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Evaluation of an attenuated strain of *Ehrlichia canis* as a vaccine for canine monocytic ehrlichiosis

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ABSTRACT

Canine monocytic ehrlichiosis is an important tick-borne disease worldwide. No commercial vaccine for the disease is currently available and tick control is the main preventive measure against the disease. The aim of this study was to evaluate the potential of a multi-passaged attenuated strain of *Ehrlichia canis* to serve as a vaccine for canine monocytic ehrlichiosis, and to assess the use of azithromycin in the treatment of acute ehrlichiosis. Twelve beagle dogs were divided into 3 groups of 4 dogs. Groups 1 and 2 were inoculated (vaccinated) with an attenuated strain of *E. canis* (#611A) twice or once, respectively. The third group consisted of naïve dogs which served as controls. All 3 groups were challenged with a wild virulent strain of *E. canis* by administering infected dog-blood intravenously. Transient thrombocytopenia was the only hematological abnormality observed following inoculation of dogs with the attenuated strain. Challenge with the virulent strain resulted in severe disease in all 4 control dogs while only 3 of 8 vaccinated dogs presented mild transient fever. Furthermore, the mean blood rickettsial load was significantly higher in the control group (27–92-folds higher during days 14–19 post challenge with the wild the strain) as compared to the vaccinated dogs. The use of azithromycin was assessed as a therapeutic agent for the acute disease. Four days treatment resulted in further deterioration of the clinical condition of the dogs. Molecular comparison of 4 genes known to express immunoreactive proteins and virulence factors (*p30*, *gp19*, *VirB4* and *VirB9*) between the attenuated strain and the challenge wild strain revealed no genetic differences between the strains. The results of this study indicate that the attenuated *E. canis* strain may serve as an effective and secure future vaccine for canine ehrlichiosis.

1. Introduction

Canine monocytic ehrlichiosis (CME) is an important tick-borne disease of dogs worldwide [1–7]. It is a multisystemic disease manifesting in acute, subclinical and chronic forms [1,8,9]. The outcome of the latter form is death [9]. Common clinical signs include anorexia, lethargy, fever, lymphadenomegaly, splenomegaly and bleeding, while thrombocytopenia and pancytopenia are common hematological findings [1]. Doxycycline and other tetracyclines are the therapeutic agents of choice for CME [10–15]. Several other antibacterial agents with variable efficiency have also been studied [15–17].

To date, no commercial vaccine for CME exists and tick control remains the main preventive measure against this disease. A previous study has shown that an inactivated vaccine was capable of provoking a rapid humoral and cellular response directed at *Ehrlichia canis* antigens, however only a partial clinical protection was achieved when the dogs were challenged with a virulent strain [18]. An earlier experimental infection, based on results from 2 dogs, suggested that an Israeli strain of *E. canis* [Attenuated #611 (#611A)] which was retrieved from a naturally infected dog in Israel has become attenuated after 8 years of alternate passages in canine (DH82) and murine (J774.A1) cell lines. It appeared to lose its virulence to dogs and failed to infect ticks [19]. The primary goal of this study was to evaluate the potential of this *E. canis* strain (#611A) to serve as a potential vaccine for CME in a larger group of dogs by following clinical, hematological and serological parameters, and using molecular methods to detect infection and find genetic differences in virulence-associated and other genes between the attenuated strain and the wild strain used for challenge. Furthermore, the potential therapeutic effect of the macrolide antibiotic azithromycin was evaluated.

2. Materials and methods

2.1. In vitro cultivation of Ehrlichia canis

An attenuated strain of *E. canis* (#611A; Deposited in the Polish Collection of Microorganisms (PCM) Ludwik Hirszfeld Institute of Immunology and Experimental Therapy, Wroclaw,

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Poland, under accession number B/00023) was cultivated in DH82 canine macrophage cell line at 37 °C and 5% CO_2 as previously described [19,20]. Infected DH82 cells were used for experimental inoculation of the dogs in groups 1 and 2 to test the potential of this strain to serve as a vaccine.

