PREVALENCE, ANTIBIOTIC RESISTANCE AND MOLECULAR CHARACTERIZATION OF *Aeromonas* spp., *Salmonella* Serovars **AND** *Listeria* spp**. ISOLATED FROM CATFISH** (*Clarias gariepinus*) **AND TILAPIA** (*Tilapia mossambica*) **OBTAINED FROM WET MARKETS AND PONDS**

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UNIVERSITI SAINS MALAYSIA

2014

PREVALENCE, ANTIBIOTIC RESISTANCE AND MOLECULAR CHARACTERIZATION OF *Aeromonas* spp., *Salmonella* Serovars **AND** *Listeria* spp. **ISOLATED FROM CATFISH (*Clarias gariepinus*) AND TILAPIA (*Tilapia mossambica*) OBTAINED FROM WET MARKETS AND PONDS**

By

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Thesis submitted in fulfillment of the requirements

for the Degree of Doctor of Philosophy

January 2014

ACKNOWLEDGEMENTS

My sincere gratitude to my supervisors Professor Gulam Rusul bin Rahmat Ali, Associate Prof. Dr. Rosma Ahmad, Associate Prof. Dr. Yahya Mat Arip for their excellence guidance, valuable advice, and encouraging attitude towards this thesis.

I am grateful to Prof. Thong Kwai Lin, Prof. Mohd. Razip Samian, Associate Prof. Dr. Rajeev Bhat, Associate Prof. Dr. Abbas FM AlKarkhi, Associate Prof. Dr. Latiffah Zakaria, Associate Prof. Dr. Liong Min Tze, Dr. Norrakiah Abdullah Sani, Puan Wan Nadiah Wan Abdullah for their useful discussions and help with the analysis, writing and statistical methods.

Special thanks to Associate Profesor Dr. Nurul Huda for introducing me about academic and social life in USM. I appreciate to Encik Ruslan Abdul Ghoni, Dr. F. Adzitey, Mr. L.C. Yung, Mr. Raja Arief, Ms. Natalie Chuah, Ms. Suguna M. for the helping, problem solving atmosphere and friendship. My gratitude also goes to my colleagues at Food Technology Division, School of Industrial Technology USM for a pleasant working atmosphere and assistance.

Last, I would like to thanks to Institute of Postgraduate Studies, Universiti Sains Malaysia for their financial support in the form of USM fellowship towards my studies. This project was supported by grants from Ministry of Science, Technology and Innovation Malaysia (305/PTEKIND/613512) and USM - Research University Postgraduate Research Grant Scheme (1001/PTEKIND/843110).

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LIST OF ABBREVIATIONS

ALOA : Agar *Listeria* According to Ottaviani and Agosti

ANOVA : Analysis of variance

AOAC : Association of Official Analytical Chemist

Azm : Azithromycin

BPW : Buffered peptone water

BSA : Bismuth Sulfite Agar

C : Chloramphenicol

CFSPH : Center for Food Security and Public Health

CLSI : Clinical and Laboratory Standards Institute

Cip : Ciprofloxacin

dNTP : Deoxyribonucleotide triphosphate

Da : Clindamycin

EC : European Commission

EPA : Environmental Protection Agency

ERIC : Enterobacterial repetitive intergenic consensus

EUCAST : European Committee on Antimicrobial Susceptibility Testing

FAO : Food and Agriculture Organization

FB : Frazer Broth

FSIS : Food Safety and Inspection Service

GAP : Good Aquaculture Practices

GSP : Glutamate Starch Phenol Red Agar

HACCP : Hazard analysis and critical control points

HFB : Half Frazer Broth

ISO : International Organization for Standardization

Kb : kilo base

LB : Luria-Bertani

MAR : Multiple antibiotics resistance

$MgCl_2$: Magnesium chloride

MLST : Multi Locus Sequence Typing

NA : Nutrient Agar

NACA : Network of Aquaculture Centres in Asia-Pacific

NTSYS : Numerical Taxonomy and Multivariate Analysis Systems

NZC : National Zoonoses Conference

OP : Operon

PALCAM : Polymixin, acriflavin, lithium chloride, ceftazidime, aesculine, mannitol

PCR : Polymerase chain reaction

PFGE : Pulsed field gel electrophoresis

PW : Peptone Water

RAPD : Random amplified polymorphic DNA

RNA : Ribonucleic acid

Rd : Rifampin

REP : Repetitive extragenic palindromic

RV : Rappaport and Vassiliadis broth

SA : Starch Agar

SAP : Sub-regional Office in the Pacific Islands

SEC : Sub-regional Office of Central Asia

Sh : Spectinomycin

SOP : Standard operating procedure

SPC : Secretariat of the Pacific Community

Sxt : Sulphamethoxazole-trimethoprim

TSB : Triptic Soya Broth

Te : Tetracycline

Tob : Tobramycin

USDA : United States Department of Agriculture

UPGMA : Unweighted Pair Group Method with Arithmetic Mean

US-FDA : United States Food and Drug Administration

UV : Ultra-violet

WHO : World Health Organization

XLD : Xylose Lysine Deoxycholate

XLT4 : Xylose-Lysine Tergitol 4

KELAZIMAN, RINTANGAN ANTIBIOTIK DAN PENCIRIAN MOLEKUL *Aeromonas* spp., *Salmonella* serovar DAN *Listeria* spp. YANG DIPENCILKAN DARIPADA IKAN KELI (*Clarias gariepinus*) DAN IKAN TILAPIA (*Tilapia mossambica*) DIPEROLEHI DARIPADA PASAR DAN KOLAM

ABSTRAK

Kajian ini melaporkan kelaziman, rintangan antibiotik dan pencirian molekul *Aeromonas* spp., *Salmonella* serovar dan *Listeria* spp. yang dipencilkan dari ikan keli (*Clarias gariepinus*) dan tilapia (*Tilapia mossambica*) diperolehi daripada pasar dan kolam (kolam tanah dan bekas lombong) di Malaysia Utara. Sebanyak 163 pencilan *Aeromonas* spp, 43 pencilan *Salmonella* serovar, dan 54 pencilan *Listeria* spp. telah dipencilkan daripada 32 bilasan ikan keli, 32 usus ikan keli, 32 bilasan tilapia, 32 usus tilapia, 44 air yang diperolehi daripada sembilan pasar dan lapan kolam di Penang, Kedah and Perak, Malaysia (2008 - 2009). Kelaziman *Aeromonas* spp., *Salmonella* serovar dan *Listeria* spp. dalam ikan keli adalah 14/32 (43.8%), 9/32 (28.1%), dan 10/32 (31.3%). Masing-masing kelaziman *Aeromonas* spp., *Salmonella* serovar dan *Listeria* spp. dalam tilapia adalah 21/32 (65.6%), 14/32 (43.8%), dan 18/32 (56.3%). *Aeromonas* spp. mempunyai kerintangan terhadap clindamycin, rifampin, dan spectinomycin. Manakala *Salmonella* serovar mempunyai kerintangan terhadap clindamycin, rifampin dan tetracycline. *Listeria* spp. pula mempunyai kerintangan terhadap ceftazidime dan clindamycin. Antibiogram utama *A. hydrophila* dipencilkan dari ikan keli dan tilapia adalah AzmDaRdSh (6/62). Antibiogram utama *Salmonella* serovar dan *Listeria* spp.

