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Oxytetracycline-resistant heterotrophic fecal bacteria from pigs of Kabalasan, Baybay City, Leyte, Philippines

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ABSTRACT

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The excessive use of oxytetracycline for the cost-efficient production of swine can increase the development of antibiotic resistance among pathogenic bacteria. This study was conducted to isolate and identify putative cultivable heterotrophic bacteria from pig feces and evaluate their resistance to oxytetracycline. Composite fecal samples were serially diluted and plated using Tryptic Soy Agar (TSA). Distinct colonies were selected and grown on TSA plates and the pure cultures were subjected to limited screening for morphological and physiological characteristics. Representative isolates were then identified and subjected to oxytetracycline susceptibility assay through the disk diffusion method. Thirteen out of 15 selected colonies from TSA plates were successfully cultured and grouped into eight groups based on the isolates' morphological and physiological characteristics. Of the eight representative isolates, three were identified as putative *Escherichia coli*, two were *Staphylococcus aureus* strains, *Staphylococcus vitulinus*, *Bacillus cereus* and *Proteus mirabilis*. Furthermore, *S. vitulinus* and *S. aureus* strains exhibited susceptibility to oxytetracycline. Meanwhile, isolates, *P. mirabilis* and the three strains of *E. coli* showed the highest resistance while *B. cereus* showed the least. These oxytetracycline-resistant isolates can be used as test organisms to determine the antibacterial effect of novel bioactive compounds or as potential sources of oxytetracycline resistance genes that could be used as selection markers in transgenic experiments.

Keywords: oxytetracycline resistance, swine fecal bacteria, *Proteus mirabilis*, *Escherichia coli*, *Bacillus cereus*

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INTRODUCTION

Antibiotics have been the most effective drug in relieving symptoms and controlling disease. They work as toxins for pathogenic microorganisms without harming the host, making them specific for a particular type of pathogen (Shlaes 2010). Even in the past, administration of antibiotics to people, animals, and crops was continually done to enable the health care systems cope efficiently with bacterial infections (Shlaes 2010, Sköld 2011). Most of the antibiotics on the market today, including penicillin, erythromycin, tetracycline and all their relatives are by-products or metabolites produced by microbial metabolism in the soil, on plants, and in the ocean for the purpose of survival and competition (Sköld 2011, D'Costa et al 2006). Various classes of antibiotics have been discovered and produced from their natural sources since the 1960s. Since then, there are only four new classes of antibiotics marketed, namely Daptomycin, Linezolid, Pleuromutilins and Fidaxomicin, which were discovered around the 1970s to the 1980s (Coleman 2011, Etebu & Arikekpar 2016).

Tetracycline is one of the early classes of antibiotics. It is the second in the tetracycline group of antibiotics discovered (Ganesan & Proudfoot 2010). Oxytetracycline is a broad spectrum antibiotic that interrupts the initiation complex required for protein synthesis in bacteria (Connell et al 2003). It is heavily used in animal production because it is the most cost-effective way to maintain or improve the health and feed efficiency of animals raised with conventional agricultural techniques such as swine (Cromwell 2002). In the Philippines, oxytetracycline is the most commonly used antimicrobial active ingredient in swine backyard farms which are mostly purchased over the counter (Barroga et al 2020). This practice contributes heavily to the very large consumption of this antibiotic, thereby exposing the pathogenic bacteria in swine and increasing their chance of developing resistance (Zhu et al 2013, Sanz et al 2021).

Antibiotic resistance of bacteria has been around long before antibiotic has ever been marketed by pharmaceutical companies. It is a natural survival characteristic of bacteria (D'Costa et al 2006). Bacterial resistance to antibiotics that develops in these pigs by exposure has been shown to transfer to humans (Datta & Hughes 1983, Thapa et al 2020). When the pigs these humans were consuming were heavily dosed with antibiotics, the number of resistant bacteria isolated from humans infected with the same bacteria isolated from pig feces increased (van den Bogaard & Stobberingh 2000). Resistance poses a threat to human health, and intensive animal husbandry is believed to be a major contributor to the increased selective pressure that causes the problem (Zhu et al 2012). For instance, swine farming contaminants like antibiotics and antibiotic resistance genes increased the abundance of pathogenic bacteria in drinking groundwater (Gao et al 2020).

With the awareness of resistant bacteria and the understanding of how they come to be, the development of new and effective drug molecules with novel bacterial molecular target sites may constitute therapeutic alternatives within the next few years. Antibiotic resistant microorganisms can also be used as test organisms for the development of better diagnostic methods, development of more potent drugs towards these resistant bacterial strains, and as a source of resistance genes that can potentially be used in various biotechnological

applications. Thus, this study aimed to isolate and putatively identify the cultivable heterotrophic bacteria present in the feces of pigs in Kabalasan, Baybay City, Leyte, Philippines and determine and compare their resistance to the antibiotic oxytetracycline.

MATERIALS AND METHODS

Fecal Sampling and Processing

Fecal samples were collected manually from three (3) oxytetracycline-fed pigs using nitrile gloves while the pigs were defecating. The pigs were 60 to 90 days old from a swine farm in Brgy. Kabalasan, Baybay City, Leyte. The fecal samples were placed in a sterile sealable plastic bag, closed tightly and kneaded for a few minutes to mix the digesta (Salanitro et al 1976, Zhu et al 2013). Two (2) grams of the composite fecal sample was placed in a sterile Erlenmeyer flask (100mL) containing 38mL of Tryptic Soy Broth (TSB) (Himedia, India) and were allowed to stand for 30mins (Thornsberry et al 1975). The supernatant was pipetted out of the solution, placed in an Erlenmeyer flask, and shaken. The supernatant was serially diluted ten-fold from 10^{-1} to 10^{-7} (Salanitro et al 1977).