2.2. Experimental infection of dogs with Ehrlichia canis

Twelve *E. canis*-free, laboratory-bred, 12–24 months old female beagle dogs, vaccinated for rabies, distemper, adenovirus type 2, parainfluenza, parvovirus and Leptospira were used in this study. The dogs were acclimatized for 4–5 weeks before the initiation of the study and divided into 3 groups of 4 dogs each. Group 1 dogs were initially inoculated subcutaneously (SQ) with 4.8×10^9 attenuated #611A *E. canis* bacteria [$\sim 1.2 \times 10^6$ infected DH82 cells; harvested 3 h before inoculation; suspended in MEM-salts base (Biological Industries, Kibbutz Beit Haemek, Israel); enriched with 15% fetal calf serum, glutamine, non-essential amino acids and 1% penicillin–streptomycin; infection rate of 80–90%; total inoculation volume of 2.4 ml] on day 0, and again on day 213 post initial inoculation with 9.6×10^9 of the attenuated *E. canis* bacteria (SQ; $\sim 2.4 \times 10^6$ infected DH82 cells; harvested 3 h before inoculation; inoculation volume of 4.8 ml). Group 2 dogs were initially inoculated subcutaneously with uninfected DH82 cells (day 0; $\sim 1.2 \times 10^6$ uninfected DH82 cells) and then with 9.6×10^9 #611A strain bacteria on day 213. The third group (group 3) consisted of naïve dogs which constituted the control group. They joined the study on day 393, acclimatized for 12 days, and were then subcutaneously inoculated with $\sim 1.2 \times 10^6$ uninfected DH82 cells on day 405 post initial inoculation of the first group. Twenty three days later (day 428 of the study), all 12 dogs (3 groups) were intravenously inoculated with 6 ml *E. canis*-infected blood containing 6×10^7 *E. canis* wild strain bacteria, drawn from a clinically acute ill, 9 years old, dog from Rishon Le-Zion, Israel; July 2011) 17 h prior to inoculation and kept refrigerated at 4 °C until inoculation. Quantitation of rickettsial load both in the cultures and the blood was determined by quantitative real-time PCR (qPCR) as will be described later. The *E. canis* infected blood was tested microscopically by stained blood smear examination and no other hemoparasites could be detected. It was also molecularly screened for the presence of *Hepatozoon canis 18S rRNA*, *Babesia* spp. *18S rRNA* and spotted fever group *Rickettsia* spp. *ompA* gene fragments, as previously described [21–23], and was found negative for all.

Monitoring of the dogs included daily inspection, physical examination at least twice weekly and a weekly body weight recording. Blood was withdrawn (5 ml from each dog in EDTA tubes, at least once weekly) and complete blood count analysis was carried out using the ADVIA 120® Hematology System (Bayer, Germany).

The study was carried out according to the Hebrew University guidelines for animal experimentation and was approved by the Institutional Animal Care and Use Committee.

2.3. Treatment

Azithromycin (Azithromycin, 200 mg/5 ml, Teva, Israel) treatment was administered to all 4 dogs in group 3. It was initiated at day 15 post challenge (day 443) when all four dogs presented fever, anorexia, lethargy and thrombocytopenia. The planned treatment protocol was 7 mg/kg, PO, q24 h for 5 days as a loading dose followed by the same dose q72 h for additional 15 days [24]. The initial loading dose was administered for 4 days, however it was discontinued due to severe clinical deterioration of 2 of the 4 treated dogs with no improvement in the other 2. At this stage (day 19 PC), azithromycin was replaced by doxycycline (10 mg/kg, PO, once daily) which was administered for 21 days.

2.4. DNA extraction

DNA was extracted from 250 μl blood and eluted in 200 μl elution buffer, using a commercial kit (Illustra blood genomicPrep mini spin kit, GE Health Care, UK), following the manufacturer instructions.

2.5. Quantitative real-time PCR

A quantitative estimation of the *E. canis* rickettsial load was performed by qPCR using the Rotor-Gene 6000 Real-time PCR analyzer (Corbett Life Science, Australia) and the *E. canis*-16S plasmid as previously described [25]. Standard curve was designed using decimal dilutions of the *E. canis*-16S plasmid. 3 μl DNA were used for each real-time PCR reaction targeting the *16S rRNA* gene using primers *E. canis*-16S-F (TCGCTATTAGATGAGCCTACGT) and *E. canis*-16S-R (GAGTCTGGACCGTATCTCAGT) and Syto 9 fluorescent nucleic acid dye.

2.6. Serology

Serological screening of all sera was performed using a commercial ELISA test kit (ImmunoComb, Biogal, Galed, Israel) as previously described [26]. The concentration of *E. canis* antibodies for each sample was recorded by a scanner designed for automatic reading of the color intensity of the reaction spots on the test kit. The results were recorded as optical density (OD) units. The following formula was used for calculation of results expressed as net absorbance: Antigen Absorbance Value/Positive Reference Absorbance × 1000.

2.7. Conventional PCR and sequencing

In order to identify possible sequence differences between the attenuated *E. canis* (#611A) and the wild strain used for challenge, four different genes including *VirB4*, *VirB9*, *gp19* and the *p30* were targeted by PCR as previously described [27–31] (Supplementary material, Table 1). All PCR procedures were carried out using a conventional thermocycler (T1 Thermocycler, Biometra, Germany). Positive and negative controls were used in each reaction.

Supplementary material related to this article is found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2012.10.003.

PCR products were run on 1% agarose gel electrophoresis, stained with ethidium bromide (1 mg/ml) and visualized using a UV illuminator (Kodak EDAS 290, USA). Positive PCR products were sequenced using the BigDye Terminator cycle sequencing chemistry from Applied Biosystems (ABI), (ABI PRISM 3730xl DNA Analyzer, CA, USA) and ABI's Data collection and Sequence Analysis software.

