ISOLATION, IDENTIFICATION AND ASSESSMENT OF ANTIBIOTIC RESISTANCE OF COMMON BACTERIA IN PORK MEAT IN KOTA BHARU, KELANTAN.

KIRTHEKKA A/P BALACHANDRAN

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CERTIFICATION

This is to certify that we have read this research paper entitled '**Isolation, Identification and Assessment of Antibiotic Resistance of Common Bacteria in Pork Meat in Kota Bharu, Kelantan'** by Kirthekka A/P Balachandran, and in our opinion, it is satisfactory in terms of scope, quality and presentation as partial fulfilment of the requirement for the course DVT 55204 – Research Project

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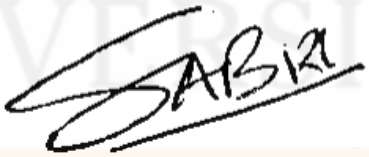
Dr. Intan Noor Aina Kamaruzaman
DVM (UMK), PhD in Infection and Global Health (Liverpool)
Senior Lecturer,

Faculty of Veterinary Medicine
Universiti Malaysia Kelantan
(Supervisor)



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Dr Mohammad Sabri Bin Abdul Rahman
DVM (UPM), MVSc (UPM)
Lecturer,
Faculty of Veterinary Medicine
Universiti Malaysia Kelantan
(Co-supervisor)

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Family

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Thank You

DEDICATIONS

I dedicate my dissertation work to my family and many friends. A special feeling of gratitude to my loving parents, Thirumagal and Balachandran, whose words of encouragement and push for tenacity ring in my ears. My sisters Sankeetha and Sangkerthana who have never left my side and are very special.

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ABSTRACT

An abstract of the research paper presented to the Faculty of Veterinary Medicine, Universiti Malaysia Kelantan, in partial requirement on the course DVT 55204 – Research Project.

Foodborne illness has been a growing curve over the past years and limited studies have been carried out to prove this in Malaysia especially in the State of Kelantan. Additionally, this is a study to detect any bacteria isolated from pork meat in Kota Bharu, Kelantan, as well as the antibiotic resistance pattern. A total of 30 pork meat samples were collected from 30 different pork vendors in wet markets in Kota Bharu, Kelantan. Based on the results from morphological colony growth on bacterial culture and biochemical tests, the isolated bacteria were *Bacillus cereus, Enterococcus* spp., *Enterobacter* spp., *Escherichia coli*, *Klebsiella* sp., *Proteus* sp., *Pseudomonas* sp., *Salmonella* sp., and *Streptococcus* spp. From the results, it was found that the isolated bacteria were resistant to various antibiotics including ampicillin, sulphonamides, trimethoprim (18%), tetracycline (14%), doxycycline (8%) and gentamicin (6%), respectively. These findings showed that the bacterial contamination in pork meat can cause foodborne illness and resistant to common antibiotics used in human and animal medicine which may potentially spread to public.

Keywords: Antibiotic resistance, Awareness, Bacteria, Pork

ABSTRAK

Abstrak daripada kertas penyelidikan dikemukakan kepada Fakulti Perubatan Veterinar, Universiti Malaysia Kelantan untuk memenuhi sebahagian daripada keperluan kursus DVT 55204 – Projek Penyelidikan.

Penyakit bawaan makanan telah meningkat sejak beberapa tahun lalu dan terdapat beberapa kajian telah dijalankan di Malaysia terutamanya di Kelantan. Ini merupakan kajian untuk mengesan bakteria yang daripada daging babi di Kota Bharu, Kelantan, serta corak rintangan antibiotik. Sebanyak 30 sampel daging babi dikumpul daripada 30 penjual di pasar pagi di Kota Bharu, Kelantan. Berdasarkan keputusan pertumbuhan koloni dan morfologi pada kultur bakteria dan ujian biokimia, bakteria yang dapat diisolasi ialah *B. cereus*, *Enterococcus* spp., *Enterobacter* spp., *Escherichia coli*, *Klebsiella* sp., *Proteus* sp., *Pseudomonas* sp., *Salmonella* sp., dan *Streptococcus* spp. Hasil keputusan menunjukkan bakteria-bakteria yang diisolasi menunjukkan kerintangan terhadap beberapa jenis antibiotik seperti ampicillin, sulphonamides, trimethoprim (18%), tetracycline (14%), doxycycline (8%) dan gentamicin (6%). Hal ini menunjukkan pencemaran bakteria pada daging babi boleh menyebabkan keracunan makanan dan kerintangan terhadap antibiotic biasa digunakan untuk perubatan manusia dan haiwan berpotensi untuk disebarkan kepada orang awam.

Kata kunci: Bakteria, Daging babi, Kesedaran, Rintangan antibiotik

1.0 Introduction

Food may be contaminated by a spread of disease-causing germs or pathogens, leading to large vary of foodborne diseases. According to Abdul-Mutalib et al. (2014), at several steps of food preparation, pathogenic germs may be introduced which include contamination at the farm level, such as milk tainted with animal faeces, or animals already infected with pathogenic microbes. Transmission can also occur during slaughtering, when meat comes into contact with animal intestine, skin, or fur, as well as in the kitchen due to inappropriate food preparation techniques. Some of the important pathogens in the pig industry that are public health concern are *Salmonella* spp., *Campylobacter* spp., *Trichinella spiralis*, *Toxoplasma gondii*, *Listeria monocytogenes*, and Methicillin-resistant *Staphylococcus aureus*. Bacterial pathogens in pork meat can be the source of food poisoning that may lead to adverse health effect in human. Additionally, recent studies reported a high level of antibiotic resistant bacteria in pigs, especially antibiotic that has been extensively used in livestock farming (Sirichokchatchawan *et al.*, 2021). Resistant bacteria causing foodborne diseases are one of the most significant public health issues associated with the risk of antibacterial resistance emergence in the food production chain.

The emergence of antibiotic resistance bacteria is driven by many factors, for which the misuse of antibiotics in livestock contributed a major factor towards development of resistance gene via selective pressure and genetic alteration (Mensah *et al.*, 2014). Antibiotic residues in food products can have a variety of negative effects on public health, such as anaphylactic reactions, liver toxicity, mutagenicity, carcinogenicity, potential toxicity, nephropathy, and antibacterial resistance (Mensah *et al.*, 2014).

The food chain can spread antibacterial resistance through direct or indirect exposure. Direct exposure occurs when a person comes into contact with an animal's blood, saliva, milk, sperm, or faeces and urine, which is a very simple and rapid method for transmitting resistant bacteria. The indirect contact is followed by the consumption of contaminated food products such as eggs, meat, and dairy, which is a more complex and extensive pathway (Chang *et al.,* 2015). These factors contribute to antibacterial resistance, which can spread globally via the food chain due to population growth, international travel, and food product trade.

2.0 Research problem

Foodborne disease causes adverse clinical conditions in humans and the main source is from animal-sourced meals. Although there have been several researches done on identification of foodborne disease-causing bacteria, there is limited research that has been done and published in Kota Bharu, Kelantan on the types of common bacteria found in pork meat. Additionally, the level of antibiotic resistance in pork meat Kelantan has never been studied.

