PREVALENCE, ANTIBIOTICS SUSCEPTIBILITY AND GENOTYPING OF FOODBORNE BACTERIA IN MEAT, EGGS AND FEEDS FROM SELECTED MARKETS

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FACULTY OF SCIENCE
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KUALA LUMPUR

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PREVALENCE, ANTIBIOTICS SUSCEPTIBILITY AND GENOTYPING OF FOODBORNE BACTERIA IN MEAT, EGGS AND FEEDS FROM SELECTED MARKETS

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PREVALENCE, ANTIBIOTICS SUSCEPTIBILITY AND GENOTYPING OF FOODBORNE BACTERIA IN MEAT, EGGS AND FEEDS FROM SELECTED MARKETS

ABSTRACT

Food safety in poultry production industry is a global concern including Malaysia. Antibiotics are used in poultry industry and consequently increases emergence of multidrug resistance foodborne pathogens. A total of 100 non-repeatitive isolates consisting 38 isolates of *Salmonella* spp., 28 isolates of *Listeria* spp., 20 isolates of *Escherichia coli* and 3 isolates of *Staphylococcus aureus* were isolated from 50 sample of chicken meat, 40 eggs and 10 types of feeds obtain from selected local markets in Klang Valley, Malaysia from 2015 to 2016. This study was aimed to assess the prevalence, antimicrobial susceptibility and genotypes of bacteria isolates. Resistance was most frequently detected to erythromycin (100%), followed by to tetracycline (76%), azithromycin (58%), streptomycin (24%), ampicillin (18%), chloramphenicol (18%), trimethoprim (18%), nalidixic acid (18%), gentamicin (13%), ciprofloxacin (5%) and nitrofuratoin (5%) in 38 *Salmonella* isolates. For the 28 *Listeria* isolates were highly resistant to clindamycin (89%), followed by tetracycline (86%), ceftriaxone (75%), ampicillin (64%) and penicillin G (50%). Thirty-five of *Salmonella* (92.1%, 35/38) isolates were resistant to at least two antibiotics, 14 (36.8%, 14/38) at least to four antibiotics, and 4 isolates (10.5%, 4/38) showed 8 multiple antibiotic resistance against 19 different antibiotics. For the 27 (96.4%, 27/28) *Listeria* isolates were resistant at least to two antibiotics, of which 11 isolates (39.3%, 11/28) showed 5 multiple antibiotic resistance against 11 different antibiotics in the study. Pulse-Field Gel Electrophoresis (PFGE) generated 6 major clusters with 12 patterns and 7 patterns in *Salmonella* and *Listeria* isolates. *Salmonella* and *Listeria* isolates

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clustered under similar PFGE patterns were resistant to similar categories of antibiotics. Of these, *tetA, tetB, sul1, sul2, qnrS, qnrB* and *mphA* and *invA* were detected in 25 (66%), 2 (5%), 20 (53%), 38 (100%), 24 (63%), 2 (5%), 4 (11%) and 38 (100%) *Salmonella* isolates using Polymerase Chain Reaction (PCR). Subsequently, the draft genome of 4 selected *Salmonella* spp. were sequenced using HiSeq 2000 Illumina platform and antibiotic resistance genes were annotated using RAST (Rapid Annotation using Subsystem Technology). The findings showed that chicken breast fillet is a potential source of antibiotic-resistant *Salmonella* and *Listeria* species. Majority of *Salmonella* and *Listeria* species isolated from meat were multidrug resistant and pose great potential hazard to public health and food safety.

Keywords: antibiotic resistance, poultry, PFGE, Whole genome sequencing

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KELAZIMAN, KECENDERUNGAN ANTIBIOTIK DAN GENOTYPING FOODBORNE BAKTERIA DALAM DAGING, TELUR DAN MAKANAN AYAM DARI PASARAN TERPILIH

ABSTRAK

Keselamatan makanan telah menjadi kebimbangan global bagi pengeluaran industri ternakan termasuk negara Malaysia. Antibiotik kerap digunakan dalam ternakan industri akibat peningkatan kemunculan pelbagai jenis antibiotik resistant patogen makanan. Sejumlah 100 non-repeatitive isolat terdiri daripada 38 *Salmonella* spp. (38%), 28 *Listeria* spp. (28%), 20 *Escherichia coli* (20%) dan 3 *Staphylococcus aureus* isolat (3%) adalah empat jenis bakteria yang dijumpai dalam 50 daging ayam, 40 biji telur ayam dan 10 jenis makanan ayam di pasaran tempatan Klang Valley, Malaysia dari tahun 2015 hingga 2016.Penyelidikan ini bertujuan untuk menilaikan kelaziman, kecenderungan antimikrob dan genotip bakteria yang dipilih. Penahanan yang kerap dikesan terhadap erythromycin (100%), diikuti oleh tetracycline (76%), azithromycin (58%), streptomycin (24%), ampicillin (18%), chloramphenicol (18%), trimethoprim (18%), nalidixic acid (18%), gentamicin (13%), ciprofloxacin (5%) dan nitrofuratoin (5%) dalam 38 *Salmonella* spp. Sejumlah 28 *Listeria* spp. memberikan penahanan yang tinggi terhadap nalidixic acid (100%), clindamycin (89%), tetracycline (86%), ceftriaxone (75%), ampicillin (64%) dan penicillin G (50%). Tiga puluh lima (92.1%) *Salmonella* spp. (92.1%, 35/38) menahan sekurang-kurangnya dua jenis antibiotik, 14 (36.8%, 14/38) yang melebihi daripada empat antibiotik dan 4 (10.5%, 4/38) menunjukkan 8 pelbagai antibiotik resistance dengan menggunakan 19 antibiotik yang berbeza. Tambahan pula, 27 (96.4%, 27/28) *Listeria* spp. menahan kepada sekurang-kurangnya dua jenis antibiotik dan 11 (39.3%, 11/28) menunjukkan 5 pelbagai antibiotik resistance dengan menggunakan 11 antibiotik yang berbeza dalam kajian ini. Pulse-Field Gel Electrophoresis (PFGE) telah menjana 6 jenis

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kelompok utama dengan 12 corak and 7 corak dalam *Salmonella* dan *Listeria* isolate. *Salmonella* dan *Listeria* isolate yang berkumpul dalam corak PFGE yang serupa adalah resistant dalam kategori antibiotik yang sama. Oleh itu, gen *tetA, tetB, sul1, sul2, qnrS, qnrB, qnr*A dan *mph*A*, inv*A dikesankan sejumlah 25 (66%), 2 (5%), 20 (53%), 38 (100%), 24 (63%), 2 (5%), 4 (11%) dan 38 (100%) *Salmonella* isolate dengan menggunakan Polymerase Chain Reaction (PCR), secara berasingan. Kemudian, 4 *Salmonella* draf genom yang dipilih telah menggunakan HiSeq 2000 Illumina platform dan menganotasi gen antibiotik dengan RAST (Rapid Annotation using Subsystem Technology). Penemuan ini menunjukkan sumber yang berpotensi bagi antibiotik-resistant *Salmonella* dan *Listeria* spp. yang didapati dalam daging ayam. Majoriti *Salmonella* dan *Listeria* dalam daging ayam adalah pelbagai jenis antimikrob resistant dan menimbulkan potensi bahaya terhadap kesihatan awam dan mengancam keselamatan makanan.

Kata Kunci: antibiotik resistance, ayam, PFGE, Whole genome sequencing

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LIST OF SYMBOLS AND ABBREVIATIONS

bp	Basepair
ºC	Degree Celsius
et al	et alia (and others)
µg	Microgram
µl	Microliter
mg	Milligram
ml	Milliliter
mm	Millimeter
%	Percent
× g	Times gravity
AMC	Amoxicillin-clavulnate
AMP	Ampicillin
AST	Antimicrobial Susceptibility test
ATM	Aztreonam
AZM	Azithromycin
CAZ	Ceftazidime
CEF	Cephalothin
CFZ	Cefazolin
CHL	Chloramphenicol
CIP	Ciprofloxacin
CLI	Clindamycin
CRO	Ceftriaxone
ERY	Erythromycin
FB	Fraser broth
GEN	Gentamicin
HEA	Hektoen Enteric agar
IMI	Imipenem

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MAC	MacConkey agar
MALDI-TOF	Matrix-Assisted Laser-Desorption-Time of Flight
MAR	Multiple Antibiotic Ratios
MCB	MacConkey broth
MHA	Muller-Hinton agar
MSA	Mannitol Salt agar
NAL	Nalidixic acid
NIT	Nitrofuratoin
OXA	Oxacillin
PMB	Polymyxin B
PC-G	Penicillin G
PCR	Polymerase Chain Reaction
PFGE	Pulse-Field Gel Electrophoresis
RIF	Rifampicin
RVS	Rappaport- Vassiliadis Soy broth
STR	Streptomycin
SXT	Trimethoprim- Sulfamethoxazole
TET	Tetracycline
TMP	Trimethoprim
TOB	Tobramycin
TSB	Tryptic Soy broth
TSYEA	Tryptone Soy Yeast Extract agar
VAN	Vancomycin

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LIST OF APPENDICES

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CHAPTER 1: INTRODUCTION

Bacteria, virus, fungi and parasites are microorganisms that cause foodborne disease. Foodborne disease is an illness due to ingestion of foods contaminated with bacteria, viruses or parasites that can pass through and survives in human digestive system. World Health Organization (WHO) (WHO, 2015a, 2015b) reported 600 million cases of illness caused by the 31 foodborne hazards in year 2010. Diarrhoeal diseases agents reported in major part (550 million) especially caused by bacteria (350 million). Foodborne diarrhoeal disease agents caused 230,000 of the 420,000 deaths due to foodborne hazards. Of these, non-typhoidal *Salmonella enterica* (*S. enterica*) accounted for 59,000 deaths, enteropathogenic *Escherichia coli* (*E. coli*) (EPEC) for 37,000 deaths, norovirus for 35,000 deaths and *Listeria monocytogenes* (*L. monocytogenes*) for 3,200 deaths. Eighteen million of disability adjusted life years (DALYs), or 54%, of the total burden was attributed to diarrheal disease agents, especially to non-typhoidal *S. enterica*, accounted for 4.0 million DALYs and *L. monocytogenes* accounted for 118, 340 DALYs (WHO, 2015c).

Center for Diseases Control and Prevention (CDC) has reported the estimated number of death in the US in 2006 caused by foodborne pathogens: non-typhoidal *Salmonella* spp. was 378 cases, STEC O157 20 cases, *Listeria* spp. 255 cases and *S. aureus* 6 cases (CDC, 2011c). Common food source contaminated with bacteria associated with foodborne illness such as raw meat and poultry, eggs, raw vegetable, raw shellfish and dairy products. In general, cholera, typhoid fever, hepatitis A and norovirus are examples of foodborne illness cases reported in Malaysia. According to non-communicable report, food poisoning cases accounted for 44.3 rates per 100,000 populations in Malaysia year 2010 (Ministry of Health, 2011b).

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Malaysia Ministry of Health (MOH) Statistic reported incidence rate and mortality rate accounted for 47.34 and 0.01 under food poisoning (foodborne disease) per 100,000 populations in year 2015 (MOH, 2016). Foodborne bacteria are easily transmitted through different routes such as contaminated with animal feces, infected animals, unclean utensils, personnel hygiene and improper food handling in food preparation; subsequently pass to human and caused foodborne illness.

Foodborne salmonellosis and listeriosis have increased significant become health concern globally and caused high fatal morbidity and mortality (WHO, 2015a). Poultry products are the main reservoir for *Salmonella* and *Listeria* spp. associated with foodborne diseases. The pathogens can be easily transferred from environment to poultry and food handlers. Consequently, the bacteria would pass from poultry products to human through the food chain especially undercooked eggs and raw poultry meat poses health risk for human. Chicken meat is widely consumed and cheaper source of animal protein poultry species in the world. The preference and consumption of chicken meat have been tremendously increasing in Malaysia whereby is acceptable by all races amongst the urban and rural residents (Jayaraman et al., 2013).

The use of antibiotic therapy is effectively against food pathogens. Emergences of antibiotic resistance in pathogenic bacteria are reported in Asia countries such as Laos, Cambodia and Thailand (Boonmar et al., 2013; Lay et al., 2011; Padungtod & Kaneene, 2006). Resistant to combinations of several classes of antimicrobials has led to the emergence of multidrug resistant strains that may pass from food to humans (White et al., 2001).

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The abuse of antibiotics in humans and domestic livestock has led to multidrug resistant (MDR) bacteria are increasingly isolated from poultry in worldwide (Aissa & Al-Gallas, 2008; Threlfall et al., 2000). Such drugs may be used either therapeutically or prophylactically, or for growth promotion (McEwen & Fedorka-Cray, 2002). Bacterial resistance is observed especially when the antibiotics are abundantly used and that the bacteria can be transmitted easily between the individuals. Consequently, resistance to antibiotics in strain of *Salmonella* and *Listeria* is being viewed as potential threat to poultry production, food safety and public health.

Numerous methods have been developed for detection; identification and molecular characterization of pathogenic bacteria include both phenotypic and genotypic methods such as serotyping and antibiotic susceptibility, PCR-based typing and Pulse-Field Gel Electrophoresis (PFGE). The present study investigates antibiotic resistant strains of *Salmonella* spp., *Listeria* spp., *E. coli* and *S. aureus* in chicken meat, chicken eggs and chicken feeds in local markets Malaysia. The scientific data significantly obtained an accurate understanding of antibiotic resistance (AMR) pattern in pathogenic bacteria isolated from chicken meat in Klang Valley, Malaysia. The pattern of antibiotic resistance is important in control of resistance to drugs in poultry industry.

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The overall aims of the study

1. To determine the microbiological safety of *Salmonella* spp., *Listeria* spp., *E. coli* and *S. aureus* from raw chicken meat, chicken eggs and chicken feeds bought from market.
2. To determine the prevalence and antimicrobial resistance profile of the bacteria isolated from chicken meat, chicken eggs and chicken feeds.
3. To conduct genotypic characterization of isolated *Salmonella* and *Listeria* spp. by PFGE.

Research Hypothesis

Poultry meats are major source of *Salmonella* and *Listeria* spp. associated with different levels of resistance to antibiotics. Antibiotics are drugs used for treatment in humans and livestock such as poultry. However, abuse uses of some antibiotic are losing effectiveness against pathogenic bacteria and consequently increase spreading of multidrug resistance within and between different *Salmonella* and *Listeria* isolates.

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CHAPTER 2: LITERATURE REVIEW

2.1 Foodborne Disease

Definition of foodborne disease derived from different organizations: according to World Health Organization (2016) define foodborne disease as "Ingestion of food that contaminated with microorganisms or chemicals and cause foodborne disease with gastrointestinal symptoms" (WHO, 2016a) as well as Food and Agriculture Organization of the United States (2004) defined foodborne illness as "Foodborne diseases are usually either infectious or toxic in nature, caused by agents that enter the body through the ingestion of food" (FAO, 2004). For Center for Disease Control and Prevention (2016) state that foodborne disease also named as foodborne illness, foodborne infection or food poisoning that causes public health concern by ingestion of contaminated foods or waters with disease-causing pathogens (CDC, 2016a).

Salmonella spp. are the main causative bacteria agents causing 34 % of foodborne outbreak has been reported in year 2013. There are several type of foods involved in foodborne illness including 50 outbreak in contaminated fish samples (24 %), 23 outbreak in contaminated mollusk (11 %), 21 outbreak in contaminated chicken meat (10 %) and 11 outbreak due to contaminated beef (5 %) as well as 3 outbreaks in contaminated eggs (1 %) were recorded. *Salmonella* spp. in chicken highlighted the highest illness (700 cases) reported as compared to other meats such as pork (436 cases) and beef (210 cases).For *Listeria* spp. was isolated from dairy products causes about 3 death cases (CDC, 2015b).

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Table 2.1: Number of cases and incidence of confirmed infections, hospitalization and deaths caused by isolated pathogens in United States, 2015. (Adapted from CDC (2016e)

Pathogens	Estimated cases illness no.	Estimated hospitalization no. (%)	Estimate death no. (%)
Salmonella spp.	7,728	2,074 (27)	32 (0.4)
Listeria spp.	116	111 (96)	15 (12.9)
Escherichia coli non O157	796	126 (16)	1 (0.1)
Campylobacter spp.	6,309	1,065 (17)	11 (0.2)
Vibrio spp.	192	47 (24)	5 (2.6)
Yersinia enterocolitica	139	37 (27)	1 (0.7)

*Pathogens causing US foodborne illnesses, hospitalizations, and deaths, 2015 per 100,000 population.

Table 2.2: Global health estimate of DALYs, YLL and YLD caused by diarrhoeal disease in year 2015. (Adapted data from WHO (2017)

Country regions	DALYs	YLL	YLD
African region	44,482,642	42,546,351	1,936,291
American region	1,804,978	1,387,190	417,788
South-East Asia region	25,978,374	23,879,035	2,099,339
European region	778,661	650,778	127,883
Eastern Mediterranean region	9,613,402	8,838,033	775,369
Western Pacific region	2,255,610	1,892,809	362,801

*DALYs, disability-adjusted life year; YLL, years of life lost; YLD, years lost due to disability.

CDC estimated number of cases of confirmed infections, hospitalization and deaths caused by pathogens associated with foodborne disease in United States in period of year 2015 (Table 2.1) and global health estimate of disability of adjusted life, year of life lost and years lost due to disability caused by diarrhoeal disease (Table 2.2). Foodborne disease has become an emerging health issues in globally. There are low number of food poisoning cases reported in Malaysia due to the complexity and lack of clarity of documentation procedures. In fact, it is not common case happened in our country but low number has been showed in statistically. The complex chain happened before reported to authority. This is due to most of the food poisoning cases are not reported.

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Diseases	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013
Cholera	0.4	1.5	0.9	0.5	0.3	0.98	1.56	2.02	0.96	0.58
Dysentry	1.4	0.5	0.4	0.5	0.3	0.54	0.37	0.15	0.29	0.28
Food poisoning	23.89	17.8	26	53.2	62.5	36.17	44.18	56.25	44.93	47.79
Typhoid	1.9	4.1	0.8	1.2	0.7	1.07	0.74	1.71	1.58	0.73
Hepatitis A	0.4	0.2	0.2	0.4	0.1	0.14	0.14	0.84	0.75	0.41

Figure 2.1: Foodborne illness reported in Malaysia year 2004-2013. (Adapted from MOH (2014)

Foodborne illnesses reported in Malaysia include typhoid, food poisoning and Hepatitis A in period year 2004 to 2013. Food poisoning case is show the highest incidence rate of 56.25 per 100,000 populations in year 2011 and typhoid is on the rise incident rate of 1.71 per 100,000 populations at the same year (Figure 2.1). The causes of foodborne disease due to inappropriate food handling procedures and poor hygiene practice of food handlers in Malaysia (Puteh et al., 2013). The number of cases and incidence rate of foodborne disease are obtained in period of year 2003 to 2009 in Malaysia (Table 2.3).

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Table 2.3: Number of cases and incidence rate of food and waterborne disease in Malaysia. (Adapted from MOH (2011a)

Year	Food Poisoning		Typhoid		Cholera	
	case	IR	Case	IR	Case	IR
2003	6624	25.4	785	3.0	135	0.5
2004	5957	23.3	484	1.9	89	0.4
2005	4641	17.8	1072	4.1	386	1.5
2006	6938	26.0	204	0.8	237	0.9
2007	14,455	53.2	325	1.2	133	0.5
2008	17,332	62.5	201	0.7	93	0.3
2009	10,238	36.2	303	1.1	276	1.0

Foods can be contaminated through from farm to fork of food chain process in Table 2.4.

Table 2.4: Contamination in food chains. (Adapted from CDC (2015a)

Process	Description
Production	Harvest growing plants or raise domestic animals on farm for food. Examples contamination in Production: Egg yolk will be contaminating before laid when reproductive organ of hen infected. Fruits and vegetables are contaminated when spray with contaminated water for irrigation.
Processing	Changing plants or animals into food involved of cleaning, sorting, slicing and packing of produce as well as slicing meat into ground and freeze before distribute. Examples of contamination in processing: Fresh produce will be contaminated with water or ice when washing and packing process. Pathogens can contaminate the meat products during slaughtering.
Distribution	Transport the packing food from farm to distributor. Examples of contamination in distribution: Fresh produce will be contaminated when the transport was not cleaned. Frozen foods are left at room temperature for period of time and promote the growth of bacteria.
Preparation	Getting the food ready-to-eat in kitchen or restaurant. Examples of contamination in preparation: The kitchen utensils are shared between raw food and cooked food will spread pathogens on food. Personal hygiene of workers will spread pathogen by prepare food.

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Ministry of Health Malaysia and Department of Veterinary Services are proposed several approaches on controlling foodborne illness caused by *Salmonella* spp., *Listeria* spp., and *Escherichia coli* O157 in poultry products including to conduct laboratory investigation surveillance on food pathogen associated with foodborne illness, propose manual on syndromic approach on infectious diseases, implement notification system for rapid response to emerging infectious diseases and regulate the national surveillance program for control and monitor the progress and spread of food pathogens in country (Mutalib et al., 2015).

2.2 Statistic in Poultry Industry

2.2.1 Poultry Meat in Malaysia

Poultry industry (chicken, geese, and duck), ruminant industry (goat, cattle, and sheep) and swine industry (pig) are categorized in livestock industry Malaysia (Department of Veterinary Service, 2015). Livestock products are preferred choices in developing countries in term of nutritional values and taste. Poultry industry plays an important role in Malaysia agricultural economics for local consumption purpose. DVS (2015) reported that consumption of poultry meat is range increases from 679 until 1286 in year 2005-2014 and for chicken egg is range from 6072 to 8457 in year 2005-2014. In Malaysia, the livestock industry is dominated by the poultry industry that contributes 50 % of the total meat requirements of the country in year 2014 (DVS, 2015).

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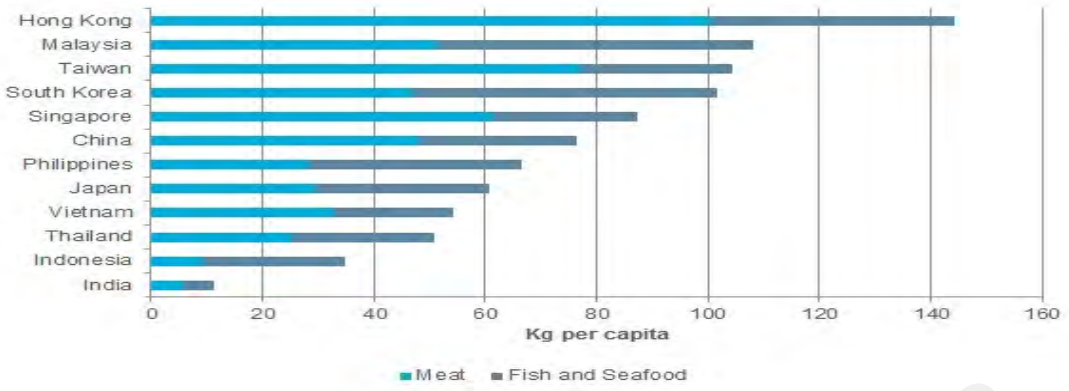

Figure 2.2: Consumption meats in Asia countries. (Adapted from Elizabeth (2015)

The consumption of meat serve as protein source in Malaysia is higher than other Asia countries such as Korea, Japan and China (Figure 2.2). Malaysia is considered having large production in poultry meat industry supply for local demand. Consumption of poultry meat is significantly high in market as compared with other meats. Poultry play role of primary protein source to be consume in majority of population. Chicken meat is accepted by consumer from different races in Malaysia population (Norimah et al., 2008). Meat consumption will reflect the economic condition of country. The variation of food habits and food consumption patterns in Malaysia influence economic growth of country (Nestle, 1999). However, chicken meat is popular serve in different ethnic groups include Chinese, Malays and Indians in Malaysia. The poultry consumption of country will increases as population increases as well as the market demand in year 2014 and 2015 (Table 2.5 & 2.6).

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Table 2.5: Livestock population in Malaysia in year 2014. (Adapted from DVS (2015)

MALAYSIA : BILANGAN TERNAKAN MENGIKUT NEGERI, 2014

Malaysia : Livestock Population By States, 2014

NEGERI *State*	Kerbau *Buffalo*	Lembu *Cattle*	Kambing *Goat*	Bebiri *Sheep*	Babi *Swine*	Ayam *Poultry*	Itik *Duck*	Burung Unta *Ostrich*	Puyuh *Quail*	Rusa *Deer*
Perlis	233	8,225	5,713	1,296	30	1,210,282	12,050	n.a	3250	285
Kedah	7,757	68,596	49,353	10,764	720	47,604,337	707,200	57	480,070	907
Pulau Pinang	566	16,091	11,005	1,540	311,791	12,452,669	495,200	n.a	150,255	145
Perak	11,669	53,007	38,077	4,374	517,163	35,256,700	6,725,600	25	65,750	4,877
Selangor	1,460	22,858	23,536	3,256	273,630	20,540,364	28,118	200	165,710	506
N. Sembilan	3,286	44,574	40,986	20,230	861	19,320,355	25,660	105	286,140	1,555
Melaka	3,740	27,935	39,507	10,710	47,195	19,690,989	124,862	25	76,000	615
Johor	3,458	106,085	49,817	26,942	270,056	63,663,879	890,039	307	1,090,800	1,681
Pahang	14,125	129,255	36,145	17,825	3,400	14,133,621	17,500	n.a	399,250	2,800
Terengganu	9,268	88,317	26,519	6,065	n.a	6,400,977	24,349	n.a	93,245	324
Kelantan	6,125	97,425	42,855	35,125	525	1,845,850	58,255	n.a	28,100	205
W. Persekutuan	n.a	450	255	n.a	n.a	n.a	n.a	n.a	n.a	95
Jumlah S. M'sia *Total For P. M'sia*	**61,687**	**662,818**	**363,768**	**138,127**	**1,425,371**	**242,120,023**	**9,108,833**	**719**	**2,838,570**	**13,995**
Sabah [P]	52,450	68,105	50,650	2,050	82,552	5,571,223	45,506	n.a	n.a	n.a
Sarawak [P]	7,122	15,860	14,980	2,258	336,180	40,613,010	347,333	n.a	155,630	2,652
JUMLAH BESAR *Grand Total*	**121,259**	**746,783**	**429,398**	**142,435**	**1,844,103**	**288,304,256**	**9,501,672**	**719**	**2,994,200**	**16,647**

P : Sementara (Provisional) n.a : Tiada maklumat *(Not available)*

Table 2.6: Livestock population in Malaysia in year 2015. (Adapted from DVS (2015)

MALAYSIA : BILANGAN TERNAKAN MENGIKUT NEGERI, 2015 [E]

Malaysia : Livestock Population By States, 2015 [E]

NEGERI *State*	Kerbau *Buffalo*	Lembu *Cattle*	Kambing *Goat*	Bebiri *Sheep*	Babi *Swine*	Ayam *Poultry*	Itik *Duck*	Burung Unta *Ostrich*	Puyuh *Quail*	Rusa *Deer*
Perlis	246	8,152	5,789	1,287	29	1,240,122	12,057	n.a	3,042	289
Kedah	7,637	66,251	50,095	10,027	613	53,862,440	761,654	49	514,107	977
Pulau Pinang	590	16,247	11,232	1,556	315,317	12,933,963	503,321	n.a	145,642	133
Perak	11,785	54,075	39,313	4,416	511,550	37,677,871	7,779,502	23	64,764	4,173
Selangor	1,465	23,352	24,044	3,343	265,888	21,552,281	25,312	225	197,228	559
N. Sembilan	3,170	45,241	42,300	19,604	557	21,361,940	24,154	117	282,535	1,533
Melaka	3,800	28,830	41,428	10,561	43,380	20,687,676	143,816	20	84,474	660
Johor	3,643	107,425	51,406	27,020	265,595	66,912,863	942,640	308	1,241,221	1,476
Pahang	14,068	129,775	37,181	17,704	3,027	15,721,309	18,247	n.a	454,026	3,151
Terengganu	9,235	89,749	27,174	5,860	n.a	6,324,664	24,242	n.a	86,979	369
Kelantan	5,982	95,576	41,852	34,417	470	1,811,088	56,670	n.a	27,527	215
W. Persekutuan	n.a	470	276		n.a			n.a	n.a	94
Jumlah S. M'sia *Total For P. M'sia*	**61,621**	**665,143**	**372,090**	**135,795**	**1,406,426**	**260,086,217**	**10,291,615**	**742**	**3,101,545**	**13,629**
Sabah	52,975	70,493	52,342	2,069	77,630	5,691,950	45,961	n.a	n.a	n.a
Sarawak	6,908	16,396	15,235	2,185	344,804	42,346,698	342,783	n.a	164,003	2,803
JUMLAH BESAR *Grand Total*	**121,504**	**752,032**	**439,667**	**140,049**	**1,828,860**	**308,124,865**	**10,680,359**	**742**	**3,265,548**	**16,432**

E : Anggaran (Estimate) n.a : Tiada maklumat *(Not available)*

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2.3 Food Pathogens Background

2.3.1 *Salmonella*

In 1879, Dr. Karl Joseph Eberth has discovered *bacillus* in abdominal lymph nodes and spleen. Sir Robert Koch confirmed the observation after a year later. The genus name "*Salmonella*" was given by Daniel Elmer Salmon in 1880. Then, Theobald Smith found *Salmonella* in hogs and named it as *Salmonella enterica* (var Choleraesuis). *Salmonella* genus belongs to *Enterobacteriacea* family has two types of species: *enterica* and *bongori* (Tindall et al., 2005). There are 2,600 types of serovar in *Salmonella* and classified into typhoid *Salmonella* includes *Salmonella enterica* Typhi usually associated with human Salmonellosis and non-typhoid *Salmonella* includes *Salmonella enterica* Enteritidis and Typhimurium associated with food from animal origin. *Salmonella* widely found in soil, water, vegetation and normal intestinal gut of animals (Agbaje et al., 2011).

2.3.1.1 Nomenclature and Taxonomy

The genus of *Salmonella* spp. is motile, aerobic, rod-shaped bacilli and Gram-negative bacteria belong to *Enterobacteriaceae* family. It is under the class of Gammaproteobacteria and enterobacteriales order in taxonomy classification (Table 2.7).

Table 2.7: Scientific classification of *Salmonella*. (Brenner et al., 2000)

Taxonomy	Classification
Domain	Bacteria
Kingdom	Eubacteria
Phylum	Proteobacteria
Class	Gammaproteobacteria
Order	Enterobacteriales
Family	Enterobacteriaceae
Genus	*Salmonella*
Species	*enterica* or *bongori*

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There are six subspecies are under *Salmonella enterica* with 2,600 serovars including *enterica* (I), *salamae* (II), *arizonae* (III), *diarizonae* (IV), *houtenae* (V), *indica* (VI) and subspecies V (Table 2.8). *Salmonella enterica* subspecies I is usually isolated from human and warm-blooded animals and most of the clinical strains are belonging to this group. *Salmonella* Typhi and Paratyphi A are common *Salmonella* strain isolated from human stool samples usually associated with human Salmonellosis. *Salmonella enterica* serovar Enteritidis and Typhimurium are under *Salmonella enterica* subspecies I can be found in poultry products and causes human illness. *Salmonella* Typhimurium is a causative agent for foodborne disease (Brenner et al., 2000).

Table 2.8: *Salmonella* subspecies classification. (Brenner et al., 2000)

Genus	Species	Subspecies
Salmonella	*enterica*	*enterica* (I)
		salamae (II)
		arizonae (IIIa)
		diarizonae (IIIb)
		houtenae (IV)
		indica (VI)
	bongori	subspecies V

2.3.1.2 Morphology and Characteristic

Salmonella is a Gram-negative, rod-shaped and facultative anaerobic bacterium. Most of *Salmonella* spp. are non-spore-forming and motile with flagella. The optimum growth temperature of *Salmonella* is 37 ℃ and suitable pH at 7. *Salmonella* spp. showed negative reaction in oxidase, urease, Voges-Proskauer and indole tests. Black color was observed on triple sugar iron agar indicate the production of hydrogen sulfide. *Salmonella* spp. showed positive reaction in citrate agar and methyl red test indicate *Salmonella* spp. able to utilize citrate as carbon source and ferment the glucose at acid condition (Ray & Bhunia, 2007).

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The cell membrane of Gram-negative bacteria contains lipopolysaccaride (LPS) in function of permeability barrier consists with outer layer of lipid A (hydrophobic region), core polysaccharide and O-specific polysaccharide (hydrophilic region). Serotyping was performed and analyzed according to Kauffman-White classification scheme used to classify the genus *Salmonella* into different serotypes based on surface antigen (Kauffmann, 1972). Three types of antigen can be expressed on *Salmonella* spp. include somatic (O) antigen, flagellar (H) antigen and capsular (Vi) antigen. O antigen is identified based on oligosaccharide attached with lipopolysaccharide of the membrane while H antigen is identified based on flagellar protein with two phase variation in motile and non-motile phenotypes present in *Salmonella*. Surface antigen (Vi) usually found in *Salmonella* Typhi, *S*. Paratyphi C and *S*. Dublin which poses capsular virulence antigen on surface membrane.

2.3.1.3 Pathogenesis

All serotypes of *Salmonella enterica* are pathogenic to human and mammals. *Salmonellae* cause a broad range of infections, including gastroenteritis, enteric fever, bacteremia, endovascular infections, and focal infections such as osteomyelitis and abscesses (Coburn et al., 2007). Young children, elderly and immunosuppressed individuals are high chance to get infected. The colonization of bacterium occur by human consume egg, poultry and meat products that contaminated with bacterium and leads to infection diseases (Mao et al., 2003). Several factors found in *Salmonella* such as plasmid virulence gene (*spv*), flagellin, surface cell structure and Pathogenicity Island (SPI) are linked to Salmonellosis (Guiney & Fierer, 2011). Some of the Pathogenicity Island includes type III secretion system (T3SS), SPI1 and SPI2 are determined to involve the invasion of bacteria into epithelial cell.

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SPI 1 is encoded for T3SS required for invasion of non-phagocytic cells in *Salmonella* (Marcus et al., 2000). The mechanism in the T3SS system started with bacterial protein that secreted from bacterial cytoplasm into host cytoplasm via needle. These needles give a highly selective and nearly impermeable membrane to let the invasion of bacteria. The base of T3SS consists of several circular rings to support the inner rod and connects needle to the base. For SPI2 is also encoded for invasion of phagocytic cell in T3SS system but important for intracellular survival and multiplying of *Salmonella* inside host cells (Hansen-Wester & Hensel, 2001). SPI2 occur in *Salmonella*-containing vacuole (SCV) for replication and survive after invasion of phagocytic cell (Coburn et al., 2007).

Table 2.9: Virulence gene in *Salmonella* T3SS system. (Coburn et al., 2007; Ohl & Miller, 2001)

Gene encoded	Name
sip	*Salmonella* invasion protein
sic	*Salmonella* invasion chaperone
spa	Surface presentation of antigen
inv	Invasion
ssp	*Salmonella*-secreted protein
prg	PhoP-repressed gene
org	Oxygen-regulated gene
iag	Invasion-associated gene

The *inv* gene is responsible for invasion of *Salmonella* spp. into intestinal epithelium cell and attributed to virulence gene in SPI. Most of the virulence gene of *Salmonella enterica* are chromosomal gene that located on SPI can acquired from other bacterial through horizontal gene transfer. *Salmonella* with fimbrial or flagella is involved in the production of endotoxins and exotoxins that associated with pathogenesis (Galán, 1996) (Table 2.9).

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2.3.1.4 *Salmonella* Serotypes in Poultry Samples

Poultry feeds are high chance to get infected with bacteria during mixing process and expose the material to microorganism. It causes high rate of poultry disease after consume poultry consume the contaminated feed. The survival time for *Salmonella* spp. in poultry feed is estimated about 98 days (Juven et al., 1984). *Salmonella* spp. is usually infected in human and animals such as poultry, livestock and birds. *S*. Enteritidis is most frequent found in poultry products associated with foodborne illness in globally (Murray et al., 2009; Rabsch et al., 2001). Inner content of eggs and birds are reported contaminate with *S*. Enteritidis (Campbell et al., 1982; Gast & Beard, 1992). Total of 133 *Salmonella* isolates include *S*. Infantis, *S*. Typhimurium and *S*. Enteritidis were recovered from retail meat samples purchased in Washington and Republic of China (Chen et al., 2004).

In the study of Van et al. (2007), it has been reported that 16 of *Salmonella* spp. (*S*. London, Havana, Hadar and Albany) were recovered from 30 poultry samples in Vietnam (Van et al., 2007). Only 7 *Salmonella* isolates were recovered in two thousand of poultry eggs and were identified as *Salmonella* Typhi, Typhimurium and Enteritidis (Begum et al., 2010). Of 24 *Salmonella* isolates were recovered and identified as *Salmonella* Enteritidis, Heidelberg and Amsterdam in Sudan (Fadlalla et al., 2012). Eight positive *Salmonella* isolates include *Salmonella* Typhimurium and Paratyphi B was recovered from 163 meat samples (Selvaraj et al., 2010). *Salmonella* Typhimurium, Newport, Montevideo and Heidelberg were most frequent identified in 57 *Salmonella* positive out of 576 meat samples in Meknes, Morocco (Abdellah et al., 2009).

