Research, Society and Development, v. 9, n. 10, e8569109183, 2020
(CC BY 4.0) | ISSN 2525-3409 | DOI: http://dx.doi.org/10.33448/rsd-v9i10.9183

Isolation and virulence characterization of pathogenic leptospires from cattle

Isolamento e caracterização da virulência de leptospiras patogênicas de bovinos

Aislamiento y caracterización de virulencia de leptospiras patógenas de bovinos

Received: 10/09/2020 | Reviewed: 10/15/2020 | Accept: 10/20/2020 | Published: 10/21/2020

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Abstract

Leptospirosis is an important veterinary disease. In Brazil, bovine leptospirosis is associated with considerable economic loss. Commercially available vaccines for livestock have serious limitations and local isolates are the best way to improve both diagnostic tests and vaccines. The objective of this work was to isolate leptospires from cattle slaughtered in local abattoirs at the city of Pelotas, in southernmost Brazil. Isolation was attempted from 250 kidneys collected from healthy bovines. Isolates suffered preliminary molecular characterization and

serogrouping, in addition to virulence test and lethal dose 50% (LD50) experiments in hamster (*Mesocricetus auratus*) model. Two pathogenic isolates were obtained. Sequencing and serogrouping revealed isolates BOV3 and BOV15 to be *Leptospira interrogans* and in the Canicola serogroup. Isolates were lethal to hamsters causing death with an inoculum of less than 250 leptospires. Necropsy showed severe lesions in hamsters inoculated with the virulent strains, such as haemorrhages in the lungs and jaundice. Two new isolates from cattle are characterized herein, being highly virulent and eligible for vaccine challenge trial and inclusion in diagnostic tests.

Keywords: Leptospirosis; Neglected tropical disease; Recombinant vaccines; Hamster.

Resumo

A leptospirose é uma importante doença veterinária. No Brasil, a leptospirose bovina está associada a consideráveis perdas econômicas. As vacinas comercialmente disponíveis para bovinos têm sérias limitações e os isolados locais são a melhor maneira de melhorar os testes de diagnóstico e as vacinas. O objetivo deste trabalho foi isolar leptospiras de bovinos abatidos em abatedouros frigoríficos da cidade de Pelotas, extremo sul do Brasil. Para tanto, a tentativa de isolamento foi realizada a partir de 250 rins coletados de bovinos saudáveis. Os isolados sofreram caracterização molecular preliminar e sorogrupagem, além de experimentos para a caracterização da virulência e dose letal 50% (DL50) em modelo de hamster (*Mesocricetus auratus*). Dois isolados patogênicos foram obtidos. O sequenciamento e o sorogrupamento revelaram que os isolados BOV3 e BOV15 estão classificados dentro da espécie *Leptospira interrogans* e do sorogrupo Canicola. Os isolados foram letais para hamsters, causando a morte com um inóculo menosr do que 250 leptospiras. A necropsia mostrou lesões graves nos hamsters inoculados com as cepas virulentas, como hemorragias nos pulmões e icterícia. Dois novos isolados de bovinos foram caracterizados neste trabalho, sendo altamente virulentos e elegíveis para teste de desafio de vacina e inclusão em testes de diagnóstico.

Palavras-chave: Leptospirose; Doença tropical negligenciada; Vacinas recombinantes; Hamster.

Resumen

La leptospirosis es una enfermedad veterinaria importante. En Brasil, la leptospirosis bovina se asocia con pérdidas económicas considerables. Las vacunas disponibles comercialmente para el ganado tienen serias limitaciones y los aislamientos locales son la mejor manera de

mejorar tanto las pruebas de diagnóstico como las vacunas. El objetivo de este trabajo fue aislar leptospiras de ganado sacrificado en mataderos locales en la ciudad de Pelotas, en el extremo sur de Brasil. Se intentó el aislamiento de 250 riñones recogidos de bovinos sanos. Los aislamientos sufrieron una caracterización molecular preliminar y un serogrupo, además de experimentos de prueba de virulencia y dosis letal al 50% (LD50) en modelo de hámster (*Mesocricetus auratus*). Se obtuvieron dos aislados patógenos. La secuenciación y el serogrupo revelaron que los aislados BOV3 y BOV15 eran *Leptospira interrogans* y pertenecían al serogrupo Canicola. Los aislamientos resultaron letales para los hámsteres y provocaron la muerte con un inóculo de menos de 250 leptospiras. La necropsia mostró lesiones graves en hámsteres inoculados con cepas virulentas, como hemorragias en los pulmones e ictericia. En este documento se caracterizan dos nuevos aislados de ganado, que son altamente virulentos y aptos para el ensayo de desafío con la vacuna y su inclusión en pruebas de diagnóstico.

Palabras clave: Leptospirosis; Enfermedad tropical desatendida; Vacunas recombinantes; Hámster.

1. Introduction

Leptospirosis is a worldwide bacterial disease that affects over 1 million humans every year (Costa et al., 2015). Leptospirosis is considered to be the most widespread zoonosis in the world, and it is recognized to be an emerging disease in developed countries and endemic in developing countries (Haake, 2015). Moreover, leptospirosis is an occupational disease for veterinarians, farmers, slaughterhouse workers, and other occupations (CDC, 2020).

