Immune response at a vaccine-challenge study using beagle dogs and locally isolated *Leptospira* spp

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Title: Immune response at a vaccine-challenge study using beagle dogs and locally isolated *Leptospira* spp.

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Abstract

Determination of the immune response of dogs by measuring the antibody levels (utilizing MAT) and levels of cytokines (TNF-α, IL-4 and IFN-γ) post-vaccination with locally produced killed whole-celled *Leptospiral* vaccine and post-challenge with a locally isolated *Leptospira icterohaemorrhagiae* Copenhageni strain. For assessment of immunity of the vaccine serum antibodies were detected before and after vaccination and challenge in three studies. The effects of the challenge were determined by a variety of parameters including reisolation of the challenge *Leptospira spp*. via blood, urine, and kidney samples. The challenge strain did not produce generalised infection but elevated circulating antibody levels in both the control and vaccinated dogs in any of the three studies, however leptospires were reisolated from the urine of the control dogs but not the vaccinated dogs. Cytokine levels (TNF-α, IFN-γ and IL-4) were detected post-challenge in the vaccinated dogs to determine the immune profile response. The whole-killed cell vaccine in this study did not prevent leptospireamia but prevented leptospiruria in vaccinated dogs after a challenge with a live *Leptospira icterohaemorrhagiea* Copenhageni. The vaccine-challenge showed increased antibody (MAT) levels due to vaccination and infection (through challenge). Cytokine production (TNF-α, IFN-γ and IL-4) by the host immune system was observed post-challenge with live leptospires.

Introduction

Veterinary vaccines should protect against test pathogens in experimental and field conditions (Fávero et al., 2018). *Leptospira* spp. can survive for prolonged periods in the environment (Mason et al.,2016). Currently, inactivated whole-cell vaccines (bacterins) are routinely used in livestock and domestic animals; however, protection is serovar-restricted and short-term only (Dellagostin et al., 2017). The most prevalent serovars in a particular region or country are not necessarily the same throughout other countries (Martins & Lilenbaum, 2013). A previous vaccine efficacy study in Trinidad was carried out by

Suepaul et al. (2010) using local serovars Copenhageni and Icterohaemorrhagiea on hamsters. The study followed up on a previous investigation of leptospirosis cases in previously vaccinated dogs (Adesiyun et al., 2006). In this study, we attempted to determine the efficacy of a locally produced vaccine and the corresponding immune response of a vaccine-challenge study on dogs by measuring the antibody levels post-vaccination 1 and 2 (locally prepared killed whole-cell *Leptospiral* vaccine) and post-challenge with a locally isolated *Leptospira icterohaemorrhagiae* Copenhageni (utilizing microscopic agglutination test, MAT) and levels of cytokines post challenge: TNF-α, IL-4 and IFN-γ.

Methodology

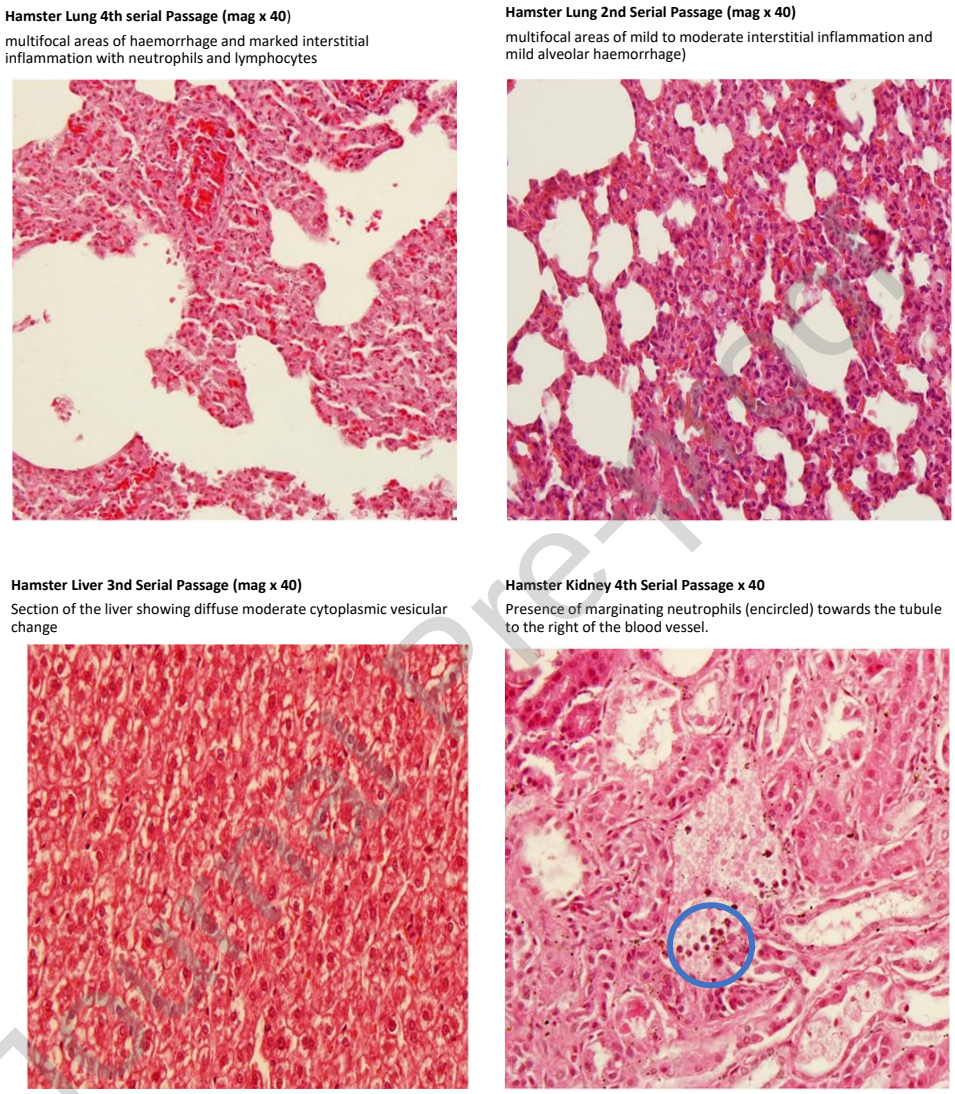
Ethics Approval

The approval for the use of dogs and hamsters for this study was granted by The University of The West Indies ethics committee before the commencement of this study.

Hamster model

The Golden Syrian Hamster, *Mesocricetus auratus*, was used in this study for the serial passage of the stored *Leptospira icterohaemorrhagiae* Copenhageni serovar. This breed is the preferred animal model for leptospiral vaccine trials (Haake, 2006). The hamsters were imported from the Charles River Laboratory (USA) and then bred and housed at The University of the West Indies (UWI) laboratory animal house. They were isolated to prevent interactions with rodents and to ensure the animals were naïve to the Copenhageni serovar. Three separate serial passages were carried out to regain the virulence of the leptospires, which were stored in culture. Figure 1 shows some of the significant lesions observed due to leptospiral challenge in the serial passage of the hamsters.

Figure 1 H&E stains to determine lesions due to Leptospiral Challenge from various organ harvesting post serial passage.



