Brief Report

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Posted Date: 30 March 2026

doi: 10.20944/preprints202603.2277.v1

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Molecular Detection and Characterization of Infectious Laryngotracheitis Virus (ILTV) Genotype VII-IX in Backyard Chickens in Lusaka and Chongwe Districts, Zambia

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Abstract

Infectious Laryngotracheitis (ILT) is a respiratory disease of chickens caused by Infectious Laryngotracheitis Virus (ILTV) characterized by mild to severe respiratory disease, poor growth rates, and variable mortality. Although the disease has been suspected and several commercial vaccines are being used for its prevention in commercial enterprises, evidence of the presence of the virus has remained speculative. We set out to investigate the presence of the disease in backyard chickens. Throat and cloacal swabs were collected from 50 free-range unvaccinated backyard chickens purchased from local markets in Lusaka and Chongwe districts. Nucleic acid extraction was followed by PCR using ILTV ICP4 gene-specific primers. The ILTV gene was detected in a single apparently healthy chicken, representing 2% (1/50). Additional primers targeting the entire ICP4 gene including the flanking UTRs were designed. Sequence comparisons and phylogenetic analysis of the complete ICP4 coding region revealed the presence of ILTV genotype VII/IX CEO cluster. This work represents the first molecular detection and characterization of ILTV in non-vaccinated free-range backyard chickens in Zambia. In conclusion, this study confirms the presence of ILTV in Zambia. Determination of the ILT burden in chickens would facilitate informed decision-making regarding vaccination of chicken strategies and ILT disease prevention.

Keywords: infectious Laryngotracheitis virus; ICP4; chicken-embryo origin; Lusaka; backyard chickens; genotype VII/IX; tissue culture origin

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1. Introduction

Infectious laryngotracheitis (ILT) is one of the important highly contagious and acute respiratory diseases of chickens, pheasants, peafowl, and turkeys caused by *Gallid herpesvirus I* (GaHV1), commonly known as infectious laryngotracheitis virus (ILTV) [1,2]. The virus belongs to the family *Herpesviridae*, subfamily *Alphaherpesvirinae*, genus *Iltovirus*, and has a linear, double-stranded DNA genome [3]. Outbreaks of the disease cause variable effects ranging from mild to severe diseases characterized by respiratory signs such as lacrimation, nasal discharge, conjunctivitis, respiratory distress, dyspnea, bloody mucus secretion, and coughing, poor growth rates, mortality, and decreased egg production [4].

Geographically, ILT has been reported in many countries, with limited reports in African countries where it has been described in Libya [5], Egypt [6], and Namibia [7]. However, ILT has only been suspected in Zambia without laboratory confirmation of the virus in chickens. The disease may be misdiagnosed with other poultry diseases that cause similar clinical signs and lesions, such as infectious bronchitis, Newcastle disease, avian influenza, infectious coryza, and mycoplasmas [3].

There are currently several commercially available live-attenuated vaccines used to prevent and control ILT. The two main categories are derived from serial passages in chicken embryos (CEO; chicken embryo origin) or in cell culture (TCO; tissue culture origin). These vaccines are commonly used in commercial layers, layer breeders, and broiler breeders [8]. Although the live vaccines have been widely used, there is evidence of reactivation of latent strains [9]. One of the vaccines available is the lyophilized live vaccine VAXXON® ILT (IZO VAXXINOVA, Italy). This vaccine is regularly used in broilers and layers. However, vaccination of free-range backyard chickens is not commonly practiced in Zambia.

The ILTV ORF encoding ICP4 is about 4386 nucleotides long [10]. The ILTV ORF encoding ICP4 is commonly used in the characterization of ILT isolates [11]. In addition, PCR-RFLP and DNA sequencing methods have been successfully used to characterize ILTV isolates, with sequencing being commonly used for finer differentiation of isolates [12,13]. This work, therefore, describes for the first time, the detection, amplification, and sequencing of the ICP4 gene for characterization of field isolates of ILTV in free-range backyard breeds of chickens in Lusaka and Chongwe districts, Zambia.

