Research article

Serological and molecular epidemiology of leptospirosis and the role of dogs as sentinel for human infection in 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 Lipl32 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 (Tm) 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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A/osanya 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 (Okeowo and Ayoolu, 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-leptosomal 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 lipL32, liga, and lipA. The LipL32 gene, which encodes the immunodominant lipoprotein located in the leptosporial 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 $N = \frac{Z^2 \pi(1-\pi)}{d^2}$ (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 (Gilman et al., 2004) was used to neutralize the urine. Both samples were centrifuged 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/VAL/141/16 FVPSC FMARD]. DNA was quantified and standardized to 0.4ng/ul using a NANO DROP2000.

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 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 15mL 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/VAL/141/16 FVPSC FMARD]. DNA was quantified and standardized to 0.4ng/ul using a NANO DROP2000.

2.6. 16S quantitative Real-Time PCR

Primers were designed to target the 16S rRNA gene region specific to Leptospira spp. Primer sequences were as follows: forward: 5’-GAGGTTGGGAGAGGCCAGTGGAAT TC-3’; reverse: 5’- GTGGCTCTACGCTAGTTT TAGGGC-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 200mM, 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 40s (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 lipL32 gene is highly conserved in pathogenic Leptospira (Group I). Primers used were described in previous studies by Cheema et al. (2007) [lipL32 178f: 5’-TCTGATCAACTATTACGGGATAC-3’; lipL32 19r: 5’-ATCCAAATATCAAACCAATGGTGG -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. Melting 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 oligonucleotide primers specific to the O-antigen region of the lipopolysaccharide of *Leptospira* spp. [wzy_zun142_169_191: 5’-AAGGGACTTTTTCAA-TACTAYGGC-3’; wzy_zun142_412_434: 5’TTRAAVGAAAGTAT-TAAACTTCC-3’]. Primer concentration and annealing temperatures were determined empirically by gradient qPCR with genomic DNA constructed using O-antigen amplification. Temperatures were compared against a standardized melt peak figure generated using 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 Puriﬁcation 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 (Tm) 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 Tm to permit visualization of gross differences between the mean melt temperatures. The means for each strain evaluated were compared to the standardized melt peak conﬁgured constructed using O-antigen ampliﬁcation of known reference strains of *Leptospira*.

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.opepi.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 4.581 (1.868, 11.234 95% 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

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

| Group I: Pathogenic | Strain Designation | Identification No. |
|---------------------|--------------------|--------------------|
| *L. interrogans* serovar Manilae L495 | NR-19816 |
| *L. interrogans* serovar Grippotyphosa Original ID: MAL-058 | NR-19434 |
| *L. interrogans* serovar Copenhageni str. Fiocruz Li-130 | * |
| *L. interrogans* serotype batisiae str. MAL-1415 | ATCC 23602 |
| *L. interrogans* serovar autumnalis str. Akyjami A | ATCC 23476 |
| *L. interrogans* serotype ausstalis str. MAL-1556 | ATCC 23605 |
| *L. interrogans* serotype wolffi str. MAL-198 | ATCC 23608 |
| *L. interrogans* serotype pomona str. Pomona | ATCC 23478 |
| *L. interrogans* serotype pyrogenes str. Salinemi | ATCC 23480 |
| *L. interrogans* serotype mansonii str. MAL-1489 | ATCC 23610 |
| *L. interrogans* serotype canicola str. Hond Utrecht IV | ATCC 23470 |
| *L. interrogans* serovar ballum | ** |
| *L. interrogans* serovar babadere | *** |
| *L. interrogans* serotype borinacica str. HS 622 | ATCC 23477 |
| *L. interrogans* serotype shermani str. LT 821 | ATCC 43286 |
| *L. interrogans* serotype taransowi str. Pereapecicin | ATCC 23481 |
| *L. interrogans* serotype javanica str. Veldra Bataviae | ATCC 23479 |
| *L. interrogans* serotype hardjostr. hardjoobovis | **** |
| *L. kirschneri* serotype cynopteri str. 3522 C | ATCC 49945 |
| *L. alexanderi* serovar manhao 3 str. L60 | ATCC 700520 |
| *Leptospira* serovar evansi | **** |

** Group II: Intermediate

| Strain Designation | Identification No. |
|--------------------|--------------------|
| *L. icterohaemorrhagiae* serovar Varillal str. VAR10 | NR-19925 |
| *L. wolfi* serovar Korat str. Korat H2T | NR-22250 |

* Strain provided as a kind gift from Dr. David Haake, UCLA.
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 serogroups was used to compare with test samples for serovars identification.

### 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 leptospirosis (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%...
in eastern State (Onyemeluwe, 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 reported 16.7%. In South Africa, Roach et al. (2010) reported 4.7% after a canine MAT serosurvey.

without manifesting clinical signs. For dogs which manifest signs of icterus, visible hemorrhages from any ori

indicators for suspicion included fever, lethargy, observable dehydration, icterus, visible hemmorhages 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 repre-
sents 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 leptospirosis 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 ob-
tained 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 pre-
sented 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 symp-
tomatic, there was significant (p < 0.05) association between unvacci-
nated 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 trans-
mision 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. Abiai Y. Elmina, Chaiboonna 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.

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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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