Canine model

The breed of dog used for this study was the Beagle, obtained from Ridglan Farms, WI, USA. Four dogs (3F and 1M) were shipped by the import requirements of the Ministry of Agriculture. At the UWI, the beagles were sero-tested for the presence of leptospiral antibodies against a battery of 23 live serovars (see Table 1). The housing for the beagles was retrofitted with an anti-rodent wire-mesh building and monthly maintenance with anti-rodent traps and lures around the building. Within the housing area, the original dogs were then bred (artificially inseminated and natural means) to obtain the pups required for

the three studies. There were two separate areas designated for housing: the breeding dogs and the experimental dogs.

Table 1 Serovars used to determine seronegative animals before the commencement of the experiment.

Australis Bratislavia	*Icterohaemorrhagiae Mankarso Mankarso*
Autumnalis Bim 1051	*Mini Georgia LT 117*
Autumnalis Autumnalis Akiyami	*Panama Panama CZ 214*
Ballum Arborea Arborea	*Pomona Kennewicki LT 1026*
Ballum Ballum Mus 127	*Pomona Pomona Pomona*
Bataviae Bataviae van Tienen	*Pyrogenes Pyrogenes Salinem*
Canicola Canicola	*Sejroe Hardjo Hardjoprajito*
Cynopteri Cynopteri 3522C	*Sejroe Sejroe M 84*
Grippotyphosa Grippotyphosa Moskva V	*Sejroe Wolfii 3705*
Hebdomadis Hebdomadis Hebdomadis	*Semaranga Patoc Patoc 1*
Icterohaemorrhagiae Copenhageni M20	*Tarassovi Tarassovi Perepelitsin*
Icterohaemorrhagiae Icterohaemorrhagiae Ictero 1	

Experimental Design

The initial experimental design for three studies required eight vaccinated dogs, with the equivalent of eight controls being used for each analysis (for a total of 48 dogs). Study 1 required the dogs to be at least 56 weeks old at challenge to determine the duration of immunity, 52 weeks after vaccination. Studies 2 and 3 required the dogs to be between 14-16 weeks of age at challenge to determine the onset of immunity two weeks after vaccination. However, due to reduced pups anticipated from breeding, the numbers of the animals were distributed accordingly. The experimental design (see Table 2) was as follows: Study 1: 6 vaccinated (3M & 3F at ages 56 weeks) and three controls (1M & 1F 40 weeks and 1F 16 weeks), Study 2: 6 vaccinated (4F & 2M at 16 weeks) and utilising the same controls as Study 1 as sampling times occurred at the same time, and Study 3: 4 vaccinated (2F and 2M at 16weeks) and two controls (2F at 16 weeks).

Study	Group	# of dogs	Vaccination		Challenge			
			Age of dogs V 1	Age of dogs V 2	Age of dogs at challenge	Antibody titre at challenge	Time after V 2	Dosage inoculum (live cells)
1	V C	6 3**	10 wk	14 wk	56 wk 40wk /16wk	0 0	52 wk	1-2.5 x 10^8
2	V C	6 3**	10 wk	14 wk	16 wk 40 wk/ 16 wk	80 0	2 wk	1-2.5 x 10^8
3	V C	4 2	10 wk	14 wk	16 wk 16 wk	80 0	2 wk	1-2.5 x 10^9

Table 2 Experimental Design

** the same controls were used as the two Studies were completed concurrently.

Vaccination

All dogs in the vaccinated group were vaccinated twice (sub-cutaneous) with the locally produced vaccine at ages 10 weeks and 14 weeks. The controls were injected (sub-cutaneous) with the sterile medium used to prepare the vaccine.

The dogs from Study 1 were aged 56 weeks at challenge (approximately 52 weeks post-vaccination 2) to determine the duration of immunity offered by the vaccine. Dogs from study 2 (onset of immunity) and three were aged 16 weeks at challenge (2 weeks post-vaccination 2), and study 3 (onset of immunity high dose) differed from Study 2 by the dosage of the challenge inoculum.

Sampling

The methods and procedures used in the current study were adapted from earlier published reports. (Klaasen et al., 2003; Minke et al., 2009) and modifications are discussed further in the sections below. Daily health checks were carried out on all experimental dogs twice daily: temperature checks, animal

appetence and weight, dehydration, conjunctivitis, jaundice and animal mood and temperance.

For the study, blood samples of each dog were taken -7 and 0 days before vaccine administration 1 and day 0 for vaccination 2 and on days 1, 5 and 7 post-vaccination 1 and 2. These samples would be used as baseline samples for each dog for the duration of the experiment. After vaccination through the subcutaneous route, the response to the vaccine was observed for the vaccinated and control dogs from all three studies. Samples were taken -2 days before the challenge and on days 0, 1 to 7, 14, 21 and 35. Whole blood samples and serum samples were collected. The whole blood samples were used to culture for leptospiraemia (directly into semi-solid and liquid EMJH media) and determine haematological parameters (in heparinised tubes). The serum samples were stored (at both -20 and -70^{o}C) to determine the biochemical and cytokine parameters and the antibody levels (utilising the gold standard of the Microscopic Agglutination Test).

Urine samples were taken on days 0, 7, 14, 21, and 35 either through catheterization or free flow. The 0.1 ml of the urine samples were then directly inoculated into 9.9 ml liquid and semi-solid EMJH medium (Ellinghausen-McCullough Johnson-Harris). The remaining urine sample was then spun down, the pellet resuspended in EMJH medium and then serially diluted in 1:10 fold, 1:100 fold and 1:1000 fold.

Euthanasia of animals was done humanely by a certified veterinarian, and kidneys were harvested for re-culture of leptospires.

ELISA KITS

Testing for cytokine levels was carried out using canine ELISA kits obtained from Biocompare EIAAB Wuhan, China. The kits tested three cytokines: Canine Interleukin-4: Serial E0056c, Canine Tumour Necrosis Factor-alpha: Serial E0133c and Canine Interferon Gamma: Serial E0049c. The detection ranges for the kits were: IL-4: 32.1 pg to 2000 pg; TNF-α: 15.6 pg to 1000 pg; IFN-γ: 32.1 pg to 2000 pg, respectively. The kits were compatible with the use of serum and tissue homogenate samples.

Cytokine levels were measured post-vaccine and post-challenge from selected stored serum samples. The samples were duplicated for each day measured and the mean values were recorded.

Microscopic Agglutination Test (MAT)

The MAT is the gold standard for detecting acute and long-term antibody responses to *Leptospira* spp. infection (Adler & de la Peña Moctezuma, 2010). Serum samples from all dogs were tested and were seronegative for the existence of antibodies for *Leptospira* spp. against 23 live serovars (see Table 1). Determination of Antibody titres post-vaccination and challenge utilizing MAT required the positive samples to be serially diluted from 1:10 to 1:10240 on a microtitre plate.

Leptospira spp. *used for Vaccination and Challenge inoculum*

Leptospira icterohaemorrhagiae Copenhageni strain 1S7 was used to produce the vaccine and challenge inoculum. The stock storage sample was thawed from liquid nitrogen (-210^{o}C), subcultured no more than three times, and then put through two serial passage (SP) experiments in hamsters to determine virulence (Figure 1). After the second SP, the live *Leptospira* spp. were purified and inoculated into two 8-month-old seronegative beagle dogs to re-culture from urine and blood samples and proceed to SP 3 in hamsters for final subculture and purification from tissue homogenates before producing the vaccine and challenge inoculum for each study.

Vaccine Preparation

The procedure was adapted from previous work by Suepaul et al. (2010) on a hamster model for vaccine efficacy utilising live *L. icteroheamorrhagiae* serovar Copenhageni, a locally cultured strain from local positive clinical samples.