Sequences were initially imported into the Sequencher 5.0 (Gene Codes® Corp., USA). Chromatograms were individually examined visually in order to confirm the quality of sequences. The forward and reverse sequences of each PCR-product were assembled into a contig, and each individual contig was visually inspected and verified. Any ambiguity was visually resolved, and a final consensus sequence was generated for further analyses. Sequences with poor quality chromatograms were excluded from the study and the whole procedure was repeated. Finally, in order to compare the sequences of the gene fragments between the attenuated and the wild virulent strain, the obtained consensus sequences were blasted using NCBI's megablast algorithm (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi).

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2.8. Statistical analysis

Mixed models for estimating associations between treatment group and body temperature, thrombocytes count, rickettsial load and antibody titers were used. Dog identification was used as a random effect, while treatment group and sample day (i.e. time effect) entered the model as fixed effects. All data management and analyses were performed using SAS version 9.3 (SAS Institute, 2006) [32]. *P*-values <0.05 were considered statistically significant.

3. Results

3.1. Clinical signs

No clinical signs were observed in any of group 1 dogs following their initial and second inoculation (days 0 and 213, respectively) with the attenuated strain of *E. canis*. Likewise, no clinical signs were observed in group 2 dogs following inoculation with uninfected DH82 cells (day 0) and following inoculation with the attenuated strain of *E. canis* (day 213).

Significant differences in body temperature were found on day 3 post second inoculation (PSI; day 216) when group 1 dogs had a higher body temperature compared to group 2 dogs ($P = 0.0291$), and on day 7 PSI (day 220) when group 1 had an elevated temperature compared to group 2 dogs ($P = 0.001$; Fig. 1).

Following challenge with the wild virulent *E. canis* strain, three dogs from groups 1 and 2 developed transient mild to moderate fever (reference body temperatures of 37.5–39.5 °C). Dog #1 (group1) had mild fever (40.1 °C, 40.0 °C and 39.6 °C) on days 17 (day 444), 19 (day 447) and 20 (day 448) post challenge (PC), respectively. Dog #3 (group 1) had mild fever (39.8 °C) on day 6 PC (day 434), and mild to moderate fever (40.4 °C and 39.9 °C) on days 19 (day 447) and 20 PC (day 448), respectively. This dog also developed mild fever (39.9 °C) on days 25 (day 453) and 27 PC (day 455; 39.7 °C). Dog #7 (group 2) experienced two episodes of mild fever (40.0 °C in both) on days 16 (day 444) and 20 PC (day 448).

Following challenge with a wild virulent *E. canis* strain, all four group 3 dogs experienced severe clinical disease with lethargy, anorexia and persistent fever. During this period the mean body temperature ranged between 40.1 °C (day 13 PC) and 39.7 °C on day 16 PC. Two of these four dogs (dogs # 9 and 11) developed severe hypothermia (35.5 °C and 35.7 °C) on day 20 PC (day 448). Intensive care treatment was initiated and included intravenous administration of pre-warmed saline solution and intra-muscular injection of oxytetracycline (Alamycin 20% LA, Norbrook, UK) at a dose of 20 mg/kg.

Following challenge, significant differences in body temperature between group 1 dogs and the naïve control dogs were detected on day 14 PC (day 442) where the naïve dogs had an increased temperature ($P = 0.0034$), on days 16 (day 444; $P = 0.0098$) and 18 PC (day 446; $P = 0.0392$) where group 3 dogs had a higher body temperature compared to group 1 dogs. This trend of higher body temperature in the group of the naïve dogs was reversed beginning on day 20 PC (day 448). On this day the body temperature of group 3 dogs was lower ($P < 0.0001$) compared to group 1, followed the next day by another reduction ($P < 0.0001$). Significant moderation in body temperature reduction was detected on days 22 ($P = 0.0002$), 23 ($P = 0.0083$) and 27 ($P = 0.00591$) PC. No significant differences were found between groups 1 and 2 dogs.

3.2. Platelet counts

3.2.1. First inoculation

Initial reduction in the number of blood platelets was recorded in group 1 dogs on day 10 post inoculation (PI) with the attenuated strain reaching a mean of $186 \times 10^3/\mu l$ (Fig. 2). The lowest mean value was observed on day 24 PI when mean platelet count reached a nadir of $105 \times 10^3/\mu L$. The platelet counts of group 1 dogs returned to normal values on day 31 PI. Dog #4 (group1) was an exception as its platelet counts remained below the normal reference range for a longer period (days 10–166 PI). The platelet counts of all group 2 dogs (inoculated with un-infected DH82 cells) remained within normal reference values (reference level of $200–500 \times 10^3$ platelets/μl) during this period. Significant differences were found on days 20 and 24 post inoculation, when the thrombocyte counts were lower in group 1 dogs compared to group 2 ($P = 0.04$ and 0.006, respectively).

