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Scarcity of *Hepatozoon americanum* in Gulf Coast tick vectors and potential for cultivating the protozoan

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Abstract

American canine hepatozoonosis (ACH) is a debilitating tick-borne disease characterized by pyrexia, body wasting, myopathy, mucopurulent ocular discharge, and periosteal proliferation. The causative agent, *Hepatozoon americanum,* is an apicomplexan that utilizes the Gulf Coast tick, *Amblyomma maculatum,* as its definitive host and vector. Unlike most tick-borne disease agents, *H. americanum* is not transmitted via a tick bite, but is transmitted when canids ingest a tick vector that contains sporulated oocysts within the tick hemocoel or paratenic hosts with cystozoites. Our understanding of *H. americanum* prevalence is based on its detection in the intermediate host, wild or domestic canids, with domestic canids often showing clinical signs at the time of diagnosis. The frequency of *H. americanum* in *A. maculatum,* on the other hand, is unknown; this gap in our knowledge hinders our understanding of transmission risk. Furthermore, current diagnostic assays are limited in efficacy, and serologic assays are not widely available. To begin to address gaps in our knowledge, we developed a TaqMan® multiplex qPCR assay for *H. americanum* detection in *A. maculatum* tick extracts and evaluated infection rates in questing adult *A. maculatum.* Additionally, we used a co-culture system to expose *H. americanum* stages to host cells for *in vitro* development. Results from qPCR analysis of over 500 tick extracts revealed no positive

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samples; this suggests both low transmission risk by adult Gulf Coast tick ingestion in the sampled areas, and that surveillance should be focused in areas where ACH has been diagnosed at higher frequencies. *Hepatozoon americanum* was detectable by qPCR in co-culture of an infected canine buffy coat with ISE6 (*Ixodes scapularis* embryonic) tick cells, and microscopic examination of samples from those days revealed some structures that were suspicious for developing stages. These data are a starting point for future work to advance our understanding of *H. americanum* transmission and mechanisms of disease in canids with ACH.

1. Introduction

The current understanding of the tick-borne protozoan, *Hepatozoon americanum*, is limited to its role as the agent of American canine hepatozoonosis (ACH). Originally believed to be caused by a virulent strain of *Hepatozoon canis*, further investigation, prompted by the consistently higher pathogenicity of canine hepatozoonosis inside the United States than cases found outside the U.S., demonstrated that ACH was a unique disease caused by a novel species, *H. americanum*. Comparison of the two agents demonstrated that the proposed *H. americanum* was distinct from *H. canis* in histopathology of tissue stages, gamont prevalence (lower in *H. americanum*) and morphology, serological assays, and the failure of the expected invertebrate host for *H. canis, Rhipicephalus sanguineus,* to be experimentally infected with *H. americanum* (Vincent-Johnson et al., 1997). Currently, the diagnosis of ACH may be achieved through molecular detection or visualization of *H. americanum* gamont stages in the blood or meronts in muscle (Li et al., 2008; reviewed in Allen et al., 2011). Canine patients present with clinical signs of pyrexia, body wasting, myopathy, periosteal proliferation, and ocular discharge. Prognosis is poor and treatment options limited; while parasite suppression is possible, no available treatment eliminates the protozoa. In contrast, infection with *H. canis,* which is common in other parts of the world, is often subclinical, with occasional reports of fever, depression, anorexia, and lethargy in canids that are immunocompromised, have a comorbidity, or present with high parasitemia (Vincent-Johnson et al., 1997).

In addition to its clinical distinction from *H. canis, H. americanum* has a different primary vector. While the brown dog tick, *R. sanguineus*, is the definitive host and primary vector for transmission of *H. canis* to canids, the Gulf Coast tick, *Amblyomma maculatum*, serves in this role for *H. americanum* (Mathew et al., 1998). Canids, considered accidental intermediate hosts, are thought to only be infected with *H. americanum* either through ingestion of the tick harboring sporulated oocysts or predation of paratenic hosts harboring cystozoites (Ewing et al., 2002; Johnson et al., 2009). Once an ingested tick vector reaches the gastrointestinal tract, *H. americanum* sporozoites are released and migrate through the blood or lymphatics until reaching tissues, predominately skeletal muscle, where host-cell invasion takes place. Merogony then occurs, leading to large "onion skin" cysts in the muscle. The release of merozoite stages from these cysts leads to the characteristic

pyogranulomatous myositis that defines much of the clinical presentation for ACH. In the U.S., the geographical distribution of ACH primarily overlaps that of *A. maculatum,* encompassing what appears to be an expanding region that currently includes the Southeast, Gulf Coast, and South Atlantic Coast. This distribution suggests that a wide population of wild and domestic canids are potentially susceptible to infection with *H. americanum.*

