Author's response to reviews

Title: Establishment of a leptospirosis model in guinea pigs using an epicutaneous inoculations route

Authors:

Yan Zhang (mavournia@gmail.com)
Xiao-Li Lou (Louxiaoli4321@yahoo.com.cn)
Hong-Liang Yang (hlyang@colostate.edu)
Xiang-Yan Zhang (joycez@sjtu.edu.cn)
Xiao-Kui Guo (xkguo@shsmu.edu.cn)
Ping He (hpatsh@sjtu.edu.cn)
Xu-Cheng Jiang (xjiang@shsmu.edu.cn)

Version: 2  Date: 16 November 2011

Author's response to reviews: see over
Dear editor,

The e-mail on 18 October 2011 and the referees’ thoughtful comments are highly appreciated. According to your comments and requests, we have made careful and extensive modifications on the original manuscript. Here, we attached revised manuscript in the formats of MS word, for your approval. A document answering every question from the referees was also summarized and enclosed.

Should you have any questions, please contact us without hesitate.

Yours Sincerely,

Ping He

13/11/2011
Reviewer's report

Title: Establishment of a leptospirosis model in guinea pigs using an epicutaneous inoculations route

Version: 1 Date: 14 October 2011

Reviewer: Mathieu Picardeau

Reviewer's report:

In the manuscript by Zhang et al., the authors have conducted a very basic set of experiments in which guinea pigs were infected with L. interrogans by epicutaneous inoculations. This is in contrast with most of the published challenge experiments where animals were infected by intraperitoneal inoculation of leptospires. As mentioned by the authors, epicutaneous inoculations may reflect conditions encountered during natural infection.

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Major Compulsory Revisions

Major concerns:

1) The authors should make sure that their study is placed in its proper context. For example, they did not cite landmark studies such as the one from Faine in 1957 on the dissemination of leptospires in infected guinea-pigs (“Virulence in Leptospira. II. The growth in vivo of virulent Leptospira icterohaemorrhagiae” British Journal of Experimental Pathology, Vol. 38, Pages 8-14). Some more recent studies (not cited in the text) have also used qPCR to follow bacterial load in animal models, including guinea pigs (ex. Lourdault et al. Use of quantitative real-time PCR for studying the dissemination of Leptospira interrogans in the guinea pig infection model of leptospirosis. 2009).

Response: Thank you for your thoughtful comments. We added 7 new references including you mentioned 2 articles. The manuscript was revised extensively according to the new context. (Background, line 73-91)

Previous: (line 67-71) It produces a lethal infection in guinea pigs and mimics the
clinical presentation of severe leptospirosis in humans. However, this route of infection does not reflect real conditions encountered during natural infection, because leptospires are presumed to enter the host via abrasions or breaches of the skin. As the route and mode of entry of leptospires via skin have been poorly studied, we evaluated epicutaneous inoculations routes in guinea pigs.

Revised: (line 73-91) The intraperitoneal (i.p.) inoculation route is the most widely applied infection routes by producing a lethal infection in experimental animals and mimicking the clinical presentation of severe leptospirosis in humans [7-10]. However, this route of infection does not reflect real conditions encountered during natural infection, because leptospires are presumed to enter the host via mucous membranes or abrasions of the skin. It has been a long time for researchers to challenge animals through alternative routes to mimic natural entry of leptospires into hosts. Even about one century ago, Ido and his colleagues attempted to reproduce natural conditions by conveying the leptospires directly to the guinea pig by the bite of rat (carrier of leptospires). The results indicated that leptospirosis is rarely transmitted by the bite of rat[11]. Since then, different infection routes such as conjunctival (c.j.) and subcutaneous (s.c.) have been employed in canine, horse, hamster and guinea pig, and resulting in acute leptospirosis in inoculated animals [11-16]. By using infection routes different from the classic i.p. route, these studies contributed to the pathogenesis of leptospirosis in experimental animals. However the route and mode of entry of leptospires directly via epidermis have been poorly studied. The methods mentioned above bypassed the epidermis of host. It is still little data on how the leptospires interact with the epidermis and if the inoculated leptospires could penetrate the skin and disseminate systemically. Cutaneous host defense mechanisms are actually quite complex and consist of a variety of active processes. Herein we examine the ability of leptospires to produce infections in guinea pigs when applied to damaged or undamaged skin.