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The 41 % of chicken carcasses were *Salmonella* positive and contaminated at retail level from different provinces of China (Zhu et al., 2014). *Salmonella* Enteritidis isolated from chicken eggs in Tennessee has been recorded (Louis et al., 1988; Mishu et al., 1991). A study in India showed prevalence of *Salmonella* Typhimurium isolated from chicken eggs (Singh et al., 2010; Williams et al., 1968).

2.3.1.5 Prevalence of *Salmonella* spp. in Chicken Meat and Eggs

The high level of prevalence of *Salmonella* spp. reported in different countries indicates the significance of chicken meat as source of food vehicle for *Salmonella* spp. (Table 2.10).

Table 2.10: Prevalence of *Samonella* spp. in chicken meat and eggs in other countries.

Countries	Prevalence (%)	References
Albania	6.5	Beli et al., 2001
Argentina	14.7	Favier et al., 2013
Iran	15.6	Salehi et al., 2005
	44.0	Fallah et al., 2013
India	4.8	Singh et al., 2010
	1.5	Saravanan et al., 2015
Malaysia	22.0	Thong & Modarressi, 2011
	51.7	Abbassi-Ghozzi et al., 2012
Morocco	44.0	Bouchrif et al., 2009
Saudi Arabia	5.9	Moussa et al., 2010
Spain	38.2	Valdezate et al., 2007
China	19.8	Lai et al., 2014
	13.0	Dahal et al., 2007
South Korea	19.8	Hyeon et al., 2011
Vietnam	34.0	Van et al., 2007
Thailand	5.3	Akbar & Anal, 2013

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2.3.1.6 Prevalence of *Salmonella* spp. in Other Food Samples

Variety of foods include raw meat, eggs, contaminated raw fruits and vegetables, unpasteurized milk and dairy products are associated with foodborne infection in different countries (Food & Drug Administration, 2011). There are 8 % of *Salmonella* spp. was isolated from 100 chicken eggs in different location of Dhaka city, Bangladesh (Ahmed et al., 2010). *S.* Typhimurium and Newport infection found in cantaloupe reported in multistate of United States in year 2012 (CDC, 2012b). Estimate 84 cases of cucumber contaminated with *S*. Saintpaul were reported in different state of United States (CDC, 2013a). Additionally, 907 people were infected by eating cucumber contaminated with *S*. Poona from 40 states (CDC, 2016b). Ground beef is contaminated with *S*. Typhimurium were reported on 22 individuals from six state of United States (CDC, 2013b). Fresh imported papaya contaminated with *S*. Agona become source of outbreak infection imported from Mexico. The outbreaks from 25 states were reported on 106 individuals infected with *S*. Agona (CDC, 2011a). Alfalfa sprouts contaminated with *S*. Enteritidis reported from five states in year 2011. Twenty-five of individuals were fall ill by consume contaminated sprouts (CDC, 2011b). There are 17 person were infected with *S*. Stanley by consume raw cashew cheese. This outbreak was reported from three states include California, Nevada and Wyoming in year 2014 (CDC, 2014). *S*. Enteritidis outbreak was reported in Canada on raw almond in year 2001 (Chan et al., 2002).

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2.3.1.7 Multidrug resistance (MDR) in *Salmonella* spp.

Antibiotic resistance is a topic that often captures headlines around the world. Antibiotic is mainly used to treat infection by inhibit the growth of microorganism. Different types of antibiotics were extensive used in animals for animal husbandry, growth promotion or prophylaxis purpose. The antibiotics will pass into intestinal tract of animals through feeds or drinking water or fecal environment. Feeds and water contaminated with *Salmonella* spp. will pass into animal via food chain and subsequently leads to zoonotic infection and causes foodborne disease after consume contaminate food animals (EFSA, 2012). Selective antibiotic stress is applied on bacteria will produce antibiotic resistance bacteria. The misuse of antibiotics in animal production will increase the emergence of MDR *Salmonella* strain and creates for public health concern and food safety issues.

Additionally, the spread of resistance *Salmonella* strain via food chain will causes the ineffective therapeutic treatment in veterinary and human medicines. It will pose high risk to human health and increased the severity of disease. The study suggests the spread of multidrug resistance *Salmonella* strain from chicken to human from Thailand to Denmark and United States. The *Salmonella* isolates showed highly resistant to nalidixic acid and decreased susceptibility to ciprofloxacin in antimicrobial susceptibility test (Aarestrup et al., 2007). Besides, 310 *Salmonella* isolates include 133 *S*. Indiana and 177 *S*. Enteritidis isolated from chicken were MDR and resistance to ampicillin, tetracycline, trimethoprim, doxycycline and sulfamethoxazole in Eastern China. Class 1 integron was used as antibiotic gene cassette detection in *Salmonella*. *S*. Indiana and Enteritidis are poses most prevalent resistance genes of *bla* $_{\text{TEM}}$, *floR*, *tetA*, *strA*, *catA1* and *aac*(*6'*)-*Ib-cr* (Lu et al., 2014).

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The increased spread of resistance *Salmonella* spp. in foods to other country indicate the appropriate usage of antimicrobial in production should be implement to reduce disseminate of the resistant strains. The 107 chicken meat were collected from different farms in Nigeria showed multiple resistance in *Salmonella* isolates were resistant to ampicillin, amoxicillin-clavulinate, tylosin, ciprofloxacin, ofloxacin, doxycycline, gentamicin, enrofloxacin, ceftazidime, nitrofurantoin, furasol and cefuroxime (Victor, 2016). The emergence of multidrug resistance *Salmonella* in chicken and importance to public health concern as the spread of resistance strain should be monitored in Nigeria.

Prevalence of multidrug resistance *S*. Enteritidis in poultry in India were highly resistant to ampicillin, penicillin-G, clindamycin, erythromycin, amikacin, rifampicin vancomycin, methicillin, nalidixic acid, and tetracycline (Bhuvaneswari et al., 2015). Of 15 *S*. Enteritidis were highly resistant to ampicillin, sulfonamides, streptomycin and tetracycline and 22 *S*. Typhimurium isolated from animals were resistant to ampicillin, chloramphenicol, streptomycin, sulfonamides, trimethoprim, nalidixic acid and tetracycline in Korea (Yang et al., 2001). In Malaysia reported *Salmonella enterica* isolated from poultry meat showed resistance to tetracycline, sulfonamides, streptomycin and nalidixic acid (Thong & Modarressi, 2011). Multidrug resistance *Salmonella* strain was reported in different countries and it is importance to monitor the spread resistance strain and usage of antibiotic in poultry production.

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2.3.2 *Listeria*

In 1924, the sudden deaths of young rabbits come out with the first report on *Listeria* spp. case was published by Murray and his researches (Murray et al., 1926). Then, Murray updated description of the bacteria and named as *Bacterium monocytogenes*. In the late 1920s, there are 2 researchers were classified *Listeria monocytogenes* from animal outbreaks. Harvey Pirie changed the genus name to *Listeria* in 1940 (Pirie, 1940). The genus of *Listerella* was proposed and named according to Joseph Lister. Initially, the genus *Listeria* was categorized under *Corynebacteriaceae* family in ninth edition of Bergey's Manual of Systematic Bacteriology (Tienungoon, 1998). However, the genus was placed under *Listeriacea*e family in 2001. All *Listeria* spp. are Gram-positive, catalase-positive and rod-shaped bacteria.

2.3.2.1 *Listeria* spp. in Nature

Listeria spp. are ubiquitous found in environment such as soil, stream water, sewage, manure, plants and foods (Ryser & Marth, 2007; Watkins & Sleath, 1981; Weis & Seeliger, 1975). It is frequently distributed in animal feces and intestinal tract of animals. The widespread of *Listeria* spp. can cause infection by ingestion of contaminated foods (Schlech & Acheson, 2000).

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2.3.2.2 Characteristic and Taxonomy

Listeria spp. are non-spore-forming, rod-shaped, Gram-positive and facultative anaerobic bacteria belongs to *Listeriaceae* family. *Listeria* spp. is catalase-positive and oxidase negative rod-shaped bacilli bacterium (Warriner & Namvar, 2009). *Listeria* spp. can grow in wide range temperature (4 ºC to 45 ºC) but the optimum growth temperature is 37 ºC. For *Listeria* spp. consists of six distinct species include *monocytogenes*, *innocua*, *ivanovii*, *seeligeri*, *welshimeri*, *grayii* and *murrayi* (Table 2.11). One of the biochemical tests, haemolysis test on blood agar can used to distinguish between the *Listeria* spp. In general, *L. monocytogenes* is pathogenic bacterium associated with human listeriolisis and animals.

Table 2.11: Scientific classification of *Listeria* spp. (McLauchlin, 1987)

Taxonomy	Classification
Domain	Bacteria
Kingdom	Bacteria
Phylum	Firmicutes
Class	Bacilli
Order	Bacillales
Family	Listeriaceae
Genus	*Listeria*
Species	*monocytogenes*, *innocua*, *ivanovii*, *seeligeri*, *welshimeri*, *grayii*, *murrayi*

Serotyping used to differentiate the subspecies of *Listeria* spp. based on flagella (H) and somatic (O) antigen. The expression of antigenic determinants on cell membrane surface O *Listeria* spp. will determine the types of subspecies. Commonly, it can be divided into two main lineage groups includes lineage I (1/2a, 1/2c, 3a and 3c), II (1/2b, 3b, 4b, 4d and 4e) and less common lineage III (4a and 4c). The serotypes of 4b are common associated with foodborne disease (Orsi et al., 2011).

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2.3.2.3 *Listeria* in Foods

The opportunistic *L. monocytogenes* strain has been reported found in wide range of raw and processed foods associated with human listeriosis. Various types of meat and meat products such as poultry, beef, pork and deli meat, dairy products such as soft cheese, raw and unpasteurized milk, fresh produces such as cabbage, salads, and fruits such as cantaloupes responsible for contamination with *L. monocytogenes* (Arslan & Özdemir, 2008; Büla et al., 1995; Fleming et al., 1985). Cooked crustaceans such as shrimp and crab could be one of the food sources for *Listeria* spp. Additionally, smoked seafood and fish products such as smoked salmon and mollusks are documented that isolate *Listeria* spp. on the food (Yücel et al., 2005). Raw seafood on sushi is one kind of ready to eat foods that increase the contamination of *Listeria* spp. in current trends (Schlech III et al., 1983). *Listeria* spp. are able to survive in frozen food products at refrigeration temperature. Foods are easy contaminated with *Listeria* spp. during processing, manufacturing and distribution process.

2.3.2.4 Foodborne Listeriosis

Listeriosis is getting high risk for elderly, pregnant women, newborn and immunocompromised individuals as compared with healthy person. Clinical manifestations in *Listeria* related to gastrointestinal tracts include nausea, vomiting, headache, high fever, diarrhea, septicaemia and meningitis. The immunocompromised individuals are getting high chance expose to meningitis and septicemia infection while pregnant women are faces spontaneous abortion or fetal death during pregnancy (Smith, 1999).

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Several foodborne listeriosis cases have been reported in United Kingdom, United States, Canada, Switzerland and France (Warriner & Namvar, 2009) (Table 2.12). These food pathogens are remaining causes problem in food industry because of the contamination occurs during food processing. The control of *L. monocytogenes* in food samples should be tighter in order to minimize hazardous contamination level expose to consumer. Listeriosis outbreaks are reported in multiple states of United States associated with frozen vegetables, raw milk, packaged salads produced in year 2016. Nine peoples were infected with *Listeria* spp. in four states by eating contaminated frozen vegetables; two illness associated with *Listeria* spp. in raw milk from organic farm and 19 persons were consumed contaminated packaged salads reported in nine states (CDC, 2016c).

In year 2015 reported thirty peoples include 28 peoples were hospitalized that consume soft cheese contaminated with *Listeria* spp. from 10 states (CDC, 2015c). Prepackaged caramel apples from Bidart were causes listeriosis outbreak in 12 states. A total of 35 peoples were infected with *L. monocytogenes* including 34 were sending for hospitalized that eating caramel apples (CDC, 2015d). *Listeria* spp. has been found in processed chicken in United States year 2002. The listeriosis outbreak causes 7 deaths, 46 fall ill and 3 miscarriages (Burros, 2002). Additionally, investigation *Listeria* spp. related to poultry was studied in Malaysia. The result shows that 42 out of 210 chicken samples (20 %) were detected with *L. monocytogenes*. Contamination of *L. monocytogenes* in chicken samples with different parts includes breast (42 %), drumstick (11 %) and thigh (7 %) (Goh et al., 2012).

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Table 2.12: Prevalence of *Listeria monocytogenes* in raw meat in different countries.

Countries	Prevalence (%)	References
China	3.3	Yan et al., 2010
	12.3	Wu et al., 2015
Thailand	15.4	Indrawattana et al., 2011
Turkey	86.4	Yücel et al., 2005
Malaysia	20 .0	Goh et al., 2012
Italy	68.9	Aureli et al., 2003
	21.8	Conter et al., 2009
	11.4	Pesavento et al., 2010
Serbia	80.0	Dimić et al., 2010
Ethiopia	15.4	Molla et al., 2004
Korea	30.2	Baek et al., 2000
Poland	61.4	Kosek-Paszkowska et al., 2005
United Kingdom	60.0	Pini & Gilbert, 1988
United States	23.0	Bailey et al., 1989
Egypt	4.0	Mohamed et al., 2016
India	8.5	Kalorey et al., 2005
Croatia	3.0	Kozačinski et al., 2006
Portugal	41.0	Antunes et al., 2002

2.3.2.5 Pathogenesis in *Listeria* spp.

Listeria spp. involved of several steps for infection: *Listeria* spp. enter the cellular bacteria cell via zipper mechanism mediated by two surface protein internalin A and B. The adhesions interact with E-cadherin and hepatocytes growth factors to initiate signaling process and leads to actin polymerization, membrane invasion and bacterial internalization. The internalization process involved of formation intracellular vacuoles with bacteria. Listeriolysin-O encoded by *hlyA* will lyse the vacuoles and release *Listeria* spp. into cytosol (Table 2.13). Actin polymerizing protein implicates actin polymerization process in bacterial cell. Most of the virulence factors contributed to pathogenicity of *Listeria* spp. is regulated by transcriptional regulator (Liu, 2006).

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Table 2.13: Virulence genes in *Listeria* spp.

Gene	Name	Functions	References
prfA	Positive regulatory factor A	Transcription function	Ramaswamy et al., 2007
hlyA	Listeriolysin-O	Virulence factor	Ramaswamy et al., 2007
plcA	Phosphatidylinositol-specific phospholipase C	Signal transduction process	Mengaud et al., 1991
plcB	Phosphatidycholine-specific phospholipase C	Promotes cell-to-cell of *Listeria*	Schlüter et al., 1998
actA	Actin polymerizing protein	Cell motility	Low & Donachie, 1997
inlA	Internalin A	Surface protein for entry human epithelial cell	Ramaswamy et al., 2007
inlB	Internalin B	Surface protein for cell internalization	Ramaswamy et al., 2007

2.3.2.6 Antibiotic Resistance in *Listeria* spp.

The first *L. monocytogenes* strain was isolated from France in 1988 (Poyart-Salmeron et al., 1990). Emergence of multidrug resistant in *Listeria* isolates have been reported in food and environment associated with listeriosis (Hadorn et al., 1993; Charpentier et al., 1995). *Listeria* spp. are commonly resistant to ampicillin, tetracycline, penicillin, gentamicin, erythromycin, sulfonamide and streptomycin (Charpentier & Courvalin, 1999). First-line antibiotic, tetracycline is extensively used in animal husbandry and consequently emergence of tetracycline resistance in *Listeria* spp. isolated in poultry (Chopra & Roberts, 2001). Therefore cause the ineffective use and effect of antibiotics in clinical treatment. *L. monocytogenes* are resistant to nalidixic acid (100 %), cephalothin (100 %), ampicillin (66 %), trimethoprim-sulfamethoxazole (66 %) and kanamycin (11 %) isolated from raw meat samples in Turkey (Yücel et al., 2005). Besides, *Listeria monocytogenes* isolates are resistant to oxacillin (100 %), clindamycin (35 %) and tetracycline (0.5 %) in meat products in Spain (Gómez et al., 2014).

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2.3.3 *Escherichia coli*

Escherichia coli (*E. coli*) is discovered by Theodor Escherich from feces of healthy person in 1885 (Friedmann, 2006). Initially, this organism named as *Bacterium coli commune* based on the shape and motility. Then, it was reclassified by Migula in 1895 as *Bacillus coli* before categorized under new genus of *Escherichia* (Alm et al., 2011). *E. coli* are a rod-shaped, non-sporulating, motile Gram-negative and facultative anaerobic bacterium under *Enterobacteriaceae* family (Labbé & García, 2001) (Table 2.14). It is commonly found in lower intestine and colon of warm-blooded organisms. *E. coli* is stained with pink color in Gram staining due to Gram-negative bacteria posses' thin layer of peptidoglycan of outer membrane.

Table 2.14: Scientific classification of *E. coli*. (Labbé & García, 2001)

Taxonomy	Classification
Domain	Bacteria
Kingdom	Eubacteria
Phylum	Proteobacteria
Class	Gammaproteobacteria
Order	Enterobacteriales
Family	Enterobacteriaceae
Genus	*Escherichia*
species	*coli*

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2.3.3.1 Virulence Factors in *E. coli*

Most of *E. coli* are not because any harm to human intestinal tract but some of pathogenic *E. coli* can causes illness and transmitted through contaminated food or water or contact with animals or person. Commonly, Gram-negative *E. coli* can cause urinary tract infections, gastroenteritis and neonatal meningitis (Besser et al., 1999; Stamm & Hooton, 1993). There are six types of pathogenic *E. coli* strains associated with diarrhea (Table 2.15) (Nataro & Kaper, 1998). Pathogenic *E. coli* are divided based on O antigen on lipopolysaccaharide (LPS), K antigen found in acidic capsular polysaccharide (CPS) and H antigen in flagellin involved in movement of *E. coli* (Nataro & Kaper, 1998).

Table 2.15: Types of pathogenic *E. coli*. (Griffin & Tauxe, 1991)

Types of pathogenic *E. coli*	Complication of infections
STEC/VTEC/EHEC	• Hemolytic uremic syndrome (HUS) decreased urination, sudden kidney failure, tiredness, losing pinking cheek and inside lower eyelids. • Common virotypes is O157: H7. • Categorized according to shiga-toxins gene (*stx*).
ETEC	• Traveler's diarrhea and major causes of diarrheal disease in undeveloped countries. • ETEC uses fimbrial adhesins to bind enterocyte cells. • Produce two protein includes heat labile enterotoxin (LT enterotoxin) and heat stable toxin (ST enterotoxin).
EPEC	• Infantile diarrhea, watery or bloody diarrhea. • Lack of LT and ST toxins but use non fimbriae adhesin named intimin to bind intestinal cell.
EAEC	• Acute diarrhea. • Poses fimbriae can aggregate tissue cells that bind to intestinal mucosa cause diarrhea by producing ST-like toxin and hemolysin.
EIEC	• Non fimbriae adhesins. • Dysentery-like diarrhea with high fever.
DAEC/AIEC	• Able to invade intestinal epithelial cells and replicate.

Shiga toxin-producing *E. coli* (STEC) or Enterohemorrhagic *E. coli* (EHEC), Enterotoxigenic *E. coli* (ETEC), Enteropathogenic *E. coli* (EPEC), Enteroaggresive *E. coli* (EAEC), Enteroinvasive *E. coli* (EIEC) and Diffusely adherent *E. coli* (DAEC)

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Other types of *E. coli* such as Uropathogenic *E. coli* (UOEC) can cause urinary tract infection. This caused by fecal contamination of urogenital by wiping the direction from back to front after defecation in female individuals or anal intercourse in male urethra introduce from anal to vaginal intercourse in female urogenital system (Manges et al., 2001).

2.3.3.2 *E. coli* in Foods

Undercooked or raw meat could be the possible contamination vehicle for *E. coli* (Zhao et al., 2001). Raw milk or unpasteurized milk and raw fruits and vegetables (lettuce or alfalfa sprouts) are easy come contact with infected animal feces and contaminated with *E. coli* (Berger et al., 2010; Soomro et al., 2002). Lakes, swimming pool or water supplies not under proper chlorination process are source contaminated with *E. coli* (Ackman et al., 1997). Improper food handling such as sharing same utensils, personal hygiene of food handlers, consume raw or undercooked foods and fresh produces, poor sanitation in food processing high risk for *E. coli* infection (Beuchat, 1996; Cohen, 2000; Redmond & Griffith, 2003).

2.3.3.3 Antibiotic Resistance in *E. coli*

Antibiotics include aminoglycoside, amoxicillin, cephalosporins, carbapenems, aztreonam, trimethoprim-sulfamethoxazole, ciprofloxacin and nitrofuratoin are used to treat *E. coli* infections (Mader et al., 1999). Antibiotic resistance in *E. coli* is become public health concern due to overuse of the antibiotics in human and growth promotion in animal feeds. Antibiotic resistance genes in *E. coli* may possible pass to other bacteria through horizontal gene transfer (Andersson & Hughes, 2010).

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2.3.3.4 *E. coli* in Foodborne Disease

E. coli is important cause of human illness in different countries. Various types of food products include chicken, salad, beef and sprouts cause *E. coli* infection in between year of 2010 to 2016. Alfalfa sprouts infected with *E. coli* O157 has investigated in Minnesota and Wisconsin in year 2016. A total of 11 peoples were infected with the outbreak strain and 2 peoples were hospitalized (CDC, 2016d). Outbreak infection related to *E. coli* O157: H7 in costco rotisserie chicken salad have been reported in year 2015. These outbreak was investigated involved 19 people were infected in 7 states include California, Colorado, Virginia, Utah, Montana, Washington and Missouri (CDC, 2015e).

Infection cases related with *E. coli* O157 was reported from 4 states of United States in year 2013. There are 33 individuals were infected with the outbreak strains by consume ready-to-eat salads (CDC, 2013c). In year 2010 reported *E. coli* O157: H7 outbreak related to beef in 16 states. Estimated 21 persons were infected with bacteria in the outbreak (CDC, 2010a). At the same year 38 persons were consume cheese that contaminated with *E. coli* O157 has been reported in 5 states (CDC, 2010b). In year 2013, a total of 35 individuals were infected with Shiga toxin-producing *E. coli* O121 reported in 19 states. They are consumed frozen food products that contaminated with *E. coli* (CDC, 2013d).

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2.3.4 *Staphylococcus aureus*

Staphylococcus was discovered by Alexander Ogston from surgical abscess in knee joint in 1880 (Licitra, 2013). It is observed as grape-like cluster under microscope and viewed as yellow halo colonies on plate. Additionally, *S. aureus* is catalase-positive using enzyme catalase to converts hydrogen peroxide (H_2O_2) into water and oxygen. Taxonomy of *S. aureus* is showed in Table 2.16. *S. aureus* is a coccal, Gram-positive facultative anaerobic bacterium commonly found in nose, respiratory tract and skin (Bennett, 2001). It is associated with skin infections, respiratory infection and food poisoning. Pathogenic strain such as methicillin-resistant *S. aureus* is a worldwide clinical problem.

Table 2.16: Scientific classification in *S. aureus*. (Bennett, 2001; Bannerman et al., 2006)

Taxonomy	Classification
Kingdom	Bacteria
Phylum	Firmicutes
Class	Bacilli
Order	Bacillales
Family	Staphylococcaceae
Genus	*Staphylococcus*
Species	*aurues*

2.3.4.1 Staphylococcal Food Poisoning

Staphylococcal food poisoning (SFP) is an intoxication caused by consumes foods contaminated with enterotoxin produced by *S. aureus* and resulting diarrhea and vomit (Gasman, 1967). Unpasteurized milk and cheese products, ham, egg, poultry, puddings and sandwiches are high risks to causes SFP. Improper cooking conditions and improper storage of raw and processed foods causes the production of enterotoxins of *S. aureus*.

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2.3.4.2 Antibiotic Resistance in *S. aureus*

Penicillin is antibiotics to inhibit the formation of peptidoglycan of cell wall in the *S. aureus* infections. Beta-lactam ring of penicillin bind to enzyme DD-transpeptidase to inhibit the formation of transpeptidase and leads to cell death (Walsh, 2000). *S. aureus* strains are isolated from clinical was mainly resistant to aminoglycosides. Methicillin-resistant *S. aureus* (MRSA) that resistant to most of the beta-lactam antibiotics are become big issue in clinical field. In fact, vancomycin is used to replace the methicillin in the treatment for MRSA. Vancomycin is a glycopeptide antibiotic able to inhibits peptidoglycan formation in the cell wall (Hiramatsu, 2001; Walsh, 2000).

Staphylococcus resistant to certain of antibiotics includes penicillin, gentamicin and streptomycin, methicillin. Aminoglycosides antibiotics were effective against staphylococcal infection by binds to 30S ribosomal subunit and inhibit protein synthesis. Staphylococcal resistant to methicillin conferred by *mecA* gene coded for altered penicillin-binding protein (PBP2a) in *mec* operon. Methicillin involved of bactericidal activity by binding PBP and inhibits bacterial cell wall synthesis (Hiramatsu et al., 2001). Vancomycin is glycopeptide antibiotic mediated by *vanA* gene to applied in the first line treatment of MRSA infections.

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2.4 Control and Prevention on Foodborne Illness

Foodborne illness can be prevented through several steps during food handling and preparation including by separate raw food from cooked or ready-to-eat food in order to avoid cross contaminate between raw and cooked foods, use separate of utensil between raw food and ready-to-eat food and wash the kitchen utensils include cutting board and knife before and after use. Proper personal hygiene and sanitation practice should be implemented during food handling. Cook the meat at the right temperature by checking the texture and color to ensure safe to consume (Table 2.17) and store the food in proper refrigerate temperature to prevent food spoilage and microbes growth.

Table 2.17: Food variety in different cooking temperature.

Cooking temperature (degree)	Types of foods
145	Roast, steak, chop of beef, pork and lamb
160	Beef, pork , lamb
165	Poultry

2.4.1 The Impact Use of Antibiotics in Poultry

Poultry is one of the reservoirs of food pathogens that cause human infection. Poultry probably contaminated during animal production, food processing and food distribution (Table 2.18). Antibiotics applied in poultry industry for treatment, prevent and control disease spread as well as growth promotion purpose (Singer & Hofacre, 2006).

Table 2.18: Antibiotic applications.

Application purpose	Descriptions
Treatment	Antibiotics are prescribed by veterinarian use to treat disease in poultry
Control and Prevention	Control the spread of the disease through water or feed sharing in the farm
Growth promotion	To enhance growth and muscle

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According to National Pharmaceutical Control Bureau (NPCB) of Ministry of Health Malaysia suggest that most of the registered antibiotics include beta-lactam, cephalosporins, tetracycline, sulphonamide, macrolides, aminoglycoside and fluoroquinolone are use in livestock in Malaysia (Table 2.19). DVS is taking responsible to monitor the antimicrobial use in poultry and feed with the Animal feed Act 2009.

Table 2.19: Registered antibiotics used in animals farming in Malaysia. (HAIAP, 2013)

Antibiotic group	Examples of antibiotics
Beta-lactam	Ampicillin, amoxycillin
Cephalosporins	Ceftiofur, cefadroxil
Tetracycline	Chlortetracycline, doxycycline
Sulphonamide	Sulfamethazine, sulfadimethoxine
Macrolide	Erythromycin, spiramycin
Aminoglycoside	Gentamicin
Fluoroquinolone	Enrofloxacin

Impact of inappropriate use of antimicrobials in poultry will develops multidrug resistance pathogenic bacteria strain and transmission of antibiotic resistance gene material in bacterial pathogens via food chain, subsequently could be potentially associated with foodborne disease (Witte, 1998). The extensive use of antibiotics will contribute the emergence of antibiotic resistance in animal and zoonotic bacterial pathogens. Bacteria that able resist to antibiotics will grow more rapidly than susceptible one, and resistance genes will passed from resistant bacteria to sensitive, consequences increase the arise of multiple antibiotic resistance bacteria (Khachatourians, 1998). In Canada, United States and New Zealand applied several ways suggests to control on the usage of antimicrobial in food animal production such as conduct food safety program, monitor antimicrobial used in poultry and surveillance of antimicrobial resistance on poultry (McEwan, 2002; USFDA, n.d.; NZFSA, 2005). The amounts of antimicrobial used should be monitor in order to reduce emerge and transmission of antibiotic resistance bacteria strain.

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2.5 Isolation, Identification and Characterization of Bacteria

2.5.1 Phenotypic Methods

2.5.1.1 Culture Media

Bacteria such as *Salmonella* spp., *Listeria* spp., *E. coli* and *S. aureus* were isolated through pre-enrichment, enrichment step and selective agar according ISO standard methods (Rose, 1998) (Table 2.20).

Table 2.20: Culture media used for bacteria isolation.

Bacteria	Pre-enrichment	Enrichment	Selective agar	Colonies color	References
Salmonella spp.	Buffered peptone water	Rappaport Vassiliadis soy peptone broth, Muller-Kauffmann Tetrathionate broth and Selenite cystine broth	Hektoen enteric agar	Blue green colonies with black center	(Rose, 1998)
			Bismuth sulphite agar	Black colonies	
			Xylose lysine deoxycocolate agar	Red colonies with black center	
			Salmonella-shigella agar	Colorless colonies with black center	
			MacConkey agar	Colorless colonies	
			Brilliance *Salmonella* agar	Purple colonies	
Listeria spp.	half Fraser broth	Fraser broth	PALCAM *Listeria* selective agar	Grey green colonies with black center	(Osaili et al., 2011)
			Brilliance *Listeria* agar	Blue- green colonies	
E. coli	Not required	MacConkey broth	MacConkey agar	Red or pink colonies	(Johnson et al., 2003)
			Eosin methylene agar	Greenish metallic sheen	
S. aureus	Not required	Tryptic soy broth	Mannitol salt agar	Yellow halo colonies	(Kitai et al., 2005)

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2.5.1.2 Gram Staining

Gram staining used to differentiate Gram-negative (GN) and Gram-positive (GP) bacteria by pink or violet stain (Table 2.21). Gram-negative has thin layer of peptidoglycan wall that does not retain crystal violet purple stain and stained with safranin pink color after decolorized. Gram-positive appeared as purple color because of the thicker layer of peptidoglycan wall that retain crystal violet-iodine complex during staining process.

Table 2.21: Gram staining in Gram-negative and positive bacteria.

Staining process	Crystal violet	Iodine	Ethyl alcohol	Safranin	References
Gram-negative bacteria	Purple	Purple	Colorless	Pink color	(Claus, 1992)
Gram-positive bacteria	Purple	Purple	Colorless	Purple color	
Functions	Primary stain	Act as mordant to fix crystal violet to cell wall	Act as decolorizer to dehydrate peptidoglycan layer	Secondary stain	

2.5.1.3 Biochemical Tests

Numerous of biochemical tests were performed to confirm the bacteria identification include IMViC test, lysine decarboxylase, nitrate reduction test, carbohydrates fermentation, triple sugar iron, urease, oxidase, ONPG, esculin hydrolysis, catalase, motility, gelatin hydrolysis and starch hydrolysis test as mentioned below.

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IMViC (Indole, Methyl Red, Voges-Proskauer, Simmon Citrate) Tests

Indole test

Indole test used to differentiate *Enterobacteriaceae* bacteria that able to break down tryptophan is hydrolyzed by tryptophanase enzyme to produce indole, pyruvate and ammonium under deamination and hydrolysis process. Indole is combined with Kovac's reagent (contains p-dimethylaminobenzaldehyde in amyl alcohol) to produce cherry red colored on the top of the broth (Yousef & Carlstrom, 2003).

Methyl Red test

Methyl red test used to determine bacteria utilize glucose in broth medium to pyruvic acid and produce stable acid in 'mixed acid pathway'. The production of stable acid will decreases the pH to 4.5 or below and changes methyl red from yellow color into red color after added into broth. This test observed bacteria performed mixed acid fermentation in glucose broth by color changes (Yousef & Carlstrom, 2003).

Voges-Proskauer test

Voges-Proskauer (VP) test for detect acetoin by adding Barritt's reagent (alpha-naphthol and 40 % potassium hydroxide) in VP broth medium containing glucose. The glucose will break down when reacted with alpha-naphthol and potassium hydroxide to form red color. The rose pink color develops indicated the presence of acetoin (Yousef & Carlstrom, 2003).

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Simmon Citrate test

Simmon citrate test used to determine bacteria able to utilize sodium citrate as carbon and energy source while inorganic ammonium salts are used as nitrogen source and produce ammonium hydroxide. The ammonium salts is break into ammonia and increase alkaline (above pH7.6) to change bromothymol blue indicator from green color to blue color. The ability of bacteria utilizes citrate into pyruvate in the presence of citrate permease enzyme. Citrate is cleaved into oxaloacetate and acetate by citrate lysate. Then oxaloacetate metabolized to pyruvate and carbon dioxide (Fairbrother & Taylor, 2014; Yousef & Carlstrom, 2003).

Lysine Decarboxylase test

Lysine decarboxylase test used to determine the bacteria used lysine as carbon source for growth. Lysine is an essential base amino acid with side chain of lysyl ((CH_2) $4NH_2$). The decarboxylases process involved of decarboxylase enzyme reacted with carboxyl (COOH) group of lysine to form cadaverine and carbon dioxide. The reaction will increase pH of the lysine decarboxylase medium and changed bromcresol purple from yellow to purple color indicated lysine decarboxylase positive reaction. The medium in yellow color after adding the reagent indicated lysine dearboxylase negative reaction (Yousef & Carlstrom, 2003).

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Nitrate Reduction test

Nitrate reduction test used to determine the ability of *Enterobacteriaceae* bacteria with nitrate reductase enzyme to hydrolyze nitrate (NO^{3-}) to nitrite (NO^{2-}) and subsequently degraded to other types of nitrogen products involved in nitrogen cycle. The nitrate broth with bacteria suspension will test for reduction of nitrate to nitrite by adding sulfanilic acid and alpha-naphthylamine. Positive reaction in reduction process when nitrate reduced to nitrite and nitrite will form nitrous acid, this nitrous acid will react with sulfanilic acid to produce diazotized sulfanilic acid and then reacted with alpha-naphthylamine to form red color compound. The medium does not turn red after addition nitrate reduction reagent will proceed to adding small amount of zinc powder. The red color develops after addition of zinc powder indicate the zinc reduced nitrate to nitrite and form nitrous acid which reacted with sulfanilic acid and alpha-naphthylamine in medium. Positive complete is defined when the medium does not turn red after zinc powder is added (Yousef & Carlstrom, 2003).

Carbohydrate Fermentation test

Carbohydrate fermentation test is used to test organism able to ferment in the given carbohydrate in purple broth medium. Some of the sugars and alcoholic derivatives that bacteria ferment includes glucose, mannitol, sorbitol, arabinose, lactose, dextrose, maltose, rhamnose, xylose and sucrose are prepared in required concentration. Bacteria utilize sugar to produce energy and growth. When the bacteria able to ferment the sugar and produces acid to reduce the purple color of bromocresol purple into yellow color (Yousef & Carlstrom, 2003).

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Triple Sugar Iron (TSI) test

TSI test used to determine Gram-negative bacilli utilize glucose, lactose or sucrose to produce hydrogen sulphite (H_2S). TSI agar contains three sugar, ferrous sulfate, peptone and pH indicator phenol red. The pH indicator phenol red is indicator the changes of acidification. The yellow color is detected under acidic condition whereas red color under alkaline condition and oxidized to carbon dioxide and water. Ferrous sulfate act as indicator for H_2S formation and will turn the slant in black color. Peptone used as nitrogen source and produced ammonia after utilized. The slant will remain red color if glucose is fermented but not ferment lactose and sucrose however the production of acid will remain butt in yellow within 18 to 24 hours. The slant and butt will turn yellow if the organism ferment lactose or sucrose in the presence of large amount of acid production. When peptone is utilized as source instead of sugar, the slant and butt is in red color under alkaline condition (Rose, 1998).

Urease test

Urease test used to determine the ability of bacteria splited urea to produce ammonia and carbon dioxide (CO_2) gas with urease enzyme in hydrolysis process. The ammonia combined with CO_2 to form ammonium carbonate and increased the alkalinity of medium containing urea. The color change of medium is observed from light orange into bright pink color indicated urease positive bacteria (Yousef & Carlstrom, 2003).

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Oxidase test

Oxidase test used to identify bacteria able to produce cytochrome c oxidase enzyme in electron transport chain. Cytochrome c oxidase oxidizes tetramethyl-p-phenylenediamine into indophenols blue. Oxidase positive bacteria will develops deep purple color as well as aerobic bacteria. Oxidase negative bacteria are lacking cytochrome c that does not able to oxidize he reagent and remain colorless (Yousef & Carlstrom, 2003).