2. Materials and Methods

2.1. Study Design

This cross-sectional study was conducted in backyard chicken markets in Lusaka and Chongwe districts, Zambia, from October to November 2024. Fifty (50), apparently healthy adult free-range backyard breeds of chickens were purchased from 4 different roadside poultry markets around Lusaka townships (Chainama, Mtendere, Ibex Hill) and Chongwe (Silverest) districts. The chickens had no known history of vaccination.

2.2. Sample Collection

The live chickens were purchased and transported to the Virology laboratory of the School of Veterinary Medicine, University of Zambia. Tracheal swabs were collected from the upper respiratory tract and eluted in 250 µL sterile normal saline.

2.3. Nucleic Acid Extraction and ILT ICP4 Gene Detection

About 200 µL of sample suspension was used for DNA extraction using the Qiagen DNA kit (QIAGEN, Germantown, MD, USA) according to the manufacturer's instructions. DNA was eluted in 60µL of the elution buffer, and split into 20µL aliquots for storage at -80°C until use.

To detect the presence of ILTV ICP4 gene, the initial PCR reaction was performed using the Q5® Hot Start High-Fidelity DNA Polymerase (New England Laboratories) or Quick Taq HS Dye Mix

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(Toyobo, Osaka, Japan) following the manufacturer's instructions. The primers for the detection of ILTV were ILT-Fw 5′-CCTCGACGCCGAGTAATTT-3′ and ILT-Rv 5′-GAGCGAGTCGATGACCGTAT-3′, yielding a 435bp amplification product [14]. The PCR conditions were 98°C for 2 min, 98°C for 10 sec, 56°C for 20 sec, 72°C for 45 sec with reaction cycles of 35 and final extension at 72°C for 5 min. The PCR products were observed on a 1% agarose gel stained with ethidium bromide. In order to minimise contamination, a commercially available live attenuated ILTV VAXXON® ILT (IZO VAXXINOVA, Italy) was subsequently acquired and processed.

2.4. ICP4 Gene Sequencing

The PCR product was Sanger sequenced and subjected to BLAST searches in the NCBI database. After confirmation of the ILT gene by PCR and sequencing, additional primers targeting overlapping fragments of the entire coding region of the ICP4 gene were prepared and used in the subsequent PCR reactions (Table 1)

Table 1. Primers used in the amplification of the entire ICP4 gene of ILTV.

No.	Segment	Primer	5'-3'	Size(bp)
1	A	ICP4_UTR226Fw	GGTTGCGGAGCTTTATTGTG	1083
2		ICP4_856Rv	CATCGGGACATTCTCCAGGTAGCA	
3	B	ICP4_241Fw	TCTGTCATCGCTGAGAATGC	997
4		ICP4_1237Rv	CCATGAACTCCTCCACAAGG	
5	C	ICP4_1163Fw	AATCAGCTTCCGTGTATCTC	757
6		ICP4_1921Rv	CATCATCGGTAGATGGCAGC	
7	D	ICP4_1746Fw	TATAGACTGCCCACCAGTTA	640
8		ICP4_2385Rv	TCTTTTCAGACCTTTACGGG	
9	E	ICP4_2304Fw	GCCATTATCTGTGTCACCGT	614
10		ICP4_2917Rv	TCACGGGTGAAATCCTCTTG	
11	F	ICP4_2788Fw	AACTATTTGCGCGGGTTGAG	779
12		ICP4_3566Rv	CTGAGAGTTACGTGAGTGAG	
13	G	ICP4_3202Fw	GCGGTAGAATGGATGCTTGA	978
14		ICP4_4179Rv	ACCAAGTAGCAATGCGAGAG	
15	H	ICP4_3815Fw	ACGAGAAAGAAGATCGTGA	791
16		ICP4_4605Rv	GCAAAGCTAAAGTCAACGGG	
17	I	ICP4_4302Fw	AAGGCTATCCGTCTCCTCAG	770
18		ICP4_UTR5071Rv	TCGCGCATTCTGTACTTTGA	

The PCR products were gel-purified using the Promega PCR Product and Gel Purification Kit (Promega, Madison, WI, USA). Each product was sequenced in the forward and reverse directions with respective primers using the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, California, USA). The nucleotide sequences were analysed using Genetyx Version 12.0 (GENETYX Corporation, Tokyo, Japan) and confirmed with BLAST search. The sequence was deposited in GenBank and assigned accession number PV433262.

