Molecular detection of *Leptospira* sp. in cattle and goats in Kelantan, Malaysia after a massive flood using multiplex polymerase chain reaction

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Abstract. Flood is a potential driver in spreading waterborne diseases including leptospirosis, which is a zoonotic disease caused by pathogenic bacteria of the genus *Leptospira*. In the case of leptospirosis, cattle and goats can be incidental hosts and potential carriers of leptospirosis. Traditionally, serology such as microscopic agglutination test (MAT) and isolation of the organisms have been commonly used as the diagnostic approaches in diagnosing leptospirosis. However, nowadays, various molecular techniques have been developed for specific detection of *Leptospira* sp. such as, polymerase chain reaction (PCR), which is sensitive, specific and rapid in detecting the species. This study detected *Leptospira* sp. directly from the blood and urine of the animals such as, cattle, goats and sheep in Kelantan after a massive flood by using multiplex PCR (mPCR). From the results collected in the study, four blood samples (0.63%; 4/635) were found to be positive with *Leptospira* sp. and one urine sample (3.23%; 1/31) was detected as positive with *Leptospira* sp. The blood and urine samples that were detected to be positive with *Leptospira* sp. were collected from cattle and goats exposed to the flood. However, no *Leptospira* sp. was detected from the sheep in this study. Multiplex PCR (mPCR) was successfully used to detect the presence of *Leptospira* sp. in animals. Apart from that, it is also suggested that flood has a significant role in transmitting the disease to animals.

INTRODUCTION

According to Levett in 2004, leptospirosis has been perceived as an infectious disease that has re-emerged in humans and animals. In addition to that, Cheema *et al.*, in their research conducted in 2007 have suggested that leptospirosis is a disease that has a significant impact on economy, as it affects domestic animals namely cattle, goats, and sheep. Leptospirosis in animals was identified by the decrease of milk produced, death, abortion, infertility, and stillbirth, but, it is mostly subclinical in cattle, goats and sheep (Haake *et al.*, 1998). Although leptospirosis is spreading globally (Hoseinpur *et al.*, 2015), it is commonly found in tropical and sub-tropical regions (Lau *et al.*, 2010). In addition, incidence and outbreak of leptospirosis were seen to be high after heavy rainfall and flooding (Huang *et al.*, 2016; Brown & Murray, 2013). Hence, the risk of leptospirosis in both humans and animals was exposed to flood waters. Several leptospirosis outbreaks were found in humans, as reported in Eco-Challenge and Beaufort in Sabah, Malaysia (Sejvar *et al.*, 2003; Koay *et al.*, 2004). Recently, a number of flash floods and the presence of leptospirosis during the floods have been reported in several places in Malaysia, Bangkok in Thailand and Jakarta in Indonesia (Bahaman, 2016). In addition to that, a massive flood in Kelantan, Malaysia was reported to increase in number of leptospirosis incidence in humans (Zainudin, 2015). According to a

report by Wan-Yussof (2015), the flood affected eight out of ten districts in Kelantan, a state of Malaysia, and most of the animals in those districts were exposed to the flood.

Leptospirosis is difficult to diagnose, especially in infected animals without showing clinical signs and these animals may excrete organisms. These animals can lead to leptospiral infection in humans, while humans are known as 'death-end' hosts for leptospirosis (Kariv *et al.*, 2001). Therefore, different laboratory tests are required to diagnose leptospirosis (Bharti *et al.*, 2003). Microscopic agglutination test is the most common diagnostic test, but live antigen must be used (Plank & Dean, 2000). According to Musso and Scola in 2013, although the technique of isolating *Leptospira* sp. consumes a lot of time, apart from it being a laborious work, this technique can be regarded as the ultimate technique in diagnosing leptospirosis. In the present days, molecular methods such as PCR are widely used for detection of leptospiral DNA in samples that were obtained from animals and humans (Ahmed *et al.*, 2012).