O-nitrophenyl-beta-D-galactopyranoside (ONPG) test

ONPG test used to differentiate *Enterobacteriaceae* bacteria in beta-D-galactosidase activity. ONPG is colorless compound and an analog of lactose. It will cleave into galactose and O-nitrophenol with enzyme beta-galactosidase in hydrolysis process. O-nitrophenol will produce yellow color to show that the hydrolysis occurs. Lactose-fermenting bacteria have two enzymes (lactose permease and beta-galactosidase) involved in lactose fermentation process. Permease enzyme is responsible for penetrate bacteria by uses lactose while beta-galactosidase enzyme will break into glucose and galactose by breaking galactoside bond. Non-fermenter bacteria do not posses any permease and beta-galactosidase enzyme will show ONPG negative reaction (Yousef & Carlstrom, 2003).

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Esculin Hydrolysis test

Bile esculin hydrolysis test used to determine the ability of bacteria utilize esculin as carbon source. Esculin is a glycosidic coumarin derivative known as 6-beta-glucoside-7-hydroxy-coumarin.Bile esculin agar is a differential selective medium for bacteria able to hydrolyzes esculin into glucose and esculetin (6,7-dihydroxycoumarin) by enzyme esculinase. This agar contains bile salt and sodium azide to inhibit growth of Gram-negative bacteria. Esculetin reacts with ferric citrate to form complex and produce dark brown color on agar. Esculin positive bacteria are able to grow in the presence of bile salt (Ryser & Marth, 2007).

Catalase test

Catalase test used to differentiate *Staphylococcus* able to produce catalase enzyme from non catalase enzyme bacteria (*streptococci*). The catalase enzyme is able to catalyses toxic oxygen metabolites hydrogen peroxide (H_2O_2) into oxygen and water. In general 3 % of catalase reagent will be used for aerobic bacteria to observe oxygen bubble on glass slide. Catalase positive bacteria mostly are strict aerobes or facultative anaerobes, able to respire using oxygen as terminal electron acceptor (Bennett et al., 2014).

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Motility test

Motility test used to identify the motile bacteria from non-motile bacteria using flagella on motility agar. Motile bacteria use flagella to diffuse spreading cloudy growth throughout the agar. Non-motile bacteria will sharply well defined growth along the stab and leave clear surrounding medium. The function of flagella in bacteria for movement enables to keep away from danger from less favorable to more favorable environment (Ryser & Marth, 2007).

Gelatin Hydrolysis test

Gelatin hydrolysis test used to determine ability of bacteria use protein gelatin as carbon source for growth using gelatinase enzyme and differentiate among *Enterobacteriaceae* bacteria. Gelatin is types of protein derived from animal and used as solidifying agent in food industry. The nature liquefy of gelatin is changes depend on the temperature; it can dissolves in water at 50 ºC, in liquid form when above 25 ºC and solidified when cooled below 25 ºC. The bacteria produce extracellular proteolytic enzyme, gelatinase to hydrolyze gelatin into polypeptide and subsequently hydrolyze into amino acid for metabolic purpose. Gelatin liquefy is observed indicate the bacteria has gelatinase to hydrolyze gelatin (Harley, 2004).

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Starch Hydrolysis test

Starch hydrolysis test used starch agar to test the ability of bacteria to hydrolyze starch with exoenzyme alpha-amylase and used as carbon source. Starch is a polysaccharide joined by glycosidic bond that produced from plants as energy storage. Iodine reagent is added to starch agar, the iodine will reacts with starch to form blue-black color on the medium. Clear halo surrounding colonies is indicating the bacteria have hydrolyzed starch in the presence of alpha- amylase on starch agar (Harley, 2004).

2.5.1.4 Serological Test

Serotyping is typing method important to characterize bacteria based on antigenic structure of lipopolysaccharide (LPS). *Salmonella* strains identified based on surface or somatic antigen (O-antigen), flagella antigen (H antigen) and virulence (Vi antigen) on LPS layer. In general, *Salmonella* strain expresses two phases of H-antigens and classified according to Kauffmann-White classification scheme developed by Philip Bruce White and Fritz Kauffmann. Slide agglutination was performed for bacteria carry antigen that can agglutinate to antibody of *Salmonella* spp. in agglutination test.

Latex agglutination or clumping of latex milky particle is observed when the specific antigen on bacteria reacted with antibody designed by manufacturer. Initially, this test is applied in clinical used for detection of infectious disease such as rheumatoid factor test (Swaminathan & Feng, 1994). Latex agglutination presumptive test was used to detect the presence of *Salmonella* spp., *Listeria* spp., *E. coli* and *S. aureus* colonies on culture media. Agglutination reaction is observed indicate positive reaction in the sample while absence of agglutination indicates the negative reaction in sample.

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2.5.1.5 Matrix-Assisted Laser Desorption/ Ionization- Time of Flight (MALDI-TOF)

Analytical microbial identification using MALDI-TOF based on protein mass molecules between 100 Da to 100 KDa. It is a high throughput technology applied on microbial identification based on protein fingerprint with reference database in system (Welker & Moore, 2011). Basically, MALDI is mixed with matrix and the matrix absorbs ultraviolet light with nitrogen laser light wavelength at 337 nm. Samples with matrix heat rapidly and vaporized to produce different charged ions. Lighter ions will have smaller mass-to-charge ratio (m/z) value and highly charged ion will move faster to reach detector. As a result, the time of ion flight is differs based on m/z value of the ion present using mass spectrometry method. TOF (time-of-flight) analyzer is measure the time taken by molecule to travel in a fixed distance.

Matrix composed of three common molecules applied on MALDI- TOF includes 2,5-dihydroxybenzoic acid (DHB), 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid) and alpha-cyano-4-hydroxycinnamic acid (CHCA) (Korfmacher, 2009). Standard solvent for matrix is prepared according to 20 mg/ml sinapinic acid in acetonitrile (ACN): water: trifluoroacetic acid (TFA) ratio (50: 50: 0.1). TFA used to generate [M+ H] ions. MALDI-TOF has three basic steps. Firstly, sample is mixed with matrix and applied on metal target plate. Then, the plate move into MALDI, matrix absorbs pulse laser irradiate sample and stimulate absorption and desorption process between sample and matrix to generate charged ion. Lastly, the molecules are ionized and move into TOF mass analyzer for analysis (Zenobi & Knochenmuss, 1998).

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2.5.2 Antibiotic Susceptibility Test

Antimicrobial is a substance in the form of natural, semi-synthetic and synthetic aims to inhibit or to kill the growth of microbial. Antibiotics are divided into bactericidal or bacteriostatic effect on the growth of bacteria. Mostly antibiotic are used for treatment in clinical, but some of antibiotic for growth promotion in livestock. The animals will given antibiotic for treatment (prophylaxis) purpose after undergone surgery and antibiotic given when expose to infectious disease or environmental conditions (metaphylaxis purpose). Several types of antibiotic action modes include inhibitors of cell wall synthesis, cell membrane function, protein synthesis, nucleic acid synthesis and metabolic process (Table 2.22) (Coyle, 2005).

Table 2.22: Antibiotic classifications and mechanisms. (Coyle, 2005)

Classifications	Antibiotics
Cell wall synthesis	Penicilin, vancomycin, beta-lactamase inhibitor, carbapenems, aztreonam, polymycin, bacitracin
Protein synthesis inhibitor (30S subunit)	Aminoglycosides, tetracycline
Protein synthesis inhibitor (50S subunit)	Macrolides, chloramphenicol, clindamycin, linezolid, streptogramins
Nucleic acid synthesis inhibitor	Fluoroquinolones, metronidazole
RNA synthesis inhibitor	Rifampin
Folic acid synthesis inhibitor	Sulphonamides, trimethoprim

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2.5.2.1 Inhibition of Cell Wall Synthesis

Beta-lactam groups

Beta-lactam antibiotic class is broad class of antibiotics that consists of beta-lactam ring in structure. Two types of spectrum activity in beta-lactam: broad-spectrum antibiotics (able to act against wide range of disease-causing bacteria in Gram-positive and Gram-negative bacteria) such as carbapenems, 2^{nd} , 3^{rd} and 4^{th} generation of cephalosporins and narrow-spectrum antibiotics (effective against specific bacteria) such as penicillin, 1^{st} generation of cephalosporins and monobactam (Table 2.23). Penicillin derived from *Penicillum chrysogenum* and *Aspergillus nidulans* while cephalosporins derived from *Acremonium chrysogenum*, *Paecilomyces persinicus*, *Streptomyces clavuligerus*, *Nocardia lactamdurans*, *Flavobacterium* sp. *Lysobacter lactamgenus* (Holten, 2000).

Table 2.23: Examples of beta-lactam groups. (Coyle, 2005)

Beta-lactam group	Examples
Penicillin	Penicillin G, penicillin V
Penicillinase-resistant penicillin	Methicillin, oxacillin, nafcillin
Extended spectrum penicillin	Ampicillin, amoxicillin, carbenicillin
Cephalosporins (first generation)	Cefalothin, cefazolin, cefalexin, cefadroxil
Second generation	Cefaclor,cefamandole, cefoxitin, cefprozil, cefuroxime
Third generation	Cefixime, cefoperazone, cefotaxime, ceftazidime, ceftriaxone, cefpodoxime, ceftibuten, ceftizoxime
Fourth generation	Cefepime
Fifth generation	Ceftaroline fosamil, ceftobiprole
Carbapenems	Imipenem
Monobactams	Aztreonam
Penicillin combination	Amoxicillin/clavulanate (Augmentin)

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Beta-lactam antibiotics are categorized based on the core ring structure (Dalhoff et al., 2006). Most of the beta-lactam act as bactericidal effect as mechanism of action by inhibition peptidoglycan bacterial cell wall synthesis. Beta-lactam antibiotic target penicillin-binding protein (PBP) enzyme in cell membrane involved in cross-linking of bacterial cell wall. Synthesis of peptidoglycan layer of cell wall by PBP is found thicker in Gram-positive bacteria and act as filter to block away unwanted molecules (antibiotic) to penetrate into cell. Beta-lactam antibiotics are analog of D-alanyl-D-alanine placed on N-acetylglucosamine and N-acetylmuramic acid (NAG/NAM) of peptidoglycan layer. The similarity makes the binding active site of PBPs and prevents cross-linking (transpeptidation) of peptidoglycan. Consequently, inhibit the cell wall synthesis and leads to bacteria cell death due to autolysis (Fisher et al., 2005).

Two main bacteria resistance mode applied on beta-lactam antibiotics include enzymatic hydrolysis of beta-lactam ring and altered PBP. For the first mode, the bacterium can synthesize beta-lactamse enzyme to hydrolyze beta-lactam ring structure of antibiotic and stop the mechanism action of antibiotic (Drawz & Bonomo, 2010). Clavulanic acid act as beta-lactamase inhibitors can overcome the resistance (Leonard et al., 2013). For second mode, some bacteria changed protein to beta-lactam antibiotic bind. Beta-lactam cannot bind to altered PBPs and causes less disrupting on cell wall synthesis. This mode of resistance can be found in MRSA.

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Vancomycin

Vancomycin was discovered by Edmund Kornfeld in 1953 from soil sample *Streptomyces orientalis.* Vancomycin is glycopeptide antibiotic used for treatment in skin infection, bone and joint infection, and meningitis caused by MRSA as well as treatment for intestine infection or bloody diarrhea caused by *Clostridium difficile*. Vancomycin involved inhibition of cell wall synthesis in Gram-positive bacteria. Vancomycin is not effectively against Gram-negative bacteria due to different mechanism and impermeable of glycopeptide into cell wall of Gram-negative bacteria except *Neisseria*. Mechanism of action in vancomycin elucidates the interaction between peptidoglycan molecules of cell wall. The hydrophilic molecule in vancomycin bind to terminal D-alanyl-D-alanine of pentapeptide N-acteylmuramic acid (NAM)/ N-acetylglucosamine (NAG) on peptidoglycan cell wall by hydrogen bonding, prevent the cross-linking by transpeptidation to form backbone strands in bacterial cell wall. Under normal circumstances, formation of peptidoglycan in call wall initiated with convertion of L-alanine into D-alanine in cytoplasm and followed by ligation of two molecules of D-alanine to form dipeptide D-alanyl-D-alanine, and added to uracil diphosphate-N-acetylmuramyl-pentapeptide that involved in formation of cross-linking by transpeptidation (Reynolds, 1989).There are two types of resistance in vancomycin includes intrinsic and acquired resistance. Most of the Gram-negative bacteria are intrinsically resistant to vancomycin due to the impermeable outer membrane to glycopeptide molecules except *Neisseria* but few of Gram-positive bacteria are intrinsically resistance to vancomycin such as *Pediococcus*. The other mechanism of resistance to vancomycin implicates alteration of terminal D-alanyl-D-alanine of NAM/NAG peptide.

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Polymyxin B

Polymyxin B is derived from *Bacillus polymyxa* under polypeptide antibiotic class discovered in 1947 and mostly used for Gram-negative infection (Storm et al., 1977). Five main compounds include polymyxin A, B, C, D and E but polymyxin B and E (colistin) are widely applied in clinical practice. It poses bactericidal effect on Gram-negative bacteria bacilli except for *Proteus* and *Neisseria*. Polymyxin B used to treat urinary tract infection and meningitis respectively. Normally, divalent cations such as magnesium (Mg^{2+}) and calcium (Ca^{2+}) will bind to lipopolysaccharide molecules of Gram-negative bacteria (Schindler & Osborn, 1979). Mechanism of action polymyxins started with bind to cell membrane by change the outer membrane structure to be more permeable. Positively charged of polymyxin B bind to negatively charge of lipopolysaccharide layer in cell wall and disrupt the cell membrane permeability; leads to cell death (Newton, 1956).

2.5.2.2 Inhibition of Protein Synthesis (30S ribosomal subunit)

Aminoglycosides

Aminoglycosides antibiotics are broad-spectrum antibiotic with part of molecules named as glycoside (sugar) in the structure. It is widely applied bactericidal (kill bacteria) effect on Gram-negative bacteria by inhibit protein synthesis process (Mingeot-Leclercq et al., 1999). Aminoglycosides are derived from *Streptomyces* (Waksman, 1964) and named with suffix *mycin* include streptomycin, tobramycin and kanamycin. In contrast, other aminoglycosides derived from *Micromonospora* (Kroppenstedt et al., 2005) and named with suffix *micin* include gentamicin and amikacin.

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Some of aminoglycosides antibiotic are share same suffix but not derived from previously bacteria such as vancomycin (Hammes & Neuhaus, 1974) and erythromycin (Tenson et al., 2003) are produced by *Saccharopolyspora erythraea* as well as clarithromycin and azithromycin in different mechanism of action. In general, aminoglycosides antibiotics involved of mechanism action mode on inhibition of protein synthesis. The aminoglycosides penetrates the cell and bind to 30S ribosomal subunit to cause misreading of genetic code in protein synthesis. Interruption of aminoglycosides binding to aminoacyl site of 30S ribosomal subunit, leads to misreading of genetic code and inhibition of translocation (Mingeot-Leclercq et al., 1999).

Tetracycline

Tetracycline is a broad-spectrum antibiotic with polyketide in structure and synthesized by *Streptomyces* genus (Gold et al., 1997). In general, tetracycline is used as treatment for Rickettsia, Lyme disease, psittacosis and lymphogranuloma venereum (Bhattacharya, 2003). Tetracycline is poses bacteriostatic (inhibit bacterial growth) effect against to some of Gram-positive and Gram-negative bacteria. Mechanism of action in tetracycline involved of inhibition of protein synthesis by blocking the binding of charged aminoacyl-tRNA to A site on the 30S subunit of microbial ribosome. The blocking of tetracycline on 30S ribosomal subunit will lead to inhibits of cell growth and translation process. Tetracycline mostly affect on small ribosomal subunit of prokaryotes and eukaryotes (30S and 40S) but not on mammalian cells (Chopra & Roberts, 2001).

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Three mechanism of cell increase resistant to tetracycline includes enzymatic inactivation of tetracycline (rarest type), efflux pump and ribosomal protection. Bacteria acquire resistance to tetracycline through horizontal gene transfer by efflux pump or ribosomal protection protein. For efflux pump will involved to pump actively tetracycline resistant gene out of the cell and prevent to increase inhibitory concentration of tetracycline in cytoplasm. Ribosomal protection proteins bind to ribosome and displace tetracycline from ribosome to allow translation process (Chopra & Roberts, 2001).

2.5.2.3 Inhibition of Protein Synthesis (50S ribosomal subunit)

Chloramphenicol

Chloramphenicol is broad-spectrum antibiotic used for treatment of meningitis, cholera in *Vibrio cholera* and typhoid fever in *S.* typhi. Chloramphenicol is derived from *Streptomyces venezuelae* discovered by David Gottlieb in 1949 for treatment of conjunctivitis (Rosenthal & Blackman, 1965). It poses bacteriostatic effect on bacteria by inhibit the protein synthesis. Chloramphenicol irreversibly binds to 50S bacterial ribosomal subunit and inhibits peptidyltransferase. Consequently, prevent protein chain elongation process in ribosome and prevent peptide bond formation (Wolfe & Hahn, 1965). When both chloramphenicol and macrolide antibiotics present and bind to ribosome to inhibit the protein synthesis, chloramphenicol will directly interferes with substrate binding while macrolide will block the elongation of growing peptide in ribosome.

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Three mechanisms of resistance in chloramphenicol includes decrease membrane permeability, involvement of chloramphenicol acetyltransferase and mutation of 50S ribosomal subunit (Jardetzky, 1963). For the first mechanism is the common occur in low level of chloramphenicol resistance by passage of bacteria. For high level resistance of chloramphenicol is conferred by *cat*-gene, encoded by enzyme chloramphenicol acetyltransferase. The function of chloramphenicol acetyltransferase is to inactivate chloramphenicol by attached one or two acetyl group (CH_3CO) from acetyl-S-coenzyme A to hydroxyl (-OH) group on chloramphenicol molecule. This process will prevent the binding of chloramphenicol to ribosome and inhibit the protein synthesis. There is rare case happen in the mutation mechanism of 50S ribosomal subunit (Wolfe & Hahn, 1965).

Macrolides

Macrolides is bacteriostatic effect (inhibit bacterial growth) and derived from natural products with large macrocyclic lactone ring to deoxy sugar. Macrolides antibiotic usually used to treat infection caused by Gram-positive bacteria such as *Streptococcus pneumoniae* and some respiratory tract infections. Azithromycin, erythromycin, clarithromycin, fidaxomicin and telithromycin are examples of macrolides antibiotics. Mode of action in macrolides are involved of inhibition of protein synthesis by prevent peptidyltransferase from adding peptide to tRNA to next amino acid and inhibit ribosomal translation (Gaynor & Mankin, 2003). Macrolides binds reversibly to P site on 50S subunit ribosome.

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Resistance of macrolides can occur by post-transcriptional methylation of 23S bacterial ribosomal RNA and acquired through chromosomal or plasmid-mediated or mutation as result of macrolides, lincosamides and streptogramins (MLS) (Gaynor & Mankin, 2003). Mechanism acquired macrolides resistance include methylases encoded by *ermA*, *ermB* and *ermC* (erythromycin ribosome methylase) to changes the macrolides-binding site on ribosomal RNA and the other is involved of active macrolides efflux pump encoded by *mef* with *msrA* and *msrB* genes confer low degree of macrolides resistance (Gaynor & Mankin, 2003).

Clindamycin

Clindamycin is antibiotic under lincosamide class that effectively against Gram-positive bacteria (*Staphyloccoci*, *Streptococci* and *Pneumococci*) and anaerobes (Dhawan & Thadepalli, 1982). It cannot penetrate Gram-negative bacteria so it does not affect endotoxin production in Gram-negative bacteria. It is widely used to treat pneumonia, joint infection, middle ear infection, acne, malaria, pelvic inflammatory infection and some cases of MRSA (Boucher & Corey, 2008). It can be inhibiting growth bacteria in most cases but also can kill bacteria at high concentration for certain species. Clindamycin can decrease bacterial toxin in *Staphylococci* and *Streptococci* toxic shock syndrome. Clindamycin involved in inhibition of protein synthesis by inhibit ribosomal translocation. It can bind to 50S bacterial ribosomal subunit and prevent the transpeptidation reaction as macrolide to inhibit the elongation process (McManus, 1997). Three mechanisms of resistance include inactivation of clindamycin by methylase (methylation), alter receptor site by methylase and mutation of ribosomal binding site (Leclercq, 2002). Clindamycin can synergistically with penicillin, cephalosporins and vancomycin in Gram-positive toxic bacteria.

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2.5.2.4 Inhibition of Nucleic Acid Synthesis

Quinolones

Quinolones are synthetic broad-spectrum antibiotics that derived from natural products (Andersson & MacGowan, 2003). Fluoroquinolones is effective treatment on bacterial infection caused by Gram-positive and Gram-negative bacteria. First generation of quinolones, nalidixic acid is used for treatment urinary tract infection in 1962 (Salim & Shupe, 1966). First, second, third and fourth generation is known as fluoroquinolones under quinolones antibiotics group (Oliphant & Green, 2002) (Table 2.24).

Table 2.24: Types of quinolones antibiotics. (Oliphant & Green, 2002; Walsh, 2003)

Generation	Types of quinolones antibiotics	Functions
First	Nalidixic acid, cinoxacin, piromidic acid, oxolinic acid, pipemidic acid, rosoxacin	To inhibit topoisomerase II ligase domain and modified of the topoisomerase II leads to DNA fragmentation through nucleasic activity of enzyme domain
Second	Ciprofloxacin, lomefloxacin, norfloxacin, enoxacin, fleroxacin, nadifloxacin, ofloxacin, pefloxacin, rufloxacin	
Third (active against *Streptococci*)	Levofloxacin, pazufloxacin, grepafloxacin, balofloxacin, tosufloxacin, sparfloxacin, temafloxacin	Selective for topoisomerase IV ligase domain
Fourth (act on DNA gyrase and topoisomerase IV)	Gemifloxacin, gatifloxacin, clinafloxacin, moxifloxacin, prulifloxacin, sitafloxacin, trovafloxacin	

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Quinolones are interacts with targets such as DNA gyrase and topoisomerase IV within bacterial cell (Robicsek et al., 2006). DNA gyrase is target for Gram-negative bacteria while topoisomerase IV is target for Gram-positive bacteria. These enzymes important in nucleic acid process help to control DNA in under winding and overwinding as well as remove the knots and tangles from bacterial chromosome (Anderson & Osheroff, 2001; Champoux, 2001). The gyrase and topoisomerase IV are involved in DNA topological state either in intact double helix or transient double-stranded break.

The mechanism of resistance action in quinolones involved of efflux pump act to reduce the intracellular quinolones concentration in chromosome-mediated quinolones (Robicsek et al., 2006). The outer membrane of Gram-negative bacteria is facilitated by protein channel via porins to enter the cell. Plasmid-mediated resistance genes produce protein bind to DNA gyrase in Gram-negative bacteria, prevent from action of quinolones. It can be acquired through horizontal or vertically transfer (conjugation) (Martínez-Martínez et al., 1998; Robicsek et al., 2006). Then, mutations in DNA gyrase or topoisomarease IV can decrease binding affinity to quinolones. DNA gyrase was first identified in nalidixic acid resistant mutant of *Escherichia coli* in *gyrA* and *gyrB* gene (Nakamura et al., 1989).

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2.5.2.5 Inhibition of Folic Acid Synthesis

Sulfonamides

Sulfonamides is a synthetic broad-spectrum antibiotics with sulfonyl group $-S(=O)_2$ attached to amine group. This sulfa drug has bacteriostatic effect on Gram-positive and Gram-negative bacteria as well as toxoplasma and protozoa agents. Examples of sulfa drugs include sulfadiazine, sulfamethoxazole and sulfadoxine. It can act synergistically in combination with trimethoprim become trimethoprim-sulfamethoxazole (SXT) used to treat urinary tract infection, MRSA infection and respiratory tract infection (Gemmell et al., 2006; Lacey et al., 1980). Sulfonamides act as competitive inhibitor involved in inhibition of folic acid synthesis (metabolic process) by stop addition of para-aminobenzoic acid (PABA) with the presence of enzyme dihydropteroate synthetase (DHPS). All cells require folic acid for growth, it can acquire through food and transported into human cell but folic acid cannot pass to bacterial cell wall by diffusion or active transport. Folate is required for bacterial cell to make nucleic acid and will synthesize folic acid from PABA with DHPS.

Two steps of folic acid synthesis in bacteria: firstly, PABA is added to pteridine to form dihydropteroic acid. Then, glutamic acid is added to form dihydrofolic acid and subsequently with the dihydrofolate reductase enzyme to form tetrahydrofolic acid, the form of folic acid required by bacterial cell. In the substitution of sulfa drug with PABA will inhibit the synthesis of folic acid in bacteria. Resistance to sulfonamides occurs in chromosomal and plasmid mediated has been reported (Lopez et al., 1984; Perreten & Boerlin, 2003; Swedberg & Sköld, 1980).

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2.5.2.6 Inhibition of RNA synthesis

Nitrofuratoin

Nitrofuratoin is under nitrafuran class of antibiotic used for treatment of lower urinary tract infection (UTI) in 1953. It has furan ring and nitro group in structure (Huttner et al., 2015). The mechanism of action involved in destruction of bacterial ribosomal protein and DNA by nitrofuran reductase enzyme. Bacterial flavoprotein alters bacterial ribosomal protein as nitrofuratoin is decrease; consequently, inactivate protein synthesis, DNA synthesis, cell wall synthesis and RNA synthesis (Tu & McCalla, 1975). Nitrofuratoin has been widely used as veterinary antibiotic in chicken. It mixed with animal feed and feed the chicken that consume by human.

Rifampicin

Rifampicin is antibiotic used to treat bacterial infections such as tuberculosis. It often involved of inhibits RNA synthesis in bacterial DNA. Rifampicins bind to DNA-dependent RNA polymerase and prevent the RNA synthesis to form the phophodiester bond in extension process (Calvori et al., 1965). Resistance of rifampicin mediated by altered the beta subunit of RNA polymerase or decreased the uptake level in Gram-negative bacteria.

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2.5.3 Genotypic Method

2.5.3.1 Polymerase Chain reaction (PCR) and Agarose Gel Electrophoresis (AGE)

PCR is a simple, rapid and sensitive molecular technique involved in DNA amplification of gene interests with the *Taq* polymerase enzyme. In brief, four PCR steps (denaturation, annealing, extension and termination steps) by separation of DNA double-stranded template in high temperature and annealing of primer formation and extension of new strand using *Taq* polymerase and dNTP.

The double-stranded DNA is heated and melted into single stranded with temperature at 95 ºC. Then, the primers will bind to the complementary strand once the strands are separated and reduce the annealing temperature in the range of 48-72 ºC. Typically the PCR will be in 25 until 35 cycles. Then extension step involved in elongation of the new strand in the range of 68-72 ºC for 5 to 10 minutes until the complete of PCR amplification (Joshi & Deshpande, 2011). AGE is used to separate the DNA fragments based on molecular weight in electrophoresis buffer by applied electric field. Most of the agarose gel is made between 0.7 % (5-10kb) and 2 % (0.2-1kb) for separation DNA purpose. Small DNA molecules fragments will migrate faster than bigger DNA molecules in the pores of agarose gel. The DNA can be visualized under UV transilluminator by high sensitive fluorescent GelStar nucleic acid stain (White et al., 1999).

2.5.3.2 Antibiotic Resistance Genes

Antibiotic genes such as tetracycline resistance genes (*tet* gene), sulfonamides resistance gene (*sul* gene), quinolones resistance gene (*qnr* gene) and macrolides resistance gene (*mph* gene) found as conferring resistance to antibiotics in *Salmonella*.

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Most common of six different classes *tet* genes, named A to E and G in Gram-negative *Enterobacteriaceae* group (Roberts, 1996; Chopra & Roberts, 2001). The *tet*(A) gene was found on plasmids and on the chromosome, *tet*(B), *tet*(C), and *tet*(D) genes were found on the chromosomes of *Salmonella enterica* (Frech & Schwarz, 2000). The *sul1* and *sul2* are the antibiotic determinants resistance to sulfonamide that inhibits dihyrdropteroate synthetase (DHPS) (Perreten & Boerlin, 2003; Mascaretti, 2003).

The genes are present on class 1 integrons (*sul1*) or plasmids (*sul2*). *Salmonella* serotypes such as Enteritidis, Hadar, Heidelberg, Orion, Rissen, Agona, Albany, Derby, Djugu, and Typhimurium have been reported found in *sul1* gene (Antunes et al., 2005; Chen et al., 2004; Doublet et al., 2004) while *Salmonella* serotypes Enteritidis, Agona, and Typhimurium isolates have been identified habouring *sul2* (Chen et al., 2004). The plasmid-specified *qnr* determinants are contribute in decreased fluoroquinolone susceptibility mechanism. Different of the *qnr* elements have been identified in Gram-negative bacteria such as *K. pneumoniae*, *E. coli* and non-Typhi *Salmonella enterica* (Martinez-Martinez et al., 1998). *Qnr* is a pentapeptide repeat family proteins involved in mediates resistance by protecting DNA gyrase and topoisomerase IV from the inhibitory action of the fluoroquinolones (Tran & Jacoby, 2002). Macrolides inactivated by modifying enzymes in *Enterobacteriaceae* are reported such as phosphotransferases encoded by *mph*(A), *mph*(B), and *mph*(D) genes. Most of the macorlides resistance was related to the expression of a macrolide 2′-phosphotransferase encoded by the *mph*(A) gene (Arthur et al., 1987; O'Hara et al., 1989).

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2.5.3.3 DNA Sequencing

DNA sequencing introduced chain-terminating dideoxynucleotides using enzyme DNA polymerase to determine the nucleotide A, T, G and C during DNA replication (Sanger et al., 1977). Four different deoxyribonucleoside triphosphates include dATP, dTTP, dCTP and dGTP are used as substrates and attached to primer which is formed hydrogen bonding with 3' end of DNA. Each DNA with primer is divided into 4 reaction mixture consists of 4 dNTPs and four dideoxyribonucleoside triphosphates (ddNTPs) include ddATP, ddGTP, ddCTP and dd TTP. This ddNTPs able to terminate the DNA polymerase reaction due to free 3'-hydroxyl (OH) group of dideoxy sugar has been replaced by hydrogen, which required for formation of phosphodiester bond between two nucleotides and causing terminate of DNA strand elongation (Anjana, 2012).

2.5.3.4 Pulsed-Field Gel Electrophoresis (PFGE)

The PFGE is "gold standard" of molecular genetic fingerprint technique used in epidemiology study of pathogenic bacteria (*Salmonella* spp., *Escherichia coli* O157: H7 and *Listeria* spp.) by separate large deoxyribonucleic acid (DNA) molecules on the gel with periodically changes in different angle of direction (Schwartz et al., 1983; Schwartz & Cantor, 1984). Genomic DNA of bacteria was digested with specific restriction enzyme and run on agarose gel with CHEF-DR II (clamped homogeneous electrical field-dynamic regulation) Mapper system to produce distinct of DNA fingerprint pattern. *Salmonella enterica* subspecies *enterica* serovar Braenderup H9812 (ATCC BAA 664) is used as molecular ladder strain. Molecular fingerprint pattern was analyzed using BioNumeric software program.

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2.5.3.5 HiSeq 2000 Platform

HiSeq 2000 system adapted sequencing by synthesis (SBS) technology, which using fluorescent labeled reversible terminator method for detection of single bases. It involved of bridge amplification to generate single-stranded DNA clusters on flow cell surface. Complementary base process occurs when the fluorescent labeled deoxyribonucleotide triphosphates (dATP, dCTP, dGTP and dTTP) are added to 3' end template strand after primer annealing. These fluorescent labeled nucleotides are preventing the addition of nucleotide at 3' hydroxyl group and nucleotides are identified by fluorophore excitation (Illumina, 2015).

There are four basic steps in NGS workflow:

a) Library preparation

   The sequencing library is prepared by tagmentation of DNA sample with adapter ligation and followed by PCR and gel electrophoresis.

b) Cluster generation

   The prepared library is loaded into flow cell. The fragments will bind on surface oligo complementary to adapter and amplified into cluster through bridge amplification.

c) Sequencing

   The sequencing are involved of four reversible terminator dNTP method to obtain single bases after introduce DNA template strands

d) Data analysis

   The new sequence are analysed and aligned with reference genome.

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CHAPTER 3: METHODOLOGY

3.1 Materials

3.1.1 Chemical Reagents

Chemical reagents used in this study were listed as follow (Table 3.1):

Table 3.1: Chemical reagents used in the study.

Materials	Supplier/Country name
Primers	1st base IDT , Singapore
Agarose analytical grade powder	Promega, United States
Loading dye	Thermo Scientific, Malaysia
Gene Ruler TM 100 bp DNA ladder	Fermentas, Canada
Gene Ruler TM 1kb DNA ladder	Fermentas, Canada
NEB *Taq* polymerase	New England Biolab, United States
Deoxynucleoside triphosphates (dNTPs) mix	New England Biolab, United States
NEB 5× *Taq* buffer	New England Biolab, United States
Qubit dsDNA HS buffer	Life Technologies, United States
Qubit dsDNA HS reagent	Life Technologies, United States
GelStar nucleic acid gel stain	Lonza, United States
Certified megabase agarose powder	BioRad lab, Spain
Seakem Gold agarose	Choice-care, Malaysia
N-Lauroylsarcosine sodium salt	Sigma, United Kingdom
Glycerol	Merck, Malaysia
Bovine serum albumin (BSA)	Thermo Scientific, Malaysia
Phosphate-buffered saline (PBS)	1st base company, Malaysia
miliQ water	Merck milipore, United States

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3.1.2 Culture Media

All the culture media were prepared according to manufacturer's instruction and sterilized by autoclaving at a temperature of 121 ºC and pressure of 15 psi for 15 minutes. The culture media used in this study were listed as follow (Table 3.2):

Table 3.2: Culture media used in the study.

Media	Supplier/Country name
Rappaport-Vassiliadis Soy Broth (RVS Broth)	Oxoid, Malaysia
Tryptic Soy Broth (TSB)	Merck, Malaysia
MacConkey Broth (MCB)	Merck, Malaysia
Fraser Broth (FB)	Oxoid, Malaysia
Hektoen Enteric Agar (HEA agar)	Oxoid, Malaysia
Mannitol Salt Agar (MSA agar)	Merck, Malaysia
MacConkey agar (MAC)	Merck, Malaysia
PALCAM selective Agar	Oxoid, Malaysia
Half Fraser Broth	Oxoid, Malaysia
Brilliance *Listeria* Agar	Oxoid, Malaysia
Brilliance *Salmonella* Agar	Oxoid, Malaysia
Nutrient Agar (NA)	Merck, Malaysia
Nutrient Broth (NB)	Merck, Malaysia
Muller-Hinton Agar (MHA)	Merck, Malaysia
Muller-Hinton Broth (MHB)	Merck, Malaysia
Tryptic Soy Agar with 5 % sheep blood (premade)	Oxoid, Malaysia
Columbia Agar (blood agar)	Oxoid, Malaysia
Tryptone Soya Yeast Extract Agar (TSYEA)	Sigma-Aldrich, United States

3.1.3 Oligonucleotide Primers

All the oligonucleotide primers were synthesized and purchased from 1st base IDT Company, Singapore. Primers were listed as follow (Table 3.3):

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Table 3.3: Oligonucleotide primers used in *Salmonella* spp.