dipencilkan dari ikan keli adalah CDaRdTe (4/13) dan CCef (10/11). Antibiogram utama *Salmonella* serovar dan *Listeria* spp. dipencilkan dari tilapia adalah DaRdSh (4/19) dan CefDa (15/23). Multiple Antibiotic Resistance (MAR) indeks *Aeromonas* spp., *Salmonella* serovar dan *Listeria* spp. dipencilkan dari ikan keli adalah 0.18-0.55, 0.18-0.46, dan 0.18-0.27. MAR indeks *Aeromonas* spp., *Salmonella* serovar dan *Listeria* spp. dipencilkan dari tilapia adalah adalah 0.18-0.55, 0.18-0.36, dan 0.18-0.23. Masing-masing plasmid (1-90 kb) telah dikesan dalam *Aeromonas* spp. (47.9%), *Salmonella* serovar (72.1%), dan *Listeria* spp. (57.4%). Gen *hly* dan *aer* hadir dalam 42.9% dan 27% *Aeromonas* spp. Masing-masing gen *hly* dan *iap* hadir dalam 70.3% dan 100% *Listeria* spp. Rawak penguatan polimorfik DNA (RAPD) mampu membezakan *Aeromonas* spp., *Salmonella* serovar dan *Listeria* spp. menggunakan primer yang berbeza. Elektroforesis gel bidang denyutan (PFGE) hanya mampu membezakan serovar yang berbeza tetapi tidak mampu membezakan serovar yang sama daripada *Salmonella* serovar. Repetitive Extragenic Palindromic (REP) dapat membezakan *L. monocytogenes*. Analisa *Aeromonas* spp., *Salmonella* serovar dan *Listeria* spp. oleh RAPD, REP dan/atau PFGE menunjukkan kewujudan kepelbagaian genetik daripada bakteria yang dipencilkan dari ikan keli, tilapia dan air daripada pasar dan kolam dengan pelbagai jenis makanan (makanan ikan buatan sendiri dan komersial) dan dipelihara dalam kolam tanah dan bekas lombong.

PREVALENCE, ANTIBIOTIC RESISTANCE AND MOLECULAR CHARACTERIZATION OF *Aeromonas* spp., *Salmonella* serovars **AND** *Listeria* spp. **ISOLATED FROM CATFISH** (*Clarias gariepinus*) **AND TILAPIA** (*Tilapia mossambica*) **OBTAINED FROM WET MARKETS AND PONDS**

ABSTRACT

This work reports on the prevalence, antibiotic resistance and molecular characterization of *Aeromonas* species, *Salmonella* serovars and *Listeria* species isolated from catfish (*Clarias gariepinus*) and tilapia (*Tilapia mossambica*) obtained from wet markets and ponds (earthen ponds and ex-mining pools) in Northern region of Malaysia. A total 163 of *Aeromonas* spp, 43 of *Salmonella* serovars, and 54 *Listeria* spp. was isolated from 32 catfish carcass rinse, 32 catfish intestines, 32 tilapia carcass rinse, 32 tilapia intestines and 44 water samples. Catfish, tilapia and water samples were obtained from nine wet markets and eight ponds in Penang, Kedah and Perak, Malaysia (2008 – 2009). Prevalence of *Aeromonas* spp., *Salmonella* serovars and *Listeria* spp. in catfish was 14/32 (43.8%), 9/32 (28.1%), and 10/32 (31.3%), respectively. Prevalence of *Aeromonas* spp., *Salmonella* serovars and *Listeria* spp. in tilapia was 21/32 (65.6%), 14/32 (43.8%), and 18/32 (56.3%), respectively. *Aeromonas* spp. was resistant to clindamycin, rifampin, and spectinomycin. *Salmonella* serovars were resistant to clindamycin, rifampin, and tetracycline. *Listeria* spp. was resistant to ceftazidime and clindamycin. The predominant antibiograms of *A. hydrophila* was AzmDaRdSh (6/62). The predominant antibiograms of *Salmonella* and *Listeria* isolated from catfish were CDaRdTe (4/13) and

CCef (10/11), respectively. The predominant antibiograms of *Salmonella* and *Listeria* isolated from tilapia were DaRdSh (4/19) and CefDa (15/23); respectively. MAR index of *Aeromonas, Salmonella* and *Listeria* isolated from catfish ranged from 0.18 to 0.55, 0.18 to 0.46, and 0.18 to 0.27, respectively. MAR index of *Aeromonas, Salmonella* and *Listeria* isolated from tilapia ranged from 0.18 to 0.55, 0.18 to 0.36, and 0.18 to 0.23, respectively. Plasmid DNA bands (1-90 kb) were detected in *Aeromonas* spp. (47.9%), *Salmonella* serovars (72.1%), and *Listeria* spp. (57.4 %). The *hly* and *aer* genes were present in 42.9 and 27% of *Aeromonas* spp., respectively. The *hly* and *iap* gene present in 70.3 and 100% of *Listeria* spp. RAPD-PCR showed good discriminatory power for *Aeromonas* spp., *Salmonella* serovars and *Listeria* spp. using different primers. PFGE was only able to differentiate between serovars but not within the same serovar of *Salmonella* spp**.** REP showed good discriminatory power for *L. monocytogenes*. The analyses of *Aeromonas* spp., *Salmonella* serovars and *Listeria* spp. by RAPD, REP and/or PFGE showed the existence of genetic diversity among the isolates in catfish, tilapia and water which were obtained from wet markets and ponds with different types of feed (homemade and commercial fish feed) and reared in earthen ponds and ex-mining pools.