Heterotrophic Bacterial Isolation and Purification

Sample dilutions from 10^{-5} up to 10^{-7} were plated into 5 Tryptic Soy Agar (TSA) (Himedia, India) plates per dilution. It was done by adding 1mL of the dilutions to their respective plates (Raymundo et al 1991, Thornsberry et al 1975). The inoculated plates were then incubated in a convection incubator (Memmert, India) for 24h at 37°C to provide maximum growth conditions for most pathogenic bacteria.

After incubation, distinct colonies were randomly selected from each of the TSA plates based on their colony characteristics. The selected colonies were inoculated into fresh TSA plates for further purification using the streaking procedure and were incubated in a convection incubator for 18–24h at 37°C. The isolates were examined for purity by checking for variations in their cultural characteristics. The isolates were then inoculated onto fresh TSA slants as stock culture for further analysis.

Cultural, Morphological, and Physiological Characterization

After establishing the purity of the selected colonies, limited characterization tests were performed. The cultural characteristics like color, shape, and margin of the isolates' colonies were recorded. Each of the isolates were then subjected to Gram staining for determining their morphological characteristics like their cell shapes and the types of their cell walls. It was done based on the procedure described by Merchant and Packer (1969).

Moreover, physiological characterization was only based on the isolates' lactose fermentation capabilities using MacConkey and Eosin Methylene Blue (EMB) agar. Only the pure cultures from the TSA slants were streaked onto the MacConkey and Eosin Methylene Blue (EMB) agar and incubated at 37°C. The cultural characteristics of the colonies of each isolate on both agar were noted (Hispanlab 2000). EMB agar produces a dark purple complex usually accompanied

by a green metallic sheen in acidic condition, which indicates lactose/sucrose fermentation. Smaller amounts of acid result in a pink coloration of the growth. The normal color of bacteria or the coloration of the medium is retained in non-fermenters while a shade of red colonies on MacConkey agar are exhibited by lactose fermenters. Both media contain pH indicator dyes that inhibit the growth of most Gram-positive bacteria (Hipanlab 2000, Leboffe & Pierce 2012).

Putative Identification of Bacterial Isolates

The isolates were compared based on the combined data on their cultural, morphological, and physiological characteristics. The isolates with the same characteristics were grouped and only one representative isolate was used for identification. The representative isolates were then inoculated into Buffered Peptone Water (BPW) vials and were submitted to the Microbiology section of the Medical Laboratory of Ormoc Doctors Hospital, Ormoc City for species identification. The hospital used the automated system VITEK® 2 Compact: Bacterial Identification and Monitoring System from bioMérieux, Inc. This system relies on the colorimetry technology which reads VITEK® test cards containing 64 wells and reads every 15mins using three different wavelengths. This system provides rapid identification of bacterial pathogen through various biochemical tests, although it is not as precise as molecular phylogenetic method of identifying bacterial species.

Oxytetracycline Resistance Assay

Isolates from the stock cultures were inoculated into Buffered Peptone Water (BPW) tubes and incubated at 37°C for 18h. The turbidity of the BPW bacterial suspensions were then checked for homogenicity based on the 0.5 McFarland standard for antibiotic susceptibility testing and were adjusted by diluting when it was necessary. This is a standard procedure used to control the density of bacterial population when inoculated to MHA (Oxoid, United Kingdom), so that its zone of inhibition is comparable to standardized data.

The bacterial suspensions for each isolate were inoculated to MHA plates by evenly streaking three planes of the BPW suspension onto the surface of the medium with a sterile cotton swab. Three of the standardized oxytetracycline disks (Oxoid, United Kingdon) containing 30µg of the antibiotic per disk were uniformly placed on the surface of the inoculated MHA plates. Sterile filter paper disks treated with sterile distilled water were also placed to check the sterility of the sterile distilled water used in the experiment (Bauer et al 1966). The plates were examined and measured for inhibition zones using a ruler. Suspensions of resistant *Escherichia coli* and susceptible *Staphylococcus aureus* from the culture collection of the College of Veterinary Medicine served as the positive and negative control, respectively. Interpreting whether an isolate is resistant, intermediate, or susceptible (non-resistant) was based on the standard interpretation criteria given by the Clinical and Laboratory Standards Institute for veterinary studies (Table 1).

Table 1. Zone Diameter Interpretive Criteria to the nearest whole millimeter of different bacterial groups (Clinical and Laboratory Standards Institute 2015)

Category	Susceptible	Intermediate	Resistant
Enterobacteriaceae	≥15	12–14	≤11
Staphylococcus spp.	≥19	15–18	≤14

RESULTS AND DISCUSSION

Putative Identities of the Bacterial Isolates

A total of 15 distinct colonies were selected from the mixed TSA plate cultures. However, only 13 of the 15 isolates grew on the TSA plates that were streaked. After comparing the isolates based on their morphological and physiological characteristics, 8 distinct groups were found. The isolates that belonged to the same group were assumed to be of the same species. The characteristics and identities of the bacterial isolates are summarized in Table 2.

Table 2. Summary of the morphological and physiological characteristics of the bacterial isolates and their putative identity

Isolate Codes	Colony Charac.	Cell Wall	Cell Shape	Lactose Fermentation		Groups	Identification
				EMBA*	MacCA		
PFB-01	White, Opaque, Round & Umbonate	Negative	Bacillus	++	-	1	*Escherichia coli*
PFB-02	White, Opaque, Round & Umbonate	Negative	Bacillus	++	++		
PFB-03	White, Opaque, Round & Umbonate	Negative	Bacillus	++	++	2	*Escherichia coli*
PFB-04	White, Opaque, Round & Umbonate	Negative	Bacillus	++	++		
PFB-05	White, Opaque, Round & Convex	Negative	Bacillus	+	-	3	*Escherichia coli*
PFB-06	Yellow, Round & Opaque	Positive	Coccus	-	-	4	*Staphylococcus aureus*
PFB-07	White, Opaque, Round, flat & Umbonate	Positive	Coccus	-	-	5	*Staphylococcus aureus*
PFB-08	White, Opaque, Entire-Margin, Round & Raised	Positive	Coccus	-	-		
PFB-09	Translucent & Punctiform	Positive	Coccus	-	-	6	*Staphylococcus vitulinus*