3.2.2. Second inoculation

One day post second inoculation with attenuated strain, group 1 (vaccinated twice) developed mild thrombocytopenia ($193 \times 10^3/\mu l$) which was reversed by day 3 PSI. During this period, significant differences were recorded on days 1 and 3 post second inoculation (days 214 and 216; $P = 0.0014$ and 0.047, respectively).

3.2.3. Challenge inoculation

Thrombocytopenia was initially observed in group 3 dogs on day 11 PC (day 439, mean platelet count $124 \times 10^3/\mu l$) (Fig. 2). Mild thrombocytopenia (mean count of $172 \times 10^3/\mu l$) in group 2 dogs was first detected on day 14 PC (day 442). On day 18 PC (day 446) groups 2 and 3 reached their lowest mean thrombocyte counts ($70 \times 10^3/\mu l$ and $47 \times 10^3/\mu l$, respectively), while group 1 reached its lowest level on day 19 PC ($52 \times 10^3/\mu l$). Three days after initiating doxycycline treatment (group 3 dogs, only), platelet counts increased to a mean of $155 \times 10^3/\mu l$. Thrombocyte counts returned to normal reference range in all three groups on day 39 PC. Significantly lower platelet counts were recorded in group 3 dogs compared with group 1 dogs on days 8 and 11 PC (days 435 and 438; $P = 0.0391$ and 0.005, respectively). No significant differences in the platelet counts were found between groups 1 and 2 throughout the post challenge trial phase.

3.3. Rickettsial load

3.3.1. First inoculation

Peak mean rickettsial load was detected in group 1 dogs on day 20 PI with 946 *16S rRNA* gene copies/μl-blood. No *E. canis* DNA could be detected in all 4 group 1 dogs on day 44. Thereafter, a single episode of DNA detection per dog occurred in 2 dogs on days 60 and 100 PI, respectively.

3.3.2. Second inoculation

The mean rickettsial load found in group 2 at the second phase was 188 copies/μl-blood on day 17 PSI (day 230), while the mean rickettsial load in group 1 was very low throughout this period (0–0.5 *16S rRNA* gene copies/μl-blood). No differences in rickettsial load were detected during this period except for day 17 PSI (day 230) in which group 2 dogs had higher load compared to group 1 dogs ($P = 0.0009$). Rickettsial DNA could not be detected in any of group 1 dogs on days 10–160 post second inoculation.

3.3.3. Challenge inoculation

No *E. canis* DNA was detected in any of the 12 dogs (3 groups) on the challenge day (prior challenge) with the wild strain. Marked differences in the rickettsial load were noticed following challenge with a wild virulent strain, when comparing groups 1 and 2 (pre inoculated with the attenuated strain) with group 3 dogs (controls). On day 14 PC, the mean rickettsial load for group 1 dogs was 243 *16S rRNA* gene copies/μl-blood, for group 2 dogs 78 *16S rRNA* gene copies/μl-blood, and for group 3 dogs 7202 *16S rRNA* gene copies/μl-blood. On day 18 PC, significant differences were

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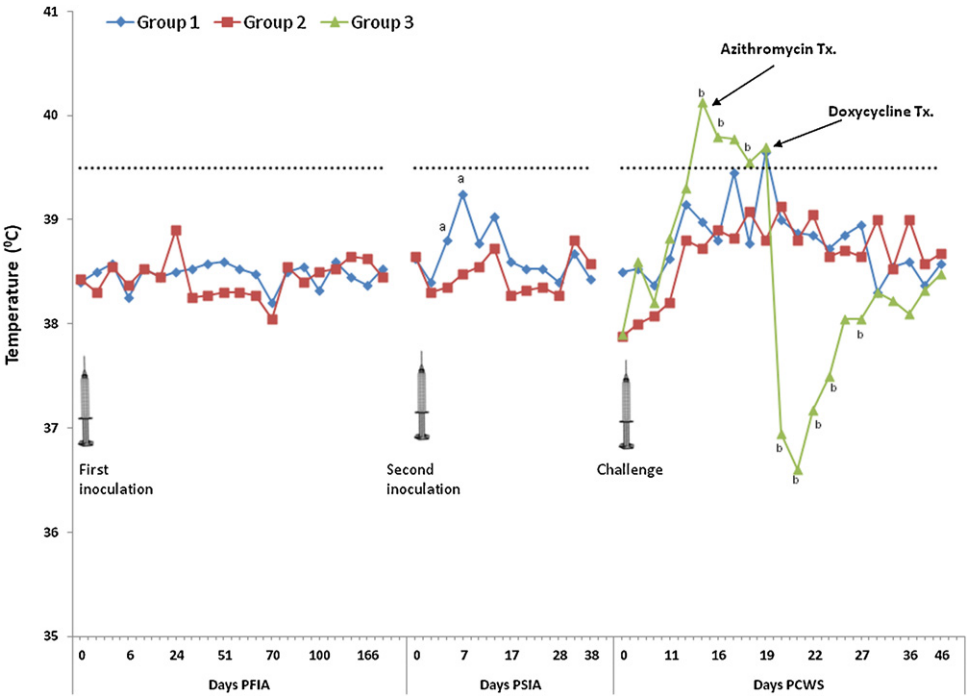