Research questions

2.1.1 What are the common bacteria found in pork meat?

2.1.2 How many antibiotics are the bacteria resistant to?

3.0 Research hypothesis

3.1.1 Several common bacteria can be found in pork meat.

3.1.2 Isolated bacteria are resistant to one or more antibiotic drug.

4.0 Objectives

4.1.1 To determine the common bacteria of pork meat.

4.1.2 To observe quantity of antibiotic drug resistant to isolated bacteria, in pork meat.

5.0 Literature Review

5.1 Overview of Food borne disease worldwide

A report from the World Health Organization (2010) (Havelaar et al., 2013), lists foodborne outbreaks as one of the greatest hazards to global public health in the twenty-first century. The World Health Organization (2010) estimates that roughly 30 percent of people in industrialised nations suffer from foodborne illness annually. In the United States of America, for instance, approximately 9.4 million cases of foodborne infections result in 55,961 documented hospitalizations and 1,351 deaths annually (Garayoa et al., 2011). In developing nations, where hundreds of millions of people suffer from diarrhoea, the most prevalent symptom of foodborne illness, the number of unrecorded cases is likely to be substantially higher (Borchers et al., 2010). In addition to human misery, foodborne infections have a tremendous economic and social impact (Low *et al.*, 2016). Major foodborne bacteria that are causing diseases from meat sourced are *B. cereus, Clostridium perfringens, Salmonella* spp., *Shigella* spp., *Staphylococcus aureus, Vibrio* spp., *Yersinia enterocolitica* (Adley & Ryan, 2016).

5.2 Overview of Food borne disease in Malaysia

Malaysia is one of the countries with a high prevalence of foodborne illnesses, as most bacteria thrive in its environment. From 2009 to 2011, cholera, food poisoning, and hepatitis A increased, but dysentery decreased. From 2011 to 2013, cholera, typhoid, and hepatitis A cases decreased, whereas dysentery cases increased. Moreover, food poisoning cases decline in 2012 but rise significantly in 2013 (MOH, 2014). Food poisoning has also proven to cause

an increased mortality rate over the years of 2006 to 2013. In Malaysia, 50% of incidents of foodborne disease are attributable to unsanitary food handling practises, such as the preparation of meals in advance, improper methods of refrigeration, and insufficient temperature when reheating (Abdul-Mutalib *et al.*, 2014). Consequently, roughly 40,000 cases were documented between 2011 and 2016. The most frequent causes of foodborne outbreaks are meat, dairy products, eggs, and vegetables, whereas the most frequent pathogens are *Salmonella enterica* serotype typhi, *S. aureus*, *E. coli*, and *C. perfringens* (Pires *et al.*, 2012).

5.3 Common bacterial pathogens found in pork meat

Multiple zoonotic agents were present, according to research conducted by the Veterinary Research Institute (VRI) to identify prevalent diseases in samples of pigs from 2014 to 2017 (Figure 1). The bacteria that harmed pigs can be categorised based on the primary lesion site. Cutaneous associated bacteria include *Staphylococcus* spp and *Treponema* sp. Gastrointestinal tract is commonly associated with *Salmonella*, *E. coli*, *Bacillus*, *Enterococcus* sp., *Clostridium* spp., *Helicobacter* spp., and *Yersinia* spp. Whereas, respiratory system is contaminated by *Streptococcus*, *Pasteurella multocida*, and *Pseudomonas*. Reproductive system associated with bacteria such as *Brucella* sp., *Leptospira* sp. and *Listeria* sp. Other non-specific bacterial infecting pigs includes *Actinobacilum* spp., *Chlamydophila* spp., and *Aeromonas* spp. (Abubakar et al., 2017).

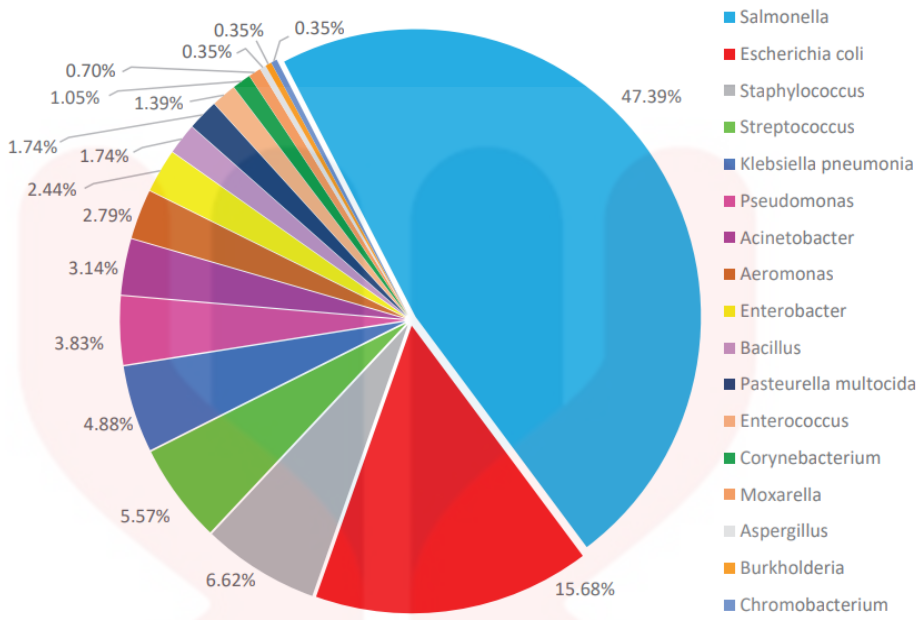

Figure 1: Percentage of bacteria identified from pig samples tested in VRI from 2014 to 2017 (Aisya Naama et al., 2018)

5.4 Overview of Antibiotic resistance among food producing animals

Penicillin, tetracyclines, quinolones, sulphonamides, and macrolides were among the antimicrobial classes used for food-producing animals in Southeast Asia in 2013. In Asia, Thailand is one of the highest antimicrobial users for livestock purposes due to the increasing number of animals each year (Boeckel et al., 2017).

A study was conducted in Mekong Delta farms, Vietnam in comparison the AMR in *E. coli* isolates from 90 pigs found that most of the isolates were resistant to various antibiotics which includes ciprofloxacin (41.4%) and gentamicin (38.1%) than European pig isolates (5.6% and 1.1%, respectively) (Nhung et al., 2014).

Meanwhile another study was done on the prevalence and AMR of *Salmonella* recovered from pig-borne food products in Henan, China. Results showed there

was high resistance towards tetracycline and sulfisoxazole. Resistance to ampicillin, amoxicillin, and streptomycin was the most prevalent, followed by resistance to quinolones and other -lactams, such as cephalosporins (Zhu *et al.*, 2018).

Antimicrobial-resistant bacteria are transmitted to humans by the ingestion of contaminated meat and meat products, leading to the development of antibiotic-resistant diseases (Bole, 2022). In a research done to detect the AMR in commensal flora of pig farmers, it was detected that pig farmers had considerably more non-groupable *Streptococci* resistant to ampicillin. In pig farmer carriers of *Enterobacteria*, isolation of *Enterobacteria* resistant to nalidixic acid, chloramphenicol, tetracycline, and streptomycin were substantially more common. In pig farmers carrying *E. coli*, the prevalence of *E. coli* resistant to cotrimoxazole, tetracycline, streptomycin, or nalidixic acid were substantially greater than in non-farmers. In *E. coli* from pig farmers, the prevalence of co-resistance to ampicillin, streptomycin, and cotrimoxazole were also much greater (Aubry-Damon, 2004).

Study of AMR so far has been limitedly performed in pork meat from Kota Bharu, Kelantan, thus we are carrying out this research to identify common bacteria in commercial pork meat and the AMR of these bacteria.

6.0 Materials and Methods

6.1 Sample collection

A total of 20-50g of pork meat from individual pig was sampled from wet market in Kota Bharu, Kelantan using sterile gloves and placed in a sterile zip lock bag. All samples were transferred directly to the lab for bacterial isolation and identification. A total of thirty samples were taken.

6.2 Isolation and identification of bacteria from pork meat

Primary bacterial culture was done using blood agar and MacConkey agar. The sample meat was sterilized by flaming to eliminate any contaminant bacteria on the surface. The instruments used were also sterilized by flaming and keeping it in 70% ethanol. The sample meat was cut into small pieces and then pressed (dabbed) onto the agar while applying gentle pressure on one spot. Streak plate method was applied to dilute the bacterial load over the surface of the agar medium (Figure 2). The agar plate was incubated at 37 °C for 18 to 24 hours. These steps were repeated for all 30 samples.