(PFB) – Pig Fecal Bacteria, (++) – Heavy lactose fermentation, (+) – Light lactose fermentation, (-) – No lactose fermentation, (*) – Can also be Acid Production

Table 2. continued

Isolate Codes	Colony Charac.	Cell Wall	Cell Shape	Lactose Fermentation		Groups	Identification
				EMBA*	MacCA		
PFB-10	White, Opaque, Irregular, Undulate-margin & Umbonate	Positive	Bacillus	-	-	7	*Bacillus cereus*
PFB-11	White, Opaque, Irregular, Undulate-margin & Umbonate	Positive	Bacillus	-	-		
PFB-12	Cream, Opaque, Round & Convex	Negative	Bacillus	+	-	8	*Proteus mirabilis*
PFB-13	Cream, Opaque, Round & Convex	Negative	Bacillus	+	-		

The isolates representing group 1 (PFB-01), 2 (PFB-02), and 3 (PFB-05) were identified as *Escherichia coli* (Figure 1). These isolates were assumed to be of different strains because of their varying growth characteristics. *E. coli* PFB-01 grew white, opaque, round, and umbonate colonies on TSA (Figure 1 Ab). The group is Gram-negative and rod-shaped (Figure 1 Aa). *E. coli* PFB-01 grew black-centered colonies with green metallic sheen on EMB agar (Figure 1 Ac), but it had no growth on MacConkey agar. *E. coli* PFB-02 (Figure 1B) has the same colonial and cellular characteristics like *E. coli* PFB-01, but unlike *E. coli* PFB-01 it has grown pink, round, opaque colonies with bile salt precipitation on MacConkey agar (Figure 1 Bd). *E. coli* PFB-05 grew white, opaque, round, and convex colonies on TSA (Figure 1 Cb), and was found to be Gram-negative and rod-shaped (Figure 1 Ca). It had also grown on EMBA, however, it exhibited white colonies with translucent margins and some having convex pink centers (Figure 1 Cc). It also did not grow on MacConkey agar.

A

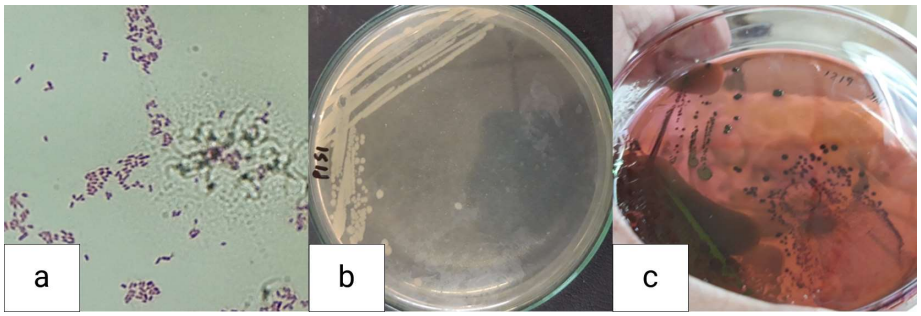

Figure 1. Characteristics of *E. coli* isolates after 24h incubation at 37°C [(A) *E. coli* PFB-01: a) Cellular morphology (1000X) after Gram-staining and its colonial growth on b) TSA and c) EMBA], [(B) *E. coli* PFB-02: (a) Cellular morphology (1000X) after Gram-staining, and its colonial growth on (b) TSA, (c) EMBA and (d) MacConkey agar], and [(C) *E. coli* PFB-05: (a) Cellular morphology (1000X) after Gram-staining, and its colonial growth on (b) TSA and (c) EMBA

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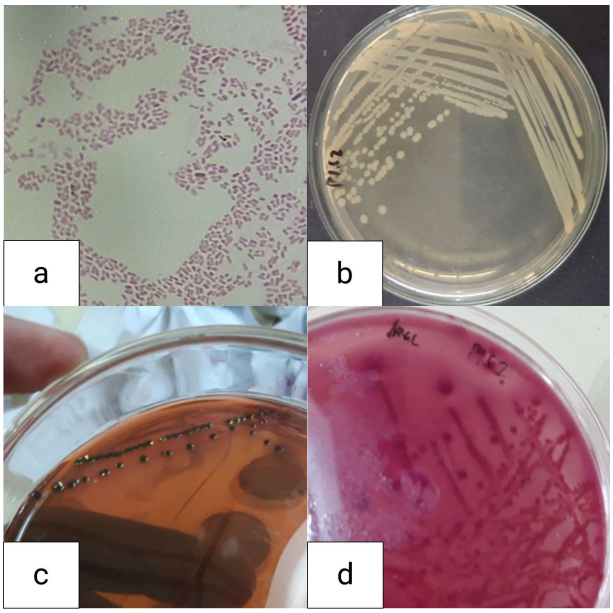

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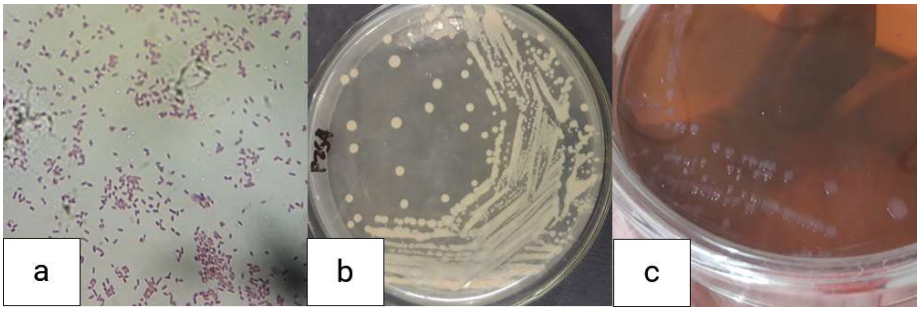