CHAPTER 1

INTRODUCTION

During the last decade, fish production in Malaysia has increased significantly (Department of Fisheries Malaysia, 2011). In 2011, freshwater fish culture in Malaysia contributed 287,057.41 tons valued at RM 2,385.64 million, which represented 17.23% of the total production and constituted 25.43% of the overall aquaculture subsector (Department of Fisheries Malaysia, 2011). Freshwater fish is cultured in pond cultures, ex-mining pools, freshwater cages, cement tanks, canvas tanks, and freshwater pen culture systems. A majority of freshwater fish is reared in earthen ponds (61.9%) and ex-mining pools (25%). Most of the catfish (58.1%) and tilapia (41.3%) are cultured in earthen ponds and ex-mining pools, respectively (Department of Fisheries Malaysia, 2011).

In the Asia–Pacific region, cultured fishes are fed both commercial and homemade feeds (fresh feed material or farm feed material). FAO (2010a) reported that homemade feeds are used to reduce cost of production. Homemade feed is usually made from chicken viscera, kitchen refuse, chicken bone, and other food waste materials (New and Csavas, 1995) which can be a source of pathogenic bacteria (Lunestad *et al*., 2007). Ultimately, this may increase the chance of food borne illness to human health since fish is a food to human.

The incidence of food borne pathogenic bacteria and drug resistant bacteria due to application of antibiotics in fish has raised serious problems. These resistances might evolve bacteria which are live in the gastrointestinal tracts to survive and thrive under pressures exerted by certain antibiotics. The release of resistant foodborne pathogenic bacteria in fish or aquatic environment may introduce a health

risk to human. The worse case may arise when the food borne illnesses occur on the susceptible human group (e.g., children, immune-compromised and elderly people).

Novotny *et al*. (2004) revealed that fish and fish products are linked with a number of human illnesses and of food borne bacterial infections or intoxications. Pathogens such as *Aeromonas hydrophila, Salmonella* Typhimurium, and *Listeria monocytogenes* (Nielsen *et al*., 2001; Novotny *et al*., 2004) have caused illness in humans. According to Centers for Diseases Control and Prevention (CDC), 29,444 outbreak-related illnesses in the United States during 2009-2010 resulted in hospitalization (1,184 cases or 4%) and death cases (23 cases or 0.08%) (Gould *et al*., 2013). In the outbreaks, *Salmonella* cases were the most outbreak-related hospitalization cases (49% or 583/1184) and *L. monocytogenes* cases were the most death cases (39% or 9/23). Consequently, the illness requires medical costs, productivity losses, and illness-related mortality (Scharff, 2012). Fish is one of the food commodities implicated to the outbreak. Fish was also linked to gastroenteritis and traveler's diarrhea in Asia, Africa and Latin America that was caused by *Aeromonas* spp. (Ghenghesh, 2008; Isonhood and Drake, 2002). The incidence of *Aeromonas, Salmonella* and *Listeria* species isolated from animals and food sources have increased in recent years. However, there is not much information on the prevalence of foodborne pathogens such as *Salmonella* serovars, *L. monocytogenes* and *A. hydrophila* in catfish and tilapia obtained from wet markets and ponds which may become potential source of foodborne illnesses in Malaysia.

Aeromonas and *Salmonella* species isolated from catfish, tilapia and their environment have been reported to be resistant to various antibiotics (Castro-Escarpulli *et al*., 2003; Efuntoye *et al*., 2012; Onyuka *et al*., 2011; Radu *et al*., 2003; Rodas *et al*., 2006; Wang and Silva, 1999). The antibiotic resistance of *L.*

monocytogenes isolated from catfish and their environment also has been studied by Chen *et al*. (2010). However, the antibiotic resistance in *Listeria* spp. isolated from tilapia is scarce. In detail, the antibiotic resistance in *Listeria* spp. isolated from tilapia obtained from ponds has not being studied by elsewhere.

Studying foodborne illnesses requires accurate and efficient methods. These can be gained with conventional and molecular methods to isolate, detect, and characterize the pathogenic bacteria (Gracias and McKillip, 2004; Gugliandolo *et al*., 2011; Mandal *et al*., 2011; van Belkum *et al*., 2000). These methods are used regularly in surveillance studies to obtain information about the potentially pathogenic bacteria and their link to human foodborne illnesses. The modification of media in conventional method to detect and isolate the pathogenic bacteria has been done by other study (Pal and Marshall, 2009). However, the application of centrifugation in sample preparation to concentrate the sample has not being studied by elsewhere.

In Malaysia, the prevalence of *Aeromonas* spp., *Salmonella* serovars and *Listeria* spp. in water, raw chicken meat, beef, raw food, cooked food, vegetables, farm, plant, obtained from wet markets, supermarkets and hypermarkets have been reported by many researchers (Goh *et al*., 2012; Radu *et al*., 2003; Thong *et al*., 2002; Wong *et al*., 2012). Antibiotic resistance among pathogens isolated from fish, salted fish, shrimp, beef, water, and sediments in Malaysia have also been reported by many investigators (Ansary *et al*., 2006; Banerjee *et al*., 2012; Odeyemi *et al*., 2012; Thong *et al*., 2002). Most of the studies on the prevalence of foodborne pathogens have focused on raw and cooked foods obtained from night markets, wet markets and super markets. There is lack of information about the presence of foodborne pathogens in fish obtained from ponds. Environment, cultural conditions,

water and feeds play an important part in the dissemination of foodborne pathogens. In Malaysia, ex-mining pool and earthen ponds are extensively used for the culture of fresh water fish. The sources of water for these bodies of water are the various rivers and streams that are also frequently contaminated with industrial waste and sewage. Feeds are also important source of contamination and commercially feeds are costly and aquaculture practitioners resort to home- prepared feeds which are cheap and normally composed of animal/poultry by-products such as blood, intestines, feathers dead carcass and discarded organs. Antibiotics are also extensively used as growth promoters and prophylactics. Therefore, the objectives of this study were:

- to determine the prevalence of *Aeromonas* spp., *Salmonella* serovars and *Listeria* spp. in catfish (*Clarias gariepinus*) and tilapia (*Tilapia mossambica*) obtained from wet markets and ponds;

- to determine the antibiotic resistance of *Aeromonas* spp., *Salmonella* serovars and *Listeria* spp. in catfish (*C. gariepinus*) and tilapia (*T. mossambica*) obtained from wet markets and ponds;

- to determine the genetic relatedness of *Aeromonas* spp., *Salmonella* serovars and *Listeria* spp. in catfish (*C. gariepinus*) and tilapia (*T. mossambica*) obtained from wet markets and ponds.

