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# Prevalence of *Salmonella* sp. in African Catfish (*Clarias gariepinus*) Obtained from Farms and Wet Markets in Kelantan, Malaysia and Their Antibiotic Resistance

(Prevalens *Salmonella* sp. dalam Ikan Keli Afrika (*Clarias gariepinus*) yang Diperoleh dari Ladang Ternakan Ikan dan Pasar Basah di Kelantan, Malaysia dan Ketahanan Antibiotiknya)

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ABSTRACT

*The present study was conducted to determine the prevalence and antibiotic resistance of* Salmonella *sp. isolated from African catfish (*Clarias gariepinus*). A total of 30 catfish were harvested from four different farms and four different wet markets. A total of 60 samples (30 catfish skins and 30 catfish intestines) were used for* Salmonella *sp. isolation (pellet-method), its biochemical and serological test. Confirmation of* Salmonella *sp. were determined by polyvalent O antisera and polymerase chain reaction (PCR) using genus specific primers for invA genes (DNA amplification showed one distinct band with molecular weight of 389 bp) and the species of isolated* Salmonella *sp. were identified by serotyping. The result showed 6/30 (20%) of fish or 6/60 (10%) of organ samples were positive for* Salmonella *sp. Among those positive for* Salmonella *sp., 4/6 were from intestine samples and 2/6 were from skin samples. No significant difference was found in the prevalence of* Salmonella *sp. isolates between fish harvested from farms and wet markets (p-value= 0.406). The* Salmonella *serovars identified were* Salmonella corvallis *(n=3),* Salmonella mbandaka *(n=2) and* Salmonella typhmurium *(n=1).* Salmonella *sp. isolates were resistance to Penicillin (P 10, 100%), Clindamycin (DA 2, 100%), Tetracycline (TE 30, 100%) and Rifampicin (RD 5, 100%) and all of the isolates were susceptible or intermediate resistance to Ceftazidime (CAZ 30) and Trimethopin (W 5). Multiple antibiotic resistance (MAR) index of all* Salmonella *sp. isolates in current study was 0.67 indicating that fish sampled in the present study was under high risk of been exposed to the tested antibiotics.*

*Keywords: Antibiotic resistance; catfish; polymerase chain reaction (PCR);* Salmonella

ABSTRAK

*Penyelidikan ini dijalankan untuk menentukan prevalens dan ketahanan antibiotik terhadap* Salmonella *sp. yang dipencil daripada ikan Keli Afrika* (Clarias gariepinus). *Sebanyak 30 ekor ikan keli dipencil dari empat ladang ternakan dan empat pasar basah yang berbeza. Jumlah 60 sampel (30 sampel kulit ikan keli dan 30 sampel usus ikan keli) telah diguna untuk menganalisis kehadiran* Salmonella *sp. (metod-pelet), ujian biokimia dan serologi. Pengesahan* Salmonella *sp. ditentukan oleh polivalen O antiserum dan tindak balas berantai polimerase (PCR) dengan berat molekul 389 bp dan* Salmonella *serovar dikenal pasti melalui seropenjenisan. Keputusan menunjukkan 6/30 (20%) daripada ikan atau 6/60 (10%) daripada sampel organ adalah positif bagi* Salmonella *sp. Antara yang positif bagi* Salmonella *sp., 4/6 adalah daripada sampel usus dan 2/6 adalah daripada sampel kulit. Tiada perbezaan yang signifikan diperoleh dalam prevalens* Salmonella *sp. yang dipencil antara ikan dari ladang ternakan dan pasar basah (nilai* p *= 0.406).* Salmonella *serovar yang dikenal pasti adalah* Salmonella corvallis (n*=3),* Salmonella mbandaka (n*=2) dan* Salmonella typhmurium (n*=1). Melalui ujian ketahanan antibiotik, 100% sampel menunjukkan kerintangan terhadap antibiotik Penisilin (P 10, 100%), Klindamisin (DA 2, 100%), Tetrasiklin (TE 30, 100%) dan Rifampisin (RD 5, 100%) dan tiada kerintangan terhadap antibotik Ceftazidim (CAZ 30) dan Trimtopin (W 5). Kesemua* Salmonella *sp. yang telah dipencil menujukkan ketahanan antibiotik berganda (MAR=0.67) dan ia berpotensi untuk menyebabkan masalah kesihatan berkaitan dengan ikan keli.*