The vaccine was prepared by growing the serial passaged and low sub-cultured *Leptospira* to densities of 1-5 x 10^{8} cells/ml. The densities were determined by counting using a cell counting chamber under a dark field microscope. After obtaining the suitable densities, the appropriate number of leptospires were then pooled to obtain 40 ml of culture and centrifuged (RC 5C Sorvall Instruments, Du Pont, Ramsey Minnesota, U.S.A) at 7500 x g for 10 minutes to obtain a pellet. The pellet was washed three times with 40 ml phosphate-buffered saline (PBS, pH @ 8.5). After the final pellet was obtained, it was re-suspended in 40 ml of buffered 10% formalin and left for 1 h to kill the leptospires. After that, the suspension was centrifuged to obtain a pellet and then washed four times using PBS. The final pellet was then re-suspended in 40 ml of PBS, and the cell count was determined as earlier described. To the final volume of the cell suspension, phenol at 2.5 mg/ml was added as a preservative, and aluminium hydroxide at 1.5 mg/ml was added as an adjuvant.

Challenge inoculum

L. icterohaemorrhagiae serovar Copenhageni strain 1S7 was sub-cultured from a virulent stored batch of a low sub-culture frequency and at peak growth phase between 7 to 10 days. The leptospires were cultured in EMJH medium and viewed under a dark-field microscope to determine the density and motility of the leptospires; they were stored in the refrigerator for no more than 2 hours before use. To standardize the exposure of each dog, the challenged dogs (all vaccinated and controls) were injected through the intraperitoneal route 2.0 ml and 0.25ml of challenge inoculum were dropped into each eye of the dog. There were two doses of inoculum of the live *L. icteroheamorrhagiae* serovar Copenhageni strain 1S7 given: a lower dose was 1-2.5 x 10^8 cells/ml was given for studies 1 and 2, and a higher dose inoculum was administered to study 3 at 1-2.5 x 10^9 cells/ml. Study 3 was necessary as the combined results obtained from studies 1 and 2 did not show typical symptoms of leptospirosis infection in both control or vaccinated dogs post-challenge. The reference studies by Minke et al., 2009 and Klaasen et al., 2003 showed typical leptospirosis after challenge in the control dogs.

Results

Antibody response to vaccine and challenge utilising the Microscopic Agglutination Test (MAT).

The mean circulating MAT titres pre-vaccination 1 (7 days before vaccination) for dogs in studies 1, 2 and 3, control 1 and 2 was 0. The highest mean circulating MAT titres achieved post-vaccination 1 for study 1 was 40, which occurred on day 7. The highest mean MAT titres for study 2 was 23.33, which also occurred on day 7 and for control 1 was 3.33, which occurred in one dog on day 7. The highest mean MAT titres for study 3 (High Dose) was 70, which occurred on day 7 and for control 2 from both dogs was 0. (Figure 2)

The mean circulating MAT titres pre-vaccination 2: for study 1 was 6.67, obtained on day 0 (before the vaccine was administered). For study 2, the mean circulating MAT titre was 21.67, which was recorded on day 1, for Study 3, the mean circulating antibody titre was 27.5, detected on day 0. For controls 1 and 2, the mean circulating MAT titres were 0, obtained on day 0.

The highest mean circulating MAT titres post-vaccination 2: for study 1, was 100 obtained on day 7, for study 2, 106.67 obtained on day 7, for study 3 was 70 obtained on day 7 and for control 1 was 3.33 on day 1 (from 1 dog) and for control 2 was 0 from both dogs. (Figure 3). It was expected that the antibody titres detected post-vaccination 2 for each study to be greatly elevated.

Immune response to Challenge (MAT titres)

The mean antibody titres detected from day 0 to day 3 post-challenge, in all dogs vaccinated in the three studies and both control groups, were below 150. Figure 4 shows the mean antibody titres detected from day 4 to day 35 post challenge.

The dogs in Study 1 displayed an increase in antibody titres following a challenge with a virulent strain of leptospires. The mean antibody titres for the vaccinated dogs in Study 1 pre-challenge was 0, whilst the maximum mean MAT titre of 320 was achieved on day 5 post-challenge. The final MAT titre at the end of the study on day 35 was 180. For the controls in Study 1, the mean pre-challenge MAT titre was also 0, whilst the highest mean MAT titre of 640 was achieved on day 6 post-challenge). The final mean MAT titre at the end of the study (day 35) was 0. The increase in antibody titres began on day 2 post-challenge and reached its peak on day 5, for the vaccinated dogs and day 6, for the controls. (Figure 4)

In Study 2, for the vaccinated dogs, the mean pre-challenge MAT titre was 80, whilst the highest mean MAT titre of 280 was detected on day 6 post-challenge. The final mean MAT titre at the end of the study (day 35) was 110. The controls were the same dogs used for Studies 1 and 2 as the two studies coincided with the challenge date post-vaccination 2. The increase in antibody titres began on day 2 post-challenge and reached its peak on day 6, for the vaccinated dogs and day 6, for the controls. (Figure 4)

In Study 3, where a higher challenge inoculum was used, for the vaccinated dogs, the mean pre-challenge MAT titre was 80, whilst the highest mean MAT titre (day 7 post-challenge) was 1280. The final mean MAT titre at the end of the study (day 35) was 220. The controls' mean pre-challenge MAT titre was 0, while the highest mean MAT titre achieved was on day 21 (post-challenge), 1920. The final mean MAT titre at the end of the study (day 35) was 480. The increase in antibody titres began on day 4 post-challenge and reached its peak on day 7, for the vaccinated dogs and the controls, the antibody titres began to increase on day 2 but reached their maximum level on day 21 post-challenge. (Figure 4)

Figure 2 Mean Circulating MAT titres post-vaccination 1 for all studies and controls.

