Neglected leptospirosis in raccoons (Procyon lotor) in Indiana, USA

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Published online: 02 May 2014.

To cite this article: Ching Giap Tan, Guha Dharmarajan, James Beasley, Olin Rhodes Jr., George Moore, Ching Ching Wu & Tsang Long Lin (2014) Neglected leptospirosis in raccoons (Procyon lotor) in Indiana, USA, Veterinary Quarterly, 34:1, 1-10, DOI: 10.1080/01652176.2014.909960

To link to this article: http://dx.doi.org/10.1080/01652176.2014.909960

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Neglected leptospirosis in raccoons (*Procyon lotor*) in Indiana, USA

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(Received 19 August 2013; accepted 26 March 2014)

Background: Leptospirosis is a globally important zoonotic disease occurring clinically and subclinically in humans and animals.

Objectives: To determine whether raccoons in Indiana carried leptospires in their kidneys.

Animals and methods: Thirty-four raccoons were live-trapped from two forest patches in central Indiana. Following euthanasia, a portion of kidney (2 cm2) from each raccoon was homogenized and used for leptospiral culture. Leptospiral cultures were subjected to DNA extraction and various polymerase chain reaction (PCR) procedures reported previously. Serum sample from each raccoon was collected and antibody titers to leptospiral serovars were determined by microscopic agglutination test (MAT).

Results: All leptospiral cultures were positive for *Leptospira* by various PCR procedures. The PCR with the primers targeting the conservative region of LipL32 gene was the most sensitive PCR in the detection of pathogenic leptospires. The variable LipL32 PCR amplicons were sequenced and compared to the reference strains available in GenBank. Twelve kidney cultures had *Leptospira interrogans*, eight had *Leptospira kirschneri* and two had *Leptospira borgpetersenii*. They were predominantly Grippotyphosa serogroup. Anti-leptospire antibodies were detected in 16 out of 34 raccoons (47.1%) by MAT. There were titers ≥ 1:80 in 16 raccoons (47.1%) and titers ≥ 1:400 in 3 raccoons (8.8%). The highest leptospiral serovar-specific seroreactivity among 34 raccoons was *L. interrogans* Bratislava (38.2%) and *L. interrogans* Grippotyphosa (32.4%).

Conclusions: Raccoons in Indiana carry leptospiral organisms in kidneys and the leptospires are predominantly *L. interrogans* species and of the Grippotyphosa serogroup.

Clinical importance: The raccoons serve as reservoir hosts that expose sources to wildlife, livestock, pets and humans.

Keywords: raccoon; *Procyon lotor*; leptospira; kidney; public health

1. Introduction

Leptospirosis is a globally important zoonotic disease occurring clinically and subclinically in humans and animals (Levett 2001; Meites et al. 2004). Leptospires are capable of renal colonization in most infected animals by replicating and persisting in renal tubular epithelial cells even in the presence of serum-neutralizing antibodies (Greene et al. 2005). Leptospirosis is transmitted by direct or indirect contact with material, especially soil or water, that has been contaminated with urine from an infected animal (Faine et al. 1999). Leptospires are maintained in nature in several subclinical wild and domestic reservoir hosts that serve as exposure sources to wildlife, livestock, pets and humans.

Raccoons (*Procyon lotor*) are a widespread and common North American wildlife species and have been recommended as sentinels for wildlife and zoonotic disease investigations (Richard & Douglas 2005). Raccoons are one of the most ideal candidates for zoonotic disease surveillance studies because they can be hosts to several infectious pathogens such as canine distemper virus, feline parvovirus and *Leptospira interrogans* that are transmissible to domestic animals, other native wildlife and exotic zoo animals. Raccoon’s habitats tend to be higher near water and agricultural areas (Jones et al. 1983). Recent work has also demonstrated that there is much higher raccoon populations in urban than rural area (Espinoza 2005). More than 40 years ago, raccoons were found to carry leptospires in their kidney (Schnurrenberger et al. 1970), and antibodies against leptospires have been detected in the serum of raccoons in United States and Canada (Schnurrenberger et al. 1970; Mikaelian et al. 1997; Mitchell et al. 1999; Warshawsky et al. 2000; Jardine et al. 2010).