*Kata kunci: Ikan keli; ketahanan antibiotik;* Salmonella*; tindak balas berantai polimerase (PCR)*

## INTRODUCTION

Salmonellosis is an important food borne zoonotic disease affecting both humans and animals (BjØrn et al. 2007). The genus *Salmonella* are facultative anaerobic, non-spore forming rod-shaped and gram negative motile bacillus belonging to the family of Enterobacteriaceae. These mesophilic organisms are distributed geographically all over the world. Aquatic environments are the major reservoirs of *Salmonella* sp. and fishery products have been recognized as a major carrier of food-borne pathogens

such as *Salmonella* sp. (Kamat et al. 2005). Fish-associated Salmonellosis in humans is an increasing public health concern.

African catfish is a type of freshwater fish, under the Order *Siluriformes*. Common family within this Order in Malaysia is *Clariidae* with genus species *Clarias gariepinus*. It is the main freshwater fish cultured in Kelantan and mainly reared in the pond culture system (72.27%), with highest production 35.42% of total aquaculture harvest. Thus, freshwater catfish has been played an important role as one of main animal protein sources in Kelantan.

The major hazards of concern for aquaculture fish include pathogenic microorganisms, antimicrobial or drug residues and environmental contaminants. It has been reported that fish can serve as vehicle of *Salmonella* transmission to public and the bacteria has a high potential to transmit its antibiotic resistance gene to other pathogen via plasmid (Hradecka et al. 2008).

In Malaysia, data regarding the prevalence of *Salmonella* in catfish cultured in ponds are limited (Budiati et al. 2012). Thus, the present study was conducted to determine the prevalence and antibiotic resistance of *Salmonella* in African catfish (*Clarias gariepinus*) obtained from ponds and wet market in Kelantan, Malaysia.

## Materials and Methods

### SAMPLES

A total of 30 adult African catfish (*Clarias gariepinus*) were collected from four different farms ($n$=16) and four different wet markets ($n$=14) from four districts in Kelantan which were Tumpat, Kota Bharu, Bachok and Pengkalan Chepa from January to March 2014. The aged of harvested fish were between 5 and 7 months old which were standard age to be sold to market or consumed. The average total body length of catfish was 36 cm, whereas the average of body weight was 425 gram. During each visit, four live catfish from farm or 3-5 catfish from wet market were placed in sterile polypropylene bags and transported in polystyrene box to laboratory. The post-mortem was done immediately in Post-mortem laboratory, Faculty of Veterinary Medicine, Universiti Malaysia Kelantan. Two organs were sampled from each catfish i.e. intestine and skin (approximately 10 g, respectively). In order to reduce contamination of sample, the intestine and skin were raised slowly with sterile distilled water immediately just after the organs were removed from body by using scalpel or knife and put in a sterile petri dish, respectively and properly labelled. To ensure that the distilled water did not enter into the intestines from both cut end, forceps were used to clamp both end during rinsing with distilled water.

### SAMPLE PREPARATION

Approximately 10 g of skin or intestine was placed on a sterile aluminum foil and chopped thoroughly with knife or scissor. Chopped skin or intestine was then placed in a stomacher bag containing 125 mL 0.1% Peptone water (PW) and homogenized using a stomacher for 3 min at 200 rpm. The homogenate was then divided equally and transferred into 50 mL centrifuge tubes and centrifuged for 15 min at 10000 × g, at 21°C to obtain a pellet. The pellet was pre-enriched by re-suspending it in 10 mL Buffered Peptone Water and incubated at 37°C for 24 h.

### ISOLATION AND IDENTIFICATION OF *SALMONELLA* SP.

After pre-enrichment, 1 mL aliquot of enriched culture was transferred into 10 mL of Rappaport and Vassiliadis broth and incubated at 42°C for 24 h. Following enrichment, 10 µL of the culture was streak-plated onto Xylose Lysine Deoxycholate agar (XLD, Merck KGaA, Darmstadt, Germany) and incubated at 37°C for 24-48 h. Well isolated colonies giving typical reactions according to manufacturer's instructions were considered as presumptive *Salmonella* sp. and were subcultured by streaking onto nutrient agar plates (Merck KGaA, Darmstadt, Germany). Pure colonies were then Gram's stained and subjected to following biochemistry tests: Catalase, Oxidase, Triple Sugar Iron, Urease, indole and motility test.