To date, the development of a quantitative (q)PCR assay has been invaluable for diagnosing ACH using blood samples from a canine host (Li et al., 2008). While this is beneficial on a case by case basis, the epidemiologic data describing *H. americanum* infection is limited to documented clinical cases in canids. The prevalence of infection in the tick vector is currently unclear; minimal to no surveillance data exists regarding *H. americanum* in wild populations of its definitive host. Previous identification of the organism in *A. maculatum* relied on dissection of ticks for visualization of oocysts with sporozoites (Mathew et al., 1998; Mathew et al., 1999). Adult *A. maculatum* that fed as nymphal stages on an infected coyote had an 80% infection rate with *H. americanum,* and nymphal stages are capable of harboring oocysts after infection as larvae, giving some insight into possible infection rates in nature (Kocan et al., 2000; Ewing et al., 2002). Insight into infection rates of *H. americanum* in *A. maculatum* populations using molecular identification would reveal areas of risk for transmission to canid hosts. However, to our knowledge, an assay to screen *A. maculatum* for *H. americanum* was not previously available. Another hindrance advancing research on *H. americanum* is the lack of an isolate. A single report of *H. canis* isolation using DH82 cells suggests that the cultivation of *H. americanum* may be possible (Harikrishnan and Ponnudurai, 2009). Isolating the organism would speed the development of diagnostic assays, such as serological assays for detection of exposure.

We had two objectives for this study. Our first was to develop a TaqMan® multiplex qPCR assay for detecting *H. americanum* in *A. maculatum* using the 18S rRNA gene target and utilize this assay on locally collected adult *A. maculatum* in Mississippi. Our second was to isolate *H. americanum* in co-culture with cell lines.

2. Methods

2.1. Development of a TaqMan® qPCR for H. americanum

We chose the 18S rRNA gene to detect *H. americanum* as this is the only gene, to our knowledge, in which partial sequences are available for *H. americanum*, and it is widely used for genetic analyses of *Hepatozoon* spp., including *H. americanum* (Baneth et al., 2000; Starkey et al., 2013). First, we aligned a portion of the 18S rRNA gene *H. americanum, H. canis,* and *A. maculatum* to identify a location for primers that maximized differences between *Hepatozoon* spp. and *A. maculatum.* Then, we identified a nucleotide probe sequence that contained one base pair difference between *H. americanum* and *H. canis.* Plasmid standards for the *H. americanum* and *A. maculatum* amplicons were generated using a pCR4™ TOPO™ vector, One Shot™ TOP10 Chemically Competent *E. coli*, and a PureLink™ Quick Plasmid Miniprep Kit. A TaqMan® multiplex quantitative PCR was designed using Brilliant® Multiplex qPCR Master Mix in a final volume of 25 μL, where 5 μL was used for template volume. Efficiencies were calculated using 10-fold dilutions of plasmid standards (10^7 - 10^0 copies), with individual assays considered acceptable only

when efficiencies fell between 90% and 110% and the R^2 was   0.985. We initially used an Agilent Mx3005P qPCR system and later moved the assay to an Agilent AriaMx qPCR system for 2019 samples, after the assay was confirmed to perform the same on the new system. See Supplementary Table for a complete list of resources used, including primers and probes.

2.2. Evaluation of field-collected Gulf Coast ticks using qPCR

We collected questing, adult *A. maculatum* via flagging and dragging areas in and surrounding Oktibbeha County, Mississippi during the summers of 2018 and 2019. DNA from *A. maculatum* was extracted using a DNeasy® Blood and Tissue Kit, whereby ticks were first macerated with a scalpel blade in the initial step of the manufacturer's protocol. DNA extracts were stored at −20 °C until analysis using the TaqMan® multiplex qPCR assay. We additionally analyzed archived DNA extracts from adult *A. maculatum* collected in Mississippi in 2010, 2014, and 2015. DNA from all individual *A. maculatum* was extracted using a DNeasy® Blood and Tissue Kit, whereby ticks were first macerated with a scalpel blade in the initial step of the manufacturer's protocol. Samples from 2014 and 2015 were extracted from half ticks, with the other half frozen (−80 °C) for potential isolation of rickettsiae, as part of another study (Lee et al., 2017). DNA extracts were stored at −20 °C until analysis using the TaqMan® multiplex qPCR assay.

