Pakistan J. Zool., vol. 56(3), pp 1117-1123, 2024 **DOI**: https://dx.doi.org/10.17582/journal.pjz/20210711080758

Use of Physiological and Clinical Biomarkers as Indicators of Antibiotic Efficacy against Lyme Borreliosis in Pet Dogs under Field Conditions

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Article Information
Received 11 July 2022
Revised 05 November 2022
Accepted 22 November 2022
Available online 14 January 2023 (early access)
Published 29 March 2024

Authors' Contribution
AZD conceptualization, methodology. MU visualization, investigation, data curation, writing original draft preparation. AZD and NM supervision. MHS software, validation. MC writing reviewing and editing.

Key words
Lyme borreliosis, Dogs, Antibiotics, Clinical biomarkers, CRP

ABSTRACT

Lyme borreliosis is caused by *Borrelia burgdorferi* sensu leto. This disease has been recently reported in Pakistan but the efficacy of different antibiotics against it has not been tested. Therefore, this study was aimed at evaluating the efficacy of different antibiotics in curing this disease under field conditions and ascertaining the role of C-reactive protein as a prognostic agent in Borrelia-positive dogs. For this purpose, twenty-four dogs of different breeds were used in the study. These dogs were found Borrelia positive on PCR and equally divided into four groups (n=6). Group A was treated with doxycycline @ 10mg/Kg, B with azithromycin @ 20mg/Kg and C with clindamycin @ 11mg/Kg, and D with amoxicillin @2mg/Kg. All drugs were administered orally in different forms, i.e., tablet, capsule, or suspension as per the availability. Five clinical biomarkers including fever, anorexia, arthritis, lameness, and lymphadenopathy were used to declare the status of the disease at days zero, 7th, 14th, 21st, and 28th. Serum samples were also collected on the same days for determining C-reactive protein (CRP) values. The results of clinical biomarkers showed that doxycycline cured the dogs immediately when checked on the 7th day followed by amoxicillin on the 21st day; whereas dogs treated with azithromycin and clindamycin did not show promising results. Besides, results of CRP showed that doxycycline had significantly ($p < 0.05$) greater efficacy than Azithromycin till the end of the trial and clindamycin till the 21st day. However, the comparative efficacy of doxycycline and amoxicillin showed a significantly ($p < 0.05$) greater efficacy of the former till the 7th day. These results have proved that doxycycline is the best drug, followed by amoxicillin, to treat dogs suffering from Lyme borreliosis. Moreover, it is also ascertained that CRP can be effectively used to monitor the prognosis of Lyme borreliosis in pet dogs. These results will be helpful for the pet clinicians and veterinarians working to diagnose, treat, and monitor Lyme borreliosis under field conditions.

INTRODUCTION

Lyme borreliosis (Lb) is an emerging vector-borne disease (Jánová, 2019). A spirochete, *Borrelia burgdorferi* sensu leto (*B. burgdorferi* s.l.), is its causative agent. There are more than 52 species of *Borrelia* (Cutler et al., 2017) but currently, 22 species are classified under *B. burgdorferi* s.l. (Springer *et al*., 2020). Dogs are highly susceptible to Lb and the most common clinical picture attributed to canine Lb includes a history of tick infestation, anorexia, lethargy, fever, arthritis, nephritis, and neurological signs (Littman *et al*., 2018).

Treatment of Lb is based on treating the infection and managing the pain. While different experiments show that the disease is self-limiting, still if it continues, can lead to neurological complications, renal failure, and ultimately death of the animal. Therefore, it is advisable not to let the infected dog untreated. Generally, rapid response to antibiotics is seen within one to two days; however, it depends on the status of infection, i.e., sole infection of Lb or coinfection with other agents like *Ehrlichia*, *Leptospira*, or *Anaplasma* sp. A long course of treatment (up to 4 weeks) is recommended due to the biological behavior of

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0030-9923/2024/0003-1117 $ 9.00/0

B. burgdorferi s.l. The duration of treatment and the best drug against *B. burgdorferi* s.l. is not known (Littman *et al*., 2018).

The diagnosis of *B. burgdorferi* s.l. is not an easy task. ELISA is most commonly used for the diagnosis of Lyme disease in dogs. Latest ELISA kits SNAP4Dx and SNAP4DxPlus [IDEXX Laboratories, Westbrook, Maine] adequately detect *B. burgdorferi* s.l. by differentiating natural Infection from the vaccinal response. According to our knowledge, due to the absence of any confirmatory report of Lb from the country, these ELISA kits are not commercially available in Pakistan yet. PCR is another parameter. It is a more sensitive and specific assay to detect different species of *B. burgdorferi* s.l. complex and it can also distinguish between active infection and past exposure (Díaz-Sánchez *et al*., 2020). Along with PCR, the presence of clinical signs can also be used as a parameter to declare the positive or negative status of a study animal.

