Retrospective Screening of Cattle Serum for Leptospirosis in the Morobe Province of Papua New Guinea

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Abstract: Leptospirosis is a significant bacterial zoonosis affecting both animals and humans across tropical regions, including Papua New Guinea (PNG). In cattle, this disease can cause abortion, decreased milk production, high fever, jaundice, and reddish-brown urine discoloration, resulting in substantial economic losses for livestock producers. This study, part of an ongoing PhD research investigating leptospirosis epidemiology in PNG livestock, retrospectively analyzed 902 cattle serum samples from 19 farms in the Morobe Province using real-time PCR (qPCR). These samples, originally collected in 2017 through a collaborative NAQIA project and stored at -80°C at PNGIMR, were accessed in 2022 for qPCR. Despite previous documentation of *Leptospira interorgans* serovars Hardjo and Tarassovi in PNG cattle, all samples tested negative for *Leptospira* spp. This unexpected outcome may potentially reflect prior vaccination practices in the sampled herds, though vaccination records were unavailable. This finding has prompted further investigation of presumed non-vaccinated cattle farms in subsequent chapters of the PhD project to better determine the true prevalence of leptospirosis in PNG. Nevertheless, this investigation provides valuable baseline data on cattle distribution, breed characteristics, and farm management practices in the Morobe Province that will inform the broader PhD research framework on leptospirosis epidemiology in PNG, contributing to improved animal health surveillance strategies and zoonotic disease control measures.

Keywords: Cattle disease, Real-time PCR, Retrospective serum, Cattle farms, Leptospirosis epidemiology

1. INTRODUCTION

Leptospirosis is a widespread zoonotic disease caused by pathogenic spirochetes of the genus *Leptospira*, affecting both humans and animals globally with high prevalence in tropical regions (Adler 2015; Costa et al., 2015). In cattle, infection with pathogenic serovars, especially *Leptospira interorgan* serovar Hardjo, manifests as abortion, decreased milk production, fever, jaundice, and hemoglobinuria (Bomfim et al., 2008; Hashimoto et al., 2017; Zelski 2007). The economic impact on livestock production in tropical countries, including Papua New Guinea (PNG), is substantial due to reproductive losses and decreased productivity (Carvalho et al., 2024; Robi et al., 2024). Clinical presentations often resemble other bacterial and viral infections, necessitating laboratory confirmation for accurate diagnosis (Bande et al., 2014; Shagfigi et al., 2014).

Control strategies for bovine leptospirosis encompass vaccination, strategic antibiotic treatment, and environmental management (Mwachui et al., 2015; Ellis 2015). Vaccination against common serovars forms the cornerstone of prevention (Hartskeerl et al., 2011), while prophylactic antibiotic therapy with streptomycin or tetracyclines can reduce urinary shedding in carrier animals (Subharat et al., 2012). Environmental measures focus on limiting exposure to contaminated water and reducing contact with wildlife reservoirs, particularly rodents that serve as maintenance hosts for various serovars (Boey et al., 2019; Mwachui et al., 2015). In regions like PNG with limited systematic control programs, understanding the

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epidemiological landscape remains essential for developing effective intervention strategies (Wynwood et al., 2014).

Diagnostic techniques for leptospirosis include serological and molecular methods with varying sensitivity and specificity profiles (Picardeau 2013; Musso et al., 2013). Serological approaches, such as microscopic agglutination test (MAT) and enzyme-linked immunosorbent assay (ELISA) detect antibodies in blood serum, with MAT considered the gold standard despite requiring specialized facilities (Hernández-Rodríguez et al., 2011; Pinna et al., 2018). Molecular techniques including conventional and real-time PCR offer higher sensitivity for detecting leptospiral DNA, particularly during early infection before seroconversion (Ahmed et al., 2009). While PCR typically shows optimal effectiveness with urine and renal tissues rather than serum due to the transient nature of leptospiremia, it remains valuable for epidemiological investigations (Stoddard et al., 2009; Subharat et al., 2011). Selection of diagnostic methods should align with specific research objectives, available resources, and infection stage (Musso et al., 2013).

