Research article

Serological and molecular epidemiology of leptospirosis and the role of dogs as sentinel for human infection in Nigeria

Nicholas N. Pilau [a,*], Aristea A. Lubar [b], Aminu I. Daneji [a], Usman M. Mera [a], Abdullahi A. Magaji [a], Elmina A. Abiayi [d], Kira L. Chaiboonma [b], Emmanuel I. Busayo [e], Joseph M. Vinetz [c], Michael A. Matthias [c]

[a] Department of Medicine, Faculty of Veterinary Medicine, Usman Danfodiyo University Sokoto, Nigeria
[b] School of Medicine, University of California San Diego, United States
[c] Department of Internal Medicine, Section of Infectious Diseases, Yale School of Medicine, United States
[d] National Veterinary Research Institute, Vom, Plateau State, Nigeria
[e] Department of Theriogenology and Animal Production, Usman Danfodiyo University Sokoto, Nigeria

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ABSTRACT

Objective: Prospective cross-sectional study of dogs in Nigeria to study leptospirosis, inferred to be endemic in all regions of the country by researchers. Aim is to generate empirical updated evidence of leptospiral infection and delineate serovars involved.
Methods: Study determined the sero-prevalence and infection rate in 342 dogs using sero-assays, culture isolation and novel qPCR. In-house designed primers targeting conserved regions were used to amplify genes in quantitative Real-Time PCR (qRT-PCR) for leptospiral detection to serogroups. Molecular analysis of the leptospiral 16S *rRNA* and *LipL*32 genes were used for identification of pathogenic *Leptospira* species. Primers targeting the O-antigen (*rfb*) region of the *Leptospira* lipopolysaccharide (LPS) were used for differentiating serovars based on comparative melting temperature (T_m) analysis against reference serogroups.
Results: Overall serological and bacteriological prevalence of 56 (16.4%) and 40 (11.7%) respectively was recorded. Vaccination, ages and season(s) were the strongest determinants of infection. Unvaccinated animals, stray dogs and symptomatic dogs presented statistically significant ($P < 0.05$) higher risk of infection: OR 25.531 (6.108, 106.712; 95% CI).
Discussion: The evidence suggests 1 of every 10 dogs is infected and could be symptomatic for the disease or a carrier of leptospires in the studied region in Nigeria with attendant public health risks.

1. Introduction

Leptospirosis is a zoonosis of global importance, caused by spirochetes of the genus *Leptospira* (de-Vries et al., 2014) Misdiagnosis often occurs due to a low index of suspicion amongst clinicians (de-Vries et al., 2014). The true burden, spread, and increase of leptospirosis remains largely unknown, as the quality and availability of diagnostic tests, testing facilities, and surveillance systems are highly variable and frequently absent in pertinent regions of the world (Hartskeerl et al., 2011).

Although globally important, leptospirosis remains under-diagnosed and under-reported in Africa and, consequentially is overlooked as a public health priority (Eshetu et al., 2004; World Health Organization,

2006). It is not considered as a priority reportable disease in most African countries, Nigeria included.

In developing countries, where the majority of people are poor and live in crowded conditions with poor sanitation, human transmission occurs through exposures to urine of *Leptospira*-infected livestock and or companion animals (dogs) (Ganoza et al., 2006). While humans are considered to be incidental hosts, animals can serve as both reservoir or incidental hosts Ganoza et al. (2010).

In Africa, approximately 154 human cases of leptospirosis occur annually, with an incidence of six human cases reported annually in Nigeria (Awosanya et al., 2013). This undoubtedly represents a gross underestimation due to the paucity of evidenced based data. Humans and livestock have been appreciably studied for leptospires in Nigeria

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Awosanya et al. (2013); de Vries et al. (2014). However, there are only very few studies on disease in dogs in Nigeria and the entire Sub-Saharan region.