Genes	Forward primer (5'-3')	Reverse primer (5'-3')	PCR product size (bp)	Annealing temperature (°C)	References
sdfI (*S*. Enteritidis)	TGTGTTTTATCTGATGCAAGAGG	TGAACTACGTTCGTTCTTCTGG	304	50	Agron et al., 2001
sefA(*S*. Enteritidis)	GCAGCGGTTACTATTGCAGC	TGTGACAGGGACATTTAGCG	310	68	Akbarmehr, 2011
fljB(*S*. Typhimurium)	ACGAATGGTACGGCTTCTGTAACC	TACCGTCGATAGTAACGACTTCGG	526	68	Akbarmehr, 2011
rfbJ(*S*. Typhimurium)	CCAGCACCAGTTCCAACTTGATAC	GGCTTCCGGCTTTATTGGTAAGCA	663	68	Akbarmehr, 2011
viaB (*S*. typhi)	CACGCACCATCATTTCACCG	AACAGGCTGTAGCGATTTAGG	738	65	Kumar et al., 2006
invA	GTGAAATTATCGCCACGTTCGGGCAA	TCATCGCACCGTCAAAGGAACC	285	50	Rahn et al., 1992
tetA	GTAATTCTGAGCACTGTCGC	CTGCCTGGACAACATTGCTT	956	60	Aarestrup et al., 2003
tetB	CTCAGTATTCCAAGCCTTTG	ACTCCCCTGAGCTTGAGGGG	414	50	Aarestrup et al., 2003
sul1	CTTCGATGAGAGCCGGCGGC	GCAAGGCGGAAACCCGCGCC	435	50	Aarestrup et al., 2003
sul2	GCGCTCAAGGCAGATGGCATT	GCGTTTGATACCGGCACCCGT	293	60	Aarestrup et al., 2003
mphA	GTGAGGAGGAGCTTCGCGAG	TGCCGCAGGACT CGGAGG TC	403	50	Nguyen et al., 2009
qnrA	GATAAAGTTTTTCAGCAAGAGG	ATCCAGATCCGCAAAGGTTA	543	50	Guillard et al., 2010
qnrS	AGTGATCTCACCTTCACCGC	CAGGCTGCAATTTTGATACC	551	50	Cattoir et al., 2007
qnrB	TCGGCTGTCAGTTCTATGATCG	TCCATGAGCAACGATGCCT	496	50	Kehrenberg et al., 2006

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3.1.4 PCR Master Mix for Amplification

The PCR master mix was prepared according to the formula. PCR master mix components were listed as follow (Table 3.4):

Table 3.4: PCR Mixture Components. (NEB, 2016)

PCR Component (Stock Concentration)	Volume per Sample (µl)	Final Concentration
deionized H_2O	18.375	
5× One *Taq* Buffer containing $MgCl_2$	5.0	1×
dNTPs (200 µM)	0.5	200 µM
One *Taq* DNA polymerase (1.25 U/µl)	0.125	1.25 units/ 50 µL reaction
Forward primer (10 µM)	0.5	0.2 µM
Reverse primer (10 µM)	0.5	0.2 µM
Genomic DNA	variable	< 1,000 ng
Total volume	25.0	

3.1.5 Agarose Gel Electrophoresis (AGE)

The agarose gel was prepared according to the formula. Agarose concentration gel range was listed as follow (Table 3.5):

Table 3.5: Agarose gel concentration for range of DNA molecules. (Rapley, 1998)

Agarose concentration in gel (%)	Range of separation DNA fragments (kb)
0.3	5-60
0.6	1-20
0.7	0.8-10
0.9	0.5-7
1.2	0.4-6
1.5	0.2-3
2.0	0.1-2

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3.1.6 Buffer Solution

All the buffer solutions were prepared according to manufacturer's instruction and sterilized by autoclaving at a temperature of 121 ºC and pressure of 15 psi for 15 minutes. Buffer solutions used in this stidy were prepared as follow:

Tris/EDTA buffer (TE buffer) (10mM Tris: 1mM EDTA, pH 8.0)

A total 10ml of 1M Tris (pH 8.0) was added with 2ml of 0.5M EDTA (pH 8.0) and then diluted to 1000 ml with distilled water.

Cell suspension buffer (100mM Tris: 100mM EDTA, pH 8.0)

A total 10 ml of 1M Tris (pH 8.0) was added with 20 ml of 0.5M EDTA (pH 8.0) and then diluted to 100 ml with distilled water.

1% (w/v) Seakem Gold agarose gel

Weight 0.5g of agarose powder into 250 ml conical flask with 50 ml of TE buffer.

Cell lysis buffer (50mM Tris: 50mM EDTA, pH 8.0 + 1% (w/v) Sarcosyl)

A total 25 ml of 1M Tris (pH 8.0) was added with 50 ml of 0.5M EDTA (pH 8.0) and 50 ml of 10% (w/v) Lauroylsarcosine, sodium salt (sarcosyl). Then diluted the buffer to 500ml with distilled water.

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3.1.7 Equipments and Consumable

Equipments and consumable were used as follow (Table 3.6):

Table 3.6: Equipments and consumable used in the study.

Equipment/apparatus	Supplier/Country name
Agarose gel electrophoresis tank	Bio-Rad, China
PCR thermocycler	Bio-Rad, Malaysia Applied biosystems, United States
UVP high performance UV transilluminator	Kinematic resource, Malaysia
Sartorius Weight machine	Stedim Biotech, Germany
Hirayama Autoclave machine	Interscience, Malaysia
Shaking incubator	Sartorius, stedim Biotech, Germany
Thermomixer	Eppendorf, Malaysia
Seward Stomacher®400 circulator	Fisher Scientific, Malaysia
Light microscope	Leica Microsystem, Singapore
Nanodrop 2000 spectrophotometer	Thermo Scientific, Malaysia
Qubit 2.0 Fluorometer	Life Technologies, United States
Belly Dancer	IBI Scientific, United States
Microwave oven	National, Malaysia
White light transilluminator	UVP, United States
Memmert waterbath	Memmert, China
-86 ºC Ultra low Temp freezer	Gala Science, Malaysia
Benchmixer	Benchmark Scientic Inc, USA
Mini spin	Biolaboratoris, Singapore
Labmart Hotplate stirrer	Copen Scientific, Malaysia
Sartorius pH meter	Stedim Biotech, Germany
ESCO Lamina flow cabinet	Team medical & Scientific, Malaysia
Centrifuge 5424R	Eppendorf, Malaysia
CHEF-DR II Mapper electrophoresis system	Bio-Rad, Malaysia
Fume Hood	Labronco, United States

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3.1.8 Commercial Kits

Commercial kits were listed as follow (Table 3.7):

Table 3.7: Commercial kits used.

Commercial kits	Supplier /country name
QIAquick ®Gel Extraction kit	Qiagen, Germany
BD™ Gram Stain Kit	Becton, Dickson and company, United State
Masterpure™ DNA Purification kit	Epicentre, United State

3.2 Bacteria Control Strains

All the bacteria were used as control strains and cultivated in nutrient broth at 37 ºC as follow (Table 3.8):

Table 3.8: Bacterial strains used.

Bacterial strains
Salmonella enterica subspecies *enterica* serovar Enteritidis ATCC 13076 ™
Salmonella enterica subspecies *enterica* serovar Typhi (Human source)
Salmonella enterica subspecies *enterica* serovar Typhimurium ATCC 14028™
Listeria monocytogenes ATCC® 15313
Listeria innocua DSMZ 20649
Listeria ivanovii subspecies *ivanovii* DSMZ 20750
Escherichia coli ATCC ® 25922™
Staphylococcus aureus subspecies *aureus* ATCC ®29213™

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3.3 Methods

3.3.1 Sampling Area and Sample Types

The sampling areas in this study are located in Klang Valley, Malaysia. A total of 100 samples were collected from 8 supermarkets and 17 local wet markets from April 2015 until January 2016. The samples comprised of chicken meat (n= 50), chicken eggs (n= 40) and chicken feeds (n= 10). All the samples were placed in ice box and transported to the laboratory for isolation within two hours.

Chicken Meat

A total of 50 breast chicken fillets were collected from different locations of wet and hypermarkets in Klang Valley area, Malaysia (Table 3.9). Two types of chickens: village and battery chickens were selected for the study. All the chicken meat were collected in sterilized bag and transported to laboratory for analysis. Total of thirty-two village chickens were collected in 16 locations of wet markets in Klang Valley area. Only 18 battery chickens were purchased in nine hypermarkets located in different area of Klang Valley.

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Table 3.9: Chicken meat collected in local markets.

No.	Location	Source	With or without skin
1	Wet Market 1	Village chicken	With skin
2	Wet Market 1	Village chicken	Without skin
3	Wet Market 2	Village chicken	With skin
4	Wet Market 2	Village chicken	Without skin
5	Wet Market 3	Village chicken	With skin
6	Wet Market 3	Village chicken	Without skin
7	Supermarket 1	Battery chicken	With skin
8	Supermarket 1	Battery chicken	Without skin
9	Supermarket 2	Battery chicken	With skin
10	Supermarket 2	Battery chicken	Without skin
11	Wet market 4	Village chicken	With skin
12	Wet market 4	Village chicken	Without skin
13	Wet market 5	Village chicken	With skin
14	Wet market 5	Village chicken	Without skin
15	Wet Market 6	Village chicken	With skin
16	Wet Market 6	Village chicken	Without skin
17	Wet Market7	Village chicken	With skin
18	Wet Market 7	Village chicken	Without skin
19	Supermarket 3	Battery chicken	With skin
20	Supermarket 3	Battery chicken	Without skin
21	Supermarket 4	Battery chicken	With skin
22	Supermarket 4	Battery chicken	Without skin
23	Wet Market 8	Village chicken	With skin
24	Wet Market 8	Village chicken	Without skin
25	Wet Market 9	Village chicken	With skin
26	Wet Market 9	Village chicken	Without skin
27	Wet Market 10	Village chicken	With skin
28	Wet Market 10	Village chicken	Without skin
29	Wet Market 11	Village chicken	With skin
30	Wet Market 11	Village chicken	Without skin
31	Wet Market 12	Village chicken	With skin
32	Wet Market 12	Village chicken	Without skin
33	Wet Market 13	Village chicken	With skin
34	Wet Market 13	Village chicken	Without skin
35	Wet Market 14	Village chicken	With skin
36	Wet Market 14	Village chicken	Without skin
37	Wet Market 15	Village chicken	With skin

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Table 3.9, continued.

38	Wet Market 15	Village chicken	Without skin
39	Wet Market 16	Village chicken	With skin
40	Wet Market 16	Village chicken	Without skin
41	Wet Market 17	Battery Chicken	With skin
42	Wet Market 17	Battery Chicken	Without skin
43	Supermarket 5	Battery Chicken	With skin
44	Supermarket 5	Battery Chicken	Without skin
45	Supermarket 6	Battery Chicken	With skin
46	Supermarket 6	Battery Chicken	Without skin
47	Supermarket 7	Battery Chicken	With skin
48	Supermarket 7	Battery Chicken	Without skin
49	Supermarket 8	Battery Chicken	With skin
50	Supermarket 8	Battery Chicken	Without skin

Battery chicken is chicken grows in battery cages; village chicken is housing village chicken; with skin mean by chicken breast fillet with skin on; without skin mean by skinless or skin is removed from chicken breast fillet.

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Chicken Eggs

A total of 40 chicken eggs were sampled from local markets and collected in sterilized bag and sent to laboratory for analysis within 2 hours. All of the chicken eggs were purchased from sixteen wet markets and four hypermarkets in Klang Valley area (Table 3.10). Surface of collected eggs were sterilized by 70 % ethanol and air dried in sterilize chamber for 10 mins. Sterilized knife was used to crack the egg surface and obtained egg contents of each single egg for bacterial isolation.

Table 3.10: Chicken eggs collected in local markets.

No.	Origin	Source
1	Wet market 1-stall 1	1 Egg
2	Wet market 1-stall2	1 Egg
3	Wet market 2-stall 1	1 Egg
4	Wet market 2-stall 2	1 Egg
5	Wet market 3-stall 1	1 Egg
6	Wet market 3-stall 2	1 Egg
7	Supermarket 1	1 Egg from 10
8	Supermarket 1	1 Egg from 10
9	Supermarket 2	1 Egg from 10
10	Supermarket 2	1 Egg from 10
11	Wet market 4-stall 1	1 Egg
12	Wet market 4-stall 2	1 Egg
13	Wet market 5-stall 1	1 Egg
14	Wet market 5-stall 2	1 Egg
15	Wet Market 6-stall 1	1 Egg
16	Wet Market 6-stall 2	1 Egg
17	Wet Market7-stall 1	1 Egg
18	Wet Market 7-stall 2	1 Egg
19	Supermarket 3	1 Egg from 10
20	Supermarket 3	1 Egg from 10
21	Supermarket 4	1 Egg from 10
22	Supermarket 4	1 Egg from 10
23	Wet Market 8-stall 1	1 Egg
24	Wet Market 8-stall 2	1 Egg
25	Wet Market 9-stall 1	1 Egg
26	Wet Market 9-stall 2	1 Egg
27	Wet Market 10-stall 1	1 Egg
28	Wet Market 10-stall 2	1 Egg
29	Wet Market 11-stall 1	1 Egg

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Table 3.10, continued.

30	Wet Market 11-stall 2	1 Egg
31	Wet Market 12-stall 1	1 Egg
32	Wet Market 12-stall 2	1 Egg
33	Wet Market 13-stall 1	1 Egg
34	Wet Market 13-stall 2	1 Egg
35	Wet Market 14-stall 1	1 Egg
36	Wet Market 14-stall 2	1 Egg
37	Wet Market 15-stall 1	1 Egg
38	Wet Market 15-stall 2	1 Egg
39	Wet Market 16-stall 1	1 Egg
40	Wet Market 16-stall 2	1 Egg

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Chicken Feeds

A total of ten bags of powder-type chicken feeds with specific descriptions were sampled from retail markets for laboratory analysis (Table 3.11).

Table 3.11: Chicken feeds collected in retail market.

No.	Company names	Descriptions
1	Cargill Malaysia	Broiler starter (22 % crude protein)
2	Cargill Malaysia	Broiler grower (19 % crude protein)
3	Cargill Malaysia	Standard layer (17 % crude protein)
4	Cargill Malaysia	Layer growth (16 % crude protein)
5	Soon Soon Oilmills Sdn Bhd	Dehulled soybean meal
6	Soon Soon Oilmills Sdn Bhd	Dehulled full fat soybean meal
7	Soon Soon Oilmills Sdn Bhd	Dehulled canola meal
8	Soon Soon Oilmills Sdn Bhd	Dehulled full fat Lupin meal
9	PBB group	Broiler feed
10	Poullive Sdn Bhd	Layer feed

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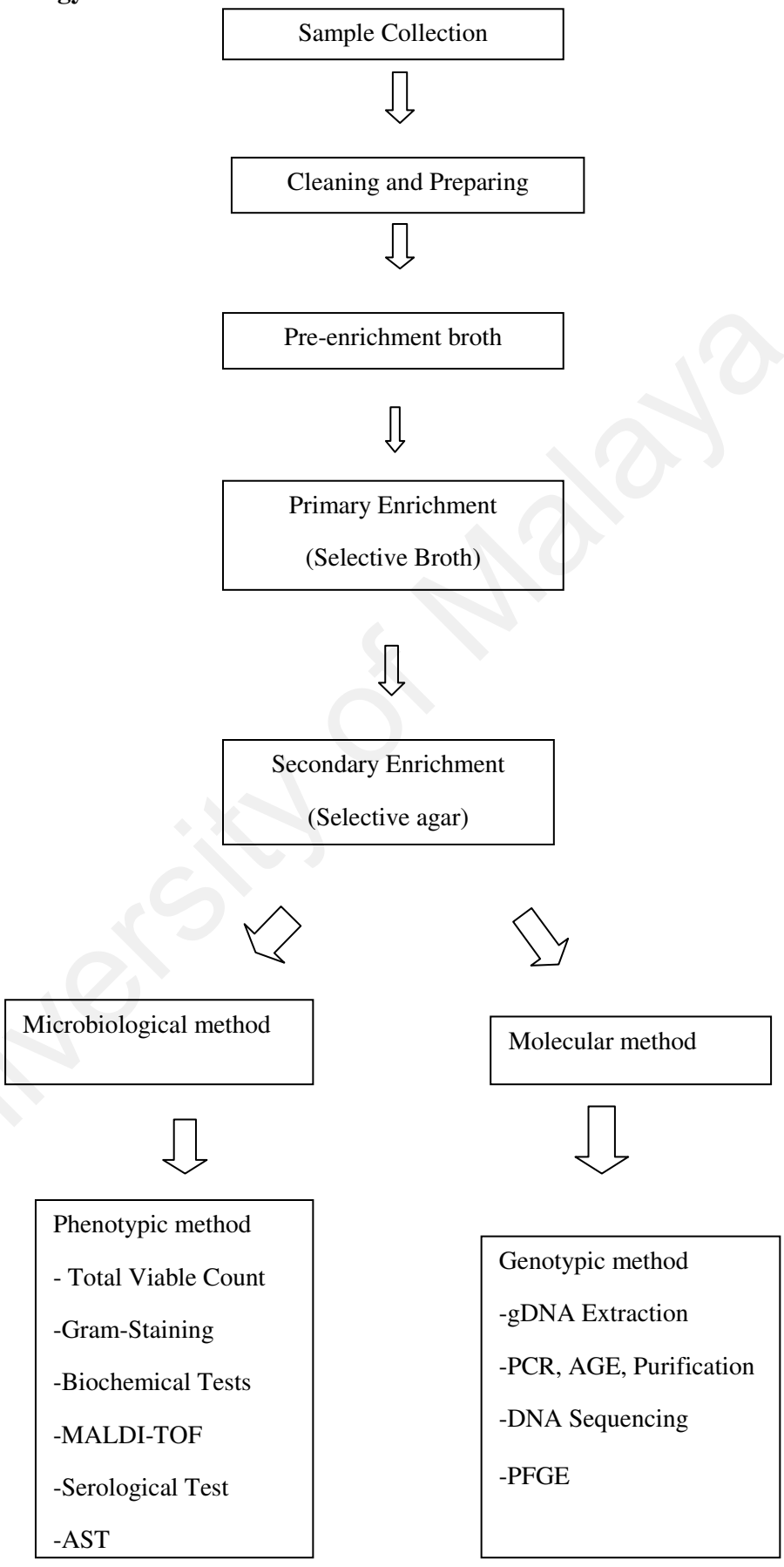
3.3.2 Methodology Workflow



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3.3.3 Bacterial Isolation, Enrichment and Identification

3.3.3.1 *Salmonella* Isolates

Salmonella isolates were isolated according to ISO 6579: 2002 (standard method for the isolation of *Salmonella* spp. for food and animal feeding stuffs, animal feces, and environmental) (ISO, 2002). Twenty-five gram of samples was aseptically transferred into stomacher bag containing 225 ml of buffered peptone water (BPW) (Oxoids). The samples were homogenized for 2 minutes at 260 rpm using Stomacher® 400 circulator (Seward). A ten-fold serial dilution (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5}) of mixture diluted with BPW was plate on nutrient agar for total viable count purpose. The homogenized samples were incubated at 37 ºC for 18-24 hours. Then, 0.1 ml of pre-enrichment culture was inoculated into 10 ml Rappaport Vassiliadis (RVS) (Oxoids) broth and incubated at 42 °C for 24 hours. Then a loopful of enriched inoculums was streaked on pre-warmed Hektoen enteric agar (HEA) (Oxoids) plate and incubated for 18-24 hours at 37 °C. Black colonies observed on the HEA plate indicated the presence of *Salmonella* due to the reduction of sulfur to hydrogen sulfide. All the pure cultures were preserved in glycerol stock consisting of 20 % (v/v) of glycerol and stored at -80 ºC for future study purpose.

3.3.3.2 *Listeria* Isolates

Listeria isolates were isolated according to ISO 11290-1: 1996 (ISO, 2004). Twenty-five gram of samples was aseptically transferred into stomacher bag containing 225 ml of half Fraser broth (pre-enrichment step). The samples were homogenized for 2 minutes at 260 rpm using Stomacher® 400 circulator (Seward). Then, ten-fold serial dilution (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5}) of mixture diluted with BPW was plate on nutrient agar for total viable count purpose.

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The homogenized samples were incubated at 30 ºC for 18-24 hours. Then, 0.1 ml of pre-enrichment culture was inoculated into 10 ml Fraser (Oxoids) broth and incubated at 35 °C for 24 hours. Then a loopful of enriched inoculums was streaked on pre-warmed PALCAM *Listeria* (Oxoids) agar plate and incubated for 18-24 hours at 37 °C. Grey green colonies with black halo observed on PALCAM *Listeria* agar indicated the presence of *Listeria*. The hydrolysis of esculin in *Listeria* species cause dark brown colonies formation on agar. All the pure cultures were preserved in 20 % (v/v) of glycerol and store at -80 ºC for future study purpose.

3.3.3.3 *Escherichia coli* Isolates

E. coli isolates were isolated according to ISO 21528: 2004 (ISO, 2005). Twenty-five gram of samples was aseptically transferred into stomacher bag containing 225 ml of buffered peptone water (BPW) (Oxoids).The samples were homogenized for 2 minutes at 260 rpm using Stomacher® 400 circulator (Seward). Then, ten-fold serial dilution (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5}) of mixture diluted with BPW was plate on nutrient agar for total viable count purpose. The homogenized samples were incubated at 37 ºC for 18-24 hours. Then, 0.1 ml of pre-enrichment culture was inoculated into 10 ml MacConkey (MCB) (Oxoids) broth and incubated at 35 °C for 24 hours. Then a loopful of enriched inoculums was streaked on pre-warmed MacConkey (MAC) (Merck) agar plate and incubated for 18-24 hours at 37 °C. *E. coli* appeared as pink colonies were observed on MAC agar indicated the presence of lactose-fermenting organism and production of acids. The decreased of pH indicated neutral red changing to pink (acidic environment). Colorless colonies produced on agar for non lactose-fermenting organism. All the pure culture of bacteria isolates were preserved in 20 % (v/v) of glycerol and store at -80 ºC for future study purpose.

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3.3.3.4 *Staphylococcus aureus* Isolates

S. aureus isolates were isolated according to ISO 6888-1 with slight modification (ISO, 1999). Twenty-five gram of samples was aseptically transferred into stomacher containing 225 ml of buffered peptone water (BPW) (Oxoids). The samples were homogenized for 2 minutes at 260 rpm using Stomacher® 400 circulator (Seward). Then, ten-fold serial dilution (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5}) of mixture diluted with BPW was plate on nutrient agar for total viable count purpose. The homogenized samples were incubated at 37 ºC for 18-24 hours. Then, 0.1 ml of pre-enrichment culture was inoculated into 10 ml tryptic soy (TSB) (Oxoids) broth and incubated at 35 °C for 24 hours. Then a loopful of enriched inoculums was streaked on pre-warmed mannitol salt (MSA) (Merck) agar plate and incubated for 18-24 hours at 37 °C. Yellow colonies with yellow halo were produced on MSA plate indicated the presence of *S. aureus* due to able to ferment mannitol and cause phenol red indicator changed from red into yellow color. All the pure culture of bacteria isolates were preserved in 20 % (v/v) of glycerol and store at -80 ºC for future study purpose.

3.3.4 Identification of Bacterial Strains

Colonies of presumptive *Salmonella* spp., *Listeria* spp., *E. coli* and *S. aureus* were purified on nutrient agar and incubated at 37 °C for 24 hours (Table 3.12). Selective culture media were used to differentiate Gram-negative and Gram-positive bacteria on agar plate (Table 3.13). Phenotypical characteristic of presumptive bacteria were determined by Gram staining and further confirmed by biochemical tests in Table 3.14 (Carpenter et al., 1966).

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Table 3.12: Bacteria isolates in the study.

Bacteria isolates	Gram reaction	Morphology
Salmonella spp.	Negative	Appeared black halo colonies on HEA agar
Listeria spp.	Positive	Appeared blue-green colonies on PALCAM agar
E. coli	Negative	Appeared pink color colonies on MAC agar
S. aureus	Positive	Appeared yellow halo colonies on MSA agar

Table 3.13: Morphology of bacteria isolates on nutrient agar.

Bacteria isolates	Surface	Elevation	Edge	Color
Salmonella spp.	Smooth	Convex	Entire	Colorless
Listeria spp.	Smooth	Flat	Entire	Colorless
E. coli	Smooth	Convex	Entire	Colorless
S. aureus	Smooth	Convex	Entire	Yellow

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3.3.4.1 Gram Staining

Morphology, size, color and shape of bacteria strains were determined by Gram staining method (Hucker & Conn, 1923). Glass slide was cleaned with 70 % (v/v) ethanol before use. A circle was drawing on the glass slide to ensure the area for the smear and bacteria name was labeled on the side of the slide. A loopful of bacteria suspension was smeared on glass slide with circular motion. The smeared slide was allowed to air dry for 1 minute before proceed to heat fixing. A few drops of crystal violet were gently flooded on the smeared area and stand for 1 minute. Iodine was added on smeared area after rinse with tap water followed by 95 % (v/v) of ethyl alcohol. At last, safranin was added for counter stain purpose. The glass slide was viewed under light microscope with oil immersion. Gram-positive bacteria were stained purple color due to the crystal violet trapped within thick cell wall. Pink color was stained in Gram-negative bacteria due to the safranin retain on the thin cell wall. *E. coli* ATCC 25922 and *S. aureus* subspecies *aureus* ATCC 29213 were used as quality control strains for Gram-negative and Gram-positive bacteria species.

3.3.4.2 Biochemical Tests for Bacteria Isolates

IMViC Test

Indole Test

Indole test was performed by inoculating overnight culture into 4 ml of tryptophan broth and incubated at 37 ºC for 24 hours. Approximately 0.5 ml of Kovac's reagent was added into broth culture and the presence or absence of pink to red color ring was observed. *E. coli* ATCC 25922 serves as positive control while *Proteus mirabilis* serves as negative control for Indole test.

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Methyl Red and Voges-Proskauer Test

Overnight pure cultures inoculate into 5 ml of MR-VP broth and transferred 2.5 ml of culture into two clean tubes. First tube was added 5 drops of methyl red reagent and observed the color changes for methyl red test. Yellow color change to red color indicates the positive result obtained. *E. coli* ATCC 25922 serves as positive control while *Enterobacter cloacae* serves as negative control in methyl red test. Another tube was added 0.6 ml of Barritt's reagent A and followed by 0.2 ml of Barritt's reagent B. The tube was shaking for 1 minute and stand for 30 minutes at room temperature to observe the color changes for Voges-Proskauer test. The development of red complex on the top of the broth indicates positive result. *Enterobacter cloacae* serve as positive control while *E. coli* ATCC 25922 serves as negative control in VP test.

Simmons Citrate Test

Single colony was inoculating on Simmon citrate agar slant and incubated at 37 ºC for 18 to 24 hours to observe the development of blue color indicates positive result. *Klebsiella pneumonia* serves as positive control while *E. coli* ATCC 25922 serves as negative control.

Lysine Decarboxylase Test

Fresh pure culture inoculates into decarboxylase broth and overlay 1 ml of mineral oil with tighten cap. The tube was incubated at 37 ºC to observe color changes for 4 days. The developments of purple color indicate positive result while yellow colors indicate negative result in the decarboxylase test. Lysine decarboxylase positive bacteria are *Klebsiella pneumonia* and *Enterobacter cloacae* as lysine decarboxylase negative bacteria.

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Nitrate Reduction Test

Fresh cultures incubated in nitrate broth at 37 ℃ for 18 to 24 hours. Then, 5 drops of reagent A (containing α-naphthylamine) and 5 drops reagent B (containing sulfanilic acid) were added into tube containing culture to be test and shake to mix well. A distinct ink or red color was observed within 5 minutes, indicates nitrate reduction reaction. Small amounts of zinc powder will add into the suspension when is colorless after addition of reagent A and B and shaked for few minutes. *E. coli* ATCC 25922 used as quality control organism reduce nitrate (NO_3) into nitrite (NO_2) after added reagent A and B.

Carbohydrate Fermentation Test

Carbohydrate types prepared in the test include glucose, mannitol, sorbitol, arabinose, lactose, dextrose, maltose, rhamnose, xylose and sucrose. Carbohydrate consumption broth was prepared and autoclaved at 121 ℃ for 15 mins. About 0.5 to 1 % of desired carbohydrate was added into tube containing carbohydrate fermentation broth. Fresh pure culture was inoculated into desired carbohydrates tubes and incubated at 37 ℃ for 24 to 48 hours. The color change from bromo cresol purple into yellow color as indicates of positive reaction in carbohydrate fermentation test.

TSI Test

An overnight culture was inoculated to triple sugar iron agar slant using inoculating needle and incubated at 37 ℃ for 18 to 24 hours. The color changes of slant and gas formation was observed to determine glucose fermentation occur and hydrogen sulfide production.

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Urease Test

Overnight pure cultures inoculate into 4 ml of urea broth and incubated with loosened cap at 35 ºC. The color change of broth was observed at 8, 12, 24 and 48 hours. The development of bright pink color from gold yellow color indicates positive result. *Proteus mirabilis* serves as positive control while *E. coli* ATCC 25922 serves as negative control.

Oxidase Test

Oxidase disc was placed on the cleaned glass slide. A fresh single pure colony was massage on the oxidase disc and observed the development of deep purple color within 10 seconds indicates positive oxidase. Oxidase positive control bacteria are *Pseudomonas* spp. and *Enterobacter cloacae* serves as oxidase negative control bacteria.

ONPG Test

ONPG tablets dissolved in 0.85 % (v/v) saline water before started the test. A single fresh culture suspended directly to tube containing ONPG tablet in saline water and incubated the tube at 35 ºC. The color changes of saline for every hour intervals up to 6 hours were examined. The formation of yellow color in tube indicated ONPG positive as active lactose fermenters. ONPG positive organism is *E. coli* ATCC 25922 and ONPG negative is *Salmonella*.

Esculin Hydrolysis Test

Fresh overnight culture was aseptically streaked on esculin agar slant and incubated at 37 ºC for 24 hours. The growth on slant and dark brown color observed indicate esculin hydrolysis occur. *Listeria* spp. showed positive reaction in esculin hydrolysis, able to hydrolyze esculin compounds as carbon source.

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Catalase Test

Fresh colony suspended with saline on glass slide using sterile loop. A drop of 3 % (v/v) hydrogen peroxide put onto slide and mix. Bubble formation within 10 seconds indicates positive reaction; no bubble formation indicates negative reaction. *S. aureus* ATCC 29213 and *Listeria* spp. was able to hydrolyze hydrogen peroxide in the presence of catalase enzyme.

Motility Test

A colony was picked using sterile needle and stabbed into motility agar medium to within 1 cm of bottom tube. The medium incubated at 37 ºC for 18 to 24 hours. Motile organism (*E. coli* ATCC 25922) was observed by diffuse growth spreading from the line of inoculation; non-motile organism (*S. aureus* ATCC 29213) grows only along the line of inoculation.

Gelatinase Test

A heavy grow inoculums of test bacteria was stabbed into gelatin agar test tube at least half inch of the tube. The tube incubated at 37 ºC for 18 to 24 hours. The tube was immersed in an ice bath for 15 minutes to check the gelatin liquefaction by tilt the tubes at 45 degree. Organism able to produce gelatinase enzyme liquefy gelatin that show complete solidification in the tube.

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Starch Hydrolysis Test

Bacteria were streaked on starch agar and incubate at 37 ºC for 24 hours. About 10% (v/v) of Gram iodine was added onto starch agar with bacteria culture. The clear halo zone appeared on blue-brown starch agar after adding iodine indicates positive reaction in starch hydrolysis test. *Bacillus cereus* DSMZ 31 serves as positive control while *E. coli* ATCC 25922 serves as negative control for starch hydrolysis test.

Table 3.14: Biochemical tests in bacteria isolated.

Biochemical tests	***Salmonella* spp.**	***Listeria* spp.**	***E. coli***	***S. aureus***
Triple sugar iron	Alkaline slant, acid butt, + H_2S	Acid slant & butt, no H_2S	Acid slant & butt, no H_2S	Acid slant & butt, no H_2S
Indole	-	-	+	-
Methyl red	+	+	+	+
Voges-Proskauer	+	-	+	+
Simmon citrate	+	-	-	+
Urease	-	-	-	+
Oxidase	-	-	-	-
Lysine decarboxylase	+	-	N/A	N/A
ONPG	-	-	+	-
Catalase	-	+	-	+
Motility	+	+	+	-
Nitrogen reduction	-	+	-	+
Esculin hydrolysis	-	+	-	-

+ positive; - negative, N/A not applicable

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3.3.4.3 MALDI-TOF- MS

A fresh single colony of bacterial isolate was smeared onto the MSP 96 target plate and 1µl of matrix (10mg/ml α-cyano-4-hydroxycinnamic acid in 50 % acetonitrile and 2.5 % trifluoroacetic acid). Then, the dry matrix will perform using MALDI-TOF- MS analysis (Bruker) with UV laser at wavelength 337nm. Each smeared spot was measured by the MBT-autoX.axe autoExecute method and analyzed in Bruker MALDI Biotyper Real Time Classification (RTC) Version 3.1 (Build 65) software. The score values were required for genus level identification (Table 3.15) (Signor & Erba, 2013).

Table 3.15: Score values in MALDI-TOF-MS.

Range	Description
2.300-3.000	Highly probable species identification
2.000-2.299	Secure genus identification, probable species identification
1.700-1.999	Probable genus identification
0.000-1.699	Not reliable identification

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3.3.4.4 Viteks2 System

Identity and antibiotic susceptibility test of *Salmonella* and *Listeria* isolates were confirmed using Viteks2 system (bioMerieux). Two types of bacteria identity cards were used in the study including GN colorimetric identification cards (GN ID) for Gram-negative fermenting and non-fermenting bacilli identification while GP colorimetric identification cards (GP ID) for Gram-positive bacteria. The preparation of the test was performed according to manufacturer's protocol. Briefly, the bacteria isolates were subcultured for 16 to 18 hours at 37 ºC. Bacteria suspension was prepared using sterile swab to transfer sufficient number of colonies of pure culture and suspended in 3 mL sterile 0.45 % saline test tube. The turbidity was adjusted to McFarland 0.5 standards using turbidity meter called Densicheck. McFarland turbidity ranges for Gram-negative and positive bacteria were 0.50 to 0.63. Test tube containing bacteria suspension was placed into cassette rack and identification cards was placed in the neighbouring slots. Then, the filled cassette placed into vacuum station for analysis (Pincus, 2006). Result with MIC interpretive was obtained in a report form based on CLSI database and breakpoint (Appendices E and F).

3.3.4.5 Serological Test

All the *Salmonella, Listeria*, *S. aureus* and *E. coli* isolates were subjected to latex agglutination. *Salmonella* isolates were subjected to slide agglutination on lipopolysaccharide somatic (O antigen), flagellar (H antigen) and Vi antigen (Oxoids) (Shipp & Rowe, 1980). Control strains used in the study are *Salmonella enterica* subspecies *enterica* serovar Enteritidis ATCC 13076 ^{TM}, *Salmonella enterica* subspecies *enterica* serovar Typhimurium ATCC 14028^{TM} strain and *Salmonella enterica* serovar Typhi. Agglutination is considered as positive reaction while no agglutination indicates negative reaction on glass slide.

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3.3.5 Antibiotic Susceptibility Test

Antibiotic susceptibility test on *Salmonella*, *Listeria*, *E. coli* and *S. aureus* isolates in this study were performed using Kirby-Bauer agar disc diffusion method on Mueller-Hinton Agar (MHA) (Oxoid) (Cockerill & Franklin, 2011). Briefly, the isolates were subculture overnight in tryptic soy broth (TSB) and incubated at 37 °C. The bacteria suspension was adjusted to McFarland 0.5 standards before spread on surface of MHA agar using cotton swab. MHA agar was inoculated with inoculum using swab over entire agar surface and repeated twice by rotating plate 60 degree for each time to ensure even distribution of entire plate. The lid was allowed to dry for 10 minutes to ensure excess surface moisture will be absorbed before apply the antibiotic discs. Each specific concentration of antibiotic discs were applied on the MHA and incubated at 37 °C for 16 to18 hours.

The following 19 antibiotics with different concentrations were selected for *Salmonella* isolates in this study: ampicillin (AMP, 10 µg), tetracycline (TET, 30 µg), erythromycin (ERY, 15 µg), streptomycin (STR, 10 µg), chloramphenicol (CHL, 30 µg), trimethoprim (TMP, 5 µg), imipenem (IPM, 10 µg), ciprofloxacin (CIP, 5 µg), ceftazidime (CAZ, 30 µg), ceftriaxone (CRO, 30 µg), nalidixic acid (NAL, 30 µg), cefazolin (CFZ, 30 µg), cephalothin (CEF, 30 µg), nitrofuratoin (NIT, 300 µg), trimethoprim-sulfamethoxazole (SXT, 1.25/23.75 µg), tobramycin (TOB, 10 µg), gentamicin (GEN, 10 µg), azithromycin (AZM, 30 µg), polymyxin B (PMB, 300 µg). The reference strains, *E. coli* ATCC 25922 and *S. aureus* subspecies *aureus* ATCC 29213 were used as quality control strains.

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Next, a total of 11 antibiotics were selected for *Listeria* isolates: ampicillin (AMP, 10 µg), tetracycline (TET, 30 µg), erythromycin (ERY, 15 µg), penicillin G (PC-G, 10 U.I or 6 µg), rifampicin (RIF, 5 µg), clindamycin (CLI, 2 µg), vancomycin (VAN, 30 µg), cefazolin (CFZ, 30 µg), chloramphenicol (CHL, 30 µg), trimethoprim-sulfamethoxazole (SXT, 1.25/23.75 µg) and ceftriaxone (CRO, 30 µg). *Listeria monocytogenes* (ATCC 15313), *Listeria innocua* (DSMZ 20649) and *Listeria ivanovii* subspecies *ivanovii* (DSMZ 20750) were used as control strains.

Then, 12 antibiotics were selected for *E. coli* isolates: ampicillin (AMP, 10 µg), ceftriaxone (CRO, 30 µg), aztreonam (ATM, 30 µg), imipenem (IPM, 10 µg), ceftazidime (CAZ, 30 µg), amoxicillin-clavulanate (AMC, 20/10 µg), nalidixic acid (NAL, 30 µg), streptomycin (STR, 10 µg), trimethoprim-sulfamethoxazole (SXT, 1.25/23.75 µg), ciprofloxacin (CIP, 5 µg), tetracycline (TET, 30 µg), chloramphenicol (CHL, 30 µg). The reference strains, *E. coli* ATCC 25922 and *S. aureus* subspecies *aureus* ATCC 29213 were used as quality control strains.