Raccoons in Indiana, USA, have been reported to be exposed to different *L. interrogans* serovars serologically with the presence of antibodies to leptospiral serovars Grippotyphosa, Autumnalis, Hardjo, Bratislava, Pomona and Icterohemorrhagiae (Raizman et al. 2009). Since serological tests may not accurately identify infective serovars, the present study was undertaken to isolate and identify leptospires from raccoons in Indiana.

2. Materials and methods

2.1. Sample collection and leptospiral isolation

Thirty-four raccoons were live-trapped from two forest patches in central Indiana. The animals were euthanized as

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soon as possible following capture by means of administering a pentobarbital overdose. A portion of kidney (2 cm²) from each raccoon was homogenized in 9 ml Ellinghausen McCullough Johnson Harris (EMJH) medium containing 100 μg/ml of 5-Fluorouracil (5FU) by using Seward Stomacher 80 Biomaster machine (Bohemia, NY, USA) and used for leptospiral culture. The samples were then serially diluted to 10 folds (10⁻¹ – 10⁻⁴) in EMJH containing 5FU and 1% bovine serum albumin, and incubated at 30 °C. The cultures were examined by dark field microscopy (DFM; ORTHOLUX®, Leitz GmbH, Wetzlar, Germany) weekly for the presence of thin slender curved spiral-shaped leptospiral organisms with hooked ends. One ml of each raccoon kidney culture with and without filtration through 0.22 and 0.45 μm was transferred to 9 ml of new EMJH media containing 5FU and/or antibiotic (bacitracin 50 μg/ml, polymixin B sulphate 5 units/ml, sulphamethofzone and trimetoprim) after one week of incubation. Serum samples were collected from individual raccoons and kept at −20 °C until use.

The protocol for using the raccoons in the present study was approved by the Purdue University Animal Care and Use Committee.

2.2. Leptospiral strains, culture conditions and DNA preparation

The leptospiral reference strains (L. interrogans serovar Autumnalis, L. interrogans serovar Bratislava, L. interrogans serovar Canicola, L. interrogans serovar Grippoyphosa, L. interrogans serovar Hardjo, L. interrogans serovar Copenhageni, L. interrogans serovar Pomona, Leptospira borgpetersenii serovar Hardjobovis, Leptospiira kirschneri serovar Grippotyphosa, Leptospira alexanderi serovar Mengla, Leptospira noguchii serovar Barbadensis, Leptospiira santarosai serovar Brasilensis, Leptospira weilii serovar Celledoni and Leptospira biflexa serovar Patoc) were obtained from National Veterinary Services Laboratories at Ames, IA, USA. The cultivation of the leptospires was in liquid EMJH medium at 30 °C (Ellinghausen & McCullough 1965).

Leptospiral reference strains were cultured in 10 ml EMJH for 7–10 days while kidney sample cultures were ended by 12 weeks. All cultures were centrifuged at 13,800 rpm for an hour and DNA was extracted using QIAamp DNAeasy kits (Qiagen, Germantown, MD, USA) from the resultant pellets. Briefly, the pellet was resuspended in 100 μl of phosphate buffered saline followed by 200 μl of a cell lysis buffer (buffer AL) and 20 μl of protease K, and incubated at 60 °C overnight. The Qiagen tissue kit was used according to the manufacturer’s instructions, with two washing steps and elution of DNA in 50 μl of the elution buffer (AE). Polymerase chain reaction (PCR) was performed on the same day on which the DNA extraction was completed.