While no confirmed negative *A. maculatum* tick was included in each assay, we tested *A. maculatum* adult ticks from a colony-reared population (purchased from Oklahoma State University) that was not exposed to canid hosts to evaluate DNA extractions performed using whole ticks and midguts removed from tick for adult *A. americanum*. However, to assess whether *H. americanum* could be amplified in the presence of *A. maculatum* DNA, we prepared ten-fold dilutions (1:5 through 1:50,000) of *H. americanum* DNA (extracted from the blood of a canine ACH case) and *A. maculatum* DNA, included undiluted extracts of *H. americanum* and *A. maculatum* as controls and tested these in triplicate. Non-template controls and dilutions of plasmid standards were included in ever qPCR assay. For a complete list of resources, see Supplementary Table.

2.3. Culture isolation for H. americanum

During our efforts to culture *H. americanum,* we initially explored two approaches, with the rationale for each approach dependent on the source of infectious material, i.e., either adult archived *A. maculatum* ticks or blood from a known or suspected *H. americanum*-infected canine. Our archived tick samples from 2014 and 2015 were initially frozen with the intent of cultivating prokaryotes (rickettsiae), not eukaryotes, and thus, were not immersed in a freezing media containing a cryoprotectant, such as dimethyl sulfoxide. For *A. maculatum* extracts of tick halves that were potential positives (i.e., Ct values were present, but outside the assay's linear range), we macerated and transferred the archived frozen tick sample onto a confluent monolayer of DH82 cells with MEM (Minimum Essential Medium) supplemented with 10% FBS (Fetal Bovine Serum) in vented flasks, maintained at 37 °C with 5% CO_2. Medium was replaced in cell culture flasks biweekly, thus the original buffy coat inoculum was considered no longer present after approximately a week, having been discarded in the process of feeding. Our rationale for choosing a canine histiocytic cell line

with morphology and phagocytic capabilities like macrophages was that potentially infected ticks would contain infectious sporozoites, which, upon ingestion by the canid, would enter leukocytes. Additionally, a previous publication suggested the development of *H. canis* in DH82 cells may have occurred (Harikrishnan and Ponnudurai, 2009).

We focused on efforts to cultivate *H. americanum* in tick cell co-cultures when blood samples from infected canines became available, as the source of *H. americanum* stages would be fresh, and stages would be more likely to be alive. Between 2017 and 2019, blood samples for any *H. americanum*-suspect or known positive cases identified in the clinics were collected in additional EDTA tubes under a protocol approved by the Institutional Animal Care and Use Committee, IACUC# 15–037. Samples were processed to collect buffy coat for cell culture (to enrich for the presence of gamont-infected mononuclear cells) and for cytospins that were stained using a HEMA 3 Stain for microscopic examination. When adequate blood volumes were obtained, additional samples were also saved for DNA extraction of the blood. Buffy coats from all cases were transferred to complete medium used for ISE6 (*Ixodes scapularis* embryonic cell) co-culture and the suspension was overlaid onto a confluent monolayer of ISE6 cells in complete L15B medium (Munderloh and Kurtti, 1989), made as described in the publication, with a modification to increase FBS to 20% and eliminate the bovine lipoprotein concentrate. ISE6 cultures were maintained in nonvented flasks at 30 °C with no additional CO_2. Our rationale for utilizing ISE6 (tick) cells was because gamonts are the infective stage for the tick definitive host. Upon ingestion by *A. maculatum*, infective gamonts continue their development in midgut epithelial cells, undergoing gametogenesis and fertilization. Oocysts containing sporocysts filled with sporozoites ultimately fill the tick hemocoel. Some co-cultures that were initiated in ISE6 cells were later passed into a new monolayer of ISE6 cells. To further evaluate the method used by Harikrishnan and Ponnudurai, 2009, the buffy coat from one canine sample was transferred as above to DH82 cells.

