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THE PREVALENCE OF *Salmonella* sp., *Listeria* sp. AND *Aeromonas* spp. IN CATFISH (CLARIAS (*Clarias gariepinus*) AND TILAPIA (*Tilapia mossambica*) BY PELLETING METHOD

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ABSTRACT

The aim of this study was to modify the isolation methods of *Aeromonas* sp., *Salmonella* spp., and *Listeria* sp. in catfish (*Clarias gariepinus*) and tilapia (*Tilapia mossambica*) obtained from wet markets and ponds in Malaysia by pelleting the sample. A total of 108 samples (32 catfish intestines, 32 tilapia intestines, and 44 water samples) were obtained from nine wet markets and eight ponds. The modified method was employed by pelleting the samples and followed by either implementing pre-enrichment or without pre-enrichment on the isolation of *Salmonella* and *Listeria* spp. The modified method (by pelleting the sample in combination with pre-enrichment) was the most efficient for *Salmonella* and *Listeria* isolation. The sensitivity of the modified *Salmonella* isolation method was 0.53 and 0.73 for fish and water samples, respectively. The sensitivity of the modified *Listeria* method was 1 and 0.92 for fish and water samples, respectively. However, the sensitivity of the method by pelleting the sample was similar to those of non-pelleting the sample on *Aeromonas* isolation. Five species of *Aeromonas* spp., seven serovars of *Salmonella* sp., and four species of *Listeria* sp. were observed in catfish, tilapia and water samples. Overall, by pelleting the sample offered the beneficial to isolate *Aeromonas* spp., *Salmonella* sp. and *Listeria* spp. in catfish, tilapia and water.

Keywords: *Aeromonas*, *Listeria*, pelleting, *Salmonella*

INTRODUCTION

The human illness caused by *Salmonella, Listeria* and *Aeromonas* have been reported to be associated with a wide variety of food products. In many studies, those pathogenic bacteria were reported to cause the human illness by the consumption of various fishes such as catfish and tilapia (**Baker and Smitherman, 1983; Chen *et al.*, 2010; Huddleston *et al.*, 2006; Jallewar *et al.*, 2007; Nawaz *et al.*, 2006; Pal and Marshall, 2009; Radu *et al.*, 2003; Sarter *et al.*, 2007**).

Salmonella spp. are Gram-negative, rod-shape bacteria that cause salmonellosis. In humans, these pathogenic bacteria caused enteric fever (only if it is Typhi or Paratyphi) and acute gastroenteritis (**Hohmann, 2001**). The symptoms include mild to severe gastroenteritis, with an incubation period of 6 to 72 h (**Hohmann, 2001**). *Listeria* sp. are Gram-positive, rod-shape bacteria that caused listeriosis for neonates, elderly, pregnant woman and immune-compromised people (**Adams and Moss, 2004**). *Aeromonas* spp. are Gram-negative, rod shape bacteria with some species of this genus to have been recognized as pathogenic in fish (**Najimi *et al.*, 2009**) and humans (**Rahim *et al.*, 2004**). *Aeromonas* spp. have also been reported to cause zoonotic diseases (wherein the disease can spread from animal to humans and vice-versa) leading to illness. They are also reported to be enterotoxigenic (**Kingombe *et al.*, 2010; Li *et al.*, 2011**). Various hazards associated with cultured fish might originate naturally from the environment or can be present due to the contamination with human or animal activities.

The detection of *Salmonella*, *Listeria* and *Aeromonas* has been introduced by elsewhere (**Andrews *et al.*, 2011; AOAC, 2000; Hitchins amd Jinneman, 2011; ISO 1996; ISO, 2004; Palumbo *et al.*, 1985; Rose and Okrend, 1998; USDA-FSIS, 2013**), however there is limited data regarding the detection or isolation method of *Salmonella*, *Listeria* and *Aeromonas* species by pelleting the sample to increase the sensitivity of method. Thus, this study was carried out to fill this gap. The aim of this study was to modify the isolation methods of *Aeromonas* sp., *Salmonella* spp., and *Listeria* sp. in catfish (*Clarias gariepinus*) and tilapia (*Tilapia mossambica*) obtained from wet markets and ponds in Malaysia by pelleting the sample.

MATERIAL AND METHODS

Samples

Catfish (*Clarias gariepinus*) samples were obtained from five local wet markets and four ponds, while tilapia (*Tilapia mossambica*) samples were purchased from two wet markets, two hypermarkets and four ponds in Penang and surrounding Penang (Malaysia) in the period 2008 to 2009. During each visit, 5-6 live catfish and tilapia were placed in sterile polypropylene bags and transported in polystyrene boxes to the laboratory. In case of live tilapia, the polypropylene bag containing water and fish was flushed with oxygen and bag was tied using rubber bands. Catfish purchased from wet markets were live and placed in sterile polypropylene bags and polystyrene box containing crushed ice. The samples were delivered and analyzed in the laboratory within 3 h. Water samples were obtained from the tanks in which live catfish were maintained in wet markets. Water from ponds where both catfish and tilapia were cultured, were also sampled.