Whilst a few studies have noted that the southwestern part of Nigeria is endemic for canine leptospirosis, with Grippotyphosa, Pomona and Bratislava identified as the predominant serogroups over the vaccinal Canicola and Icterohaemorrhagiae based on Microscopic Agglutination Test (MAT) screening (Okewole and Ayoola, 2009), there are no record of parallel studies in the northern region of the country for canine leptospirosis.

Most studies in Nigeria on canine leptospirosis are based on serological surveys, often enzyme linked immuno assay (ELISA) or MAT employed for detecting antibodies against predetermined serovars. Bearing in mind limitations associated with MAT, and possible cross-reacting anti-leptospiral antibodies leading to false positive or negative results, it has become imperative to have a definitive approach through molecular assays.

Previous qPCR assays have traditionally targeted genes common to all *Leptospira* species, including *16S* rDNA, *gyrB,* and *secY* genes (Chagas et al., 2012), or pathogen-specific genes including *lipL*32, *lig*A, and *lig*B. The *LipL*32 gene, which encodes the immunodominant lipoprotein located in the leptospiral outer membrane, is highly conserved among the pathogenic serovars and absent among saprophytes (Haake et al., 2004). This genetic feature of *Leptospira* has been exploited for detection of pathogenic *Leptospira* in population-based prospective studies. Our study is the first broad scale epidemiological survey of leptospirosis in dogs using up-to-date molecular diagnostic techniques in the region in Nigeria.

2. Methods

2.1. Sample size determination

A prevalence of 33.6% reported by (Pilau et al., 2011) in dogs was used to compute the required sample size using formula $N = Z^2P (1-P)/d^2$ (Thursfield, 2007). Minimum sample size (N) was calculated to be 342.

2.2. Sample collection

The study was carried out in Sokoto State, northwestern Nigeria (12–13.9667, 4.1333–6.9) on the border of the Sahel. Samples were collected from febrile and non-specific symptomatic dogs presenting at three veterinary clinics, and former patient dogs from these clinics that had owner information on file. Samples were batch-collected during each of the three regional seasons: rainy (July–October), dry windy (*harmattan*; November–February), and hot dry (March–June). From each dog, 5mL of blood was collected through recurrent tarsal venipuncture according to methods described by Gatley (2009), and 5mL of urine collected by cystocentesis with a 23G needle and transferred to a sterile 15ml tube. The pH of the urine was immediately tested with litmus paper (Apex-90QK, China). In acidic samples, equal volume of buffered saline was added. The urine samples were immediately transported at ambient temperature to the Center for Advanced Medical Research and Training (CAMRET) in Usmanu Danfodiyo University Sokoto, Nigeria and stored at -20 °C until removed for culture.

Each aseptically collected blood sample was emptied into a labeled bijou bottle (Becton Dickinson[R]) without anticoagulants, and immediately transported to CAMRET Sokoto, Nigeria and left slanted at room temperature for 10m, after which it was centrifuged at 3,000 rpm for 30 min as described by Gatley (2009). The separated serum was pipetted with a Pasteur pipette into sterile cryovials and subsequently preserved at -20 °C in the CAMRET until analysis. Demographic data (sex, breed, age, season, pet or stray, and vaccination status) on each dog sampled was recorded. Physical examination with attention for overt clinical signs of leptospirosis was recorded in symptomatic dogs. An ethical clearance numbered UDUS/IACUC/2014/AUP-R0-11 was obtained from the Institutional Animal Care and Use Committee of the Usman Danfodiyo University Sokoto in Nigeria for this research.

2.3. Serological screening

The DAI IgG *Leptospira* Microwell Elisa test kit, sourced from Difco Laboratories, Detroit Michigan was used for serological screening of serum samples according to the manufacturer's protocol.