2) The authors should compare the dissemination (kinetics, bacterial load in target organs), histopathology, LD50 (not determined in this study), ... in guinea pigs infected
by epicutaneous and the classical intraperitoneal inoculations.

**Response:** Thanks a lot for the thoughtful comment. In the revised manuscript, we compared our findings with the bacterial load in blood and histopathology of guinea pigs infected by intraperitoneal inoculations. (Discussion, line 276-291, line 298-308)

**Revised:** (line 276-291) When *L. interrogans* strain Lai was inoculated on the abraded skin, localized changes around the inoculated site were detected. All of the guinea pigs showed hemorrhage at the dermis around the site-inoculation before the appearance of internal organs hemorrhage. Skin hemorrhage was rarely reported in animals infected experimentally through the i.p. route, and little attention has been called for. The pathogenesis of hemorrhage caused by leptospirosis has not been elucidated yet. Factors contributing to the hemorrhage might involve direct action of toxins and autoimmune process. Nicodemo and coworkers detected the intact leptospires in capillary endothelial cells, indicating the lung injury is directly triggered by leptospires and/or by their toxic products [30]. Another study demonstrated the deposition of antibodies and complement along the alveolar basement membrane of infected guinea pigs, indicating pulmonary hemorrhage might be led by autoimmune process [8]. Our data showed that leptospires were detected abundantly in the dermis and subcutaneous of hemorrhagic area and were rarely detected in adjacent none hemorrhagic areas, confirming the high burden of leptospires in the dermis is an important factor to cause hemorrhage. Humoral immune response seems not be associated with the pathogenesis of skin hemorrhage, as dermis hemorrhage developed as early as 8-24 hours p.i.. Further examination of the local hemorrhage may give a clue to understand the mechanism of hemorrhage in this disease.

**Revised:** (line298-308) Recently, Lourdault and his colleagues compared different routes (i.p., c.j. and s.c. inoculation) of infection and the dissemination of leptospires in blood and tissues of guinea pigs using multiple methods including real-time PCR [16]. The results showed infected guinea pigs developed similar physical signs and pathological changes after i.p., s.c. and c.j. inoculation with leptospires, and the bacterial burden in tissues and histopathology revealed no major differences between
the three routes of infections[16]. In the guinea pigs with abraded skin inoculations, our real-time PCR results showed that the bacteraemia peaked at 96 hours p.i. and then quickly decreased at 144 hours p.i., which were consistent with the result of i.p. inoculated guinea pigs or s.c. inoculated hamsters reported by Lourdault and Truccolo respectively[15, 16]. It is interesting to note that the high leptospires burden ($3 \times 10^5$ leptospires ml$^{-1}$) detected in the blood at 2 hours p.i., and then quickly dropped by 1 log at 8 hours p.i..

3) subcutaneous infections: how long do the the filter discs with the bacterial suspension are put in contact with the skin? Any idea of the true inoculum dose? This could be evaluated by qPCR of filter discs before and after contact with the skin.

Response: Thanks for your suggestion. The filter discs were removed 2–24 h p.i. We added this in the revised manuscript. At different time points after inoculation, we have checked the number of leptospires left on the filter discs by extensively wash the filter discs by PBS and then counted in a Petroff-Hausser counting chamber. The result showed that only about $5 \times 10^4$ leptospires left on the filter discs at 2h p.i., and there was no leptospires detected after 24h p.i.. It was supposed that all the leptospires were inoculated on the skin/or abrade skin at 24h p.i..

Previous: (line 109) The guinea pigs were euthanized at 2, 8, 24, 48, 72, 96 and 144 hours post-infection (p.i.).