Figure 1. continued

The three *E. coli* isolates each exhibited variation in colony characteristics in TSA, EMB agar, and MacConkey agar. The eosin Y and methylene blue dyes in EMB agar that produce a dark purple complex usually accompanied by a green metallic sheen is caused by the increase in acidity on the media due to heavy lactose fermentation. Typical *E. coli* strains manifest the characteristics on EMB agar. Smaller amounts of acid production result in a pink coloration of the growth on EMB agar (Leboffe & Pierce 2012) which was exhibited by the *E. coli* PFB-05. A study by Barcella et al (2016) showed that only 6% of the 885 *E. coli* isolates from in-patients manifested the weakest lactose fermentation compared to the typical and dominant *E. coli* strains, making this an uncommon strain to find. The difference between *E. coli* PFB-01 and PFB-02 was the presence and absence of growth on MacConkey agar, respectively, but this is not conclusive, thus more tests are needed in order to confirm that the *E. coli* isolates were really of different strains.

Furthermore, representative isolates of group 4 (PFB-06) and 5 (PFB-07) were both identified as *Staphylococcus aureus* (Figure 2). They were also assumed to be of different strains because of their varying characteristics. *S. aureus* PFB-06 grew yellowish, round, and opaque colonies on TSA (Figure 2 Ab), and was found to be Gram-positive and coccus (Figure 2 Aa). Unlike the *E. coli* isolates, *S. aureus* PFB-06 did not grow on EMB and MacConkey agar. Some *S. aureus* PFB-07 colonies grew white, round, opaque, flat, and umbonate (Figure 2 Bb), while the other colonies grew white, opaque, entire-margin, round, and raised colonies on TSA (Figure 2 Bc). *S. aureus* PFB-07 was different from *S. aureus* PFB-06 in terms of colony color. Both isolates were found to be Gram-positive and coccus (Figure 2 Aa & Ba) and did not grow on EMB and MacConkey agar.

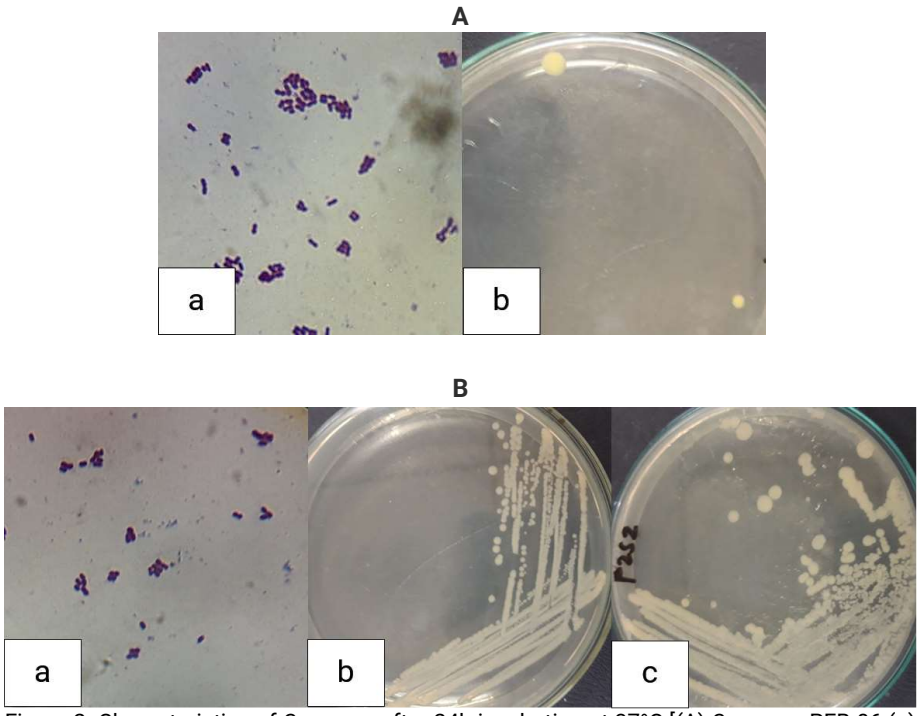

Figure 2. Characteristics of *S. aureus* after 24h incubation at 37°C [(A) *S. aureus* PFB-06 (a) Cellular morphology (1000X) after Gram-staining, and its colonial growth on (b) TSA], [(B) *S. aureus* PFB-07 after 24h incubation at 37°C (a) Cellular morphology (1000X) after Gram-staining, and colonial growth on (b) TSA for the first isolate and (C) for the second isolate

The representative isolate of group 6 (PFB-09) was identified as *Staphylococcus vitulinus* (Figure 3). It grew translucent and punctiform colonies on TSA (Figure 3B), and was found to be Gram-positive and (Figure 3A). Unlike the previous species of *Staphylococcus, S. vitulinus* PFB-09 grew on EMB agar with translucent and punctiform colonies (Figure 3C), but did not grow on MacConkey agar.

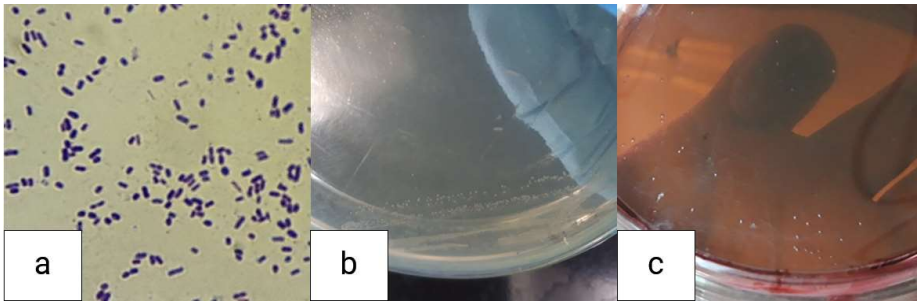

Figure 3. Characteristics of *S. vitulinus* PFB-09 after 24h incubation at 37°C (A) Cellular morphology (1000X) after Gram-staining, and its colonial growth on (B) TSA and (C) EMBA