C-reactive protein (CRP) belongs to the pentraxin family of proteins and increases 1000-fold or more in concentration in blood during the occurrence of an injury, inflammation, or tissue death. CRP is known to be involved in the conjugation of pathogens to induce their destruction by the complement system and is also studied as a marker of inflammation, infection, and a diagnostic adjunct (S 2014). CRP is present below 10 mg/L in the blood of healthy dogs, but spikes during ongoing inflammation, even above 600 mg/L depending on the severity of the inflammatory process, and may increase up to 1000 folds in response to inflammation followed by a surgical procedure in dogs (Yamashita *et al*., 1994). Expression of CRP is successfully studied in human patients having early to late manifestations of Lb (Uhde *et al*., 2016). So, it can also be used as a diagnostic tool in dogs suffering from Lb.

Keeping the aforementioned background in view, the current study was designed to determine the efficacy of different antibiotics in the field conditions in four groups of pet dogs. Initially, Lb-positive dogs were selected based on polymerase chain reaction (PCR). Later, clinical biomarkers were used to declare the status of Lb. In addition, CRP values were also determined to check their relation with Lb and the efficacy of our treatment regimens. This study would be helpful for clinicians and pet owners in the country and beyond to understand the clinical picture, diagnosis, and treatment of Lb.

MATERIALS AND METHODS

Ethical statement

Institutional Review Committee for Biomedical Research, University of Veterinary and Animal Sciences, Lahore approved the sample collection methodology (Letter number DR/894; dated 22/08/2017). Furthermore, blood samples were collected after seeking consent from pet owners.

Study design

Lb-positive dogs (n=24) were included in the field trials. These dogs were found positive on PCR and reported in another study (Usman, 2022). These dogs were divided into four different groups, each comprising 6 dogs. Group A was treated with doxycycline @ 10mg/Kg, B with azithromycin @ 20mg/Kg and C with clindamycin @ 11mg/Kg PO, and D with amoxicillin @2mg/Kg PO. Moreover, supportive therapy consisting of NSAID and glucose was also used in all four groups according to the clinical picture of each dog.

Sampling strategy

6 ml of blood was collected from each dog from cephalic vein. This blood was preserved in plain vacutainers. These tubes were properly labeled earlier and after blood collection transported immediately to the Post-graduate Laboratory of Veterinary Medicine Department, University of Veterinary and Animal Sciences, Lahore, Pakistan for further analysis. These samples were collected on 0, 7th, 14th, 2st, and 30th days.

Qualitative and semi-quantitative determination of C-reactive protein (CRP)

For this purpose, the HumaTex® CRP kit was used according to the manufacturer's instructions. Initially, one drop of serum, drawn at zero, 7th, 14th, 21st, and 30th days, was used against the same quantity of reagent from the CRP kit. Agglutination was observed within two minutes if the CRP value was lower than 12mg/L. This was just for the qualitative determination of CRP. For semi-quantitative determination, the dilution given in Table I.

Table I. Measurement of C-Reactive Protein (CRP) at different dilutions.

Dilution	CRP values (milligram/Liter)
1 + 1 (1:2)	12
1 + 3 (1:4)	24
1 + 7 (1:8)	48
1 + 15 (1:16)	96
1 + 31 (1:32)	192

Record of clinical biomarkers

The main clinical biomarkers which were observed and noted in this study included anorexia, lymphadenopathy, lameness, arthritis, and fever. Fever

was measured using a digital thermometer, anorexia was recorded from the history provided by owners, while the rest of the biomarkers were clinically observed at zero, 7th, 14th, 21st, and 30th days.

Statistical analysis

The data was compiled into Microsoft Excel spreadsheets and validated using Epi Info™ software. The data was statistically analyzed using Minitab® 17.1.0 and SPSS version 20.0. Data related to clinical biomarkers could not be statistically tested because it contained zero value, therefore it was presented as such. Two statistical models were used for estimating drug efficacy based on CRP values. Two sample t-test was used for comparing the mean difference between CRP values of group A with other groups individually. For evaluating the efficacy among all groups at different time intervals repeated measures ANOVA was used. *p* values less than 0.05 were considered significantly different in both models.

RESULTS

The results of reversal of clinical biomarkers on days 0, 7th, 14th, 21st, and 28th are given in the Table II for a comprehensive understanding of the clinical picture of Lb-positive dogs at different stages of the treatment.