And also 12 antibiotics were selected for *S. aureus* isolates: Penicillin G (PC-G, 10 U.I or 6 µg), oxacillin (OXA, 1 µg), cefazolin (CFZ, 30 µg), vancomycin (VAN, 30 µg), ciprofloxacin (CIP, 5 µg), tetracycline (TET, 30 µg), erythromycin (ERY, 15 µg), tobramycin (TOB, 10 µg), imipenem (IPM, 10 µg), clindamycin (CLI, 2 µg), ceftazidime (CAZ, 30 µg), trimethoprim-sulfamethoxazole (SXT, 1.25/23.75 µg). *E. coli* ATCC 25922 and *S. aureus* subspecies *aureus* ATCC 29213 were used as quality control strains.

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The result zone of inhibition will appeared uniform circular on the agar plate. The zone of inhibition were measured using ruler and categorized as susceptible (S), intermediate (I) and resistant (R) according to Clinical and Laboratory Standards Institute (CLSI) guideline (CLSI, 2012).

3.3.6 Multiple Antibiotic Resistances Index

Multiple Antibiotic Resistances (MAR) index of each isolate was ratio calculated based on formula:

MAR index = a / b

Where 'a' is number of resistance antibiotics to isolate was resistant
'b' number of antibiotics tested

Isolates were tested for antibiotic resistance on different antibiotics used for prophylactic in livestock. Isolates were recorded as resistant to an antibiotic if MAR index values are greater than 0.2 indicate high risk contamination of source (Hinton & Linton, 1983).

3.3.7 Molecular Analysis of Bacteria Isolates

3.3.7.1 Genomic DNA Extraction

Genomic DNA (gDNA) of *Salmonella* isolates were extracted using Masterpure™ DNA Purification kit (Epicentre) according to manufacturer protocol with slight modifications. Briefly, bacterial pellets were re-suspended in 300 µL of Tissue and Cell Lysis Solution containing proteinase K followed by incubation at 65ºC for 15 minutes with 300 rpm shaking using Thermomixer (Eppendorf) for every 5 minutes and placed on ice to cool down for 5 minutes. Then, 1 µl of RNase A was added into sample and incubated at 37 ºC for 30 minutes.

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After 30 minutes of incubation time, 175 µL of MPC Protein Precipitation Reagent was added into the lysed cells to remove presence of protein and subjected to centrifugation (13,000 × *g*, 10 minutes, 4 ºC). Supernatant was collected in clean 1.5 ml tubes and 500 µL chilled isopropanol was added for total nucleic acid precipitation followed by centrifugation (13,000 × *g*, 10 minutes, 4 ºC). The extracted DNA was washed twice with 500 µL of 70% (v/v) ethanol and 30 µl elution buffer was use to re-suspend the extracted genomic DNA. The quality and concentration of the gDNA was qualified and quantified using Nanodrop 2000 spectrophotometer (Thermo Scientific) and Qubit 2.0 Fluorometer (Life Technologies).The extracted DNA was stored at -20 °C for further analysis.

3.3.7.2 PCR Amplification, Agarose Gel Electrophoresis and Purification

Briefly, 1 µl of genomic DNA of *Salmonella* isolates was used as template DNA and added in total volume of 25 µl PCR reactions. The primers used and PCR reaction mixture were showed in Table 3.3 & 3.4. The antibiotic resistance genes as mentioned in Table 3.3 were detected in *Salmonella* isolates according to PCR condition: initial denaturation 94 °C for 2 minutes, followed by 30 cycles of 94 °C for 30s, 50 °C for 30s, 68 °C for 1 minute and final extension step of 68 °C for 5 minutes in thermal cycler (Bio-Rad, USA). *Salmonella enterica* subspecies *enterica* serovar Enteritidis ATCC 13076 ™ and *Salmonella enterica* subspecies *enterica* serovar Typhimurium ATCC 14028™ strain were used as control strain in the PCR reaction. Amplified PCR products of each samples (5 µl) was mixed with 1 µl of 6 × loading dye (Thermo Scientific) and loaded on 2.0 % (w/v) agarose gel with prestained 0.5 mg/ml of GelStar nucleic acid gel stain and visualized. The 100 bp ladder (Thermo Scientific) was used as reference molecular size and reaction mixture without DNA template serve as negative control.

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Electrophoresis was carried out in 1× Tris-borate-EDTA buffer at 80 V, 400 mA for 55 minutes. Electric current was migrate from anode (negative charge) to cathode (positive charge) and separate the band according to molecular weight. The electrophoresis was running until the loading dyes approaches the edge of the gel. The bands were visualized under UV light transilluminator (UVP) and photographed. Amplified products were excised from gel and purified using QIAquick® Gel Extraction kit (Qiagen) according to manufacturer protocol. About 300 µl of QG buffer was added into 1.5 ml tube containing 100 mg of gel and incubated at 50 °C for 10 minutes to dissolve the gel. Isopropanol was added into tubes and transferred into 2.0 ml spin column and followed by centrifugation (10,000 × *g*, 1 minute). The flow through was discard and 750 µl of PE buffer was added to wash the column and followed by centrifugation (10,000 × *g*, 1 minute).

Purified product was eluted with 30 µl of elution buffer. Concentration of purified PCR products was measured using by Nanodrop 2000 spectrophotometer (Thermo Scientific) before sending to DNA sequencing (1st Base sequencing). The gene sequences data was analyzed using Applied Biosystems Sequence Scanner software tool (version 2.0, Applied Biosystems). DNA sequence was analyzed using NCBI server (National Center for Biotechnology Information)-BLASTN program with the website link (http://www.ncbi.nlm.nih.gov).

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3.3.7.3 PFGE of *Salmonella* and *E. coli* Isolates

PFGE was performed for 38 *Salmonella* spp. and 20 *E. coli* genetic subtyping according to PulseNet CDC protocol (standard operating procedure for PulseNet PFGE of *E. coli* O157: H7, *E. coli* non-O157 (STEC), *Salmonella* serotypes, *Shigella sonnei* and *Shigella flexneri*) (CDC, 2013e). In brief, *Salmonella* isolates were cultured on tryptic soy agar (Merck) at 37 ºC overnight. Desired optical density (OD) 0.8 of bacteria suspension was adjusted with cell suspension buffer (100mM Tris: 100mM EDTA, pH 8.0) and measured with Nanodrop 2000 spectrophotometer (Thermo Scientific) at 610 nm. About 200 µl of bacteria suspension was added into 1.5 ml tubes that containing 10 µl proteinase K (20 mg/ml stock). Then, the 200 µl of 1 % (w/v) Seakem gold agarose was added into tube and make up to 4 plugs (Table 3.16). The agarose plug was allowed to solidify for 10 minutes at room temperature.

The gDNA plugs were lysed into 5 ml of cell lysis buffer (50mM Tris: 50mM EDTA, pH 8.0 + 1% (w/v) sarcosyl) and 10 µl of proteinase K in a 50 ml tube at 55 ºC with shaking speed of 175 rpm for 2 hours. After 2 hours of lysis process, the plugs were washed with sterile ultrapure clinical laboratory reagent water twice and washed with 1× TE buffer (10mM Tris: 1mM EDTA, pH 8.0) in four times at every interval of 10 minutes. About 1mm of plugs was cut and pre incubated with restriction buffer for 10 minutes and then digested with restriction enzyme, 25 U XbaI (NEB) for 2 hours (Table 3.17). Subsequently, the digested plugs was removed TE buffer and replaced with 200 µl of 0.5 × TBE buffer for 10 minutes before run on 1% certified megabase agarose gel for 18 hours using CHEF-DR II Mapper electrophoresis system (Bio-Rad, USA).

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Salmonella enterica serovar Braenderup H9812 (ATCC BAA-664) is used as PFGE standard molecular marker ladder. The running condition was showed in Table 3.18 for *Salmonella* spp. and for non *E. coli* O157 showed in Table 3.19. The gel was stained with gel red stain for an hour in a closed container after the electrophoresis is over and DNA bands were visualized under UV transilluminator. Fingerprint profile was analyzed using BioNumeric fingerprint software (version 7, Applied Biomath). A dendrogram was constructed and genotypes were determined with cut off value of 80 % in similarity based on unweighted pair group method with arithmetic mean (UPGMA) after background subtraction and gel normalization. Similarity index was calculated using DICE correlation coefficient option with tolerance of 0.5 % and optimization of 0.5 %.

Table 3.16: Pre-incubation step for *Salmonella* and *E. coli* isolates.

Reagent	µl/Plug slice	µl/10 plug slice	µl/15 plug slice
CLRW	180	1800	2700
10× NEB Restriction Buffer	20	200	300
Total volume	200	2000	3000

Table 3.17: Restriction enzyme step for *Salmonella* and *E. coli* isolates.

Reagent	µl/Plug slice	µl/10 plug slice	µl/15 plug slice
CLRW	173	1730	2595
10× Restriction buffer	20	200	300
BSA (10 mg/ ml)	2	20	30
XbaI (10U /µl)	5	50	75
Total volume	200	2000	3000

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Table 3.18: Auto algorithm condition in CHEF-DR II for *Salmonella* isolates.

Components	Running condition
Low molecular weight	30kb
High molecular weight	700kb
Initial switch time	2.2s
Final switch time	63.8s
Running time	18 hours
Voltage	6V
Angles	120º
Ratio	1.0

Table 3.19: Auto algorithm condition in CHEF-DR II for non *E. coli* O157:H7.

Components	Running condition
Low molecular weight	50 kb
High molecular weight	400 kb
Initial switch time	6.76 s
Final switch time	35.38 s
Running time	18 hours
Voltage	6V
Angles	120º
Ratio	1.0

3.3.7.4 PFGE of *Listeria* Isolates

PFGE was performed for 28 *Listeria* genetic subtyping according to PulseNet CDC protocol (standard operating procedure for PulseNet PFGE of *Listeria monocytogenes*). *Listeria* isolates were cultured on tryptic soy agar with 5 % sheep blood agar (Merck) at 37 ºC overnight. Desired optical density (OD) 2.0 of bacteria suspension was adjusted with cell suspension buffer (100mM Tris: 100mM EDTA, pH 8.0) and measured with Nanodrop 2000 spectrophotometer (Thermo Scientific) at 610 nm. The 200 µl of bacteria suspension was added into 1.5 ml tubes that containing 10 µl of lysozyme (20 mg/ml) and incubated at 55 ºC for 10 minutes. After 10 minutes, 10 µl of proteinase K (20 mg/ml stock) was added into tube and mix gently.

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Then, the 200 µl of 1 % (w/v) Seakem gold agarose + 0.5% (w/v) sodium dodexxyl sulfate (SDS) was added into tube and makes up to 4 plugs (Table 3.20). The agarose plug was solidified for 10 minutes at room temperature. The genomic DNA plugs were lysed into 5 ml of cell lysis buffer (50mM Tris: 50mM EDTA, pH 8.0 + 1% (w/v) sarcosyl) and 10 µl of proteinase K in a 50 ml tube at 55 ºC with shaking speed of 175 rpm for 2 hours. After 2 hours of lysis process, the plugs were washed with sterile ultrapure clinical laboratory reagent water twice and washed with 1 × TE buffer (10mM Tris: 1mM EDTA, pH 8.0) in four times at every interval of 10 minutes. About 1 mm of plugs was cut and pre incubated with restriction buffer for 10 minutes and then digested with restriction enzyme, AscI for 1.5 hours (Table 3.21). Subsequently, the digested plugs was removed TE buffer and replaced with 200 µl of 0.5 × TBE buffer for 10 minutes before run on 1% (w/v) certified megabase agarose gel for 18 hours using CHEF-DR II Mapper electrophoresis system (Bio-Rad, USA).

Salmonella Braenderup H9812 used as PFGE standard molecular marker digested with XbaI. The PFGE running condition was showed in Table 3.22. The gel was stained with gel red stain for an hour in a closed container after the electrophoresis is over and DNA bands were visualized under UV transilluminator. Fingerprint profile were analyzed using BioNumeric software (version 7, Applied Biomath) and the genotypes were determined with cut off value of 80 % in similarity coefficient based on unweighted pair group method with arithmetic mean (UPGMA).

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Table 3.20: Pre-incubation step for *Listeria* isolates.

Reagent	µl/Plug slice	µl/10 plug slice	µl/15 plug slice
CLRW	180	1800	2700
10× NEB Restriction Buffer	20	200	300
Total volume	200	2000	3000

Table 3.21: Restriction enzyme step for *Listeria* isolates.

Reagent	µl/Plug slice	µl/10 plug slice	µl/15 plug slice
CLRW	175.5	1755	2632.5
10× NEB Restriction Buffer	20	200	300
BSA (10 mg/ ml)	2	20	30
AscI (10U/ µl)	2.5	25	37.5
Total volume	200	2000	3000

Table 3.22: Auto algorithm condition in CHEF-DR II for *Listeria* isolates.

Components	Running condition
Low molecular weight	49kb
High molecular weight	450kb
Initial switch time	4.0s
Final switch time	40.0s
Running time	18 hours
Voltage	6V
Angles	120º
ratio	1.0

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3.3.7.5 Genome Sequencing

A total of four *Salmonella* strains named S1, S7, S12 and S13 were selected for genome sequencing and renamed to PS01, PS07, PS12 and PS13. The next generation sequencing (NGS) was applied sequencing by synthesis technology uses four fluorescent labeled nucleotide on flow cell surface. Reversible terminator based method able to detect single bases. Quality of the library was determined using Agilent 2100 bioanalyzer with high sensitivity DNA chip kit. Template library was prepared using Nextera DNA library kit and index kit (Illumina, USA) according to manufacturer's protocol.

3.3.8 Genome Annotation

Quality of collected raw data from sequencer will firstly analyze using FastQC software followed by trimming and *de novo* assembly using CLC Genomic Workbench version 7.5 (CLC Bio, Denmark). *Salmonella* genome in fasta file will loaded to SISTR online tool and obtained predicted *Salmonell*a serovars based on the database (Yoshida et al., 2016). The genome will be annotated using software listed as follow (Table 3.23):

Table 3.23: Software for genome annotation.

Softwares	Descriptions	References
Prodigal server	Genome annotation	Hyatt et al., 2010
PROKKA (Rapid Prokaryotic genome annotation)	Genome annotation	Seemann, 2014
RAST (Rapid annotation server)	Genome annotation	Aziz et al., 2008
RNAmmer 1.2 server	RNA prediction	Lagesen et al., 2007
tRNAscan-SE	tRNA prediction	Lowe & Eddy, 1997
UniProt (universal Protein resource)	Protein sequence and function	Consortium, 2014

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CHAPTER 4: RESULTS

4.1 Bacteria Isolation and Characterization

4.1.1 Total Viable Count of Bacteria Isolates

Table 4.1 showed total viable count of bacteria isolated for chicken meat, eggs and feeds using nutrient agar. The result indicated poultry meat serve as food reservoir of bacteria. Contamination levels of bacteria in chicken meat were higher bacterial count than those in the chicken eggs. A high bacterial count was found in condition with chicken skin as compared without chicken skin. Chicken collected from wet markets were indicating higher bacterial counts than chicken purchased in hypermarkets.

Table 4.1: Total viable count of bacteria isolated for chicken meat, eggs and feeds.

Sample types	Source	Range of total aerobic count (CFU/g)
Chicken (with skin)	Hypermarket	2.7×10^3 - 3×10^4
Chicken (with skin)	Wet market	$5.4 \times 10^3 - 8 \times 10^4$
Chicken (without skin)	Hypermarket	$1.6 \times 10^3 - 1 \times 10^4$
Chicken (without skin)	Wet market	$2.6\times 10^3 - 1 \times 10^4$
Chicken eggs	Hypermarket	$2.4 \times 10^1 - 4 \times 10^1$
	Wet market	$3.6 \times 10^1 - 8 \times 10^1$
Chicken feeds	Retail market	0

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4.1.2 MALDI-TOF identification of Gram-negative bacteria

Morphology and Gram reaction of all the presumptive bacteria isolates were identified and the genus identification were confirmed by using MALDI-TOF, latex agglutination and numerous of different biochemical tests.

Genus identification was performed in all Gram-negative isolates using MALDI-TOF-MS (Table 4.2). Based on the score value were interpreted according to Table 3.15, all 38 *Salmonella* and 20 *E. coli* isolates were able to identify up to genus level.

Table 4.2: Genus identification of Gram-negative isolates using MALDI-TOF-MS.

Isolate label	Organism Identification	Score
S1	*Salmonella* spp.	2.409
S2	*Salmonella* spp.	2.254
S3	*Salmonella* spp.	2.415
S4	*Salmonella* spp.	2.437
S5	*Salmonella* spp.	2.365
S6	*Salmonella* spp.	2.353
S7	*Salmonella* spp.	2.410
S8	*Salmonella* spp.	2.337
S9	*Salmonella* spp.	2.256
S10	*Salmonella* spp.	2.342
S11	*Salmonella* spp.	2.368
S12	*Salmonella* spp.	2.438
S13	*Salmonella* spp.	2.441
S14	*Salmonella* spp.	2.368
S15	*Salmonella* spp.	2.357
S16	*Salmonella* spp.	2.423
S17	*Salmonella* spp.	2.401
S18	*Salmonella* spp.	2.396
S19	*Salmonella* spp.	2.431
S20	*Salmonella* spp.	2.393
S21	*Salmonella* spp.	2.459
S22	*Salmonella* spp.	2.296
S23	*Salmonella* spp.	2.348
S24	*Salmonella* spp.	2.330
S25	*Salmonella* spp.	2.330
S26	*Salmonella* spp.	2.335

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Table 4.2, continued.

S27	*Salmonella* spp.	2.276
S28	*Salmonella* spp.	2.301
S29	*Salmonella* spp.	2.236
S30	*Salmonella* spp.	2.225
S31	*Salmonella* spp.	2.394
S32	*Salmonella* spp.	2.314
S33	*Salmonella* spp.	2.368
S34	*Salmonella* spp.	2.406
S35	*Salmonella* spp.	2.363
S36	*Salmonella* spp.	2.394
S37	*Salmonella* spp.	2.350
S38	*Salmonella* spp.	2.329
E1	*Escherichia coli*	2.090
E2	*Escherichia coli*	2.268
E3	*Escherichia coli*	2.109
E4	*Escherichia coli*	2.143
E5	*Escherichia coli*	2.200
E6	*Escherichia coli*	2.174
E7	*Escherichia coli*	2.115
E8	*Escherichia coli*	2.189
E9	*Escherichia coli*	2.175
E10	*Escherichia coli*	2.279
E11	*Escherichia coli*	2.045
E12	*Escherichia coli*	2.047
E13	*Escherichia coli*	2.238
E14	*Escherichia coli*	2.380
E15	*Escherichia coli*	2.207
E16	*Escherichia coli*	2.270
E17	*Escherichia coli*	2.292
E18	*Escherichia coli*	2.264
E19	*Escherichia coli*	2.271
E20	*Escherichia coli*	2.380

MALDI-TOF-MS only identified until genus level, species level required subtyping confirmation.

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4.1.3 MALDI-TOF identification of Gram-positive bacteria

All the presumptive of Gram-positive bacteria were subjected to MALDI-TOF-MS for genus confirmation. *Listeria* isolates were grows well on tryptone soya yeast extract agar than nutrient agar at 37 ºC.

All *Listeria* isolates were streaked on TYSEA agar while *S. aureus* isolates were streaked on nutrient agar to obtain single pure colony. Based on the score value were interpreted according to Table 3.15, all 28 *Listeria* and 3 *S. aureus* isolates were able to identify up to genus level (Table 4.3).

Table 4.3: Genus identification of Gram-positive isolates using MALDI-TOF-MS.

Isolate label	Organism Identification	Score
L1	*Listeria* spp.	1.864
L2	*Listeria* spp.	1.892
L3	*Listeria* spp.	1.745
L4	*Listeria* spp.	1.914
L5	*Listeria* spp.	2.165
L6	*Listeria* spp.	1.845
L7	*Listeria* spp	2.165
L8	*Listeria* spp.	1.845
L9	*Listeria* spp.	1.949
L10	*Listeria* spp.	2.320
L11	*Listeria* spp.	2.192
L12	*Listeria* spp.	2.219
L13	*Listeria* spp.	2.029
L14	*Listeria* spp.	2.045
L15	*Listeria* spp.	2.036
L16	*Listeria* spp.	1.979
L17	*Listeria* spp.	1.951
L18	*Listeria* spp.	1.937
L19	*Listeria* spp.	2.238
L20	*Listeria* spp.	2.070
L21	*Listeria* spp.	1.978
L22	*Listeria* spp.	1.977
L23	*Listeria* spp.	1.899
L24	*Listeria* spp.	1.980
L25	*Listeria* spp.	1.997
L26	*Listeria* spp.	1.946

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Table 4.3, continued.

L27	*Listeria* spp.	2.038
L28	*Listeria* spp.	1.934
SA1	*Staphylococcus aureus*	2.010
SA2	*Staphylococcus aureus*	2.232
SA3	*Staphylococcus aureus*	2.118

MALDI-TOF-MS only identified until genus level, species level required subtyping confirmation.

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4.1.4 Prevalence of Bacteria Isolated

In the study, a total of 100 samples (chicken meat, eggs and feeds) were collected from local wet and supermarket in Klang Valley, Malaysia. Thirty-eight of *Salmonella* spp., 28 *Listeria* spp., 20 *E. coli* and 3 *S. aureus* were obtained among 100 samples (Table 4.4).

Table 4.4: Prevalence of *Salmonella* spp., *Listeria* spp., *E. coli* and *S. aureus* isolated from chicken meat, eggs and feeds in Klang Valley.

Food samples	Source	No. of samples	No. of sample positive for *Salmonella* spp. (%)	No. of sample positive for *Listeria* spp. (%)	No. of sample positive for *E. coli* (%)	No. of sample positive for *S. aureus* (%)
Chicken meat (with skin)	Hypermarket	8	4 (50)	6 (75)	8 (100)	0
Chicken meat (with skin)	Wet market	17	15 (88.5)	8 (47.1)	6 (35.3)	3 (17.6)
Chicken meat (without skin)	Hypermarket	8	4 (50)	6 (75)	2 (25)	0
Chicken meat (without skin)	Wet market	17	15 (88.5)	8 (47.1)	4 (23.5)	0
Chicken eggs	Hypermarket	20	0	0	0	0
	Wet market	20	0	0	0	0
Chicken feeds	Retail market	10	0	0	0	0
Total		100	38	28	20	3

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4.2 Antibiotic Resistance Profile in Bacteria Isolates

4.2.1 Antibiotics Resistance Profiles of *Salmonella* Isolates

As shown in Table 4.5, ten antibiotic classes (beta-lactam, phenicols, quinolones and fluoroquinolones, tetracycline, aminoglycosides, marcolides, sulfonamides, carbapenem, nitrofurans and polymyxin groups) were selected for *Salmonella* isolates. All of the *Salmonella* isolates were resistant to erythromycin (100 %), and majority of them were resistant to tetracycline (76 %), azithromycin (58 %) and streptomycin (24 %). Low percentage resistances to some of the antibiotics include ampicillin (18 %), chloramphenicol (18 %), trimethoprim (18 %), nalidixic acid (18 %), gentamicin (13 %) and ciprofloxacin (5 %). There are 34 % of *Salmonella* isolates were resistant to sulfonamides and synergistic agents, 21 % and 50 % of *Salmonella* isolates intermediate to nalidixic acid and ciprofloxacin. All the isolates were susceptible to polymyxin B, cefazolin, cephalothin, ceftriaxone, ceftazidime and imipenem.

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Table 4.5: Antibiotics resistance of *Salmonella* isolates in the study.

Antibiotic types	Number of isolates (%)		
	Resistant (R)	**Intermediate (I)**	**Susceptible (S)**
Beta-lactams			
Ampicillin (10 µg)	7 (18)	0	31 (82)
Ceftriaxone (30 µg)	0	0	38 (100)
Cefazolin (30 µg)	0	0	38 (100)
Cephalothin (30 µg)	0	0	38 (100)
Ceftazidime (30 µg)	0	0	38 (100)
Phenicols			
Chloramphenicol (30 µg)	7(18)	0	31 (82)
Tetracycline			
Tetracycline (30 µg)	29 (76)	0	9 (24)
Quinolones and Fluoroquinolones			
Nalidixic acid (30 µg)	7 (18)	8 (21)	23 (61)
Ciprofloxacin (5 µg)	2 (5)	19 (50)	17 (45)
Aminoglycosides			
Streptomycin (10 µg)	9 (24)	16(42)	13 (34)
Gentamicin (10 µg)	5 (13)	0	33 (87)
Tobramycin (10 µg)	2 (5)	0	36 (95)
Macrolides			
Erythromycin (15 µg)	38 (100)	0	0
Azithromycin (30 µg)	22 (58)	12 (32)	4 (10)
Sulfonamides and synergistic agents			
Trimethoprim-sulfamethoxazole (25 µg)	6 (16)	1 (2)	31 (82)
Trimethoprim (5 µg)	7 (18)	0	31 (82)
Carbapenems			
Imipenem (10 µg)	0	0	38 (100)
Nitrofurans			
Nitrofuratoin (300 µg)	2(5)	0	36(95)
Polymyxin			
Polymyxin B (300 µg)	0	0	38 (100)

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Table 4.6: Antibiotics resistance patterns of *Salmonella* isolates.

Multiple resistance patterns	Resistance patterns	No. of isolates (%)
One type of antibiotic	ERY	3 (7.9)
Two type of antibiotics	ERY- AZT	3 (7.9)
	ERY- TET	7 (18.4)
Three type of antibiotics	ERY- TET- AMP	1 (2.6)
	ERY- GEN- NAL	1 (2.6)
	ERY- TET- AZT	6 (15.8)
	ERY- TET- STR	2 (5.3)
	ERY- STR- AZT	1 (2.6)
Four type of antibiotics	ERY- TET- TMP- NAL	1 (2.6)
	ERY- TET- AZT- STR	4 (10.5)
	ERY- TET- AZT- CHL	1 (2.6)
	ERY- TET- AZT- CIP	1 (2.6)
	ERY- TET- AZT- GEN	1 (2.6)
Seven type of antibiotics	ERY- AZT- AMP- STR- SXT- TMP- CHL	1 (2.6)
	ERY- TET- AMP- TMP- SXT- CHL- NAL	1 (2.6)
Eight type of antibiotics	ERY- TET- AMP- AZT- TMP- SXT- CHL- NAL	2 (5.3)
≥Eight type of antibiotics	ERY- TET- AMP- AZT- TMP- SXT- CHL- GEN- TOB	1 (2.6)
	ERY- TET- AMP- AZT- TMP- SXT- CHL- GEN- TOB-STR- CIP- NAL	1 (2.6)
Total		38

ERY, erythromycin; AZT, azithromycin; TET, tetracycline; AMP, ampicillin; GEN, gentamicin; NAL, nalidixic acid; STR, streptomycin; TMP, trimethoprim; CIP, ciprofloxacin; SXT, trimethoprim- sulfamethoxazole; CHL, chloramphenicol; TOB, tobramycin.

Antibiotic resistance patterns in *Salmonella* spp. were showed in Table 4.6. Antibiotic resistance patterns were recorded including 3 *Salmonella* isolates (7.9 %) were resistant to at least one type of antibiotics, 10 *Salmonella* isolates (26.3 %) were resistant to two type of antibiotics, 11 *Salmonella* isolates (28.9 %) were resistant to three types of antibiotics, 8 *Salmonella* isolates (20.9 %) were resistant to four types of antibiotics and 6 *Salmonella* isolates (15.7 %) were resistance at least seven antibiotics.

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Table 4.7: MAR indexes of *Salmonella* isolates.

MAR index	Resistance patterns	*Salmonella* isolates	Percentages of isolates (%)
0.63 (12/19)	ERY- TET- AMP- AZT- TMP- SXT- CHL- GEN- TOB-STR- CIP- NAL	S22	2.6
0.47 (9/19)	ERY- TET- AMP- AZT- TMP- SXT- CHL- GEN- TOB	S1	2.6
0.42 (8/19)	ERY- TET- AMP- AZT- TMP- SXT- CHL- NAL	S19 & 20	5.3
	ERY- AMP- AZT- TMP- SXT- CHL- NAL- STR	S37	2.6
0.37 (7/19)	ERY- TET- AMP- TMP- SXT- CHL- NAL	S10	2.6
0.2 (4/19)	ERY- TET- AZT- CHL	S2	2.6
	ERY- TET- AZT- NIT	S3,S4	5.3
	ERY- TET- TMP- NAL	S8	2.6
	ERY- TET- AZT- STR	S12,S29,S30, S35	10.5
	ERY- TET- AZT- CIP	S11	2.6
	ERY- TET- AZT- GEN	S14	2.6

ERY, erythromycin; AZT, azithromycin; TET, tetracycline; AMP, ampicillin; GEN, gentamicin; NAL, nalidixic acid; STR, streptomycin; TMP, trimethoprim; CIP, ciprofloxacin; SXT, trimethoprim- sulfamethoxazole; CHL, chloramphenicol; TOB, tobramycin.

Multiple antibiotic resistance (MAR) values of 38 *Salmonella* isolates were showed in Table 4.7. Only 16 *Salmonella* isolates were classified under MAR index varied from 0.2 to 0.63 in the study. A MAR index value was calculated according to section 3.36. Strain S22 was the highest MAR value of 0.63 (2.6 %) and followed by MAR value of 0.47 in 1 isolate (2.6 %) and 0.42 in 3 isolates (10.5 %), respectively. The finding obtained six resistance patterns with MAR index value of 0.2 in 10 *Salmonella* isolates.

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4.2.2 Antibiotics Resistance Profiles of *Listeria* Isolates

Antibiotics resistance profiles of 28 *Listeria* isolates were tested with 11 selected antibiotics in Table 4.8. Three categories were used to classify the isolates as resistant, intermediate and susceptible. The breakpoint of the *Listeria* isolates was based on *Staphylococcus aureus* in CLSI guideline. *Listeria* isolates were resistant to clindamycin, tetracycline, ceftriaxone and ampicillin in 89 %, 86 %, 75 % and 64 % respectively. Fourteen of each *Listeria* isolates were resistant and susceptible to penicillin G (50 %). A total of 14 % *Listeria* isolates were categorized as resistant to erythromycin, whereas 4 % isolates were intermediate and 82 % susceptible respectively. All 28 *Listeria* isolates were susceptible to trimethoprim- sulfamethoxazole and rifampicin.

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Table 4.8: Antibiotics resistance profile of *Listeria* isolates in the study.

Antibiotic types	Number of isolates (%)		
	Resistant (R)	**Intermediate (I)**	**Susceptible (S)**
Beta-lactams			
Ampicillin (10 µg)	18 (64)	0	10 (36)
Ceftriaxone (30 µg)	21 (75)	6(21)	1 (4)
Cefazolin (30 µg)	0	1(4)	27 (96)
Penicillin G (6 µg)	14 (50)	0	14 (50)
Phenicols			
Chloramphenicol (30 µg)	3(11)	0	25 (89)
Tetracycline			
Tetracycline (30 µg)	24 (86)	0	4 (14)
Macrolides			
Erythromycin (15 µg)	4 (14)	1 (4)	23 (82)
Sulfonamides and synergistic agents			
Trimethoprim-sulfamethoxazole (25 µg)	0	0	28 (100)
Glycopeptides			
Vancomycin (30 µg)	0	0	28 (100)
Lincosamides			
Clindamycin (2 µg)	25 (89)	3 (11)	0
Others			
Rifampicin (5 µg)	0	0	28 (100)

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Table 4.9: Antibiotics resistance patterns of *Listeria* isolates.

Multiple resistance patterns	Resistance patterns	No. of isolates (%)
One type of antibiotic	TET	1 (3.6)
Two type of antibiotics	TET- ERY	1 (3.6)
Three type of antibiotics	TET- AMP- CLI	2 (7)
	TET- CLI- CHL	1 (3.6)
	TET- CLI- CRO	3 (10.7)
	TET- AMP- PC-G	1 (3.6)
	AMP- CLI- CRO	1 (3.6)
	CLI- CRO- PC-G	1 (3.6)
Four type of antibiotics	TET- AMP- CLI- CRO	1 (3.6)
	TET- ERY- CLI- CRO	1 (3.6)
	TET- AMP- CLI- CRO	1 (3.6)
	TET- PC-G- CLI- CRO	1 (3.6)
	AMP- PC-G- CLI- CRO	2 (7)
Five type of antibiotics	TET- AMP- ERY- CLI- CHL	1 (3.6)
	TET- ERY- CLI- CRO- CHL	1 (3.6)
	TET- AMP- PC-G- CLI- CRO	9 (32.1)
TOTAL		28(100)

ERY, erythromycin; TET, tetracycline; AMP, ampicillin; CHL, chloramphenicol; PC-G, penicillin G; CLI, clindamycin; CRO, ceftriaxone.

Analysis of antibiotics resistance pattern in *Listeria* isolates were showed in Table 4.9. It categorized as five pattern classes including resistance to two types, three types, four types, five types and six types of antibiotics were tested in 28 *Listeria* isolates. In the finding observed 16 multiple antibiotic resistance patterns in *Listeria* isolates. One isolate (3.6 %) was resistance to two types of antibiotics, 1 isolate (3.6 %) was resistance to three types of antibiotics and 26 isolates (92.8 %) were define as multidrug resistance isolates showed resistant to more than four types of antibiotics.

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Table 4.10: MAR index of *Listeria* isolates.

MAR index	Resistance patterns	*Listeria* isolates	Percentage of isolates (%)
0.18 (2/11)	TET- ERY	L18	3.6
0.27 (3/11)	TET- AMP- CLI	L1, L8	32.1
	PC-G- CLI- CRO	L11	
	AMP- CLI- CRO	L12	
	TET- AMP- PC-G	L13	
	TET- CLI- CHL	L14	
	TET- CLI- CRO	L15, L19, L20	
0.36 (4/11)	TET- AMP- CLI- CRO	L3	21.4
	TET- ERY- CLI- CRO	L4	
	TET- AMP- CLI- CRO	L5	
	TET- PC-G- CLI- CRO	L17	
	AMP- PC-G- CLI- CRO	L27, L28	
0.45 (5/11)	TET- AMP- ERY- CLI- CHL	L2	39.3
	TET- ERY- CLI- CRO- CHL	L6	
	TET- AMP- PC-G- CLI- CRO	L7, L9, L10, L21, L22, L23, L24, L25, L26	

ERY, erythromycin; TET, tetracycline; AMP, ampicillin; CHL, chloramphenicol; PC-G, penicillin G; CLI, clindamycin; CRO, ceftriaxone.

MAR index value was showed in the range of 0.18 until 0.45 with 15 resistance patterns in 28 *Listeria* isolates (Table 4.10). A MAR index value was calculated according to section 3.36. The highest index value 0.45 was obtained that displayed four different types resistance patterns in 11 *Listeria* isolates (39.3 %). In contrast, the lowest index 0.18 was observed in one isolate (3.6 %). Nine *Listeria* isolates (32.1 %) were showed six resistance patterns with MAR index value of 0.27 and 6 isolates (21.4 %) were showed five resistance patterns with MAR index of 0.36 respectively.

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4.2.3 Antibiotics Resistance Profiles of *Escherichia coli* Isolates

Antibiotics resistance profiles of 20 *E. coli* isolates were tested with 12 selected antibiotics showed in Table 4.11. All of the *E. coli* isolates were resistant to tetracycline and majority of *E. coli* isolates were resistant to ampicillin, nalidixic acid and amoxcillin-clavulanic acid in 95 %, 90 % and 80 % respectively. Each of 15 *E. coli* isolates was resistant to chloramphenicol, trimethoprim- sulfamethoxazole and streptomycin. Majority of *E. coli* isolates (>90 %) were susceptible to aztreonam, imipenem and ceftazidime.

Table 4.11: Antibiotic resistance profile of *E. coli* isolates in the study.

Antibiotic types	Number of isolates (%)		
	Resistant (R)	**Intermediate (I)**	**Susceptible (S)**
Beta-lactams			
Ampicillin (10 µg)	19 (95)	0	1(5)
Ceftriaxone (30 µg)	2 (10)	3(15)	15 (75)
Ceftazidime (30 µg)	0	2(10)	18 (90)
Amoxcillin-Clavulanic acid (30 µg)	16 (80)	3(15)	1(5)
Phenicols			
Chloramphenicol (30 µg)	15(75)	0	5 (25)
Tetracycline			
Tetracycline (30 µg)	20 (100)	0	0
Quinolones and Fluoroquinolones			
Nalidixic acid (30 µg)	18 (90)	1(5)	1 (5)
Ciprofloxacin (5 µg)	9 (45)	7 (35)	4 (20)
Sulfonamides and synergistic agents			
Trimethoprim-sulfamethoxazole (25 µg)	15 (75)	1 (5)	4 (20)
Carbapenems			
Imipenem(10 µg)	0	1(5)	19(95)
Aminoglycoside			
Streptomycin (10 µg)	15 (75)	3 (15)	2 (10)
Monobactams			
Aztreonam (30 µg)	0	0	20 (100)

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Table 4.12: Antibiotics resistance patterns of *E. coli* isolates.

