An imprint method for detecting leptospires in the hamster model of vaccine-mediated immunity for leptospirosis

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INTRODUCTION

Leptospirosis is a widespread zoonosis with a global distribution, caused by pathogenic spirochaetes of the genus Leptospira (Adler & de la Pena Moctezuma, 2009; Levett, 2001). The major impact of leptospirosis is the high rate of case fatality due to its most severe complications: Weil’s disease (>10%) (Bharti et al., 2003) and severe pulmonary haemorrhage syndrome (>50%) (Gouveia et al., 2008; Marotto et al., 1999; Park et al., 1989). A priority in current research on leptospirosis is the development of a vaccine that is able to elicit long-term immunity and to induce cross-protection against the serovars that are of greatest importance to public health (Adler & de la Pena Moctezuma, 2009; Koizumi & Watanabe, 2005; McBride et al., 2005).

Several groups have reported on the use of various animal models, including mice, hamsters and gerbils, for vaccine candidate evaluation studies (Haake et al., 1999; Koizumi & Watanabe, 2004; Palaniappan et al., 2006; Silva et al., 2007; Sonrier et al., 2000). The primary goal of any vaccine is to induce a specific immune response such that the initial infection is prevented or subsequently eliminated (Seder & Mascola, 2003). Therefore, an important evaluation of vaccine efficacy is carriage status, as a successful vaccine should confer sterilizing immunity to the vaccinated animal. However, the gold standard protocol is culture isolation (CI), with the potential problems of contamination, incubator space requirements and delayed results (up to 8 weeks) (Palaniappan et al., 2005).

In other disease conditions, tissue imprints (or touch preparations) are commonly used for the direct detection of micro-organisms. In leishmaniasis, the direct observation of trypanosomes in tissue imprints of canine liver and spleen samples is regarded as the definitive laboratory diagnostic test (Bahamdan et al., 1996; Berger et al., 1987;
Kar, 1995; Roscoe, 2005; Teixeira et al., 2008; Volpini et al., 2006). The technique has been applied to several infectious diseases (Jan et al., 2008; Olsen & Stenderup, 1990; Silverman & Gay, 1995). The aim of this study was to adapt and evaluate the imprint method (IM) for the direct observation of leptospires in samples from experimentally infected hamsters, comparing its performance against CI and immunohistochemistry (IHC).

METHODS

Leptospira strain and culture conditions. Leptospira interrogans serovar Copenhageni strain Fiocruz L1-130, isolated from a patient during an outbreak of leptospirosis in the city of Salvador, Brazil (Ko et al., 1999; Nascimento et al., 2004), was cultivated in liquid Ellinghausen–McCullough–Johnson–Harris medium (EMJH, Difco) at 29 °C (Ellinghausen & McCullough, 1965; Johnson & Harris, 1967) and counted in a Petroff–Hausser counting chamber (Fisher Scientific), as described by Faine et al., (1999). The strain was passaged four times in Golden Syrian hamsters (Mesocricetus auratus) and stored at −70 °C. Before use, the aliquots were thawed and passaged in liquid EMJH seven times prior to use as a low-passage isolate in the infection experiments.

Experimental infection of hamsters. Female, 9-week-old hamsters (Fiocruz) were used in leptosporial dissemination studies and in evaluating the performance of the three methods for the direct detection of Leptospira spp. (Silva et al., 2008). Groups of hamsters (n=5, days 3 and 6 post-infection (p.i.); n=15, day 9 p.i.) were inoculated intraperitoneally with 250 leptospires in 1 ml PBS, equivalent to 2.5 x LD₅₀ (Silva et al., 2007), and were sacrificed at intervals ranging from 1 day to 21 days p.i. Control animals (n=5) were injected intraperitoneally with 1 ml sterile PBS. Kidney, liver, lung and blood samples were collected aseptically for evaluation. Collection of blood samples during the dissemination study was by phlebotomy of the retro-orbital venous plexus. The Ethical Committee of the Oswaldo Cruz Foundation approved all animal protocols used in this study.