Revised: (line 127-128) The filter discs were removed after 2–24 h and the guinea pigs were euthanized at 2, 8, 24, 48, 72, 96 and 144 hours post-infection (p.i.).

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Minor Essential Revisions

4) references for the EMJH medium should include Ellinghausen et al. (Nutrition of Leptospira pomona and growth of 13 other serotypes: fractionation of oleic albumin complex and a medium of bovine albumin and polysorbate 80. Am J Vet Res. 1965;26:45-51) and Johnson et al. (Differentiation of pathogenic and saprophytic

**Responses:** Thank you for your suggestion. Two references about EMJH medium were added in the revised manuscript.

5) Discussion: again, authors should compare their findings with previous papers on the use of PCR (or other methods) to detect Leptospira in a range of tissues in a range of animal species by different routes of inoculations.

**Responses:** Thank you for your thoughtful comments. In the revised manuscript, we extensively compared our findings with the qPCR results of i.p. inoculated guinea pigs or s.c. inoculated hamsters reported by Lourdault and Truccolo respectively. (Discussion, line 298-308)

**Revised:** (line 298-308) Recently, Lourdault and his colleagues compared different routes (i.p., c.j. and s.c. inoculation) of infection and the dissemination of leptospires in blood and tissues of guinea pigs using multiple methods including real-time PCR [16]. The results showed infected guinea pigs developed similar physical signs and pathological changes after i.p., s.c. and c.j. inoculation with leptospires, and the bacterial burden in tissues and histopathology revealed no major differences between the three routes of infections[16]. In the guinea pigs with abraded skin inoculations, our real-time PCR results showed that the bacteraemia peaked at 96 hours p.i. and then quickly decreased at 144 hours p.i., which were consistent with the result of i.p. inoculated guinea pigs or s.c. inoculated hamsters reported by Lourdault and Truccolo respectively[15, 16]. It is interesting to note that the high leptospires burden (3×10^5 leptospires ml^{-1}) detected in the blood at 2 hours p.i., and then quickly dropped by 1 log at 8 hours p.i..
Reviewer's report

Title: Establishment of a leptospirosis model in guinea pigs using an epicutaneous inoculations route

Version: 1 Date: 4 October 2011

Reviewer: Daniel athanazio

Reviewer's report:

1. Background. Second paragraph. The authors adequately state that “intraperitoneal inoculation route is the most commonly used in these models of leptospirosis”. However, if the main point of the study is to search for alternative ways to establish experimental infection, some references are lacking on:

Conjunctival infusion, mainly in dogs


Subcutaneous route in hamsters


Attempts to reproduce natural conditions, such as inoculation of rat urine or exposure to
rat bites (in guinea pig model).


Thus, a more detailed discussion of alternative methods of inoculation is warranted to clearly introduce what is the background of the issue addressed by the authors.

**Response:** Thank you for your thoughtful comments. We revised our manuscript according to your comments. (Background, line 77-91)

**Revised:** (line 77-91) It has been a long time for researchers to challenge animals through alternative routes to mimic natural entry of leptospires into hosts. Even about one century ago, Ido and his colleagues attempted to reproduce natural conditions by conveying the leptospies directly to the guinea pig by the bite of rat (carrier of leptospires). The results indicated that leptospirosis is rarely transmitted by the bite of rat[11]. Since then, different infection routes such as conjunctival (c.j.) and subcutaneous (s.c.) have been employed in canine, horse, hamster and guinea pig, and resulting in acute leptospirosis in inoculated animals [11-16]. By using infection routes different from the classic i.p. route, these studies contributed to the pathogenesis of leptospirosis in experimental animals. However the route and mode of entry of leptospires directly via epidermis have been poorly studied. The methods mentioned above bypassed the epidermis of host. It is still little data on how the leptospires interact with the epidermis and if the inoculated leptospires could penetrate the skin and disseminate systemically. Cutaneous host defense mechanisms are actually quite complex and consist of a variety of active processes. Herein we examine the ability of leptospires to produce infections in guinea pigs when applied to damaged or undamaged skin.