Fig. 1. Mean body temperature (◦C) measured in 3 groups of dogs during three trial phases. The *X*-axis represents sampling days (the distance between sampling days is not proportional on a true time scale). Group 1 – vaccinated twice with attenuated *E. canis* bacteria. Group 2 – vaccinated once with an attenuated *E. canis*. Group 3 – control non-vaccinated group. Syringes indicate time of inoculation of the attenuated *E. canis* strain (#611A) (first inoculation – day 0; second inoculation – day 213) and challenge with wild virulent strain (day 428). Arrows indicate the initiation of treatment with azithromycin on day 15 post challenge (day 443) and doxycycline on day 19 post challenge (day 447) in group 3 dogs. Dotted horizontal line represents the upper normal reference range of body temperature. PFIA – post first inoculation with attenuated strain; PSIA – post second inoculation with attenuated strain; PCWS – post challenge with wild strain; "a" represents significant statistical differences between groups 1 and 2; "b" represents significant statistical differences between groups 1 and 3.

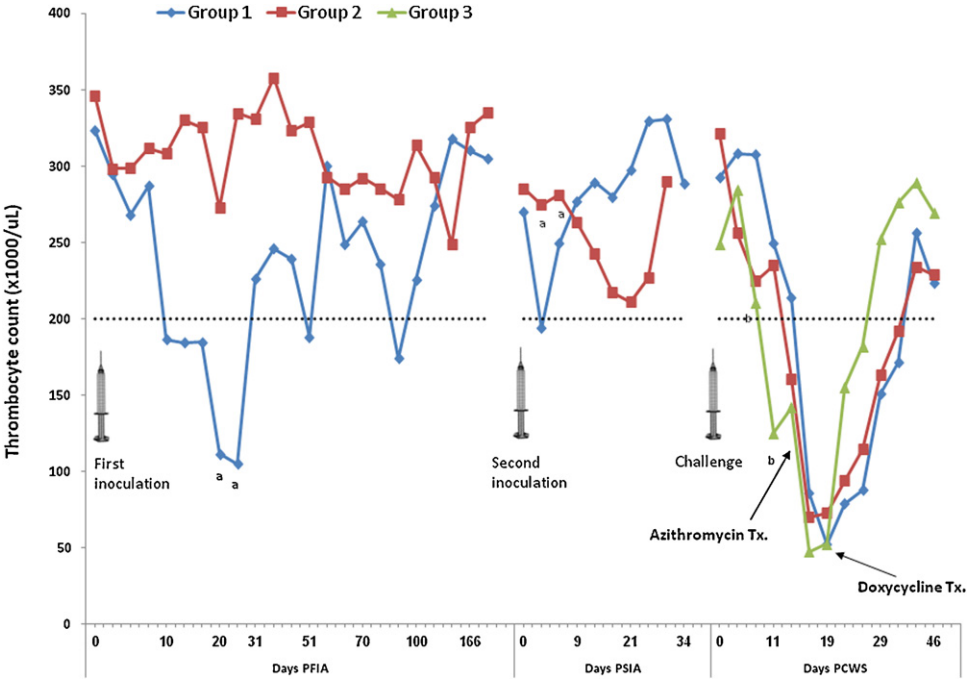

Fig. 2. Mean thrombocyte counts measured in 3 groups of dogs during three trial phases. The *X*-axis represents sampling days (the distance between sampling days is not proportional on a true time scale). Group 1 – vaccinated twice with attenuated *E. canis* bacteria. Group 2 – vaccinated once with an attenuated *E. canis*. Group 3 – control non vaccinated group. Syringes indicate time of inoculation of the attenuated *E. canis* strain (#611A) (first inoculation – day 0; second inoculation – day 213) and challenge with wild virulent strain (day 428). Arrows indicate the initiation of treatment with azithromycin on day 15 post challenge (day 443) and doxycycline on day 19 post challenge (day 447) in group 3 dogs. Dotted horizontal line represents the lower normal reference range of thrombocyte count. PFIA – post first inoculation with attenuated strain; PSIA – post second inoculation with attenuated strain; PCWS – post challenge with wild strain; "a" represents significant statistical differences between groups 1 and 2; "b" represents significant statistical differences between groups 1 and 3.

