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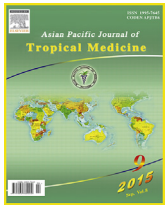

Original research http://dx.doi.org/10.1016/j.apjtm.2015.07.029

Genetic variation of *Leptospira* isolated from rats catched in Yogyakarta Indonesia

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ARTICLE INFO

Article history:
Received 15 Jun 2015
Received in revised form 20 Jul 2015
Accepted 20 Jul 2015
Available online 29 July 2015

Keywords:
Pathogenic *Leptospira*
16S rRNA
Rats
Genetic variation

ABSTRACT

Objective: To detect genetic variations among pathogenic *Leptospira* isolated from rats using 16S rRNA gen as chronometer.
Methods: This is an observational study with cross sectional design. Rats samples were taken in Yogyakarta Special Region of Indonesia. *Leptospira* in the rats was detected by two methods *i.e.* real time PCR (qPCR) by using primers correspond to16S rRNA gene of *Leptospira*, and standard PCR by using different set of primer correspond to the 16S rRNA gene of *Leptospira*. The standard PCR amplicon then subjected for DNA sequencing. Analysis genetic variation was performed using MEGA 6.2. Software.
Results: There were 99 DNA samples from rats included in this study. Detection of *Leptospira* by using qPCR revealed 25 samples positive for pathogenic *Leptospira*, while only 6 samples were able to be detected using standard PCR. The new primer set correspond to 16S rRNA gene was able to detect specifically pathogenic *Leptospira* in the rats. Sequencing analysis of 6 PCR amplicons showed that the *Leptospira* which infect the rats catched in Yogyakarta genetically close related with pathogenic *Leptospira* which were isolated from human, animal, rodents, and environment.
Conclusions: It can be considered that rats are the most important vector and reservoir of *Leptospira*.

1. Introduction

Leptospirosis is an infectious disease caused by a direct zoonotic *Leptospira* [1–3]. *Leptospira* is transmitted through direct contact with urine and body fluids of an infected animal, or through indirect contact with water and soil environments contaminated with *Leptospira* [4–6]. The urine of infected animals and rodents can pollute the environment such as soil, water, and vegetation [7,8]. *Leptospira* may reside in the kidney of reservoir and when its passing urine, it may contaminate the environment [9–11].

Rats are reservoir with chronic leptospirosis infection. The infection is transmitted from rats to another through direct contact at a young age or older [12]. There were reported that some *Leptospira* species has specific susceptibility to particular rodents. *Leptospira icterohaemorrhagiae* species commonly infect *Bandicota indica* and *Rattus diardii*, whereas *Leptospira ballum* infect *Mus musculus*. *Rattus norvegicus*, *Rattus diardii*, *Rattus exulans* and *Suncus murinus* allegedly having an important role in the incidence of leptospirosis [13].

Leptospira has three types of ribosomal RNA *i.e.* 5S, 16S, and 23S rRNA. Among the three, 16S rRNA gene is the most frequently used molecular markers [14–18]. The 16S rRNA is building block of the 30S subunit, which is important for translation. The 16S rRNA gene is the most commonly used and accepted to study the genetic relationship among bacteria [19].

The 16S rRNA gene of *Leptospira* can be detected using standard polymerase chain reaction (PCR). Detection may be

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Peer review under responsibility of Hainan Medical College.

done by using a primer set, targeting the 16S rRNA gene [14–18]. It was also reported that *Leptospira* could be detected by using real time-PCR (qPCR), which was employed primer set and probe correspond to 16S rRNA gene [20,21].

There is limited information about genetics of *Leptospira* which are circulating in Indonesia. Our work gives new information concerning the genetics variation in *Leptospira* which was isolated from rats. It is important also to understand its relationship with previously reported *Leptospira* which were isolated from human, rodents, animal and environment.

2. Materials and methods

2.1. DNA samples

Rats were catched at Bantul District, Yogyakarta, Indonesia. Rats were sacrificed and kidney tissue was obtained. DNA was isolated by using PCR High Pure Template Preparation Kit (Roche®). DNA samples were stored at −80 °C before subsequent analysis. This work has been approved by Medical and Health Research Ethics Committee of Faculty of Medicine, Universitas Gadjah Mada.