2.4. Culture

Each urine sample was cultured according to methods described by Freitas et al. (2004) at the Central Diagnostic Laboratory, National Veterinary Research Laboratory (NVRI), Vom-Plateau State, Nigeria. The Ellinghausen-McCullough-Johnson Harris (EMJH) (Difco) basal media was prepared by dissolving 2.3g of powdered media per liter, 10% glycerol, pH 7.4. Fetal calf serum was added to commercial leptospiral enrichment (Difco) prepared according to methods described by Freitas et al. (2004) and Wuthiekanun et al. (2013). Final liquid EMJH was prepared by dissolving 100ml enrichment and 10ml fetal calf serum per liter of EMJH basal media. Media was sterilized through 0.22um filtration system. A total of 2mL of urine was inoculated into 18mL EMJH liquid media and incubated at room temperature.

2.5. DNA extraction and real-time PCR

The GeneJET[®] Genomic DNA purification kit (Thermo Scientific) was used for the extraction according to the manufacturer's protocol. 84 urine samples [40 culture-positive and 44 randomly selected among the culture negative samples] were used. These samples fully represented all discrete demographic categories used for the study. Samples were spun at 5000 x *g* followed by subsequent digestion of the pellet with 20uL proteinase K and incubation in a rocking thermo mixer at 56 °C for 30 min 20uL RNase A was used to degrade RNA in the sample. 200uL of Lysis buffer was added, vortexed 15 s, followed by 400uL 50% EtOH and mixing. The lysate was transferred to a kit purification column and spun at 6000 x *g* for 1 min. Sequential washes were performed with 500uL of kit wash buffers I and II and genomic DNA was eluted in 200uL of kit elution buffer. Extracted DNA was transported to our laboratory, School of Medicine, University of California, San Diego, USA [CDC import permit 2015-12-040; CDC0728F13:40REV.4-13; Nigerian export permit: FDVP/ VAI/141/16 FDVPCS FMARD]. DNA was quantified and standardized to 0.4ng/uL using a NANODROP2000.

2.6. 16S quantitative Real-Time PCR

Primers were designed to target the 16S *rRNA g*ene region specific to *Leptospira* spp. Primer sequences were as follows: fLIP: 5′-GAGTTTGG-GAGAGGCAAGTGGAAT TC-3′; rLIP: 5′-GTGCCTCAGCGTCAGTTT TAGGCC-3′. qPCR was performed using Real-Time System CFX96 C1000 Touch thermocycler (BioRad) and Perfecta SYBR Green FastMix (Quanta). Each PCR reaction contained 2uL of genomic DNA and 200nM, 16S primers. Blank template reactions (no DNA) were used as negative controls, and genomic DNA from cultured *Leptospira interrogans* serovar Manilae as positive controls. qPCR conditions consisted of 3 min at 95 °C, followed 39 cycles at 95 °C for 31s and 65 °C for 10s (data collection step). Following amplification, a melt curve analysis was performed to check for amplification of a single product. Samples were run in triplicate, all runs were repeated in duplicate.

2.7. LipL32 quantitative real time PCR

The *lipL*32 gene is highly conserved in pathogenic *Leptospira* (Group I). Primers used were described in previous studies by Cheema et al. (2007) [lipl32_178f: 5′-TCTGTGATCAACTATTACGGATAC-3′; lipl32_4 19r: 5′-ATCCAAGTATCAAACCAATGTGG -3′]. qPCR was carried out

using same instruments, reagents and reaction conditions as above. Blank template reactions without DNA were used as negative controls and genomic DNA from cultured *Leptospira interrogans* serovar Manilae as positive controls. Melt curve analysis was performed as a quality check for false positives. Samples were run in triplicate and runs were repeated.