The use of PCR is currently achieving recognition in the detection of micro-organisms involving *Leptospira* and in the diagnosis of many infectious diseases (Mullan & Panwala, 2016). In addition to that, Ahmad *et al.*, as according to their research in 2012 has suggested that this technique proposed substantial advantages over the isolation and MAT techniques, as it produces less impurity, fast turn-around time, and refute the need to maintain hyperimmune antisera for the identification of cultures. In this study, multiplex PCR (mPCR) was used to detect *Leptospira* sp. in animals after a massive flood. Two important genes of *Leptospira* sp. were targeted by the mPCR, which are 16S rRNA and LipL32. The 16S rRNA gene was preserved throughout the bacterial domain, while, LipL32 gene is highly specific to pathogenic *Leptospira* sp. (Haake *et al.*, 2000). These two genes have been commonly used to detect and differentiate pathogenic and non-pathogenic *Leptospira* sp. (Gokmen *et al.*, 2016). However in this study, all the animals were tested to be healthy, and the detection of *Leptospira* sp.

is required in order to confirm that the animals are antigen free or infected with or without exposing to the flood.

MATERIALS AND METHODS

Samples collection
In the state of Kelantan, the collected samples were managed by the Department of Veterinary Services (DVS), which complied with their standard requirements and fixed procedural instructions. DVS is a governmental organisation responsible for health of the animals, productivity and welfare of animals in Malaysia.

Blood samples
A total of 635 blood samples were collected from 280 cattle, 239 goats and 116 sheep in Kelantan after a massive flood via venipuncture. From the total, 585 animals were exposed to the flood, while 50 other animals were not exposed to the flood.

Urine samples
A total of 31 urine samples were collected from 21 cattle, 4 goats and 6 sheep in Kelantan after a massive flood via spontaneous micturition. From the total, 27 animals were exposed to the flood, while 4 other animals were not exposed to the flood.

Positive control
Leptospira borgpetersenii serovar *hardjobovis* strain 117123 was grown for 7 days in liquid medium of Eliinghausen, McCullough, Johnson and Harris (EMJH) at 30°C.

DNA Extraction from Blood Samples, Urine Samples and Positive Control
DNA extraction from the samples and positive control of *Leptospira borgpetersenii* serovar *hardjobovis* strain 117123 were performed by using DNeasy® Blood & Tissue Kit (QIAGEN, Germany). The procedures were followed as instructed by the manufacturer and the end products (DNA template) were inspected using 1.5% agarose gel for purity.

Table 1. Primers for mPCR amplification of *Leptospira* sp.

Primer	Sequence 5' – 3'	Length (bp)	Amplicon size (bp)
16S rRNA – forward	GAACTGAGACACGGTCCAT	19	541
16S rRNA – reverse	GCCTCAGCGTCAGTTTTAGG	20	541
LipL32 – forward	ATCTCCGTTGCACTCTTTGC	20	756
LipL32 – reverse	ACCATCATCATCATCGTCCA	20	756

Polymerase Chain Reaction (PCR) Primers

Based on previous studies, two sets of primers were selected in which targeted 16S rRNA gene and LipL32 gene with amplicon size 541 base pair (bp) and 756 bp were targeted respectively (Varni *et al.*, 2014; Boonsilp *et al.*, 2013; Ahmed *et al.*, 2011) as shown in Table 1.

Polymerase Chain Reaction (PCR) Amplification

A total of 25.00µl reaction volume has been optimised as follows: 12.50µl TopTaq Master Mix 2x (QIAGEN, Germany), 1.50µl for each of all four primers mentioned above, 2.50µl RNase-free water (QIAGEN, Germany) and 5.00µl DNA template. Amplification was optimised and performed in a Mastercycler Pro S (Eppendorf, Germany) with initial denaturation of 94°C for 3 minutes followed by 30 cycles of denaturation at 94°C for 30 seconds, primer annealing at 63°C for 30 seconds, and DNA extension at 72°C for 30 seconds before the final extension step at 72°C for 5 minutes to complete the synthesis of all strands.

Detection of Amplified DNA's

The amplicons were analysed in tris-borate-EDTA (TBE) buffer at 80 volts for 1.5 hours by using 1.5% gel electrophoresis. The gel was pre-stained with SYBR® Safe DNA gel stain (Invitrogen™, North America) and examined using Gel Documentation (AlphaImager™, USA). The amplicons were identified by theirs band sizes.

