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Detection and antibiotic susceptibility profiles of *Listeria monocytogenes* in wildlife and water samples in Kubah National Park, Sarawak, Malaysia

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Abstract

This study was conducted to detect the presence of *Listeria monocytogenes (L. monocytogenes)* and screen for its antibiotic susceptibility characteristic from wildlife and water samples at Kubah National Park, Sarawak, Malaysia. Samples collected were incubated and streaked on selective medium PALCAM agar to confirm the presence of *Listeria* spp. before they were further tested using molecular analysis. Specific Polymerase Chain Reaction (PCR) assay were performed to target specific virulence gene, haemolysin gene, *hlyA* to further distinguish the presence of this pathogenic bacteria in the samples. Overall, out of the 30 samples tested, 10 samples were confirmed as to contain *L. monocytogenes* strains and selected to subsequent antibiotic susceptibility test. Susceptibility patterns to 10 antibiotics were investigated among the *L. monocytogenes* strains. All strains were uniformly resistant to tetracycline and erythromycin. On the other hand, all strains were sensitive to gentamycin and tobramycin. The multiple antibiotic resistance shown by the strains in this study indicate the potential health hazard associated with the possible transmission between wildlife and water to its surrounding environment especially visitors and workers of Kubah National Park, Sarawak, Malaysia.

Introduction

Listeria monocytogenes (L. monocytogenes) is a pathogen regarded as public health concern and zoonotic disease. *Listeria monocytogenes* and *Listeria ivanovii* are classified as pathogenic species while other type of *Listeria* spp. are considered as avirulent and seldom to cause any disease (Liu, 2006). Infection with *L. monocytogenes* can result in septicemia, meningoencephalitis and abortion in humans and animals, primarily affecting pregnant, newborn and immunocompromised individuals (Hage *et al*., 2014). The common symptoms of listeriosis include fever, muscle aches and gastrointestinal symptoms such as diarrhea or nausea. Among the effective antibiotics used to treat infection by *Listeria* (listeriosis) are vancomycin, sulfamethoxazole-trimetophrim (SXT), rifampin, combination of ampicillin and an aminoglycoside (Zulema *et al*., 2011). Additionally, it has been stated that antibiotic treatment against listeriosis showed susceptibility to most antibiotics but it can be slowed and may even be untreatable or persistent (Zulema *et al*., 2011).

Nowadays, there are trends of emergence of pathogenic bacteria from wildlife (Emilio, 2014). Animals in wildlife can act as reservoir and pose threat to global biodiversity and human population. This is due to population expansion which will lead to human invasion and risking themselves towards pathogenic bacteria. Intestinal tract of animal such as wild and feral mammals, crustaceans, birds and fish act as reservoir of infection. Besides humans, at least 42 species of wild and domestic mammals, rodents and 17 avian species, including domestic and game fowl, can act as a vector host for *L. monocytogenes* (Lloyd, 2008).

Apart from wildlife, *L. monocytogenes* has been implicated in infection originality from environment samples such as soil, water, manure and vegetation (Strawn *et al*., 2013). Therefore in the study, environment samples such as water and sediments from Sungai Rayu were included in to measure the rate of occurrence in the surrounding area around Kubah National Park, Kuching. Molecular technique such as Polymerase Chain Reaction (PCR) is used for virulence gene detection after the preliminary and identification method. One of the advantages of this technique are they offer accurate, reliable and time saving procedure to obtain result. The *hlyA* gene is used as a PCR target as it is species-specific

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to identify *L. monocytogenes* (Day *et al.*, 2015). Previously, several local findings have reported on the isolation and detection of *L. monocytogenes* from foods in Malaysia (Hassan *et al.*, 2001; Lesley *et al.*, 2003; Jeyaletchumi *et al.*, 2010). Nonetheless, to the best of our knowledge, there has been no study on *L. monocytogenes* from animals and birds in Sarawak, Malaysia. Thus, this study was carried out to investigate the occurrence of *L. monocytogenes* at Kubah National Park by analyzing wildlife feces waste and swab samples from their anal, cloacal, faecal wastes. Environmental samples such as water and sediments were also been analyzed. This area is chosen as it is one of the popular areas and received many visitors each year for recreational purpose such as camping and hiking. At this level, there is possibility of direct transmission of *Listeria* spp. especially *L. monocytogenes* from wildlife to human.

Materials and Methods

Sample collection and bacteria identification

Small mammals and bird specimens were collected during an expedition to Kubah National Park, Kuching, Sarawak, during April 2012. Two sampling sites had been chosen which was Matang Wildlife Center for animal samples collection and Sungai Rayu for water sampling site. Matang Wildlife Centre which is situated in Kubah National Park consists of large ecosystem area for endangered wildlife located 35 km from Kuching while Sungai Rayu is a river situated along the human settlement area, Kampung Rayu. Bats and birds samples were captured using mist nets while rodents, mongoose and tree shrew were captured using cage traps. The process of identification of mammals and birds was carried out according to Payne *et al.* (1985) and Francis (2005), respectively.