This study analysed 902 retrospective cattle serum samples from 19 farms across PNG's Morobe Province. These samples were originally collected in 2017 through collaborative efforts by the National Agriculture Quarantine and Inspection Authority (NAQIA), then stored at -80°C at the PNG Institute of Medical Research (PNGIMR) facilities until 2022 when retrieved for quantitative real-time PCR analysis. This retrospective screening complements the current PhD research project on the "Incidence and Distribution of Leptospirosis in Cattle Population in the Morobe Province of PNG". Integrating historical data with current studies enhance understanding of regional leptospirosis dynamics, potentially revealing temporal patterns and ecological factors influencing transmission (Goarant 2016; Wynwood et al., 2014).

This study aimed to: (1) screen retrospective cattle serum samples for the presence of leptospirosis using qPCR; (2) establish the incidence and distribution of leptospirosis across different cattle farms in PNG's Morobe Province; and (3) generate reliable baseline information on leptospirosis to completement the current PhD research on disease epidemiology and control. Results will contribute significantly to developing targeted surveillance programs and preventive measures for leptospirosis in PNG livestock systems.

2. MATERIALS AND METHODS

2.1 Research Ethics

The retrospective samples were part of a previous collaborative project by NAQIA on animal disease surveillance that met all ethical requirements. As PNG's mandated biosecurity and animal health authority, NAQIA conducted sampling according to standard operating procedures.

2.2 Sampling Location

Cattle blood samples were collected in 2017 across commercial and smallholder farms throughout the Morobe Province. The study encompassed 19 cattle farms (Table 1), with access facilitated through prior coordination with NAQIA staff. Most farms were situated within the Markham Valley, with two exceptions: Ramu Agri Industry Limited, located in Gusap in the Ramu Valley, and Evangelical Brotherhood Church (EBC) and Pelgens farm, positioned 6 and 9 km outside of Lae City, respectively.