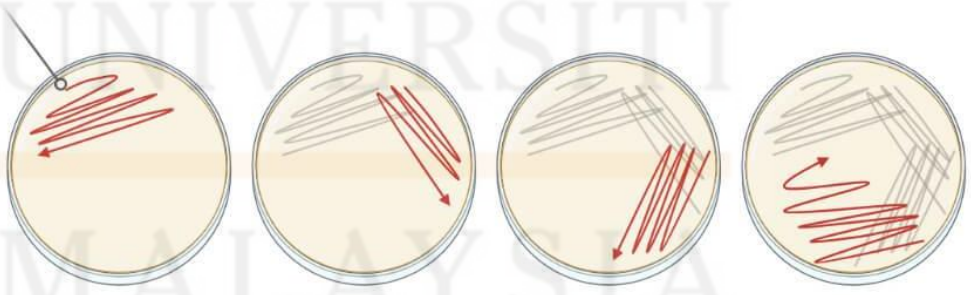

Figure 2: Streak plate method (Dahal, 2022)

Thirty of the incubated agar cultures were observed after 18 to 24 hours of incubation. The colonies were observed based on size, surface appearance, colour, haemolysis on the blood agar, shape. As for the colonies on the MacConkey agar, it was observed for the colour changes on the agar where non-lactose fermenting bacteria changes agar to yellow.

Isolated colonies from the primary culture were cultured onto nutrient agar as secondary culture to grow pure culture of the isolated bacteria. A total of 38 distinct colonies were selected from primary cultures to perform secondary culture on nutrient agar. This was done by streak method on an agar divided into two halves. Thus, two isolated bacteria colonies were streaked onto each agar plate. The agar plate is incubated at 37 °C for 18 to 24 hours. These steps were repeated for all 38 colonies.

Gram staining was performed on 38 colonies that were isolated onto secondary culture and the results were recorded. This was done by diluting a sample from the isolated colony in a drop of sterile physiological saline on a clean glass slide. The dilution is then heat fixed onto the slide. Next, crystal violet was placed on the slide and left for 60 seconds. The slide was then rinsed under a slow flow of water and followed by staining with iodine and left for 60 seconds and again rinsed under a slow flow of water. Next, 95% ethyl alcohol was used to decolourise the sample and left for 15 seconds before rinsing off with water. Lastly, safranin is used to stain for 60 seconds before rinsing the slide. This protocol were repeated for all 38 colonies that were isolated. The morphology of all the bacteria were observed under 100x magnification.

After identification of Gram staining and also the morphology of each colony, the results were tabulated and similar findings were shortlisted. These bacteria were then proceeded to biochemical tests for further identification. The Gram-positive bacteria were tested using urease agar, oxidase test and catalase test. The gram-negative bacteria were tested using a set of biochemical tests for *Enterobacteriaceae* which consist of triple sugar iron agar (TSI), Simmons citrate agar, urease test, sulphur indole motility media (SIM), Methyl Red and Vogus-Proskauer (MRVP), catalase test and oxidase test. All cultures were incubated at 37 °C for 24 hours and results were recorded.

6.3 Antibiotic sensitivity testing

Kirby-Bauer Disk diffusion susceptibility test protocol was used to determine the sensitivity or resistance of the bacteria that were isolated. The bacteria were diluted in 0.9% normal saline (physiological saline) using a sterile inoculating loop in a sterile test tube. The dilution was measured using a McFarland densitometer. Dilution was maintained between 0.5 McFarland units. Sterile cotton swab was used to dip into the diluted bacteria solution and then inoculated onto the Mueller Hinton Agar (MHA). It was made sure that the whole agar medium surface was inoculated by streaking multiple times and rotating the plate a little every time. Antibiotic disks were placed after all the agar were inoculated with isolated bacteria. Since there were 7 different antibiotic disks, each bacterium was inoculated onto two MHA plates. Four antibiotic discs were placed on one inoculated plate and another three discs were placed on the second inoculated plate for each bacterium. The MHA plates were incubated, at 37°C for 24 hours.

Antibiotic disks that were used are; Ampicillin (AMP10), Amoxicillin (AML25), Doxycycline (DO30), Gentamicin (CN30), Compound Sulphonamides (S3300), Tetracycline (TE30), and Trimethoprim (W5). This list of antibiotics was chosen based on similar antibiotic drug used in both livestock and human medicine. The diameter of zone of inhibition by the antibiotics which is shown by the clear area around the antibiotic disk on the agar was observed and measured after 24 hours of incubation. The diameter of zone of inhibition, which is the clear zone around the antibiotic disc, was measured using a ruler and their resistance and susceptibility diameter was compared to CLSI and EUCAST guidelines (Table 1-5). Each diameter is recorded according to the sample grown on the agar. A table is generated to assess the susceptibility of each antimicrobial towards the bacteria.

Tables of Zone diameter breakpoints.

Table 1 Zone diameter breakpoints of Enterobacterales based on CLSI.

Antibiotic	Disc content (µg)	Zone diameter breakpoints (mm)			Notes
		S	I	R	
Ampicillin	10	≥ 17	14-16	≤ 13	Ampicillin test results can be used to predict amoxicillin results.
Amoxicillin		≥ 17	14-16	≤ 13	
Gentamicin	10	≥ 15	13-14	≤ 12	
Tetracycline	30	≥ 15	12-14	≤ 11	
Doxycycline	30	≥ 14	11-13	≤ 10	
Trimethoprim	5	≥ 16	11-15	≤ 10	
Sulphonamide	300	≥ 17	13-16	≤ 12	

Table 2 Zone diameter breakpoints of Enterococcus spp. based on EUCAST and CLSI

Antibiotic	Disc content (µg)	Zone diameter breakpoints (mm)			Notes
		S	I	R	
Ampicillin	10	≥ 17		≤ 16	Ampicillin test results can be used to predict amoxicillin results. (CLSI)
Amoxicillin		≥ 17		≤ 16	
Gentamicin	10				If zone diameter is <8 mm, the isolate is high-level resistant to gentamicin and other aminoglycosides, except streptomycin. (EUCAST)
Tetracycline	30	≥ 19	15-18	≤ 14	CLSI
Doxycycline	30	≥ 16	13-15	≤ 12	CLSI
Trimethoprim	5				The activity of trimethoprim is uncertain against enterococci. (EUCAST)

Table 3 Zone diameter breakpoints of Streptococcus spp. based on EUCAST

Antibiotic	Disc content (µg)	Zone diameter breakpoints (mm)			Notes
		S	I	R	
Ampicillin	10	≥ 18	-	≤ 18	Susceptibility testing of penicillins and other β-lactams approved by the US Food and Drug Administration for treatment of β-hemolytic streptococcal infections does not need to be performed routinely,as isolates are extremely rare and have not been reported.
Amoxicillin		≥ 18	-	≤ 18	
Gentamicin	10	-	-	-	
Tetracycline	30	≥ 23	-	≤ 20	
Doxycycline	30	≥ 23	-	≤ 20	
Trimethoprim	5	IP		IP	

Isolates may be reported as R without prior testing with the indication of "-".

Susceptibility testing is not recommended as the species is a poor target for therapy with the agent.

IP = In Preparation

Table 4 Zone diameter breakpoints of Pseudomonas spp. based on EUCAST & CLSI

Antibiotic	Disc content (µg)	Zone diameter breakpoints (mm)			Notes
		S	I	R	
Ampicillin	10	-	-	-	EUCAST
Amoxicillin		-	-	-	EUCAST
Gentamicin	10	≥ 15	13-14	≤ 12	CLSI
Tetracycline	30	-	-	-	EUCAST
Doxycycline	30	-	-	-	EUCAST
Trimethoprim	5	-	-	-	EUCAST

Isolates may be reported as R without prior testing with the indication of "-". Susceptibility testing is not recommended as the species is a poor target for therapy with the agent.