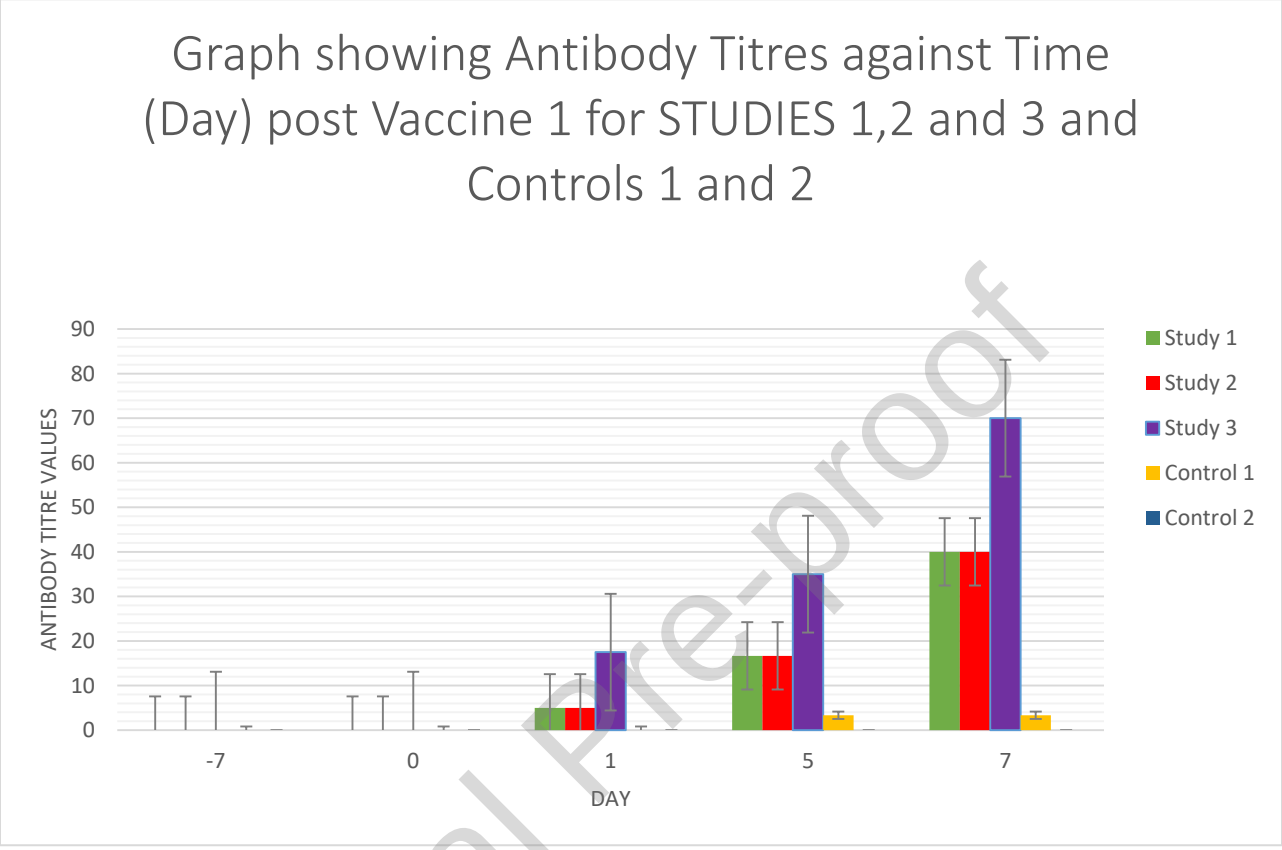


Figure 3 Mean circulating MAT titres post-vaccination 2 for all studies and controls.

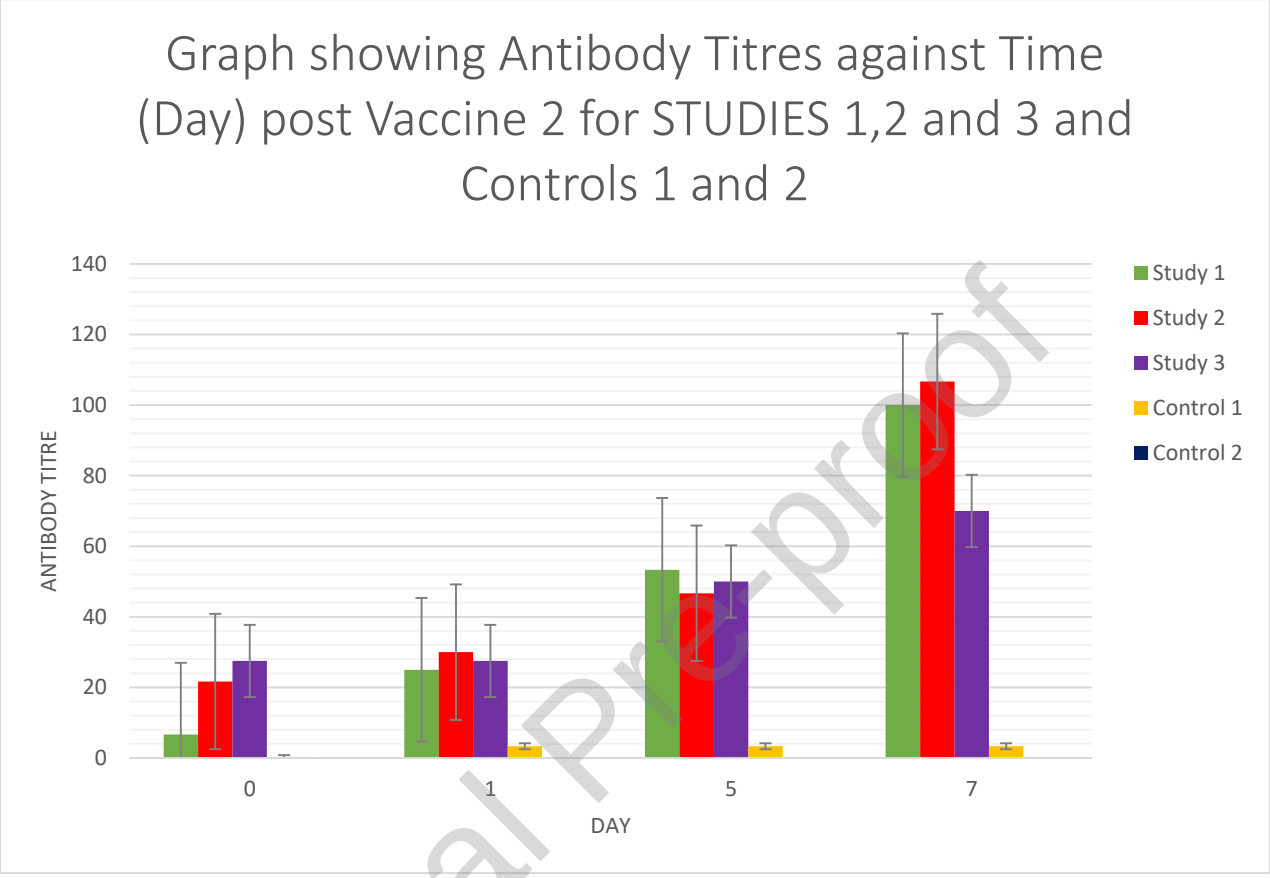
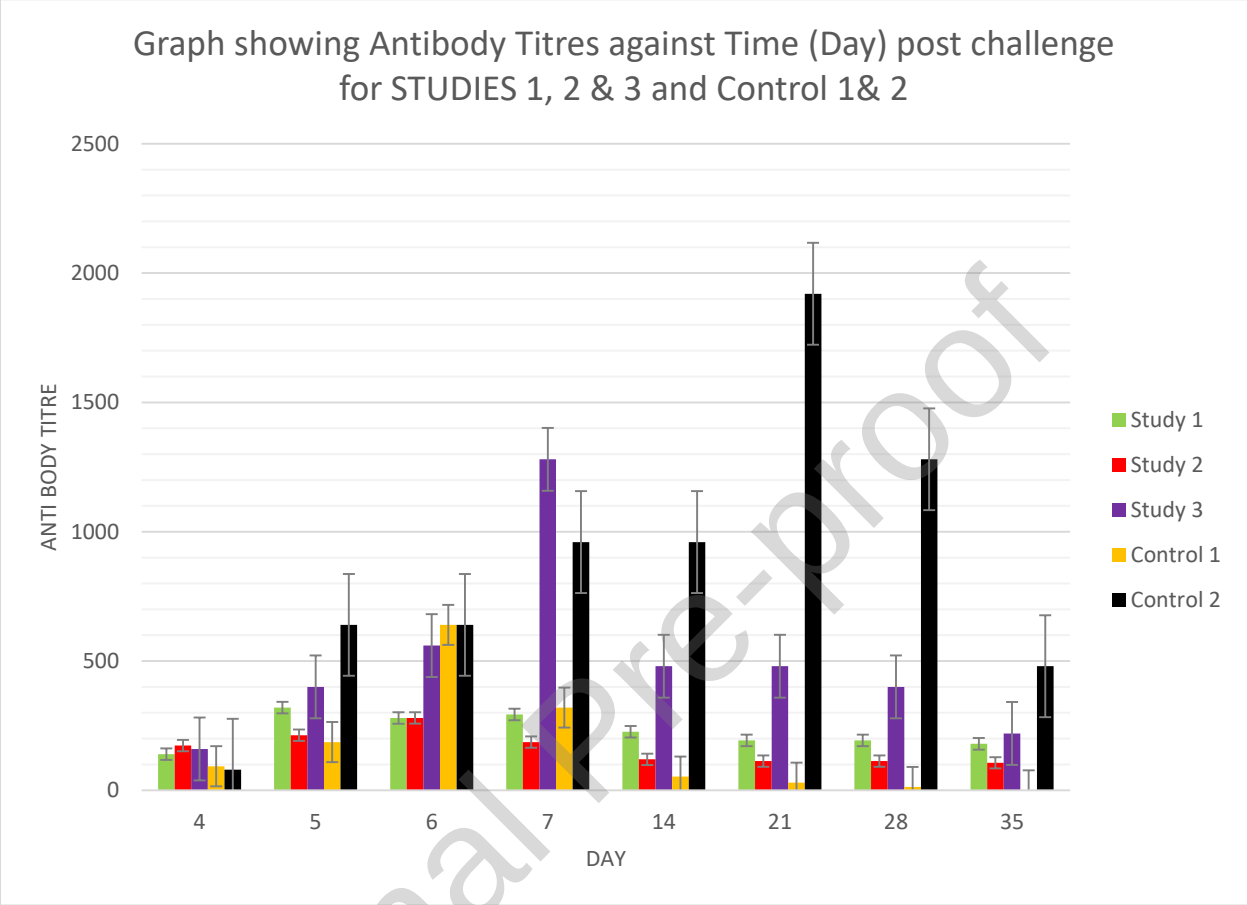