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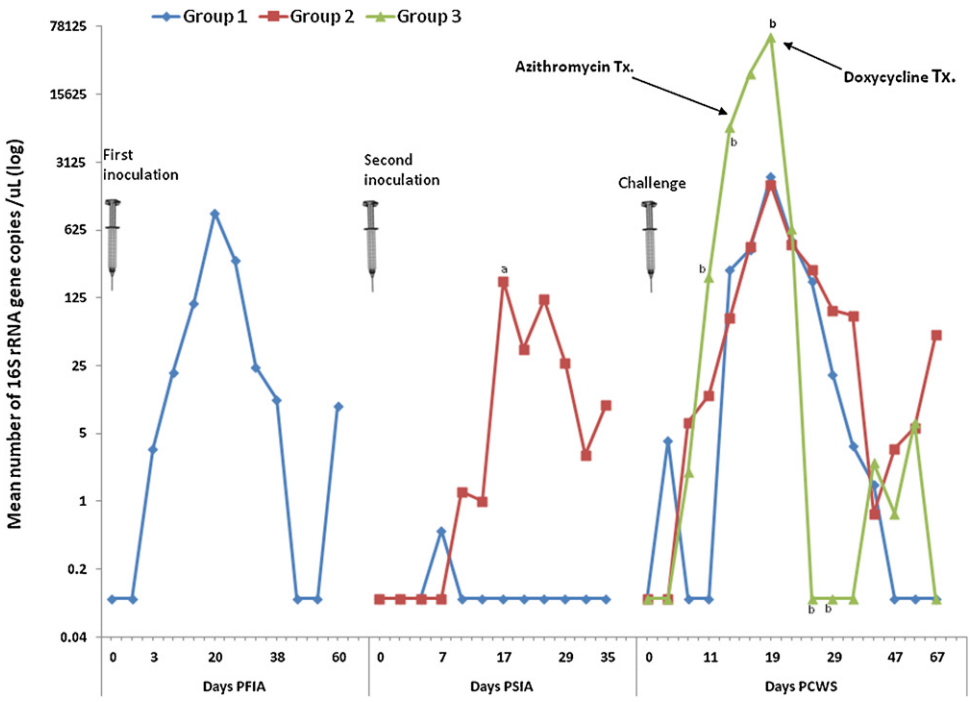

Fig. 3. Mean number of *16S rRNA* gene copies/μl measured in 3 groups of dogs during 3 trial phases. The *X*-axis represents sampling days (the distance between sampling days is not proportional on a true time scale). Group 1 – vaccinated twice with attenuated *E. canis* bacteria. Group 2 – vaccinated once with an attenuated *E. canis*. Group 3 – control non vaccinated group. Results are expressed in log of the absolute copy numbers. As log 0 reflects an error, results equal to zero copy number were considered as 0.1. Syringes indicate time of inoculation of the attenuated *E. canis* strain (#611A) (first inoculation – day 0; second inoculation – day 213) and challenge with wild virulent strain (day 428). Arrows indicate the initiation of treatment with azithromycin on day 15 post challenge (day 443) and doxycycline on day 19 post challenge (day 447) in group 3 dogs. PFIA – post first inoculation with attenuated strain; PSIA – post second inoculation with attenuated strain; PCWS – post challenge with wild strain; "a" represents significant statistical differences between groups 1 and 2; "b" represents significant statistical differences between groups 1 and 3.

recorded between the rickettsial loads of groups 1 and 2 versus group 3 (396, 424, and 25,906 mean *16S rRNA* gene copies/μl-blood, respectively. Marked differences between the 3 groups were observed in the mean blood rickettsial load on day 19 PC (2,237, 1,847 and 61,586 *16S rRNA* gene copies/μl-blood in groups 1, 2 and 3, respectively). On this day, severe clinical signs were noticed in group 3 dogs which included severe hypothermia, anorexia and lethargy. After doxycycline administration (day 19 PC), marked reduction in rickettsial load was observed (Fig. 3).

Comparison of modeled results by log of rickettsial load, revealed significantly higher loads in group 3 dogs compared with group 1 dogs on days 11, 14 and 19 PC (days 439, 442 and 447; $P < 0.0001$, =0.0088 and =0.0151, respectively). Significant lower rickettsial loads were detected in group 3 compared to group 1 dogs on days 25 and 29 PC (days 453 and 457; $P = 0.0003$ and 0.0107, respectively). No significant differences were found in the rickettsial loads between groups 1 and 2 dogs throughout the post challenge period.

3.4. Serology

3.4.1. First inoculation

First significant increase in mean antibody titers in group 1 dogs compared to day 0 was noticed on day 6 post inoculation. This trend continued throughout this period.

3.4.2. Second inoculation

Significant differences in mean antibody titers were noticed between group 1 and 2 dogs from day 0 of second inoculation through day 9 PSI ($P < 0.0001$ and =0.046, respectively). Thereafter no significant differences were found in this phase (Fig. 4).

3.4.3. Challenge inoculation

Post challenge, significant higher mean antibody titers were detected in the sera of group 1 dogs (vaccinated twice) compared to group 3 dogs ($P < 0.05$) throughout this period. When groups 1 and 2 were compared, higher mean antibody titers were detected in group 2 on day 4 PC ($P = 0.014$), while higher mean antibody titers were detected in group 1 on day 33 PC ($P = 0.039$).