Table 5 Zone diameter breakpoints of Proteus spp. based on EUCAST & CLSI.

Antibiotic	Disc content (µg)	Zone diameter breakpoints (mm)			Notes
		S	I	R	
Ampicillin	10	-	-	-	EUCAST
Amoxicillin		-	-	-	EUCAST
Gentamicin	10	≥ 15	13-14	≤ 12	CLSI
Tetracycline	30	-	-	-	EUCAST
Doxycycline	30	-	-	-	EUCAST
Trimethoprim	5	-	-	-	EUCAST

6.4 Statistical analysis

Data was recorded and statistically analysed manually by using manual recording and tabulation from Microsoft Word and Excel.

7.0 Results

Table 6 shows colony morphology of bacteria that grew on blood agar from all pork meat. The colony morphology and Gram staining results were based on the pure culture growth observed on the secondary culture. Secondary culture was done from isolated colonies that showed same colony morphology as listed in the Table 6. Six of colonies that were pale yellowish on the blood agar with beta haemolysis was isolated and stained negative with bacilli shape. Five of colonies that were whitish colour on the blood agar with beta haemolysis was isolated and stained positive with coccus shape. Six of colonies that were white colour on the blood agar with beta haemolysis was isolated and stained positive with coccus shape.

Table 7 shows colony morphology of bacteria that grew on MacConkey agar from all pork meat. The colony morphology and Gram staining results were based on the pure culture growth observed on the secondary culture. Single colony from sample P10, was pinkish in colour on the MacConkey agar with lactose fermenting properties, had bipolar stain with coccus shape. Four of colonies that were raised, purple colour on the MacConkey agar with lactose fermenting properties was isolated and stained negative with bacilli shape. Seven of colonies that were purple colour on the MacConkey agar with non-lactose fermenting property was isolated and stained negative with bacilli shape. Single colony from sample P18, was purple in colour with a white centre on the MacConkey agar. It also had lactose fermenting properties and stained negative with coccobacilli shape. Four of colonies that were translucent purplish in colour on the MacConkey agar with non-lactose fermenting property was isolated and stained negative with bacilli shape.

After performing Gram staining of the secondary cultures, biochemical tests were done and the results were then interpreted to what bacteria grew on the agar. Table 9 shows the biochemical tests and the result interpretation of bacteria present in the pork meat.

The bacteria that were isolated and identified in this study are; *E. coli*, *Streptococcus* spp., *Enterococcus* spp., *B. cereus*, *Salmonella* spp., *Proteus* spp., *Pseudomonas* spp., *Enterobacter* spp., and *Klebsiella* spp.

Table 6 Colony morphology on Blood agar and Gram staining results.

Sample ID	Colony morphology	Gram staining
P1, P2, P3, P7, P19, P26	Pale yellowish colony with Beta haemolysis	Negative bacilli
P3, P10, P11, P12, P21,	Whitish colony with Beta haemolysis	Positive coccus
P1, P3, P7, P22, P25, P29	White colony, Gamma haemolysis	Positive coccus

Table 7 Colony morphology on MacConkey agar and Gram staining results.

Sample ID	Colony morphology	Gram staining
P10	Pinkish colony, Lactose fermenting	Bipolar, coccus
P6, P15, P18, P28	Raised Purple colony, Lactose fermenting	Negative, bacilli
P6, P15, P18, P23, P26, P29, P28	Purple colony, Non-lactose fermenting	Negative bacilli
P18	Purple colony with white centre, Non-lactose fermenting	Negative coccobcilli
P15, P18, P23, P29	Translucent purplish colony, Non-lactose fermenting	Negative bacilli

Table 8 Biochemical test results of the isolated bacterial colonies.

Sample ID	TSI	Sulphur	Indole	Motility	Citrate	Urease	MR	VP	Oxidase	Catalase	Interpretation / Suspected bacteria
P19-B2	A/A	-	-	-	-	-	-/+	-	+	-	*E. coli*
P3-B4						-			-	-	*Streptococcus* spp.
P22-B1						-			-	-	*Enterococcus* spp.
P25-B2	A/A	-	-	-	-	-	+/-	-	+	-	*E. coli*
P29-B2	A/A	-	+	-/+	-	-	+	-	-	-	*E. coli*
P1-B3						-			+	-	*B. cereus*
P6-M2	A/A	-	+	-/+	-	-	+/-	-	+	-	*E. coli*
P28-M1	K/A, Gas	-	-	-/+	-	+	-	-	+	-	*Salmonella* spp.
P2-M4	A/A, Gas, H2s +	+	-	+	+	-	-	-	+	-	*Proteus* spp.
P1-M6	K/A, Gas	-	+	-/+	-	-	+	-	+	-	*Pseudomonas* spp.
P28-M2	A/A, Gas	-	+	-/+	-	-/+	+	-	-	-	*E. coli*
P18-M1	A/A, Gas	-	-	-/+	+	+	-	-	-	-	*Klebsiella* spp.
P15-M4	A/A, Gas	-	-	-	+	+	-	-	-	-	*Klebsiella* spp.

(A=Acidic, K= Alkaline)

General comment: Since the biochemical tests done were not complete and inconclusive, confirmation of the bacterial species should be confirmed by either additional biochemical test or PCR.

7.1 Antibiotic sensitivity and resistance

Based on the results from Kirby Bauer disc diffusion method in Antibiotic sensitivity testing the diameter of zone of inhibition was measured using a ruler. The diameter is referred with breakpoints from CLSI and EUCAST guidelines to assess the susceptibility of the bacteria towards list of antibiotics used.

7.1.1 *Escherichia coli*

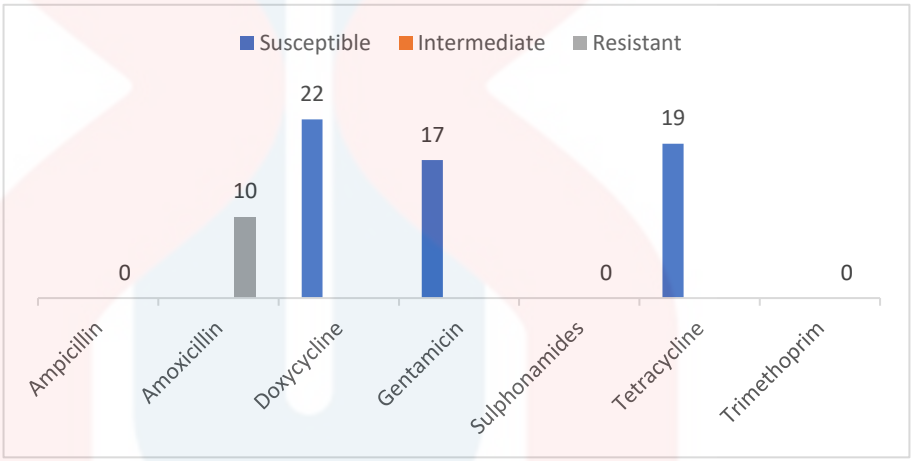

Figure 3: Antibiotic sensitivity of *Escherichia coli.*

Figure 3 shows that *E. coli* is susceptible to doxycycline (22 mm), gentamicin (17 mm), and tetracycline (19 mm). *E.coli* isolated in this study was found resistant to ampicillin (0 mm), amoxicillin (10 mm), sulphonamides (0 mm) and trimethoprim (0 mm).

7.1.2 *Streptococcus* spp.