CHAPTER 2

LITERATURE REVIEW

2.1 Freshwater aquaculture

Freshwater aquaculture is the farming of aquatic organism that enhances production of freshwater food fish using interventions (e.g., controlled breeding, feed, medications, and containment) (Sapkota *et al*., 2008). In Malaysia, the annual production of freshwater fish is estimated to be 287,057.4 tons valued at RM 2,385.6 million, (Department of Fisheries Malaysia, 2011). This is representing 17.2% of the total aquaculture production in 2011 (Department of Fisheries Malaysia, 2011). In Malaysia, freshwater fish is cultured using ponds ex-mining pools, freshwater cages, cement tanks, canvas tanks, and freshwater pen culture systems. Most of the freshwater fish is reared using pond culture system (59.5%) and ex-mining pools (25%). Catfish (58.1%) and tilapia (41.3%) are reared in earthern ponds and examining ponds, respectively, (Department of Fisheries Malaysia, 2011).

2.1.1 Characteristic of catfish and tilapia

2.1.1.a Characteristic of catfish

Catfish (*Clarias gariepinus*) is different from ray-finned fish and belongs to the order Siluriformes, family Clariidae (ITIS, 2013a). Catfish has smooth body with scaleless skin and the air chamber connected directly to the gill-chamber and pharynx, allowing the fish to live out of water for many weeks in muddy marshes. The size of male catfish can range from 90 to 170 cm in length when caught (FAO, 1996).

Catfish was a part of traditional capture-based aquaculture in North Africa. The farming activities started in the early 1970 in Central and Western Africa. The improvement of farming technology (e.g., artificial propagation based on hormonal stimulation, improvement of technical skill) was conducted to *increase* the yield and productivity (FAO, 2012a).

2.1.1.b Characteristic of tilapia

Tilapia is a freshwater fish that can inhabit ponds, shallow streams, lakes and river, and unusually inhabit brackish water. This fish belongs to the order Perciformes, family Cichlidae (ITIS, 2013b). Tilapia originates from Africa and this is characterized by the presence of lateral line and nostril on the head (Nelson, 2006). El-Sayed (2006) reported that tilapia can start to breed at the age of two to three months and the length is usually eight to ten cm. The optimal growing temperatures of tilapia ranged between 22 to 29°C (Mjoun *et al*., 2010). Tilapia is primarily herbivores which feed on phytoplankton or other aquatic vegetation. Tilapia can feed on complete pelleted feeds which contain plant and/or animal proteins (Mjoun *et al*., 2010).

2.1.2 Importance of catfish and tilapia

Catfish and tilapia are the important sources of high quality protein, vitamins, minerals and omega-6-fatty acids (Lim *et al*., 2010; Ng *et al*., 2012). Catfish and tilapia are sold mainly in fresh fish or live fish, but it can be sold in many forms such as chill-packed, fillet, shank fillet, smoked, and sashimi (Silva and Dean, 2001). Catfish and tilapia production have a positive effect on the national economy as it

provides employment, increase household income thus alleviating poverty and ensuring food security (FAO, 2012b).

2.1.3 Catfish and tilapia production and statistics

World aquaculture production increased from 13,074,379 tons in 1990 to 59,872,600 tons in 2010 (78.2%) (FAO, 2012c). The current percentage of aquaculture production by different continents was shown in Table 2.1.

Table 2.1 The percentage of aquaculture production in different continents

Continent	1990 (tons)	2010 (tons)	Growth (%)
Africa	81,015	128,8320	93.7
America	548,479	2,576,428	78.7
Asia	10,801,356	53,301,157	79.7
Europe	1,601,524	2,523,179	36.5
Oceania	42,005	183,516	77.1

Source : FAO (2012c)

FAO (2012c) reported that the highest aquaculture production was in Asia which accounted for 89% of the total world aquaculture production. This was followed by America (4.3%), Europe (4.2%), and Africa (2.2%).

Malaysia was among the top 12 aquaculture producers in Asia with a total production of 243,081 tons in 2008 (FAO/SEC/FIEL, 2009; FAO/SAP/SPC, 2010). Aquaculture in Malaysia consisted of brackish water and freshwater aquaculture. In 2011, the total production of brackish water and fresh water were 164,838.7 tons (57.4%) and 122,218.7 tons (42.6%), respectively (Department Fisheries Malaysia, 2011). The freshwater fish cultured in ponds, ex-mining pools, freshwater cages, cement tanks, canvas tanks, and freshwater pen culture systems for 75,721.4 tons or

61.9% , 30,590.3 tons or 25%,11,080.5 tons or 9.1%, 4,104.9 tons or 3.4%,400.8 tons or 0.3%, and 320.9 tons or 0.2%, respectively (Department Fisheries Malaysia, 2011). Thus, the majority of freshwater fish was reared in ponds and ex-mining pools. The earthen pond and ex-mining pool were shown in Fig. 2.1 and Fig. 2.2.

Figure 2.1. Earthen pond

Figure 2.2. Ex-mining pool

Catfish and tilapia production in Malaysia from 2005 to 2011 are presented in Table 2.2. Catfish production increase annually but it decreased in 2010 and 2011. Tilapia production also increased annually until it decreased in 2010 and 2011

Table 2.2 Catfish and tilapia production in Malaysia

Year	Catfish (tons)	% increase or decrease	Tilapia (tons)	% increase or decrease
2005	17,684.5		3,609.3	
2006	18,107.1	2.3	4,826.2	25.2
2007	21,406.1	15.4	8,129.6	40.6
2008	39,981.3	46.5	9,033.4	10.0
2009	81,041.0	50.7	10,211.5	11.5
2010	60,255.5	-34.5	10,087.9	-1.2
2011	44,016.1	-36.9	12,633.1	20.1

Source: Department of Fisheries Malaysia, 2011

In the Asia–Pacific region, cultured fish are fed by both commercial and homemade feeds (fresh feed or farm feed material). According to FAO (2010a),

homemade feeds are used to reduce cost of production (New and Csavas, 1995; FAO, 2010a). Homemade feed is usually made from chicken viscera, kitchen refuse, chicken bone, and other food waste materials (New and Csavas, 1995). In Malaysia, the feed is made from chicken offal and carcasses, spoiled eggs, leftover from restaurant' and food service establishments. The chicken offals and carcasses are minced before fed to the catfish (Fig. 2.3). The raw spoiled eggs are mixed with other ingredients. All materials were blended; formed into pellets and dried. The pelleting equipment is presented in Fig. 2.4.