### CONFIRMATION OF *SALMONELL*A SP. AND SPECIES DETECTION

*Salmonella* sp. isolates were phenotype confirmed by polyvalent O antisera and while genotypic confirmation by polymerase chain reaction (PCR) using genus specific primers for *inv*A genes (DNA amplification showed one distinct band with molecular weight of 389 bp). DNA extraction was followed as the guideline stated in Vivantis Nucleic Acid Extraction Kit Handbook by GF-1 Bacterial DNA Extraction User Guide (Version 2.1) The PCR protocol was as follows: Initial denaturation (10 min at 95°C), 35 amplification cycles (30 s at 95°C, 45 s at 55°C, 30 s at 72°C) and the final extension (10 min at 72°C). A volume of 10 µL of the PCR product was analyzed by electrophoresis in 1% agarose gel. Species of isolated *Salmonella* sp. were also sent to Veterinary Research Institution (VRI), Ipoh, Perak, for serotyping.

### ANTIBIOTIC SUSCEPTIBILITY TESTS

The antibiotic susceptibility test was performed by using disc diffusion method on Muller-Hinton agar, as described by Kirby-Bauer (Bauer et al. 1966), in accordance with the guidelines of the Clinical and Laboratory Standard Institute. Six antibiotics were selected for tests on isolates *Salmonella* sp., which were Ceftazidime (CAZ 30), Trimethoprim (W 5), Penicillin (P 10), Clindamycin (DA 2), Tetracycline (TE 30) and Rifampicin (RD 5). For each isolate, the zone of inhibition around each disk was measured after incubation at 37°C for 24 h. The results were interpreted as sensitive, intermediate or resistant according to Performance Standards for Antimicrobial

Disk Susceptibility Tests (CLSI 2010). Multiple Antibiotic Resistances (MAR) index was calculated based on the formulae: number of antibiotic to which the isolate was resistant: divided by the number of antibiotics to which the isolates were subjected to; A MAR index value equal or less than 0.2 was considered to indicate that the bacteria samples were from where antibiotics were seldom or never used. MAR index value higher than 0.2 was considered to have originated from high exposure to antibiotics.

### STATISTICAL ANALYSIS

The differences in the prevalence of *Salmonella* sp. isolates between ponds and wet markets were determined by using one-way ANOVA (SPSS Statistics version 21) at a significance level of $p<0.05$.

## RESULTS

### PREVALENCE OF *SALMONELLA* SP. IN CATFISH OBTAINED FROM FARMS AND WET MARKETS

The prevalence of *Salmonella* sp. in catfish obtained from four different farms and four different wet markets is presented in Table 1. Catfish samples was considered positive either it was positive only in intestines or only in skin or both organs. Overall prevalence of *Salmonella* sp. isolated in term of number of fish and organ samples were 6/30 (20%) and 6/60 (10%) respectively (Table 1). The results showed that 2/4 (50%) of fish from farms were positive for *Salmonella* sp. while other farms were negative. In the wet markets 25% - 66.67% fish samples and 10% - 30% organs were positive for *Salmonella* sp. presence (Table 1). *Salmonella* sp. was detected in 1/3 catfish (1/6 organ samples), 1/5 catfish (1/10 organ samples) and 2/3 catfish (2/6 organ samples) obtained from wet market 1, 2 and 3, respectively. *Salmonella* sp. was not detected in catfish samples obtained from wet market 4. The results showed that the prevalence of *Salmonella* sp. in wet market (28.57%) were higher than in farms (12.5%) although statistical analysis showed no significant difference ($p>0.05$) between the catfish obtained from farms and wet markets.

In addition, it was found that 6/60 (10%) organs were positive *Salmonella* sp. isolation. The results showed that the prevalence of *Salmonella* sp. in intestine was higher than on skin which were 4/6 (66.67%) for intestine and 2/6 (33.33%) for skin sample, respectively.

### ANTIBIOTIC RESISTANCE *SALMONELLA* SP. ISOLATES OBTAINED FROM AFRICAN CATFISH

The antibiotic resistance among *Salmonella* serovars isolated from catfish is presented in Table 2. All *Salmonella* sp. isolates were resistance to Penicillin (P 10, 100%), Clindamycin (DA 2, 100%), Tetracycline (TE 30, 100%) and Rifampicin (RD 5,100%) and all of the isolates were susceptible or intermediate resistance to Ceftazidime (CAZ 30) and Trimethopin (W 5). Multiple Antibiotic Resistance (MAR) index of the *Salmonella* isolates was 0.67.