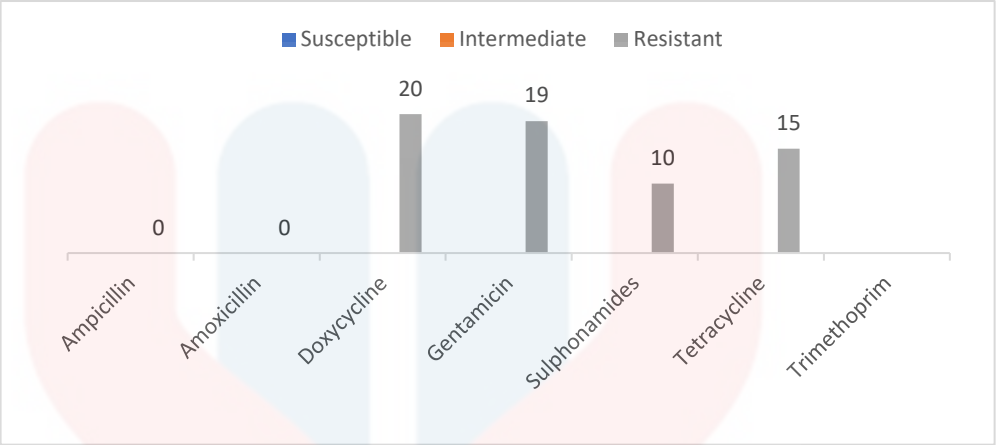

Figure 4: Antibiotic sensitivity of *Streptococcus* spp.

Figure 4 shows that *Streptococcus* spp. is resistant to ampicillin (0 mm), amoxicillin (0 mm), doxycycline (20 mm), and tetracycline (15 mm). Susceptibility testing is not recommended using gentamicin as the bacteria is a poor target for therapy with this agent. According to EUCAST, the breakpoint for sulphonamides (10 mm) and trimethoprim (16 mm) is in preparation.

7.1.3 *Enterococcus* spp.

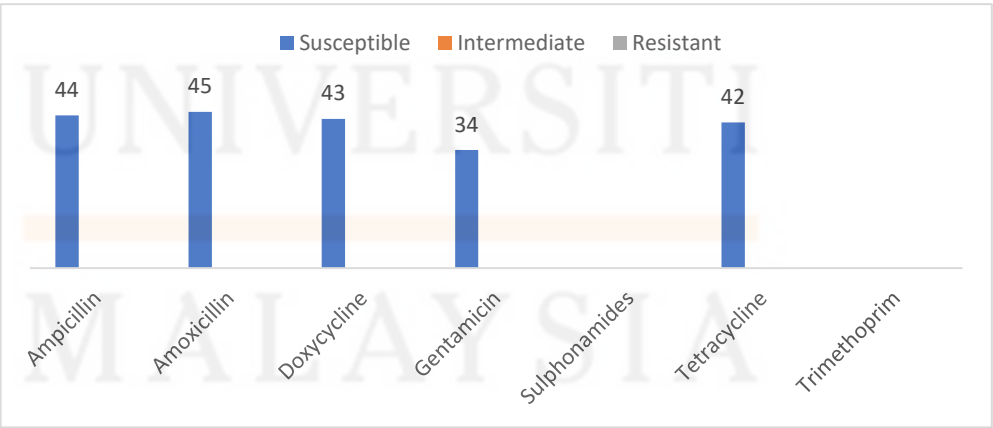

Figure 5: Antibiotic sensitivity of *Enterococcus* spp.

Figure 5 shows that *Enterococcus* spp. is susceptible multiple antibiotics; ampicillin (44 mm), amoxicillin (45 mm), doxycycline (43 mm), gentamicin

(34 mm) and tetracycline (42 mm). There is no data of antibiotic susceptibility against sulphonamides and there is uncertain activity with trimethoprim according to EUCAST.

7.1.4 *Bacillus cereus*

There are no zone diameter breakpoints for any of the antibiotic used in this study. Guidelines for *Bacillus* sp. breakpoints are reported in minimum inhibitory concentration (MIC, µg/mL) from CLSI and there was no data for the antibiotics used in this study from EUCAST.

7.1.5 *Salmonella* spp.

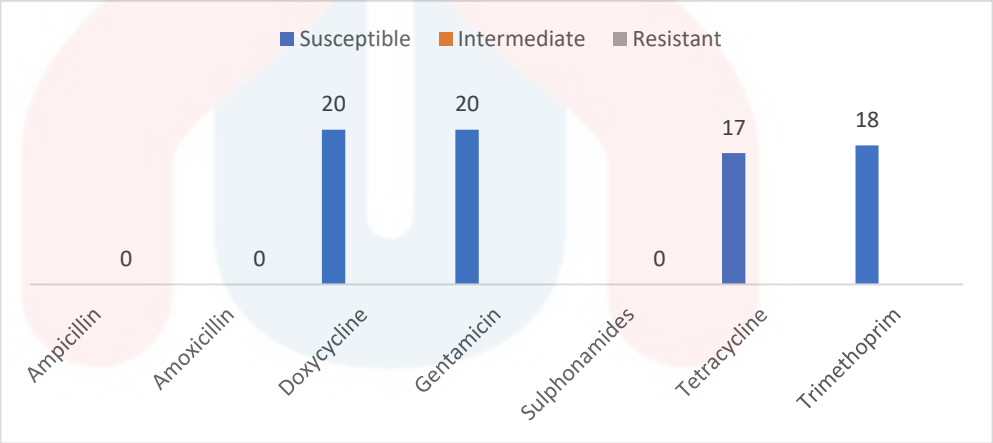

Figure 6: Antibiotic sensitivity of *Salmonella* spp.

Figure 6 shows *Salmonella* spp. is resistant to ampicillin (0 mm) and amoxicillin (0 mm). *Salmonella* spp. is susceptible to doxycycline (20 mm), gentamicin (20 mm), tetracycline (17mm) and trimethoprim (18 mm). However, there is no breakpoints for sulphonamides (0 mm) but *Salmonella* spp. shows resistance towards it.

7.1.6 *Proteus* spp.

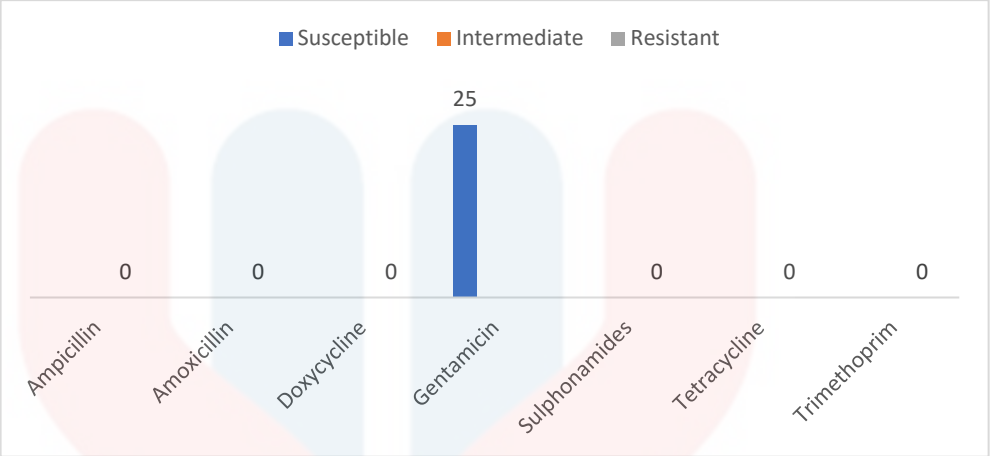

Figure 7: Antibiotic sensitivity of *Proteus* spp.

Figure 7 shows that *Proteus* spp. is resistant to almost all antibiotics except gentamicin (25 mm).

7.1.7 *Pseudomonas* spp.