RESULTS

Four blood samples (0.63%; 4/635; 95% Confidence Interval (CI):0. 00%-1.20%) were tested positive for *Leptospira* sp., which comprised of two pathogenic *Leptospira* sp. and two non-pathogenic *Leptospira* sp. Figure 1 presents the direct detection of *Leptospira* sp. in blood from two cattle and two goats by using mPCR. Two bands were observed at 541bp and 756bp in positive control *L. hardjobovis* and in the blood sample from two cattle. The results obtained have indicated that the two cattle were infected by pathogenic *Leptospira* sp. One band was observed at 541bp in the blood samples collected from two goats, which specified that the respective goats were infected by non-pathogenic *Leptopsira* sp.

Figure 2 presents the direct detection of *Leptospira* sp. in urine from cattle by using mPCR. One urine sample (3.23%; 1/31; 95% CI: 0.00%-9.40%) was tested positive for *Leptospira* sp. in which two bands were observed at 541bp and 756bp in positive control *L. hardjobovis*. This result denotes that these cattle were infected by pathogenic *Leptospira* sp.

All the samples were tested positive for *Leptospira* sp. by using mPCR in which all samples were extracted from animals that have been exposed to the flood.
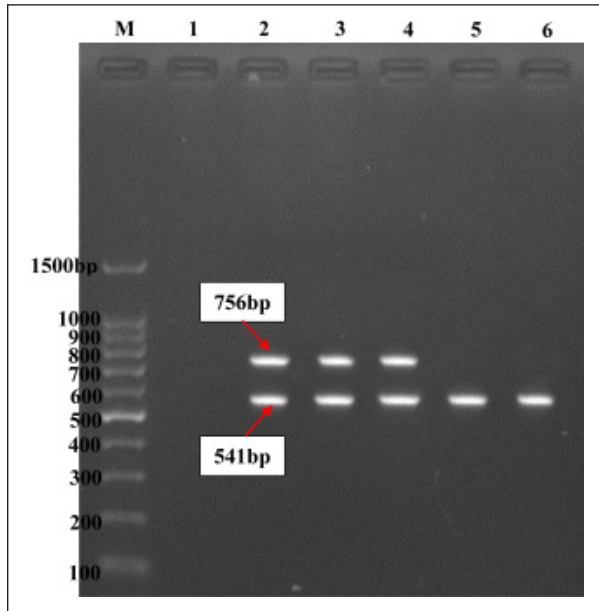
Figure 1. Direct detection of *Leptospira* sp. in blood from cattle and goat using mPCR. (M) 100bp DNA ladder; (3)-(6) blood samples; (1) negative control (RNase-free water); (2) positive control *L. hardjobovis*; (3)-(4) *Leptospira* sp. in blood of cattle; (5)-(6) *Leptospira* sp. in blood of goats.
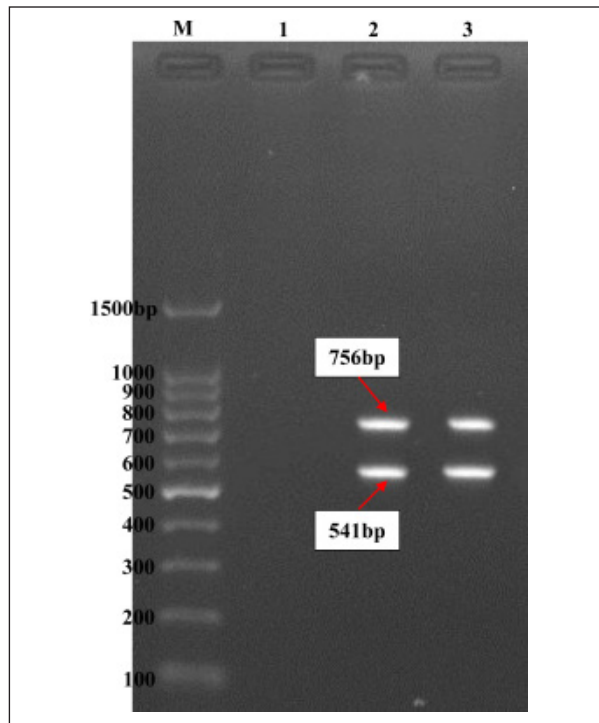

Figure 2: Direct detection of *Leptospira* sp. in urine from cattle using mPCR. (M) 100bp DNA ladder; (1) negative control (RNase-free water); (2) positive control *L. hardjobovis*; (3) *Leptospira* sp. in urine of cattle.

