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Comparison of sample preparation for the isolation of *Listeria* species in naturally contaminated catfish and tilapia samples

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Abstract

In this study, two preparation sample (pelleted and non-pelleted) and two pre-enrichment (with and without pre-enrichment in Half Frazer Broth) were assessed on their efficacy to isolate *Listeria* sp. from naturally contaminated catfish and tilapia samples. A total of 108 samples (32 catfish intestines, 32 tilapia intestines, and 44 water samples) were examined for the presence of *Listeria* sp. Out of 16 *Listeria* sp. were observed on pelleted sample and 5 *Listeria* sp. were observed on non-pelleted sample. All samples were preceded without pre-enrichment. Nineteen (19) isolates were observed on pelleted sample 17 on non- pelleted sample. Those were combined with pre-enrichment. The study revealed that, the isolation of *Listeria* sp. by pelleting the sample in the combination with pre-enrichment from naturally contaminated catfish, tilapia and water samples were relatively higher than their opponents. By improving the efficacy of isolating *Listeria* sp. is beneficial for reporting the presence of these pathogenic bacteria due to public health purposes.

Key words: Catfish, Isolation method, *Listeria*, Pelleted, Tilapia

Introduction

Catfish and tilapia are the highest fresh water fishes reared in the ponds in Malaysia. Based on the recent data, total production of catfish and tilapia were 64.9% and 18.2%, respectively (Fisheries Department Malaysia 2010). However, catfish and tilapia are potential agents of pathogenic bacteria such as *Listeria* sp. (Chen et al., 2010; Jaleewar et al., 2007).

Listeria sp. is a gram positive and grows in a wide temperature range, from -1.5 to 45ºC (Adam and Moss, 2004). The growth of the organism at -1.5ºC was very slow, with a lag time of 174 h (Hudson et al., 1994). *Listeria* could move with flagella and polymerizing actin comet tails with a protein called ActA. Some studies suggested that 1 to 10% of humans may carry *L. monocytogenes* in their intestines (EMLab, 2009).

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Listeriosis, caused by *L. monocytogenes*, is a food borne infection of great public health concern due to its clinical severity and high fatality. Mostly affected by severe disease are people who are elderly or immunocompromised, pregnant women and neonates (younger than four weeks). *L. monocytogenes* could cause meningoencephalitis and septicemia in newborns, elderly, immunocompromised patients and abortion in pregnant women (Marchant, 2003). The infective dose of *L. monocytogenes* is unknown but it is believed to vary with the strains and susceptibility of the victim. Some studies has reported to find the antibacterial to against this pathogenic bacteria (Mattana et al., 2012; Satorres et al., 2012, 2013).

The conventional isolation method of Listeria sp. was established by ISO 11290 (ISO 2004; ISO, 1998; ISO, 1996). However, there is limited data regarding the isolation method of *Listeria* species by pelleted sample to increase the yield Thus, this study was carried out to fill this gap. The aims of this study were to compare on two preparation sample (pelleted and non-pelleted) and two pre-enrichment (with and without pre-enrichment in Half Frazer Broth) and to determine their efficacy on *Listeria* isolation from naturally contaminated catfish and tilapia samples.

Material and Methods

Samples

The microbial quality of the catfish and tilapia were investigated by taking samples of intestines. The fish were taken from local wet markets and ponds on November 2008 to September 2009. The fishes were sampled from each wet market and pond in Penang and surrounding Penang Malaysia. The samples were placed in sterile bags. Water samples were collected in sterile test tubes, covered, and transferred to the laboratory within minutes.

Isolation of *Listeria* sp.