Cell culture samples from inoculated flasks and uninoculated control stock flasks were periodically collected for microscopic examination using stained cytospins and TaqMan® qPCR analysis, as described above or without the additional *A. maculatum* primers and probe. For a complete list of resources, see Supplementary Table.

3. Results

3.1. Evaluation of questing adult A. maculatum for H. americanum

We evaluated 531 *A. maculatum* tick extracts from Mississippi for *H. americanum* (Table 1). While several tick extracts were "potentially positive" based on initial Ct values, they remained outside the assay's linear range on re-evaluation. While the standard curve ranged from 10^7 to 10^0 copies, 10^0 was not consistently achieved even when efficiencies were within 90–110%, and the limit of detection was sometimes 10^1 copies. For some extracts initially tested using 3 μL of template, with Cts falling outside the linear range of the assay, some were re-analyzed using 5 μL of the template but still fell outside the linear range preventing classification as "positive." No tick extracts from 2019 were considered potential positive. All extracts were positive for the *A. maculatum* MIF gene target, confirming successful DNA extractions.

All *A. maculatum* ticks from Oklahoma State University midgut and whole tick samples demonstrated similar levels of tick DNA (copies of the amplified MIF gene target), and all were negative for *H. americanum* DNA. We were unable to obtain any known *H. americanum*-infected *A. maculatum* to test extraction and qPCR, either within our resources or from colleagues. When mixing DNA from *H. americanum*-infected blood (gamont stages) and adult *A. maculatum* ticks, we were able to amplify the 18S rRNA target for *H. americanum* in samples that had approximately 73 copies of the *H. americanum* 18S rRNA gene in the presence of 8.13×10^5 copies of the *A. maculatum* MIF gene.

3.2.   Culture isolation for H. americanum

None of three frozen *A. maculatum* "half" ticks that were "potential positives" based on initial qPCR results of DNA extracts, and which were introduced into DH82 co-cultures, generated evidence of *H. americanum* isolation. Samples collected from these co-cultures were negative by qPCR and we observed no suspect organisms in stained cytospins. We received blood samples from four canine ACH cases at Mississippi State University College of Veterinary Medicine that were processed for cell co-culture. One case was later confirmed negative by diagnostic laboratory results; culture aliquots from that sample were also negative by qPCR; no suspect organisms were observed in stained cytospins. We recognize that since we did not perform cytospins on the same sample material that was used towards DNA extraction, the *H. americanum* detected by qPCR in that aliquot may not have been intact or present in the sample used for cytospin. Alternatively, a sample negative for *H. americanum* by qPCR assay may have had an organism on cytospin that was missed. A summary of qPCR results (copy number of *H. americanum*) and relevant images are provided in Table 2 and Fig. 1 for cell co-cultures taken from one of the canine cases that was known to be infected with *H. americanum* (diagnosed). Of the two remaining cases that were used for co-culture, one sample extract that was inoculated into an ISE6 flask had *H. americanum* copy numbers of 3.66×10^4 and 1.15×10^4 after 4 days and 12 days in culture but was lost to contamination after approximately 3 weeks. The remaining buffy coat sample from the fourth case was transferred to two flasks of ISE6 cells, neither of which were positive for *H. americanum* based on qPCR of samples taken six days to ~ two months in co-culture. This case was considered likely negative and had no additional diagnostic data available to confirm ACH.

4.   Discussion

The primary source of *H. americanum* infection, whether tick vector or paratenic host, has not yet been identified; this makes it challenging to prioritize preventative measures for ACH. While epidemiological data derived from the detection of *H. americanum* in the diseased canine demonstrate active cases, we lack an understanding of disease risk from infected tick vectors and paratenic hosts. In this study, we focused on addressing infection rates of *H. americanum* in Gulf Coast tick vectors. Further, with a single report of cultivation of another canine *Hepatozoon* sp. (*H. canis*) using a continuous cell line, to our knowledge (Harikrishnan and Ponnudurai, 2009), we believed it was necessary to repeat co-culture attempts using *H. americanum* stages in appropriate cell lines.