Figure 2.3. Equipment for mincing chicken offals and carcasses in catfish farm

Figure 2.4. Equipment for pelleting spoiled eggs in tilapia farm

Based on investigation, the farmers in this study informed that fish is fed twice a day and the amount of feed given is approximately to 3 and 2% body weight of catfish and tilapia respectively. The feed should be consumed by the fish within

the first 15 minutes in order to avoid the feed from sinking. Therefore, the farmers must take into account the number of fish in the ponds, the actual needs at each meal, growth on daily basis, suitability of feed (e.g., smell, texture, and taste), water quality, and other stress factors.

At the wet market in Malaysia, the containers of live catfish are placed on the floor (Fig. 2.5) and fresh tilapias are displayed on benches (Fig. 2.6). Tilapia sold in hypermarkets was displayed on ice cubes (Fig. 2.7). Equipments use to process catfish and tilapia are not cleaned frequently and used for processing multiple batches of fish before being clean. Similarly water use for washing degutted or descaled fish is not replaced often (Fig. 2.8). At the hypermarket tilapia was cleaned under running tap water (Fig. 2.9). In general, it was observed that catfish and tilapia sold in wet markets were processed in poor hygienic conditions.

Fig. 2.5. The container of catfish placed on the floor in wet market

Fig. 2.6 The display of tilapia in wet market

Fig. 2.7 The display of tilapia in hypermarket

Figure 2.8. Fish cutting equipment in wet market

Figure 2.9. Fish cutting equipment in hypermarket

At wet markets and domestic kitchens, potentially pathogenic bacteria present in rupture intestines of catfish and tilapia might be transmitted to the fish carcass and/or to others (e.g. knife, cutting board, hand worker) via cross contamination. At fish farm, pond water might be contaminated with pathogens from fish when the fish are swimming in ponds. This may introduce pathogens to persist in soil for long

time. Water from ponds usually is used to keep live fish in containers during their distribution to wet markets. This may spread the pathogens to the wider area.

2.2 *Aeromonas* species

2.2.1 Description and infection of *Aeromonas* spp.

Aeromonas spp. is a member of *Aeromonadaceae*. These bacteria are Gram-negative, rods, catalase-positive, oxidase-positive, typically 0.3 to 1 μm in width and 1 to 3.5 μm in length, facultative anaerobic, and is motile by single polar flagellum, except for *A. salmonicida* (Adams and Moss, 2004; Carnahan and Joseph, 2005) Some species have peritrichous or lateral flagella (Carnahan and Joseph, 2005). The growth of *Aeromonas* spp. has been reported as low as -0.1 °C and grows optimally at 22 to 35°C (Ghenghesh *et al*., 2008). They are tolerant to natrium chloride (<5%) (Adams and Moss, 2004) and pH 4.5 to 9 (Isonhood and Drake, 2002). *Aeromonas* is unable to grow in the presence of 6.5% natrium chloride, or on TCBS Agar (thiosulfate-citrate-bile-salts-sucrose agar), resistant to O/129, able to ferment sucrose and D-mannitol but do not ferment inositol (USDA/FSIS, 1998).

Aeromonas hydrophila is predominant in human illnesses (Adams and Moss, 2004; Janda and Abbott, 2010). However, it has been reported that *A. caviae, A. sobria* and *A. aquariorum* are also human pathogens (Beatson *et al*., 2011; Janda and Abbott, 2010; Wu *et al*., 2012). *A. hydrophila* is able to cause gastroenteritis in healthy persons, and septicaemia in immunocompromised persons or persons with malignancies (Khajanchi *et al*., 2010). Gastroenteritis is usually accompanied by watery or bloody diarrhoea. Non-gastrointestinal infection may lead to peritonitis, cellulitis, wound infection, meningitis, soft-tissue infection, hepatobiliary tract

infections, septicaemia, urinary tract infection (Khajanchi *et al*., 2010). The duration of diarrhea is 1 to 14 days after infection occurs (Janda and Abbot, 2010).

The host of *Aeromonas* can be human, animals, fish, birds, freshwater reptiles and cold-blooded marine (Horneman *et al*., 2007). *A. hydrophila* is transmitted through the fecal-oral route infection, the direct ingestion or consumption of contaminated fish, water or other foods such as meat, dairy, or shrimp (Horneman *et al*, 2007).

The infections of *A. hydrophila* to humans due to fish consumption have been reported in some studies. Granum *et al*. (1998) reported the disease of three people in Norway after consumption of raw fermented fish that was contaminated by *A. hydrophila* (up to 10^7 CFU/g food sample). Daskalov (2006) reported a case of illness in Sweden after direct ingesting fish that was contaminated by *A. hydrophila* (10^6–10^7 CFU/g food sample).

The pathogenicity of *Aeromonas* spp. is due to extracellular enzymes (e.g., β-lactamases, lipases, hemolytic enterotoxins, proteases, chitinases, nucleases and amylases) which are released during the multiplication of *Aeromonas* in the host cells (Gavin *et al*., 2003; Pemberton *et al*., 1997). *Aeromonas* also produces sidephores, S-layer and lipopolysaccharide during the multiplication in the host cells. Other virulent factors are cytotoxic, cytotonic enterotoxins, and hemolysins (Khajanchi *et al*., 2010). *A. hydrophila* harbour the genes that regulate enterotoxigenic or cytotoxic activity (Ørmen and Østensvik, 2001).

Production of enterotoxins by *A. hydrophila* is influenced by temperature. Environmental strains of *A. hydrophila* produce more enterotoxin when incubated at 28°C compared to 37°C but clinical isolates, on the other hand, produced more

enterotoxins when incubated at 37°C than those incubated at 28°C (Mateos *et al*., 1993).

2.2.2 Prevalence and antibiotic resistance of *Aeromonas* spp. in catfish and tilapia in different studies.

Prevalence of *Aeromonas* spp. in catfish and tilapia and the antibiotic resistance of *Aeromonas* spp. isolated from catfish and tilapia from different studies is presented in Table 2.3 and Table 2.4.

In Bangladesh, Rahman *et al*., (2007) reported the prevalence of *Aeromonas* spp. in fish ponds with and without duckweed. The incidence of *Aeromonas* was higher in water and sediments of fish pond with duckweed (23/23) compared to fish pond (27/68) without duckweed.