In method A (pellet method), the intestines of fish were removed using a sterile knife and were pooled by using sterile forceps. The intestines were placed on a sterile tray wrapped in aluminum foil and chopped thoroughly with sterile knife. Twenty five grams of the intestines were placed in a stomacher bag containing 225 mL 0.1% Peptone Water (PW, Oxoid, Basingtoke, Hampshire, United Kingdom) and homogenized using a stomacher (Interscience, France) for 2 min. The homogenate was divided equally, placed in 50 mL centrifuge tubes and centrifuged for 15 min at 10,000 x g to obtain a pellet. The pellet was pre-enriched by re-suspending it in 10 mL Half Frazer Broth (HFB, Merck KGaA, Darmstadt, Germany) and incubated at 37^{o}C for 24 h. In method B (non-pelleted method), 25 g of chopped intestines were placed in a stomacher bag and pre-enriched by homogenizing with 225 mL HFB using a stomacher for 2 min and incubated at 37^{o}C for 24 h. Twenty five g of intestines were also directly pre-enriched by homogenizing in 225 mL of HFB, using a stomacher for 2 min and incubated at 37^{o}C for 24 h. After pre-enrichment, 1 mL portions were transferred into 10 mL of Frazer Broth (FB, Merck KGaA, Darmstadt, Germany) and incubated at 37^{o}C for 24 h. Following enrichment, 10 µL of the culture was streak-plated on to ALOA (Merck KGaA, Darmstadt, Germany) and PALCAM (Merck KGaA, Darmstadt, Germany) and were incubated at 37^{o}C for 24-48 h.

Twenty five mL of water samples were pelleted by centrifuging at 10,000 x g for 15 min, the pellet was re-suspended and pre-enriched in 10 mL of HFB and incubated at 37^{o}C for 24 h. Twenty five mL of water samples were also directly pre-enriched by homogenizing in 225 mL of HFB, using a stomacher for 2 min and incubated at 37^{o}C for 24 h. After pre-enrichment, 1 mL of both pelleted and non-pelleted methods was enriched in 10 mL of FB and incubated at 42^{o}C for 24 h. After enrichment, 10 µL of culture was streak-plated onto ALOA (Merck KGaA, Darmstadt, Germany) and PALCAM (Merck KGaA, Darmstadt, Germany) and were incubated at 37^{o}C for 24-48 h. The protocol was based on the modification of EN ISO 11 290-1. Presumptive *Listeria* colonies were picked, purified Gram stained and subjected to following biochemical tests; gram staining, catalase, cytochrome oxidase, microscopic observation, motility test and haemolysis test. Microbact *Listeria* Identification System 12L (Oxoid, Basingtoke, Hampshire, United Kingdom) was used to identify *Listeria* sp.

Preparation of genomic DNA

Single colony of pure *Listeria* culture was inoculated into 5 mL Triptic Soya Broth (TSB, Merck KGaA, Darmstadt, Germany) and incubated in orbital shaker (with vigorous shaking) at 37°C for 16 to 18 h. The overnight culture (5 mL) was centrifuged for 5 min at 1000×g to obtain pellets. Pellets were dried and subjected to plasmid DNA extraction and purification using Wizard Genomic Purification Kit (Promega, Madison, USA) by following the manufacturer's instructions (Anonymous, 2012).

Detection iap gene of *Listeria* species by PCR

The PCR was set for 25 µl reaction volume. Initially, for the detection of individual *iap* gene of *Listeria* sp., PCR conditions were used with modification method of Chen and Knabel (2007). The reaction mixture for PCR was optimized as follows; 10× PCR buffer (Sigma), 0.2 mM dNTP mix (Promega), 2 mM $MgCl_2$ (Promega) and 0.1 µmol of forward and reverse primer of each set, 1.6 unit of Taq DNA Polymerase (Promega), 2.5 µl of cell lysate and sterilized water free nuclease (Promega) to make up the reaction volume. The primers were used according to Bubert (1992); IAPF (5'- ATG AAT ATG AAA AAA GCAAC-3') and IAPR (5'-TTA TAC GCG ACC GAA GCC AAC-3'). Temperature cycling was performed according to Chen and Knabel (2007) using a thermocycler (Biometra, Germany) with an initial activation step for 5 min at 95°C prior to 15 cycles of 1 min at 94°C, 1 min with a touchdown from 55°C to 51°C (3 cycles per temperature), and 1 min at 72°C, followed by 15 cycles of 1 min at 94°C, 1 min at 50°C, and 1 min at 72°C and 1 final cycle for 8 min at 72°C. The PCR products (10 µl reaction mixture mixed with 2 µl loading dye) were separated by electrophoresis on a 1.5% agarose gel (Promega, USA) and was run at 90V for 1h 20 min. Ladder 100 bp (Promega, USA) was used for molecular weight marker.