The sample preparation was carried out by using 2 different methods: Method A (pelleting the sample) and B (without pelleting the sample). In method A, the intestines (the contents of the intestines were not removed) of catfish and tilapia were removed using a sterile knife and were pooled by using sterile forceps. The intestines with the contents were placed on a sterile tray wrapped in aluminum foil and chopped thoroughly with sterile knife.
Twenty five grams intestines (or 25 mL water) were placed in a stomacher bag which were containing 225 mL 0.1% PW 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 re-suspended in 10 mL PW. In method B, 25 g of chopped intestines (or 25 mL water) were placed in a stomacher bag which were containing 225 mL 0.1% PW and homogenized using a stomacher (Interscience, France) for 2 min.

Detection and isolation of *Salmonella* sp.

The pellets obtained from methods A was re-suspended using 10 mL Buffered Peptone Water (BPW, Merck KGaA, Darmstadt, Germany) and homogenizing using vortex for 2 min. About 1 mL of homogenate was directly pre-enriched using 10 mL Rappaport and Vassiliadis broth (RV, Merck KGaA, Darmstadt, Germany). The incubation of sample in BPW was 37 °C for 24 h and those in RV was 42 °C for 24 h.

In method B (non-pelleting the sample), 25 g of chopped intestines (or 25 mL water) were re-suspended using 225 mL BPW and homogenized using stomacher for 2 min. About 1 mL of homogenate was directly transferred into 10 mL RV. The incubation of sample in BPW was 37 °C for 24 h and those in RV was 42 °C for 24 h.

After pre-enrichment in BPW, 1 mL portions were transferred into 10 mL RV and incubated at 42 °C for 24 h. After enrichment in RV, 10 µL of the culture was streak-plated onto Rambach (Merck KGaA, Darmstadt, Germany), Xylose Lysine Deoxycholate (XLD, Merck KGaA, Darmstadt, Germany), Xylose-Lysine-Tergitol 4 (XLT4, Merck KGaA, Darmstadt, Germany), and Bismuth Sulfite Agar (BSA, Merck KGaA, Darmstadt, Germany) and were incubated at 37 °C for 24-48 h.

Well isolated colonies giving typical reactions according to manufacturer's instructions, were considered as presumptive *Salmonella* were purified by streaking onto nutrient agar plates (Merck KGaA (Darmstadt, Germany). Well isolated colonies were Gram stained and subjected to following biochemical tests; catalase, cytochrome oxidase, triple sugar iron, lysine iron, urease, indole, motility test. The biochemical test materials were obtained from Merck KGaA (Darmstadt, Germany). *Salmonella* was confirmed by using polyvalent O and H antisera (BD, Franklin Lakes, USA) according to the Bacteriological Analytical Manual (**Andrews *et al.*, 2011**). *Salmonella* isolates were serotyped by Institute for Medical Research, Kuala Lumpur as the WHO Reference Laboratory located in Malaysia.

Detection and isolation of *Listeria* sp.

The pellets obtained from methods A was re-suspended using 10 mL Half Frazer Broth (HFB, Merck KGaA, Darmstadt, Germany) and homogenizing using vortex for 2 min. About 1 mL of homogenate was directly pre-enriched using 10 mL Frazer Broth (FB, Merck KGaA, Darmstadt, Germany). The incubation of samples in HFB was 30 °C for 24 h and those in FB was 37 °C for 24 h. In method B (non-pelleting the sample), 25 g of chopped intestines (or 25 mL water) were re-suspended using 225 mL HFB and homogenized using stomacher for 2 min. About 1 mL of homogenate was directly transferred into 10 mL FB. The incubation of samples in HFB was 30 °C for 24 h and those in FB was 37 °C for 24 h. After pre-enrichment in HFB, 1 mL portions were transferred into 10 mL FB and incubated at 37 °C for 24 h. After enrichment in FB, 10 µL of the culture was streak-plated onto ALOA (Merck KGaA, Darmstadt, Germany) and PALCAM (Merck KGaA, Darmstadt, Germany). These were incubated at 37 °C for 24-48 h. Well isolated colonies giving typical reactions according to manufacturer's instructions, were considered as presumptive *Listeria* were purified by streaking onto nutrient agar plates (Merck KGaA (Darmstadt, Germany). Well isolated colonies were Gram stained and subjected to following the biochemical tests: catalase, cytochrome oxidase, and motility test. The biochemical test materials were obtained from Merck KGaA (Darmstadt, Germany). *L. monocytogenes* obtained from Department of Chemistry Malaysia was used as a positive control. The isolates were identified using Microbact *Listeria* Identification System 12L (Oxoid, Basingtoke, Hampshire, UK).