We developed a TaqMan® multiplex qPCR to evaluate the infection prevalence of *H. americanum* in *A. maculatum*. Although we detected evidence of *H. americanum* at low copy numbers in some extracts, none could be confirmed by retesting as all were outside the linear range of our assay, where the minimum detectable copies of *H. americanum* was 10^0 or 10^1, in cases when 10^0 copy number would not amplify. There is a possibility that *H. americanum* was present below the limit of detection for our assay, and future optimization of the assay would include attempting to maintain 10^0 copy number limit by increasing efficiency requirements to 95% - 105%. Although experimental transmission studies of *H. americanum* from canids to *A. maculatum* suggest higher infection rates in tick populations are possible compared to rates detected in our study (Mathew et al., 1998; Kocan et al., 2000; Ewing et al., 2002), lack of confirmed positive ticks in this study may reflect limitations in the study or low natural prevalence in nature due to alternative routes of infection. We were limited by not having known positive ticks that could be subjected to the same extraction technique and qPCR assay as unknown ticks. In place of this, we used a mixture of *H. americanum* DNA extract and *A. maculatum* DNA extracts that allowed us to detect *H. americanum* in *A. maculatum* DNA at the equivalent of 3.5 copies to 1000 copies, respectively. In the future, we will process any known positive adult Gulf Coast ticks (e.g., collected from an ACH case, experimentally infected canine, or wild canine known to have *H. americanum*) through our extraction protocol, then test with our optimized assay, to evaluate a true positive control.

In addition, the ability of naïve immature *A. maculatum* tick populations to acquire *H. americanum* gamonts and become infected adult ticks is dependent on the availability of infected, parasitemic canids as a host for immature tick feeding (Ewing et al., 2002). As *H. americanum* infection has been detected from the blood of resident dogs, shelter (free-roaming) dogs, and canine with clinical signs of ACH, in an endemic area of Oklahoma, it is not unlikely that Gulf Coast ticks in that area should harbor sporozoites of *H. americanum* (Allen et al., 2008). Similar surveillance of canids is lacking in Mississippi at this time, and future studies should focus on surveying Gulf Coast ticks in highly endemic areas, such as Oklahoma, as well as canids in Mississippi. Furthermore, information on immature stages, particularly nymphal ticks, as well as paratenic hosts, may offer more insight on the primary transmission route for ACH, at least in Mississippi. However, paratenic hosts or nymphal Gulf Coast ticks may contribute a more significant source of infection to domestic canids, with paratenic hosts likely reservoirs of *H. americanum*. Common vertebrate hosts for immature ticks include ground-dwelling birds and rodents (particularly cotton rats), as well as coyotes, though not typically domestic canids (as reviewed in Teel et al., 2010). Thus, the role of infected immature ticks in the transmission of *H. americanum* to canids is more likely as a source of infection to potential paratenic hosts. Predation of paratenic hosts was linked to two ACH outbreaks in Oklahoma canids, with cystozoites identified in rodents and rabbits from experimental trials conducted during the investigation or naturally infected in the area; no infected ticks were detected on the paratenic hosts or premises (Johnson et al., 2009). These findings support our current data, which suggest that adult *A. americanum* in a region with reported ACH cases are not the primary source of *H. americanum* infection to domestic dogs.

Our efforts to propagate *H. americanum* were promising when using fresh canine blood from ACH cases that presented to the clinic, with visualization of circulating gamonts in stained buffy coats at the time of preparation. This was based on qPCR data demonstrating evidence of *H. americanum* DNA in cultures maintained after the initial inoculum was presumed to be removed, and on the evidence of potential developing intracellular gamonts as they differentiated to gametes; visualization of a zygote was not confirmed, though at least one of the images showed a potentially early zygote with a diffuse nucleus (Fig. 1B) (Mathew et al., 1999). Additional optimization includes passage of archived cultures that have since been frozen and use of these passages for processing to continue testing by qPCR and for further examination by light microscopy as well as electron microscopy. As no specific cryoprotectant was used for the preservation of protozoa when tick halves were initially frozen, it is likely that freezing conditions used as part of a previous study did not support the survival of any protozoa that may have been present. While unlikely that protozoa would have survived long-term freezing in archived tick halves from this study, our initial attempts using co-cultures with DH82 cells were useful as an exercise of the procedure for future application. Using appropriate cryopreservation medium (e.g., containing 10% dimethyl sulfoxide) for protozoa in ticks used for archiving, and trying other cell lines, including other embryonic tick cell lines or perhaps primary cells, may lead to successful isolation as we address future efforts to cultivate this canine protozoan.