### PCR IDENTIFICATION AND SEROTYPING

In the present study, *Salmonella* sp. was confirmed by polymerase chain reaction (PCR) using genus specific primers for *inv*A genes (DNA amplification showed one distinct band with molecular weight of 389 bp). The result of PCR is present as Figure 1. The result of serotyping showed 3/6 of samples were *Salmonella Corvallis* and 2/6 were *Salmonella mbandaka* and 1/6 were *Salmonella typhimurium*.

## DISCUSSION

### PREVALENCE OF *SALMONELLA* IN CATFISH OBTAINED FROM FARMS AND WET MARKETS

*Salmonella* sp. was successfully isolated from African catfish in this study with a prevalence of 6/30 (20%) positive for *Salmonella* sp. Thus it was proven that cultured African catfish could definitely serve as carrier of *Salmonella* transmission to the public in Kelantan. Baker and Smitherman (1983) had shown that *Salmonella* serovars were isolated from catfish and proved that cold-blooded animals are potential hosts for *Salmonella* species.

All selected farms in this study practiced giving home-mixed feed to fish consisting of commercial fish feed, chicken offals and local ingredients. Local ingredient of

TABLE 1. Prevalence of *Salmonella* sp. isolated in catfish obtained from farms and wet markets

| | Farms | | | Wet market | |
|---|---|---|---|---|---|
| No. of farms | % of positive fishes | % of positive organs | No. of wet markets | % of positive fishes | % of positive organs |
| Farm 1 | 2/4 (50) | 2/8 (25) | Market 1 | 1/3 (33.33) | 1/6 (16.67) |
| Farm 2 | 0/4 (0) | 0/8 (0) | Market 2 | 1/5 (25.00) | 1/10 (10.00) |
| Farm 3 | 0/4 (0) | 0/8 (0) | Market 3 | 2/3 (66.67) | 2/6 (30.00) |
| Farm 4 | 0/4 (0) | 0/8 (0) | Market 4 | 0/3 (0) | 0/6 (0) |
| Total | 2/16 (12.5) | 2/32 (6.25) | Total | 4/14 (28.57) | 4/28 (14.29) |

Farm 1 and Market 1: Tumpat district; Farm 2 and Market 2: Kota Bharu district; Farm 3 and Market 3: Bachok district; Farm 4 and Market 4: Pengkalan Chepa district

TABLE 2. Antibiotic resistance tests of *Salmonella* serovars from catfish

| Antibiotic | Sensitive (%) | Intermediate resistance (%) | Resistance (%) |
|---|---|---|---|
| Ceftzaidine (CAZ 30) | 5 (83.33) | 1 (16.67) | 0 (0) |
| Trimethopin (W 5) | 6 (100) | 0 (0) | 0 (0) |
| Penicillin (P 10) | 0 (0) | 0 (0) | 6 (100) |
| Clindamycin (DA 2) | 0 (0) | 0 (0) | 6 (100) |
| Tetracycline (TE30) | 0 (0) | 0 (0) | 6 (100) |
| Rifampicin (RD 5) | 0 (0) | 0 (0) | 6 (100) |

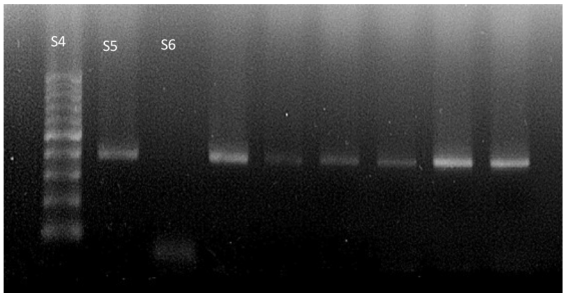


FIGURE 1. PCR confirmation. Polymerase chain reaction (PCR) using genus specific primers for *inv*A genes (DNA amplification showed 389 bp band in all samples ($S_1$-$S_6$). In the present graph S1-S3 are positive for *Salmonella corvallis*, S4-S5 are positive for *Salmonella mbandaka* and S6 is for *Salmonella typhimurium*