Figure 4. Mean circulating MAT titres post-challenge for all studies and controls.



Leptospiraemia

Blood samples were taken pre-and post-challenge to determine leptospiraemia. Blood samples were taken daily for culture for the first week and then weekly to determine leptospiraemia.

Studies 1 and 2 leptospiraemia was detected on day 1 to day 3 post-challenge with the live inoculum in both *vaccinated* and *control* dogs. After the third day, the leptospires were cleared from the bloodstream and were no longer detectable in the vaccinated and non-vaccinated (controls).

Study 3 leptospiraemia was detected on day 3 to day 6 in the vaccinated dogs after being challenged with the live higher dose inoculum. In the control dogs, leptospiraemia was detected from day 1 to day 6 post-challenge.

For all three studies there was no significant differences between vaccinated dogs and controls for leptospiremia. (Table 3)

Leptospiuria

Urine samples were taken weekly until 5 weeks post-challenge to determine the presence of renal carriage/ chronic disease caused by the challenge inoculum. For the three studies, none of the vaccinated dogs showed evidence of detectable leptospires in their urine or kidney cultures. However, for the three studies, statistically significant ($P<0.05$) differences were detected in the frequencies of leptospiruria between the vaccinated and unvaccinated controls (Table 3). Of all the euthanised dogs, colonization of the kidneys (detection of leptospires in homogenized kidney tissues, 1:1000 fold) was detected only in the unvaccinated control dogs.

Table 3 shows the statistical significance using χ2 for vaccinated vs non-vaccinated dogs for the frequency of positive cultures for leptospiremia, leptospiuria and the colonisation of *Leptospira* from kidney cultures.

Study	Leptospiremia (blood samples)			Leptospiruria (urine samples)			Isolation from kidney
	Positive Samples	Negative samples	χ 2	Positive samples	Negative samples	χ 2 Fisher exact test	Leptospiral load/ (number of euthanized dogs/ total dogs in the group)
V1	18	54		0	31		N / (2/6)
C1	11	25	0.646*	5	9	<0.05*	+++ / (2/3)
V2	18	54		0	27		N / (2/6)
C1	11	25	0.646*	5	9	<0.05*	+++ / (2/3)
V3	12	36		0	20		N / (2/6)
C2	12	12	0.034**	9	2	<0.01**	+++ / (2/2)

*p-values set at 0.05 **p-values set at 0.01

+: 1:10 Positive for leptospires ++: 1:100 Positive for leptospires

+++: 1:1000 Positive for leptospires N : Negative for leptospiruria

Cytokine assay

The cytokine assay for TNF-alpha, IFN-gamma and IL-4 was measured in vaccinated dogs post-vaccination for each study carried out. The results did not produce detectable levels of cytokine

response to the vaccination in any of the studies. The cytokine assay post-challenge for each study had detectable levels as the sampling period progressed. The mean values were used to represent the data as the samples were measured in duplicate. For TNF-α, the values on days 0 to 3 were relatively constant but began to rise on Day 5 for studies 1 and 3. For study 1, the values remained at this elevated level between 336 pg/ml to 353 pg/ml, in study 2 the TNF-α values remained undetectable until day 7 when they reached a high value of 145 pg/ml and study 3, the TNF-α values continued to increase 232 pg/ml to 653 pg/ml.

IFN-γ for days 0 and 1, the values were undetectable for all three studies and then began to rise on Day 3 for studies 2 and 3 with mean values of 474 and 680 pg/ml, respectively. On day 5, the IFN values for all three studies continued to rise, with Study 1 showing up with a value of 197 pg/ml. On day 6, the IFN-γ values rose to, 402 pg/ml, 833 pg/ml and 1188 pg/ml for studies 1, 2 and 3, respectively. On day 7, the values; increased from 565 pg/ml for study 1 and study 2 to 1058 pg/ml and levelled off for study 3 at 1208 pg/ml

IL-4 values were measured from Day 1 to Day 6 post-challenge. On Day 3, mean IL-4 values were 325 pg/ml and 352 pg/ml for studies 2 and 3, while for study 1, no detectable levels of IL-4 were observed. On day 5, IL-4 levels increased to 106, 356 and 673 pg/ml for studies 1,2 and 3, respectively. Day 6 produced values of 278, 546 and 1018 pg/ml for studies 1, 2 and 3, respectively.

Figure 5 Cytokine concentration (pg/ml) vs Time (day of sampling) for mean TNF-Alpha Levels Post Challenge in Studies 1, 2 and 3.