Culture isolation of leptospires. CI was performed as previously described (Athanazio et al., 2008). Briefly, tissue samples (kidney, liver and lung) were homogenized in 10 ml EMJH, cell debris was allowed to settle for 10 min and 0.5 ml cleared homogenate was inoculated into 5 ml EMJH. Blood samples (1–2 drops) were inoculated directly into 5 ml EMJH, and all cultures were incubated at 29 °C. The cultures were examined regularly for growth, by darkfield microscopy, for up to 8 weeks.

Immunohistochemistry. Necropsies were performed immediately upon sacrifice and kidney, lung and liver samples were stored at −70 °C in mounting medium (Tissue-Tek, Sakura Finetek). Frozen sections (4–5 μm) were thawed out onto poly-l-lysine-coated glass slides and incubated (1 h) with a primary polyclonal anti-leptospiral antibody (prepared by immunizing New Zealand White rabbits with a whole-cell preparation of L. interrogans serovar Icterohaemorrhagiae strain RGA (Athanazio et al., 2008) at a dilution of 1:200. After three washes in PBS, the sections were incubated (1 h) with goat anti-rabbit IgG-FITC conjugate (Jackson ImmunoResearch) at 1:500, and washed three times in PBS and dried before visualization of stained organisms by fluorescence microscopy. Tissue imprints from non-infected hamsters and normal (uninfected) rabbit serum (NRS) were used as negative controls. Counterstaining of nucleic acids or cytoplasm was achieved with 1 mg ml⁻¹ 4',6-diamidino-2-phenylindole (DAPI, Sigma Aldrich) or 0.1 % Evans Blue (Sigma Aldrich) at room temperature for 10 min.

Hamster immunization and challenge studies. Recombinant LigA C-terminal protein fragments were cloned, expressed and purified as described previously (Silva et al., 2007). The subunit vaccine preparations were prepared by adsorption of the recombinant leptospiral immunoglobulin-like (Lig) proteins to aluminium hydroxide (alhydrogel) adjuvant. Immunization protocols were performed as described previously (Silva et al., 2007) with the following modifications. Briefly, female, 4-week-old hamsters were immunized by intramuscular injection on day 0 with a second immunization on day 14; control animals were injected with alhydrogel at the same intervals. The hamsters were challenged with an intraperitoneal inoculum of 250 leptospires (strain Fiocruz L1-130) in 1 ml PBS. Hamsters were monitored three times a day during the post-challenge period and euthanized when clinical signs (prostration or jaundice or pulmonary haemorrhage) of terminal disease appeared. Survivors were euthanized 21 days p.i. Kidney samples were collected from all surviving hamsters in eight independent experiments. Blood, liver and lung samples were collected from a random selection of immunized animals. The kidney samples were analysed using both CI and the IM detection methods. The IM was used to screen the remaining blood, liver and lung samples.

Statistical analysis. Statistical analysis was carried out using Epi Info, ver 6.04 (Centers for Disease Control and Prevention, USA). The chi-squared test was used to compare proportions; P<0.05 was considered significant. Concordance between detection methods was calculated using Cohen’s Kappa (Landis & Koch, 1977).

RESULTS AND DISCUSSION

Direct observation of leptospires in a dissemination study

The IM permitted the direct observation of leptospires by using specific anti-Leptospira whole-cell-antigen antibodies and an FITC conjugate or DAPI in kidney, liver, lung and blood samples (Fig. 1a, b). Table 1 summarizes how the three different methods performed in the detection of leptospires in kidney, liver, lung and blood samples collected from infected hamsters. Leptospires, although present, could not be detected until day 6 p.i. The IM had a higher, but not significant, rate of detection than CI on day six p.i. in the kidney (100 vs 60 %, respectively), liver (100 vs 80 %) and lung (40 vs 20 %) from infected hamsters, while the two methods exhibited a similar capacity to detect leptospires in blood samples (80 vs 80 %). By day 9
p.i., the methods detected leptospires in all 15 of the kidney samples analysed. Both the IM and IHC offered a significant improvement in detection over CI in liver (100 and 100 % vs 53.3 %, respectively, \( P<0.01 \)) and lung samples (80.0 and 73.3 % vs 18.2 %, \( P<0.01 \)). The IM and CI performed similarly for blood samples (53.3 vs 46.6 %, respectively). When blood samples were collected daily from day 2 to day 10 p.i., leptospires were detected by day 6 p.i. and overall IM performance was equal or superior to CI. On day 9 p.i., the IM detected leptospires in 80 % (4/5) compared to 20 % (1/5) for CI; however, this difference was not significant (data not shown). Concordance was good for all three tests (\( \kappa=0.61 \)), and for CI and the IM (\( \kappa=0.64 \)), agreement between CI and IHC was moderate (\( \kappa=0.49 \)), while between the IM and IHC concordance was very good (\( \kappa=0.96 \)). When stratified according to sample type, the agreement between CI and the IM was very good in kidney (\( \kappa=0.86 \)) and blood samples (\( \kappa=0.93 \)), moderate in liver samples (\( \kappa=0.50 \)) and fair in lung samples (\( \kappa=0.23 \)).