Water and sediment samples were collected along the river at the study sites. Approximately 10 g of sediment were collected using scoop and placed in the sterile plastic bags. Approximately 5 mL of water were collected sterile universal bottles by dipping the bottles into selected water source. All samples were kept at 4°C until further processed at the laboratory.

Animal samples were acquired using swabbing method where by anal swabs and cloacal swabs were collected using sterilized cotton buds. For feces and water samples, the specimens were collected directly from the respective sources. All specimens were immediately placed into 1 mL of phosphate buffered saline (PBS) and stored at 4°C inside the ice box throughout the field trip. Samples were immediately processed at laboratory within the same day of sampling to avoid sample degradation and contamination.

All of the samples were cultured directly on PALCAM agar (Oxoid, England) and incubated at 37°C for overnight. Plates were examined for the presence of grey-green colony with diameter around 2 mm and surrounded with black zone. Subsequently, colonies that exhibited this characteristic were stored in nutrient agar (Oxoid, England) and were further tested using gram-staining. *L. monocytogenes* reference cultures ATCC 15313 was included as a positive control.

DNA isolation

Bacterial total DNA of the *L. monocytogenes* isolate was extracted by using boiling extraction method as described by Apun *et al.* (2011) with minor modification. For the extraction of bacterial genomic DNA, *L. monocytogenes* isolates was cultured in 3 mL Tryptone-Soy broth at 37°C incubator with agitation at 150 rpm. Overnight broth suspensions (1.5 mL) were then centrifuged at 10,000 rpm for 5 min to collect the pellets. After that, the pellets were re-suspended in 400 µL of distilled water and boiled for 20 min. The lysed cells were then cooled in ice for another 20 min and centrifuged at 10,000 rpm for 5 min. The supernatant containing DNA was used as template in the specific Polymerase Chain Reaction (PCR).

Specific PCR assay for the detection of L. monocytogenes

The primers used for the detection of the hemolysin gene, *hlyA* gene were as previously reported by Gouws and Liedemann (2005) while the condition for the specific PCR assay performed was as described by Kargar and Ghasemi (2009) with modification. Single primer, hlyA-F 'CAT TAG TGG AAA GAT GGA ATG' and hlyA-R 'GTA TCC TCC AGA GTG ATC GA' were used for detecting the hemolysin gene, *hlyA* gene. Each PCR was performed in a total reaction volume of 25 µL containing 2.5 µL of 10x PCR buffer (Invitrogen, Brazil), 2.0 µL of 25 mM Magnesium Chloride (MgCl2) (Invitrogen, Brazil), 0.5 µL of 10 mM dNTP mix (RBC Bioscience Corp, Taiwan), 1.0 µL of 10 mM hlyA-F and hlyA-R primers (MWG-Biotech, Germany), 0.5 µL of 2.5 mM Taq DNA polymerase (Fermentas International Inc, Canada), 16.5 µL of sterile distilled water (ddH_2O) and 1 µL of DNA template. *L. monocytogenes* reference cultures ATCC 15313 was included as a positive control. On the other hand, PCR amplification was performed using iCycler™ Thermal Cycler (Bio-Rad, USA) as

follows: Initial denaturation at 95°C for 5 min; 35 cycles of denaturation at 95°C for 1 min; annealing at 62°C for 45 sec; and extension at 72°C for 1.5 min; followed by final extension at 72°C for 5 min.

Gel electrophoresis

A 5 µL aliquot of each amplification product was analyzed using electrophoresis on 2% agarose gels cast and run in 1 X TAE buffer. A 100-bp marker (Vivantis, Malaysia) was included in the gel. Gel was stained with ethidium bromide (0.5µg/ml) and visualized using transmitted ultraviolet illumination and photographed using gel documentation system (AlphaDigiDoc RT).

Antibiotic susceptibility testing

Antibiotic susceptibility test were carried out using Kirby-Bauer disk diffusion method according to Clinical and Laboratory Standards Institute (CLSI) (CLSI, 2006a and 2006b; Mauro *et al.*, 2007). The medium used for the testing is Mueller Hinton agar (Oxoid, England). The antibiotics used were Ampicillin (10 µg), Cephalotin (30 µg), Chloramphenicol (30 µg), Clindamycin (2 µg), Erythromycin (30 µg), Gentamicin (10 µg), Rifampicin (5 µg), Streptomycin (10 µg), Tetracycline (30 µg) and Tobramycin (10 µg). L. monocytogenes reference cultures ATCC 15313 acted as internal control.