3.5. Sequence comparison

No differences were found in the sequences of the 4 membrane- and virulence associated gene fragments between the attenuated and the wild virulent strain analyzed.

4. Discussion

This study confirmed that the attenuated *E. canis* strain (#611A) could potentially protect dogs from CME and serve as a vaccine for the disease. It supports a limited previous study which suggested that this strain might serve as a vaccine for CME [19]. While the previous study used only 2 dogs and no controls were included, a larger group of dogs participated in the current study including a control group. No clinical signs were presented in this study by groups 1 and 2 dogs following the inoculations with the attenuated strain using 2 different inocula suggesting its relative safety. When all three groups were intravenously challenged with a high dose of a virulent wild strain, significant differences were observed between the dogs inoculated with the attenuated strain (groups #1 & #2; vaccinated dogs) and the control group (group #3). All four control group dogs developed severe clinical signs including lethargy, anorexia and persistent fever beginning at day 13 PC (day 441) and reaching peak fever on days 17–18 PC (days 445–446).

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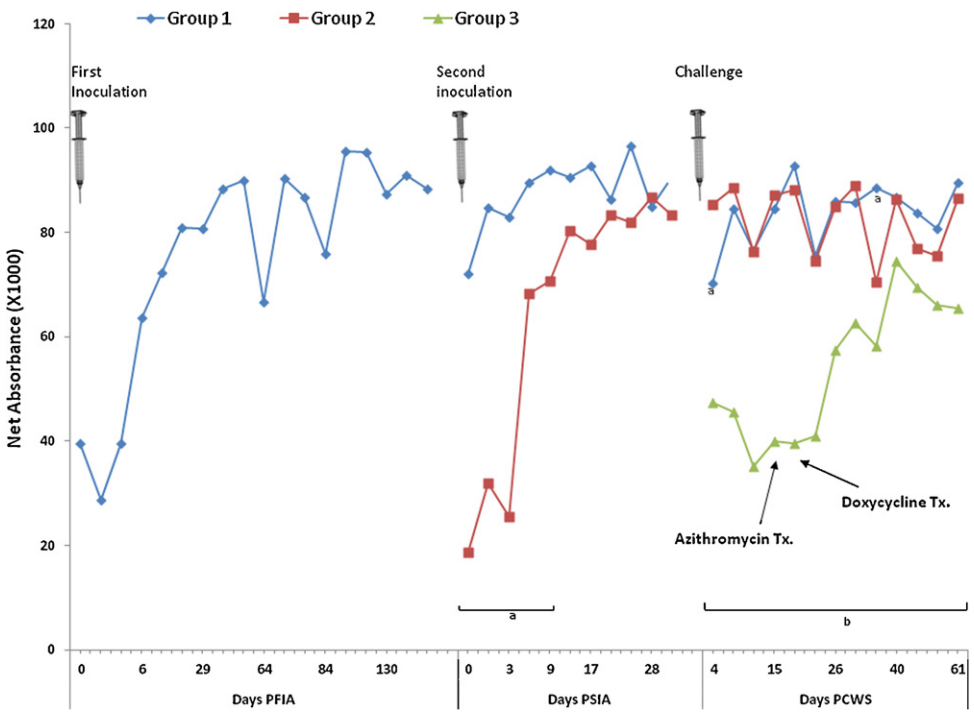

Fig. 4. Mean antibody titers measured in 3 groups of dogs during 3 trial phases. The *X*-axis represents sampling days (the distance between sampling days is not proportional on a true time scale). Group 1 – vaccinated twice with attenuated *E. canis* bacteria. Group 2 – vaccinated once with an attenuated *E. canis*. Group 3 – control non vaccinated group. Results are expressed in net absorbance. Syringes indicate time of inoculation of the attenuated *E. canis* strain (#611A) (first inoculation – day 0; second inoculation – day 213) and challenge with wild virulent strain (day 428). Arrows indicate the initiation of treatment with azithromycin on day 15 post challenge (day 443) and doxycycline on day 19 post challenge (day 447) in group 3 dogs. PFIA – post first inoculation with attenuated strain; PSIA – post second inoculation with attenuated strain; PCWS – post challenge with wild strain; "a" represents significant statistical differences between groups 1 and 2; "b" represents significant statistical differences between groups 1 and 3.

Furthermore, 2 of the control dogs developed life threatening clinical disease with severe hypothermia (35.5 °C and 35.7 °C), lethargy and anorexia which necessitated urgent intervention to spare their lives. At the same time, only 3 of the 8 vaccinated dogs (groups 1 and 2) showed fever which was mild in most cases and transient in all dogs.