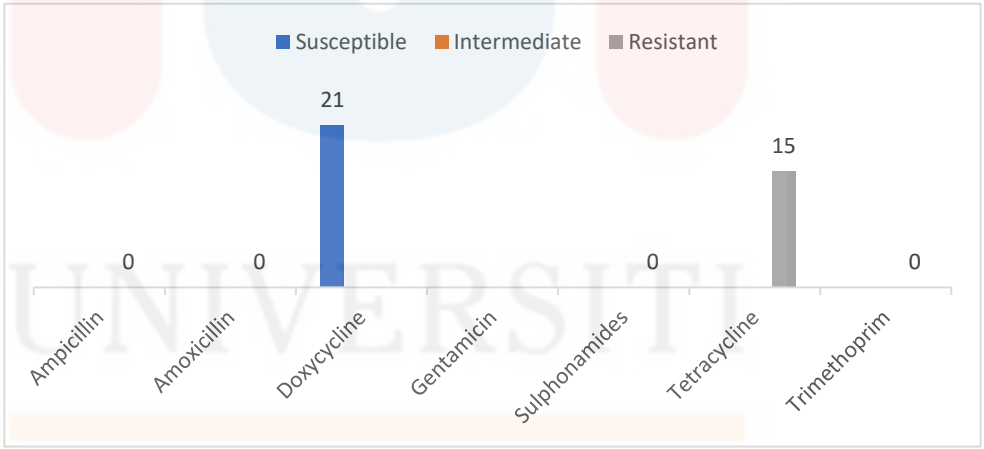

Figure 8: Antibiotic sensitivity of *Pseudomonas* spp.

The figure shows that *Pseudomonas* spp. is resistant to ampicillin (0 mm), amoxicillin (0 mm), tetracycline (15 mm), and trimethoprim (0 mm). *Pseudomonas* spp. is susceptible to doxycycline (21 mm) only. There is insufficient evidence towards gentamicin according to EUCAST. There is

also no evidence of breakpoints towards sulphonamides (0 mm) however it is resistant towards it.

7.1.8 Enterobacter spp.

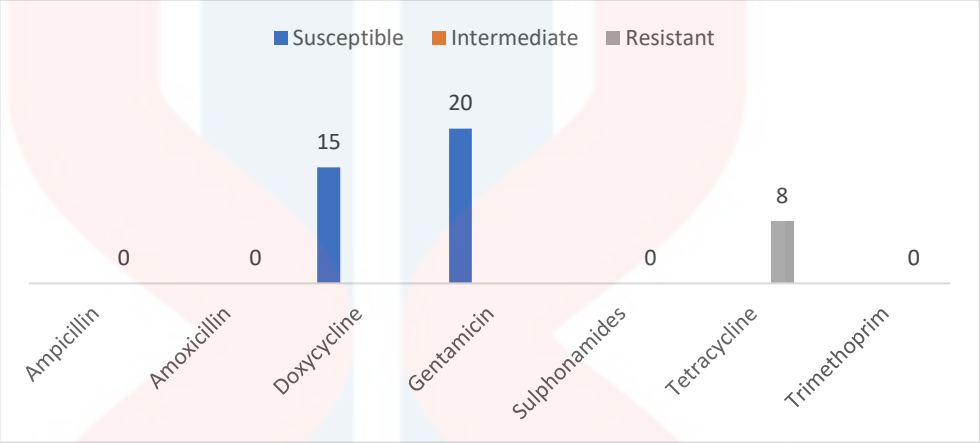

Figure 9: Antibiotic sensitivity of *Enterobacter* spp.

The figure shows that *Enterobacter* spp. is resistant to ampicillin (0 mm), amoxicillin (0 mm), sulphonamides (0 mm), tetracycline (4mm), and trimethoprim (4 mm). It is however susceptible towards doxycycline (15 mm) and gentamicin (20 mm).

7.1.9 Klebsiella spp.

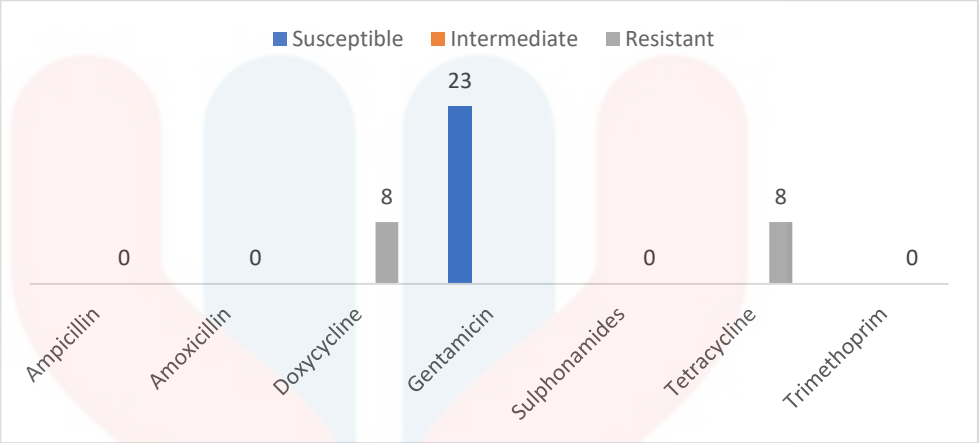

Figure 10: Antibiotic sensitivity of *Klebsiella* spp.

The figure shows that *Klebsiella* sp. is resistant to ampicillin (0 mm), amoxicillin (0 mm), doxycycline (8 mm), sulphonamides (0 mm), tetracycline (0mm), and trimethoprim (0 mm). It is only susceptible towards gentamicin (23mm).

7.1.10 Antibiotic Resistance Pattern

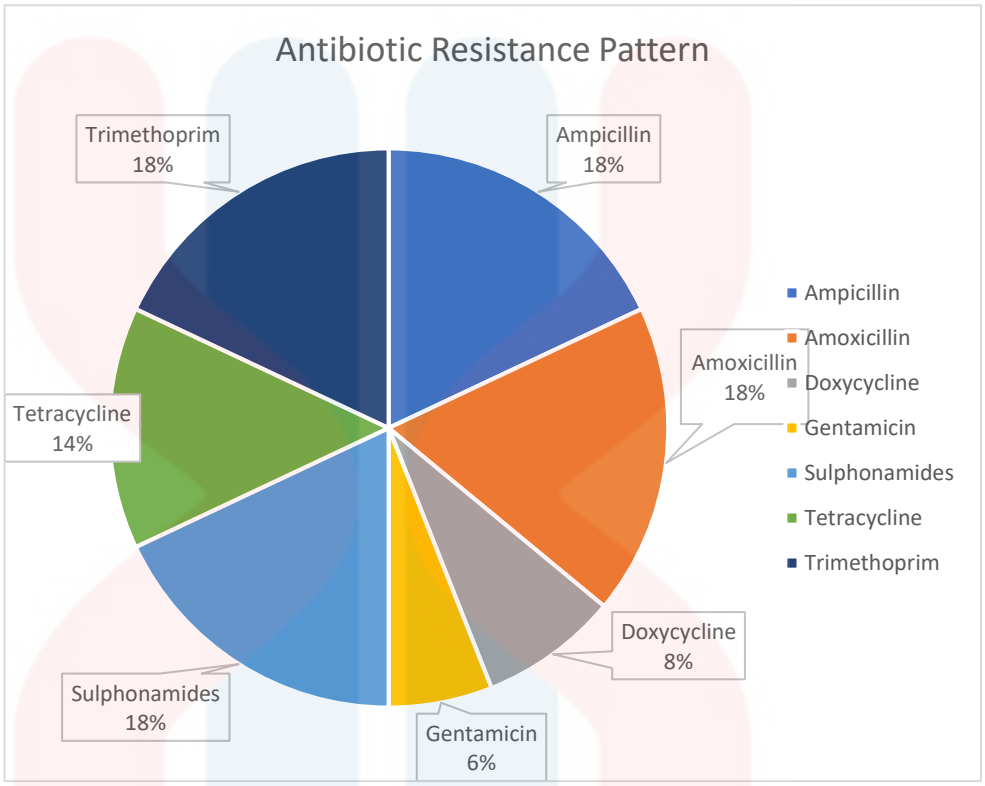

Figure 11: This chart shows the number of bacteria identified that are resistant to the antibiotic listed.