Bovine leptospirosis is associated with important economic loss. However, it is impracticable to assess what the total cumulative loss is either in an individual herd or in national herds (Ellis, 2015). In Brazil, it is frequently encountered in dairy and beef cattle (Fávero et al., 2017; Guedes et al., 2019). It causes of reproductive shortcomings such as abortion, stillbirth, premature birth, lost weight, among others (Lilenbaum, 2017). Primarily, bovine leptospirosis is caused by serovar Hardjo, although the infected animals rarely present clinical signs of disease. Nonetheless a variety of other serovars may cause infection in cattle with varying clinical outcomes (Zuerner et al., 2012, Guedes et al., 2019).

The Golden Syrian hamster (*Mesocricetus auratus*) is the main model for leptospirosis pathogenesis studies because of its susceptibility to infection and the reproducibility of the

results. Furthermore, acute disease in the hamster mimics the severe form of human leptospirosis (Haake, 2006).

In this study, we report the isolation and virulence characterization of two leptospiral strains from beef cattle slaughtered in the city of Pelotas (Brazil). We also performed LD50 experiments to demonstrate the suitability of virulent strains for challenge assays in the hamster model.

2. Material and Methods

In this work, we carried out a study of a quantitative nature (Pereira et al., 2018), which bacterial isolation attempts were carried out through bovine kidney tissue cultures. The kidneys used in this study came from of apparently healthy animals, which were slaughtered in three abattoirs located in Pelotas, Rio Grande do Sul state, Brazil. The animals, male and female cattle, came from seven municipalities (Pelotas, Capão do Leão, Pedro Osório, Rio Grande, Canguçu, Turuçu, and Morro Redondo) in the southern region of the state.

Each kidney was aseptically removed immediately after slaughter, and transported to the laboratory under refrigerated conditions in individual sterile polypropylene bags. In the laboratory, tissue samples were removed aseptically. Excised kidney material was macerated and suspended in Ellinghausen-McCullough-Johnson-Harris (EMJH) liquid medium (Difco laboratories) for isolation. All cultures were incubated at 29°C and checked weekly for growth. When growth was detected, successive transfers were made in liquid and semisolid EMJH media until growth was sufficiently abundant. Density of the leptospiral challenge strain was determined by dark-field microscopy using a Petroff–Hausser counting chamber (Fisher Scientific) as described previously (WHO, 2003).

In virulence characterization, groups of two 28-day-old Golden Syrian hamsters (*Mesocricetus auratus*), provide by Biotério Central-UFPel, were inoculated intraperitoneally with approximately $1x10^8$ leptospires of each isolate in a final volume of 1mL. Animals were confined in isolator cages and monitored daily for the presence of clinical signs, including evidence of external haemorrhage, dehydration, ruffled hair coat, decreased activity and isolation. When moribund, they were euthanized and subjected to necropsy. Fragments of kidneys, liver and lungs were aseptically removed for re-isolation. Animals that survived infection were euthanized on the 25th day after challenge to collect tissues and determine sub-lethal infection (Silva et al., 2008).

Preliminary molecular characterization, to determine genomic species, was accomplished by sequencing of the 16S rRNA gene in Biomanguinhos (FIOCRUZ/RJ, Brazil). In addition, serogrouping was performed using a standard panel of 20 rabbit antisera at the Gonçalo Moniz Research Center (FIOCRUZ/BA, Brazil).

For all LD50 experiments, female and male 9-week-old hamsters (Biotério Central-UFPel) were infected intraperitoneally with 10-fold serial dilutions. Inocula of $1x10^4$ to $1x10^0$ organisms were tested. The number of animals for each inoculum was eight according to the availability of animal facilities and the recommendation of the Committee for Animal Care and Use (UFPel). Hamsters were inoculated intraperitoneally with 1mL. Animals were monitored daily for clinical outcome until 28 days post infection (DPI). Moribund animals were euthanized and necropsied, gross lesions were observed and noted, and kidney tissue was cultured at 29°C in liquid EMJH. After this, LD50 was calculated (Reed, 1934).

Animal procedures carried out in this study were reviewed and approved by the Committee for Animal Care and Use of UFPel (Protocol nº 4657).