homemade feed was usually made from chicken viscera, kitchen refuses, chicken bone and other food waste materials. Prevalence of *Salmonella* sp. in chicken, eggs and feed has been reported by many researches previously (Arshad et al. 2006; Singh et al. 2010; Veldman et al. 1995). Thus it was postulated that, this is one of the possible pathway how *Salmonella* sp. was introduced to catfish aquaculture in Kelantan. This finding was similar to an earlier report which stated that feed made from chicken offals, spoiled eggs and commercial fish feed could transfer *Salmonella* sp. to the aquaculture environment (Budiati et al. 2012) and feed serves as a niche for *Salmonella* sp. growth in fish and aquaculture water (Lunestad et al. 2007). Budiati et al. (2012) stated that, the prevalence of *Salmonella* sp. in catfish fed with chicken offal or homemade food was relatively higher than those fed with only commercial fish feed. Besides that, this also support the finding in this study which showed the prevalence of *Salmonella* sp. in intestine was higher than on skin samples from African catfish. *Salmonella* sp. was successfully isolated on the skin of African catfish could be due to contamination from numerous sources during harvest, slaughter, dressing, processing and packaging. Poor water quality, farm runoff, feeds, insanitary processing conditions and poor distribution, handling and preparation practices can be part of the contamination pathway on skin.

The water sources for all the selected farms and selected wet markets were ground water and only farm 1 was from stream water. Both water sources could be considered as another route of *Salmonella* sp. being transferred to the fish. Previous studies in Taiwan (Li et al. 2009), concluded that stream water and ground water could be easily contaminated by *Salmonella* sp. Organic polluted water could contributed to the colonization of fish by *Salmonella* sp. and transmitted to human (Iwamoto et al. 2010).

Stocking density and post-harvest handling of catfish are some of the important issues in aquaculture and they could be related to how easy the cultured fish can be exposed to *Salmonella* sp. bacteria. All catfish obtained from wet market were put in a small container without water change. Andrews et al. (1977) found that *Salmonella* sp. levels were enhanced by high stocking densities and warm water temperature. And, water pollution could be a result of overstocking the fish (Amagliani et al. 2012). Thus, unhygienic and improper handling of the fish during dressing could be resulted in cross contamination of fish eat from viscera. Thus, this could have explained the prevalence of *Salmonella* sp. obtained from wet market was higher than catfish obtained from farms. *Salmonella* sp. is a contaminant in the environment which could be transferred to the fillets during processing and it also can become establish in the processing facility. Good sanitation and processing practices greatly reduced the frequency and levels of contamination of the fillets (Andrews et al. 1977).

The outbreak of fish-related *Salmonellosis* in human was believed to be due to improperly cook of contaminated fish. An outbreak of *Salmonellosis* after consumption of smoked eel in Germany has been reported which indicated that fish could become a vector of *Salmonella* sp. infection and smoking process may not eliminate bacterial contamination from raw fish (Novotny et al. 2004). Improper storage or cross-contamination of food by contaminated raw fish or utensil was also the factors of *Salmonellosis* outbreak in human. An epidemiological investigation proved that there was no estimate available for foodborne illness attributed to channel catfish meat because catfish are usually cooked to sufficient temperature to kill microbial pathogen. The other likely pathways of cross-contamination from raw fish to another food was eating utensil. Several studies reported and demonstrated that raw or undercooked animal-source proteins such as catfish may be contaminated with a variety of pathogenic organism including *Salmonella* sp. and *Campylobacter* sp.

(Finley et al. 2006; Stiver et al. 2003; Weese et al. 2005). Cats and dogs may develop foodborne illness after being fed with animal-source protein contaminated with these organisms and secondary transmission of these pathogen to human (such as pet owner) has been reported by Joffe and Schlesinger (2002) and LeJeune and Hancock (2001). Therefore, fish-associated foodborne *Salmonellosis* outbreak could happen primarily in animal and human due to consuming the improper cooking of contaminated raw catfish or improper handling of contaminated raw catfish, or, it could happen as secondary *Salmonellosis* outbreak in human (owner) from infected animals due to consumption of contaminated raw catfish. This is related to 'One-Health' issues.