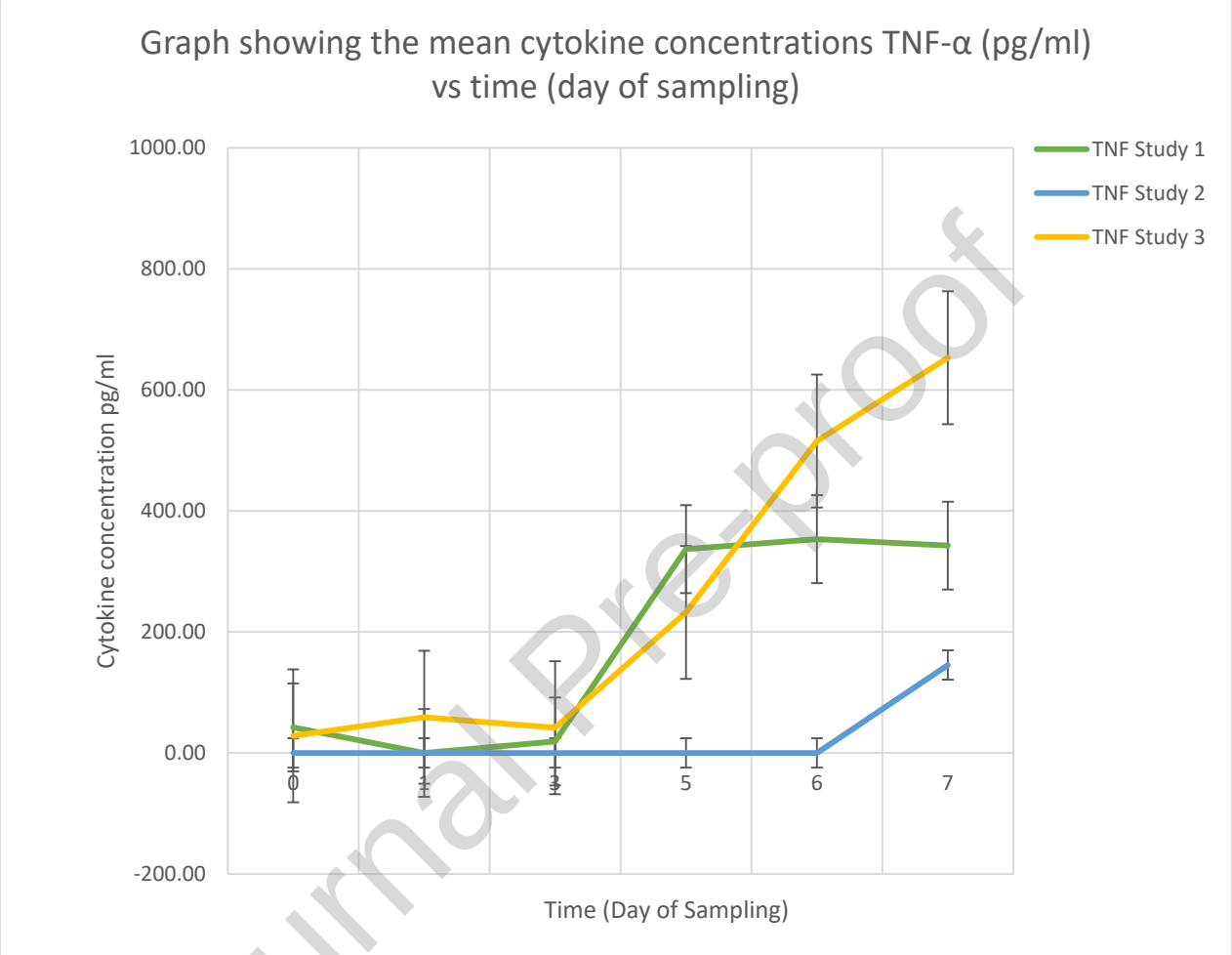


Figure 6 Cytokine concentration (pg/ml) vs Time (day of sampling) for mean IFN Gamma levels Post challenge in Studies 1, 2 and 3

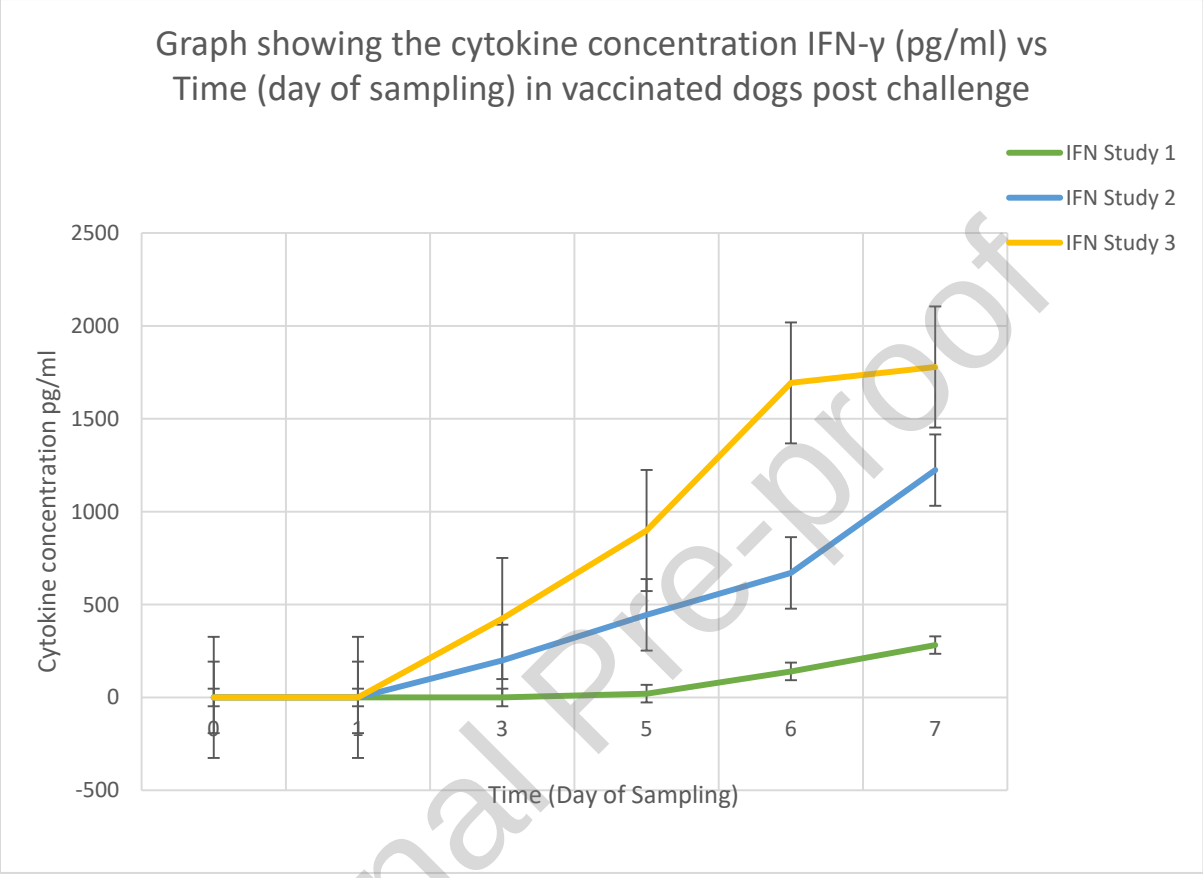
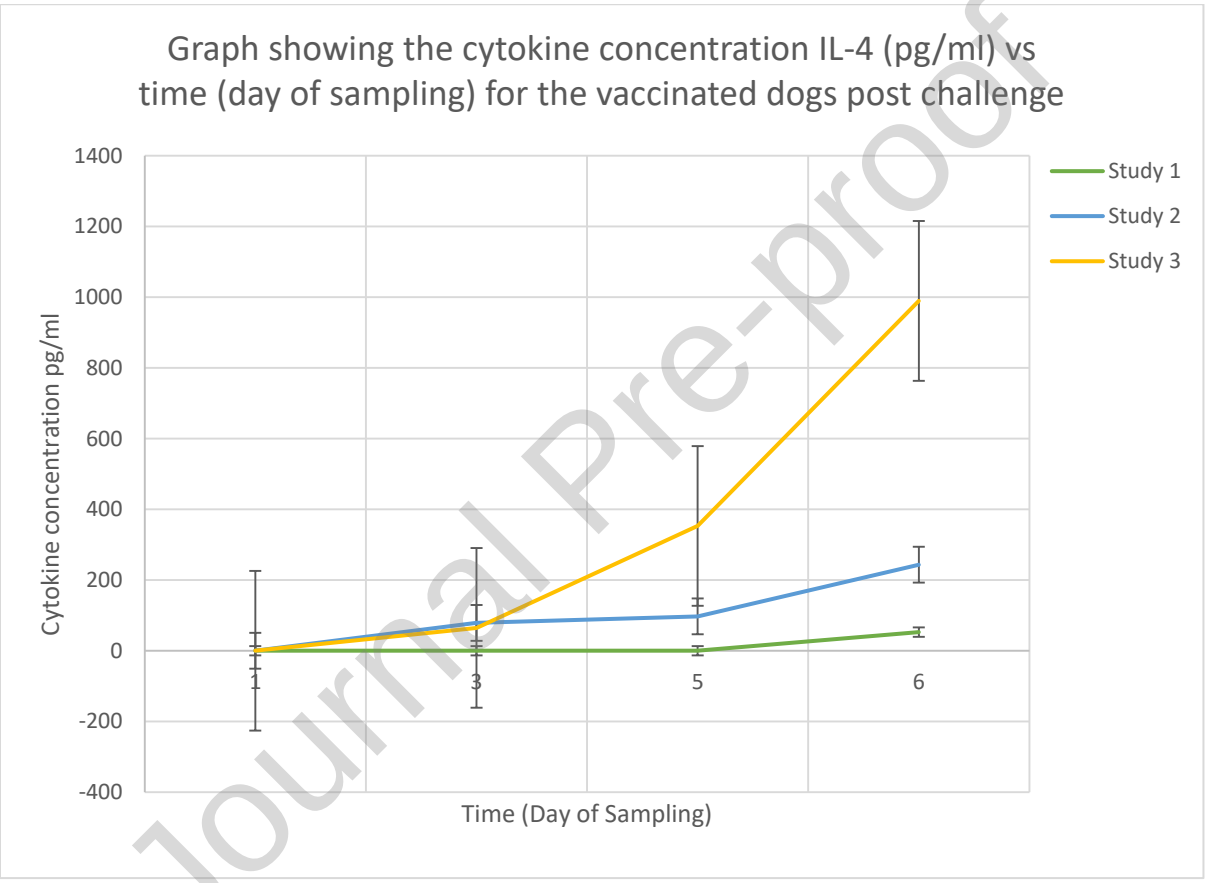