S. aureus PFB-06 and PFB-07 had no growth on EMB and MacConkey agars. They were assumed to be of different strains due to their different growth characteristics in TSA. The presence of the eosin Y and methylene blue dyes in EMB agar as well as bile salts and crystal violet in MacConkey agar inhibits the growth of most Gram-positive bacteria (Leboffe & Pierce 2012). This implies that *S. aureus* PFB-06 and PFB-07 and *B. cereus* PFB-10 are Gram-positive, which is also supported by the Gram staining results. This is not the case with *S. vitulinus* PFB09 which is a Gram-positive bacteria that grew on EMB agar. Its growth on EMB agar made it distinct from the other *Staphylococcus* species. Some species and strains of *Staphylococcus*, along with some *Enterococcus* species, has been shown to grow on EMB agar, but only in translucent and punctiform colonies due to the toxicity of the dyes in EMB agar, thereby decreasing acid production that causes lesser coloration (Hispanlab 2000). Furthermore, *S. vitulinus* and other Gram-positive bacteria grow the way they do on EMB agar since they are known to proliferate in dairy which enables them to ferment lactose (Piessens et al 2011).

Moreover, a representative isolate of group 7 (PFB-10) was identified as *Bacillus cereus* (Figure 4). It grew white, opaque, irregular, irregular-margined and umbonate colonies on TSA (Figure 4B), and was found to be Gram positive and rod-shaped (Figure 4A). *B. cereus* PFB-10 did not grow on both EMB and MacConkey agar.

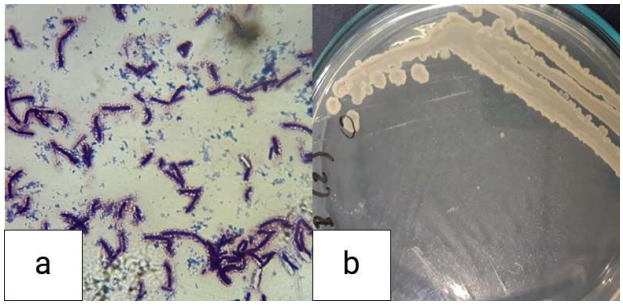

Figure 4. Characteristics of *B. cereus* PFB-10 after 24h incubation at 37°C (A) Cellular morphology (1000X) after Gram-staining, and its colonial growth on (B) TSA

Lastly, the representative isolate from group 8 (PFB-12) was identified as *Proteus mirabilis* (Figure 5). This bacterium grew cream-colored, opaque, round, and convex colonies on TSA (Figure 8B) and was found to be Gram-negative and rod-shaped (Figure 5A). *P. mirabilis* PFB-12 exhibited growth of translucent and flat colonies with some elevated white centers on EMB agar (Figure 5C), and had pinkish, translucent, and confluent growth on MacConkey agar (Figure 5D).

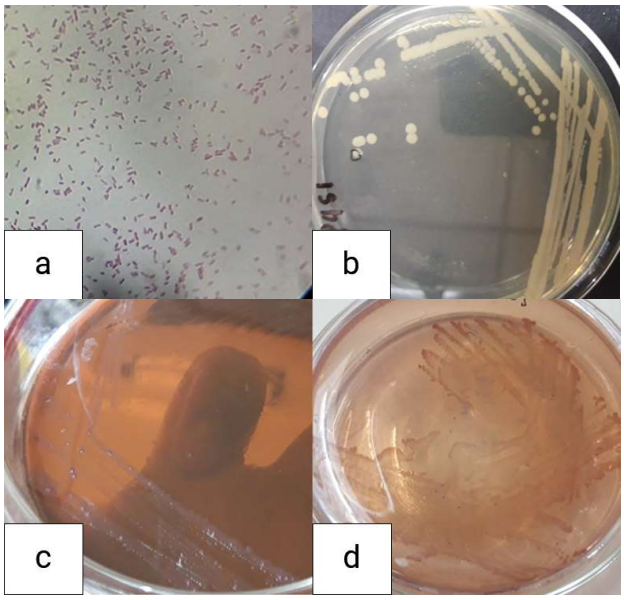

Figure 5. Characteristics of *P. mirabilis* PFB-12 after 24h incubation at 37°C (A) Cellular morphology (1000X) after Gram-staining, and its colonial growth on (B) TSA, (C) EMBA and (D) MacConkey agar

P. mirabilis PFB-12 showed similar characteristics to that of *E. coli* PFB-05 on EMB agar. The formation of pink zones on MacConkey agar indicates its capability to ferment lactose. Absence of lactose fermentation on MacConkey agar which was shown by *P. mirabilis* PFB-12 implies that they are sucrose fermenters, rather than lactose fermenters. This was assumed since standard formulations of EMB agar contains sucrose. Light sucrose fermentation makes the media acidic, resulting to pink colonies (Hipanlab 2000, Leboffe & Pierce 2012).

Oxytetracycline Resistance of the Bacterial Isolates

Results of the oxytetracycline susceptibility assay showed that *S. aureus* PFB-06, PFB-07 and *S. vitulinus* PFB-09 were susceptible to oxytetracycline (Table 3, Figure 6A-C). This is because the mean zones of inhibition of oxytetracycline treatment against each of the isolates were more than 19mm as described in the *Staphylococcus* category of the Clinical and Laboratory Standards Institute (CLSI) standardized interpretation table for disk diffusion. This result is consistent with that of the susceptible control (Figure 6J).

Table 3. Resistance of the bacterial isolates to oxytetracycline.

