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Short communication

Isolation of naturally infecting *Leishmania infantum* from canine samples in Novy-MacNeal-Nicolle medium prepared with defibrinated blood from different animal species

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ABSTRACT

The most commonly used culture medium for the in vitro isolation of *Leishmania* spp. from canine biological samples is biphasic Novy-MacNeal-Nicolle (NNN) medium, whose solid phase is prepared using rabbit blood. *Leishmania infantum* parasites from natural infections are highly sensitive and demanding for growth in axenic conditions when firstly obtained from the dog's body. The objective of this study was to evaluate whether NNN medium (NNN-test) prepared with chicken blood (NNN-C), ox blood (NNN-O), horse blood (NNN-H) or sheep blood (NNN-S) was viable for the isolation of parasites from naturally infected dogs, in an endemic area for visceral leishmaniasis caused by *L. infantum*. Spleen aspirates from six dogs previously diagnosed as infected by parasitological methods were simultaneously inoculated in each NNN-test medium, including the conventional medium prepared with rabbit blood (NNN-R), and the cultures were examined for three weeks under optic microscopy. Spleen samples were also analyzed for parasite loads by quantitative PCR (qPCR). Cultures from three of the six dogs (50%) were positive in at least one of the NNN-test media: one sample presented the highest spleen parasite load by qPCR (1.19×10^4 parasites/mL) and was positive in all test media; the second sample presented parasitic isolation in the first week of culture in all inoculated media, of which the NNN-C medium had the highest mean parasite count (NNN-C = 23.5×10^4/mL vs. NNN-R = 3.25×10^4/mL); the third sample was positive only in the NNN-S medium besides the conventional control NNN-R. Cultures from the three remaining dogs were negative in all NNN media, including the control and test media; of those three dogs, two presented the lowest spleen parasitic loads according to qPCR. Blood from chicken, ox, horse and sheep shown to be viable for the preparation of NNN culture medium for the primary isolation of *L. infantum* from samples of naturally infected dogs and can be considered as an alternative to rabbit blood when necessary.

1. Introduction

Visceral leishmaniasis (VL) is a systemic, chronic and severe zoonosis caused by *Leishmania* protozoa (WHO, 2017), and in the Americas the disease is caused by *Leishmania infantum* (Gontijo and Melo, 2004; Kuhls et al., 2011). The culture of *Leishmania* spp. is essential for obtaining an adequate number of live and viable parasites for diagnostic and research purposes, studies on host-parasite interaction, improvement of diagnostic methods, production of vaccines, and the determination of a parasite's biological characteristics and its sensitivity to antiparasitic drugs (Visvesvara and Garcia, 2002). The classic and still most commonly used culture medium for the isolation and growth of the promastigote stages of *Leishmania* spp. from biological samples is the biphasic Novy-MacNeal-Nicolle (NNN) medium (Nicolle, 1908), in which defibrinated rabbit blood is the main ingredient of the blood agar solid phase (Palomino, 1982; Schuster and Sullivan, 2002). The classic literature on *Leishmania* parasitology recommends the use of NNN medium with rabbit blood, which was considered the best and most consistent method for the purpose of *Leishmania* isolation since the initial studies (Evans et al., 1989; Evans, 1993). Nevertheless, rabbits used for supplying blood need to be raised in a vivarium, where they are susceptible to fungal and bacterial infections, trauma, and stress, among other problems that may render blood collection from these animals unfeasible thus sometimes discontinuing its provision (Fallahi,

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2014; Kaliste et al., 2002; Whary et al., 1993). In addition, blood collection from rabbits is usually done by cardiac puncture to obtain an adequate amount of blood, which negatively affects the welfare of these animals (Limoncu et al., 1997).

Some studies have sought less expensive alternative culture media that are effective for the in vitro cultivation of *Leishmania* spp., using cow, goat and sheep milk in medium 199 (Muniaraj et al., 2007), chicken serum in RPMI 1640 medium (Nasiri et al., 2013), or human plasma and serum in liver tryptose medium (Mahamoud et al., 2013). Another study evaluated sheep blood in agar base medium to isolate and cultivate *L. major* from skin lesions of experimentally infected mice (Nasiri, 2013). However, in the case of the initial isolation of parasites from biological samples, NNN medium with rabbit blood stands as the most recommended medium (Ladopoulos et al., 2015). Nevertheless, there might be situations in which rabbit blood is not readily available, necessitating alternative blood sources for the preparation of NNN medium that have at least a similar efficiency to rabbit blood for the isolation and cultivation of *Leishmania* spp. (WHO, 2010).

Despite the importance of the subject, there is scarce information on the isolation and cultivation of *Leishmania* spp. using blood from other animal species than rabbits in the solid phase of NNN medium. Only one study has demonstrated the efficacy of the in vitro culture of *Leishmania* spp. in NNN media prepared with sheep, goat, asinine and equine blood; the authors used cultures of *L. infantum, L. donovani, L. tropica* and *L. major*, which have been isolated from human patients with classical NNN medium and previously expanded in laboratory (Ladopoulos et al., 2015). To the best of our knowledge, no literature data on *L. infantum* primary isolation or using dog samples have been published concerning the use of NNN medium so far. Accordingly, the objective of the present study was to experimentally determine the feasibility of using the defibrinated blood of chicken, ox, horse and sheep as a substitute for rabbit blood in the preparation of the blood agar solid phase of NNN culture medium for the primary isolation of *Leishmania* spp. from splenic aspirates of naturally infected dogs from an endemic area for *L. infantum*.