Marked differences were observed in the rickettsial loads between the control dogs (group 3) and dogs vaccinated with the attenuated strain (groups 1 and 2) post challenge with a wild virulent strain (Fig. 3). A positive association between rickettsial loads and clinical presentation in CME was demonstrated before in another experimental study [30]. In our study, the mean rickettsial load reached a level that was 92 folds higher during peak disease (day 14 PC) in the control dogs when compared to rickettsial load found in the vaccinated groups. These findings, together with the absence of clinical disease in most group 1 and 2 dogs, indicate that the latter dogs were immunized by inoculation with the attenuated strain. The marked differences between the rickettsial load post challenge with the wild strain indicated the efficiency of the attenuated strain and its potential to serve as a vaccine. It can also explain the differences in the clinical outcome between the groups. The significantly lower rickettsial loads in groups 1 and 2 are associated with the absence of clinical signs in 5 of 8 dogs in these groups. Reduction of bacteremia as judged from the qPCR assay in both vaccinated groups (#1 and #2) was rapid, while the reduction in rickettsial load in the control group (#3) was achieved only after the initiation of doxycycline treatment. This also indicates the immunization effect of the attenuated strain in the vaccinated dogs.

Thrombocytopenia is considered as the most common and consistent hematological abnormality in dogs infected with *E. canis* [35,36]. Thrombocytopenia occurred in all dogs included in this study. Nevertheless, following challenge with the virulent strain, the onset of thrombocytopenia in the control dogs (group 3) appeared 4–5 days earlier and significant lower counts were recorded on days 8 and 11 PC in this group compared to the vaccinated group 1 dogs. In addition, the platelet counts of groups 1 and 2 dogs returned to normal reference range without therapeutic intervention following the inoculation with the attenuated and the challenge strains. In contrast, the thrombocytopenic phase experienced by the control group was reversed only after initiation of doxycycline treatment. The transient decrease in platelet counts in the vaccinated dogs in the different study phases might be associated with post vaccinal immune thrombocytopenia mediated by the injection of high-dose intracellular bacteria or with the possible capability of the attenuated strain to induce the production of antiplatelet antibodies [36].

It is important to note that the dogs in this study were challenged intravenously in contrast to intradermal administration of the rickettsiae by ticks in natural cases [34]. Moreover, the infectious dose used in this study was high. Although there is no published information on the rickettsial load transmitted by the ticks in natural *E. canis* infections, it is likely that the transient mild clinical signs, and the hematological signs, that occurred in some of the vaccinated dogs post challenge could have been due to this high dose and may have been milder if intradermal inoculation with a lower inocula were used for challenge [33].

Azithromycin was evaluated in this study as a potential alternative to tetracyclines and other suggested therapeutic agents for the treatment of CME [12,15,16,37,13]. This was done based on previous trials indicating the efficacy of this drug in the treatment of other rickettsial diseases such as Rocky Mountain Spotted Fever, Mediterranean Spotted Fever and Scrub Typhus [38–41]. The failure to alleviate clinical and hematological abnormalities following the administration of azithromycin, the further deterioration in the clinical condition with life-threatening situation in 2 of 4 dogs, and the further increase in rickettsial load during treatment in the

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4 control dogs led us to switch treatment to doxycycline which proved effective. Thus, it is concluded that azithromycin was not effective in the treatment of *E. canis* infection.

Molecular comparison of 4 genes known to express immunoreactive proteins and virulence factors (*p30*, *gp19*, *VirB4* and *VirB9*) between the attenuated and the challenge wild strain was carried out. No sequence changes that could explain the differences in the virulence between these 2 strains were found. Our results indicate that the tested gene fragments were still present and unchanged in the attenuated strain, potentially contributing to the protective immune response seen in the vaccinated dogs. Other methods, such as whole genome sequencing, comparing these two strains should be employed in future studies to detect possible attenuation at the DNA level and potentially elucidate the mechanism of attenuation.

The role of humoral antibody response in CME immunity is poorly understood [42]. However, it is likely to play an important role in the response against *E. canis* alongside cellular mechanisms. The attenuated strain used as a vaccine in this study elicited high antibody response that persisted throughout the study. The latter phenomenon was in contrary to the reduction and disappearance of ehrlichial DNA in group 1 dogs, as judged by the qPCR assay used in this study. The difference between the antibody titers and molecular results post vaccination will have to be taken into account in evaluation of dogs vaccinated with the attenuated *E. canis* strain (#611A).

5. Conclusions

This study provides initial results indicating that the attenuated strain of *E. canis* (#611A) reduced severity of clinical signs and bacterial loads in dogs post challenge with a wild strain. Our results suggest that it may serve as a future vaccine for CME subject to further fine tuning of the vaccination dose and evaluation of challenge via tick infection. Although no evidence of reversion of the attenuated strain to a pathogenic state occurred in the vaccinated dogs participating in this study, future longer studies will have to exclude this optional phenomenon. A vaccine for CME is of utmost importance due to the worldwide distribution, high prevalence, and severity of this disease. Furthermore, treatment with azithromycin was shown not to be effective against *E. canis*.

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