2.2. PCR amplification

DNA was amplified using Dream *Taq*™ Green PCR Master Mix (2×). The PCR reaction was done according to the manufacturer protocol. Reaction mixture was consist of green buffer, dATP, dCTP, dGTP, dTTP, $MgCl_2$, distilled water, and mineral oil (Promega®). One set of primer correspond to 16S rRNA gene were employed. Primer sequences are 508F (5′-GGCGGACATGTAAGT CAGGT-3′) and 1217R (5′-GGGACCGGATTTTTGAGATT-3′). The PCR amplification generated 709 bp PCR products. Amplification products were mounted and run on 2% agarose gel.

2.3. DNA sequence analysis

Amplicon of PCR product then subjected to be sequenced by using direct sequencing method. Multiple alignment analysis was done to analyze the genetic diversity between our results and 16S rRNA gene sequences deposited in the gene bank. Phylogenetic analysis was performed by using MEGA 6.2 software.

3. Results

There were 99 rats involved in this study. DNA samples obtained from kidney tissue of rats. *Leptospira* detection by using qPCR was showed positive in 25 samples [21]. However, only 6 samples were confirmed as pathogenic *Leptospira* by using standard PCR (Figure 1). Cohen's Kappa test between the two methods showed value of 0.32 ($P < 0.01$). Detection of *Leptospira* using qPCR and standard PCR, which was targeted 16S rRNA gene, showed weak agreement.

DNA sequencing correspond to the 16S rRNA gene of 6 *Leptospira* were obtained. By using BLAST analysis, it was noticed that all 6 *Leptospira* isolates are closely similar with 16S rRNA of pathogenic *Leptospira*. This means that the primer set for standard PCR is fully specific to the pathogenic *Leptospira*, though less sensitive than qPCR methods.

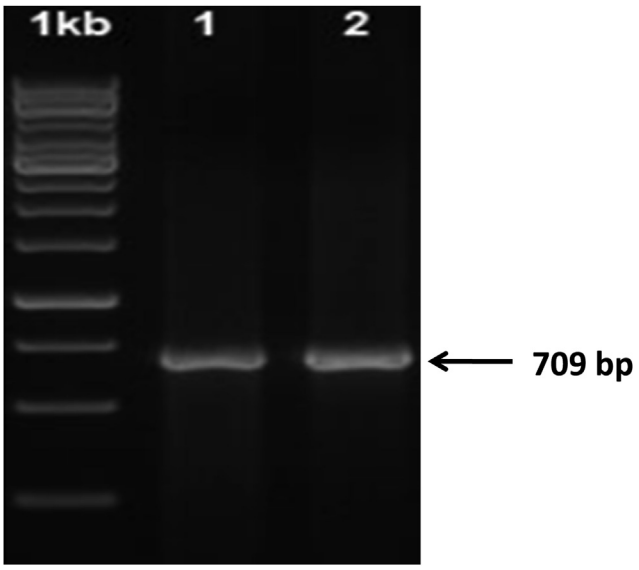

Figure 1. Electrophoresis of standard PCR product on 2% agarose gel. Marker is a 1 kb ladder, Lane 2 and 3 are representative of PCR products. The PCR amplification produced 709 bp length PCR products.

The phylogenetic tree of 6 DNA sequence of 16S rRNA showed that the *Leptospira* isolated from rats catched in Bantul district, Yogyakarta has close relationship with pathogenic *Leptospira* reported in gene bank, which is isolated from various host and environment. *Leptospira* isolated from rats: CP001221,

