Identification of Pathogenic and Saprophytic Leptospira spp from the Rice Fields of Tonekabon Township Using PCR Technique

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Abstract

Leptospirosis is one of the most significant common diseases between human and livestock created by the various species of Pathogenic Leptospira. Pathogenic Leptospiras are shed in the environment through urine of the infected animals, including rodents or domestic animals (Dog, Cow, Sheep and Pig) and cause disease in human through direct and indirect contact with contaminated environment. This disease is to be construed as a native disease in the Northern provinces of Iran (Mazandaran and Gilan) and risk of infection with the strains of pathogen is high. 115 samples of water (67), soil (36) and feces of the rodents (12) were collected from the rice fields of suburbs of Tonekabon township situated in the Northern part of Iran. In order to identified sample was inoculated to liquid EMJH medium and incubated for 1 month at 30°C. After incubation time, PCR technique was used to identify the saprophytic and pathogenic species of this bacterium. It was in such a way that, after initial enrichment, DNA extraction of bacterium was carried out by DNA extraction kit and PCR technique was executed using the specific primers. Instead, 92 strains of Leptospira were identified using the PCR technique, out of which 54 species
were diagnosed as saprophytic ones (46.95%) and 38 species as pathogenic ones (33.04%). PCR technique can be used to identify the *Leptospira* species in the water and soil samples. Due to usage of mentioned primers, this method is able to differentiate between the saprophytic and pathogenic *Leptispiras*.

**Keywords:** *Leptospira*, environmental samples, Tonekabon, rice farms Iran

**Introduction**

*Leptospiras* are gram negative, rod like and helical bacteria which move actively, and they are usually curved in one or two ends. They are very thin and 6-20 µm in length and 0.2-0.15 µm in width (Faine, 1982, 1994; Levett, 2001). Genus of *Leptospira* includes the pathogenic and nonpathogenic species (Magalhaes et al., 2010). Nonpathogenic *Leptospiras* are the native of surface waters and there is not a parasitic feature for them (Johnson & Harris, 1967). Reservoir of pathogenic leptospirosis is rodents and wild animals (Laurichesse et al., 2007; Aviat et al., 2009; Monahan et al., 2009). Transfer of pathogenic *Leptospiras* carries out when the direct relationship with infected mammals or contact with the water contaminated by urine of rodents occurs (Levett, 2001; Laurichesse et al., 2007). Agricultural lands, particularly rice and grains lands are ideal places for survival and reproduction of mice as the first carriers of the *Leptospira* (Natarajaseenivasan et al., 2002). Agricultural environment are contaminated constantly by urine of mice, and possibility of transfer and occurrence of Leptospirosis in the rice farms workers increases by them (Natarajaseenivasan & Ratnam., 1997).

At the end, it must be reminded that Leptospirosis, as a problem of the public health, is raised all around the world (World Health Organization, 2003), and Leptospirosis is the main factor of various clinical syndromes, including jaundice, renal failure (Muthusethupathy et al., 1995), myocarditis and atypical pneumonia in the native regions.

Today, culture Technique, serology methods (IFA, ELISA, MAT) and, also, molecular methods are used on the basis of PCR in order to diagnose *leptospira*. It is required to mention that usage of serologic methods depends on passage of, at least, 1 to 2 weeks and creation of the hemoral response in the patient and this time increase up to a few weeks regarding culture too. With regard to important of the untimely premature diagnosis of the disease's factor, it seems that only speed and high sensitivity of the PCR method for the above problem is to be effective. Thus, it was tried in this research that possibility of the untimely diagnosis of *leptospira* is to be provided by PCR technique (Brown et al., 1995; Guidugli et al., 2000; Wagenaar et al., 2000; Levett et al., 2001).

For this reason and on the basis of the available documents and witnesses regarding presence of the pathogenic and saprophytic *Leptospiras* in various geographical regions, this study was executed in order to identity the presence
Identification of pathogenic and saprophytic Leptospira spp and rate of frequency of them in water, soil and feces of rodents in the rice farms of Tonekabon Township located in the Northern part of Iran (Mazandaran province).

Materials and methods

Sample collection

In spring and summer of 2012, 67 water, 36 soil and 12 feces were collected from the rice farms of Northern part of Iran located in Tonekabon area. At first, some parts of agricultural land which had stagnant waters such as: streams, water channels, soil and inside of land water were referred, and after observance of safety points, including wearing of glove and boot, the samples were collected directly from rice farms by 50 ml sterile falcom tubes. Also, soil and feces samples were collected from agricultural lands and transferred to the sterile falcom tubes. Then, after 2 hours, the samples in the flask containing the dry ice were transferred to laboratory.