Multiple antibiotic resistances (MAR) analysis

MAR index of the isolates was determined as described by Krumperman (1983). The MAR index is defined as a/b, where "a" where 'a' represents the number of multiple antibiotics to which the particular isolates are resistant, and 'b' the number of multiple antibiotics to which the particular isolates are tested.

Results and Discussion

In this study, swab samples collected from 22 individuals of wildlife in Matang Wildlife Centre, Kubah National Park and 8 water samples from Sungai Rayu, Matang were tested for the occurrence of *Listeria* spp. A total of 15 bats (five species), 2 squirrels (one species), 2 birds (two species), 1 mongoose (one species), 1 tree shrew (one species) and a rodent (one species) were successfully captured. Table 1 shows the list of animals captured with its type of species.

Our study showed 33% (10/30) isolates were positive with *hlyA* gene indicating the presence of *L. monocytogenes*. The targeted gene, *hlyA* gene (730 bp) was detected in 7 animal samples and 3 water

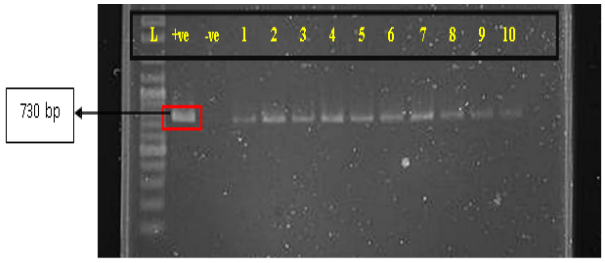

Figure 1. Amplicon obtained from Specific PCR for 10 *L. monocytogenes* isolates from Kubah National Park, Kuching with virulence gene, *hlyA* with expected size of 730bp fragmented by 1.25% agarose gel electrophoresis. Ladder: 100kb, Lane +ve (Positive control): *L. monocytogenes* ATCC 15313, Lane –ve (Negative control): none, Lane 1: Isolate TK172786 (F) for bat sample (faecal), Lane 2: TK172787 (F) from bat sample (faecal), Lane 3: TK172792 (F) from bat sample (faecal), Lane 4:TK172803 (F) from bat sample (faecal), Lane 5: CS1-W from water sample, Lane 6: LCS2-S1 from water sample (sediment), Lane 7: UCS2-W from water sample, lane 8: TK172820 from rodent sample (anal), Lane 9: TK172811 from bat sample (anal) and Lane 10: TK172818 from tree shrew sample (anal)

samples (Figure 1). Previous study by Nitaya *et al*, (2011) showed that 35 out of 84 of *Listeria* isolates (42%) from raw meats carried the gene coding for hlyA, while the remaining 58% isolates harboured only some of the other virulence traits (*act*A, *iap* and *fla*A). In our study, the animal samples were obtained from four fecal samples and three anal samples. The animal samples consist of 5 bats; 1 rodent and a tree shrew while for water samples, 2 samples were obtained from water source and 1 from sediment. The result obtained suggested that there is possibility of cross contamination between these animals. Bats, rodent and tree shrew may share similar food sources such as fruit that already been contaminated with *L. monocytogenes* strains. In addition, there is possibility that they come in contact with the bacterium through indirect contact such as through water source and sharing habitats.

Presence of *Listeria* spp. in bat is considered high compared to other animal as most of the animal samples source were from bats. Several species of bats captured indicates that there is source of foods available such as fruits and vegetation sources to allow it to survive. Previous study related to *Listeria* spp. in bats is quite limited; however the possibilities of transmission can be understood through its feeding habits. According to Hubalek and Rudolf (2011), bats usually become the agent of dispersal or pollinator for fruit by foraging it and disperse the pollen or fruits. Therefore, there is possibility of transmission occur during feeding process or interaction with other type of wildlife.

Table 1. Number of wildlife hosts tested for the occurrence of *L. monocytogenes* in Matang Wildlife Centre, Kuching

Type of wildlife hosts	Species	Number of samples examined	*L. monocytogenes* N (%)
Bat	*Cynopyterus*	6	2 (33.3%)
	Hipposideros	4	3 (75.0%)
	Balionycteris	2	-
	Kerivoula	2	-
	Penthetor	1	-
			-
Bird	*Ceyx*	1	
	Otus	1	-
Mongoose	*Herpestes*	1	-
Rodent	*Maxomys*	1	1 (100.0%)
Squirrel	*Callosciurus*	2	-
Tree shrew	*Tupaia*	1	1 (100.0%)
Total		**22**	

Table 2. Antibiotic resistant patterns and MAR index of 10 confirmed *L. monocytogenes* isolates

Field number	Antibiotic resistant pattern	MAR index
TK172786 (F)	AmpERdTe	0.4
TK172787 (F)	C	0.1
TK172792 (F)	AmpETe	0.3
TK172803 (F)	ETe	0.2
CS1-W	EKfSTe	0.4
LCS2-S1	AmpETe	0.3
UCS2-W	AmpEKfTe	0.4
TK172820	ETe	0.2
TK172811	EKfRdTe	0.4
TK172818	ESTe	0.3

Keys: Ampicilin (AMP) (10 µg), Cephalotin (KF) (30 µg), Chloramphenicol (C) (30 µg), Clindamycin (DA) (2 µg), Erythromycin (E) (30 µg), Gentamicin (CN) (10 µg), Rifampicin (RD) (5 µg), Streptomycin (S) (10 µg), Tetracycline (TE) (30 µg) and Tobramycin (TOB) (10 µg).