DISCUSSION

Four blood samples (0.63%; 4/635; 95% CI: 0.00%-1.20%) were tested positive for *Leptospira* sp. by using mPCR in this study. In the previous study conducted by Doosti *et al.* (2012), the results of the blood samples were reported high, 14.61% (19/130; 95% CI: 8.50%-20.70%) due to the target population of only one animal species (camel) compared to in this study, which targeted three animal species (cattle, goat and sheep). One urine sample (3.23%; 1/31; 95% CI: 0.00%-9.40%) was tested positive for *Leptospira* sp. by using mPCR in this study. Moreover, urine sample from the previous study conducted by Cetinkaya *et al.* (2000) also showed a low result of 4.02% (19/473; 95% CI: 2.20%-5.80%). However, studies by Baquero *et al.* (2010) and Shafighi *et al.* (2014) detected high results of 13.53% (74/547; 95% CI: 10.70%-16.40%) and 43.00% (42/98; 95% CI: 33.10%-52.70%) respectively due to the target population of only cattle and the number of urine samples collected in previous studies was higher compared to in this study. Furthermore, in this study, urine samples were collected via spontaneous micturition since this method was the accessible way to collect midstream of urine. In addition, collecting urine via spontaneous micturition provides less stress to the animals and to ensure the purity of the samples. Hence, it benefited from preventing the occurrence of contamination and mixing in the urine samples when using the urinary catheter instead of free catch.

Detection of *Leptospira* sp. in the blood and the urine suggested that the animal was in leptospiraemia and leptospiruria phase respectively. Despite assuming all the animals were tested healthy, they could hypothetically yield the organisms, thus contaminating the environment or directly infecting other animals and humans. Infected animals are potential source of leptospiral infections to other animals and may be imposed to public health concerns. In this study, the infected animals were found in cattle and goats, but no leptospiral infection detected in sheep. Cattle is the serovar *L. hardjobovis* conservation host and can be infected by a wide range of serovars without showing clinical signs (Zuerner *et al.*, 2011; Ellis, 2015). The infected cattle in particular can develop leptospiral infection after flood, as these animals were exposed to the flood. Relatively to the cattle, sheep have been considered resilient to leptospiral infection. The leptospiral infection in goats was significantly lesser than the cattle and the sheep (Ellis, 2015). However, in this study, non-pathogenic *Leptospira* sp. was detected in two goats that may have contacted with the contaminated environment. The non-pathogenic *Leptospira* sp. can be either saprophytic or intermediate species. Saprophytic and intermediate *Leptospira* sp. is commonly found in the environment (Pui *et al.*, 2017; Benacer *et al.*, 2013). Apart from that, it is also suggested that flood has a significant role in the distribution of the *Leptospira* sp. throughout the affected areas.

The effectiveness of mPCR in the detection of *Leptospira* sp. has been proven in many studies. This method had been implemented to detect *Leptospira* sp. in water in Rio de Janeiro, Brazil (Vital-Brazil *et al.*, 2010) and had detected, 3.00% (3/100) of *Leptospira* sp. in water by mPCR. This method also has been optimised and can be applied as rapid diagnosis of leptospirosis (Ahmed *et al.*, 2012). In a recent study, the mPCR was used to detect *Leptospira* sp. in dogs (Khor *et al.*, 2016). In this study, the detection of pathogenic *Leptospira* sp., along with the process of distinguishing pathogenic and non-pathogenic *Leptospira* sp., was done rapidly as this study implemented the method of mPCR, which held extra advantage over conventional PCR. In the method of mPCR, two sets of primers were used concurrently, which contributed to the increase of specific amplification (541bp and/or 756bp).

CONCLUSION

Based on history and locality, all of the animals that were positively tested with *Leptospira* sp. by using mPCR have experienced flood. To conclude, flood could be the major risk factor in transmitting and distributing leptospirosis the disease to the

animals. In addition to that, mPCR was shown to be a promising adjunct to detect *Leptospira* sp. particularly in animals along with its ability to differentiate between pathogenic and non-pathogenic *Leptospira* species.

Conflict of interest

All authors report no conflict of interest relevant to this article.

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