**Detection of leptospires in immunized hamsters post-challenge**

Summarizing the eight experiments performed (Table 2), survival was 85.0 % (68/80) among immunized hamsters compared to 2.5 % (2/80) among control hamsters immunized with adjuvant only. Among the immunized hamsters which survived, cultures from eight animals could not be evaluated due to contamination. In an evaluation of sterilizing immunity, leptospires were isolated (CI) from
the kidneys of 23.3% (14/60) of surviving hamsters, whereas leptospires were detected by the IM in 26.5% (18/68) of surviving hamsters. In a random evaluation of the IM, leptospires were detected in 42.8% (9/21) liver samples and 20.0% (1/5) lung samples, while none of the blood samples (0/43) were positive. Based on the kidney samples, there was very good agreement between CI and the IM (κ=0.84). It is of note that all samples that were CI positive were also positive by the IM.

Sterilizing immunity is an important secondary end point in vaccine candidate evaluations, particularly since it is known that commercial vaccines for animal use do not prevent leptospiuria (Adler & de la Pena Moctezuma, 2009). The gold standard for the direct detection of leptospires in biological samples is culture isolation (Faine et al., 1999; WHO & ILS, 2003). However, this method is not ideal due to the fastidious nature of pathogenic Leptospira spp., the need to incubate the cultures for up to 8 weeks, the considerable demand on laboratory resources when large numbers of cultures are involved, and the risk of contamination (Palaniappan et al., 2005). The main aim of this study was to adapt and evaluate the imprint method for the direct detection of leptospires in biological samples. To our knowledge, this is the first report on the application of this technique to the field of leptospirosis.

A potential limitation in the use of the IM is that although the technique identifies intact Leptospira spp. it may not discriminate between intact-viable and intact-non-viable leptospires. However, the IM only demonstrated significantly increased detection of leptospires, when compared to CI, in the dissemination study. It is of note that in the dissemination study the presence of leptospires can be presupposed, as 100% of the infected animals died. It is known that leptospires are cleared from the blood during the first 2 weeks of infection (Faine et al., 1999; Levett, 2001); therefore if the IM was detecting intact non-viable leptospires it should have identified more positive samples by day 9 p.i. and this was not the case (Table 1).

Table 1. Direct detection of leptospires in a dissemination study of hamsters infected with L. interrogans strain Fiocruz L1-130

<table>
<thead>
<tr>
<th>Days p.i.</th>
<th>Method*</th>
<th>Number of positives/total (% positive)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Kidney</td>
</tr>
<tr>
<td>3</td>
<td>CI</td>
<td>0/5 (0)</td>
</tr>
<tr>
<td></td>
<td>IM</td>
<td>0/5 (0)</td>
</tr>
<tr>
<td>6</td>
<td>CI</td>
<td>3/5 (60)</td>
</tr>
<tr>
<td></td>
<td>IM</td>
<td>5/5 (100)</td>
</tr>
<tr>
<td>9</td>
<td>CI</td>
<td>15/15 (100)</td>
</tr>
<tr>
<td></td>
<td>IHC</td>
<td>15/15 (100)</td>
</tr>
<tr>
<td></td>
<td>IM</td>
<td>15/15 (100)</td>
</tr>
<tr>
<td>Uninfected controls</td>
<td>CI</td>
<td>0/5 (0)</td>
</tr>
<tr>
<td></td>
<td>IHC</td>
<td>0/5 (0)</td>
</tr>
<tr>
<td></td>
<td>IM</td>
<td>0/5 (0)</td>
</tr>
</tbody>
</table>

ND, Not determined.
*CI, culture isolation; IHC, immunohistochemistry; IM, imprint method.
†Four cultures were excluded due to contamination.
‡One culture was excluded due to contamination.