2.5. Phylogenetic Analysis

The evolutionary tree based on the full-length ICP4 gene (4,449bp) was constructed on MEGA X software using the neighbor-joining method with sequences from Australia, Canada, Chile, China,

 doi:10.20944/preprints202603.2277.v1

Italy, Peru, Russia, South Korea and USA. The data were subjected to bootstrap analysis with 1000 replicates to determine the reliability values at each internal node.

3. Results

3.1. Detection and Sequencing of ILT ICP4 Gene

The ILTV was successfully amplified (Table 1, Figure 1) in 1/50 free-range backyard breeds of chickens (ILT_ZAM_08_2024). Subsequently, the entire ORF of ICP4 (4,449bp) was successfully amplified and sequenced. The commercial VAXXON® ILT vaccine was similarly processed. The sequence was deposited in GenBank with accession number PV433262.

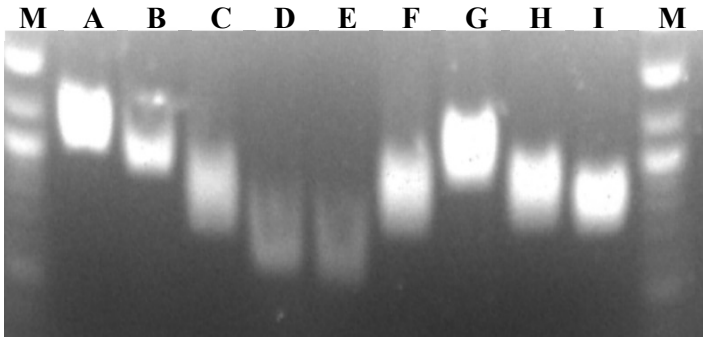

Figure 1. PCR detection of ICP4-gene of ILTV using ICP4 primers (Table 1). Lane M (1kb ladder) Lane A, Lane B, Lane C, Lane D, Lane E, Lane F, Lane G, Lane H, Lane I, Lane M.

3.2. Phylogenetic Analysis of the ICP4 Gene

Phylogenetic analysis based on the ILTV ICP4 gene with other sequences produced several genotype clusters, namely, IV, VI-4, VI-5, I/III and VII/IX genotypes. The Zambian ICP4 sequence (PV433262) was grouped in genotype VII/IX . In addition, the commercial vaccine sequence of Vaxxon® vaccine appeared to cluster with genotype IV TCO clade (Figure 2). A comparison of the nucleotide of the ICP4 gene with the sequence of accession number: NC075683.1 SA2 of genotype VII/IX showed similar nucleotide polymorphism [15]. The ILTV genotype was closer to the TCO genotype (I/III) than the CEO genotype (IV) suggesting different origins of the virus.