2.8. O-antigen (rfb) quantitative Real-Time PCR

qPCR was carried out using the same instruments as described above and Precision Melt Supermix (Biorad). Each PCR reaction contained 2uL of purified genomic DNA from canine urine samples [40 culture positive and 44 culture negative] and 10.8uM final concentration of oligonucle-otide primers specific to the O-antigen region of the lipopolysaccharide of *Leptospira* spp. [wzy_zun142_169_191: 5'-AACGGACTTTTTCAA-TACTAYGC-3'; wzy_zun142_412_434: 5'-TTRAAVGAAAGTA-TAAAACTTCC-3']. Primer concentration and annealing temperatures were determined empirically by gradient qPCR with genomic DNA extracted from cultured *L. interrogans* serovar Manilae L495 [NR-19816] (data not shown). qPCR run conditions were as follows: 3 min at 95 °C, followed by 45 cycles of 95 °C for 10s and 60.7 °C for 30s (annealing) and 72 °C for 1min (extension and data collection) to allow for full extension of the PCR product. Amplification was followed by melt curve analysis, with data collection performed at 0.1 °C increments, in order to maxi-mize melt temperature resolution for serovar identification. Genomic DNA extracted from *L. interrogans* serovar Manilae was used as a positive control. Blank template reactions (no DNA) were run, and mixed extracted gDNA from cultured and frozen bacteria selected as common contaminants of canine urine: *Citrobacter rodentium* [ATCC DBS 100], *Escherichia coli* (EPEC E2348/69), *Clostridium difficile* (ATCC BAA-9689) and *Staphylococcus aureus* (MARSA, USA 300) was used as a negative control. Samples were run in triplicate and runs were repeated. Melt temperatures were compared against a standardized melt peak figure constructed using O-antigen amplification of known reference strains of *Leptospira*.

2.9. Construction of standardized melt peak figures using known reference strains

Twenty-one (21) known *Leptospira* strains taken from the Centers For Disease Control (CDC) reference panel [Table 1] were maintained in liquid culture in EMJH media as described above (Difco).

Genomic DNA was extracted using the GeneJet Genomic DNA Puri-fication Kit (Thermo). qPCR was carried out using the same instruments, reagents, run conditions, primers, and primer concentrations used for the O-antigen assay described above. Both samples and runs were repeated in triplicate. Cq values of ≤40 cycles were considered to be positive. For strains with positive amplification, the resulting melt temperatures (T$_m$) were compiled, outliers were removed using a ROUT analysis (Q = 5%; Graphpad Prism ver. 7.0a), and the remaining values used to generate a box-and-whisker plot. Strains were plotted via smallest to largest mean T$_m$ to permit visualization of gross differences between the mean melt temperatures. The means for each strain evaluated were compared to each other using a nonparametric t-test (Kolmogorov-Smirnov, Graphpad Prism) and strains resolved into discrete categories based upon temper-ature differentials. One strain was selected from each discrete T$_m$ cate-gory and a melt curve figure generated.

2.10. Statistical analysis of clinical samples

Generated data were analyzed using Chi square (The OpenEpi: Open Source Epidemiologic Statistics for Public Health, version 7: www.op enepi.com, accessed 22/02/18) to test difference in means with level of significance at P < 0.05. Odd Ratio (OR) and statistical significance between sero-positivity and sero-negativity was determined using Fisher's exact test at 95% Confidence level. We used a bivariate and

multivariate logistic regression to determine predictors of risks for infection and disease using a dichotomous variable as a reference.

3. Results

3.1. Serological screening

This study recorded an overall serological prevalence of 56 (16.4%). The local breed was the most represented with serological prevalence at 27 (15.6%) (P > 0.05). The strongest determinants of seropositivity were vaccination status, with unvaccinated animals representing the most statistically significant (P < 0.05) seropositivity rate [OR 25.531 (6.108, 106.712; 95% CI)]. Puppies between one day to 5-months old being most prominently represented [OR 3.203 (1.140, 8.669; 95% CI)]; and the prevailing season, with most seropositive cases recorded in the rainy season [OR 0.307 (0.104, 0.760; 95%CI)] [Table 2].

3.2. Culture

Results of bacteriological culture indicated unvaccinated status [34 (16.9%); OR 4.581 (1.868, 11.234 95% CI)], stray dogs [33 (25.0%); OR 9.669 (4.132, 22.622 95%CI)], and symptomatic dogs [37 (24.7%); OR 0.048 (0.015, 0.16195%CI)] were all significantly (P < 0.05) associated with the disease. Stray dogs were ten times more likely to be infected and propagate disease than the domesticated category. The rainy season presented the highest prevalence in the three seasons at 28 (13.4). However, this was not statistically significant (P > 0.05) [Table 3].