Figure 7 Cytokine concentration (pg/ml) vs Time (day of sampling) for mean InterLeukin-4 Levels Post Challenge in Studies 1, 2 and 3



Discussion

To assess the efficacy of a vaccine to prevent canine leptospirosis, it has been reported that it should be able to prevent clinical and chronic diseases (Klaasen et al., 2003; Minke et al., 2009; Schreiber et al., 2005). It is also known that several factors, including the age of dogs, vaccination schedule, selection of challenge strain, and challenge method, are important in vaccine trials (Minke et al., 2009). In Study 1, the challenge of vaccinated and non-vaccinated controls took place 52 weeks after the booster vaccine. Studies 2 and 3 used two doses of the challenge strain (Copenhageni 1S7) in both vaccinated and unvaccinated controls at 2 weeks post booster vaccine. The results of the three studies of this experiment showed the presence of leptospiraemia as the leptospires were able to transfer out of the peritoneum into the bloodstream, thus pointing to the two possibilities: a) the vaccine did not induce a great enough immunity to prevent the proliferation of the *Leptospira* or b) the very high numbers of leptospires in the challenge inoculum was sufficient for the leptospires to escape into the bloodstream as the immune response was just about to begin.

One of the methods to determine the immunity of a vaccine is to assess the presence of antibodies against the pathogen. Utilising the MAT we could detect agglutinating antibodies in response to the vaccine and the challenge of live *Leptospira* spp. albeit to a limited extent based on the results of vaccination followed by a challenge.

The immune system's interactions are coordinated by the innate, humoral and cell-mediated immunity. It has also been previously documented that immunity to leptospirosis is predominantly humoral (Adler & Faine, 1978; Adler & Faine, 1977; Adler et al., 1989) when the host's immune system commences clearing the *Leptospira* via the production of antibodies. The serological response to leptospiral infection has been established (Minke et al., 2009). In the case of the vaccinated dogs from this study, it was expected that humoral antibodies would have been produced in response to the challenge inoculum because the dogs were previously exposed to the leptospiral antigens through vaccination.

In this current investigation, we observed the production of antibody response to inoculation and leptospiraemia. There was a gradual increase in antibody levels for the vaccinated dogs during the first week post-vaccination, with the maximum titre being at day 7 post-vaccination in all three studies. During the challenge period, the maximum MAT titre frequency was achieved on day 6, post-challenge, in Studies 1 and 2 in both vaccinated and unvaccinated control dogs; however, the unvaccinated controls achieved a higher mean MAT titre of 640 compared with 320 and 280 for the vaccinated dogs in Study 1 and 2 respectively. A similar pattern was detected in Study 3, where higher challenge doses

were administered with the control dogs achieving the maximum mean MAT of 1920 on day 21 post-challenge compared with the maximum mean titre of only 1280 obtained in vaccinated dogs on day 7 post-challenge. Similar findings have been reported where vaccinated dogs produced a weaker serological response than the control dogs (André-Fontaine et al., 2003; Klaasen et al., 2003). The authors suggested that the leptospires could have been cleared via humoral immunity (other than agglutinating antibodies) and cell-mediated immunity.

Due to the lack of typical clinical signs during Studies 1 and 2 for both controls and vaccinated dogs, Study 3 was done at a higher challenge dosage. There was a delay (approximately 5 days compared to 2 days in Studies 1 and 2) for the initial mean MAT titre increase in both vaccinated and control dogs post-challenge. Andre-Fontaine (2013) similarly reported that two dogs showed a delayed response to the challenge with live leptospires. Possible explanations were the presence of undetectable non-agglutinating antibodies circulating in the bloodstream and the leptospires evading the host's immune system. In this investigation: for the findings in Study 3, leptospiraemia was detected in vaccinated dogs by day 3 post-challenge but cleared by day 5. This could have possibly been due to the host's complement system, resulting in the opsonisation of the leptospires and the presence of circulating non-agglutinating antibodies. However, in the control dogs, leptospiraemia was detected from day 1 until day 6 post-challenge, attributable to the naivety of their immune system. Similar findings by Klaasen et al. (2003) showed a more extended period for leptospiraemia post-challenge for the control vs the vaccinated dogs using Canicola serovars. Two vital studies (Bouvet et al., 2016; Minke et al., 2009) showed none of the vaccinated dogs had leptospiraemia, but leptospiraemia was observed in all control dogs post-challenge.

Leptospiraemia was detected in all dogs in the current study, although there were no significant differences ($P < 0.05$ values of 0.646, 0.646 for studies 1 and 2, and $P<0.01$ for 0.034 for study 3 respectively) in the frequency (Table 3- 16/16 vaccinated dogs and 5/5 control dogs) and duration (minimum of 3 to a maximum of 6 days) among the vaccinated and control dogs, an indication that the vaccine and protocol used did not offer sufficient protection against leptospiraemia possibly due to its low immunogenicity or the very high inoculum doses used. Previous studies (André-Fontaine et al., 2003; Klaasen et al., 2003; Bouvet et al., 2016) have reported similar findings, albeit to a lesser extent, using serovar Canicola.