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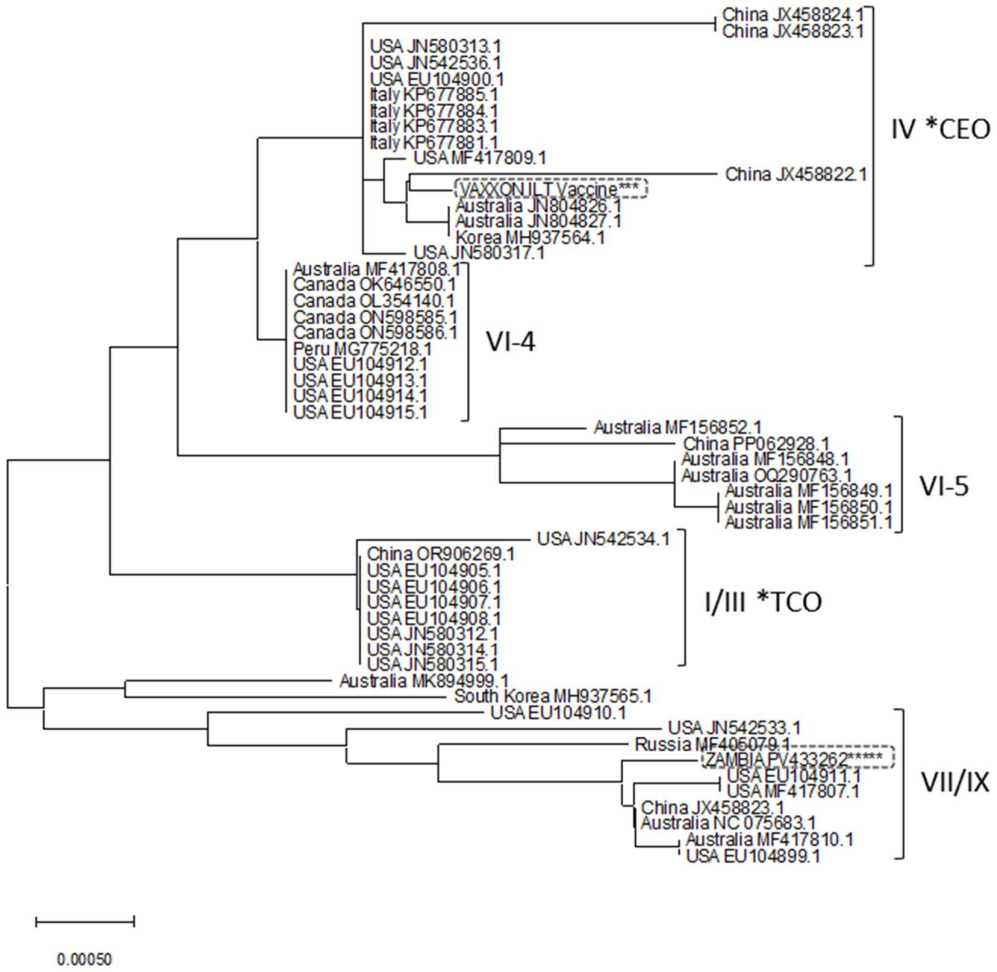

Figure 2. The evolutionary history of ILTV ICP4 gene sequences inferred using the Neighbor-Joining method [16]. The phylogenetic tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method [17] and are in the units of the number of base substitutions per site. This analysis involved 54 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). A total of 4483 positions in the final dataset were used. Evolutionary analyses were conducted in MEGA X [18]. The two ILTV sequences obtained in the study (Zambia PV433262 and the Vaxxon® Vaccine) are shown (****) in clade VII/IX and IV, respectively. [*CEO; chicken-embryo origin, (TCO; tissue culture origin].

4. Discussion

Infectious laryngotracheitis is an important respiratory disease of poultry with serious economic losses worldwide. The ILTV has not yet been reported in Zambia. This is the first report of molecular detection and characterization of ILTV in apparently healthy free-range backyard chickens. There is currently no scientific data on ILTV surveillance in poultry farms available in the country. Despite this lack of confirmation of the virus circulating, commercial vaccines are available and are regularly administered in medium and commercial flocks. In Zambia, ILT is occasionally suspected during incidences of unexplained respiratory diseases in poultry. Until now, the presence or extent of ILT distribution in Zambia is unknown. The detection of ILTV in this study is strengthened by the lack of vaccination of the chickens before sampling. In addition, the ILTV had not been previously

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detected nor manipulated in the laboratory before. The vaccine control from the vaccine virus was only processed subsequent to the detection and sequencing of the chicken samples.