Figure 1. The standard curve generated using gDNA from *L. interrogans* serovar Manilae L495 demonstrated minimal to absent off-target amplification and consistent melt temperatures [all replicates 80.8°C] over a decreasing gradient of DNA template availability. The

Table 1. List of *Leptospira* reference strains evaluated.

	Strain Designation	Identification No.
Group I: Pathogenic	*L. interrogans* serovar Manilae L495	NR-19816
	L. interrogans serovar Grippotyphosa Original ID: MAL-058	NR-19434
	L. interrogans serovar Copenhageni str. Fiocruz L1-130	*
	L. interrogans serotype bataviae str. MAL 1415	ATCC 23602
	L. interrogans serovar autumnalis str. Akiyami A	ATCC 23476
	L. interrogans serotype australis str. MAL 1556	ATCC 23605
	L. interrogans serotype wolffii str. MAL 198	ATCC 23608
	L. interrogans serotype pomona str. Pomona	ATCC 23478
	L. interrogans serotype pyrogenes str. Salinem	ATCC 23480
	L. interrogans serotype mankarso str. MAL 1489	ATCC 23610
	L. interrogans serotype canicola str. Hond Utrecht IV	ATCC 23470
	L. interrogans serovar ballum	**
	L. interrogans serovar babuderi	***
	L. santarosai serotype borincana str. HS 622	ATCC 23477
	L. santarosai serotype shermani str. LT 821	ATCC 43286
	L. borgpetersenii serotype tarassovi str. Perepelicin	ATCC 23481
	L. borgpetersenii serotype javanica str. Veldrat Bataviae *L. borgpetersenii* serotype hardjo, str hardjobovis	ATCC 23479 ****
	L. kirschneri serotype cynopteri str. 3522 C	ATCC 49945
	L. alexanderi serovar manhao 3 str. L60	ATCC 700520
	Leptospira serovar evansi	****
Group II: Intermediate	*L. licerasiae* serovar Varillal str. VAR10	NR-19925
	L. wolffii serovar Korat str. Korat H2T	NR-22250

* Strain provided as a kind gift from Dr. David Haake, UCLA.

Table 2. Serological prevalence of Leptospiral infection in dogs in Nigeria.

Variables	Seropositive n = 56 (%)	Seronegative n = 286 (%)	OR (95% Cl)	P
Sex				
Male	31 (15.3%)	171 (84.7%)	1.199 (0.673, 2.136)	0.555
Female	25 (17.9%)	115 (82.1%)	1.00	
Vaccination				
Unvaccinated	54 (26.9%)	147 (73.1%)	25.531 (6.108, 106.7)	0.00*
Vaccinated	2 (1.4%)	139 (98.6%)	1.00	
Dog Ownership				
Stray	25 (18.9%)	107 (81.1%)	1.349 (0.76, 2.41)	0.37
Domesticated	31 (14.8%)	179 (85.2%)	1.00	
Signs				
Asymptomatic	26 (13.5%)	166 (86.5%)	0.627 (0.352, 1.114)	0.14
Symptomatic	30 (20.0%)	120 (80.0%)	1.00	
Age(s)				
0-5Mths	8 (33.3%)	16 (66.7%)	1.199 (0.673, 2.136)	0.03*
6-12Mths	31 (16.2%)	160 (83.8%)	1.253 (0.664,2.419)	0.59
>12Mths	17 (13.4%)	110 (86.6%)	1.00	
Breed(s)				
Caucasian	6 (14.0%)	37 (86.0%)	0.877 (0.309, 2.211)	0.97
Rottweiler	6 (12.0%)	44 (88.0%)	1.253 (0.664, 2.419)	0.68
G/shepherd	17 (22.4%)	59 (77.6%)	1.555 (0.777, 3.063)	0.27
Local breed	27 (15.6%)	146 (84.4%)	1.00	
Season(s)				
Rainy	42 (20.15%)	167 (79.9%)	0.307 (0.104, 0.76)	0.02*
Harmattan	9 (14.3%)	54 (85.7%)	0.664 (0.288, 1.419)	0.39
Hot	5 (7.1%)	65 (92.9%)	1.00	

[a] Reference category.
* Significant, OR = Odds Ratio, CI = Confidence Interval.