2.3. Polymerase chain reaction for leptospiral detection

Ten sets of previously published primers for conventional PCR (Gravekamp et al. 1993; Kee et al. 1994; Léon et al. 2006; Merien et al. 2006; Scola et al. 2006; Kositannot et al. 2007; Djadid et al. 2009; Stoddard et al. 2009) used for the detection of leptospiros from the cultures are listed in Table 1. In addition, three sets of primers targeting the hypervariable regions of LipL32, gyrB and FlaB1 genes whose nucleotide sequences are available in GenBank were designed for conventional PCR (Table 1). The PCR amplification was performed with 25 μl of reaction mixture containing 5X PCR buffer, 25 mM MgCl₂, 100 mM deoxynucleoside triphosphates, 0.1 μM of each primer, 2.5 units Taq DNA polymerase (Promega, Madison, WI, USA) and PCR-grade water. The PCR conditions were set as follows: initial denaturation at 94 °C for 5 min; 40 cycles of 1 min at 94 °C (denaturation), 1 min at 50–60 °C according to the melting temperature (Tm) of the primer pair (annealing) and 1.5 min at 72 °C (extension); final extension was at 72 °C for 5 min. Amplification products were analyzed by electrophoresis on a 1.5% (weight/volume) agarose gel in 0.5X Tris Borate ethylenediaminetetraacetic acid (TBE) buffer at 90 V for 40 min.

2.4. Sequencing and sequence analysis for differentiation of leptospiral species

A nested PCR targeting hypervariable region of LipL32 gene was used for PCR amplification, direct sequencing and sequence analysis to differentiate leptospiral species. Briefly, DNA extracted from the kidney cultures and reference strains were subjected to the first round of PCR amplification with 10 pmol of each of the two primers (Table 1) amplifying the full-length LipL32 gene in a total volume of 25 μl with the following cycling parameters: denaturation at 94 °C for 5 min, followed by 40 cycles of denaturation at 94 °C for 1 min, annealing at 58 °C for 1 min, extension at 72 °C for 1.5 min and ending with a final extension at 72 °C for 5 min. An aliquot (2 μl) of the first PCR reaction mixture added to 23 μl of a new mastermix (Phusion, Keilara, Espoo, Finland) containing 10 pmol of the nested primers flanking the hypervariable region of LipL32 gene was subjected to the following cycling parameters: denaturation at 94 °C for 5 min, followed by 40 cycles of a ‘touchdown’ procedure starting with 30 cycles of denaturation at 94 °C for 1 min, annealing at 57 °C for 1 min and extension at 72 °C for 1.5 min, followed by 5 cycles with an annealing temperature of 56 °C for 1 min and continued with another 5 cycles with an annealing temperature of 55 °C for 1 min. A final extension step consisted of 5 min at 72 °C. Following amplification, the amplicons were also resolved in a 1.5% agarose gel in TBE buffer at 90 V for 40 min. The PCR products were purified using the gel DNA recovery kit (Zymoclean, Orange, CA, USA) and sequenced by the Purdue University Genomics Core facility. The nucleotide sequences were aligned in Bioedit using CLUSTAL W (http://www.mbio.ncsu.edu/bioedit/bioedit.html) and compared with each other and with those previously available in GenBank. Phylogenetic trees were generated from DNA sequences by the neighbor-joining method using the MEGA 4 software (http://www.megasoftware.net).
2.5. Leptospiral serogroup-specific PCR

All samples positive for leptospiral species and confirmed by direct sequencing were subjected to five leptospiral serogroup-specific PCRs as described previously (Cai et al. 2010). The PCR amplification was performed on 25 μl of reaction mixture containing 5X PCR buffer, 25 mM MgCl₂, 100 mM deoxynucleoside triphosphates, 0.1 μM of each primer (Table 1), 2.5 units Taq DNA polymerase (Promega, Madison, WI, USA) and PCR-grade water. The PCR cycling parameters’ set conditions...
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2.6. Microscopic agglutination test (MAT) for antibody to leptospires

The microscopic agglutination test (MAT) was performed using a battery of the reference strains of serovars Autumnalis (Akiyami A), Bratislava (Jez Bratislava), Canicola (Hond Utrecht IV), Grippotyphosa (Andaman), Hardjo (Hardjoprajitino), Copenhageni (M20) and Pomona.
(Pomona) for detection of anti-leptospiral antibodies in raccoon serum samples. Briefly, serially diluted serum samples (final dilution of serum in 1:40, 1:80, 1:100, 1:200, 1:400, 1:800 and 1:1600) were mixed with live leptospiral serovars followed by DFM, and the reciprocal of the highest dilution with 50% agglutination was determined as the antibody titer (Raizman et al. 2009). Results were recorded as seronegative if the titer was <1:80. For reporting, the serovar with the highest titer was recorded as the important serovar and was the only serovar reported for that raccoon, unless multiple serovars had equivalent high titers.