Petersen and Dalsgaard (2003) reported that most of the bacteria isolates present in shrimp, pond water, and pond sediment samples obtained from an integrated fish farm and control fish farm in Antique, Iloilo, Aklan, Capiz, and Negros Oriental (Philippines) belong to the genus *Aeromonas.* These authors also observed that the mean levels of resistance were below 20% for most antimicrobials in the integrated farms (chloramphenicol, ciprofloxacin, erythromycin, sulfamethoxacole) except oxytetracycline (37%). The mean levels of resistance in isolates from the control fish farms were generally lower compared to isolates from integrated farms with the exception of resistance to sulfamethoxazole (22% in control farms, 19% in integrated farms). All studies did not have any information about the antibiotic resistance in fish during cultivation at farm level with different ponds types, different feeds types, and different water types.

Table 2.3 Prevalence of *Aeromonas* spp. in catfish and tilapia in different studies

Country	Sample	Number (%)	*Aeromonas* spp.	References
Malaysia	Catfish	2/20 (10) 10/20 (50)	*A. hydrophila,* *A. sobria*	Radu *et al*., 2003
USA	Catfish fillet	86/238 (36.1) 85/238 (35.7) 26/238 (10.9)	*A. hydrophila* *A. sobria* *A. caviae*	Wang and Silva, 1999
Malaysia	Tilapia	5/32 (15.6) 18/32 (56.3)	*A. hydrophila,* *A. sobria*	Radu *et al*., 2003
Egypt	Tilapia	25/800 (3.1)	*A. hydrophila*	Ibrahem *et al*., 2008
Trinidad	Tilapia	33/75 (44)	*Aeromonas* spp.	Newaj-Fyzul *et al*., 2008
Mexico City	Tilapia flesh	52/250 (20.8) 16/250 (6.4) 4/ 250 (1.6) 3/250 (1.2) 2/250 (0.8)	*A. salmonicida* *A. bestiarum* *A. sobria* *A. encheleia* *A. hydrophila*	Castro-Escarpulli *et al*., 2003

Table 2.4 Antibiotic resistance among *Aeromonas* isolated from catfish and tilapia in different studies

Country	Samples	Antibiotic resistance	Weakness	References
Malaysia	A total of 23 strains of *Aeromonas* spp. isolated from 32 samples of tilapia and 12 strains of *Aeromonas* spp. isolated from 20 samples of catfish samples were purchased from retail market in Sri Serdang, Selangor, Malaysia	The predominant antibiograms were BCbCfErGmSm (2/20) for *Aeromonas* isolated from catfish and BCbErSmKm (2/32) for *Aeromonas* isolated from tilapia. *Aeromonas* isolates were resistant to carbenicillin, erythromycin and streptomycin and susceptible to ceptazidime	This study did not have any information about the antibiotic resistance in fish during rearing in different ponds types and fed with different feed types	Radu *et al*., 2003
USA	A total of 197 strains of *Aeromonas* spp. isolated from 238 samples of channel catfish fillet obtained three processing plants in the Mississippi Delta	Most isolates were susceptible to chloramphenicol, neomycin, streptomycin, and trimethoprim-sulfamethoxazole and resistant to ampicillin and bacitracin	same above	Wang and Silva, 1999
Mexico City	A total of 82 strains of *Aeromonas* spp. were isolated from 250 samples of frozen fish tilapia purchased in local markets in Mexico City.	All strains showed 100% of resistance to ampicillin, carbenicillin, cephalothin, clindamycin, and penicillin. The highest resistances encountered were 85.7% to polymyxin B, 75.3% to streptomycin, 58.4% to gentamicin, 57.1% to rifampin, 54.5% to erythromycin. In contrast, all the strains were susceptible to nalidixic acid, cefotaxime, cefuroxime and nitrofurantoin.	same above	Castro-Escarpulli *et al*., 2003
Trinidad	A total of 52 stains of *Aeromonas* spp. was isolated from 75 samples of tilapia obtained from four commercial tilapia fish farms in Trinidad	*Aeromonas* isolates were resistance to ampicillin (88.5%), oxytetracycline (55.8%), erythromycin (57.7%), and susceptible to nalidixic acid, chloramphenicol, gentamycin, sulphamethazole/trimethoprim, norfloxacin.	same above	Newaj-Fyzul *et al*., 2008

BCbCfErGmSm: bacitracin, carbenicillin, cefoperazone, erythromycin, gentamicin, streptomycin;
BCbErSmKm: bacitracin, carbenicillin, erythromycin, streptomycin, kanamycin

2.3 *Salmonella* species

2.3.1 Description and infection of *Salmonella* spp.

Salmonella is a member of family *Enterobacteriaceae*. *Salmonella* is Gram-negative, rods, non spore–forming, typically 0.5 μm by 1-3 μm, facultative anaerobic, oxidase-negative, catalase positive, and are generally motile by peritrichous flagella (Adams and Moss, 2004) except *S.* Pullorum and *S.* Gallinarum (Habib-ur-Rehman *et al*., 2004). *Salmonella* spp. are able to grow at temperatures ranging from 5 to 45 °C with a minimum water activity (a_w) at 0.93. and pH between 4.4 to 9.4 (Adams and Moss , 2004). The genus *Salmonella* consists of two species namely *S. enterica* and *S. bongori* (Adams and Moss, 2004). Ellermeier and Slauch (2006) and Bhunia (2008) reported that *S. enterica* consists 2,443 serovars and *S. bongori* consists of 20 serotypes.

The predominant *Salmonella* serovars responsible for foodborne illness are *S.* Typhimurium and *S.* Enteritidis (Miller and Pegues, 2005). Other serovars such as *S.* Corvalis, *S.* Stanley, *S.* Agona. *S.* Bovis-mobificans, *S.* Albany have also being implicated in foodborne illnesses (Hamada and Tsuji, 2001; Zaidi *et al*., 2006). According to Gunn (2011) there are two major *Salmonella* diseases: typhoid fever and non-typhoidal *Salmonella*. Non-typhoidal *Salmonella* causes gastroenteritis, bacteremia, and subsequent fecal infection (Hohmann, 2001). The infection is characterized by diarrhoea, abdominal pain and cramps, and sometimes fever. The onset of symptoms is 6 to 72 hours after exposure of bacteria and the duration of illness is 5 to 7 days (Onwuezobe *et al*., 2012).

The infectious dose depends on the virulence of the serovar, age of patients, physical condition of patients, immune-supressive illness, level of acidity in human stomach, bacterial number in the contaminated food, physiological status of bacterial

cells, and nature of food matrix (Bronze and Greenfield, 2005; Kothary and Babu, 2001). The infectious dose of non-typhoidal salmonellosis and enteric fever is approximately 10^3 cells and 10^5 cells, respectively (Bronze and Greenfield, 2005; Kothary and Babu, 2001).