Statistical analysis

The General Linear Model Procedure (SPSS version 13, USA) at the significance level ($P<0.05$) was used to assess differences of pellet and non-pellet.

Results and Discussion

Listeria species are well known pathogenic bacteria in most food-borne diseases which cause the highest mortality rate. Symptoms of *Listeria* infection are gastroenteritis, meningitis, abortion, and death in some cases (Adams and Moss, 2004). Thus, the methods which ensure the highest possible recovery of these pathogens are important. In this present study, centrifuging sample to obtain the pellet was shown to be relatively higher to isolate *Listeria* species. The material in the suspension was against the walls and separated from the solution by centrifugal force. The samples which may be contaminated by *Listeria* can be more concentrated in to the pellet form. Thus, the number and chance to isolate *Listeria* might be increased.

This study found that pelleted method with pre-enrichment was significant ($P<0.05$) with non-pelleted method on *Listeria* isolation (Table 1). The highest yield using pelleted method in fish and water were 8/64 and 11/44. The yields obtained from pelleted method were relatively higher than those using non-pelleted method. The sensitivity was relatively higher on pelleted method compared to non-pelleted method. The sensitivity of pelleted method in combination with pre-enrichment was 1 and 0.92 for fish and water samples. The sensitivity of non-pelleted method in combination with pre-enrichment was 1 and 0.75 for fish and water samples. Even though the centrifuged bacteria has been reported to be damaged on cell membrane (Bell, 2005), the bacteria might be repaired in enrichment broth with or without pre-enrichment step.

The sensitivity of pelleted method without pre-enrichment in HFB was found to be high for both fish (1) and water (0.75) samples. The pelleted method with pre-enrichment in HFB yielded relatively high for fish (1) and water (0.92) samples. HFP has a high buffering capacity which may repair the injured Listeria (Holbrook et al., 1992). HFB contained lithium, acriflavin and nalidixic acid (Frazer and Sperber, 1988). The Half Frazer Broth, which contained of half concentration of acriflavin and antibiotics, was aimed to allow the better growth of injured *Listeria* (Holbrook et al., 1992).

This present study isolation of *Listeria* without pre-enrichment step was observed (Table 1). The enrichment for *Listeria* use FB which was effective for the growth of *Listeria* sp. and the growth of enterococci was inhibited (Frazer and Sperber, 1988). Thus, the combination of pre-enrichment and enrichment could increase the growth of *Listeria* species.

The presence of *Listeria* sp. was detected by PCR to observe iap gene. The genus *Listeria* sp. contained the genetic marker such as iap gene which encoded a major extracellular protein p60 (Bubert et al., 1992). In this present study, this gene was observed on all *Listeria* sp. isolates. The representative gel was shown in Figure 1.

Table 1. Positive sample of *Listeria* sp. using two preparation sample (pelleted and non-pelleted sample) and two pre-enrichment (with and without pre-enrichment in Half Frazer Broth).