3. Results

3.1. Isolation of the leptospires
Twenty-nine out of 34 (85.3%) raccoons’ kidney cultures were observed to have thin slender curved spiral-shaped leptospiral organisms with hooked ends under DFM. All 29 raccoons’ kidney cultures were heavily contaminated with other bacteria and pure cultures could not be obtained.

3.2. Molecular detection of leptospires
All 34 raccoons’ kidney cultures including those negative for leptospires by DFM were positive for leptospires by various PCR procedures (Table 2). The PCR with the primers targeting the conservative region of LipL32 gene (Figure 1) was the most sensitive PCR in detecting pathogenic leptospires (23/34). The newly designed LipL32 primer sets targeting the hypervariable region of LipL32 (22/34) was more sensitive than gyrB (4/34) and FlaB1 (2/34) primer sets in leptospiral detection (Table 2).

3.3. Leptospiral species identification by sequencing
Twenty-two raccoons’ kidney cultures were positive for leptospires by PCR amplification with the primers flanking the hypervariable region of LipL32 gene (Figure 2).
The PCR amplicons were directly sequenced and the nucleotide sequences were compared to those of the reference strains available in GenBank. Twelve kidney cultures had *L. interrogans*, eight had *L. kirschneri* and two had *L. borgpetersenii* (Figure 3).

3.4. **Serogroups identification by PCR**

Nineteen leptospiral species identified above were revealed to contain three serogroups (Canicola, Grippotyphosa and Sejroe) by serogroup-specific PCR (Figure 4). There were 2 Canicola and 9 Grippotyphosa serogroups in 11 *L. interrogans* containing kidney cultures; 6 Grippotyphosa and 1 Sejroe in 7 *L. kirschneri* containing kidney cultures and 1 Grippotyphosa serogroup in 1 *L. borgpetersenii*.

3.5. **Serology**

Anti-leptospiral antibodies (reciprocal serum titers above or greater than 80) were detected in 16 out of 34 (47.1%) raccoons' serum by MAT (Table 3), three raccoons had titers ≥ 1:400 (8.8%). The highest leptospiral serovar-specific seroreactivity among 34 raccoons was *L. interrogans* Bratislava (38.2%) and *L. interrogans* Grippotyphosa (32.4%). One raccoon serum had a titer of 1:200 for *L. interrogans* Bratislava and that of 1:200 for *L. interrogans* Grippotyphosa.
4. Discussion

The protocol for leptospiral culture as described in the World Health Organization (WHO) guidelines involves a five-tube dilution procedure. No pure culture could be obtained by five-tube dilution procedure from the present study although raccoons’ kidney cultures were observed to have thin slender curved spiral-shaped leptospiral organisms with hooked ends under DFM. All cultures were heavily contaminated with bacteria. Attempts to get rid of bacterial contamination from the cultures by filtration of sample cultures using syringe filter (0.22 and 0.45 μm) by force or using a surfactant-free cellulose acetate filter device by gravity force after one week of incubation were unsuccessful. In addition, selective culture media containing 5 FU with a combination of bacitracin 50 μg/ml, polymixin B sulphate 5 units/ml, sulphanethoxazole and trimethoprim were used to prevent bacterial contamination but pure cultures were still unable to be obtained. However, those antibiotics do not have an effect on the growth of leptospiral reference strains.