Reversal of clinical signs in pet dogs treated with four different antibiotics in different groups

As it can be observed in these results that dogs in all groups had arthritis and lameness while fever, anorexia, and lymphadenopathy were variably present. In group A, all clinical signs vanished after 7 days of treatment with doxycycline. Whereas in Group D, which was treated with amoxicillin, one dog recovered after 7 days, 4 dogs after 14 days, and the whole group recovered after 21 days. Contrarily, groups B and C, which were treated with Azithromycin and Clindamycin, respectively, showed poor results. In both groups, dogs showed recovery from some clinical signs after 14 and 21 days. However, at the end of the trial, one dog in group B and three dogs in group C still had arthritis and lameness as given in Table II.

CRP in different Lb positive dogs during the therapeutic trial

For CRP serum samples were used and analyzed using humatex® CRP kit according to manufacturer's instructions. The results of clinical biomarkers, CRP, showed that at day zero, i.e., when no treatment was administered the CRP values were very high. These means of these values were greater than 160 mg/L. However, after 7 days the values of CRP dropped to below 12mg/L and these values remained normal till the end of the trial. The same trend was observed in group D after 21 days. Contrarily, dogs in groups B and D did not show these trends and these dogs had higher CRP values till the end of the trial, i.e., 28 days. These results were in line with the results of reversal of arthritis described in Table II. When these results were analyzed using repeated measures ANOVA, *p* value was found below 0.05, and thus significant difference was observed (Table III).

Table II. Reversal of clinical biomarkers in four groups after treating with specific therapeutic regimens.

Clinical biomarkers	Group A	Group B	Group C	Group D
Day zero				
Fever	2	3	2	2
Anorexia	2	3	2	2
Arthritis	6	6	6	6
Lameness	6	6	6	6
Lymphadenopathy	2	2	2	2
Day 7th				
Fever	0	2	2	1
Anorexia	0	2	2	1
Arthritis	0	6	6	5
Lameness	0	6	6	5
Lymphadenopathy	0	2	2	0
Day 14th				
Fever	0	2	2	0
Anorexia	0	2	2	0
Arthritis	0	4	5	2
Lameness	0	4	5	2
Lymphadenopathy	0	0	0	0
Day 21st				
Fever	0	1	1	0
Anorexia	0	1	1	0
Arthritis	0	3	5	0
Lameness	0	3	5	0
Lymphadenopathy	0	0	0	0
Day 28th				
Fever	0	0	0	0
Anorexia	0	0	0	0
Arthritis	0	1	3	0
Lameness	0	1	3	0
Lymphadenopathy	0	0	0	0

Table III. C-reactive protein (CRP, Mean ± SD) in four groups of dogs at four consecutive weeks and their statistical analysis.

Variables	0 day	7th day	14th day	21th day	28th day	p[a] value
Group A	192±0	10.7±1.03	10.7±1.03	10.7±1.03	10.7±1.03	0.004
Group B	160±45.8	160±49.6	152±63.8	115.3±89.7	101±99.7	
Group C	176±39.2	160±49.6	129.7±75.2	129.7±75.2	85.3±89.3	
Group D	176±39.2	113.7±69.2	23.3±19.1	11±1.1	10.7±1.03	

[a]$p < 0.05$ is considered significantly different.

Group-wise comparison of different drugs to ascertain their efficacy against Lb

More specifically, group A treated with doxycycline was compared with each group B, C, and D individually using two-samples t-test. The results were very interesting and confirmed the greater efficacy of doxycycline as compared to azithromycin, clindamycin, and amoxicillin.

Comparative efficacy of doxycycline and Azithromycin against Lb

Table IV showed the results of this comparative efficacy. At day zero, dogs in both groups had maximum CRP values as well as the almost equal status of clinical biomarkers (Table II). However, on day 7, clinical biomarkers, as well as CRP values (10.7±1.03) in dogs of group A, fully reverted to a normal level. On the other hand, dogs treated with azithromycin showed higher CRP values (160±49.6) and approximately the same picture of clinical biomarkers with the exception of one dog in which fever and anorexia vanished on the 7th day. On day 14th, CRP values in group B slightly lowered (152±63.8) along with slight improvement in their clinical presentation. The same trend was observed on day 21st and day 28th with higher CRP levels, i.e., 115.3±89.7 and 101±99.7, respectively. On all days these CRP values significantly differ ($p < 0.05$) showing the higher efficacy of doxycycline as compared to azithromycin (Table V).

Table IV. Comparison of C-reactive protein (CRP) values (Mean±SD) in dogs of groups A and B at different days.