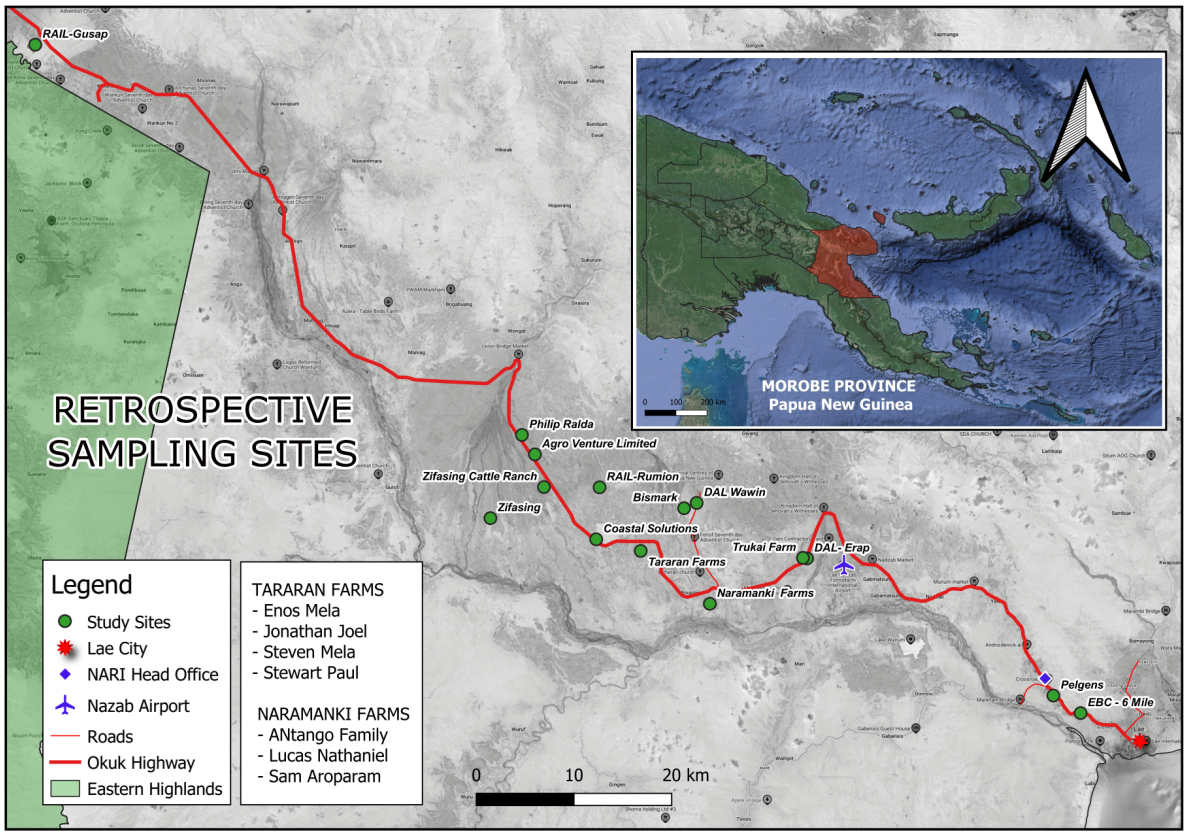

Figure 1. Geographical distribution of cattle serum sampling sites across Morobe Province, PNG. *(Map generated using QGIS software)*

Table 1. Name and locations of retrospective sampling sites.

#	Owner/Farm	Specific Location	Area
1	Antango Family	Naramanki	Markham Valley
2	Bismark	Warwin	Markham Valley
3	Coastal Solutions	Klin Wara	Markham Valley
4	DAL	Warwin	Markham Valley
5	DAL	Erap	Markham Valley
6	EBC	Six Mile/Lae	Lae City
7	Enos Mela	Tararan	Markham Valley
8	Jonathan Joel	Tararan	Markham Valley
9	Lucas Nathaniel	Naramanki	Markham Valley
10	Pelgens	Singawa/Lae	Lae City
11	Philip Ralda	Sasieng	Markham Valley
12	RAIL- Gusap	Gusap	Ramu Valley
13	RAIL Rumion	Rumion	Markham Valley
14	Sam Aroparam	Naramanki	Markham Valley
15	Steven Mela	Tararan	Markham Valley
16	Stewart Paul	Tararan	Markham Valley
17	Trukai Cattle Farm	Erap	Markham Valley
18	Zifasing	Zifasing	Markham Valley
19	Zifasing Cattle Ranch	Zifasing	Markham Valley

RAIL – Ramu Agri Industry Limited, DAL – Department of Agriculture and Livestock, EBC – Evangelical Brotherhood Church

2.3 Data Collection and Handling

NAQIA personnel, including veterinarians and certified animal health officers with specialized training in animal blood collection, obtained 902 blood samples from 19 cattle farms across the Morobe Province. Sampling was conducted randomly without regard to age or breed, with farm owners and workers helping with animal restraint when needed. Research personnel systematically documented environmental and operational characteristics at each site, recording secondary information such as farm type, cattle breed, and gender and age distribution of the herds.

Blood was aseptically collected from the jugular or tail vein of each animal using 10 ml syringes and 24-gauge butterfly needles, with approximately 4-5 ml drawn directly into red vacutainer tubes. Samples were maintained in an upright position in insulated containers with ice packs, preserving the cold chain during same-day transport to the nearby laboratory. After overnight storage at -4°C, samples were centrifuged at 3,000 rpm for 15 minutes to separate serum from whole blood. The separated serum was stored at -20°C until transportation. Upon completion of the field sampling, all serum specimens were repacked with ice packs and transported by road from Lae to the PNGIMR Goroka branch laboratory in the Eastern Highlands Province (a 5–6-hour journey), where they were immediately sorted and stored at -80°C until laboratory analysis. All subsequent procedures, including qPCR testing, were performed at PNG IMR research facilities.