5. Conclusion

Based on the infection rates from this study, adult Gulf Coast ticks in Mississippi may not be the primary source of *H. americanum* in canids. However, evaluating known positive ticks that have fed on experimentally infected canids is necessary to better assess the qPCR assay. Co-culture in tick or mammalian cells may support *H. americanum* cultivation if we optimize the conditions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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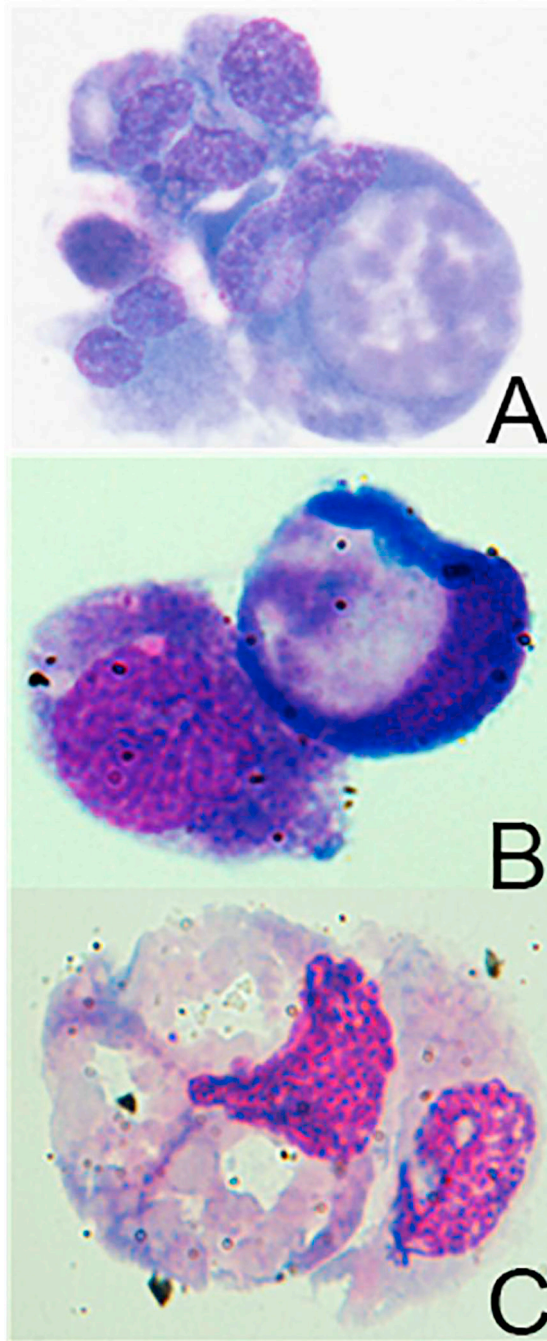
Fig. 1.
Images of intracellular gamont-like organisms *H. americanum* from samples taken after (A)
18 days in culture; (B) 8 days in co-culture after passage (ISE6 P1–1) and (C) 53 days in
co-culture (ISE6 P/S 1 + 2). Cytospin preparations were stained using a Hema 3 Stain Kit.

Table 1

Adult *A. maculatum* tick extracts (archived and new collections) tested by *H. americanum* TaqMan® multiplex qPCR.

Year	No. tick extracts tested	Reference
2010	48	Ferrari et al., 2012
2014	150	Lee et al., 2017
2015	102	Lee et al., 2017
2018	129	*This study*
2019	102	*This study*

Table 2

Hepatozoon americanum copy numbers detected in cell culture aliquots removed from co-cultures of canine buffy coats with ISE6 cells.

Sample	Days in culture	Number of *H. americanum* copies per 5μL sample
ISE6 P/S[a] 1	18	2.077×10^2
ISE6 P/S 2	18	2.442×10^2
ISE6 1	18	9.043×10^1
ISE6 2	18	1.924×10^2
ISE6 1	39	1.58
ISE6 P1[b] −1	8	1.32
ISE6 P/S 1 + 2[c]	53	7.38
ISE6 1 + 2	53	3.55

P/S, denotes addition of penicillin-streptomycin to these flasks.

[a]ISE6, *Ixodes scapularis* embryonic tick cell line (additional number after ISE6 was used to identify individual flasks, or when samples from flasks were pooled for extraction).

[b]P1, denotes a passage.

[c]1 + 2, denotes a pooled sample from two flasks.