The gold standard for the detection of the leptospires is by culture but this often requires considerable skill and extended periods of incubation. Besides that, the low sensitivity in culturing leptospires is largely due to the fastidious growth of the organism in the artificial media and the contamination that may be present in the culture. Thus, the utilization of PCRs for the detection of leptospires in the samples is warranted. Various PCRs that targeted leptospiral different genes have been developed in detecting leptospires for a wide range of clinical samples such as blood, serum, aqueous humor, cerebrospinal fluid, aborted fetus, semen, kidney, urine and water in the environment (Fearnley et al. 2008). However, only few PCRs were able to amplify leptospiral DNA from human and veterinary clinical samples and only two methods were extensively subjected to clinical evaluation (Brown et al. 1995; Merien et al. 1995). There were variations in the sensitivity and specificity in detecting leptospiral organisms by various PCR methods (Wagenaar et al. 2000). In addition, some PCR methods exhibited false positives in detecting pathogenic leptospires in clinical samples which cross-reacted with saprophytic leptospires (Merien et al. 1992; Wagenaar et al. 1994; Harkin et al. 2003). Selection of PCR methods is highly critical for the detection of
leptospires in various samples. It has been shown in the present study that the result of leptosomal detection by PCR cannot be totally relied on only one PCR method.

LipL32 gene codes for the major outer membrane lipoprotein of Leptospira and also acts as one of the virulence factors (Yang et al. 2002). The nucleotide sequences of LipL32 are highly conserved among the pathogenic leptospires but absent in saprophytic leptospires and other bacterial species. Thus, LipL32 is highly specific for the pathogenic leptospires. The hypervariable region of the LipL32 gene is suitable for differentiation of leptosporial species. There were 22 raccoons (67.1%) positive for the pathogenic leptospires. The hypervariable region sequences of LipL32 gene. L. interrogans, L. kirschneri and L. borgpetersenii were identified in raccoons in Indiana. Such findings were similar to previous findings that raccoons act as maintaining host for these leptosporial species in North America (Galton 1959; Alexander 1960; Carbrey et al. 1963; Martin et al. 1967; Schnurrenberger et al. 1970; Falk 1985; Vinetz et al. 2005). The raccoons in the present study were trapped around the agricultural and livestock area, suggesting that there may be a high prevalence of leptospirosis in central Indiana animal population.

The relationship between the maintenance host and serovar is often associated with the efficiency of transmission, high seroprevalence and asymptomatic carriage of leptospirosis. There is evidence that specific molecular interaction between the host and the specific serovar occurs to allow the specific pairing of the specific mammal serovar (Nally et al. 2005). Twenty-five pathogenic leptosomal serovars have been isolated from wildlife, namely raccoons in the United States, consisting of serogroups Grippotyphosa, Pomona, Autumnalis, Bratislava, Ballum, Hebdomadis and Mini (Michigan DNR... 2011). Serogroups Canicola, Grippotyphosa and Sejroe were found in raccoons in Indiana in the present study; serogroup Grippotyphosa was predominant. One study reported previously has also shown that the raccoons act as the natural reservoir for L. interrogans, especially serovar Grippotyphosa (Shotts et al. 1975; Mitchell et al. 1999). Therefore, a large sampling of raccoons in Indiana for the surveillance of leptospirosis around the residential and livestock area may reveal leptosporial serovars involved in zoonotic or public health concern.

One raccoon might have dual exposure or sequential infection to L. interrogans serovar Bratislava and L. interrogans serovar Grippotyphosa in the present study. Because serogroup-specific PCR for serogroup Australis has not been available for use, correlation of the findings between serology and serogroup-specific PCR is not possible.

The overall seroprevalence of the leptospirosis infection in raccoons in this study was 46.1%, which is generally comparable to the other studies in North America such as 50% in Maryland (Alexander et al. 1972), 58.8% in Georgia (Shotts et al. 1975), 48% in Illinois (Mitchell et al. 1999), 45% in Illinois (Hungerford et al. 2000), 36% in Connecticut (Richardson & Gauthier 2003), 11% in Nebraska (Bischof & Rogers 2005), 19.1% in Washington (Davis et al. 2007) and 47% in Indiana (Raizman et al. 2009). However, the serological results may greatly underestimate infection with leptosomal serovars in the reservoir hosts.

In conclusion, the raccoon samples in Indiana carry leptosomal organisms in their kidneys and the leptospires are predominantly L. interrogans species and Grippotyphosa serogroup.

Acknowledgements
The authors thank Dr Ron Gillespie, Dr Bong-Suk Kim, Dr Chun-Yu Tung and Mr Seth Nahrwold for their invaluable assistance in sampling, culture or PCR in the present study.

References