2.4 DNA Extraction

DNA was aseptically extracted from each cattle serum sample, using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions conducted in a type A2 biosafety cabinet. One hundred microliters of each serum sample were used for the extraction. Purified DNA from each sample was equally aliquoted into 2 x 2 ml screw-cap tubes and stored at -80°C until required for qPCR testing.

2.5 Real-Time PCR Amplification and Detection of DNA

Extracted DNA samples were screened for *Leptospira* spp. using real-time PCR methodology as described by Smythe et al. (2002). The assay utilized specific oligonucleotides: forward primer (5′-CCC GCG TCC GAT TAG-3′), reverse primer (5′-TCC ATT GTG GCC GRA CA-3′), and a fluorogenic probe (5′-[FAM] CT CAC CAA GGC GAC GAT CGG TAG C[BHQ1]-3′). The qPCR master mix was prepared with nuclease-free water, Quantitech DNA master mix, forward and reverse primers (20 μM each), and probe (10 μM), with reagent volumes adjusted according to sample numbers per reaction. Amplification was performed using a CFX96 real-time PCR system (Bio-Rad, USA) with thermal cycling parameters consisting of initial activation at 95°C for 15 min, followed by 40 cycles of denaturation (94°C for 60 s) and annealing/extension (60°C for 90 s). *Leptospira* spp. detection was determined by amplification curves crossing threshold values (Ct-values) in real-time. All primers, probes, and positive controls were sourced from previous zoonotic disease studies conducted at PNGIMR (Javati et al., 2022; Robby et al., 2017).

2.6 Data Analysis

Primary data in this study were collected from field sampling and qPCR results. Information generated from qPCR was entered into Microsoft Excel 2021 spreadsheets (Microsoft, Redmond, USA) and analysed accordingly. The main variable for analysis was the presence of leptospirosis in each retrospective cattle serum sample across different farms. The presence (positive) or absence (negative) of leptospirosis was determined from the qPCR output. Additional survey information including geographical farm locations, number of samples collected from each farm, and cattle breed details are presented in graphs and tables. Graphical distribution of cattle samples, age, and gender across different farms was created using the ggplot package in RStudio.

2.7 Quality Management

Sample collection was conducted by qualified and experienced NAQIA personnel using appropriate standard operating procedures. Samples were stored under reliable and consistent temperatures to maintain freshness and quality over time. DNA quantification and measurement of impurities were performed using a spectrophotometer (Nanodrop) for quality assessment. Positive controls (*Leptospira* spp. from other studies) and negative controls (nuclease-free water) were included in all real-time PCRs runs to ensure reliability. Extracted DNA was stored in a -80°C freezer with consistent power supply to maintain temperature stability. The extracted DNA could be easily accessed and retested when required to ensure validation and reliability of the screening techniques.

3. RESULTS

Table 2. Real-time PCR results for leptospirosis screening in cattle serum collected from different farms in Morobe Province

Cattle Farm/Owner	Number of Samples	qPCR Ct Values	Results
Antango Family	34	0.00	Negative
Bismark	50	0.00	Negative
Coastal Solutions	63	0.00	Negative
DAL - Warwin	44	0.00	Negative
DAL – Erap	26	0.00	Negative
EBC	39	0.00	Negative
Enos Mela	50	0.00	Negative
Jonathan Joel	65	0.00	Negative
Lucas Nathaniel	31	0.00	Negative
Pelgens	56	0.00	Negative
Philip Ralda	63	0.00	Negative
RAIL -Gusap	60	0.00	Negative
RAIL Rumion	65	0.00	Negative
Sam Aroparam	24	0.00	Negative
Steven Mela	40	0.00	Negative
Stewart Paul	57	0.00	Negative
Trukai Cattle	68	0.00	Negative
Zifasing	41	0.00	Negative
Zifasing Cattle Ranch	25	0.00	Negative

**Positive control qPCR Ct value = 25.00 and Negative control qPCR Ct value = 0.00*

RAIL – Ramu Agri Industry Limited, DAL – Department of Agriculture and Livestock, EBC – Evangelical Brotherhood Church

The qPCR results indicated that 100% of retrospective cattle samples tested negative for *Leptospira* spp., showing no prevalence of leptospirosis in any of the cattle farms examined. DNA extracted from each blood serum sample was pooled in batches of five for qPCR analysis. The positive control yielded a threshold Ct value of 25.00, while the negative control had a Ct value of 0.00, confirming the validity of the real-time PCR runs and verifying that the results obtained for each farm were true negatives.