Detection and isolation of *Aeromonas* spp.

The pellets obtained by methods A was re-suspended using 10 mL of 0.1% PW. Serial dilutions (10^{-4} to 10^{-6}) of 2 methods were done by transferring and mixing 1 mL of the homogenate into 9 mL of 0.1% PW. About 0.1 mL of the appropriate dilution was spread-plated onto selective agar media which were Glutamate Starch Phenol Red Agar (GSP, Merck KGaA, Darmstadt, Germany) supplemented with Penicillin (Merck KGaA, Darmstadt, Germany) and Starch Agar (SA, BD, Franklin Lakes, USA) supplemented with Ampillicin (Oxoid, Baringstoke, Hampshire, UK) (**Palumbo *et al.*, 1985**), in duplicate. These were incubated at 28 °C for 24 to 48 h. Bright yellow colonies measuring 2-3 mm in diameter on GSP agar plates were considered as presumptive *Aeromonas* spp. Colonies with 3-5 mm in diameter appearing as yellow to honey color and positive for amylase activity (after being flooded with 5 mL of Lugol iodine solution on SA) were considered as presumptive *Aeromonas* spp. (**Palumbo *et al.*, 1985**). These colonies were purified by streaking onto Nutrient Agar (NA, Merck KGaA, Darmstadt, Germany). Well isolated colonies were Gram stained, morphology and biochemical tests (**Colakoglu *et al.*, 2006**). *Aeromonas hydrophilla* ATCC 7966 was used as a positive control. The isolates were identified using API 20 NE (BioMerieux, France).

Statistical analysis

The statictical analysis was used to assess differences of pellet and non-pellet in by using General Linear Model procedure (SPSS version 13, USA) at the significance level (P<0.05).

RESULTS AND DISCUSSION

By pelleting the sample, isolation of *Aeromonas, Salmonella* and *Listeria* were relatively higher than those of non-pelleting the sample. By pelleting the sample, the material in the suspension can be separated from the solution and the sample in pellet form will be more concentrated. Thus, the chance to isolate *Aeromonas, Salmonella* and *Listeria* species will be higher. Moreover, the sensitivity of the method by pelleting the sample will be relatively higher than those of non-pelleting the sample (Table 1).

According to **Bell (2005)**, bacteria cell membranes can be damaged due to centrifugation. But bacteria can be recovered in pre-enrichment and enrichment broth. BPW has a high buffering capacity, which may repair the injured *Salmonellae* (**Baylis *et al.*, 2000**). **D'Aoust *et al.* (1990)** and **Stephens *et al.* (1997)** also reported that *Salmonella* can be recovered by the addition of certain metabolic enzymes to repair the injured and increase the low numbers of *Salmonellae.*

These were also observed in *Listeria* isolation, which used HFB as a pre-enrichment broth to 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 a one half concentration of acriflavin and antibiotics, was intended to allow for better growth of injured *Listeria* (**Holbrook *et al.*, 1992**).

The present study found that isolation of *Salmonella* and *Listeria* can be carried out without pre-enrichment (Table 1). Rappaport and Vassiliadis broth (RV) was used as enrichment broth in *Salmonella* isolation. RV contained of tryptone as carbon and a nitrogen source to enhance the growth of *Salmonella* when compared with other *Enterobacteriae* (**van Schothorst and Renaud, 1983**). Other studies reported that RV was the most effective enrichment broth for *Salmonella* compared to other broths (**Beckers *et al.*, 1986; Hammack *et al.*, 1999; June *et al.*, 1995**). The enrichment broth of *Listeria* isolation was Frazer Broth which can inhibit the growth of enterococci (**Frazer and Sperber, 1988**). Overall, the combination of pre-enrichment and enrichment could increase the growth of *Salmonella* or *Listeria* species.