Preparation of samples to culture

Each one of the water samples into the falcom tube was mixed completely and transferred to 4 sterile short test tubes. Then, the tubes were centrifuged with 4000 rpm for 10 minutes. Surface contents of the tubes were extracted and poured away through a dish containing the savlon. Then, we took out the contents of a few tubes by sterile syringe and passed them through a 0.45 µm filter and transferred them to the sterile 1.5 ml Micro tube. Then, we took out the contents from 1.5 ml microtube by another sterile syringe and inoculated against the liquid EMJH medium (Ellinghausen & McCullough, 1965; Johnson & Harris, 1967) after passage from 0.2 µm filter. For the soil and feces samples, the sample was first transformed into suspension with aid of buffer phosphate salin and incubated in 30°C with in 30 days.

PCR

DNA extraction from bacterium was executed according to instruction of manufacturer company (Kiagen-Germany). In order to confirm the DNA extraction, of sample in the range of 260 and 280 nm was studied by biophotometer system (Eppendrof-Germany) and remaining samples were placed in -20°C.

In order to identify the saprophytic and pathogenic Leptospira spp. the PCR molecular test was used. For this purpose, the specific primers (Magalhaes...
et al., 2010) were synthesized by the tag Copenhagen company of Denmark (Table 1).

Lep16srRNA primer was able to identify both the saprophytic and pathogenic species of the *Leptospira*. But, *LipL32* primer identified only the pathogenic species. The PCR technique was carried out to identify the saprophytic and pathogenic species with the final reaction volume of 25 µl. All reactions included 14.5 µl DNase free water (Cinagen-Iran), 1X PCR buffer (Cinagen-Iran), 1 µl of 10 pmol from each one of the primers, 1 µl of 10 mM dNTPs (Cinagen-Iran), 1 µl of 50 mM MgCl₂ (Cinagen-Iran), 0.2 µl Taq polymerase enzyme (Cinagen-Iran) and 5 µl of the sample DNA.

Thermal program of PCR apparatus for saprophytic and pathogenic species includes one denaturation cycle at 95°C for 5 min, 40 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 30 sec and extension at 72°C for 1 min, and a final extension at 72°C for 7 min.

**TABLE 1. The specific primers of the *Leptospira***

<table>
<thead>
<tr>
<th>Name of primer</th>
<th>Type</th>
<th>Sequence of primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>16S rRNA</td>
<td>Forward</td>
<td>5'GAACCTGAGACACGGTCCAT3'</td>
</tr>
<tr>
<td>16S rRNA</td>
<td>Reverse</td>
<td>5'GCCTCAGCGTCAGTTTTAAGG3'</td>
</tr>
<tr>
<td>LipL32</td>
<td>Forward</td>
<td>5'ATCTCCGTTGCACTCTTTGC3'</td>
</tr>
<tr>
<td>LipL32</td>
<td>Reverse</td>
<td>5'ACCATCATCATCATCGTCCA3'</td>
</tr>
</tbody>
</table>

**Electrophoresis**

In order to perform electrophoresis, 5 µl from the 100 bp DNA ladder (Fermentas-Russia) and each one of the samples, were transferred to 1.5% agarose gel. Then, voltage was regulated on 75 V and a 60 min considered. After passage of above time, the gel was transferred into the UV transilluminator apparatus (UV Dock England) so that the formed bands are to be observed.

**Results**

**Identification of the saprophytic species of *Leptospira* with the PCR technique**

Of 67 water samples and 36 soil samples, 35 and 16 strains of saprophyte *Leptospira* were identified, respectively. It is required to mention that, of 12 fecal samples, no saprophytic strain was recognized by the PCR technique. Totally, of 115 studied samples, 54 samples (46.95%) contained the saprophytic species (Table 2). Observation of band in the 430 bp region specified the saprophytic species of *Leptospira* (Figure 1).
Identification of pathogenic species of *Leptospira* with PCR technique

Of 67 water samples and 36 soil samples, 29 (43.28%) and 9 strains (25%) of pathogenic *Leptospira* were recognized. Meanwhile, of total 12 fecal samples, no pathogenic strain was discerned by the PCR technique. Totally, of 115 studied samples, 38 samples (33.04%) contained the pathogenic species (Table 2). Observation of band in the 474 bp region specified the pathogenic species of the *Leptospira* (Figure 2). By use of PCR technique and specific primers, LipL32 and Lep16srRNA, differentiation of the saprophytic species from pathogen of *Leptospira*.