Table 3. Farm details comprising of farming system and distribution of cattle breed on each farm.

#	Cattle Farm/Owner	Farm Type	Cattle Breed
1	Antango Family	Small holder	Brahman
2	Bismark	Small holder	Brahman
3	Coastal Solutions	Large holder commercial	Brahman
4	DAL Warwin	Small holder	Brahman
5	DAL Erap	Small holder	Brahman

6	EBC	Small holder	Holstein
7	Enos Mela	Small holder	Brahman
8	Jonathan Joel	Small holder	Brahman
9	Lucas Nathaniel	Small holder	Brahman
10	Pelgens	Small holder	Brahman
11	Philip Ralda	Small holder	Brahman
12	Ramu Agri Industry Limited - Gusap	Large holder commercial	Brahman
13	Ramu Agri Industry Limited - Rumion	Large holder commercial	Brahman
14	Sam Aroparam	Small holder	Brahman
15	Steven Mela	Small holder	Brahman
16	Stewart Paul	Small holder	Brahman
17	Trukai Cattle Farm	Large holder commercial	Brahman
18	Zifasing	Small holder	Brahman
19	Zifasing Cattle Ranch	Small holder	Brahman

RAIL – Ramu Agri Industry Limited, DAL – Department of Agriculture and Livestock, EBC – Evangelical Brotherhood Church

Four farms, Ramu Agri Industry (Gusap and Rumion), Coastal Solutions, and Trukai cattle farms are large commercial holders while rest of the farms (n=15) are small holder based. The common cattle breed reared in all the farms are Brahman except in EBC farms where Holstein breeds are purposely raised for dairy (milk) production.

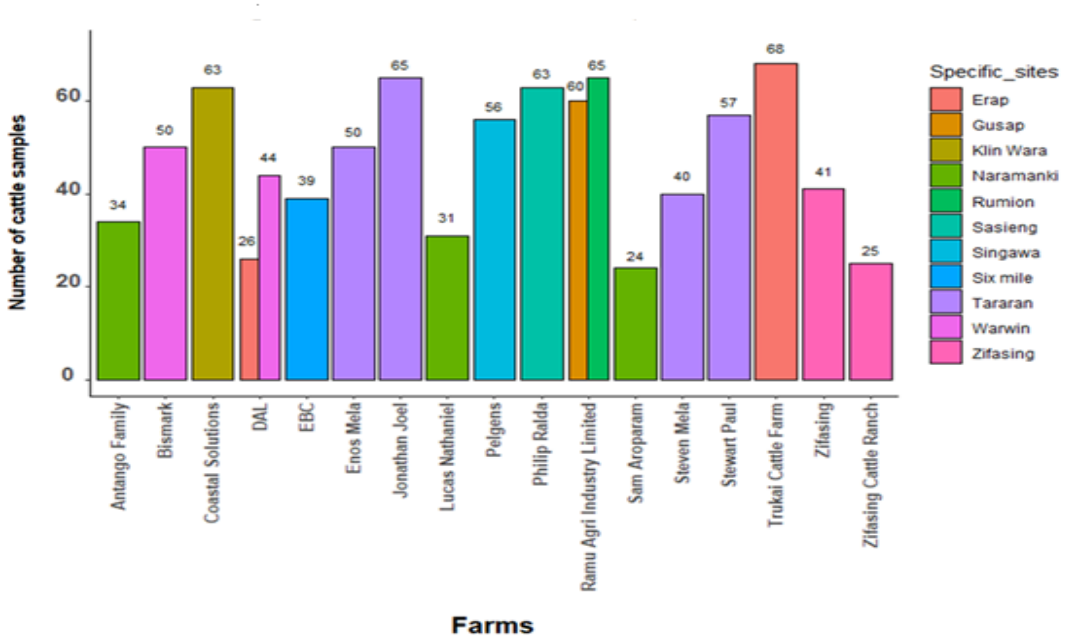

Figure 2. Distribution of cattle at each farm. *The figures on top of each bar plot show the number of cattle. For example, DAL farm is located at two sites; Erap and Warvin, however, the number of cattle sampled was higher in Warwin (n=44) than Erap (n=26).*