Table 3. Bacteriological prevalence of leptospirosis in dogs in Nigeria.

Variables	Culture + ve n = 40 (%)	Culture –ve n = 302 (%)	OR (95% Cl)	P
Sex				
Male	26 (12.9%)	176 (87.1%)	0.752 (0.378, 1.498)	0.49
Female[a]	14 (10.0%)	126 (80.0%)	1.00	
Vaccination				
Non-vaccinated	34 (16.9%)	167 (83.1%)	4.581 (1.868, 11.234)	0.00*
Vaccinated[a]	6 (4.3%)	135 (95.7%)	1.00	
Dog Ownership				
Stray	33 (25.0%)	99 (75.0%)	9.667 (4.132, 22.632)	0.00*
Domesticated	7 (3.3%)	203 (96.7%)	1.00	
Signs				
Asymptomatic[a]	3 (1.6%)	189 (98.4%)	1.00	
Symptomatic	37 (24.7%)	113 (75.3%)	0.048 (0.015, 0.161)	0.00*
Age(s)				
0-5Mths	1 (4.2%)	23 (95.8%)	0.198 (0.009, 1.155)	0.16
6-12Mths	16 (8.4%)	175 (91.6%)	0.415 (0.206, 0.821)	0.02*
>12Mths[a]	23 (18.1%)	104 (81.9%)	1.00	
Breed(s)				
Caucasian	3 (7.0%)	40 (93.0%)	0.359 (0.083, 1.131)	0.15
Local breed	30 (17.3%)	143 (82.7%)	0.306 (0.071, 0.956)	0.05
G/shepherd	4 (5.3%)	72 (94.7%)	0.266 (0.071, 0.956)	0.02*
Rottweiler[a]	3 (6.0%)	47 (94.0%)	1.00	
Season(s)				
Rainy	28 (13.4%)	181 (86.6%)	0.307 (0.14, 0.74)	0.12
Harmattan	8 (12.7%)	55 (87.3%)	0.941 (0.382, 2.136)	0.95
Hot[a]	4 (5.7%)	66 (94.3%)	1.00	

[a] Reference category.
* Significant, OR = Odds Ratio, CI = Confidence Interval.

consistent detection limit of the primers was determined to be 10^4 leptospires/mL, though partial and single-replicate amplification were observed at concentrations as low as 10^3 and 10^2 (data not shown). The melting temperature T_m of representative strains from each of 8 discrete categories resolved by O-antigen (*rfb*) RT-PCR on reference serovars was used to compare with test samples for serovars identification.

4. Discussion

Endemic zoonoses affect impoverished and developing communities worldwide, often causing significant mortality and morbidity, but are frequently overshadowed in public and clinician's awareness by high profile pathogens causing diseases such as malaria and HIV/AIDS (World Health Organization, 2006; Maudlin et al., 2009).

A cohort study in Nairobi, Kenya reported extensive serological and molecular evidence for the presence of leptospires in domestic dogs (Halliday, 2010). The study concluded that there was high potential risk of leptospiral transmission from the canine to human population and that, canine serological surveillance could be used as a tool for the determination of broad scale patterns of pathogen presence and relative levels of pathogen exposure in populations (Halliday, 2010).

To date, the few studies in Nigeria and SSA at large are based on antibody detection in a variety of conjugation systems. The most common is the MAT, hitherto used as the gold standard in most of these studies. Serological diagnosis by MAT has important limitations, including the requirement of a panel of live leptospires that have sufficient diversity of antigens to be able to detect specific anti-leptospiral antibodies (Matthias et al., 2008). Bacteriological culture of blood, urine or tissue specimen is the definitive method for the diagnosis of leptospirosis (Bolin, 1996).