3. Results and Discussion

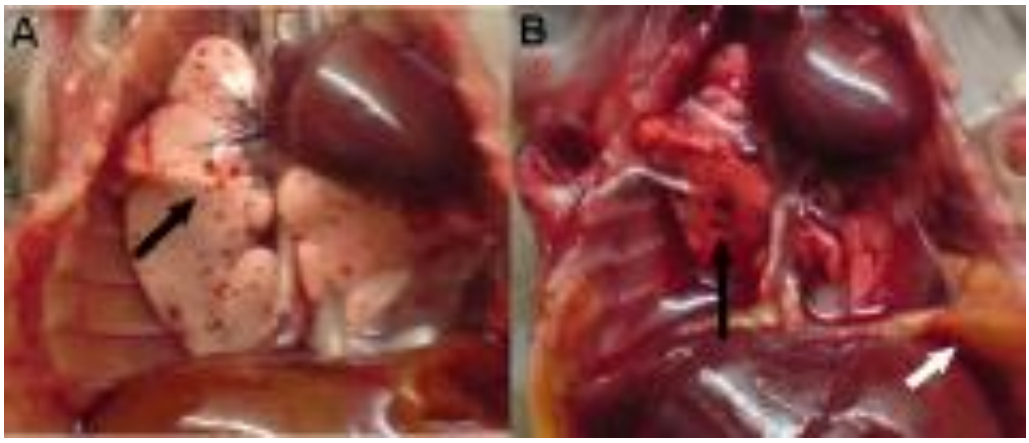
Leptospira isolation was obtained from two bovine kidneys, out of 250 attempts. Isolates BOV3 and BOV15 required 3 weeks of incubation before leptospires could be detected by dark-field microscopy examination.

Strains BOV3 and BOV15 were recovered from kidneys of experimentally inoculated hamsters 2 weeks after inoculation. Both isolates caused clinical signs, including dehydration, piloerection, tremors, decreased activity, isolation, and death shortly afterwards.

Necropsy showed severe lesions in hamsters inoculated with the two virulent strains, such as petechial haemorrhages in the lungs and jaundice (Figure 1). Staining with hematoxylin and eosin showed alveolar haemorrhage and nephritis.

Sequencing and serogrouping revealed isolates BOV3 and BOV15 to be *Leptospira interrogans* and in the Canicola serogroup, respectively. These virulent strains induced disease in hamsters and led to death with an inoculum containing less than 250 leptospires. Strain BOV3 had a LD50 of 215 leptospires in females and 58.8 in males, and strain BOV15 had a LD50 of 58.8 leptospires in females and 17 in males (Table 1).

Figure 1. Histopathologic findings of leptospirosis in hamster tissue after intraperitoneal inoculation in virulence test. A. Haemorrhages in the lungs (black arrow) of hamsters five days after infection with BOV3. B. Haemorrhages in the lungs (black arrow) and jaundice (white arrow) of hamsters six days after infection with BOV15.



Source: Authors.

Signs of leptospirosis were observed from the 5th day post infection and the mean period of death of hamsters was 10 DPI. However, animals experimentally infected with BOV3 strain died between 8 and 16 DPI while BOV15 strain caused death in hamsters between 8 and 15 DPI.

In this study, we report the isolation of two *Leptospira* strains from bovine kidney tissue in slaughterhouses in the city of Pelotas. In addition, we characterized the virulence of isolates belonging to Canicola serogroup. Although the animals came from municipalities close to Pelotas, the isolations demonstrate that the ecosystem of the southern region is rich in diversity of microorganisms, which had already been evidenced in previous studies with the isolation of different serogroups (Silva et al., 2008; Silva et al., 2009; Diniz et al., 2011).

The inoculation of leptospires in hamsters remains an essential model in many aspects for the study of leptospirosis pathogenesis (Haake, 2006). In our study, the virulence test revealed two strains that cause experimental leptospirosis in hamsters, with clinical and pathological manifestations similar to those described in humans and other animals.

Table 1. LD50 of virulent *Leptospira* strains.

Isolate	LD50 experiment			
	Female (215 leptospires)		Male (59 leptospires)	
	Inocula	Days until death(%)	Inocula	Days until death(%)
BOV3	10^4	9,9,10,12 (100)	10^4	8,9,10,10 (100)
	10^3	10,10,13,15 (100)	10^3	9,10,10,12 (100)
	10^2	15 (25)	10^2	10,12 (50)
	10^1	- (0)	10^1	16 (25)
	10^0	- (0)	10^0	- (0)
	Female (59 leptospires)		Male (17 leptospires)	
	Inocula	Days until death(%)	Inocula	Days until death(%)
BOV15	10^4	9,9,9,11 (100)	10^4	8,9,9,11 (100)
	10^3	12,12,13,15 (100)	10^3	10,11,11,11 (100)
	10^2	14,14 (50)	10^2	10,11,14 (75)
	10^1	14 (25)	10^1	13,14 (50)
	10^0	- (0)	10^0	- (0)

Source: Authors.

Compared with previous studies, the isolate BOV15 is similarly virulent to *L. interrogans* strain Fiocruz L1-130, widely used in immunoprotection experiments, but like BOV3, it is substantially less virulent than *L. interrogans* serovar Canicola strain Kito and *L. noguchii* serovar Autumnalis strain Bonito (Silva et al., 2008).

Virulent strains with small LD50 are required to simulate acute lethal leptospirosis in the hamster model. Moreover, as recommended, when local isolates are used in MAT panel for human and animal serological studies the sensitivity can be increase (WHO, 2003; Pinto et al., 2016). In this light, future experiments will be planned to carry out a complete characterization of this isolates.

4. Conclusion

Acute infections described here by isolates provide a basis for prospective pathogenesis studies. Moreover, the isolates may now be applied in future vaccine challenge trials and diagnostic tests.

Declaration of Interest

The authors declare that they have no conflict of interest.

Acknowledgements

We would like to thank the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), and Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS).

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