2.3.2 Prevalence and antibiotic resistance of *Salmonella* spp. in catfish and tilapia in different studies.

Prevalence of *Salmonella* spp. in catfish, tilapia and freshwater fish in different studies is presented is in Table 2.5. The incidence of *Salmonella* in catfish and tilapia obtained from China, USA, Kenya, Nigeria and Botswana range from 3.3 to 50% (Awuor *et al*., 2011; Efuntoye *et al*., 2012, Mhango *et al*., 2010; Onyuka *et al*., 2011; Pal and Marshall, 2009).

Presence of *Salmonella* in catfish and tilapia reared in fish farms can be attributed to farm runoff, poor water quality, feed, faecal contamination from livestock or wild animals (Amagliani *et al*., 2012), poor sanitation in processing (Novotny *et al*., 2004) and poor handling practices during distribution (Novotny *et al*., 2004; Zhao *et al*., 2003). Pal and Marshall (2009) revealed that intestinal tracts of animals, farm animals, birds and humans were primary sources of *Salmonella.* These may result in the contamination to farm-raised fish.

The antibiotic resistance among *Salmonella* serovars isolated from catfish and tilapia from different studies is presented in Table 2.5. Data presented in Table 2.6 shows most of the *Salmonella* were resistant to gentamycin, erythromycin but susceptible to augmentin, carbenicillin, cephalothin, novobiocin, norfloxacin, ciprofloxacin, and rifampin.

Table 2.5 Prevalence of *Salmonella* spp. in catfish and tilapia in different studies

Country	Sample	Number (%)	Serovars	
Nigeria	Catfish (mixed of intestines and gills)	5/56 (8.9) 2/56 (3.6)	*S.* Typhimurium *S.* Enteritidis	Efuntoye *et al*., 2012
USA	Catfish	13/153 (8.4)	*Salmonella* spp.	Wyatt *et al*., 1979
USA	Frozen channel catfish fillet Frozen basa fish fillet	10/30 (33.3) 15/30 (50)	*Salmonella* spp. *Salmonella* spp.	Pal and Marshall, 2009
Botswana	Catfish Tilapia Gutted tilapia	13/42 (30.9) 20/50 (40) 22/101 (21.8)	*Salmonella* spp. *Salmonella* spp. *Salmonella* spp.	Mhango, *et al*., 2010
Kenya	Tilapia	24/54 (44.4)	*S.* Typhimurium	Onyuka *et al*., 2011
Kenya	Tilapia	9/120 (7.5) 4/120 (3.3) 7/120 (5.8)	*S.* Typhimurium, *S.* Enteritidis *S.*Typhi	Awuor *et al*., 2011

Table 2.6 Antibiotic resistance among *Salmonella* isolated from catfish and tilapia in different studies

Country	Samples	Antibiotic resistance	Weakness	References
Nigeria	A total of 7 stains of *Salmonella* serovars were isolated from 90 samples of catfish collected randomly from 3 fish farms located in Ago-Iwoye, Southwestern Nigeria.	*Salmonella* spp. was resistant to erythromycin (85.7%), gentamicin (71.4%), amoxicillin (57.1%), chloramphenicol (57.1%), and sulphamethoxazole (57.1%). *Salmonella* spp. was susceptible to ciprofloxacin, novobiocin, ofloxacin, tetracycline, nalidixic acid.	This study did not have any information about the antibiotic resistance in fish during rearing in different ponds types and fed with different feed types	Efuntoye *et al*., 2012
USA	A total of 4 strains of *Salmonella* serovars were isolated from catfish and tilapia obtained from USA markets which were imported from Taiwan and Thailand	*Salmonella* spp. was resistant to nalidixic acid and decreasing susceptibility to ciprofloxacin (a fluoroquinolone drug that is valuable to treat human as well as animal infectious diseases). Antibiograms of *Salmonella* isolated from catfish were KanNalStrTe and NalStrTet	same above	Zhao *et al*. 2003
Kenya	A total of 24 strains of *S.* Typhimurium were isolated from 54 samples of tilapia obtained from three fish landing beaches namely Dunga, Luanda Rombo and Sirongo and from three markets: Kisumu Municipality, Luanda and Bondo within L. Victoria basin of western Kenya	*S.* Typhimurium was resistant to tetracycline (70.8%), co-trimoxazole (66.7%) and susceptible to ampicillin, chloramphenicol, gentamicin erythromycin, norfloxacin , ciprofloxacin, methicillin	same above	Onyuka *et al*., 2011
Malaysia	A total of 28 isolates of *S.enteritidis* was isolated from 10 gills of tilapia obtained from several wet markets in Selangor, Malaysia	Isolates were resistant to nalidixic acid (21.1%), penicillin (39.3%) and streptomycin (71.4%), and were susceptible to carbenicillin, cephalothin, kanamycin, rifampin and tetracycline. Seventeen isolates (60.7%) were resistant to a single antibiotic and eleven (39.3%) were resistant to two antibiotics; thus separating the isolates into six antibiotic resistance patterns	same above	Radu *et al*., 2000

KanNalStrTe : Kanamycin-nalidicix acid-streptomycin-tetracycline; NalStrTet : Nalidicic acid-streptomycin-tetracycline

In Iran, *S.* Typhimurium and *S.* Newport were isolated from 125(4.2%) of ungutted cultivated silver and common carp obtained from fish farm located near the city and polluted river. The polluted river may introduce the *Salmonella* spp. contamination to the fish farm via run-off water. The fish farm was fertilized by large animal manure (Basti *et al*., 2004). Jenkins *et al*. (2008) reported the *Salmonella* spp. levels in pond waters located near pasture and crop fields fertilized with poultry manure ranged from 0.1 to 3.4 MPN/L.

Banerjee *et al*. (2012) examining water samples and shrimps from 3 different shrimp ponds observed that *S.* Corvalis was only present in water sample obtained from one pond and in shrimps obtained from 2 ponds. The serovar isolated from water was resistant to erythromycin but was susceptible to norfloxacin, doxycycline hydrochloride, sulfamethoxazole, trimethoprim, nitrofurantoin, sulphonamides, ampicillin, oxolinic acid, chloramphenicol, and tetracycline, while the 2 isolates of serovar isolated from shrimps was resistant to all the antibiotics except for nitrofuratoin and norfloxacin.