Multiple resistance patterns	Resistance patterns	No. of isolates (%)
Four type of antibiotics	TET- STR-NAL- CIP	1 (5)
Five type of antibiotics	AMP- TET- AMC- SXT- CHL	1 (5)
	AMP- TET- AMC- NAL- CIP	1 (5)
	AMP- TET- AMC- STR- CHL	1 (5)
	AMP- TET- NAL- SXT- CHL	1 (5)
Six type of antibiotics	AMP- TET- AMC- NAL- SXT- CHL	1 (5)
	AMP- TET- AMC- NAL- CRO- STR	1 (5)
	AMP- TET- AMC- NAL- STR- CHL	1 (5)
	AMP- TET- NAL- SXT- STR- CHL	1 (5)
Seven type of antibiotics	AMP- TET- AMC- NAL- STR- SXT- CHL	4 (20)
	AMP- TET- AMC- NAL- STR- SXT- CIP	2 (10)
	AMP- TET- NAL- STR- SXT- CHL- CIP	1 (5)
Eight type of antibiotics	AMP- TET- AMC- NAL- SXT- CHL- CRO- CIP	1 (5)
	AMP- TET- AMC- STR- NAL- SXT- CHL- CIP	3 (15)
TOTAL		20 (100)

TET, tetracycline; AMP, ampicillin; NAL, nalidixic acid; CHL, chloramphenicol; AMC, amoxcillin-clavulanic acid; STR, streptomycin; CRO, ceftriaxone; CIP, ciprofloxacin; SXT, trimethoprim- sulfamethoxazole

Analysis of antibiotics resistance pattern in 20 *E. coli* isolates showed in Table 4.12. It categorized under five pattern classes including resistance to four types, five types, six types, seven types and eight types of antibiotics. Fourteen of multiple antibiotic resistance patterns in *E. coli* isolates. One isolates (5 %) were showed resistant to four types of antibiotics, 4 isolate (20 %) was resistant to five types of antibiotics and 15 isolates (75 %) were define as multidrug resistance isolates shown resistant to more than six types of antibiotics.

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Table 4.13: MAR index of *E. coli* isolates.

MAR index	Resistance patterns	*E. coli* isolates	Percentages of isolates (%)
0.67	AMP- TET- AMC- NAL- SXT- CHL- CIP- CRO	E5	5
(8/12)	AMP- TET- AMC- STR- NAL- SXT- CHL- CIP	E8 ,E13,E19	15
0.58	AMP- TET- AMC- STR- NAL- SXT- CHL	E1,E6,E7,E9	20
(7/12)	AMP- TET- AMC- STR- NAL- SXT- CIP	E4	5
	AMP- TET- STR- NAL- SXT- CHL- CIP	E15	5
	AMP- TET- AMC- STR- NAL- SXT- CIP	E20	5
0.5	AMP- TET- AMC- NAL- SXT- CHL	E2	5
(6/12)	AMP- TET- AMC- STR- NAL- CRO	E10	5
	AMP- TET- AMC- STR- NAL- CHL	E14	5
	AMP- TET- STR- NAL- SXT- CHL	E17	5
0.41	AMP- TET- AMC- SXT- CHL	E3	5
(5/12)	AMP- TET- AMC- NAL- CIP	E12	5
	AMP- TET- AMC- STR- CHL	E16	5
	AMP- TET- NAL- SXT- CHL	E18	5
0.33	TET- NAL- STR- CIP	E11	5
(4/12)			

TET, tetracycline; AMP, ampicillin; NAL, nalidixic acid; CHL, chloramphenicol; AMC, amoxcillin-clavulanic acid; STR, streptomycin; CRO, ceftriaxone; CIP, ciprofloxacin; SXT, trimethoprim- sulfamethoxazole

The range of MAR index 0.33 until 0.67 with 15 resistant patterns in 20 *E. coli* isolates showed in Table 4.13. A MAR index value was calculated according to section 3.36. Five *E. coli* isolates (20 %) with the highest MAR index 0.67, followed by 4 isolates (20 %) with MAR index of 0.41, 4 isolates (20 %) with MAR index of 0.5, 7 isolates (35 %) with MAR index of 0.58 and 1 isolate (5 %) with the lowest MAR index of 0.33.

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4.2.4 Antibiotics Resistance Profiles of *Staphylococcus aureus* Isolates

Antibiotics resistance profiles of 3 *S. aureus* isolates were tested with 12 selected antibiotics showed in Table 4.14. In the study, one *S. aureus* isolate was resistant to oxacillin, penicillin G, tetracycline, erythromycin, trimethoprim- sulfamethoxazole and clindamycin.

Table 4.14: Antibiotic resistance profile of *S. aureus* in the study.

Antibiotic types	Number of isolates (%)		
	Resistant (R)	Intermediate (I)	Susceptible (S)
Penicillins			
Oxacillin (1 µg)	1 (33.3)	0	2 (66.7)
Ceftazidime (30 µg)	0	0	3 (100)
Cefazolin (30 µg)	0	0	3 (100)
Penicillin G (6 µg)	1 (33.3)	0	2 (66.7)
Quinolones and Fluoroquinolones			
Ciprofloxacin(5 µg)	0	1 (33.3)	2 (66.7)
Tetracycline			
Tetracycline (30 µg)	1 (33.3)	0	2 (66.7)
Carbapenems			
Imipenem (10 µg)	0	0	3 (100)
Macrolides			
Erythromycin (15 µg)	1 (33.3)	0	2 (66.7)
Sulfonamides and synergistic agents			
Trimethoprim-sulfamethoxazole (25 µg)	1 (33.3)	0	2 (66.7)
Glycopeptides			
Vancomycin (30 µg)	0	0	3 (100)
Lincosamides			
Clindamycin (2 µg)	1 (33.3)	0	2 (66.7)
Aminoglycosides			
Tobramycin (10 µg)	0	0	3 (100)

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Table 4.15: Antibiotic resistance patterns of *S. aureus* isolated.

Multiple resistance patterns	Resistance pattern	*S. aureus* isolate
Six type antibiotics agents	OXA- TET- ERY- CLI- SXT- PC-G	SA1

PC-G, penicillin G; OXA, oxacillin; TET, tetracycline; ERY, erythromycin; CLI, clindamycin; SXT, trimethoprim- sulfamethoxazole.

Table 4.16: MAR index value of *S. aureus*.

MAR index	Resistance Pattern	*S. aureus* isolate	Percentages of isolate (%)
0.5 (6/12)	OXA- TET- ERY- CLI- SXT- PC-G	SA1	33.3

PC-G, penicillin G; OXA, oxacillin; TET, tetracycline; ERY, erythromycin; CLI, clindamycin; SXT, trimethoprim- sulfamethoxazole.

Antibiotic resistance pattern of *S. aureus* was showed in Table 4.15. Only one isolate is resistant to six types of antibiotics in the study. One *S. aureus* isolate (33.3 %) resistant to six types of antibiotics showed in Table 4.16. A MAR index value was calculated according to section 3.36.

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4.3 Molecular Identification on Antibiotic Resistance Genes in *Salmonella* Isolates

4.3.1 Distribution of Antibiotic Resistance and Virulence Gene in *Salmonella* Isolates

All 38 *Salmonella* isolates (100%) were harbored *invA* gene, which encoded for invasion function of *Salmonella* groups in host (Table 4.17).

Table 4.17: Virulence gene in *Salmonella* isolates.

Virulence gene	Number of *Salmonella* isolates (%)
invA	38 (100)

Distribution of antibiotic resistance genes including *tetA* and *tetB* gene encoding for tetracycline resistance, *sul1* and *sul2* gene encoding for sulfonamide resistance, *mphA* gene encoding for marcolides resistance and *qnrS*, *qnrA* and *qnrB* gene encoding for quinolones resistance have been detected using PCR. In Table 4.18 and 4.19 showed out of total 38 *Salmonella* isolates, 66 % of *Salmonella* harbored *tetA* gene (n= 25 isolates) and 5 % of *tetB* gene (n= 2 isolates) only. All of the *Salmonella* isolates harbored *sul2* gene and 53 % (n= 20 isolates) *sul1* gene were detected. Sixty-three percent of *Salmonella* harbored *qnrS* gene (n= 24 isolates) and 5 % of *qnrB* gene (n= 2 isolates). Only 4 (11 %) *mphA* genes were detected positive in *Salmonella* isolates.

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Table 4.18: PCR amplification of antibiotic resistance genes in *Salmonella* isolates.

Antibiotic resistance genes	***tetA***	***tetB***	***sul1***	***sul2***	***qnrS***	***qnrB***	***mphA***
Number of *Salmonella* isolates (%)	25 (66 %)	2 (5 %)	20 (53%)	38 (100 %)	24 (63 %)	2 (5 %)	4 (11 %)

Table 4.19: Antibiotic resistance genes in *Salmonella* isolates.

Antibiotic resistance genes	***Salmonella* isolates harbor the gene**
tetA	S1,2,3,4,5,6,7,8,11,12,13,17,18,22,23,24,25,26,29,30,31,32,33,34,36
tetB	S14,35
sul1	S1,2,3,4,5,6,8,10,11,12,13,14,15,16,19,20,21,22,27,37
sul2	All
qnrA	None
qnrB	S14,35
qnrS	S1,2,5,7,8,11,13,18,22,23,25,26,27,28,29,30,31,32,33,34,35,36,37,38
mphA	S1,2,3,4

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4.4 Molecular Typing Pulse-Field Gel Electrophoresis

4.4.1 PFGE in *Salmonella* Isolates

PFGE is a molecular tool to produce distinct of DNA fingerprint pattern from different source for epidemiology analysis. Genetic relatedness of 38 *Salmonella* isolates were analyzed using PFGE dendrogram with different band patterns. The genomic DNA was digested with restriction enzyme XbaI and produces 6 major clusters with 14 different Xbal PFGE pattern in the analysis.

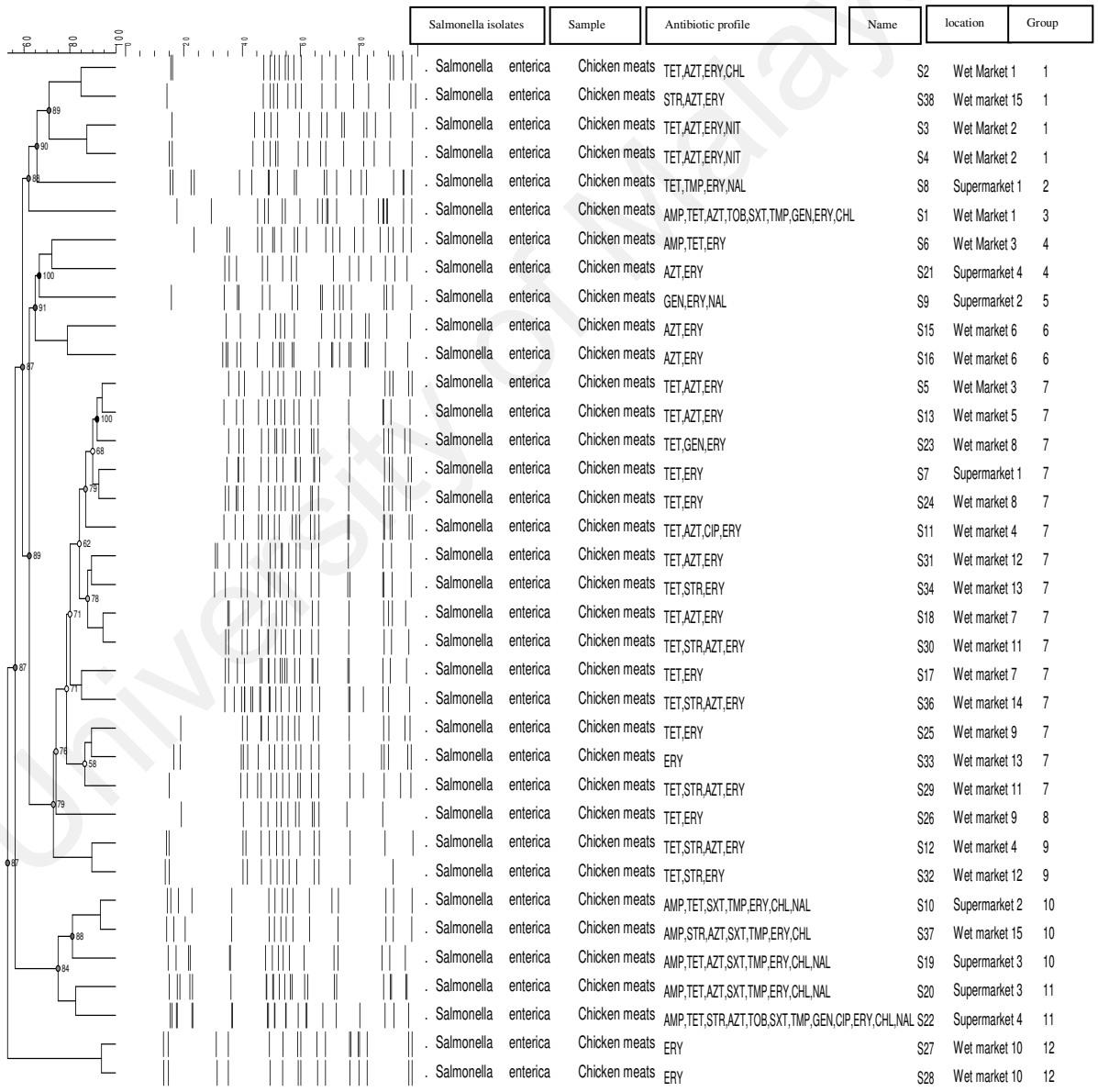

Figure 4.1: PFGE fingerprint in *Salmonella* isolates with 80 % similarity coefficient using clustered UPGMA algorithm and DICE coefficient.

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PFGE pattern among 36 *Salmonella* isolates were analyzed with BioNumeric software and a dendrogram was constructed using UPGMA algorithm. *Salmonella* isolate pulsotypes were categorized into cluster A, B, C, D, E and F with an overall similarity of 80 %. The 36 *Salmonella* isolates were grouped into 6 major clusters with 12 different Xbal PFGE pattern by PFGE analysis. Two *Salmonella* isolates were failed to type with PFGE so it is not show in the data image (Data not show). These 36 isolates were grouped into 6 clusters named A-F, respectively (Figure 4.1). The PFGE profile were named to Cluster D (18 isolates, 50 %) and followed by cluster C (5 isolates, 14 %), cluster E (5 isolates, 14 %), cluster A (4 isolates, 11 %), cluster B (2 isolate, 6 %), and cluster F (2 isolates, 6 %). Majority of XbaI profile were assigned to cluster D which represented 18 isolates from different location of wet markets.

Most of the isolates in cluster D showed resistant to tetracycline and erythromycin. Majority of *Salmonella* isolates are isolated from wet market. This indicates the isolates closely related to each other and showed similar pattern of PFGE DNA fingerprint although isolated from different location of wet market. *Salmonella* isolates classified under cluster E showed resistant to more than six types of antibiotics agents. Multidrug resistant *Salmonella* strain was observed in cluster E at which resistant to ampicillin, trimethoprim, erythromycin, chloramphenicol, trimethoprim- sulfamethoxazole and nalidixic acid.

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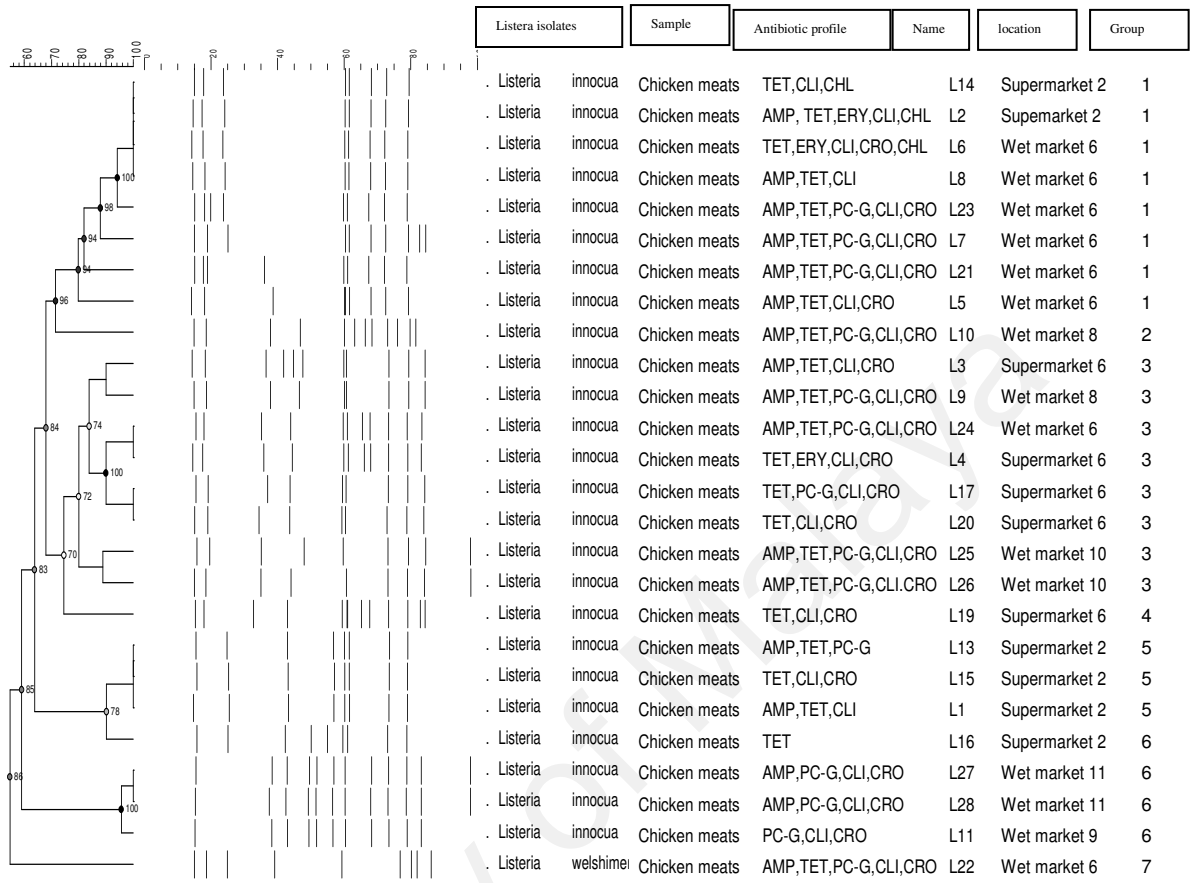
4.4.2 PFGE in *Listeria* Isolates



Listera isolates	Sample	Antibiotic profile	Name	location	Group
. Listeria innocua	Chicken meats	TET,CLI,CHL	L14	Supermarket 2	1
. Listeria innocua	Chicken meats	AMP, TET,ERY,CLI,CHL	L2	Supemarket 2	1
. Listeria innocua	Chicken meats	TET,ERY,CLI,CRO,CHL	L6	Wet market 6	1
. Listeria innocua	Chicken meats	AMP,TET,CLI	L8	Wet market 6	1
. Listeria innocua	Chicken meats	AMP,TET,PC-G,CLI,CRO	L23	Wet market 6	1
. Listeria innocua	Chicken meats	AMP,TET,PC-G,CLI,CRO	L7	Wet market 6	1
. Listeria innocua	Chicken meats	AMP,TET,PC-G,CLI,CRO	L21	Wet market 6	1
. Listeria innocua	Chicken meats	AMP,TET,CLI,CRO	L5	Wet market 6	1
. Listeria innocua	Chicken meats	AMP,TET,PC-G,CLI,CRO	L10	Wet market 8	2
. Listeria innocua	Chicken meats	AMP,TET,CLI,CRO	L3	Supermarket 6	3
. Listeria innocua	Chicken meats	AMP,TET,PC-G,CLI,CRO	L9	Wet market 8	3
. Listeria innocua	Chicken meats	AMP,TET,PC-G,CLI,CRO	L24	Wet market 6	3
. Listeria innocua	Chicken meats	TET,ERY,CLI,CRO	L4	Supermarket 6	3
. Listeria innocua	Chicken meats	TET,PC-G,CLI,CRO	L17	Supermarket 6	3
. Listeria innocua	Chicken meats	TET,CLI,CRO	L20	Supermarket 6	3
. Listeria innocua	Chicken meats	AMP,TET,PC-G,CLI,CRO	L25	Wet market 10	3
. Listeria innocua	Chicken meats	AMP,TET,PC-G,CLI.CRO	L26	Wet market 10	3
. Listeria innocua	Chicken meats	TET,CLI,CRO	L19	Supermarket 6	4
. Listeria innocua	Chicken meats	AMP,TET,PC-G	L13	Supermarket 2	5
. Listeria innocua	Chicken meats	TET,CLI,CRO	L15	Supermarket 2	5
. Listeria innocua	Chicken meats	AMP,TET,CLI	L1	Supermarket 2	5
. Listeria innocua	Chicken meats	TET	L16	Supermarket 2	6
. Listeria innocua	Chicken meats	AMP,PC-G,CLI,CRO	L27	Wet market 11	6
. Listeria innocua	Chicken meats	AMP,PC-G,CLI,CRO	L28	Wet market 11	6
. Listeria innocua	Chicken meats	PC-G,CLI,CRO	L11	Wet market 9	6
. Listeria welshimei	Chicken meats	AMP,TET,PC-G,CLI,CRO	L22	Wet market 6	7

Figure 4.2: PFGE fingerprint in *Listeria* isolates constructed with 80 % similarity coefficient using clustered UPGMA algorithm and DICE coefficient.

A total of twenty-eight of *Listeria* isolates were digested with AscI enzyme for PFGE fingerprint pattern in the study (Figure 4.2). The electrophoresis running conditions were applied according Table 3.22. *Salmonella enterica* serovar Braenderup H9812 was used as molecular size standard for PFGE. Twelve patterns were observed and another two strains (p and 6) are untypeable (data not show). Majority of *Listeria* isolates were resistant to ampicillin, tetracycline and clindamycin from different location site. In PFGE obtained six cluster groups including cluster A (9 isolates, 32 %), cluster B (9 isolates, 32 %), cluster C (4 isolate, 14 %), cluster D (3 isolates, 11 %), cluster E (1 isolates, 4 %).

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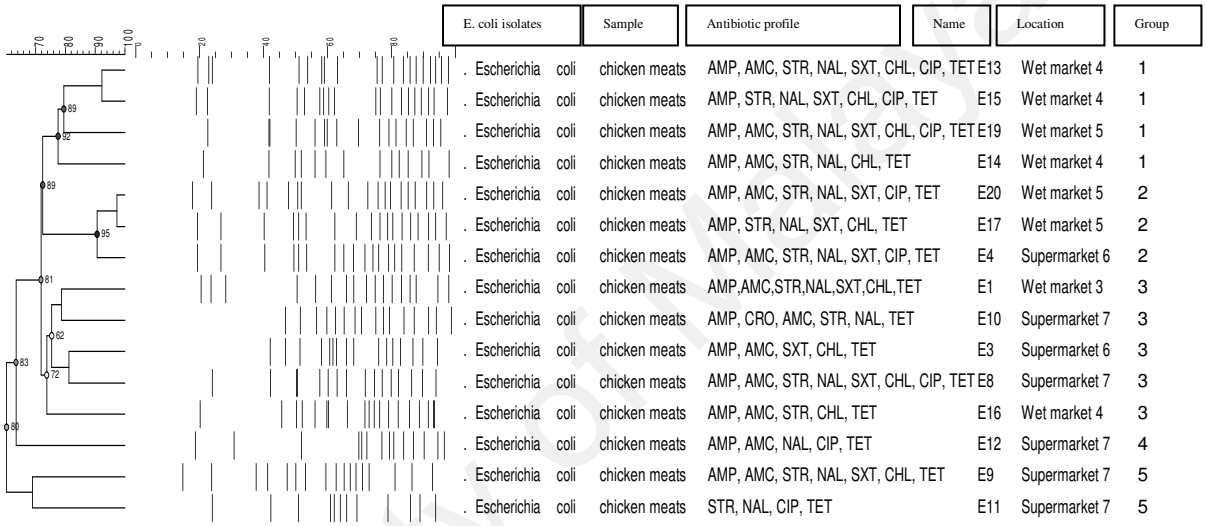
About 18 isolates were categorized under cluster A and B isolated from different location of local market showed high similarity pattern and resistant to tetracycline. Eight *Listeria* isolates were resistant to five types of antibiotics with resistant pattern of AMP, TET, PC-G, CLI and CRO were mostly found in cluster A and B.

4.4.3 PFGE in *Escherichia coli*



E. coli isolates	Sample	Antibiotic profile	Name	Location	Group
. Escherichia coli	chicken meats	AMP, AMC, STR, NAL, SXT, CHL, CIP, TET	E13	Wet market 4	1
. Escherichia coli	chicken meats	AMP, STR, NAL, SXT, CHL, CIP, TET	E15	Wet market 4	1
. Escherichia coli	chicken meats	AMP, AMC, STR, NAL, SXT, CHL, CIP, TET	E19	Wet market 5	1
. Escherichia coli	chicken meats	AMP, AMC, STR, NAL, CHL, TET	E14	Wet market 4	1
. Escherichia coli	chicken meats	AMP, AMC, STR, NAL, SXT, CIP, TET	E20	Wet market 5	2
. Escherichia coli	chicken meats	AMP, STR, NAL, SXT, CHL, TET	E17	Wet market 5	2
. Escherichia coli	chicken meats	AMP, AMC, STR, NAL, SXT, CIP, TET	E4	Supermarket 6	2
. Escherichia coli	chicken meats	AMP,AMC,STR,NAL,SXT,CHL,TET	E1	Wet market 3	3
. Escherichia coli	chicken meats	AMP, CRO, AMC, STR, NAL, TET	E10	Supermarket 7	3
. Escherichia coli	chicken meats	AMP, AMC, SXT, CHL, TET	E3	Supermarket 6	3
. Escherichia coli	chicken meats	AMP, AMC, STR, NAL, SXT, CHL, CIP, TET	E8	Supermarket 7	3
. Escherichia coli	chicken meats	AMP, AMC, STR, CHL, TET	E16	Wet market 4	3
. Escherichia coli	chicken meats	AMP, AMC, NAL, CIP, TET	E12	Supermarket 7	4
. Escherichia coli	chicken meats	AMP, AMC, STR, NAL, SXT, CHL, TET	E9	Supermarket 7	5
. Escherichia coli	chicken meats	STR, NAL, CIP, TET	E11	Supermarket 7	5

Figure 4.3: PFGE fingerprint in *E. coli* isolates with similarity coefficient of 80 % using clustered UPGMA algorithm and DICE coefficient.

PFGE pattern of 20 *E. coli* isolates were digested with XbaI enzyme showed in Figure 4.3. The electrophoresis running conditions were applied according to Table 3.19. *Salmonella enterica ser* Braenderup H9812 was used as molecular size standard for PFGE. The data showed five different PFGE pattern for 15 *E. coli* isolates and another 5 isolates (E2, 5, 6, 7, 18) were untypeable in the data (data no shown). The *E. coli* isolates were obtained different antibiotic resistance patterns collected from six sampling location of local wet and supermarkets in Klang Valley.

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Most of the *E. coli* isolates were resistant to ampicillin and tetracycline collected from different sampling site. Five cluster groups were obtained including most dominant group consists of 5 *E. coli* isolates (33 %), followed by cluster 1 consists of 4 *E. coli* (27 %), cluster 2 consists of 3 *E. coli* (20 %), cluster 5 consists of 2 *E. coli* (13 %) and cluster 4 consists of 1 *E. coli* isolate (7 %). For cluster group 3 observed similarity pattern in five *E. coli* isolates were isolated from four different locations. These indicate the *E. coli* isolated from chicken meats were collected in different location assume that high possibly contaminated from same source. High similarities patterns in *E. coli* isolates were obtained in different geographical location.

Three *E. coli* isolates (E8, 9 and 13) were observed same antibiotic resistance patterns however isolated from different isolation places and categorized under different cluster in PFGE. They are resistant to eight antibiotics include ampicillin, amoxicillin-clavulanate, streptomycin, nalidixic acid, trimethoprim-sulphamethoxazole, chloramphenicol, ciprofloxacin and tetracycline. Another highly similar PFGE pattern was observed in three *E. coli* strain (E13, 14 and 15) under cluster 1 isolated from same location (wet market 2) but obtained different antibiotic resistance pattern.

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4.5 Genome Analysis

The summary of genome sequencing showed in *Salmonella* strain PS01, PS07, PS12 and PS13 using HiSeq 2000 platform (Table 4.20).

Table 4.20: Genome sequencing information of *Salmonella* strain PS01, 07, 12 and 13 using HiSeq 2000 platform.

***Salmonella* strains**	**PS01**	**PS07**	**PS12**	**PS13**
Finishing quality	Draft genome	Draft genome	Draft genome	Draft genome
Assemblers	CLC Genomics	CLC Genomics	CLC Genomics	CLC Genomics
Genbank ID	LQZK01	LRYA01	LWMB01	LWMC01
Size	5,036,442	4,952,228	4,849,073	4,955,801
Fold coverage	116 ×	138 ×	114 ×	140 ×
GC content	52%	52%	52%	52%

4.5.1 *In silico* Typing Resource (SISTR) of *Salmonella* Serovar

Using SISTR, four *Salmonella* serovar strains were determined (Table 4.21). Serotyping of *Salmonella* PS01, 07, 12 and 13 (IMR lab No: 94, 95, 96 and 97) were confirmed with Infectious Diseases Research Centre Malaysia in agreement with result of SISTR.

Table 4.21: Predication of *Salmonella* serovars in SISTR.

Genome	**Subspecies**	**Serovar**	**Serogroup**	**H1**	**H2**	**MLST ST**
PS01	*enterica*	Brancaster	B	$_z$29	-	2133
PS07	*enterica*	Corvallis	C2-C3	$_z$4 $_z$23	-	1541
PS12	*enterica*	Corvallis	C2-C3	$_z$4 $_z$23	-	1541
PS13	*enterica*	Corvallis	C2-C3	$_z$4 $_z$23	-	1541

4.5.2 RNAmmer 1.2 Server

RNAmmer server used the 16S rRNA gene predicted bacteria identification in PS01, 07, 12 and 13 strains are *Salmonella* spp. using RNAmmer server (Appendices A to D).

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4.5.3 Gene Annotation using RAST Server

Genome feature of *Salmonella enterica* strain PS01, 07, 12 and 13 from Rapid Annotation using Subsystem Technology (RAST) annotation system (Table 4.22).

Table 4.22: Genome feature of *Salmonella enterica* PS01, 07, 12 and 13 in RAST.

Strain	Source	Size(bp)	No. of Contig(s)	G+C (%)	No. of CDS	No. of RNA(s)	No. of Subsystem
PS01	Poultry	5,036,442	142	52	4976	68	581
PS07	Poultry	4,952,228	125	52	4928	75	581
PS12	Poultry	4,849,073	109	52	4817	78	580
PS13	Poultry	4,955,801	106	52	4959	73	581

The pie chart in RAST indicates the abundance of subsystem category distribution. All the subsystem are covered by subsystem coverage include green color bar indicate percentages of protein analyzed in the subsystem whereas blue color bar indicate percentages of protein not analyzed in the subsystem.

4.5.3.1 *Salmonella enterica* Strain PS01

Subsystem feature counts are significant annotated based on RAST server in PS01 strain (Figure 4.4). For instance, carbohydrates (737 genes); amino acid and derivatives (441 genes); cofactors, vitamins, prosthetic groups, pigments (315 genes); cell wall and capsule (286 genes); RNA metabolism (257 genes); membrane transport (254 genes) and protein metabolism (243 genes). They are essential of basic need for bacteria to sustain their life. There are 122 genes responsible for virulence, disease and defense function and 96 genes are resistance to antibiotics and toxic compounds under the categories.

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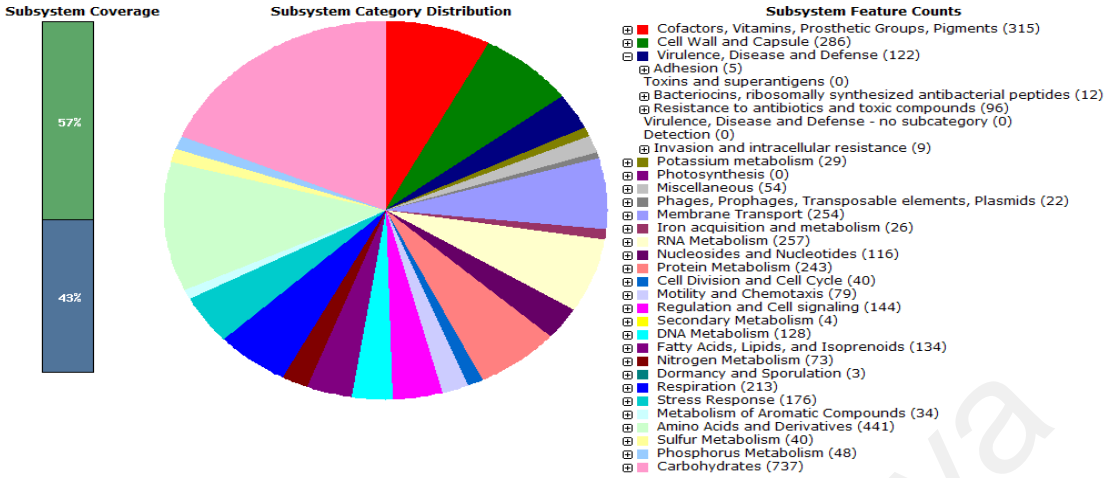

Figure 4.4: Subsystem distributions of *Salmonella* genome strain PS01.

4.5.3.2 *Salmonella enterica* Strain PS07

Subsystem feature counts are significant annotated based on RAST server in PS07 strain (Figure 4.5). For instance, carbohydrates (700 genes); amino acid and derivatives (446 genes); cofactors, vitamins, prosthetic groups, pigments (323 genes); protein metabolism (271 genes); cell wall and capsule (269 genes); RNA metabolism (258 genes) and membrane transport (249 genes). They are essential of basic need for bacteria to sustain their life. There are 101 genes responsible for virulence, disease and defense function and 75 genes are resistance to antibiotics and toxic compounds under the categories.

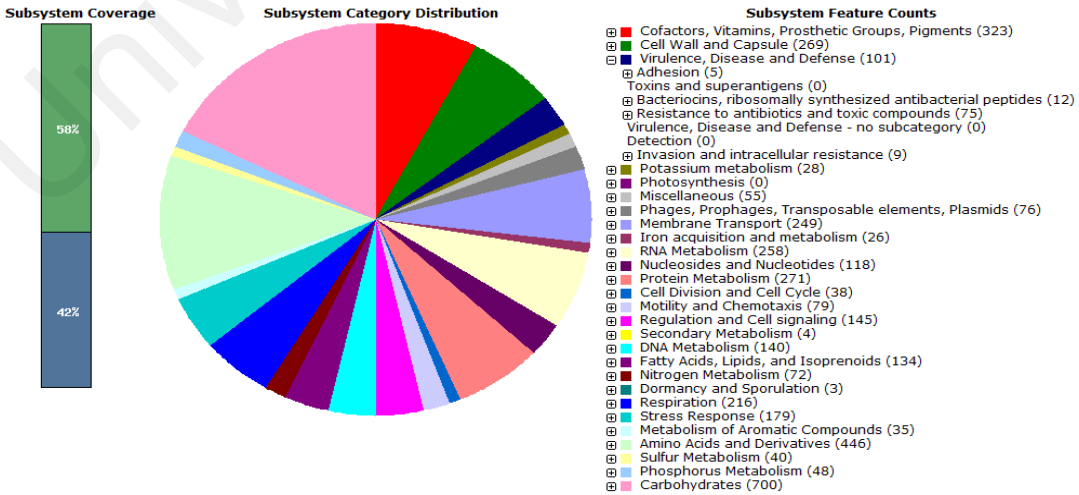

Figure 4.5: Subsystem distributions of *Salmonella* genome strain PS07.

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4.5.3.3 *Salmonella enterica* Strain PS12

Subsystem feature counts are significant annotated based on RAST server in PS12 strain (Figure 4.6). For instance, carbohydrates (696 genes); amino acid and derivatives (448 genes); cofactors, vitamins, prosthetic groups, pigments (322 genes); cell wall and capsule (272 genes); protein metabolism (269 genes); RNA metabolism (258 genes) and membrane transport (246 genes). They are essential of basic need for bacteria to sustain their life. There are 101 genes responsible for virulence, disease and defense function and 75 genes are resistance to antibiotics and toxic compounds under the categories.