Test Material	Mean (±SD) ZOI (mm)	Description
S. aureus PFB-06	24.50±1.740	Susceptible
S. aureus PFB-07	25.44±0.192	Susceptible
S. vitulinus PFB-09	23.44±0.385	Susceptible
B. cereus PFB-10	13.11±0.385	Resistant
P. mirabilis PFB-12	-	Resistant
E. coli PFB-01	-	Resistant
E. coli PFB-02	-	Resistant
E. coli PFB-05	-	Resistant
S. aureus (- control)	+	Susceptible
E. coli (+ control)	-	Resistant
Sterile Distilled Water	-	-

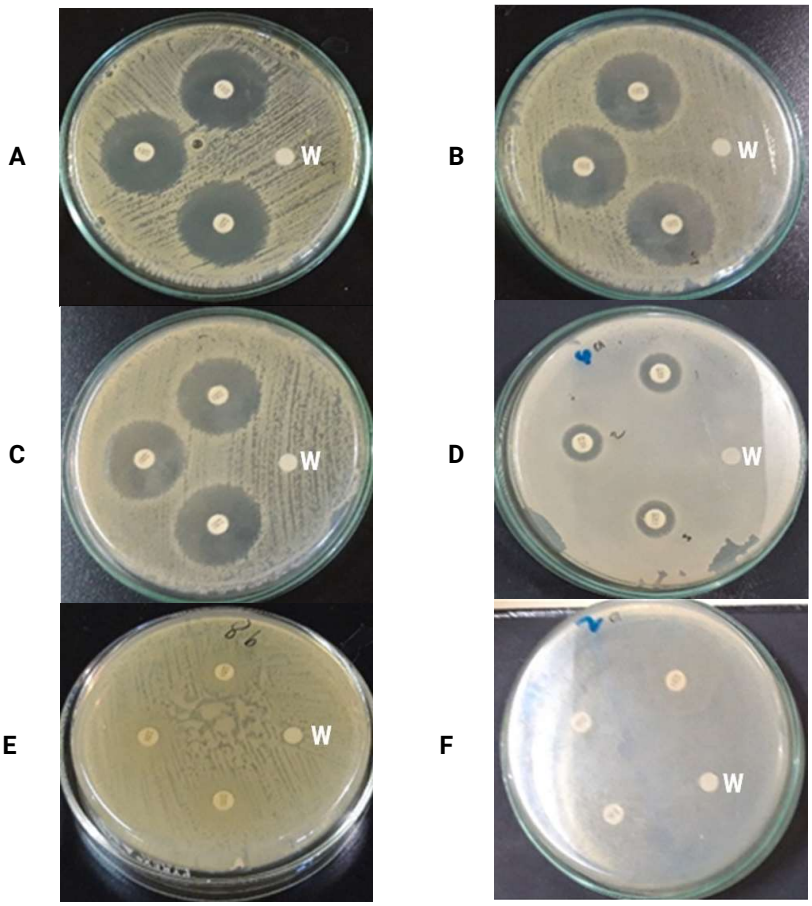

Figure 6. Representative plates after 18h incubation at 37°C showing the response of (A) *S. aureus* PFB-06, (B) *S. aureus* PFB-07, (C) *S. vitulinus* PFB-09, (D) *B. cereus* PFB-10, (E) *P. mirabilis* PFB-12, (F) *E. coli* PFB-01, (G) *E. coli* PFB-02, (H) *E. coli* PFB-05, (I) Positive control *E. coli* (Resistant) and the J) Negative control *S. aureus* (non-resistant) to Oxytetracycline. (W) shows the negative control disks treated with sterile distilled water

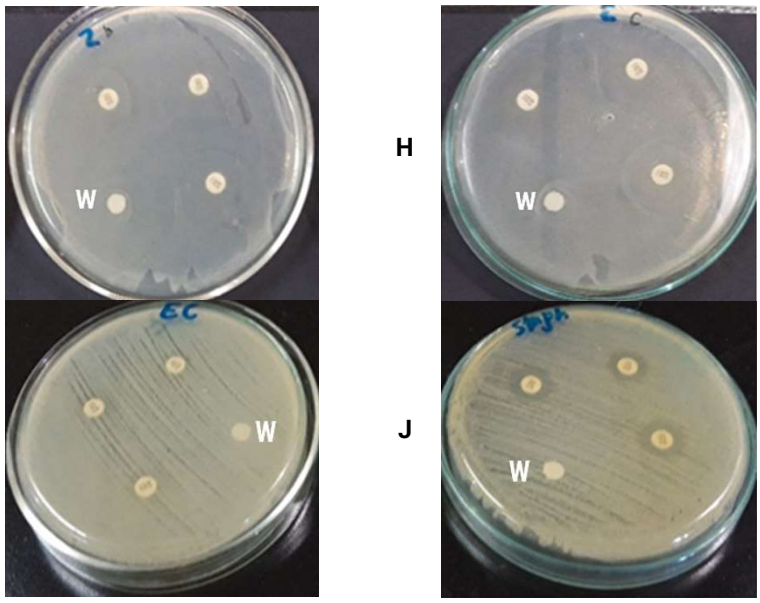

Figure 6. continued

Furthermore, oxytetracycline also showed zone of inhibition against *B. cereus* PFB-10 (Table 3, Figure 6D). However, there was no standardized zone diameter criteria in the CLSI manual that was used specifically for *B. cereus*. But since *Bacillus* spp. and *Staphylococcus* spp. belong to the same Order, which is Bacillales, and both of them are Gram-positive bacteria, the data could be interpreted in terms of the *Staphylococcus* spp. criteria. It was revealed that *B. cereus* PFB-10 could already be considered resistant to tetracycline since the zone of inhibition recorded was less than 14mm (Table 3). Conversely, the three *E. coli* (PFB-01, PFB-02, PFB-05) and *P. mirabilis* PFB-12 isolates were found to be completely resistant to oxytetracycline. No zone of inhibition was observed on the inoculated plates treated with the antibiotic (Figure 6E-H). These results are the same with the oxytetracycline-resistant *E. coli* that served as the positive resistant control (Figure 6I).