Days	CRP level in group A (mg/L)	CRP level in group B (mg/L)	t value	p[b] value
7th	10.7±1.03	160±49.6	-7.38	0.001
14th	10.7±1.03	152±63.8	-5.43	0.001
21st	10.7±1.03	115.3±89.7	-2.86	0.02
28th	10.7±1.03	101±99.7	-2.22	0.04

[a] Standard error of mean; [b] $p < 0.05$ is considered significant and made bold.

Comparative efficacy of doxycycline and clindamycin against Lb

Table VI presented the results of the comparative efficacy of doxycycline and clindamycin. On day zero, both groups had almost the same status of clinical and physiological biomarkers (Tables II and VI). However, on the 7th day, CRP values (10.7±1.03), as well as the clinical picture in dogs of group A, became normal in contrast to group C which was treated with clindamycin. In group C, CRP values dropped slightly from 160±49.6, 129.7±75.2, to 129.7±75.2 on days 7th, 14th, and 21st, respectively. These results were statistically different ($p < 0.05$) confirming the greater efficacy of doxycycline as compared to clindamycin till the 21st day. Nonetheless, on the 28th day, the clinical picture in group C presented promising results with only one dog suffering from arthritis and lameness (Table II). Similarly, results of CRP values in group C dropped significantly (85.3±89.3) and the statistical difference between the two groups became nonsignificant on the 28th day (Table VI).

Table V. Comparison of C-reactive protein (CRP) values (Mean±SD) in dogs of groups A and C at different days.

Days	CRP level in group A (mg/L)	CRP level in group C (mg/L)	t value	p[b] value
7th	10.7±1.03	160±49.6	-7.38	0.000
14th	10.7±1.03	129.7±75.2	-3.88	0.01
21st	10.7±1.03	129.7±75.2	-.388	0.01
28th	10.7±1.03	85.3±89.3	-2.04	0.05

[a] Standard error of mean; [b] $p < 0.05$ is considered significant and made bold.

Comparative efficacy of doxycycline and amoxicillin against Lb

The same trend of non-significant difference among clinical and physiological biomarkers of both study groups A and D were observed on the zero-day. The trend remained consistent till the 7th day with a slight modification (Tables II and VI). The CRP values in group A dropped to normal at first observation as previously mentioned. In group

D, this level of CRP dropped (113.7±69.2) but it was still significantly ($p < 0.05$) different confirming greater efficacy of doxycycline as compared to amoxicillin on the 7th day. Nevertheless, clinical biomarkers reverted to normal on day 14th in group D (Table II). Besides, the level of CRP in group D also became normal on day 14th and remained so till the end of the trial. Therefore, a non-significant difference among means of CRP values of groups A and D were observed on days 14th, 21st, and 28th. These results confirmed that doxycycline had greater efficacy till the 7th day and after that, both drugs showed equal results against Lb (Table VI).

Table VI. Comparison of C-Reactive Protein (CRP) values (Mean±SD) in dogs of groups A and D at different days.

Days	CRP level in group A (mg/L)	CRP level in group D (mg/L)	t value	p[b] value
7th	10.7±1.03	113.7±69.2	-3.64	0.01
14th	10.7±1.03	23.3±19.1	-1.62	0.08
21st	10.7±1.03	11±1.1	-0.54	0.3
28th	10.7±1.03	10.7±1.03	0.00	0.5

[a] Standard error of mean; [b] $p < 0.05$ is considered significant and made bold.

DISCUSSION

Lb has many unsolved questions and the question of antibiotic therapy is among one those questions. It is also debated whether to use a single antibiotic group or a combination of many groups. Nevertheless, leaving a patient untreated after confirmation of infection is unethical. Without antibiotic therapy, the clinical signs may suppress for a while but the underlying silent infection will persist and a relapse of infection may occur (Straubinger *et al*., 2000). However, treatment of Lb is a difficult task and many scientists believed that this disease cannot be fully treated. As far as therapeutic trials of Lb-positive dogs in this study were concerned, the selected dogs were those which were found positive during the first phase of the study (Usman, 2022). Mainly, selected positive dogs were from Bulldog, Greyhound, German Shepherd, Rottweiler, and Labrador species. PCR can be used to check the negative results, however, it is not a reliable marker to check the recovery from Lb (Arvikar and Steere, 2015). Moreover, it was already proved in various other studies that *B. burgdorferi* s.l. did not fully remove from the body of animals and it was sustained even after complete therapy and reversal of clinical signs (Sapi *et al*., 2019; Straubinger *et al*., 1997). In a recent study in Lb-positive humans, it was found that the DNA of *B. burgdorferi* sensu stricto, a member of *B. burgdorferi* s.l., can persist for years despite extensive antibiotic therapy (Sapi *et al*., 2019). So, our study focused on treating animals under field conditions and evaluating results based on clinical biomarkers rather than focusing on eradicating the pathogen from the body of the animal.