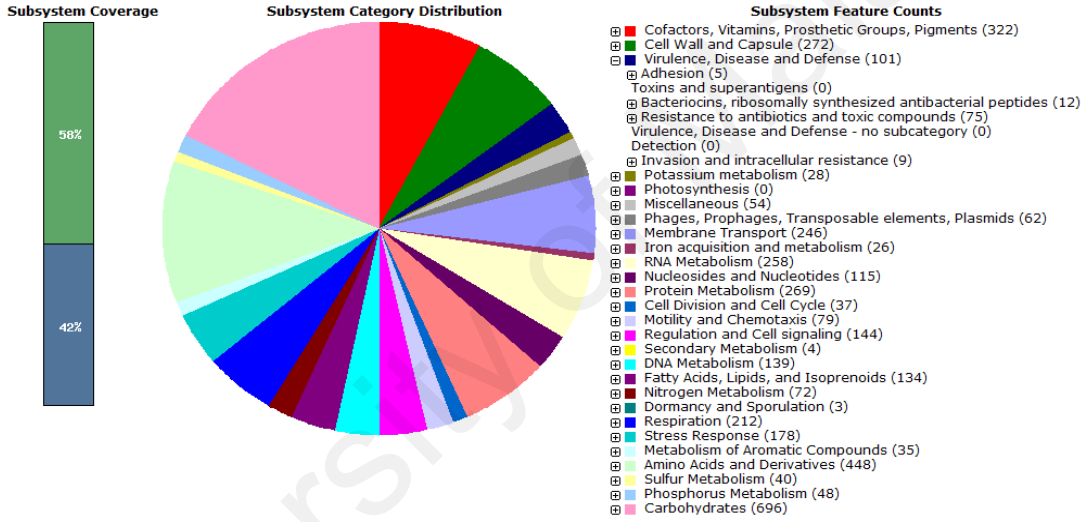

Figure 4.6: Subsystem distributions of *Salmonella* genome strain PS12.

4.5.3.4 *Salmonella enterica* Strain PS13

Subsystem feature counts are significant annotated based on RAST server in PS13 strain (Figure 4.7). For instance, carbohydrates (696 genes); amino acid and derivatives (446 genes); cofactors, vitamins, prosthetic groups, pigments (323 genes); cell wall and capsule (269 genes); protein metabolism (270 genes); RNA metabolism (258 genes) and membrane transport (248 genes). They are essential of basic need for bacteria to sustain their life.

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There are 102 genes responsible for virulence, disease and defense function and 75 genes are resistance to antibiotics and toxic compounds under the categories.

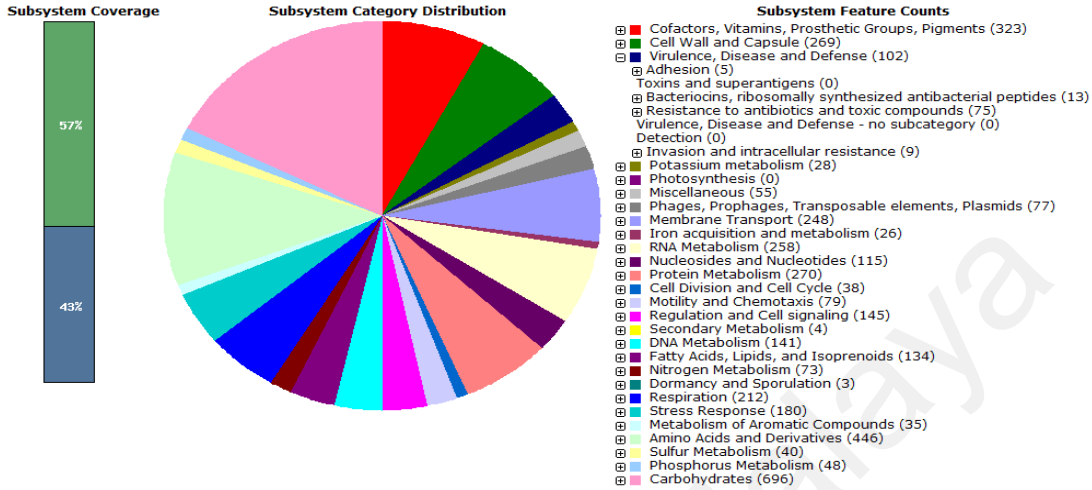

Figure 4.7: Subsystem distributions of *Salmonella* genome strain PS13.

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4.5.4 Resfinder 2.0

Antibiotic resistance gene of *Salmonella* strains were annotated using Resfinder 2.0 server. The online web tool, Resfinder uses BLAST to identify acquired antibiotics resistance genes in whole genome data. Antibiotics resistance genes are important to identify for better understanding underlying mechanism and epidemiology of antibiotics resistance. Four different sequencing platforms include preassembled, complete or draft genome and short sequence reads can be used as input (Kleinheinz et al., 2014). Predicted antibiotic resistance gene showed in PS01, 07, 12 and 13 strains (Table 4.23).

Table 4.23: Predicted antibiotic resistance genes in strain PS01, 07, 12 and 13.

Strain	Resistance gene	% identity	Predicted phenotypes
PS01	*aph(4)-Ia*	100	Aminoglycosides resistance
	aac(3)-IVa	100	
	aadA1	99.87	
	aph(3)-Ic	99.14	
	$bla_{\text{TEM-176}}$	100	Beta-lactam resistance
	QnrS1	100	Quinolone resistance
	mph(A)	100	Macrolide resistance
	flo(R)	98.11	Phenicol resistance
	sul3	100	Sulphonamide resistance
	tet(A)	100	Tetracycline resistance
	dfrA14	99.59	Trimethoprim resistance
PS07	*aph(3")-Ib*	99.88	Aminoglycosides resistance
	aph(6)-Id	100	
	qnrS1	100	Quinolone resistance
	sul2	100	Sulphonamide resistance
	tet(A)	99.83	Tetracycline resistance
PS12	*aph(3")-Ib*	100	Aminoglycosides resistance
	aph(6)-Id	100	
	sul2	100	Sulphonamide resistance
	tet(A)	99.92	Tetracycline resistance
PS13	*aph(3")-Ib*	99.88	Aminoglycosides resistance
	aph(6)-Id	100	
	qnrS1	100	Quinolone resistance
	sul2	100	Sulphonamide resistance
	tet(A)	99.83	Tetracycline resistance

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CHAPTER 5: DISCUSSION

5.1 Isolation of Foodborne Bacteria from Chicken Meat

In the decade of 19th century, foodborne disease outbreak occurs in globally. Foodborne illness is usually increase health risk to human population and causes high mortality rate. *Salmonella* and *Listeria* spp. are bacterial pathogens associated with foodborne disease that affects both animal and human in worldwide. Undercooked of poultry meat and eggs are potential source food vehicle transmission of food pathogens (Newell et al., 2010; WHO, 2002). Poultry meat and eggs are common food vehicle for pathogens in transmission of human illness and increase risk for public health (Zdragas et al., 2012). Some reports on poultry meat serve as reservoir for *Salmonella* spp. (Antunes et al., 2003; Bouzoubaa et al., 1992; de Oliveira et al., 2005; Humphrey et al., 1988) and *Listeria* spp. (Dhama et al., 2013; Elmali et al., 2015; Genigeorgis et al., 1989; Jay, 1996; Lawrence & Gilmour, 1994, 1995). Consequently consumption of undercooked chicken products may contribute to foodborne illness and increase public health risk. In this work chicken meat and eggs were selected for isolation source to assess on prevalence and antibiotics resistance profile of *Salmonella* spp., *Listeria* spp., *E. coli* and *S. aureus*. Extensive reports have shown the emergence of multiple antibiotic resistances in food pathogens (Mathew et al., 2007; McDermott et al., 2002; Singh, 2016; Walsh & Fanning, 2008). Most of the *Salmonella* and *Listeria* spp. are well known as foodborne pathogenic bacteria associated with foodborne illness (Newell et al., 2010; Scallan et al., 2011; Tauxe, 2002).

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Total viable count can use as sanitary and quality index in foods when produced, handled and stored (Saad et al., 2015). In the present study, contamination levels and frequency of isolation of bacteria in chicken meat samples were higher than egg samples. High bacterial count reported in other study (Hossain et al., 2015; Bhandari et al., 2013). Generally, the high bacterial counts of chicken meat may be due to meat come in contact with contaminated of equipments and knives during processing (Eyi & Arslan, 2012). Moreover, the high storage temperature at the retail level is one of factors contributing to high bacterial counts (Saad et al., 2015).

5.2 Prevalence of Bacteria Isolated from Chicken Meat

In the present study showed 38 % of positive *Salmonella* samples (38/ 100 total samples), 28 % of *Listeria* isolates (28/ 100 total samples), 20 % of *E. coli* isolates (20/ 100 total samples) and 3 % of *S. aureus* isolates (3/ 100 total samples) in Klang Valley regions. Notably, the present study is close agreement with prevalence research of 39.7 % *Salmonella* spp. from retail meats in Mexico (Norimah et al., 2008); 38.9 % from retail chickens in Sichuan Province (Yang et al., 2011) and 23.02 % of *Salmonella* spp. from retail chicken in China (Cui et al., 2016). Similarly, some prevalence of *Salmonella* spp. in Thailand in 48 % (Minami et al., 2010) and North Vietnam in 42.9 % (Thai et al., 2012) are reported higher percentages than the finding in this study. In contrast, the prevalence of 13 % *Salmonella* spp. from poultry in Vietnam is lower than the present finding (Ellerbroek et al., 2010).

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The presence of *Listeria* spp. in raw chicken meat could be attributed to food handler (Yücel et al., 2005). *Listeria* spp. in present finding is in close agreement with prevalence research in Italy (Pesavento et al., 2010). According to previous some reports, the percentages of *Listeria* spp. in this study are lower than *Listeria* spp. (54.1 %) reported in Turkey (Yücel et al., 2005), 56.6 % in Japan (Ryu et al., 1992) and 55.3 % in Switzerland (Brackett, 1988). In contrast, the prevalence of *Listeria* spp. from meat in Thailand showed lower percentages than present study (Stonsaovapak & Boonyaratanakornkit, 2010). Tables 5.1 and 5.2 showed the prevalence of *Salmonella* and *Listeria* spp. from chicken meat in Malaysia and in other countries (Table 5.3).

E. coli is known as coliform bacteria are commonly used as hygiene indicator (Feng et al., 2002) for possible presence of other enteric pathogens (Akbar et al., 2014). Three of *S. aureus* was isolated from chicken meat's skin. *S. aureus* is normally found in skin and soft tissues (Frazee et al., 2005).

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Table 5.1: Prevalence of *Salmonella* spp. in Malaysia.

Food samples	Prevalence (%)	References
Broiler carcass	85.5	Rusul et al., 1996
Chicken pieces	39	Arumugaswamy et al., 1995
Chicken parts	26.7	Thung et al., 2016
Retail meat	22	Thong & Modarressi, 2011
Chicken meat	10	Azizan & Abdul Aziz, 2011

Table 5.2: Prevalence of *Listeria* spp. in Malaysia.

Food samples	Prevalence (%)	References
Chicken	60	Arumugaswamy et al., 1994
Raw chicken	40	Goh et al., 2012
Chicken fillets	25	Jamali et al., 2013
Chickens	50	Ali et al., 1991

Table 5.3: Prevalence of *Listeria* and *Salmonella* spp. in several countries.

Countries	Bacteria	Prevalence (%)	References
Egypt	*Listeria* spp.	56.0	Abdelazeem & Elsayh, 2010
Iran	*Listeria* spp.	20.0	Sohrabi et al., 2013
Turkey	*Listeria* spp.	79.0	Yücel et al., 2005
Japan	*Listeria* spp.	37.0	Inoue et al., 2000
Spain	*Listeria* spp.	95.0	Capita et al., 2001
	Salmonella spp.	35.83	Domınguez et al., 2002
Poland	*Listeria* spp.	51.4	Kosek-Paszkowska et al., 2005
Nigeria	*Listeria* spp.	70.0	Ikeh et al., 2010
Vietnam	*Salmonella* spp.	40.0	El-Aziz, 2013
		48.9	Huong et al., 2006
Colombia	*Salmonella* spp.	27.0	Donado-Godoy et al., 2012
Russian Federation	*Salmonella* spp.	31.5	Alali et al., 2012
India	*Salmonella* spp.	23.7	Kaushik et al., 2014
China	*Salmonella* spp.	45.2	Bai et al., 2015
Korea	*Salmonella* spp.	22.4	Kim et al., 2012

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Chicken eggs suggest as food vehicle for transmission of *Salmonella* and *Listeria* spp. Some of previous study in Malaysia reported on low percentages of *Salmonella* spp. (Ong et al., 2014) and *Listeria* spp. in eggs and egg products (Jamali et al., 2013). Low percentages of *E. coli* were observed in chicken eggs reported in other studies (Loongyai et al., 2011; Cader et al., 2014). The spread of pathogens in poultry mainly come from water, feed additives, faeces, equipments and rodents in the farm (Poppe, 2000). Feeds can be contaminated through feed formulation from feed mills or on farm and feeds preparation, subsequently eaten by poultry (Whyte et al., 2003). In the study showed no *Salmonella* spp., *Listeria* spp., *E. coli* and *S. aureus* were isolated from chicken feeds purchase from retail market. These indicate the sterilization and handling processes of feeds performed by manufacturer are controlled before sending to market.

Several factors contribute to the prevalence of *Salmonella* and *Listeria* spp. in chicken meat including climate condition, cross contamination on kitchen utensils and awareness of food handling and practice. Malaysia is located in equatorial region with hot and humid climate that provides suitable temperature and growing condition for bacteria. The bacteria can grows on surface of the kitchen utensils such as knife, cutting board, cleaning sponge and cloth (Kusumaningrum et al., 2003; Mattick et al., 2003). Open wet market is an open space public area to sell daily goods for consumer. Among the *Salmonella*-positive chicken meat, 4 were obtained from retail markets and the remaining 15 were from wet markets.

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Chicken meat and eggs are possibly cross contaminated during handling, processing, preparation and distribution stages (Sousa, 2008). Food handlers using unclean kitchen utensils (knife and cutting board) that easy to spread bacteria by fecal-oral route or skin lesions during food handling process (Linscott, 2011). Besides, lack of awareness on personnel hygiene practice and using unclean water supply from environment to wash the materials (Campos et al., 2009; Meftahuddin, 2002) could enhance the risk of foodborne bacteria grow. Hence, the neglect of food handlers on personnel and kitchen utensils hygiene practice could contribute the risk of bacterial contamination in foods.

The small samples size selected may causes high chances of bias. Low number of sample size may involved biased selection and leads to draw erroneous conclusion. In this study, small number of sample size from one sampling area of Malaysia is limited reliability and accuracy of the truly representative sample produces in the whole group. The low number of sample size may bring units of population are not in homogenous. The small or heterogeneous in sample size possibly due to limited data can be drawn to representative of whole group and high chance of sampling errors are identified. Sampling error is occurred by chance and caused by using a sample based on random selection procedure, result in a possibly different value of estimation. However, relative small samples properly selected may be much more reliable than large samples poorly selected. Sampling design by increasing sample size can reduce the sampling error (Beer & Miller, 1964; Onwuegbuzie & Leech, 2007).

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Prevalence of *E. coli* in this study showed atypical percentage as compared to other studies (Gwida & El-Gohary, 2015; Cook et al., 2012; Wilfred et al., 2012; Zhao et al., 2001). This may be due to the sampling scheme size which may have missed a relevant subgroup of target population. The relatively small number of samples in this study limited the ability to utilize sophisticated statistical methodologies and thus contribute to atypical results. In addition, the laboratory isolation methods, different types of poultry sources and processing methods are varied from the various studies may limit the comparability of these studies (Cook et al., 2012).

5.3 Antibiotic Resistance Profile in *Salmonella* spp., *Listeria* spp., *E. coli* and *S. aureus*

Enterobacteriaceae bacteria particularly *Salmonella* and *E. coli* are significant causes of serious infections in human and animals, becoming increasingly resistant to multiple antibiotics. Poultry meat is food vehicle that easy contaminated with foodborne pathogen has grab attention of public and lead to emerge and spread of antibiotics resistance bacteria associated with foodborne illness (Khan et al., 2007; Threlfall et al., 2000). Studies on multidrug resistant in *Salmonella* spp. have been reported (Abouzeed et al., 2000; Van Duijkeren et al., 2003). The present study showed *Salmonella* isolates highly resistance to tetracycline, sulfamethoxazole-trimethoprim, ampicillin, nalidixic acid and ciprofloxacin are commonly used antibiotics in veterinary medicine for animal production and human treatment. The result in agreement with study reported in China (Zhang et al., 2010). Presently, all of the *Salmonella* isolates from chicken meat were resistant to erythromycin. Same results obtained from research in Korea (Hyeon et al., 2011a) and Pakistan (Shaheen et al., 2003).

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Tetracycline and ampicillin are common antibiotics used for growth promotion, animal husbandry and human treatment (Kowalski & Konieczna, 2007). The result concur with research in Malaysia (Thong & Modarressi, 2011), China (Yang et al., 2016; Yin et al., 2016), Argentina (Favier et al., 2013) and Iran (Dallal et al., 2010). Poultry and poultry products are considered reservoir for antibiotics-resistant *Salmonella* strain (de Oliveira et al., 2005).

In the last two decades, the emergence of multidrug resistant in *Salmonella* strains had led to withdrawal of chloramphenicol and replacement with fluoroquinolones (Bhan et al., 2005). Quinolones and fluoroquinolones have been used for treatment of *Salmonella* invasive infection (Hopkins et al., 2005). However, the emergence of quinolones and fluoquinolones resistant *Salmonella* isolates were reported in Japan and United State (Izumiya et al., 2005; Olsen et al., 2001). In present study, 18 % of *Salmonella* isolates were resistant to nalidixic acid and 5 % to ciprofloxacin. Nalidixic acid commonly antibiotic used in human and veterinary medicine (Stevenson et al., 2007). The result in present study is slightly lower percentages as compared with finding in Thong & Modarressi 2011. Spain, Portugal, South Brazil, Mexico, Thailand and China were reported on resistance of nalidixic acid in *Salmonella* spp. (Álvarez-Fernández et al., 2012; Antunes et al., 2003; de Oliveira et al., 2005; Miranda et al., 2009; Padungtod & Kaneene, 2006; Yan et al., 2010). Some research reported on increase resistant *Salmonella* isolates to fluoroquinolones in chicken meat (Angulo et al., 2000; Randall et al., 2005). The usage of these antibiotic classes should be controlled although low percentages have been observed. The extensive use of fluoroquinolones will increase the bacteria resistance and lead to treatment failure (Gagliotti et al., 2008).

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The present study showed 5 % of *Salmonella* isolates resistant to nitrofurantoin. Nitrofutantoin has been banned to be used in poultry due to its carcinogenic and mutagenic properties (Antunes et al., 2006). The main usage of these furans groups for therapy in the poultry breeding and to medicate animals (Bada-Alambedji et al., 2006). The result is disagreeing with the finding in South Brazil (de Oliveira et al., 2005). The usage of nitrofurantoin should be monitored due to its carcinogenic properties will effect on human.

In present study showed *E. coli* isolates were resistant to 100 % tetracycline, 90 % nalidixic acid, 95 % ampicillin, 80 % amoxcillin-clavulanic acid, 75 % chloramphenicol, 75 % trimethoprim-sulfamethoxazole and 75 % streptomycin. The study in agreement with research conducted in Malaysia (Akbar et al., 2014; Apun et al., 2008; Tin, 2003), Spain (Sáenz et al., 2004) and United State (Tadesse et al., 2012). Resistance to trimethoprim-sulfamethoxazole and nalidixic acid in *E. coli* were also observed. These sulfonamides and fluoroquinolones groups are commonly used for urinary tract infection treatment (Hooton & Stamm, 1997). In this work, 28 *Listeria* isolates were resistance to 89 % clindamycin, 86 % tetracycline, 75 % ceftriaxone, 64 % ampicillin, 50 % Pencillin G and 14 % erythromycin. Ampicillin or penicillin G, tetracycline and erythromycin were antibiotics choice for most listeriosis treatment and veterinary medicine (Charpentier & Courvalin, 1999; Conter et al., 2009; Hof et al., 1997; Teuber, 1999). The result is in agreement with the other studies (Davis & Jackson, 2009; Harakeh et al., 2009; Lesley et al., 2003). The data showed the concern of these antibiotics as choices therapy for listeriosis and possibility causes of failure in treatment. The susceptibility category of data was assigned based on breakpoint criteria for *Staphylococci*.

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Several studies were reported on antibiotic resistance in *L. monocytogenes* (Al-Nabulsi et al., 2015; Cetinkaya et al., 2014; Doğruer et al., 2015; Gómez et al., 2014; Shi et al., 2015) whereby *L. monocytogenes* isolates were highly resistance to oxacillin, clindamycin, ceftriaxone and tetracycline. *Listeria* isolates in the study were highly sensitive to chloramphenicol (89 %).

Similarly, research in Italy, India and Malaysia indicated that *Listeria* spp. isolated from meat products were sensitive to chloramphenicol (Barbuti et al., 1992; Dhanashree et al., 2003; Marian et al., 2012). Present study, almost all the *Listeria* isolates were resistant to ceftriaxone as expected, *Listeria* spp. exhibits intrinsic resistance to cephalosporin (Yücel et al., 2005). Similarly, *Listeria* spp. was naturally and intrinsic resistant to nalidixic acid (Ennaji et al., 2008). Therefore, nalidixic acid is used in selective media for *Listeria* isolation. Antibiotics resistance of *Listeria* spp. reported in other studies (Table 5.4).

Table 5.4: Antibiotic resistance of *Listeria* spp. in other studies.

Antibiotics	Food sources	References
Ampicillin	Retail food products	Yan et al., 2010
	Raw meat and retail foods	Pesavento et al., 2010
	Meat products	Yücel et al., 2005
Clindamycin	Retail meat and environment	Conter et al., 2009
	Raw meat and retail foods	Pesavento et al., 2010
Rifampicin	Retail meat and environment	Conter et al., 2009
	Retail food products	Yan et al., 2010
Tetracycline	Chicken meats and retail foods	Zhang et al., 2005
	Retail food products	Yan et al., 2010
	Chicken meats and meat products	Osaili et al., 2011
Vancomycin	Raw meat and retail foods	Pesavento et al., 2010
Erythromycin	Retail food products	Yan et al., 2010
	Raw meat and retail foods	Pesavento et al., 2010
Trimethoprim-sulfamethoxazole	Raw meat and retail foods	Pesavento et al., 2010
	Meat products	Yücel et al., 2005
Nalidixic acid	Meat products	Yücel et al., 2005

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The frequent and unnecessary use of antibiotics in poultry for prophylaxis and growth promotion purposes will increase the resistance in pathogenic strains and leads to resistance in the endogenous flora of human and animals applied in the poultry industry (Marshall & Levy, 2011). The emergence of resistant bacteria pathogen in chicken meat has pose failure in treatment (Poppe et al., 1995; Van Duijkeren & Houwers, 2000). Chickens are administered different antibiotics through chicken feed or drinking water in the farm (Van Duijkeren & Houwers, 2000). The source of bacteria antibiotic resistance may come from feed whereby antibiotic act as feed additive is added into feed to promote the growth of poultry (Tabatabaei & Nasirian, 2003). Inappropriate use of antibiotic has given selective pressure for multidrug bacteria resistance in poultry.

Multiple antibiotic resistances (MAR) index is a valid method to investigate the source of contamination in resistance isolates (Krumperman, 1983; Mthembu, 2008). High MAR index found in *Enterobacteriaceae* bacteria revealed that high risk present of multiple antibiotic bacteria resistant and poses highly contamination of chicken in the wet market.

5.4 Antibiotic Resistance Genes in *Salmonella* spp.

The increasing of emergence bacteria resistance to beta-lactam, macrolide and sulfonamide groups used in treatment for *Salmonella* infections (Sjölund-Karlsson et al., 2011). The oligonucleotide primers of *rfbJ* and *fljB* gene were used to target *S*. Typhimurium, *sefA* and *sdfI* were target specific identification for *S*. Enteritidis (Madadgar et al., 2008) and *viaB* gene for detection of *S*. Typhi from food samples (Kumar et al., 2006).

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Salmonella spp. is gram negative *Enterobacteriaceae* bacteria commonly used Type III secretion system (T3SS) to cause diseases in animals and plants. The *invA* is the core to function the T3SS in *Salmonella* spp. The *invA* is responsible for invasion of bacteria into epithelial cell of the host (Galan et al., 1992). The *invA* gene is responsible for invasion into epithelial cell of the host and normally use to specific identifies *Salmonella enterica* serovar in PCR (Darwin & Miller, 1999; Madadgar et al., 2008). The result concurs with other studies (Aabo et al., 1993; Chiu & Ou, 1996; Salehi et al., 2005).

Tetracycline, sulfonamide and macrolides groups are usually used in veterinary industry for growth promotion purpose (Landers et al., 2012).The high tetracycline resistance phenotypes observed in *Salmonella* isolates were linked to presence of *tetA* gene and *tetB* gene. Result showed positive 66 % in *tet*A gene and 5 % in *tet*B gene in *Salmonella* isolates. The result is in agreement with previous research in Canada and Korea (Aslam et al., 2012; Dessie et al., 2013). Transfer rate of tetracycline resistance gene between bacteria strains from chicken to human was high (Levy et al., 1976). Plasmid, transposon and integrons are mobile genetic elements involved of transfer resistance antibiotic determinants and contribute to the multi resistant bacteria strain (Van Hoek et al., 2011). Transfer of resistance genes present in zoonotic bacteria from food to human through consumes contaminated food with resistant bacteria strain (Sørum & L'Abée-Lund, 2002). Subsequently, antibiotics resistant in pathogenic bacteria are a significant public health concern.

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Resistance to sulfonamide phenotype in *Salmonella* isolates were observed in the study. The sulfonamide resistance genes usually acquisition of *sul1* and *sul2* genes were encoding for dihydropteroate synthase (Antunes et al., 2005). *Salmonella* isolates in agreement with earlier studies that reported *sul1* and *sul2* gene were common among bacteria in *Enterobacteriaceae* family (Antunes et al., 2005). However, the result showed *sul2* gene detected in all *Salmonella* isolates and only 20 isolates were detect *sul1* gene. The result disagrees with the findings in Korea reporting that *sul1* gene was the mechanism of resistance in *Salmonella* isolates (Antunes et al., 2005). The spread of *sul*2 gene is increased reported in United Kingdom and Poland (Lucarelli et al., 2010; Mąka et al., 2015).

The finding showed 5 % of *qnrB* and 63 % of *qnrS* gene were detected in *Salmonella* isolates. Plasmid-mediated quinolones resistance (PMQR) genes include *qnrA*, *qnrB* and *qnrS* are detected in *Enterobacteriaceae* bacteria (Poirel et al., 2012). The first *qnrA* gene was identified from *Klebsiella pneumoniae* reported in 1998, USA (Martínez-Martínez et al., 1998). Three well studied *qnr* genes, *qnrA*, *qnrB* and *qnrS* were widely detected in clinical and animal source of *Salmonella* isolates (Robicsek et al., 2006). PMQR genes in *Salmonella* isolates are emergence public health concern (Alcaine et al., 2007; Asai et al., 2010; Cattoir et al., 2007; Hopkins et al., 2007).

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The high macrolide resistance phenotypes observed in *Salmonella* isolates were linked to *mphA* gene. Result showed 11 % of *mph*A gene detected in *Salmonella* isolates. The *mphA* gene encoded as macrolide phosphotransferase that confer resistance to macrolides antibiotic groups in enteric bacteria such as *E. coli* (O'Hara et al., 1989). *MphA* gene involved in phosphorylates and inactivates erythromycin in *Enterobacteriaceae* due to efflux transporter (Leclercq, 2002). The *mphA* gene confers resistance to azithromycin that usually used as treatment for *Salmonella* infection (Hill & Beeching, 2010). The antibiotic resistance genes are exhibited in chromosomal, plasmid or *Salmonella* genomic island that consists of multidrug resistant region for antibiotic resistance phenotypes (Beutlich et al., 2011). Monitoring the multidrug resistance bacteria isolated from foods is important on appropriate antibiotics use in veterinary and human medicine (Cummings et al., 2013).

5.5 PFGE

PFGE is a gold standard molecular fingerprinting subtyping technique using nuclease digestion of genomic DNA to determine the relatedness and diversity of bacteria isolates (Sabat et al., 2013). It provides information in order to track the sources of infection and epidemic control (Neves et al., 2008; Roussel et al., 2014; Yang et al., 2010). In this study, PFGE was used for identify clonal relatedness of *Salmonella* spp., *Listeria* spp. and *E. coli* strains. The use of XbaI as restriction enzyme of *Salmonella* isolates in PFGE (Ammari et al., 2009; Sandt et al., 2013; Tsen & Lin, 2001) and *E. coli* isolates (Gai et al., 2015; Van den Bogaard et al., 2001) whereas *Listeria* isolates are used AscI as restriction enzyme to cut the DNA fragment in PFGE (Fox et al., 2012; Mendonça et al., 2015).

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Dice similarity coefficient is used to quantify the similarity between PFGE band patterns of bacteria isolates; the dendrogram showed that PFGE differentiated into groupings that correlated with antibiotic resistance patterns. Moreover, PFGE analysis revealed matching between *Salmonella* spp., *E. coli* and *Listeria* spp. isolated from chicken meat samples, suggesting chicken meat can be reservoir and source of *Salmonella* dissemination in the region. It can show high possibility of come from same original supplier source and distribute to different location of market place in Klang Valley area. Different isolates under same cluster were recovered from different time and sampling places.

High similarities of *E. coli* strain pattern were obtained in different geographical location. PFGE is a molecular technique used to determine the relatedness of *E. coli* strain isolated from different isolation places (Davis et al., 2003). *E. coli* are coliform bacteria responsible for the hygiene indicator in food samples, the emergence of multidrug resistant pattern among the bacterial flora of chicken meat (Talukdar et al., 2013). Identical PFGE pattern is suggests clonally relationship and high possibly originate from same ancestor (Gaul et al., 2007). The similarity observed in PFGE profile within each *Salmonella* spp., *E. coli* and *Listeria* spp. regardless of sampling place, suggested the possibility of transmission of clones in Klang Valley regions.

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5.6 Genome Analysis of *Salmonella* Strains

In the effort to identify antibiotic genes, genome sequencing was performed using HiSeq 2000 sequencer platform. To be more effective determine the gene, next generation sequencing (NGS) provide faster and accurate method on species characterization (Illumina, 2015). NGS able to assemble *Salmonella* strain PS01, 07, 12 and 13 genomes into 142 contigs, 125 contigs, 109 contigs and 106 contigs with many gaps. This may causes many crucial genes might fall in between the gaps and loss of valuable genes to analyze. Several studies were reported on HiSeq sequencing of antibiotic genes in *Salmonella* strains (Dhanani et al., 2015; Chiou et al., 2015; Labbé et al., 2016).

Salmonella spp. has two main species with approximately 2,600 known serovars. Serovar of the *Salmonella* is challenge for research work. Most of the serotyping methods are time consuming and difficult to differentiate among variety of *Salmonella* serovars. Most of *Salmonella* spp. under *enterica* species level is highly close related to each other. Due to diversify and complexity of *Salmonella serovar*, online servers such as RNAmmer 1.2 server and *in silico* typing resource (SISTR) were used for accurate *Salmonella* identification. RNAmmer server used to predict 16S, 23S and 5S ribosomal RNA in full genome sequence of prokaryotes whereas 18S, 28S and 8S normally found in eukaryotes (Lagesen et al., 2007). The 16S rRNA gene in prokaryotes is used to determine the identity of bacteria species. Hyper variable regions of 16S rRNA gene provide unique sequence for bacterial identification (Woo et al., 2008). The reasons include the house keeping genes almost found in all bacteria, the function of 16S rRNA gene seldom changed and it contains of 1,500 bp sufficient to provide informatics purpose (Patel, 2001).

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With the advance in whole genome sequencing, SISTR platform is an online software tool to determine the serovar prediction for *Salmonella* species. SISTR platform is an open web bioinformatics tool to perform *in silico* molecular typing analysis on draft genome of *Salmonella* assemblies. The software tool provides shorter time and accurate output as compared with serology-based method which is expensive and time consuming (Yoshida et al., 2016). Other studies reported on use of RNAmmer server (Ogunremi et al., 2014; Quainoo et al., 2017) and SISTR platform in *Salmonella* strains (Yachison et al., 2017).

Furthermore, Resfinder2.0 and RAST annotation system are server used to annotate genes of interest. Resfinder 2.0 server is a web-based method uses BLAST to identify acquired antibiotic resistance genes with best matching of more than 90 % identical index. The assembled genome serves as input and blast against Resfinder database to generate best matching gene as output.The higher percentages (% ID) in identity indicate the more identical between resistance gene in database and the genome. Resfinder is a bioinformatics tool specifically for analyze genome sequence data (Zankari et al., 2012). RAST server was used to determine gene function and provide to browse annotated genome within SEED integration. It also provides prediction of annotation for bacterial and archaeal genome service (Aziz et al., 2008).

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Annotation involved of raw data sequence is added to genome database process. The system will predict and identify protein- encoding, rRNA and tRNA gene with function and metabolic mechanism presented in genome as output. Two main classes of gene function include subsystem-based assertions (only using functional variants of subsystem) and non subsystem-based assertions (integration of numerous tools) (Aziz et al., 2008). Some research reported on use of Resfinder online tool (Moura et al., 2017; Toro et al., 2016; Folster et al., 2015) and RAST annotation system in *Salmonella* strains (Tasmin et al., 2017; Hindermann et al., 2017; Matthews et al., 2015).

5.7 Future Work

Multiple antibiotic resistant pathogenic bacteria are causes of significant mortality rate globally. Further research should be conducted to assess the risk of the consumption of chicken meat and chicken eggs contaminated with antibiotic resistant *Salmonella*, *Listeria* and *E. coli* strains. The need for continued surveillance on prevalence and on emerging antibiotics resistance to ensure the effective treatment of infection was recognized. Detailed genome analysis on pathogenicity and antibiotic resistance genes in *Salmonella* and *Listeria* spp. will aid in understanding of the antibiotic mechanisms and gene regulation.

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CHAPTER 6: CONCLUSION

In conclusion, the data presented in the study demonstrated that chicken meat collected in Klang Valley regions were detected with *Salmonella*, *Listeria* and *E. coli* isolates as confirmed by MALDI-TOF-MS. The number of samples size in this study limited the ability to give clear overview picture of analysis and thus contribute to atypical result. Future research would benefit from the use of a larger sample size. The findings in this work occurred may by chance due to low number of sample size. In this work presented low percentages of detection with *Salmonella*, *Listeria* and *E. coli* isolates in local markets. The prevalence and antimicrobial resistance profile of the bacteria isolated were determined in this work. The isolates exhibited multidrug resistance patterns indicates that chicken meat could be a source of infectious exposure for consumers. The high level of antibiotic resistance and multiple MDR patterns observed in *Salmonella*, *Listeria* and *E. coli* isolates suggest that control measures for antibiotic usage and surveillance should be enforced. PFGE and next generation analysis were useful tools for determining genetic diversity of *Salmonella* isolates. Of these, the draft genome of 4 selected *Salmonella enterica* isolates were sequenced using HiSeq 2000 Illumina platform and antibiotic resistance genes were annotated using RAST. Presence of potentially transferable antibiotic resistance genes in *Salmonella* highlights the health risk for consumers. Contamination of bacteria in poultry may originate from farm, food handler or from food process environments. High prevalence of *Salmonella*, *Listeria* and *E. coli* from chicken meat emphasizes the need for education on proper food handling and cooking practice to decrease the risk of transmission of bacteria from contaminated chicken meat and egg products. The detection of antibiotic resistance in this study provides clear picture for Ministries of Health and Agriculture on restriction usage of antibiotic in veterinary sector.

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Nonetheless, more continue surveillance need to be conducted on prevalence and antibiotic resistance in *Salmonella* and *Listeria* isolates for better understanding of the epidemiology of the isolates.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

LIST OF PUBLICATION

Chin, P. S., Yu, C. Y., Ang, G. Y., Yin, W. F., & Chan, K. G. (2017). Draft genome sequence of a multidrug resistant *Salmonella enterica* serovar Brancaster strain PS01 isolated from chicken meat, Malaysia. *Journal of Global Antimicrobial Resistance, 9,* 41-42.

LIST OF PRESENTATION

Chin, P. S., Tan, G. Y. A., & Chan, K. G. (2016, Nov). *Pathogenic bacteria in raw chicken meat obtained from Malaysian market*. Poster presented at Monash Science International Symposium 2016, Monash University, Malaysia.