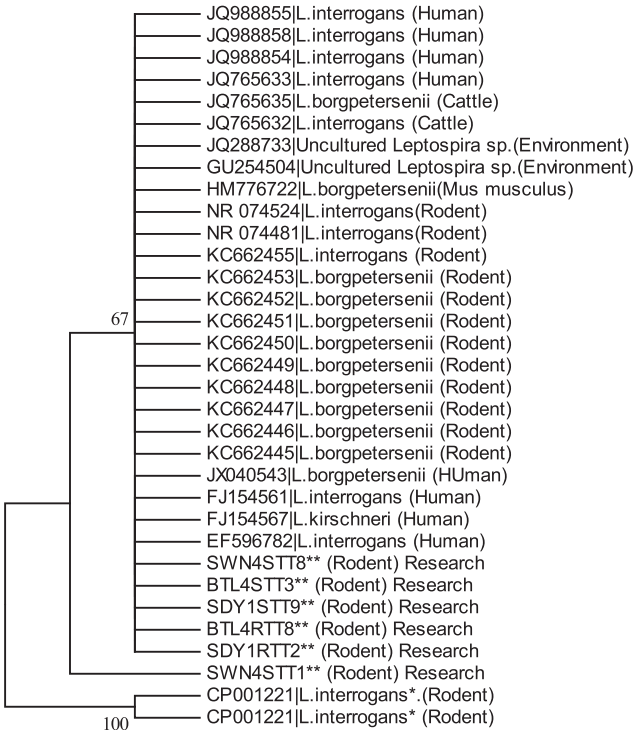

Figure 2. *Leptospira* phylogenetic tree based on 16S rRNA gene sequences.

Phylogenetic tree were drawn by using Neighbor Joining Methods with 1 000 bootstrap replication. * is denoted for reference sequence. ** is denoted for current research isolates. The 16S rRNA gene sequences were extracted from gene bank to represent the origin of *Leptospira* isolates *i.e.* from human (EF596782, FJ154561, FJ154567, JQ765633, JQ988854, JQ988855, JQ988858, JX040543); from rats (CP001221, KC662445, KC662446, KC662447, KC662448, KC662449, KC662450, KC662451, KC662452, KC662453, KC662455, NR_074524, NR_074481); from cattle (JQ765632, JQ765635); from *Mus muculus* (HM776722); and from environment (GU254504, JQ288733).

KC662445, KC662446, KC662447, KC662448, KC662449, KC662450, KC662451, KC662452, KC662453, KC662455, NR_074524, and NR_074481. *Leptospira* isolated from human: EF596782, FJ154561, FJ154567, JQ765633, JQ988854, JQ988855, JQ988858, and JX040543. *Leptospira* isolated from Cow: JQ765632 and JQ765635. *Leptospira* isolated from *Mus muculus* HM776722, and environment (GU254504, JQ288733) (Figure 2).

4. Discussion

Leptospira detection from rat's tissues samples is commonly done by using standard PCR and qPCR methods. Our result showed that qPCR is more sensitive compare to the standard PCR. Previous report by Desvars *et al*., showed similar result in which detection by using qPCR successfully detect 29.8% of the sample, whereas by using standard PCR was able to detect 14.18% of the samples [22].

Rats as reservoir, plays an important role for *Leptospira* infection to humans. Although dogs, pigs, cows, horses, cats, rabbits, bats, squirrels, raccoons can also serve as a reservoir. However, rats are the most prominent reservoir among others. Rats are the most potential vector and reservoir for *Leptospira* transmission to human being [21]. Furthermore, Barcellos *et al*. reported that the distribution of leptospirosis in human were concentrated in areas where rats were highly populated, as well as areas with unfavorable trash management and poor sanitary conditions [23].

Certain *Leptospira* serovar has been associated with particular animals such as *Leptospira icterohaemorrhagiae* and *Leptospira copenhageni* were associated with rats, *Leptospira canicola* with dogs, *Leptospira pomona* with pigs, *Leptospira hardjo* with cows, and *Leptospira gryppotyphosa* with voles [8,24]. However, it is interesting that our result showed pathogenic *Leptospira* isolated from rats have close relationship with *Leptospira* isolated from other host and environment [25–30]. This means that the *Leptospira* circulating in the rats, animals, humans, and environment were genetically similar. There is no evidence that particular genetics constitution has tropism for specific host and reservoir. In conclusion, we should be considered rats are the most important vector and reservoir of *Leptospira*. Further studies are needed for detail elaboration of transmission pathway from rats to other animals, environment, and human.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

This paper is part of the dissertation of HS.

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