swine and poultry farms (commercial and backyard farms) followed by Amoxicillin, Colistin, and Oxytetracycline (6). Extensive use of these agents will lead to deleterious effects on public health. Antibiotic residues can cause mutations, bone marrow impairment, damage to reproductive systems, it has also carcinogenic effects, and such (14).

In recent data from Statista Research Department, chicken, and pork-derived products are the leading produce consumed in high levels per capita in the Philippines. Wherein pork consumption per person was 14.9 kg and chicken had 13.74 kg. Beef and veal (3.15 kg per capita consumption) were also one of

several meat types found to be consumed at high levels in the Philippines (15), hence the threat of antibiotic residues in beef is possible. Several studies found antibiotic residues in their samples as well. In the research of Samandoulougou et al, published in the African Journal of Food Science, 31% of samples contained Aminoglycosides, Quinolones, Macrolides, Beta-lactam, Sulfonamides, and Tetracyclines when they utilized Premi® Test which is also a Microbiological Inhibition Test in Ouagadougou, Burkina Faso (16). In Madagascar, antibiotic residues in pork meat were found at an alarming rate with 37.2% of the samples tested positive (17). It was reported in the study of Liousia et al, conducted in Greece, that 26% of pork meat samples tested positive for antibiotic residues (18). Hence, surveillance, monitoring, and control measures should be employed in the production process (4).

In screening antibiotic residues using plate tests, the use of different test organisms is of paramount importance. Due to the reason that, test organisms have varying degrees of sensitivity to each type of antibiotic. *Geobacillus stearothermophilus* was used as a test organism in the study of Sophila et al, and it was found to detect a wide range of antibiotics (3). *Micrococcus luteus* was highly sensitive to detecting β-lactam antibiotics while *Bacillus cereus* can detect tetracycline up to its maximum residue limits. Additionally, fluoroquinolones can be detected by *Escherichia coli* and all of these mentioned antibiotics can be detected sensitively by *Bacillus subtilis* (19). Moreover, different test organisms can be combined to detect antibiotic residues in meat.

In the study of Hind et al, the combination of *B. subtilis* and *S. aureus* was found to be more sensitive in detecting antibiotic residues using meat as a matrix than *E. coli* (10). This justifies the use of *B. subtilis* and *S. aureus* in this study. Further, *B. subtilis* is more sensitive in detecting antibiotic residues present in beef, chicken, and pork samples than *S. aureus* in this study.

5. Conclusion

Detection of antibiotic residues is imperative in securing food safety due to its notable side effects such as antibiotic resistance. With the popularity of meat-based products in the Philippines, strengthening animal husbandry is therefore crucial. Because this might contribute to the abusive use of antimicrobial drugs and disregarding proper withdrawal periods. In the country, regulatory agencies continuously strengthen measures on ensuring food safety from farm to fork. In this study, the result shows that the prevalence of antibiotic residue in retailed meats was low. The use of microbiological inhibition assay for detecting antibiotic residue qualitatively, using the principle of inhibition, requires a test organism or combination of indicator organisms that has wide detection capabilities. *B. subtilis* was found to be an ideal test organism for *S.aureus* due to its higher detection capacity. Detecting food contaminant metabolite is critical in securing food quality. Therefore monitoring antibiotic residues by using cost-effective testing methods is needed.

Conflict of interest

The authors declare that they do not have any conflict of interest.

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