Salmonella can be found in soil that is determined by the presence of water and its flow paths. Therefore, the path of the water flow will determine the direction of the bacterial transport (Jacobsen and Bech, 2012). *Salmonella* introduced to the soil environment with waste are either attached to waste particles or planktonic cells that may attach to soil particles upon introduction to the soil environment (Jacobsen and Bech, 2012).

Lunestad *et al.* (2007) reported *Salmonella* are found in fish feed for 29 out of 8778 (0.33%). The authors also reported that fish feed was contaminated by *S.*Agona, *S.* Senftenberg, *S.* Montevideo, *S.* Mbandaka, *S.* Meleagridis, *S.* Havana, *S.* Cubana, *S.* Kentucky, *S.* Livingstone, *S.* Worthington, *S.* Indiana, *S.* Ohio, *S.*

Schwarzengrund, *S.* Anatum, *S.* Rissen, *S.* Duisburg, *S.* Heidelberg, *S.* Lexington, *S.* Lille, *S.* Nille. All *Salmonella* isolates were susceptible to all antibiotics except for one isolate which was resistant to streptomycin (Norwegian Scientific Comitee For Food Safety, 2006). The use of antimicrobials in feeds may lead to the emergence antibiotic resistance among bacteria present in fish and aquatic environment (Nesse *et al*., 2005).

2.4 *Listeria* species

2.4.1 Description and infection of *Listeria* spp.

Listeria spp. is a member of the family *Listeriaceae*. The bacteria are Gram-positive, cocoid to rod-shaped cells, catalase-positive, oxidase-negative, non spore-forming, facultative anaerobic, and are motile by peritrichous flagella, which may disappear when the bacteria penetrate the human cell (Adams and Moss, 2004). The width and length of *Listeria* spp. ranged from 0.4 to 0.5 µm and 0.5 to 2.0 µm, respectively. *Listeria monocytogenes* is able to grow at 4 °C, and the optimum growth temperature ranges from 30 to 35°C (Adams and Moss, 2004). They are tolerant to salt (10%) and are able to grow at 4.4 to 9.4 (Adams and Moss, 2004). The genus *Listeria* currently consists of ten species which are *L. monocytogenes, L. grayi, L. innocua, L. ivanovii, L. marthii, L. welshimeri, L. seeligeri* (Milillo *et al*., 2012), *L. rocourtiae* (Leclercq *et al*., 2010), *L. weihenstephanensis* (Halter *et al.*, 2013), and *L. fleischmanii* (den Bakker *et al*., 2013)

L. monocytogenes is widely distributed in the environment and can be isolated from soil, water, sewage, silage, decaying plant materials, both domestic and wild animals (Budzinsha *et al*., 2012). *L. monocytogenes* is able to survive in the environment of food processing plants and contaminate a variety of processed meats

(Budzinsha *et al*., 2012). The presence of *L. monocytogenes* in rivers, lakes seawater and ground water can be attributed contamination with sewage contaminated with this pathogen (Budzinsha *et al*., 2012). *L. monocytogenes* is the main cause of listeriosis and can cause severe disease in neonates, elderly people, pregnant woman, and immune-compromised persons (Freitag *et al*., 2009). *L. monocytogenes* is able to migrate to the intestinal, blood-brain, and fetoplacental barriers (Jacquet *et al*., 2004). *L. monocytogenes* causes gastroenteritis, meningitis, septicemia, meningoencephalitis, abortion, or perinatal infection. *L. monocytogenes* is reported to be a highly invasive intracellular pathogen. It is able infect macrophages via phagocytosis, or epithelial cells, which cause changes to the cytoskeletal and plasma membrane (Edelson and Unanue, 2000).

Freitag *et al*. (2009) reported that the invasion of *L. monocytogenes* is facilitated by bacterial surface proteins (InlA and InlB). Once internalized, *L. monoytogenes* escaped from the membrane-bound vacuole secreting listeriolysin O (LLO or *hly*) that acts as a poreforming cytolysin (Freitag *et al*., 2009). The nutrient of host will be used by *L. monocytogenes* for replication. Using actin polymerization, *L. monocytogenes* can move through the cell and adjacent cells. *L. monocytogenes* secretes LLO and phosphatidyl-choline phospholipase C (PC-PLC) to escape from the double-membraned secondary vacuoles that are formed as a result of cell-to-cell spread (Freitag *et al*., 2009).

The symptoms of listeriosis include fever, muscles aches, and occasionally nausea or diarrhea. If the infection spreads to the nervous systems, the symptoms are headache, stiff neck, loss of balance or convulsions. Infected pregnant women usually experience only mild, flu-like symptoms (Baltimore, 2007; Bortolussi, 2008). Newborn babies suffering from listeriosis usually exhibit irritability, fever, vomiting,

and lack of interest in feeding (Baltimore, 2007; Bortolussi, 2008). The duration of incubation periods ranged from 8 to 67 days except for incubation periods of gastroenteritis cases ranged from 6 to 240 hours (Goulet *et al.,* 2013).

Infective dose depends on the health status (age and immune status) of the individual, virulence of the pathogen, type and amount of food ingested (Leggett *et al*., 2012). Foods containing 10^2 to 10^4 CFU per g of *L. monocytogenes* have reported to cause listeriosis (Vázquez-Boland *et al*., 2001).

L. monocytogenes has been recognized as significant foodborne pathogen with mortality rates around 50% (Gahan and Hill, 2005). In USA, the annual prevalence of *Salmonella* and *Campylobacter* were observed to be over 500 and 700 times higher than *L. monocytogenes*, but the medical costs and productivity loses due to *L. monocytogenes* were much higher compared to *Salmonella* and *Campylobacter*. Roughly, the loss caused by *Salmonella, Campylobacter* and *L. monocytogenes* was $3.3 billion, $ 1.7 billion and $2.6 billion, respectively (Hoffmann *et al.*, 2012)

Swaminathan and Gerner-Smidt (2007) reported that listeriosis has lethality cases of 20-30% for epidemic and sporadic cases. At the EU in 2007, smoked fish was the food item most often containing *L. monocytogenes* (18.3%), and at levels exceeding 100 cfu/g (2.4%) (Todd *et al*., 2011).

2.4.2 Prevalence and antibiotic resistance of *Listeria* spp. isolated from catfish and tilapia

Prevalence of *Listeria* spp. in catfish and tilapia, in different studies is presented in Table 2.7. Chen *et al*. (2010) reported that 221 strains of *Listeria* isolates, 86 (50 in catfish fillets and 36 in catfish processing facilities) were identified as *L. monocytogenes*, 41 (22 in catfish fillets and 19 in processing