Several farms are located within specific sites. Farms owned by Antango Family, Lucas Nathaniel and Sam Aroparam are in Naramankib; Bismark and DAL in Warwin; Coastal Solutions in Klin Wara; DAL and Trukai Cattle Farm in Erap; EBC in Six Mile; Enos Mela, Jonathan John, Steven Mela and Stewart Paul in Tararan; Pelgens in Singawa; Philip Ralda in Sasieng; Ramu Agri Industry Limited in Gusap and Rumion; and Zifasing and Zifasing Cattle Ranch in Zifasing. Trukai Cattle Farm had the highest number of cattle samples (n=68) followed by Ramu Agri Industry Limited (n=65) and Jonathan Joel (n=65) with the least number found in Sam Aroparam farm (n=24). *Farm distribution and distance between farms are indicated in Fig. 2.*

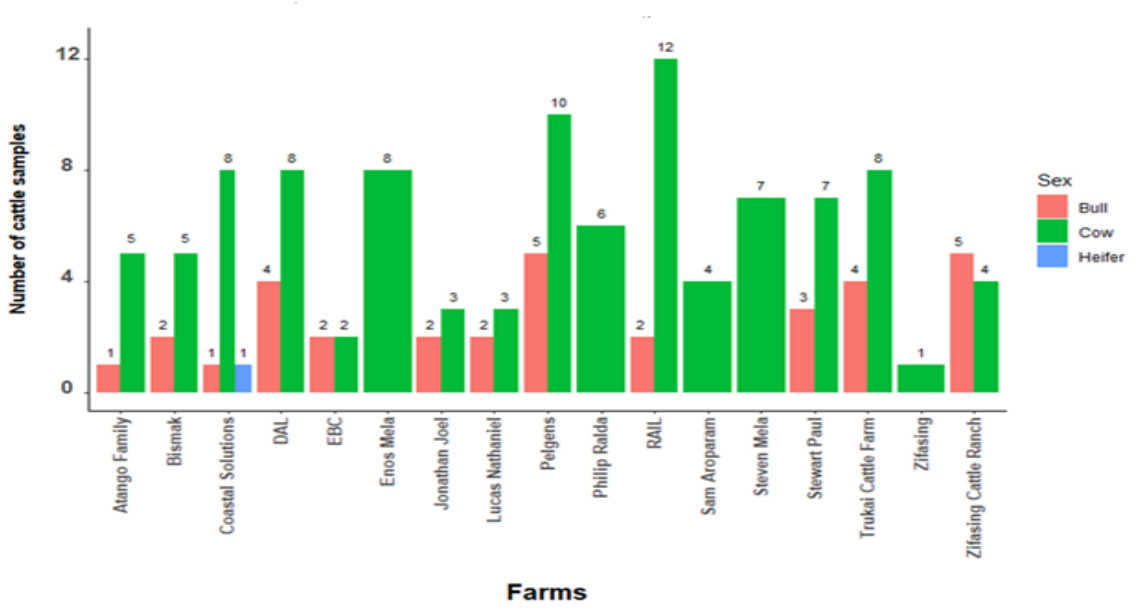

Figure 3. Distribution plot comparing the number of cattle at each farm and their sex or gender composition. *Some farms had both the male and female while others had only one gender. The figures on top of each bar plot shows the number of cattle.*