Among the susceptible isolates of *Staphylococcus*, *S. aureus* is the more virulent strain compared to *S. vitulinus* since it causes most cases of staphylococcus related diseases like food poisoning, scalded skin syndrome, and nosocomial infections including staphylococcal meningitis (Bien et al 2011). However, this does not mean that all *Staphylococcus* species covered in this study are susceptible to oxytetracycline. It was shown that 100% of the Methicillin-resistant *S. aureus* (MRSA) that were isolated by Schmithausen et al (2015) from swine farms in Germany were also resistant to oxytetracycline. There might have been *S. aureus* and *S. vitulinus* strains that are present in pig feces from the farm that are resistant, but were not isolated in this study.

All the *E. coli* strains isolated were completely resistant to oxytetracycline. It is not surprising to find that *E. coli* is resistant to various forms of tetracycline, but there are still strains of *E. coli* that are susceptible which is rare nowadays. The first tetracycline-resistant bacterium, *Shigella dysenteriae*, was isolated in 1953 and is in the same taxonomic family as *E. coli* (Chopra & Roberts 2001). It has also been

known that horizontal gene transfer can happen between the two bacterial genera (van Passel et al 2008). A study by Makita et al (2016) showed that resistance to tetracycline and oxytetracycline in *E. coli* had the highest resistance rate among the other antibiotics they used for the study which was 64%. In the same study, it was stated that it was the most commonly used antibiotic for therapy by 18% of the 250 farms they collected fecal samples from.

P. mirabilis also belongs to the same taxonomic family as *E. coli*, the Family Enterobacteriaceae. Although this study found that *P. mirabilis* was completely resistant to Oxytetracycline, it is still plausible that there are susceptible strains of *P. mirabilis* present in swine feces. A study by Kim et al (2005) showed that not all of the 64 *P. mirabilis* they have isolated were resistant to ampicillin, tetracycline, gentamycin, and kanamycin. Moreover, they showed that the multidrug-resistant *P. mirabilis* exhibited conjugal gene transfer of the multidrug-resistance with susceptible *E. coli*. The same can happen in reverse. This could be the case with the *P. mirabilis* and *E. coli* strains isolated in this study. Sharing genes within bacteria of the same family and more so of the same genus is a form of commensal to mutualistic relationship which improves survivability in bacteria from exposure to antibiotics including oxytetracycline (Chopra & Roberts 2001).

The oxytetracycline resistance assay revealed that 50% (4 out of 8) of the tested bacterial isolates are resistant to oxytetracycline. The high incidence of the oxytetracycline-resistant bacterial strains could be an indication of the excessive use of this antibiotic in the farm. This is because the presence of excess antibiotics in the environment makes it possible for non-target bacterial species to develop antibiotic resistance. Bacteria use three strategies for becoming resistant to tetracycline. First is that they limit the access of the tetracycline molecule to the ribosome. Tetracycline has to enter the cell and bind to the bacterial ribosome to inhibit protein synthesis. Some gram-negative bacteria alter their porin proteins present in its periplasm to limit diffusion of the molecule into the bacterial cell wall (Speer et al 1992). Another way is through a mechanism called tetracycline efflux. It is done by pumping the antibiotic out of the cell at a rate equal to or greater than its uptake, thereby reducing the tetracycline concentration in the cell. Another strategy for bacterial resistance to tetracycline is by altering the ribosome to prevent efficient binding to tetracycline. The mechanism involves a cytoplasmic protein that interacts with the ribosome, making the bacteria and its ribosomes somewhat resistant to tetracycline (Burdett 1991). Lastly, some bacteria develop resistance to tetracycline by enzymatic inactivation of the drug. These bacteria produce an NADPH-requiring oxidoreductase that inactivates tetracycline in the presence of oxygen and NADPH. It has only been found in obligate anaerobes, Bacteroides, where oxygen is present (Chopra & Roberts 2001). Bacteroides strains do not only show natural resistance, but they also require high levels of aeration to function as a resistance factor in *E. coli*, where resistance genes are present in most strains. This may be the case of how *E. coli* strains isolated in this study developed their oxytetracycline-resistant abilities.

CONCLUSION

This study demonstrates the occurence of oxytetracycline-resistant bacteria in a swine farm in Kabalasan, Baybay City, Leyte. The results of this study can be the

basis of generating solutions to deal with oxytetracycline resistance in swine farms and in the community. For instance, the identity of the resistant bacterial isolates can help in strategizing rotation or sequential use in the use of antibiotic groups that could inhibit the growth of specific pathogenic bacterial species. Furthermore, the oxytetracycline-resistant bacteria isolated in this study can be used as test organisms for the development of better diagnostic methods, the development of more potent drugs for these resistant bacterial strains, and as a source of resistance genes that can potentially be used as selection markers in transgenic manipulation studies. Finally, it is recommended that the bacterial isolates be tested for their resistance to other kinds of antibiotics and that their species identification be further confirmed through molecular phylogenetic approach.

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REFERENCES

Barcella L, Barbaro AP & Rogolino SB. 2016. Colonial morphology of *Escherichia coli*: Impact of detection in clinical specimens. *Microbiological Medica* 31(2):51-55

Barroga TRM, Morales RG, Benigno CC, Castro SJM, Caniban MM, Cabullo FB, Agunos A, de Balogh K & Dorado-Garcia A. 2020. Antimicrobials used in backyard and commercial poultry and swine farms in the Philippines: A qualitative pilot study. *Frontiers in Veterinary Science* 7(329):1-8

Bauer AW, Kirby WM, Sherris JC & Turck M. Antibiotic susceptibility testing by a standard single disk method. *American Journal of Clinical Pathology* 45(4):493-496

Bien J, Solovoka O & Bozko P. 2011. Characterization of virulence factors of Staphylococcus aureus: Novel function of known virulence factors that are implicated in activation of airway epithelial proinflammatory response. *Journal of Pathogens* 2011:1-13

Burdett V. 1991. Purification and characterization of Tet (M), a protein that renders ribosomes resistant to tetracycline. *Journal of Biological Chemistry* 266(5):2872-2877