On the other hand, the occurrence of *Listeria* in bird captured at Kubah National Park was in similar range of previous study conducted by Hellstrom *et al.* (2006) and Crowly *et al.* (2013). The data suggested that birds are normally not the main reservoir harbouring this pathogenic strain and usually being infected transiently. The possible transmissions for bird most likely come in food source such as by pecking *Listeria*-contaminated soils, faeces, fruits, carcass or through other animal faecal waste. In addition, the presence of *Listeria* was also detected in rodents. In the rehabilitation environment, rodent may obtain foods from nature like bats and birds or through human wastes as public can access the reserved forest through hiking trails or camp sites nearby.

Other three *hlyA* amplicons for *L. monocytogenes* isolates originated from water were suspected to carry similar risk to wildlife source. Sungai Rayu flows between human settlement area; Kampung Rayu to lower level of land before reaching the sea. The presence of the gene in the sample collected may indicate there is probability of interaction between water to animal and human at surrounding areas. Animals may obtained this isolates when drinking or getting food from Sungai Rayu while human may have possibility of infection through their close contact with the river. Thus, early detection may signify the chances of transmission between animal and water to human and subsequently increases

the potential of these pathogenic bacteria to be transmitted zoonotically.

In this study, the bacteria were tested for susceptibility to 10 antibiotics (Table 2). All of *L. monocytogenes* strains, showed resistance to at least one of the 10 tested antibiotics. *Listeria* is still considered to have high percentage of susceptibility towards many antibiotics. Highest percentages of susceptibility (100%) were seen in all samples towards gentamicin and tobramycin whereas rifampicin, streptomycin, ampicillin, clindamycin and chloramphenicol has range of susceptibility varied from 60% to 90%. The most common resistance was resistance towards tetracyline and erythromycin, which was detected in 9 out of 10 sample isolates. In contrast, the previous study showed that most of the *L. monocytogenes* isolates were resistant to cefotaxime, ceftazidime and ceftriaxone. Based on Christensen *et al*. (2011), listeriosis is commonly treated using antibiotics such as ampicillin or penicillin G with combination between an aminoglycoside such as gentamicin. Chloramphenicol which is derived from Streptomyces venezuelae work against both grams positive and negative (Vining and Stuttard, 1995). However, Chloramphenicol is no longer effective to treat listeria meningitis and not recommended in current treatment. When data were analyzed based on the samples source of origin, 30% of the isolates from water and 70% of the isolates from animals were found to be resistant to at least one antibiotic. Interestingly, four (40%) *L. monocytogenes* isolates from different sample sources showed similar resistance characteristics which might be due to contact between animal and water. In general, evident differences in antibiotic resistance patterns were observed among isolates tested.

According to Krumperman (1983), the multiple antibiotic resistances (MAR) can be used to determine the resistance of isolates studied. MAR indices for the *L. monocytogenes* isolates were observed to be in the range between 0.1 and 0.4. There were seven isolates (70%) with MAR index more than 0.2. On the other hand, three isolates showed MAR index reading lower than 0.2 (30%). Index value more than 0.2 indicates that the isolates manage to recover from high risk source while value lower than 0.2 indicates vice versa. High risk source can be defined as the source which contains risk that can be considered as significant and the consequence of this risk can hold possibility of having disease outbreak. Nevertheless, Matang Wildlife is considered as protected area and no antibiotic usage has been applied to the wildlife. As observed from antibiotic analysis, water samples have range of 0.3 to 0.4; which can be considered as alarming. MAR index suggested that human activities may affect the resistant properties of bacteria compared to natural habitats. Therefore, precaution steps need to be taken especially for any possible contacts in order to prevent or treat any potential health problems to manifest.

Conclusion

Overall, this study provides overview of distribution and presence of *Listeria* spp., specifically *L. monocytogenes* in animals and water samples from Kubah National Park. This finding shows that external factor such as different type of carrier, habitats and adaptation to living host may affect the dispersion of these bacteria in nature. The existence of multiresistant *Listeria* spp. strain in the nature has raised concern about the possible transmission between wildlife and water to human either directly or through indirect contact which might include chances of emergence of zoonotic disease from nature.

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