The sex and respective number of cattle samples varied across the farms. The number of samples corresponds to the size of farm and its status whether it is commercial or smallholder. The number of cows sampled was highest in Ramu Agri Industry Limited (RAIL) (n=12) followed by Pelgens (n=10) and lowest in Atango Family, Coastal Solutions and Zifasing (n=1) (Fig. 3). Only cows were sampled in Enoc Mela, Ralda, Sam Aroparam, Steven Mela and Zifasing. Both bulls and cows were sampled in Atango Family, Bismak, DAL, EBC, Jonathan Mela, Lucas Nathaniel, Pelgens, RAIL, Stewart Paul, Trukai Cattle Farm and Zifasing Cattle Ranch. All three age groups (bull, cow, heifer) were only sampled in Coastal Solutions.

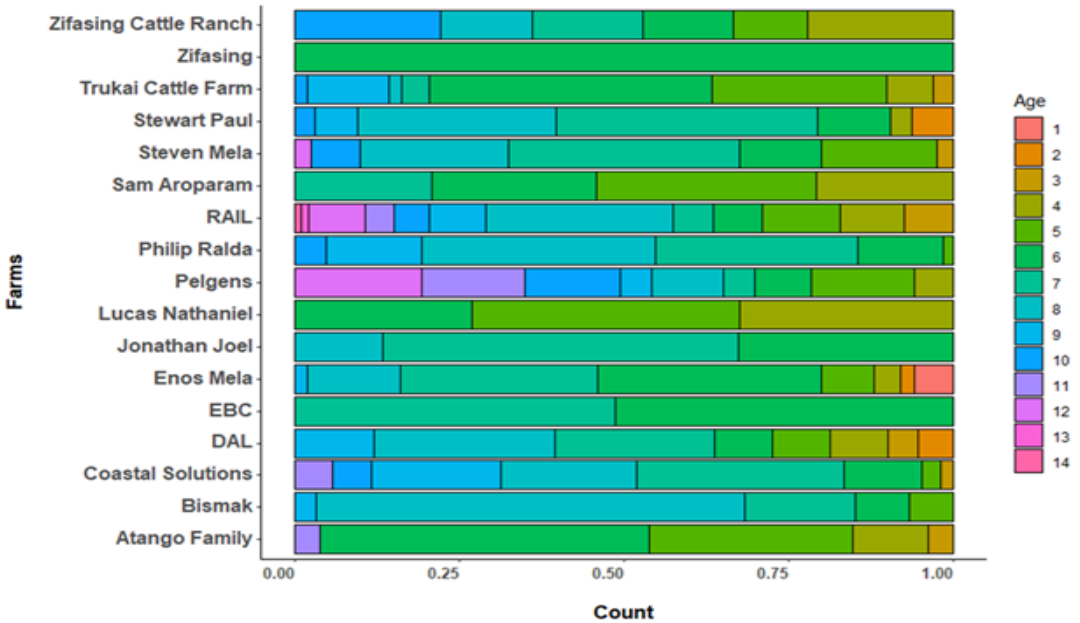

Figure 4. Age distribution of cattle across different farm. *To read the graph/figure for example: Atango Family farm had five age group which are 3, 4, 5, 6 and 11. Each age group contribution was 4%, 12%, 31%, 50% and 4%, respectively*

The distribution of age groups also varied across the farms (Fig. 4). We sampled the highest range of age groups at RAIL farm (n=12) with Age 8 being dominant (28%). The second highest range of age groups was sampled at Pelgens farm (n=9) with Age 12 being common (19%). In contrast, just two age groups, Age 6 and 7 were sampled at EBC farm respectively (51%, 49%) while a single age group (Age 8) was sampled at Zifasing farm (100%).

4. DISCUSSION

Retrospective screening for leptospirosis provides valuable understanding about past prevalence and distribution of the disease, enabling appropriate intervention and preventative measures (Garba et al., 2017). The screening of 902 retrospective serum samples from cattle populations in the Morobe Province revealed no positive results for leptospirosis through real-time PCR screening, despite employing a robust scientific approach and standard screening assay.