The virus detected in the present study shares a high nucleotide similarity with strains from other regions. While clustering in a clade VII/IX, the virus was genetically distant from the VAXXON® vaccine strain. Since these birds had no known history of vaccination against ILT, the extent of virulence of the virus could not be established. Whether the genetic difference with the vaccine has implications for the protective efficacy of the vaccine cannot be deduced. It was however evident from the genetic difference that the strain in the chickens was not TCO vaccine-derived, as the virus was CEO derived. Therefore, there is a need for more investigation to facilitate appropriate selection as vaccines can cause disease in vaccinated flocks [19].

Beyond Zambia, ILT has been confirmed in Namibia and Egypt [7,20]. The prevention of ILT focuses on the administration of attenuated live-virus of chicken embryo origin and/or tissue culture vaccine strains [21]. Although generally safe, these vaccines have also been associated with outbreaks in vaccinated flocks through reversion to virulence [7,22]. During outbreaks, the virus is known to persist for a considerable period of time in the environment thus, facilitating spread even when biosecurity measures are in place [7]. Although useful in mitigating the effects of the disease, vaccination strategy alone is not effective at eliminating the virus. In the present study, there was no evidence of vaccination of the chickens. The full spectrum of commercially available vaccines was not available. CEO-based vaccines may also available on the Zambian market.

Infectious Laryngotracheitis causes severe disease and mortality in commercial birds [4,7]. However, the disease situation in backyard chickens remains unknown and scanty, with a few reported cases in some countries [23]. The birds in this study were apparently healthy, with no available history of vaccination against ILT. Vaccination of backyard chickens is not a common practice and is rarely administered. The absence of clinical disease in the backyard chickens could suggest a mild strain of the virus or low susceptibility of these chickens to ILT, possibly influenced by their genetic makeup as previously observed [24].

Infectious Laryngotracheitis has been suspected in both commercial broiler and layer flocks, evidenced by the use of commercial vaccines [7]. More data on the epidemiology of the ILT is required in order to establish the direction of the disease transmission between commercial and backyard flocks [25]. The importance of molecular characterization of ILT and the roles of live vaccines in precipitating ILT outbreaks should also be considered in order to establish the origins and direction of infection [26].

5. Conclusion

The present study reveals the presence of ILTV genotype VII/IX in asymptomatic backyard chickens. The detection of ILTV in backyard chicken form a foundation for a broader understanding of the disease epidemiology. The molecular investigation is important to understand the origin, molecular and biological characteristics of ILTV in Zambia as more vaccines are introduced in the poultry sector.

Author Contributions: For research articles with several authors, a short paragraph specifying their individual contributions must be provided. The following statements should be used "Conceptualization, N.S., R. M., A.N.M., O.J. and J.S.; methodology, O.J., C.C., and A.N.M.; software, N.S.; validation, N.S., R.M. and J.S.; formal analysis, K.M, N.S, A.N.M.; investigation, N.S., O.J, R.M.,; resources, J.S., K.M., H.S., and A.T., ; data curation, N.S. J.S. and K.M.,.; writing—original draft preparation, N.S., O.J., and R.M.,; writing—review and editing, N.S., O.J., R.M., J.S, A.T, K.M, H,S., A.N.M., and C.C.; visualization, N.S., and K.M.; supervision, N.S., R.M. J.S.; project administration, N.S.; funding acquisition, J.S,. K.M., A.T., H.S. All authors have read and agreed to the published version of the manuscript.

Funding: This study was supported by the Japan Agency for Medical Research and Development (AMED) under grant number JP20wm0125008.

 doi:10.20944/preprints202603.2277.v1

Institutional Review Board Statement: The animal study protocol was approved by University of Zambia Health Sciences Research Ethics Committee of The University of Zambia (protocol code 2023270273 and 20th October, 2024).

Informed Consent Statement: Not applicable.

Data Availability Statement: The sequence generated in this study was deposited in GenBank, under accession number PV433262. The sequence generated in this study was deposited in GenBank under accession number PV433262.

Acknowledgments: Not applicable.

Conflicts of Interest: The authors declare no conflicts of interest.

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