Virulent serotypes of *Leptospira* spp. have been known to cause clinical disease and classical gross and microscopic pathological changes in naturally or experimentally exposed dogs (Greenlee et al., 2004;

Klaasen et al., 2003; Minke et al., 2009; Bouvet et al., 2016). It has been reported that Icterohaemorrhagiae serovars of *Leptospira* spp. display different levels of virulence in challenge studies (Schreiber et al., 2005), and that long-term storage and adaptation of *Leptospira* strains to culture media may occur during storage (Truper, 1992). It has been reported that human and animal infections by microorganisms, including *Leptospira* spp., may not result in clinical disease or histopathological lesions, or they may be mild or undetectable (Brown et al., 1996; Schreiber et al., 2005).

In our study, we observed an absence of clinical disease and lesions during the experiment and post-mortem examination. The histopathological findings of all the euthanised vaccinated and unvaccinated control dogs in the three studies were within recommended limits indicating no significant visible damage to the organs by the *Leptospira spp*. It was found that the none of the vaccinated dogs (0/16) produced leptospiruria, whereas 4/5 control dogs produced leptospiruria. However, upon euthanasia (8 of the 21 dogs), cultures from sections of the liver, kidney and lung were done. There was 3+ positive for leptospiral bacterial loads after serial dilution (1:10, 1:100 and 1:100) from the kidney cultures. Cultures from the liver and lung were negative for *Leptospira* spp., demonstrating the non-systemic nature of the disease in this study, which could have accounted for the lack of clinical manifestations (Barbosa et al., 2019), whereas the culture from the kidneys was positive, suggesting the presence of the renal carriage state or chronic infection.

In this study, we investigated the vaccinated dog's immune system's response when vaccinated (with a killed-whole cell vaccine) and challenged with live *Leptospira interrogans icterorrahiagea* Copenhageni. A limitation of the study was the failure to determine the cytokine response in the control dogs which was primarily due to inadequate available funding for the assay. The results for the vaccination cytokine assays were undetectable, possibly due to the samples' age and the vaccine's lack of immunogenicity due to being adapted to the culture. When leptospires enter the body, they are confronted by the innate immune system, and the leptospires activate the alternative pathway of complement (Meri et al., 2005). Severe or acute leptospirosis is usually associated with toxic shock syndrome with sepsis, and a promising biomarker of patients suffering from the systemic disease is the production of TNF-α (Diament et al., 2002; Strasser et al., 2003; Goris et al., 2011). The highest mean TNF-α values reached were 342.0 pg/ml, 145.3 pg/ml and 653.0 pg/ml post-challenge for Studies 1, 2 and 3, respectively. It has been determined that TNF-α is an early-phase cytokine and its levels are reduced to undetectable levels as the progression of disease occurs, as demonstrated by Diament et al. (2002). However, in our study, the results of study 2 for TNF-α were undetectable, with a very delayed increase in cytokine levels at day

7. Two possible reasons could have resulted in the outcomes: possible adaptation to culture media by our inoculum serovar or the period between sampling times for TNF-α was too long to detect notable changes. There has been documented a significant increase in TNF- α for the virulent strains over the culture-adapted strains (Goris et al., 2011). The possible explanation is the lack of antigenicity of the leptospires because of low strain virulence (Vernel-Pauillac & Goarant, 2010; Maissen-Villiger et al., 2016), as they were present in the bloodstream but did not cause system organ damage. Instead, they were cleared slowly from the bloodstream by the immune system. However, in the unvaccinated control dogs, infection led to the subclinical and renal carriage with leptospires cultured from the kidney tissues upon euthanasia.

The detection of interferon-gamma (IFN-γ), a cytokine critical to both innate and adaptive immunity, is known to induce the activation of primary histocompatibility class II (MHCII) cells, which induce cell-mediated immunity (Brown et al., 2003; Faisal et al., 2008, 2009). Determining the cytokine response in dogs post-challenge after being vaccinated was not previously explored. A study done by Strasser et al., 2003 was conducted in dogs and post-vaccination and the author documented the findings of 1548 pg/ml pre-vaccination and 1931 pg/ml post-vaccination when foreign antigens were introduced. The current study was conducted to determine the cytokine response to challenge with live *Leptospira* spp. in vaccinated dogs to ascertain the type of immunity offered by a killed whole-celled vaccine. The mean IFN-γ cytokine level in the Study 1 was the lowest, with a value of 565.0 pg/ml. Interestingly, the cytokine levels for challenged dogs in Studies 2 and 3 reached maximum levels of 1058.2 pg/ml and 1208.5 pg/ml, respectively. Comparing the changes in the IFN-γ levels in the study by Strasser et al., 2003, the dogs' response to foreign antigens was increased. Studies 2 and 3 were compared with the increase in IFN-γ levels when the antigens were re-introduced as a challenge as live *Leptospira* spp. Indicating a cellular type of immunity was being mounted. However, the findings of lower IFN-γ cytokine levels in Study 1 (highest levels were 282pm/ml--see Figure 6) are at contradictory to the results in both Studies 2 and 3 (highest levels 1223.85 pg/ml and 1778.95 pg/ml—see Figure 6) and further studies may be required to determine the type of immunity in dogs aged 56 weeks or older (Duration of Immunity) versus younger dogs 16 weeks (Onset of Immunity) after vaccination and challenge.

The presence of interleukin-4 (IL-4) is known to indicate T_h-2 cell maturity and differentiation of B-cells into plasma cells. The maximum mean IL-4 cytokine levels detected were lower post-challenge in Studies 1 and 2 at 278.4 pg/ml and 545.8 pg/ml, respectively, for vaccinates, accounting for the relatively low

mean maximum MAT titres, 320 and 280, respectively. The notably higher IL-4 level at 1017.9 pg/ml detected post-challenge in Study 3, with a higher challenge dose compared with Studies 1 and 2, was also detected along with a high antibody titre of 1280, which is indicative of higher humoral immune mounted by both the vaccinates and controls in Study 3.

The presence of elevated levels of IFN-γ (indicating a cell-mediated type of response) and elevated levels IL-4 (indicating a humoral response to infection) can help bring further discussion into the coordinated type of immune response to a *Leptospira app* infection in dogs. Additional work into the actions of TNF-α in response to acute infection to *Leptospira spp* (possibly hourly sampling) as elevated cytokine levels can describe the severity of disease.

Limitations of the study

Insufficient sample size for each study for vaccinated and control dogs.

Lack of a virulent local serovar(s) to vaccinate and challenge the dogs.

Time and funding constraints for sample collection, storage, and sample processing.

Conclusion

The whole-killed cell vaccine in this study did not prevent leptospireamia but prevented leptospiruria in vaccinated dogs after a challenge with a live *Leptospira icterohaemorrhagiea* Copenhageni. The vaccine-challenge showed increased antibody (MAT) levels due to vaccination and infection (through challenge). Cytokine production (TNF-α, IFN-γ and IL-4) by the host immune system was observed post-challenge with live leptospires.

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Conflicts of interest

There are no conflicts of interest regarding this study and research paper.

Highlights

- Lack of typical signs of leptospirosis was observed possibly due to low antigenicity of the strain.
- Antibody titres were shown to be lower in vaccinated dogs than control dogs.
- There was a delay in the antibody production of both control and vaccinated dogs at the higher dose used for challenge.
- Immune response produced by the vaccinated dogs post vaccine was particularly low for vaccine 2 and challenge.
- Leptospiraemia was present in all the dogs post challenge.
- Leptospiruria was only present in only the control dogs post challenge.
- The cytokine response showed to increase post challenge with live *Leptospira spp*.