Chopra I and Roberts M. 2001. Tetracycline antibiotics: Mode of action, applications, molecular biology, and epidemiology of bacterial resistance. *Microbiology and Molecular Biology Reviews* 65(2):232-260

Clinical and Laboratory Standards Institute. 2015. Performance standards for antimicrobial susceptibility testing: 25th Informational Supplement. CLSI Document M100-S25. Clinical Laboratory Standards Institute, Wayne, PA

Coleman K. 2011. Diazabicyclooctanes (DBOs): a potent new class of non-β-lactam β-lactamase inhibitors. *Current Opinion in Microbiology* 14(5):550-555

Connell SR, Tracz DM, Nierhaus KH & Taylor DE. 2003. Ribosomal protection proteins and their mechanisms of tetracycline resistance. *Antimicrobial Agents and Chemotherapy* 47(12):3675-3681

Cromwell GL. 2002. Why and how antibiotics are used in swine production. *Animal Biotechnology* 13(1):7-27

Datta N and Hughes VM. 1983. Plasmids of the same Inc groups in Enterobacteria before an after the medicinal use of antibiotics. *Nature* 306(5943):616-617

D'Costa VM, King CE, Kalan L, Morar M, Sung WWL, Schwarz C, Froese D, Zazula G, Calmels F, Debruyne R, Golding GB, Poinar HN & Wright GD. 2011. Antibiotic resistance is ancient. *Nature* 477(7365):457-461

Etebu E and Arikekpar I. 2016. Antibiotics: Classification and mechanisms of action with emphasis on molecular perspectives. *International Journal of Applied Microbiology and Biotechnology Research* 4:90-101

Gao FZ, Zou HY, Wu DL, Chen S, He LY, Zhang M, Bai H & Ying GG. 2020. Swine farming elevated the proliferation of Acinetobacter with the prevalence of antibiotic resistance genes in the groundwater. *Environment International* 136)105484):1-9

Hispanlab SA. 2000. *Manual for Microbiology* (3rd edn). Pronadisa; Culture media and Reagents for Diagnostics. Torrejon de Ardoz, Madrid: Labroatorios Conda

Leboffe MJ and Pierce BE. 2011. A photographic atlas for the microbiology laboratory (4th edn). Morton Publishing, Eaglewood, Colorado, USA

Kim SH, Wei CI & An H. 2005. Molecular characterization of multidrug-resistant proteus mirabilis isolates from retail meat products. *Journal of Food Protection* 68(7):1408-1413

Makita K, Goto M, Ozawa M, Kawanishi M, Koike R, Asai T & Tamura Y. 2016. Multivariable analysis of the association between antimicrobial use and antimicrobial resistance in *Escherichia coli* isolated from apparently healthy pigs in Japan. *Microbial Drug Resistance* 22(1):28-39

Merchant IA and Packer RA. 1969. Veterinary and virology (7th edn). The Iowa State University Press, Ames, Iowa, USA

Piessens V, Van Coillie E, Verbist B, Supre K, Braem G, Van Nuffel A, de Vuyst L, Heyndrickz M & de Vliegher S. Distribution of coagulase-negative Staphylococcus species from milk and environment of dairy cows differs between herds. *Journal of Dairy Science* 94(6):2933-2944

Salanitro JP, Blake IG & Muirhead PA. 1997. Isolation and identification of fecal bacteria from adult swine. *Applied and Environmental Microbiology* 33(1):79-84

Sanz C, Casado M, Navarro-Martin L, Tadić D, Parera J, Tugues J, Bayona JM & Piña B. 2021. Antibiotic and antibiotic-resistant gene loads in swine slurries and their digestates: Implications for their use as fertilizers in agriculture. *Environmental Research* 194(110513):1-10

Schmithausen RM, Kellner SR, Schulze-Geisthoevel SV, Hack S, Engelhart S, Bodenstein I, Al-Sabti N, Reif M, Fimmers R, Körber-Irrgang B & Harlizius J. 2015. Eradication of methicillin-resistant *Staphylococcus aureus* and of *Enterobacteriaceae* expressing extended-spectrum beta-lactamases on a model pig farm. *Applied and Environmental Microbiology* 81(21):7633-7643

Shlaes DM. 2010. The perfect storm. In Shlaes DM (ed) *Antibiotics* (pp1-7). Springer, Dordrecht

Sköld O. 2011. Antibiotics and antibiotic resistance. John Wiley & Sons, Inc., Hoboken, New Jersey

Speer BS, Shoemaker NB & Salyers AA. 1992. Bacterial resistance to tetracycline: Mechanisms, transfer, and clinical significance. *Clinical Microbiology Reviews* 5(4):387-399

Thapa SP, Shrestha S & Anal AK. 2020. Addressing the antibiotic resistance and improving the food safety and food supply chain (farm-to-fork) in Southeast Asia. *Food Control* 108(106809):1-7

Thornsberry C, Gavan TL, Sherris JC, Balows A, Matsen JM, Sabath LD, Schoenknecht F, Trupp & Washington JA. Laboratory evaluation of a rapid, automated susceptibility testing system: Report of a collaborative study. *Antimicrobial Agents and Chemotherapy* 7(4):466-480

Van den Bogaard, Anthony E & Stobberingh EE. 2000. Epidemiology of resistance to antibiotics: Links between animals and humans. *International Journal of Antimicrobial Agents* 14(4):327-335

Van Passel MW, Marr PR & Ochman H. 2008. The emergence and fate of horizontally acquired genes in *Escherichia coli*. *PLos Computational Biology* 4(4):1-8

Yong-Guan Z, Johnson TA, Su JQ, Qiao M, Gou GX, Stedtfeld RD, Hashsham SA & Tiedje JM. 2013. Diverse and abundant antibiotic resistance genes in Chinese swine farms. *Proceedings of the National Academy of Sciences* 110(9):3435-3440