### ANTIBIOTIC RESISTANT AMONG *SALMONELLA* SEROVARS ISOLATED FROM AFRICAN CATFISH

Antimicrobial residues in foods are emerging worldwide issue. Antimicrobials that are used in aquaculture to control diseases included antibiotics and antifungals. Antibiotics are used for treatment and as growth promoters in the animal husbandry and aquaculture and the improper use of antibiotics can lead to development of resistance (Serrano 2005). In the current study, it was observed that all the *Salmonella* isolates were resistant to Penicillin (P 10), Clindamycin (DA 2), Tetracycline (TE 30) and Rifampicin (RD 5) and all of the isolates were susceptible or intermediate resistance to Ceftazidime (CAZ 30) and Trimethopin (W 5). In a recent study in Saudi Arabia, by Elhadi (2014), it was found that *Salmonella* isolates from fish were mainly resistant to Tetracycline followed by Ampicillin and Amoxicillin-clavulanic acid. This findings indicated the wide spread use of antibiotics in fish farming. In Malaysia, the use of antibiotics in aquaculture was not well regulated and enforcement of the regulation was weak especially in small fish farms. In a recent study in Penang, Malaysia, by Budiati et al. (2012), they found that *Salmonella* sp. isolated from catfish were found resistant to antibiotics such as Clindamycin, Tetracycline, Rifampicin and Penicillin. It thus reflected the wide spread indiscriminate use of antibiotics in catfish farming in Malaysia.

Geidam et al. (2012) stated that *Salmonella* sp. isolated in chicken from poultry farm in Selangor, Malaysia showed multiple antibiotics resistance and all of the *Salmonella* sp. were found to be resistant to Tetracycline (100%) and Clindamycin (100%). As mentioned by Budiati et al. (2012), catfish fed with chicken offals and spoiled eggs could be a potential source of *Salmonella* sp. and have a high risk of spreading antibiotic resistant genes among bacteria associated with catfish. It was supported by Carattoli (2003) that plasmid in *Salmonella* sp. has been proven to transfer antibiotics resistance and virulence traits. Therefore, the origin of antibiotic resistant in catfish in the current study could be due to the feeding of chicken offal and spoiled eggs which contained antibiotic resistant genes. The great concerns are about on the human consumption of the residual antibiotics in fish meat and on the pathogenic microorganisms becoming resistance to these antibiotics resulting from adapting to the widespread exposure to antibiotics (Sapkota et al. 2008). This could cause the treatment of human infections to become more difficult as the antibiotics lose their effectiveness.

MAR index of all the *Salmonella* isolates in the current study was 0.67. The high value of MAR that was observed in this study indicated that the fish sampled in the present study was under high risk of been exposed to the tested antibiotics in which the results could give us information on the existence or contamination of the tested antibiotics residue in the sampling area.

### SPECIES OF *SALMONELLA* ISOLATED

The species of *Salmonella* isolated in this study were *Salmonella corvallis*, *Salmonella mbandaka* and *Salmonella typhimurium*. *Salmonella* sp. contains approximately 2500 strains. All the serotype can cause disease in human and some species are host specific, for example, *Salmonella dublin* found only in cattle and *Salmonella choleraesuis* found for only in pig. According to the report by Thong (2006) on the surveillance and subtyping of *Salmonella* sp. in Malaysia, it was found that the *Salmonella corvallis* was the top three species and *Salmonella typhimurium* was the top four which caused non-typhoidal *Salmonellosis* in human in Malaysia during the year 2005. Kocabiyik et al. (2006) proved that 13.41% of stray dogs have been infected by *Salmonella corvallis* and 45% of infected dog showed clinical signs of diarrhea. In addition, in an outbreak of *Salmonella mbandaka* in Manitoba, Canada in 2007, it was reported that all the human patients showed manifested clinical signs of diarrhea, vomiting, nausea and abdominal pain.

## CONCLUSION

This study demonstrated that the prevalence of *Salmonella* sp. in African catfish (*Clarias gariepinus*) obtained from farms and wet markets in Kelantan, Malaysia was categorized as mild since six out from 30 catfish were positive (20%). The isolates of *Salmonella* species were identified as *Salmonella corvallis*, *Salmonella mbandaka* and *Salmonella typhimurium*. The current study also showed that the occurrence of *Salmonella* sp. in catfish obtained from wet market was higher than catfish obtained direct from farms although statistical analysis showed no significant difference ($p>0.05$) Lastly, there was a multi-antibiotic resistance in *Salmonella* sp. isolated from cultured African catfish in Kelantan and this issue must be given attention by authorities to formulate appropriate laws and regulation to avoid worsening this condition in the near future. We must play role in executing the concept of One-Health, One Medicine and One World: Animal, Human and Environment health.

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