The results indicate that the IM is a sensitive and specific method for detecting leptospires in biological samples.

Table 2. Evaluation of the IM for the detection of sterilizing immunity in the hamster model of leptospirosis

<table>
<thead>
<tr>
<th>Outcome</th>
<th>Sample</th>
<th>Immunized hamsters*</th>
<th>Non-immunized hamsters*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CI</td>
<td>IM</td>
<td>Total</td>
</tr>
<tr>
<td>Survival</td>
<td>Kidney</td>
<td>23 (14/60)‡</td>
<td>26 (18/68)</td>
</tr>
<tr>
<td></td>
<td>Liver</td>
<td>ND</td>
<td>43 (9/21)</td>
</tr>
<tr>
<td></td>
<td>Lung</td>
<td>ND</td>
<td>20 (1/5)</td>
</tr>
<tr>
<td></td>
<td>Blood</td>
<td>ND</td>
<td>0 (0/43)</td>
</tr>
<tr>
<td>Death</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

ND, Not determined; NA, not applicable.
*Pooled results from eight independent experiments.
‡Eight cultures lost to contamination.
possible that the antigenic determinants targeted in the IM are not readily detected in intact non-viable leptospires, possibly due to loss of the outer membrane or changes in conformation or degradation of the antigens.

The leptospiral immunoglobulin-like (Lig) proteins are highly conserved, unique to pathogenic *Leptospira* spp. (Matsunaga et al., 2003; Cerqueira et al., 2009; McBride et al., 2009), and their use in vaccine preparations has been reported by several groups (Koizumi & Watanabe, 2004; Palaniappan et al., 2006). We reported that hamsters immunized with a LigA C-terminal fragment in Freund’s adjuvant were significantly protected (67–100%), but that all survivors exhibited evidence of renal colonization (Silva et al., 2007). In addition, LigA vaccine formulations were reported to confer 75–100% protection, with sterilizing immunity ranging from 25 to 90% (Faisal et al., 2008, 2009; Palaniappan et al., 2006). A vaccine preparation of LigB and alhydrogel induced significant protection in hamsters (54–83%) but did not confer sterilizing immunity (Yan et al., 2009). In C3H/HeJ mice, immunization with Lig proteins elicited significant protection (100%), but not sterilizing immunity (Koizumi & Watanabe, 2004). Haake et al., (1999) found that hamsters immunized with recombinant OmpL1 and LipL41 were significantly protected (71–100%) and sterilizing immunity was 100%. Such variability in sterilizing immunity may be associated with the virulence of the challenge strain, as the more virulent challenge strains resulted in kidney colonization (Silva et al., 2007; Faisal et al., 2008, 2009; Yan et al., 2009); however, this was not always the case (Haake et al., 1999). Yet another factor could be the poor reproducibility of CI, highlighting the urgent need for an improved standardized method. Among the eight cultures that were contaminated during CI, three were imprint positive. This suggests that the imprint technique could provide more reproducible results than CI in evaluating sterilizing immunity.

The IM proved to be a robust and sensitive method for the direct observation of intact leptospires in blood and tissue samples. Although the technique was equal in terms of diagnostic accuracy correlating to protective immunity. V vaccine preparations of recombinant OmpL1 and LipL41 incorporated in liposomes and PLGA microspheres produces a robust immune response correlating to protective immunity. Vaccine 27, 378–387.

In conclusion, we report the successful adaptation and evaluation of the imprint method for the direct detection of pathogenic *Leptospira* spp. in blood and tissue samples collected from an experimental animal model. Furthermore, the technique was readily applied to the evaluation of sterilizing immunity in vaccine-mediated immunity studies. We therefore propose that the imprint technique should be considered as a complementary method for the detection of leptospires in animal models of leptospirosis.

**ACKNOWLEDGEMENTS**

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**REFERENCES**


Detection of leptospires in infected hamsters