This non-detection, however, does not necessarily indicate the pathogen's complete absence in PNG's cattle population. Previous studies conducted within the Morobe Province have documented leptospirosis infection in cattle, confirming the presence of *Leptospira interorgan* serovar Hardjo and Tarassovi as dominant serovars (Wai'in 2007; Yombo 2006). These earlier investigations predominantly employed serological methodologies, specifically MAT and ELISA, for detection in blood and serum specimens. Notably, Yombo (2006) sero-epidemiological research revealed statistically significant variations in infection prevalence between agricultural establishments, with Markham Farm demonstrating a markedly higher incidence of active leptospirosis infection compared to Trukai Farm.

The absence of detectable leptospirosis may be partially attributed to vaccination status of the farms. Commercial farms and some smallholder operations may implement routine vaccination against endemic diseases, potentially reducing the likelihood of leptospirosis detection. Recent incursions of exotic animal diseases in PNG, such as African Swine Fever in pigs (PHARMAPlus 2021) and Newcastle Disease in poultry (Raitano 2010), may have enforced enhanced on-farm biosecurity measures and disease management strategies. However, information regarding vaccination and biosecurity protocols was unavailable for the sampled cattle farms. This limitation has prompted further investigation of presumed non-vaccinated cattle farms in subsequent chapters of this PhD project to better determine the true prevalence of leptospirosis in PNG.

4.1 Valuable Epidemiological Insights

Despite these limitations, our study yielded valuable epidemiological data regarding farm locations and cattle distribution patterns. Most cattle farms are centrally located along the Markham Valley, with some in proximity and others sharing land boundaries. This geographical clustering potentially facilitates disease transmission within or between farms. The Markham Valley's flat topography, high rainfall, and seasonal flooding create conditions favorable for leptospirosis transmission, as *Leptospira* spp. can survive up to 200 days under ideal environmental conditions (Pastre et al., 2020; Zelski 2007).

The study also revealed that 15 of the participating farms are smallholders, while four operate large-scale commercial enterprises. While farm management systems likely influence disease transmission, the retrospective data unfortunately did not provide sufficient information to evaluate management practices or disease control programs. Brahman cattle emerged as the dominant breed across all farms except EBC, which raises Holstein breeds primarily for dairy production. This breed preference is significant as Brahman cattle demonstrate better adaptation to tropical climates (Blackshaw et al., 1994; Hansen 2004), potentially affecting disease susceptibility patterns.

Cattle distribution varied considerably among farms, with nine operations (Bismarck, Coastal Solutions, Enos Mala, Jonathan Joel, Pelgens, Philip Ralda, Ramu Agri Industry Limited, Steward Paul, and Trukai) each providing 50 or more serum samples. Although the number of samples collected does not necessarily represent the total cattle population on each farm, densely populated operations generally face increased risk of disease transmission without proper management strategies. The study's analysis of gender and age distribution of cattle across farms further enhances the epidemiological value of this research, providing potential insights for

understanding how leptospirosis might distribute across different demographic groups, even though the pathogen was not detected in this study.

5. CONCLUSION

This retrospective investigation of cattle serum samples from the Morobe Province revealed no positive detections of *Leptospira* spp. through qPCR analysis, contrasting with previous documentation of *Leptospira interorgan* serovars in PNG cattle. This unexpected outcome may reflect prior vaccination practices in the sampled herds, though vaccination records were unavailable. Despite the absence of positive leptospirosis cases, the study generated valuable epidemiological data on farm distribution, cattle demographics, and environmental conditions potentially facilitating disease transmission. As part of our broader PhD research on leptospirosis in PNG livestock, these findings underscore the importance of investigating non-vaccinated farms in future studies. The discrepancy between our results and previous research warrants additional sampling to determine the true prevalence of leptospirosis in PNG cattle populations. The demographic data and sampling framework established through this investigation will inform ongoing epidemiological research and support the development of targeted surveillance strategies for this significant zoonotic disease, addressing both the economic impacts on livestock productivity and the associated public health concerns.

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