From the study, 18% of the samples that were tested for antibiotic sensitivity were resistant to ampicillin, amoxicillin, sulphonamides and trimethoprim. Additionally, 14% of the samples were resistant to tetracycline. An additional of 8% of the samples are resistant towards doxycycline and 6% of the samples are resistant towards gentamicin (Figure 11). However, the results for antibiotic sensitivity towards *B. cereus* was not interpreted as there was only reference breakpoints in minimum inhibitory concentration.

8.0 Discussion

The purpose of this study was to identify the common bacterial pathogens of pork meat and the antibiotic resistance pattern of it. In this study, 9 bacteria were isolated from the samples. The results obtained in this study showed similarity with the bacteria identified from pig samples in Veterinary Research Institute from year 2014 to 2017 (Aisya Naama et al., 2018). Similar bacteria that was identified in this study includes; *B. cereus*, *Enterococcus* spp., *Enterobacter* spp., *Escherichia coli*, *Klebsiella* spp., *Pseudomonas* spp., *Salmonella* spp., and *Streptococcus* spp, respectively. The only newly identified bacteria from this research is the *Proteus* spp. This proves that there might be more unidentified species of bacteria that can be isolated from meat samples given the sample size is larger and a sample collection area is used. Additionally, *Proteus* spp. can also be a contaminant from the laboratory itself thus, better sterile methods should be used in future studies. To our knowledge, this is the first study to be done on pork meat in Kota Bharu, Kelantan.

B. cereus is one of the bacteria isolated and identified in this study. *B. cereus* produces two separate forms of foodborne illness through the synthesis of unique toxins: a diarrheal syndrome and an emetic syndrome (Griffiths & Schraft, 2017). Unfortunately, the pattern of antibiotic resistance cannot be discussed as there is insufficient data in this study.

Enterococcus spp. on the other hand is a nosocomial gram-positive bacterium that is commonly identified in meat and foodborne transmission of enterococci affects larger part of the population. *Enterococcus faecalis* and *Enterococcus faecium* are the species of enterococcus found most commonly in clinical and dietary samples that pose the greatest risk to human health. Both species are

responsible for several illnesses in immunocompromised patients. They showed resistance to antimicrobials like Beta-lactams, aminoglycosides, and glycopeptides (Kim & Koo, 2020). Except for clindamycin, erythromycin–nitrofurantoin and erythromycin–tetracycline was the most often found resistance patterns against two antimicrobials (Kim & Koo, 2020). This coincides with the data from this study that shows enterococci susceptibility towards ampicillin, amoxicillin, doxycycline, gentamicin and tetracycline.

Numerous *Enterobacteriaceae* species have been documented to pose a threat to consumer health. A study by ElGendy et al. (2014) shows *Enterobacter aerogenes*, *Enterobacter intermedium* and *Enterobacter gergoviae* were isolated from beef meat products which are proven to be a major cause of foodborne illness. High *Enterobacteriacea*e counts in minced meat point to unhygienic conditions inside butcher shops, particularly for the mincing machines that were used to mincemeat without routine cleaning or washing, as well as for the hands of the workers, who carry heavy contamination and contaminate meat through improper handling.

Salmonellosis is the most common foodborne diseases elicited with signs of diarrhoea, fever and abdominal cramps. *Salmonella thypimurium* and *Salmonella enteritidis* are the most common causes of foodborne illness in humans, and *S. enteritidis* is one of the most commonly documented causes of foodborne illness in humans. According to a CDC report covering the years 2009 through 2015, the FDOSS received reports of 5,760 outbreaks resulting in 100,939 illnesses, 5,699 hospitalizations, and 145 deaths (Dewey-Mattia *et al.*, 2018). Based on a review of outbreaks of foodborne disease in the United States, pork meat may be responsible for between 8 and 13 percent of the

approximately 1 million cases of human salmonellosis that are caused by food each year (USDA, 2022). Another study based in Greece proved that *Salmonella enterica* subsp. *Arizonae* was isolated from 13 out of 123 swine slaughtered (Evangelopoulou et al., 2013). Resistance of *Salmonella* spp. in pork meat according to the Malaysian Action Plan on Antimicrobial Resistance 2017-2021 showed increase in ampicillin, chloramphenicol, ciprofloxacin, gentamicin, trimethoprim/sulfamethoxazole and erythromycin (Ministry of Health Ministry, 2017). From this study we can see, *Salmonella* isolates are susceptible to gentamicin which proves there is positive pattern changes. However, in this study, further identification of *Salmonella* spp. was not done and further study should be warranted to identify the serotype associated with foodborne illness.

Studies shown that *Klebsiella pneumoniae* is multi-drug resistant as well as causing infections to humans via foodborne transmission. The incidence of resistance to all antibiotics except trimethoprim-sulfamethoxazole and nalidixic acid was considerably higher among meat-source isolates, with tetracycline and gentamicin being the most resistant (Davis *et al.*, 2015). *K. pneumonia* causes foodborne illnesses that results with septicaemia, liver abscesses and diarrhoea. From this study additionally, we know that the pathogen is only susceptible to gentamicin hence proving that it is a multi-drug resistant bacterium.

E. coli is part of the natural intestinal microbiota of many animals, including humans, and was discovered in this investigation. There are two types of pathogenic *E. coli* strains: intestinal and extraintestinal (Lorenz *et al.*, 2020). *E. coli* is commonly found in meat products. In poultry, pork, and beef, the

prevalence of extended spectrum beta-lactamase (ESBL)-producing E. coli was 51.2% (109/213), 26.9% (58/216), and 7.3% (15/205), respectively. From 184 samples, a total of 225 ESBL-producing *E. coli* was isolated (Guo *et al.*, 2020). In this study we can observe that *E. coli* was completely resistant to ampicillin, amoxicillin, sulphonamides and trimethoprim. This coincides with the data from Malaysian Action Plan on Antimicrobial Resistance 2017-2021 (Ministry of Health Ministry, 2017), showing a hike in resistance of *E. coli* in pork meat towards ampicillin, chloramphenicol, ciprofloxacin, gentamicin, tetracycline, trimethoprim/sulfamethoxazole, and erythromycin. Pathotyping for the isolated *E. coli* was not done in this study. It is significant as different type of *E. coli* causes different disease progression as well as showing different AMR patterns. Based on a few literatures, types of diarrhoeagenic *E. coli* includes Enterotoxigenic *E. coli* (ETEC), Enterohaemorrhagic *E. coli* (EHEC), Enteropathogenic *E. coli* (EPEC), Enteroinvasive *E. coli* (EIEC) include Shigella, Enteroaggregative *E. coli* (EAEC), Diffusely Adherent *E. coli* (DAEC) (Shah et al., 2018) and Adherent Invasive E. coli (AIEC) (Martinez-Medina & Garcia-Gil, 2014). EHEC has the most prominent effect in human foodborne diseases resulting in haemorrhagic diarrhoea (Shah et al., 2018).

Another *Enterobacter* that was isolated in this study was, *Proteus* spp. Some strains of *P. mirabilis* have been linked to food poisoning outbreaks, but the pathogenesis is largely unknown. A study conducted by Wang et al. (2010) based on food poisoning events in a group of 13 people, confirmed that the source of *P. mirabilis* infection was from stewed pork balls. Integrons primarily mediate the spread of antibiotic-resistant genes among intestinal bacteria in food animals and the environment. The class 1 and class 2 integrons

were detected in roughly 46% and 14% of the poultry and cattle *Enterobacteriaceae* isolates, respectively (Kim et al., 2005).

