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Brief Note

A glass bead protocol for recovery of host cell free *Ehrlichia canis* and quantification by Sybr-green real-time PCR

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ABSTRACT: *E. canis* infection of the canine cell line DH82 is a routine in studies with this bacteria. A protocol for isolation of host cell free bacteria was developed based on the use of glass beads. Improvement of infection with *E. canis* isolated by this method was detected by real-time PCR.

The *Anaplasmataceae* family of Gram-negative endobacteria are tick-borne pathogens of human and veterinary interest. *Ehrlichia canis*, the causative agent of canine monocytic ehrlichiosis, is one of the most important species affecting dogs worldwide. The *in vitro* cultivation of these bacteria in the canine macrophage cell line DH82 is a powerful tool for studies of host-pathogen interaction and validation of susceptibility to antibiotics (Branger *et al*., 2004; Cheng and Ganta, 2008). Moreover, studies of gene expression of *in vitro* cultured *E. canis* have the potential of identifying targets for the development of new drugs to combat endobacteria. Methods for purifying host cell-free *E. canis* have been described and are an important prerequisite for such studies (Zhang *et al*., 2007; Cheng and Ganta, 2008). In this communication we present our protocol, modified from Cheng and Ganta (2008) for isolation and storage by freezing of host cell free *E. canis*. We also present a Sybr-Green real-time PCR, based on the major surface protein 4 gene sequence (msp4), for detection of the bacteria.

The *E. canis* São Paulo strain (Aguiar *et al*., 2008) was used for infection of DH82 cells cultured in 25 cm^2 flasks, incubated at 37ºC, 5% CO_2. After reaching 90 to 100% infection, the cells were detached from the flask bottom with the help of a disposable scraper and homogenized. A 5 ml aliquot was transferred to a sterile centrifuge conical tube containing about 200 glass beads. After homogenization, tubes were centrifuged for 1 minute at 150 g. The supernatant was divided into 1 ml aliquots and transferred to 1,5 ml microtubes. After centrifugation for 15 minutes, at 15.500 g and 4ºC, the supernatant was discarded and the cell pellet ressuspended in 500 µl of freezing medium consisting of 70% culture media, 20% fetal bovine serum and 10% DMSO. Stocks were frozen at -80ºC.

The defrosted content of a microtube was used for infecting healthy DH82 cells and the presence of morulae was confirmed by Diff-Quick staining

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(LaborClin®, Brasil), after five days infection. These results demonstrate that our isolation and freezing method generates viable *E. canis* cells for post infection of DH82 cells.

A Sybr-Green real time PCR assay was also employed for quantification of *E. canis* after isolation by the glass beads method. The DNEasy Blood & Tissue kit (Qiagen) was used for the extraction of genomic DNA of the isolated bacteria. Two newly designed primers p28fwd3 5'-CAAGCATGTCCTCCGCAAG-3' and p28rev3 5'-ATCAGTACCAACACCTGCAC-3' were used for amplification and were based in the msp4 gene sequence of the *E. canis* Jake strain (GenBank: CP000107). The amplicon genereated was 146 pbs long and encompassing positions 1279114 to 1279259 of the reference sequence. PCR reactions and fluorescence detection were performed using a Stratagene Mx3000P (Stratagene, La Jolla, CA) and a Brilliant SYBR Green QPCR Master Mix (Stratagene, La Jolla, CA). The final volume of reactions were 25 µl and contained 150 nM of each primer. The thermal cycler program was 95ºC for 10 min, followed by 40 cycles of 95ºC for 15 s, 60ºC for 20 s, 72ºC for 20 s, followed by a dissociation curve.

The comparison of Ct values in the samples with known amounts of DNA indicates that the concentration of isolated bacteria are $1{,}63 \times 10^5$ bacterial cells/µL, when glass beads were used for isolation, and $1{,}3 \times 10^3$ bacterial cells/µL when glass beads were not used. The protocols described in the literature for isolation of *Ehrlichia* sp. use sonication for homogenization and release of the bacteria from host cells (3, 6). Alternativelly, density gradient centrifugation is also used (5). Ganta *et al*. (2007) used glass beads for dispersing *E. chaffeensis*, a related *E. canis* species, and immediate use without freezing in the infection of mice. Zhang *et al*. (2004) also used sonication and storage by freezing of *E. chaffeensis* for infection of the human monocyte cell line THP1. In the case of *E. canis*, only the methods using sonication were described until now and without freezing the isolated bacterial cells before infection.

Our method uses glass beads in the step of homogenization to increase the release and concentration of isolated bacterial cells. Further optimization of this protocol will allow sinchronization of infection of DH82 cells. This will facilitate, for example, *in vitro* experiments for screening of drugs which can block adherence of the bacteria to the host cell or the process of lysosome and phagosome fusion during the life cycle of *Ehrlichia spp* (Zhang *et al*., 2007).

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