2. Material and methods

2.1. Animals, biological samples and ethics

Six dogs were selected without regard to breed, sex or age from an area endemic for human and canine VL caused by *L. infantum* in Camaçari City, State of Bahia, at Northeastern Brazil. Dogs were sedated with acepromazine (0.1 mg/kg) for splenic aspirates, whose procedures for sample collection and handling was performed as previously described (Barrouin-Melo et al., 2006a). The reason why spleen was chosen for sampling is that previous studies have demonstrated that it provides superior sensitivity results in culture when compared with other organs in the dog, namely lymph nodes (Barrouin-Melo et al., 2004; Solcà et al., 2014), skin, blood, bone marrow or the eye's conjunctiva (Solcà et al., 2014). The selection criterion for dogs was to present a natural *Leishmania* spp. infection confirmed by a positive result in direct parasitological examination by cytology of the bone marrow and/or lymph nodes.

For the sources of blood, one specimen each of ovine (*Ovis aries*), bovine (*Bos taurus*), equine (*Equus caballus*), chicken (*Numida meleagris*), and rabbit (*Oryctolagus cuniculus*) were selected among herds created in a semi-extensive system, kept in the facilities of the School of Veterinary Medicine and Zootechny of UFBA, Salvador and Oliveira dos Campinhos, Bahia State. All animals were clinically examined to assure that they were healthy and free of ectoparasites. Blood samples were collected using the most appropriate method for each species: a maximum blood volume of 15 mL was collected from the large animals (sheep, ox and horse) by puncture of the jugular vein, and 8 mL of blood was collected by puncture on the brachial vein in chickens or the marginal ear vein in rabbits.

The present study underwent ethical review and was given approval by the Committee of Ethics on Animal Use of the School of Veterinary Medicine and Zootechny of the Federal University of Bahia (CEUA-UFBA Nº 19/2011).

2.2. DNA extraction and quantitative PCR

For DNA extraction of the canine splenic aspirates, a commercial PureLink® Genomic DNA kit (Invitrogen®, USA) was used according to the protocol provided by the manufacturer. The quality and concentration of each eluate were evaluated using an L-Quant (Loccus®, Brazil) spectrophotometer for an average absorbance value of 1.8 at 260/280 wavelengths. The detection and quantification of *L. infantum* was performed with a qPCR reaction using the hydrolysis probe technology (TaqMan®, USA), according to Rolão et al. (2004). The sequences used for detection were sense – 5′-GGTTAGCCGATGGTGGT CTT-3′ and anti-sense – 5′-GCTATATCATATGTCCAAGCACTTACCT-3′ and a TaqMan® probe (5′-ACCACCTAAGGTCAACCC-3′). Reactions were developed for the StepOnePlus™ thermocycler (Applied Biosystems, USA), at a final volume of 25 µL, containing DNA (100 ng), 0.5 µL of custom probe, 1.25 µL of primers and 12.5 µL of 2x TaqMan® Universal PCR Master Mix, No AmpErase® UNG (Applied Biosystems, USA). The thermal cycling conditions were as follows: a 10 min initial incubation at 95 °C, followed by 44 cycles of 95 °C for 15 s and 60 °C for 1 min. The parasitic DNA load was determined in each sample by comparing the data with a specific standard curve based on the number of *Leishmania* per µL. Ten-fold serial dilutions of the DNA stock solution were performed to obtain the six points of the curve spanning from 10^6 to 10^1 DNA equivalent parasites.

2.3. Preparation of the Novy-MacNeal-Nicolle (NNN) culture medium

The blood samples were mechanically defibrinated by slow agitation in Erlenmeyer flasks containing glass beads for 10 min at room temperature.

The NNN medium was prepared according to the manufacturer's instructions by diluting the agar base (Columbia blood Acumedia®, USA) in distilled water. The agar solution was autoclaved for 15 min at 121 °C and cooled to 45–50 °C. Next, in sterile conditions, the defibrinated blood of each animal species was added to a cooled aliquot of the 1.4% agar solution at a proportion of 5 mL of blood to 20 mL of agar solution, similarly to the procedure adopted to prepare the classical NNN-rabbit medium (Evans et al., 1989; Evans, 1993; WHO, 2010). The mixture was homogenized until a uniform color was obtained and then distributed into plastic tubes with lids (Greiner®, USA) at a volume of 1.5 mL per tube and set on a slope at a 45° angle. As a control, conventional NNN was prepared with rabbit blood.

2.4. Cultivation of canine spleen samples and Leishmania spp. isolation

Samples of spleen aspirates from the dogs were individually diluted in fluid Schneider's culture medium (Sigma®, USA) supplemented with 20% fetal bovine serum (Gibco®, USA) and 100 mg/mL gentamicin antibiotic (Sigma®, USA), comprising a 15 mL volume that was mixed to form a macroscopically homogeneous suspension. Each suspension was divided into 1.5 mL fractions; each fraction was designated for one of the NNN-test media prepared with the blood of the different animal species: NNN-Chicken, NNN-Ox, NNN-Horse, NNN-Sheep, and NNN-Rabbit. All canine samples were tested in duplicate for each NNN test, totaling 10 fractions of simultaneous cultivation for each splenic aspirate sample.

All of the cultures were incubated at 24–26 °C and analyzed weekly using 40× optical microscopy in a Neubauer chamber for the visualization and counting of *Leishmania* promastigotes. Readings were made for three weeks.