Pseudomonas spp. does not constitute a serious risk to public health concerns but is considered a specific spoilage organism causing the spoilage of meat. According to Elbehiry *et al.* (2022) there are seven pseudomonas species that are identified from chicken meat samples where *P. ludensis* appeared with highest incidence rates. It was also reported in the same paper that, the *Pseudomonas* isolates were resistant against nitrofurantoin, ampicillin, cefuroxime, ceftriaxone, aztreonam and ciprofloxacin (Elbehiry *et al.*, 2022). Results of this study does coincide with other findings.

Based on a study by Hassanien & Abdel Aziz (2021), *Streptococcus. pyogenes*, *Streptococcus mitis*, and *Streptococcus pneumoniae* were isolated from 120 throat swab samples and 200 meat products. Streptococcal infection causes pharyngitis, skin infections, rheumatic fever and toxic shock syndrome in humans, which makes it a concern of public health (Bush, 2022). The majority of streptococci strains isolated from humans and meat products were resistant to more than one antimicrobial agent, including tetracycline and amoxicillin/clavulanic acid, which exhibited the highest prevalence of resistance (Hassanien & Abdel Aziz, 2021). As compared to the results in this study, *Streptococcus* spp. are resistant towards amoxicillin, ampicillin, doxycycline and tetracycline as well.

This research is crucial to increase the awareness of AMR among public and pork meat consumers of Kota Bharu, Kelantan. Antibiotic resistance is directly related to the food safety in which diseases are able to spread faster and without

limited treatment. Disease spread that occurs in the pork meat due to antibiotic resistance will lead to a shortage of meat supply and thus inflation. Since these antibiotics are commonly used in human medicine as well as animal medicine, it shows that humans are also at risk of getting infected with multiple drug resistant bacteria without a cure at hand. Proper interventions by the authorities involved need to be performed now to curb the spread of resistance. This starts by raising awareness of the issue among the public itself. Antibiotic and AMR is only actively discussed within the human and animal medical community itself, but has a limited success towards alerting the public towards this issue.

9.0 Conclusion and Recommendation

In conclusion, the number and type of bacteria that were isolated could pose a risk to the public that aren't aware of zoonoses and zoonotic transmission of diseases. Most of the bacteria showed resistance towards amoxicillin, ampicillin, sulphonamides, tetracycline and trimethoprim. Better monitoring procedures must be implemented by manufacturers, veterinarians, and feed producers in order to comprehend the dosage, quantity of doses, and adverse reactions of monitors in animals. Additional testing of animal drugs involving the examination of animal resistance and animal by-products. Importantly, campaigns should be implemented to educate the public about the dangers of food poisoning and AMR. Further studies should be done using wider geographic distribution of sample size to be able to identify other bacteria that may be present in pork meat.

As for the recommendation in further studies, the identification of common bacteria can be done using wild boar meat in the sample are and this result can be compared with pre-existing results from research done on pork meat. Pathotyping and serotyping of *Salmonella* spp. and *E. coli* to identify the serotypes causing foodborne illness. This is also important to know any new identification of foodborne disease-causing bacteria. Furthermore, antibiotic sensitivity testing should be performed in a wider antibiotic group especially those that are widely used in both human and also livestock medicine. Added investigations are to be done using knowledge, attitude and practices (KAP) survey, to quantitatively analyse food safety awareness among pork meat consumers can be done to assess the antibiotic resistance awareness among public or pork meat consumers.

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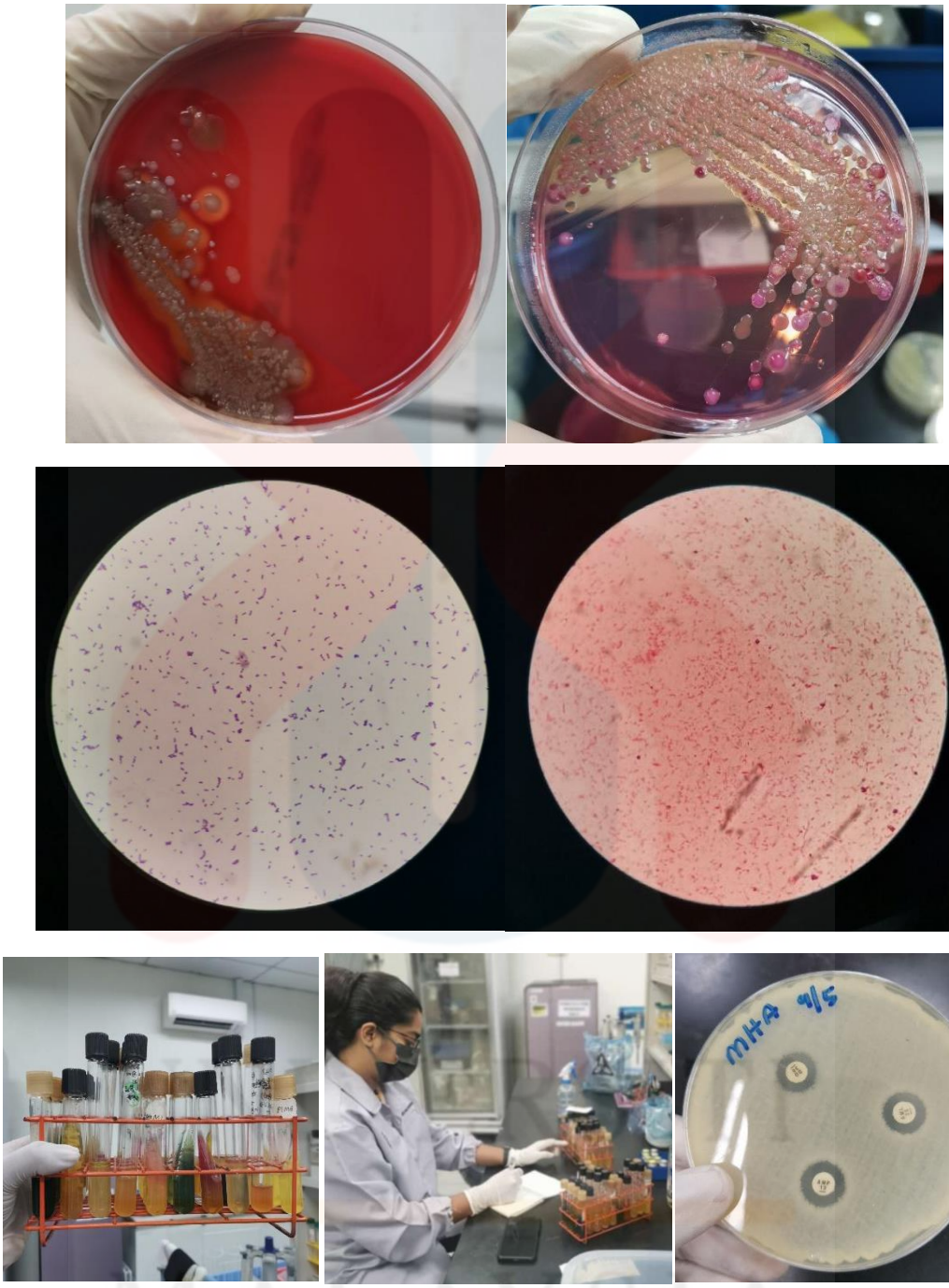
Appendix A

Chinese Wet Market, Kota Bharu, selling pork meat on display.

Flaming method was used to sterilize all the equipment used.



Colony observation, gram staining slide observation, biochemical testing and AST results.