Sample	Method	Pelleted method		Non-pelleted method	
		Total positive	Sensitivity[c]	Total positive	Sensitivity
Fish	Without Pre-enrichment (*n*=64)	6[b]	0.75	1[a]	0.13
	With Pre-enrichment (*n*=64)	8[a]	1	8[a]	1
	Total positive	8		8	
Water	Without Pre-enrichment (*n*=44)	10[b]	0.83	4[a]	0.33
	With Pre-enrichment (*n*=44)	11[a]	0.92	9[a]	0.75
	Total positive	12		12	

a,b = different alphabet means significant different at $P<0.05$ in the same row; c = sensitivity is calculated in relation to the total number of positive sample

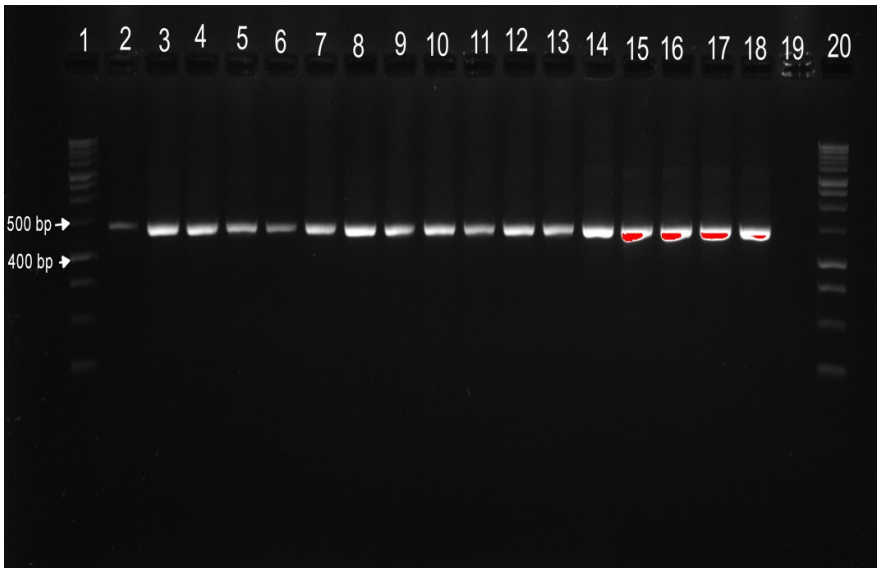

Figure 1. The representative gel of iap gene in *Listeria* isolates isolated from catfish, tilapia and water.
Lane 1,20: Marker 100 bp; Lane 2 : positive control L. monocytogenes; Lane 3-18 : samples; Lane 19 : negative control

The prevalence of *Listeria* sp. in catfish and tilapia were 3/32 and 5/32. The prevalence of *Listeria* sp. in tilapia and its water was relatively higher than catfish and its water (Table 1). Other study found that *Listeria* was found in tilapia and catfish (Chen et al., 2010; Jaleewar et al, 2007). This study found 3 isolates of *L. ivanovii* isolated from catfish, 2 isolates of *L. ivanovii*, 2 isolates of *L. grayi* and 1 isolates of *L. welshimeri* isolated from tilapia. The prevalence of *Listeria* sp. in water obtained from catfish tank and ponds were 5/32. These were 4 isolates of *L. ivanovii* and 1 isolates of *L. welshimeri*. The prevalence of Listeria spp. obtained from tilapia pond were 7/12 which were 3 isolates of *L. ivanovii*, 2 isolates of L. grayi, 1 isolates of *L. welshimeri* and 1 isolates of *L. welshimeri*.

L. ivanovii was the predominant in tilapia, catfish and water. Chen et al. (2010) found that *L. ivanovii* and *L. monocytogenes* were observed in catfish. The important finding of this study was the presence of *L. ivanovii* and *L. monocytogenes*. Guillet et al. (2010) revealed that *L. ivanovii* and *L. monocytogenes* could make human disease.

Conclusion

Pelleted method can be the new alternative to isolate *Listeria* species. This new method can be combined with and without pre-enrichment to isolate *Listeria* sp. Pelleting sample in combination with pre-enrichment yielded higher sensitivity compared to non-pelleting sample and other. The presence of *L. monocytogenes* and *L. ivanovii* in catfish and tilapia become food safety concern for the public health.

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