The results of clinical signs could not be compared statistically. The reason for this lies in zero value in various groups when a clinical sign disappears (Table II). So, clinical signs were used in general to declare the status of disease or recovery. However, CRP values were compared and analyzed using repeated measures ANOVA (Table III). It was found that doxycycline, azithromycin, clindamycin, and amoxicillin had significantly different ($p < 0.05$) efficacy. Doxycycline had the greatest efficacy as it helped in the recovery of animals after 7 days, followed by Amoxicillin (group D), Azithromycin (group B), and Clindamycin (group C) as shown in Table II. The group-wise comparison of antibiotic therapy confirmed these results more efficiently. Doxycycline had significantly ($p < 0.05$) greater efficacy than azithromycin till the end of the trial (Table IV). It had significantly ($p < 0.05$) greater efficacy than clindamycin till the 21st day (Table V) and amoxicillin till the 7th day (Table VI). There was an exceptional study in the past that reported the greater susceptibility of *B. burgdorferi* to other antibiotics (FK037, clarithromycin, 14-OH-clarithromycin, and dirithromycin) rather than Doxycycline and Amoxicillin (Levin *et al*., 1993). With the exception of that study, the results in this study are in agreement with previous studies (Boerner *et al*., 1995; Johnson *et al*., 1987; Straubinger *et al*., 2000). However, more recent studies report the development of resistance in *B. burgdorferi* s.l. (Cabello *et al*., 2022; Sapi *et al*., 2019). According to these studies, clinical signs of Lb diminish after antimicrobial therapy, yet, the disease persists and reoccurrence of the disease occurs. However, such a finding requires long-term follow-up of the cases which is out of the scope of this study. The important point about this study is the report of the existence of *B. burgdorferi* s.l. in the study area for the first time and the use of reported antibiotics against it to recover positive animals clinically. Therefore, further studies are imperative to study resistant patterns of *B. burgdorferi* s.l. and the reoccurrence of the disease.

Clinical signs in Lb-positive dogs markedly reduced in group A treated with doxycycline on the 7th day. The corresponding results of CRP levels in the same group also showed that CRP values also decreased on the 7th day and became normal (<12mg/L). CRP values increase due to some inflammation in the body (Hillström *et al*., 2016; Keany *et al*., 2021). All Lb-positive cases included in this study had arthritis, i.e., inflammation of joints, so the trend of rising and falling CRP values depended upon

the corresponding degree of inflammation of joints. The same trend of levels of CRP and arthritis was observed in all four groups (Table II). This finding is in line with previous studies in which CRP was used to monitor disease progression, arthritis, and prognosis of arthritic cases (Christensen *et al*., 2015; Hillström *et al*., 2016; Keany *et al*., 2021; Ohno *et al*., 2006). This study confirmed that CRP can be used to monitor Lb-positive cases and their prognosis during treatment. The use of CRP in Lb-positive humans was reported in the past and this study has widened the scope of CRP to Lb-positive dogs (Uhde *et al*., 2016).

In conclusion, the study confirmed that doxycycline had significantly greater efficacy against Lb in dogs followed by amoxicillin under field conditions. However, azithromycin and clindamycin had no significant efficacy against Lb. Moreover, CRP can be used effectively as a prognostic agent in Lb-positive dogs.

ACKNOWLEDGEMENTS

The research was supported by HEC Indigenous Scholarship Phase II, Batch VI Pin number 520-147912-2AV6-164 (50093565). The authors are also grateful to Dr. Muhammad Zahid Iqbal, Dr. Abid Hussain, Dr. Zia Ullah as well as all pet clinicians for their support in sample collection and processing.

Funding

The research was supported by HEC Indigenous Scholarship Phase II, Batch IV Pin number 520-147912-2AV6-164 (50093565)

IRB approval

The research was approved by Advance Studies and Research Board in its 53rd meeting held on 19-02-2020 at University of Veterinary and Animal Sciences, Lahore (Letter No. DAS/320; dated 10/03/2020).

Ethical statement

Institutional Review Committee for Biomedical Research, University of Veterinary and Animal Sciences, Lahore approved the sample collection methodology (Letter number DR/894; dated 22/08/2017).

Statement of conflict of interest

The authors have declared no conflict of interest.

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