2. Results. “Tissue distribution…” “A pronounced acute inflammatory response were induced in site inoculation”. Please note that this is not usually described in clinical settings. No local response seems to follow human infection after penetration of
leptospires in superficial tissues. Since this observation is of more interest than histopathology of classical lesions in target organs, the reader would benefit of having a more detailed description of such findings with the correlation of skin lesions and the presence of leptospires.

**Response:** Thank you for your thoughtful comments. We revised our manuscript based on your suggestion. (Result, line 204-208, 212-216; Discussion, line 276-297)

**Previous:** (line 184-186) In the guinea pigs with abraded skin inoculations, leptospires were seen to have invaded from the inoculation site into the dermis and subcutaneous by immunohistochemistry as early as 2 hours p.i. (Figure 5a).

**Revised:** (line 204-208) In the guinea pigs with abraded skin inoculations, leptospires were seen to have invaded from the inoculation site into the dermis and subcutaneous by immunohistochemistry as early as 2 hours p.i. (Figure 5a). At 48 hours p.i., leptospires were detected abundantly in the dermis and subcutaneous of hemorrhagic area and were rarely detected in adjacent none hemorrhagic areas (data not shown).

**Previous:** (line 190-193) In addition, a pronounced acute inflammatory response were induced in site inoculation and surrounding tissues characterized by the presence of neutrophils, macrophages, lymphocytes and histiocytes from 48 hours p.i. (data not shown).

**Revised:** (line 212-216) In addition, a pronounced acute inflammatory response were induced in site inoculation and surrounding tissues characterized by the presence of neutrophils, macrophages, lymphocytes and histiocytes at 48 hours p.i.. At the late stage (from 72 to 144 hours), inflammatory cells in the subcutaneous tissue and muscular layers decreased gradually, and replaced by fibroblasts proliferation.

**Revised:** (line 276-297) When *L. interrogans* strain Lai was inoculated on the abraded skin, localized changes around the inoculated site were detected. All of the guinea pigs showed hemorrhage at the dermis around the site-inoculation before the appearance of internal organs hemorrhage. Skin hemorrhage was rarely reported in animals infected experimentally through the i.p. route, and little attention has been called for. The pathogenesis of hemorrhage caused by leptospirosis has not been
elucidated yet. Factors contributing to the hemorrhage might involve direct action of toxins and autoimmune process. Nicodemo and coworkers detected the intact leptospires in capillary endothelial cells, indicating the lung injury is directly triggered by leptospires and/or by their toxic products [30]. Another study demonstrated the deposition of antibodies and complement along the alveolar basement membrane of infected guinea pigs, indicating pulmonary hemorrhage might be led by autoimmune process [8]. Our data showed that leptospires were detected abundantly in the dermis and subcutaneous of hemorrhagic area and were rarely detected in adjacent none hemorrhagic areas, confirming the high burden of leptospires in the dermis is an important factor to cause hemorrhage. Humoral immune response seems not be associated with the pathogenesis of skin hemorrhage, as dermis hemorrhage developed as early as 8-24 hours p.i.. Further examination of the local hemorrhage may give a clue to understand the mechanism of hemorrhage in this disease.

Hemorrhage in the skin is produced as one of the general symptoms in clinical cases [4]. However, Hemorrhage localized at infected site was rarely recognized clinically. Local hemorrhage in our experiment model might caused by high dose inoculation of leptospires. When in nature infection, it seems like that low dose leptospires in the cuts or abrade skin will not cause skin hemorrhage until large amount of pathogen proliferated in the circulation, and then extensive skin hemorrhage will be produced.

3. Discussion . The main concern on the whole article is that it describes a clever way to reproduce skin abrasions that could better reproduce a natural way to acquire leptospirosis, however, this new model was not compared to established ones. Since intraperitoneal inoculation is much easier to handle than this new abrasion route, intraperitoneal infection would be still the route of choice except if the authors can show that intraperitoneal infection result in artificial outcomes such as different patterns of dissemination of leptospires in tissues, different immune responses or different clinical outcomes. If all of these are the same, intraperitoneal infection will still be the route of choice. If
intraperitoneal and abrasion routes are different in those results they provide, than that would be a strong point favoring the new method.