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APPENDICES

Appendix A The 16S rRNA gene sequence of PS 01 strain

Accession number: PRJNA308552

>rRNA_S1R1__paired__trimmed__paired__contig_9_153-1682_DIR+ /molecule=16s_rRNA /score=1970.2
AGAGTTTGATCATGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAAC
GGTAACAGGAAGCAGCTTGCTGCTTCGCTGACGAGTGGCGGACGGGTGAGTAATGTCTGG
GAAACTGCCTGATGGAGGGGGATAACTACTGGAAACGGTGGCTAATACCGCATAACGTCG
CAAGACCAAAGAGGGGGACCTTCGGGCCTCTTGCCATCAGATGTGCCCAGATGGGATTAG
CTTGTTGGTGAGGTAACGGCTCACCAAGGCGACGATCCCTAGCTGGTCTGAGAGGATGAC
CAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATAT
TGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTT
GTAAAGTACTTTCAGCGGGGAGGAAGGTGTTGTGGTTAATAACCGCAGCAATTGACGTTA
CCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAA
GCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTCTGTCAAGTCGGATGTGA
AATCCCCGGGCTCAACCTGGGAACTGCATTCGAAACTGGCAGGCTTGAGTCTTGTAGAGG
GGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCG
AAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGA
TTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCTACTTGGAGGTTGTGCCCTTGAGG
CGTGGCTTCCGGAGCTAACGCGTTAAGTAGACCGCCTGGGGAGTACGGCCGCAAGGTTAA
AACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGC
AACGCGAAGAACCTTACCTGGTCTTGACATCCACAGAACTTTCCAGAGATGGATTGGTGC
CTTCGGGAACTGTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTG
GGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCGGTTAGGCCGGGAAC
TCAAAGGAGACTGCCAGTGATAAACTGGAGGAAGGTGGGGATGACGTCAAGTCATCATGG
CCCTTACGACCAGGGCTACACACGTGCTACAATGGCGCATACAAAGAGAAGCGACCTCGC
GAGAGCAAGCGGACCTCATAAAGTGCGTCGTAGTCCGGATTGGAGTCTGCAACTCGACTC
CATGAAGTCGGAATCGCTAGTAATCGTGGATCAGAATGCCACGGTGAATACGTTCCCGGG
CCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTTGCAAAAGAAGTAGGTAGCTTAA
CCTTCGGGAGGGCGCTTACCACTTTGTGATTCATGACTGGGGTGAAGTCGTAACAAGGTA
ACCGTAGGGGAACCTGCGGTTGGATCACCT

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Appendix B The 16S rRNA gene sequence of PS 07 strain

Accesion number: PRJNA310709

>rRNA_S7R1__paired__trimmed__paired__contig_39_50-1579_DIR- /molecule=16s_rRNA /score=1966.4
AGAGTTTGATCATGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAAC
GGTAACAGGAAGCAGCTTGCTGCTTTGCTGACGAGTGGCGGACGGGTGAGTAATGTCTGG
GAAACTGCCTGATGGAGGGGGATAACTACTGGAAACGGTGGCTAATACCGCATAACGTCG
CAAGACCAAAGAGGGGGACCTTCGGGCCTCTTGCCATCAGATGTGCCCAGATGGGATTAG
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Appendix C The 16S rRNA gene sequence of PS 12 strain.

Accesion number: PRJNA318665

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CT

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Appendix D The 16S rRNA gene sequence of PS 13 strain.

Accesion number: PRJNA318666

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Appendix E CLSI breakpoint of *Enterobacteriaceae*.

Test/Report Group	Antimicrobial Agent	Disk Content	Zone Diameter Interpretive Criteria nearest whole mm S	I	R	MIC Interpretive Criteria (µg/mL) S	I	R	Comments
PENICILLINS									
A	Ampicillin	10 µg	≥17	14–16	≤13	≤8	16	≥32	(4) **Results of ampicillin testing can be used to predict results for** amoxicillin. See comment (2).
B	Piperacillin	100 µg	≥21	18–20	≤17	≤16	32–64	≥128	
O	Mecillinam	10 µg	≥15	12–14	≤11	≤8	16	≥32	(5) For **testing and reporting of** *E. coli* urinary tract isolates only.
β-LACTAM/β-LACTAMASE INHIBITOR COMBINATIONS									
B	Amoxicillin-clavulanic acid	20/10 µg	≥18	14–17	≤13	≤8/4	16/8	≥32/16	
B	Ampicillin-sulbactam	10/10 µg	≥15	12–14	≤11	≤8/4	16/8	≥32/16	
B	Piperacillin-tazobactam	100/10 µg	≥21	18–20	≤17	≤16/4	32/4–64/4	≥128/4	
B	Ticarcillin-clavulanate	75/10 µg	≥20	15–19	≤14	≤16/2	32/2–64/2	≥128/2	
CEPHEMS (PARENTERAL) (including cephalosporins I, II, III, and IV. Please refer to Glossary I.)									
	(6) **WARNING:** For *Salmonella* spp. and *Shigella* spp., first- and second-generation cephalosporins and cephamycins may appear active *in vitro*, but are not effective clinically and should not be reported as susceptible.								
	(7) Following evaluation of PK-PD properties, limited clinical data, **and MIC distributions,** revised interpretive criteria for cephalosporins (cefazolin, cefotaxime, ceftazidime, ceftizoxime, and ceftriaxone) and aztreonam were first published in January 2010 (M100-S20) and are listed in this table. Cefazolin interpretive criteria were revised again in June 2010 and are listed below. Cefepime and cefuroxime (parenteral) were also evaluated; however, no change in interpretive criteria was required for the dosages indicated below. When using the **current** interpretive criteria, routine ESBL testing is no longer necessary before reporting results (ie, it is no longer necessary to edit results for cephalosporins, aztreonam, or penicillins from susceptible to resistant). **However,** ESBL testing may still be useful for epidemiological or infection control purposes. **For laboratories that have not implemented the current interpretive criteria, ESBL testing should be performed as described in Table 2A Supplemental Table 1.**								
	Note that interpretive criteria for drugs with limited availability in many countries (eg, moxalactam, cefonicid, cefamandole, and cefoperazone) were not evaluated. If considering use of these drugs for *E. coli, Klebsiella,* or *Proteus* spp., ESBL testing should be performed (see Table 2A **Supplemental Table 1**). If isolates test ESBL positive, the results for moxalactam, cefonicid, cefamandole, and cefoperazone should be reported as resistant.								
	(8) *Enterobacter, Citrobacter,* and *Serratia* may develop resistance during prolonged therapy with third-generation cephalosporins as **a result of derepression of AmpC β-lactamase**. Therefore, isolates that are initially susceptible may become resistant within three to four days after initiation of therapy. Testing of repeat isolates may be warranted.								
A	Cefazolin	30 µg	≥23	20–22	≤19	≤2	4	≥8	(9) Interpretive criteria are based on a dosage regimen of 2 g every 8 h. See comment (7).
U	Cephalothin	30 µg	≥18	15–17	≤14	≤8	16	≥32	(10) Cephalothin interpretive criteria can be used only to predict results to the oral agents, cefadroxil, cefpodoxime, cephalexin, and loracarbef. Older data that suggest that cephalothin results could predict susceptibility to some other cephalosporins may still be correct, but there are no recent data to confirm this.

Test/Report Group	Antimicrobial Agent	Disk Content	Zone Diameter Interpretive Criteria nearest whole mm S	I	R	MIC Interpretive Criteria (µg/mL) S	I	R	Comments
CEPHEMS (PARENTERAL) (including cephalosporins I, II, III, and IV. Please refer to Glossary I.) (Continued)									
B	Cefepime	30 µg	≥18	15–17	≤14	≤8	16	≥32	(11) Interpretive criteria are based on a dosage regimen of 1 g every 8 h or 2 g every 12 h. See comment (7).
B	Cefotaxime or	30 µg	≥26	23–25	≤22	≤1	2	≥4	(12) Interpretive criteria are based on a dosage regimen of 1 g every 24 h for ceftriaxone and 1 g every 8 h for cefotaxime. See comment (7).
B	ceftriaxone	30 µg	≥23	20–22	≤19	≤1	2	≥4	
B	Cefotetan	30 µg	≥16	13–15	≤12	≤16	32	≥64	
B	Cefoxitin	30 µg	≥18	15–17	≤14	≤8	16	≥32	**(13) The interpretive criteria for cefoxitin are based on a dosage regimen of at least 8 g per day (eg, 2 g every 6 h).**
B	Cefuroxime (parenteral)	30 µg	≥18	15–17	≤14	≤8	16	≥32	(14) Interpretive criteria are based on a dosage regimen of 1.5 g every 8 h. See comment (7).
C	Ceftazidime	30 µg	≥21	18–20	≤17	≤4	8	≥16	(15) Interpretive criteria are based on a dosage regimen of 1 g every 8 h. See comment (7).
O	Cefamandole	30 µg	≥18	15–17	≤14	≤8	16	≥32	See comment (7).
O	Cefmetazole	30 µg	≥16	13–15	≤12	≤16	32	≥64	**(16) Insufficient new data exist to reevaluate interpretive criteria listed here.**
O	Cefonicid	30 µg	≥18	15–17	≤14	≤8	16	≥32	See comment (7).
O	Cefoperazone	75 µg	≥21	16–20	≤15	≤16	32	≥64	See comment (7).
O	Ceftizoxime	30 µg	≥25	22–24	≤21	≤1	2	≥4	(17) Interpretive criteria are based on a dosage regimen of 1 g every 12 h. See comment (7).
O	Moxalactam	30 µg	≥23	15–22	≤14	≤8	16–32	≥64	See comment (7).
CEPHEMS (ORAL)									
B	Cefuroxime (oral)	30 µg	≥23	15–22	≤14	≤4	8–16	≥32	
O	Loracarbef	30 µg	≥18	15–17	≤14	≤8	16	≥32	(18) Because certain strains of *Citrobacter, Providencia,* and *Enterobacter* spp. have been reported to give false-susceptible results **when tested by disk diffusion** with cefdinir and loracarbef, strains of these genera should not be tested by disk diffusion with these **agents**.
O	Cefaclor	30 µg	≥18	15–17	≤14	≤8	16	≥32	
O	Cefdinir	5 µg	≥20	17–19	≤16	≤1	2	≥4	See comment (18).
O	Cefixime	5 µg	≥19	16–18	≤15	≤1	2	≥4	(19) For disk diffusion, not applicable for testing *Morganella* spp.
O	Cefpodoxime	10 µg	≥21	18–20	≤17	≤2	4	≥8	See comment (19).
O	Cefprozil	30 µg	≥18	15–17	≤14	≤8	16	≥32	(20) Because certain strains of *Providencia* spp. have been reported to give false-susceptible results **when tested by disk diffusion** with cefprozil, strains of this genus should not be tested **by** disk **diffusion with this agent**.
Inv.	Cefetamet	10 µg	≥18	15–17	≤14	≤4	8	≥16	See comment (19).
Inv.	Ceftibuten	30 µg	≥21	18–20	≤17	≤8	16	≥32	(21) **For testing and reporting of** urine isolates only.

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Table 2A. (Continued)

Test/Report Group	Antimicrobial Agent	Disk Content	Zone Diameter Interpretive Criteria nearest whole mm S	I	R	MIC Interpretive Criteria (µg/mL) S	I	R	Comments
FLUOROQUINOLONES									
(31) **NOTE: Reevaluation of fluoroquinolones is ongoing.** See comment (2).									
B	Ciprofloxacin	5 µg	≥21	16–20	≤15	≤1	2	≥4	**(32) For testing and reporting against *Enterobacteriaceae* other than *S. typhi* and extraintestinal *Salmonella* spp.**
B	Levofloxacin	5 µg	≥17	14–16	≤13	≤2	4	≥8	
B	**Ciprofloxacin**	**5 µg**	**≥31**	**21-30**	**≤20**	**≤0.06**	**0.12–0.5**	**≥1**	**(33) For reporting against *S. typhi* and extraintestinal *Salmonella* spp. only.** **(34) Because of limited clinical experience in the treatment of infections caused by *S. typhi* and extraintestinal *Salmonella* spp. with ciprofloxacin MICs or zone diameters in the intermediate range, clinicians may wish to use maximal oral or parenteral dosage regimens. See comment (36).**
U	Lomefloxacin or	10 µg	≥22	19–21	≤18	≤2	4	≥8	
U	ofloxacin	5 µg	≥16	13–15	≤12	≤2	4	≥8	
U	Norfloxacin	10 µg	≥17	13–16	≤12	≤4	8	≥16	
O	Enoxacin	10 µg	≥18	15–17	≤14	≤2	4	≥8	
O	Gatifloxacin	5 µg	≥18	15–17	≤14	≤2	4	≥8	
O	Gemifloxacin	5 µg	≥20	16–19	≤15	≤0.25	0.5	≥1	(35) FDA-approved for *Klebsiella pneumoniae*.
O	Grepafloxacin	5 µg	≥18	15–17	≤14	≤1	2	≥4	
Inv.	Fleroxacin	5 µg	≥19	16–18	≤15	≤2	4	≥8	
QUINOLONES									
O	Cinoxacin	100 µg	≥19	15–18	≤14	≤16	32	≥64	See comment (21).
O	Nalidixic acid	30 µg	≥19	14–18	≤13	≤16	–	≥32	(36) In addition to testing urine isolates, nalidixic acid may be used to test for reduced fluoroquinolone susceptibility in isolates from patients with extraintestinal *Salmonella* infections. **Strains of *Salmonella* that test resistant to nalidixic acid may be associated with clinical failure or delayed response in fluoroquinolone-treated patients with extraintestinal salmonellosis.** **However, nalidixic acid may not detect all mechanisms of fluoroquinolone resistance. Therefore, *Salmonella* strains may also be tested with ciprofloxacin and reported using the *Salmonella* spp. interpretive criteria above. See comments (32) and (33).** See comments (21) and (31).
FOLATE PATHWAY INHIBITORS									
B	Trimethoprim-sulfamethoxazole	1.25/23.75 µg	≥16	11–15	≤10	≤2/38	–	≥4/76	See comment (2).
U	Sulfonamides	250 or 300 µg	≥17	13–16	≤12	≤256	–	≥512	(37) Sulfisoxazole can be used to represent any of the currently available sulfonamide preparations.
U	Trimethoprim	5 µg	≥16	11–15	≤10	≤8	–	≥16	

Table 2A. (Continued)

Test/Report Group	Antimicrobial Agent	Disk Content	Zone Diameter Interpretive Criteria nearest whole mm S	I	R	MIC Interpretive Criteria (µg/mL) S	I	R	Comments
MONOBACTAMS									
C	Aztreonam	30 µg	≥21	18–20	≤17	≤4	8	≥16	(22) Interpretive criteria are based on a dosage regimen of 1 g every 8 h. See comment (7).
CARBAPENEMS									
(23) Following evaluation of PK-PD properties, limited clinical data, and MIC distributions that include recently described carbapenemase producing strains, revised interpretive criteria for carbapenems were first published in June 2010 (M100-S20-U) and are listed below. Because of limited treatment options for infections caused by organisms with carbapenem MICs or zone diameters in the intermediate range, clinicians may wish to design carbapenem dosage regimens that use maximum recommended doses and possibly prolonged intravenous infusion regimens, as has been reported in the literature.[1-6] Consultation with an infectious diseases practitioner is recommended for isolates for which the carbapenem MICs or zone diameter results from disk diffusion testing are in the intermediate or resistant ranges. Until laboratories can implement the current interpretive criteria, the MHT should be performed as described in the updated Table 2A **Supplemental Table 3**. After implementation of the **current** interpretive criteria, the MHT does not need to be performed other than for epidemiological or infection control purposes (refer to Table 2A **Supplemental Table 2**). The following information is provided as background on carbapenemases in *Enterobacteriaceae* that are largely responsible for MICs and zone diameters in the new intermediate and resistant ranges, and thus the rationale for setting revised carbapenem breakpoints: • The clinical effectiveness of carbapenem treatment of infections produced by isolates for which the carbapenem MIC or disk diffusion test results are within the new intermediate (I) range is uncertain due to lack of controlled clinical studies. • Imipenem MICs for *Proteus* spp., *Providencia* spp., and *Morganella morganii* tend to be higher (eg, MICs in the new intermediate or resistant range) than meropenem or doripenem MICs. These isolates may have elevated MICs by mechanisms other than production of carbapenemases.									
B	Doripenem	10 µg	≥23	20–22	≤19	≤1	2	≥4	(24) Interpretive criteria are based on a dosage regimen of 500 mg every 8 h.
B	Ertapenem	10 µg	**≥22**	**19–21**	**≤18**	**≤0.5**	**1**	**≥2**	(25) Interpretive criteria are based on a dosage regimen of 1 g every 24 h.
B	Imipenem	10 µg	≥23	20–22	≤19	≤1	2	≥4	(26) Interpretive criteria are based on a dosage regimen of 500 mg every 6 h or 1 g every 8 h.
B	Meropenem	10 µg	≥23	20–22	≤19	≤1	2	≥4	(27) Interpretive criteria are based on a dosage regimen of 1 g every 8 h.
AMINOGLYCOSIDES									
(28) **WARNING:** For *Salmonella* spp. and *Shigella* spp., aminoglycosides may appear active *in vitro* but are not effective clinically and should not be reported as susceptible.									
A	Gentamicin	10 µg	≥15	13–14	≤12	≤4	8	≥16	
A	Tobramycin	10 µg	≥15	13–14	≤12	≤4	8	≥16	
B	Amikacin	30 µg	≥17	15–16	≤14	≤16	32	≥64	
O	Kanamycin	30 µg	≥18	14–17	≤13	≤16	32	≥64	
O	Netilmicin	30 µg	≥15	13–14	≤12	≤8	16	≥32	
O	Streptomycin	10 µg	≥15	12–14	≤11	–	–	–	(29) There are no MIC interpretive standards.
TETRACYCLINES									
(30) Organisms that are susceptible to tetracycline are also considered susceptible to doxycycline and minocycline. However, some organisms that are intermediate or resistant to tetracycline may be susceptible to doxycycline, minocycline, or both.									
C	Tetracycline	30 µg	≥15	12–14	≤11	≤4	8	≥16	
O	Doxycycline	30 µg	≥14	11–13	≤10	≤4	8	≥16	
O	Minocycline	30 µg	≥16	13–15	≤12	≤4	8	≥16	

Table 2A. (Continued)

PHENICOLS									
C	Chloramphenicol	30 µg	≥18	13–17	≤12	≤8	16	≥32	(38) Not routinely reported on isolates from the urinary tract.
FOSFOMYCINS									
O	Fosfomycin	200 µg	≥16	13–15	≤12	≤64	128	≥256	(39) **For testing and reporting of** *E. coli* urinary tract isolates only. (40) The 200-µg fosfomycin disk contains 50 µg of glucose-6-phosphate. 41) The **only** approved MIC **method for** testing is agar dilution **using** agar media supplemented with 25 µg/mL of glucose-6-phosphate. Broth dilution **MIC testing** should not be performed.
NITROFURANS									
U	Nitrofurantoin	300 µg	≥17	15–16	≤14	≤32	64	≥128	

Abbreviations: ATCC, American Type Culture Collection; CAMHB, cation-adjusted Mueller-Hinton broth; ESBL, extended-spectrum β-lactamase; FDA, US Food and Drug Administration; MHA, Mueller-Hinton agar; MHT, modified Hodge test; MIC, minimal inhibitory concentration; PK-PD, pharmacokinetic-pharmacodynamic; QC, quality control.

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Appendix F CLSI breakpoint of *Staphylococcus aureus*.

Table 2C. Zone Diameter and MIC Interpretive Standards for *Staphylococcus* spp. M02 and M07

Testing Conditions

Medium: Disk diffusion: MHA
Broth dilution: CAMHB; CAMHB + 2% NaCl for oxacillin, methicillin, and nafcillin; CAMHB supplemented to 50 µg/mL calcium for daptomycin
Agar dilution: MHA; MHA + 2% NaCl for oxacillin, methicillin, and nafcillin. Agar dilution has not been validated for daptomycin.
Inoculum: Direct colony suspension, equivalent to a 0.5 McFarland standard
Incubation: 35±2°C; ambient air;
Disk diffusion: 16 to 18 hours; 24 hours (coagulase-negative staphylococci and cefoxitin);
Dilution methods: 16 to 20 hours;
All methods: 24 hours for oxacillin, methicillin, nafcillin, and vancomycin. Testing at temperatures above 35°C may not detect MRS.

Minimal QC Recommendations (See Tables 3A and 4A for acceptable QC ranges.)

Staphylococcus aureus ATCC® 25923 (disk diffusion)
Staphylococcus aureus ATCC® 29213 (MIC)
Escherichia coli ATCC® 35218 (for β-lactam/β-lactamase inhibitor combinations)

Test/Report Group	Antimicrobial Agent	Disk Content	Zone Diameter Interpretive Criteria nearest whole mm S	I	R	MIC Interpretive Criteria (µg/mL) S	I	R	Comments
PENICILLINS									
(9) Penicillin-susceptible staphylococci are also susceptible to other penicillins, β-lactam/β-lactamase inhibitor combinations, antistaphylococcal cephems, and carbapenems approved for use by the FDA for staphylococcal infections. Penicillin-resistant, oxacillin-susceptible strains are resistant to penicillinase-labile penicillins, but susceptible to other penicillinase-stable penicillins, β-lactam/β-lactamase inhibitor combinations, antistaphylococcal cephems, and carbapenems. Oxacillin-resistant staphylococci are resistant to all currently available β-lactam antimicrobial agents, with the exception of the newer cephalosporins with anti-MRSA activity. Thus, susceptibility or resistance to a wide array of β-lactam antimicrobial agents may be deduced from testing only penicillin and either cefoxitin or oxacillin. Routine testing of other penicillins, β-lactam/β-lactamase inhibitor combinations, cephems, or carbapenems is not advised. (10) If a penicillinase-stable penicillin is tested, oxacillin is the preferred agent and results can be applied to the other penicillinase-stable penicillins, cloxacillin, dicloxacillin, flucloxacillin, methicillin, and nafcillin. See comment (4).									
A	Penicillin	10 units	≥29	–	≤28	≤0.12	–	≥0.25	(11) Penicillin-resistant strains of staphylococci produce β-lactamase, and the testing of penicillin instead of ampicillin is preferred. Penicillin should be used to test the susceptibility of all staphylococci to all penicillinase-labile penicillins, such as ampicillin, amoxicillin, azlocillin, carbenicillin, mezlocillin, piperacillin, and ticarcillin. Perform **test(s) to detect** β-lactamase **production** on **staphylococci** for which the penicillin MICs are ≤ 0.12 µg/mL or zone diameters ≥ 29 mm before reporting the isolate as penicillin susceptible. Rare isolates of staphylococci that contain genes for β-lactamase production may **appear negative by** β-lactamase tests. Consequently, for serious infections requiring penicillin therapy, laboratories should perform MIC tests and β-lactamase testing on all subsequent isolates from the same patient. PCR testing of the isolate for the *blaZ* β-lactamase gene may be considered. See Table 2C **Supplemental Tables 1 and 3** at the end of Table 2C. **(12) For oxacillin-resistant staphylococci report penicillin as resistant or do not report.**
A	Oxacillin For *S. aureus* and *S. lugdunensis*.	1 µg oxacillin	≥13	11–12	≤10	≤2 (oxacillin)	–	≥4 (oxacillin)	For **use with** *S. aureus*. **(13) If oxacillin-intermediate results (disk diffusion testing) are obtained for *S. aureus*, perform testing for *mecA* or PBP 2a, the cefoxitin MIC or cefoxitin disk test, an oxacillin MIC test, or the oxacillin-salt agar screening test. Report the result of the alternative test rather than the oxacillin intermediate result [see comment (14) for reporting oxacillin when using cefoxitin as a surrogate test].**
		1 µg oxacillin	–	–	–	≤2 (oxacillin)	–	≥4 (oxacillin)	For **use with** *S. lugdunensis*.
		30 µg cefoxitin	≥22	–	≤21	≤4 (cefoxitin)	–	≥8 (cefoxitin)	For **use with** *S. aureus* and *S. lugdunensis*. (14) Cefoxitin is used as a surrogate for oxacillin resistance; report oxacillin susceptible or resistant based on the cefoxitin result. (15) If both cefoxitin and oxacillin are tested against *S. aureus* or *S. lugdunensis*, and either result is resistant, the organism should be reported as oxacillin resistant. See comments **(5) and** (9).

Test/Report Group	Antimicrobial Agent	Disk Content	Zone Diameter Interpretive Criteria nearest whole mm S	I	R	MIC Interpretive Criteria (µg/mL) S	I	R	Comments
PENICILLINS (Continued)									
A	Oxacillin For coagulase-negative staphylococci except *S. lugdunensis*.	1 µg oxacillin	–	–	–	≤0.25 (oxacillin)	–	≥0.5 (oxacillin)	For **use with** coagulase-negative staphylococci except *S. lugdunensis*. (16) Oxacillin interpretive criteria may overcall resistance for some coagulase-negative staphylococci, because some non–*S. epidermidis* strains for which the oxacillin MICs are 0.5 to 2 µg/mL lack *mecA*. For serious infections with coagulase-negative staphylococci other than *S. epidermidis*, testing for *mecA* or for PBP 2a or with cefoxitin disk diffusion may be appropriate for strains for which the oxacillin MICs are 0.5 to 2 µg/mL.
		30 µg cefoxitin	≥25	–	≤24	–	–	–	See comments **(5),** (9), **and (14).**

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Test/Report Group	Antimicrobial Agent	Disk Content	Zone Diameter Interpretive Criteria nearest whole mm			MIC Interpretive Criteria (µg/mL)			Comments
			S	I	R	S	I	R	
PENICILLINS (Continued)									
O	Ampicillin	10 µg	≥29	–	≤28	≤0.25	–	≥0.5	(17) Class representative for ampicillin and amoxicillin. (18) For oxacillin-resistant staphylococci, report ampicillin as resistant or do not report.
O	Methicillin	5 µg	≥14	10–13	≤9	≤8	–	≥16	For use with *S. aureus* only.
O	Nafcillin	1 µg	≥13	11–12	≤10	≤2	–	≥4	For use with *S. aureus* only.
β-LACTAM/β-LACTAMASE INHIBITOR COMBINATIONS (19) For oxacillin-resistant staphylococci, report as resistant or do not report. See comments (4) and (9).									
O	Amoxicillin-clavulanic acid	20/10 µg	≥20	–	≤19	≤4/2	–	≥8/4	
O	Ampicillin-sulbactam	10/10 µg	≥15	12–14	≤11	≤8/4	16/8	≥32/16	
O	Piperacillin-tazobactam	100/10 µg	≥18	–	≤17	≤8/4	–	≥16/4	
O	Ticarcillin-clavulanic acid	75/10 µg	≥23	–	≤22	≤8/2	–	≥16/2	
CEPHEMS (PARENTERAL) (Including cephalosporins I, II, III, and IV. Please refer to Glossary I.) See comments (4), (9), and (19).									
O	Cefamandole	30 µg	≥18	15–17	≤14	≤8	16	≥32	
O	Cefazolin	30 µg	≥18	15–17	≤14	≤8	16	≥32	
O	Cefepime	30 µg	≥18	15–17	≤14	≤8	16	≥32	
O	Cefmetazole	30 µg	≥16	13–15	≤12	≤16	32	≥64	
O	Cefonicid	30 µg	≥18	15–17	≤14	≤8	16	≥32	
O	Cefoperazone	75 µg	≥21	16–20	≤15	≤16	32	≥64	
O	Cefotaxime	30 µg	≥23	15–22	≤14	≤8	16–32	≥64	
O	Cefotetan	30 µg	≥16	13–15	≤12	≤16	32	≥64	
O	Ceftazidime	30 µg	≥18	15–17	≤14	≤8	16	≥32	
O	Ceftizoxime	30 µg	≥20	15–19	≤14	≤8	16–32	≥64	
O	Ceftriaxone	30 µg	≥21	14–20	≤13	≤8	16–32	≥64	
O	Cefuroxime (parenteral)	30 µg	≥18	15–17	≤14	≤8	16	≥32	
O	Cephalothin	30 µg	≥18	15–17	≤14	≤8	16	≥32	
O	Moxalactam	30 µg	≥23	15–22	≤14	≤8	16–32	≥64	
CEPHEMS (ORAL) See comments (4), (9), and (19).									
O	Cefaclor	30 µg	≥18	15–17	≤14	≤8	16	≥32	
O	Cefdinir	5 µg	≥20	17–19	≤16	≤1	2	≥4	
O	Cefpodoxime	10 µg	≥21	18–20	≤17	≤2	4	≥8	
O	Cefprozil	30 µg	≥18	15–17	≤14	≤8	16	≥32	
O	Cefuroxime (oral)	30 µg	≥23	15–22	≤14	≤4	8–16	≥32	
O	Loracarbef	30 µg	≥18	15–17	≤14	≤8	16	≥32	

Test/Report Group	Antimicrobial Agent	Disk Content	Zone Diameter Interpretive Criteria nearest whole mm			MIC Interpretive Criteria (µg/mL)			Comments
			S	I	R	S	I	R	
CARBAPENEMS See comments (4), (9), and (19).									
O	**Doripenem**	**10 µg**	**≥30**	–	–	**≤0.5**	–	–	**(20) For use with methicillin-susceptible staphylococci only.** **See comment (7).**
O	Ertapenem	10 µg	≥19	16–18	≤15	≤2	4	≥8	
O	Imipenem	10 µg	≥16	14–15	≤13	≤4	8	≥16	
O	Meropenem	10 µg	≥16	14–15	≤13	≤4	8	≥16	
GLYCOPEPTIDES									
B	Vancomycin	–	–	–	–	≤2	4–8	≥16	For **use with** *S. aureus*. (21) MIC tests should be performed to determine the susceptibility of all isolates of staphylococci to vancomycin. The disk test does not differentiate vancomycin-susceptible isolates of *S. aureus* from vancomycin-intermediate isolates, nor does the test differentiate among vancomycin-susceptible, intermediate, and resistant isolates of coagulase-negative staphylococci, all of which will give similar size zones of inhibition. (22) The vancomycin 30-µg disk test detects *S. aureus* isolates containing the *vanA* vancomycin resistance gene (VRSA). Such isolates will show no zone of inhibition around the disk (zone = 6 mm). The identification of isolates showing no zone of inhibition should be confirmed. Isolates of staphylococci producing vancomycin zones of ≥ 7 mm should not be reported as susceptible without performing a vancomycin MIC test. (23) Send any *S. aureus* for which the vancomycin is ≥ 8 µg/mL to a reference laboratory. See Appendix A. (24) Disk diffusion testing is not reliable for testing vancomycin. Also refer to Table 2C **Supplemental Table 2** for *S. aureus* at the end of Table 2C, Section 12.1.3 in M07-A9, and Section 11.1.3 in M02-A11.

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Test/Report Group	Antimicrobial Agent	Disk Content	Zone Diameter Interpretive Criteria nearest whole mm			MIC Interpretive Criteria (µg/mL)			Comments
			S	I	R	S	I	R	
GLYCOPEPTIDES (Continued)									
B	Vancomycin	–	–	–	–	≤4	8–16	≥32	For **use with** coagulase-negative staphylococci. See comments (21) and (24). (25) Send any coagulase-negative *Staphylococcus* for which the vancomycin MIC is ≥ 32 µg/mL to a reference laboratory. See Appendix A. See also Section 12.1.3 in M07-A9 and Section 11.1.3 in M02-A11.
Inv.	Teicoplanin	30 µg	≥14	11–13	≤10	≤8	16	≥32	(26) Teicoplanin disk diffusion interpretive criteria were not reevaluated concurrent with the reevaluation of vancomycin disk diffusion interpretive criteria. Therefore, the ability of these teicoplanin interpretive criteria to differentiate teicoplanin-intermediate and teicoplanin-resistant staphylococci from teicoplanin-susceptible strains is not known.
LIPOPEPTIDES									
B	Daptomycin	–	–	–	–	≤1	–	–	(27) Disk diffusion testing is not reliable for testing daptomycin. (28) Daptomycin should not be reported for isolates from the lower respiratory tract. See comment (7).
AMINOGLYCOSIDES									
C	Gentamicin	10 µg	≥15	13–14	≤12	≤4	8	≥16	
O	Amikacin	30 µg	≥17	15–16	≤14	≤16	32	≥64	
O	Kanamycin	30 µg	≥18	14–17	≤13	≤16	32	≥64	
O	Netilmicin	30 µg	≥15	13–14	≤12	≤8	16	≥32	
O	Tobramycin	10 µg	≥15	13–14	≤12	≤4	8	≥16	
MACROLIDES (29) Not routinely reported on organisms isolated from the urinary tract.									
A	Azithromycin or	15 µg	≥18	14–17	≤13	≤2	4	≥8	
A	clarithromycin or	15 µg	≥18	14–17	≤13	≤2	4	≥8	
A	erythromycin	15 µg	≥23	14–22	≤13	≤0.5	1–4	≥8	
B	Telithromycin	15 µg	≥22	19–21	≤18	≤1	2	≥4	
O	Dirithromycin	15 µg	≥19	16–18	≤15	≤2	4	≥8	

Test/Report Group	Antimicrobial Agent	Disk Content	Zone Diameter Interpretive Criteria nearest whole mm			MIC Interpretive Criteria (µg/mL)			Comments
			S	I	R	S	I	R	
TETRACYCLINES (30) Organisms that are susceptible to tetracycline are also considered susceptible to doxycycline and minocycline. However, some organisms that are intermediate or resistant to tetracycline may be susceptible to doxycycline, minocycline, or both.									
B	Tetracycline	30 µg	≥19	15–18	≤14	≤4	8	≥16	
B	Doxycycline	30 µg	≥16	13–15	≤12	≤4	8	≥16	
B	Minocycline	30 µg	≥19	15–18	≤14	≤4	8	≥16	
FLUOROQUINOLONES (31) *Staphylococcus* spp. may develop resistance during prolonged therapy with quinolones. Therefore, isolates that are initially susceptible may become resistant within three to four days after initiation of therapy. Testing of repeat isolates may be warranted.									
C	Ciprofloxacin or	5 µg	≥21	16–20	≤15	≤1	2	≥4	
C	levofloxacin or	5 µg	≥19	16–18	≤15	≤1	2	≥4	
C	ofloxacin	5 µg	≥18	15–17	≤14	≤1	2	≥4	
C	Moxifloxacin	5 µg	≥24	21–23	≤20	≤0.5	1	≥2	
U	Lomefloxacin	10 µg	≥22	19–21	≤18	≤2	4	≥8	
U	Norfloxacin	10 µg	≥17	13–16	≤12	≤4	8	≥16	
O	Enoxacin	10 µg	≥18	15–17	≤14	≤2	4	≥8	(32) FDA approved for *S. saprophyticus* and *S. epidermidis* (but not for *S. aureus*).
O	Gatifloxacin	5 µg	≥23	20–22	≤19	≤0.5	1	≥2	
O	Grepafloxacin	5 µg	≥18	15–17	≤14	≤1	2	≥4	
O	Sparfloxacin	5 µg	≥19	16–18	≤15	≤0.5	1	≥2	
Inv.	Fleroxacin	5 µg	≥19	16–18	≤15	≤2	4	≥8	

Test/Report Group	Antimicrobial Agent	Disk Content	Zone Diameter Interpretive Criteria nearest whole mm			MIC Interpretive Criteria (µg/mL)			Comments
			S	I	R	S	I	R	
NITROFURANTOINS									
U	Nitrofurantoin	300 µg	≥17	15–16	≤14	≤32	64	≥128	
LINCOSAMIDES									
A	Clindamycin	2 µg	≥21	15–20	≤14	≤0.5	1–2	≥4	(33) Inducible clindamycin resistance can be detected by disk diffusion using the D-zone test and by broth microdilution using a single well containing a combination of erythromycin and clindamycin. See Table 2C **Supplemental Tables 2 and 3 for the most current recommendations, and** Section 12 in M02-A11, and Section 13 in M07-A9 for **general** recommendations. See comment (29).
FOLATE PATHWAY INHIBITORS									
A	Trimethoprim-sulfamethoxazole	1.25/23.75 µg	≥16	11–15	≤10	≤2/38	–	≥4/76	
U	Sulfonamides	250 or 300 µg	≥17	13–16	≤12	≤256	–	≥512	(34) Sulfisoxazole can be used to represent any of the currently available sulfonamide preparations.
U	Trimethoprim	5 µg	≥16	11–15	≤10	≤8	–	≥16	
PHENICOLS									
C	Chloramphenicol	30 µg	≥18	13–17	≤12	≤8	16	≥32	See comment (29).
ANSAMYCINS									
B	Rifampin	5 µg	≥20	17–19	≤16	≤1	2	≥4	(35) ***Rx:*** Rifampin should not be used alone for antimicrobial therapy.
STREPTOGRAMINS									
C	Quinupristin-dalfopristin	15 µg	≥19	16–18	≤15	≤1	2	≥4	(36) For reporting against methicillin-susceptible *S. aureus*.
OXAZOLIDINONES									
B	Linezolid	30 µg	≥21	–	≤20	≤4	–	≥8	(37) When testing linezolid, disk diffusion zones should be examined using transmitted light. Organisms with resistant results by disk diffusion should be confirmed using an MIC method.

Abbreviations: ATCC, American Type Culture Collection; CAMHB, cation-adjusted Mueller-Hinton broth; FDA, US Food and Drug Administration; MHA, Mueller-Hinton agar; MIC, minimal inhibitory concentration; MRS, methicillin-resistant staphylococci; MRSA, methicillin-resistant *S. aureus;* MOD-SA, modified *S. aureus;* PBP, penicillin-binding protein; PCR, polymerase chain reaction; QC, quality control; VRSA, *vanA* vancomycin resistance gene.

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