In African countries where the disease is known to cause sporadic outbreaks, the absence of reported cases of leptospirosis in human and animals does not reflect the absence of the disease, but a complex network of low clinical suspicion, lack of special reference centers for leptospiral diagnosis, and basic laboratory facilities for diagnosis of leptospires (Pappas et al., 2008). Although researchers in and outside Africa agree on the ubiquity of leptospirosis in Sub-Saharan Africa (de Vries et al., 2014), the epidemiological pattern, risks factors and the complex dynamics of disease and environmental factors remains unclear and is not being systematically studied. Leptospirosis is neither commonly reported in most Nigerian cities, nor is it captured as differential diagnosis in classical cases of acute undifferentiated fever in animals.

Most cities in West Africa are prone to flooding during the rainy season (de Vries et al., 2014). In Nigeria, three background serological surveys in healthy people yielded leptospiral prevalence ranging 13.5%
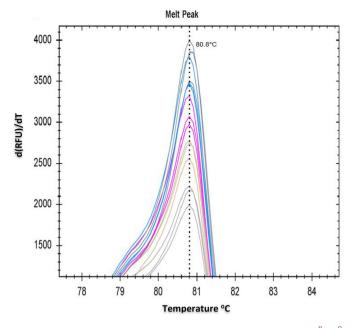
Figure 1. Melt curve from standard curve, DNA concentration range 10^8–10^0

in eastern State (Onyemelukwe, 1993), 18.0% in the Central (Ezeh et al., 1990) and 20.4% on a country wide survey (Agunloye et al., 2001). However, as even the most recent of this data is nearly two decades old, it is hardly reflective of the current situation. Our study recorded an overall prevalence of 16.7%, this is similar to a study, although entirely based on MAT in the southern region of Nigeria, where Agunloye et al. (2001) reported 16.7%. In South Africa, Roach et al. (2010) reported 4.7% after a canine MAT serosurvey.

Iwamoto et al. (2009) reported that dogs may shed leptospires without manifesting clinical signs. For dogs which manifest signs of systemic disease, there are no pathognomonic signs, but instead subtle indicators Aedo et al. (2013). In our study, overt clinical signs used as indicators for suspicion included fever, lethargy, observable dehydration, icterus, visible hemorrhages from any orifices. Prevalence was highest among symptomatic dogs, with 30 (20.0%) sero-prevalence and 37 (24.7%) bacteriological prevalence respectively (Tables 2 and 3), relative to 26 (13.5%) sero-prevalence and 3 (1.6%) bacteriological prevalence seen among asymptomatic dogs sampled.

The findings of this research of leptospirosis prevalence amongst symptomatic and asymptomatic dogs are important as they document an overlooked source of infection. With an ever-growing proportion of un-differentiated febrile disease documented in animals, combined with typical cultural and ecological predisposing factors, leptospirosis represents an important differential diagnosis and substantial risks if subsets of canine population continue shedding leptospires indefinitely.

In the United States, Ward (2002) reported male dogs are at significantly higher risk of infection and supported his claim by demonstrating a steady rise in prevalence among male dogs compared to females, in a retrospective analysis of hospital cases between 1970 and 1998. The author also emphasized that working dogs, such as those involved in guarding, hunting, forensic, and herding, as well as stray dogs are at high risk compared with companion dogs. This pattern was supported in our study, with male dogs presenting a serological and bacteriological prevalence of 31 (15.3)% and 26 (12.9%) respectively, compared to 25 (17.9%) and 14 (10.0%) in females (Tables 2 and 3).

The results of our study verified general opinions and observation amongst leptospiral researchers on the role of season as a determinant of disease in canine and human populations and specifically highlighted that claim in studied region of northwestern Nigeria. The highest serological and bacteriological prevalence, 42 (20.1%) and 28 (13.4%) respectively, were recorded during the rainy season (Tables 2 and 3). Ward (2002), in a

retrospective survey of the seasonality of canine leptospirosis in the United States and Canada, used time series analysis to determine variables that influence and describe disease occurrence. The study reported clustering of cases between the Months of August to November every year over the 15-year period studied. The author concluded that leptospirosis has a seasonal distribution and rainfall can be used to predict risks.