Response: Thank you for your thoughtful comments. The intraperitoneal inoculation is easy to handle and allows reproducible amounts of leptospires to be introduced. It is still the most used model to study the systemically infection of leptospirosis due to its easy to handle feature. The guinea pigs leptospirosis model with an epicutaneous inoculation route described here revealed epicutaneous inoculation might be an alternative route to investigate the pathogenesis of leptospirosis, especially when focus on the early steps of infection. Our epicutaneous inoculations model would be a new choice when applying to 1) a better understanding of the mechanisms involved in cutaneous barriers and epidermal interactions with this organism; 2) characterization of *Leptospira* mutants that are deficient in protein with binding affinity for skin. We revised our manuscript extensively according to your comment. (Discussion, line 320-328, line 331-336)

Revised: (line 320-328) The traditional intraperitoneal inoculation is easy to handle and allows reproducible amounts of leptospires to be introduced. It is still the most widely used model to study the systemically infection of leptospirosis. However, there are some shortages of i.p. or other non-epicutaneous route when apply on the pathogens causing infection through skin. In study performed by Bischof and colleagues, the subcutaneous injection of *B. anthracis* (Sterne strain, which lacks the pX02 capsule plasmid) caused lethal infection in C57BL/6 mice, while quite resistant to epicutaneous inoculation of *B. anthracis* onto abraded skin [20]. This study suggested that our epicutaneous inoculation model would be an alternative way to apply the characterizations of *Leptospira* mutants that are deficient in protein with binding affinity for skin.

Revised: (line 331-336) The guinea pigs leptospirosis model with an epicutaneous inoculation route described here replicated a natural course of infection and revealed epicutaneous inoculation might be an alternative route to investigate the pathogenesis of leptospirosis, especially when focus on the early steps of infection while the intraperitoneal inoculation is still a classic and main infection route due to its easy to
handle feature.

Only establishing infection resulting in acute lethal disease is not sufficient to support a new route of infection to the scientific community devoted to study leptospirosis. Please note that there is already a study comparing different routes of infection and the dissemination of leptospires in tissues.


All these concerns have not been discussed in the manuscript.

Response: Thank you for your suggestion. In the revised manuscript, we described the work did by Lourdault and coworkers, and also compared our findings with the qPCR results of i.p. inoculated guinea pigs or s.c. inoculated hamsters reported by Lourdault and Truccolo respectively. (Background, line 82-85; Discussion, line 298-308)

Revised: (line 82-85) Since then, different infection routes such as conjunctival (c.j.) and subcutaneous (s.c.) have been employed in canine, horse, hamster and guinea pig, and resulting in acute leptospirosis in inoculated animals [11-16]. By using infection routes different from the classic i.p. route, these studies contributed to the pathogenesis of leptospirosis in experimental animals.

Revised: (line 298-308) Recently, Lourdault and his colleagues compared different routes (i.p., c.j. and s.c. inoculation) of infection and the dissemination of leptospires in blood and tissues of guinea pigs using multiple methods including real-time PCR [16]. The results showed infected guinea pigs developed similar physical signs and pathological changes after i.p., s.c. and c.j. inoculation with leptospires, and the bacterial burden in tissues and histopathology revealed no major differences between the three routes of infections[16]. In the guinea pigs with abraded skin inoculations, our real-time PCR results showed that the bacteraemia peaked at 96 hours p.i. and then quickly decreased at 144 hours p.i., which were consistent with the result of i.p. inoculated guinea pigs or s.c. inoculated hamsters reported by Lourdault and Truccolo.
respectively[15, 16]. It is interesting to note that the high leptospirse burden ($3 \times 10^5$ leptospires ml$^{-1}$) detected in the blood at 2 hours p.i., and then quickly dropped by 1 log at 8 hours p.i..