**FIG 1.** Agarose gel (1.5%) analysis of a PCR test for environmental sample. Lane 1, 3, 5-8 species of *Leptospira*, Lane 9 *leptospira interrogans* as positive control, Lane 10 negative control and Lane M size marker 100 bp.
TABLE 2. Results of recognition of saprophytic and pathogenic species of *Leptospira* with PCR technique

<table>
<thead>
<tr>
<th>Type of Sample</th>
<th>saprophytic <em>Leptospira</em></th>
<th>pathogenic <em>Leptospira</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Samples</td>
<td>Positive</td>
</tr>
<tr>
<td>water</td>
<td>67</td>
<td>35</td>
</tr>
<tr>
<td>soil</td>
<td>36</td>
<td>19</td>
</tr>
<tr>
<td>feces</td>
<td>12</td>
<td>0</td>
</tr>
<tr>
<td>total</td>
<td>115</td>
<td>54</td>
</tr>
</tbody>
</table>

Discussion

Rice consists of most part of the feeding resource of Iranian northern people, and most of lands are used to culture the rice. As a result, occupation of most villagers is agriculture and stock breeding. Agricultural lands are an ideal habitat for the rodents and they are the main reservoir to transfer the pathogenic leptospiras to environment. Instead, these lands are considered to be a working environment for farmers because they are forced to be in contact with the rice.
farms for long times in order to do business and achieve economical prosperity and, some times, stock breeders set free their own live stocks in order to graze in the rice fields. Therefore, human and livestock are disposed to the risk of affliction with Leptospirosis constantly.

In this research, the agricultural lands of Tonekabon were sampled in order to isolate and identify the pathogenic and saprophytic leptospiras and determine rate of contamination of these regions which the obtained results were as following: Of total 67 water, 36 soil and 12 feces samples, 92 strains of leptospira were identified with the aid of PCR technique. Of this number, 54 specied were recognized to be saprophyte (46.95%) and 38 species were discerned to be pathogen (33.04%).

Studied aiming at this research have been carried out in the different countries. In 2010, a research was conducted by Ridzlan et al with aim of identification of pathogenic Leptospira from the selective medium in Kelantan and Terengganu of Malaysia. Totally, 145 soil and water samples were collected from National Center for Educational Services (NSTC) in Kelantan and Terengganu. The samples were transferred to the Semisolid culture medium (EMJH), incubated under the room temperature for 1 month and studied by the dark field microscope. Positive growth of samples of Leptospira was confirmed by 8-Azaguanine test, polymerase chain reaction (PCR) and Microscopic Aglotimation Test (MAT). 15 cultures showed the positive growth upon seeing under the dark field microscope, while only 20% of them were identified as the pathogenic species (Ridzlan et al., 2010)

In 2010, a research was carried out by Magalhaes et al., in the dirty precincts of Petropolis city, a part of Rio deJaneiro. Purpose of this research is to identify the Leptospira DNA in the environmental water samples obtained from the dirty places on the basis of multiplex PCR. Of 100 studied samples, 3 samples were positive multiplex PCR, 2 cases showed the saprophytic Leptospira and 1 case showed the pathogenic Leptospira. Although the discerned molecular task applied in our research was PCR and Magalhaes et al used the multiplex PCR technique, rate of prevalence of pathogenic and saprophytic Leptospiuras (33.04% and 46.95%, respectively) in our research has been very higher, but both two techniques applied in these researches are able to identify the Leptospira spp and differentiate the saprophytic and pathogenic Leptospiuras (Magalhaes et al., 2010).

With the aim of distribution of frequency of the pathogenic leptospiiras in 22 coastal river of the tropical region, a research was carried out using the quantitative PCR (qPCR) by viau and boehm. Statistical study to perceive the relationship of pathogenic leptospiiras with water, land and feces was performed by use of reagent markers of the microscopic resources. Results suggested that pathogenic Leptospira genomes were widespread in O'ahu coastal streams during the Hawai'ian rainy season (Viau & Boehm, 2011).
Conclusion

The conducted studies all around the world put emphasis very much on the survival of Leptospiras in the warm and humid geographical conditions. Since the North of Iran, Gilan and Mazandaran provinces, specially Tonekabon township in which cases of death as a result of Leptospirosis are observed clearly locate in the mild and humid region from viewpoint of geographical and climatic region and there are plentiful rainfalls in most seasons of year in the mentioned provinces, it can be said that it has provided an appropriate habitat for survival of Leptospiras because rainfalls of the seasonal rains has led to the soil shedding phenomenon.

References


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