In Sokoto State, Nigeria where the study was carried out, the rainy season occurs between June to October and averages about 600mm annually (Mohammed and Baba, 2013) with the rains peaking between July and August. Although our study did not measure the variability of rainfall from month-to-month, the high numbers of positive samples obtained in the rainy season relative to other seasons serve as an indication of elevated risk during the rainy season. Straying in dogs is very common in developing countries, where legislation that protects pets and ensures their welfare may be absent or rarely enforced. Stray dogs that roam free in urban and suburban areas are especially important in the transmission of leptospirosis (Batista et al., 2004). In Nigerian cities, in both urban and rural areas, there are abundant, and largely unquantified populations of owned and stray dogs (Oboegbulem, 1989). In the study, stray dogs presented higher serological and microbiological prevalence with significant association to leptospirosis, accounting for 33 (25.0%) sero-prevalence against 7 (3.3%) of domesticated dogs that received care (Tables 2 and 3). The Odds Ratio of infection (OR) was 22.6, with stray dogs about 23 times more likely to acquire infection than domesticated dogs. Roach et al. (2010) reported an odd ratio of 4.347 amongst dogs in South Africa.

Agunloye et al. (2001) reported a sero-prevalence of 16.7% amongst unvaccinated dogs, significantly lower than the results of our study at 54 (26.9%; OR 25.531 95% CI). Serological screening showed unvaccinated dogs were 25 times more likely to be infected. *Leptospira* was isolated in 34 (16.9%; OR 4.581 95% CI) stray dogs most of which were symptomatic, there was significant (p < 0.05) association between unvaccinated dogs and disease. Unsurprisingly, the subsets of vaccinated canine population sampled presented far lower sero-prevalence and culture positivity at 2 (1.4%) and 6 (4.3%) respectively. Presently in Nigeria, vaccination statutes and liabilities are not enforced and awareness of risks has diminished in the populace.

A number of studies have inferred the role of dogs as sentinels of human infection and the complex ecological and transmission dynamics between these two closely bonded species. Gay et al. (2014) reported that in dog populations studied in New Caledonia, MAT results confirmed the circulation of the same *Leptospira* serogroups involved in human cases. He reported Icterohaemorrhagiae as accounting for 50%–60% of human cases, Pomona 5%, Pyrogenes 5%, Australis 5%, Bataviae 5% and Ballum 10%, all of which were serovars found in dogs by MAT.

Whilst our study is not comprehensive regarding horizontal transmission dynamics between dogs and humans in Nigeria, certainly, with evidence of pathogenic zoonotic leptospiral serovars in circulation in symptomatic and apparently healthy dogs, there is a quantitative and qualitative risk of dogs serving as active and potential sources of human infections.

Understanding the epidemiology of leptospirosis in any region is a critical step in designing interventions for reducing risks of transmission (Assimina Zavitsanou, 2008). Results from this study we hope will, sensitize health authorities and other stakeholders. With the paucity of research-based data, this study can reasonably only scratch the surface of the many unanswered and unasked questions about the epidemiology of leptospirosis in Africa.

Declarations

Author contribution statement

Pilau N. Nicholas: Conceived and designed the experiments; Performed the experiments; Wrote the paper.

Lubar A. Aristea: Performed the experiments; Wrote the paper.

Daneji A. Ibrahim: Conceived and designed the experiments.

Mera U Mohammed, Magaji A. Abdullahi, Emmanuel I Busayo: Analyzed and interpreted the data.

Abiayi A. Elmina, Chaiboonma L. Kira: Performed the experiments.

Joseph M. Vinetz: Contributed reagents, materials, analysis tools or data.

Matthias A. Michael: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Data availability statement

Data associated with this study has been deposited at SSRN.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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