Table 1 Prevalence of *Salmonella*.spp., *Listeria* sp. and *Aeromonas* spp. by using different isolation methods

Bacteria	Sample	Method	Pelleting the sample		Without pelleting the sample	
			Total positive	Sensitivity[c]	Total positive	Sensitivity
Salmonella spp.	Fish	Without Pre-enrichment (n=64)	7[b]	0.47	0[a]	0
		With Pre-enrichment (n=64)	8[a]	0.53	7[a]	0.47
		Total	15			
	Water	Without Pre-enrichment (n=44)	7[b]	0.63	0[a]	0
		With Pre-enrichment (n=44)	8[b]	0.73	5[a]	0.45
		Total	11			
Listeria sp.	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	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	12			
Aeromonas spp.	Fish	n=64	26[a]	0.84	21[a]	0.68
		Total	31			
	Water	n=44	9[a]	0.69	7[a]	0.54
		Total	13			

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

For *Aeromonas* isolation, the sensitivity of the method by pelleting the sample was 0.84 and 0.69 for fish and water,respectively. The sensitivity of the method by pelleting the sample was observed to be lower than those of the method by pelleting the sample. There is no significant difference (P > 0.05) between pelleting and non-pelleting the sample in the isolation of *Aeromonas* spp. In this present study, *Aeromonas* spp. was isolated without pre-enrichment and with enrichment in the broth. Thus, the possibility of injured bacteria because of centrifugation might be higher. **Bell** (**2005**) reported that the application of centrifugation could cause the damage to the extracellular polysaccharides (EPS) layer of bacteria.

By pelleting the sample, the prevalence of *Salmonella* was 8/64 and 8/44 for fish and water, respectively. In the same preparation sample method, the prevalence of *Listeria* was 8/64 and 11/44 for fish and water, respectively. In similar method, the prevalence of *Aeromonas* was 26/64 and 9/44 for fish and water, respectively. Overall, the prevalence of *Salmonella*, *Listeria* and *Aeromonas* in catfish was 21.88%, 9.38% and 43/75%, respectively (Table 2). The prevalence of *Salmonella*, *Listeria* and *Aeromonas* in tilapia was 25%, 15.63% and 53.13%, respectively (Table 2). This is in agreement with other studies. *Aeromona*s spp., *Salmonella* spp., and *Listeria* have been observed in catfish and tilapia (**Baker and Smitherman, 1983; Chen *et al.*, 2010; Jaleewar *et al*, 2007; Pal and Marshall, 2009; Radu *et al.* , 2003**).

Table 2 Prevalence of *Salmonella*.spp., *Listeria* sp. and *Aeromonas* spp. in catfish, tilapia and water obtained from wet markets and ponds in Malaysia

Samples	*Salmonella* spp. number (%)	*Listeria* sp. number (%)	*Aeromonas* spp. number (%)
Catfish	7/32 (21.88)	3/32 (9.38)	14/32 (43.75)
Water	8/32 (25)	5/32 (15.63)	7/32 (21.88)
Tilapia	8/32 (25)	5/32 (15.63)	17/32 (53.13)
Water	3/12 (25)	7/12 (58.33)	6/12 (50)

In this present study, 7 serovars of *Salmonella*, 4 species of *Listeria* and 5 species of *Aeromonas* were found (Table 3). *A. hydrophila, S.* Corvallis, and *L. ivanovii* are predominant in catfish, tilapia and water. These were similar to other studies. **Radu *et al.* (2003)** reported that *A. hydrophilla* has been observed in catfish and tilapia. **Chen *et al.* (2010)** found that *L. ivanovii* and *L. monocytogenes* were observed in catfish. However, the presence of *S.* Corvalis in tilapia and catfish has not been reported elsewhere.

The important finding of this study was the presence of *S.* Typhimurium in catfish and tilapia (Table 3). Thus, catfish and tilapia can be potential source of *S.* Typhimurium that can adversely affect human health. According to **Stan Bailey and Maurer** (**2001**), 70% of all the reported cases of salmonellosis world-wide are due to *S.* Typhimurium and *S.* Enteritidis. **Guillet *et al.* (2010**) revealed that *L. ivanovii* and *L. monocytogenes* could make human disease.

Table 3 Distribution of *Salmonella*.spp., *Listeria* sp. and A*eromonas* spp. in catfish, tilapia and water obtained from wet markets and ponds in Malaysia

Samples	Catfish (n)	Water (n)	Tilapia (n)	Water (n)
Salmonella spp. (n = 26)				
S. Corvallis	1	2	7	2
S. Albany	3	4	na	na
S. Agona	1	1	na	na
S. Stanley	1	1	na	na
S. Mikawashima	na	na	1	na
S. Bovis-mobificans	na	na	na	1
S. Thypimurium	1	na	na	na
Listeria sp (n = 20)				
L. ivanovii	3	4	2	3
L. grayi	na	na	2	2
L. welshimeri	na	1	1	1
L. monocytogenes	na	na	na	1
Aeromonas spp. (n=44)				
A. caviae	4	2	7	2
A. hydrophila	7	4	6	2
A. sobria	1	1	2	2
A. schubertii	1	na	na	na
A. trota	1	na	2	na

na = not avalaible

CONCLUSION

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

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