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Application of ATP6 as Genetic Marker for the Detection of Migratory *Trichinella spiralis* Larvae in the Blood of Slaughtered Pigs in Kaduna Metropolis, Nigeria

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Trichinellosis, a re-emerging zoonotic disease with global prevalence, affects mammals, birds, and reptiles through the consumption of raw or undercooked pork containing *Trichinella* larvae. This study aimed to evaluate the efficacy of the ATP6 synthase F0 subunit (ATP6) gene as a genetic marker for detecting migratory *Trichinella spiralis* larvae in the blood of slaughtered pigs in Kaduna Metropolis, Nigeria. Whole blood samples from nine pigs, sourced from backyard farms and slaughtered at a local abattoir, were analysed using Polymerase Chain Reaction (PCR) targeting the ATP6 gene, producing an amplicon of 250 base pairs (bp). The molecular analysis revealed a prevalence rate of 33.3% for *T. spiralis* larvae. The PCR-based approach, utilising species-specific primers for ATP6, enables real-time diagnosis of trichinellosis, offering earlier detection compared to conventional methods such as microscopy, pepsin digestion, or serology.

Keywords: ATP6, Polymerase Chain Reaction, Trichinellosis, Migratory, *Trichinella*, Larvae, Pigs, Kaduna, Nigeria

Introduction

Trichinella, a genus of parasitic roundworms, is responsible for trichinellosis, a zoonotic disease with global prevalence in humans and animals. Trichinellosis manifests primarily in humans with clinical symptoms. Historically, swine have been the primary intermediate hosts of concern for human infections due to their close association with the biology and epidemiology of *Trichinella* (Diaz et al., 2020). Transmission occurs through predation or consumption of carrion, involving both domestic and sylvatic cycles. Human infections typically result from consuming raw or undercooked infected meat, often linked to household pig slaughter or unregulated game meat consumption, practices that pose challenges for control (Ojodale et al., 2020b; Echeverry et al., 2021). Infections are now predominantly associated with unregulated sources, such as backyard pork production and game animals (Kärssin et al., 2017). *Trichinella spiralis*, the most prevalent species, employs a sophisticated biological mechanism to meet its energy demands across its complex life cycle, involving multiple animal and human hosts (Atterby et al., 2009; Pozio et al., 2009; Ribicich et al., 2010). Understanding the parasite's reservoir is critical for effective public health strategies (Echeverry et al., 2021). Mitochondrial genes, including mitochondrial small subunit ribosomal DNA (mt SSUrDNA), mitochondrial large subunit ribosomal DNA (mt-lsrDNA), 5s rRNA, and the ATP6 synthase F0 subunit (ATP6) gene, have been characterised in *Trichinella* (Lavrov and Brown, 2001; Golab et al., 2009; Attia et al., 2016). The ATP6 gene, the focus of this study, is conserved in *Trichinella* and readily amplified (Golab et al., 2009; Attia et al., 2016). Also known as ATP synthase 6, ATPase-6, or MTATP6, among other names, it is a key component of the mitochondrial ATP synthase complex, crucial for energy production (GHR, 2018; Del Dotto et al., 2024).

The *Trichinella* life cycle begins when encysted larvae in raw or undercooked meat are ingested by humans or animals. These larvae are released during digestion, mature into adult worms in the small intestine, and, after mating, female worms produce newborn larvae. These larvae penetrate the intestinal wall, enter the bloodstream, and disseminate to various tissues, particularly muscle, where they encapsulate, establishing chronic infections. The cycle continues if the infected host is consumed by a suitable predator (Mohammed et al., 2022). The ATP6 gene, located in the mitochondrial genome, encodes a vital subunit of ATP synthase, a molecular machine that synthesises ATP, the universal energy currency, using a proton gradient across the mitochondrial inner membrane during oxidative phosphorylation. The ATP6 subunit serves as a proton channel, driving ATP synthesis (GHR, 2018). In *Trichinella*, the ATP6 gene is highly conserved across species, underscoring its essential role in parasite survival. Its protein sequence suggests adaptations to the parasite's unique environmental challenges, and variations in ATP6 expression across developmental stages reflect the parasite's dynamic energy

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needs (Del Dotto et al., 2024). Exploring the ATP6 gene's role could reveal insights into *Trichinella*'s energy metabolism and adaptation strategies, potentially identifying novel therapeutic targets (Del Dotto et al., 2024).

Diagnosis of *Trichinella* infections is critical to eliminate infected animals from the food chain and prevent human disease (Mohammed et al., 2022). Polymerase Chain Reaction (PCR) has been widely used to amplify DNA from *T. spiralis* muscle larvae and detect larvae in blood or muscle of various hosts (Pozio and Murrell, 2006; Caballero-Garcia and Jimenez-Cardoso, 2001; Atterby et al., 2009; Attia et al., 2016). PCR-based methods have simplified *Trichinella* identification across host species and geographic regions, confirming the taxonomy of the genus (Pozio and Murrell, 2006). Using PCR, *T. spiralis* was identified, overcoming limitations of digestion techniques, which are impractical for large sample sizes (Diaz et al., 2020; Ojodale et al., 2020a). PCR-based methods with genetic markers have proven effective for species identification and phylogenetic analysis of all 12 *Trichinella* genotypes (Borsuk et al., 2006). Studying the ATP6 gene and its proteins could enhance diagnostic tools, such as molecular markers, for more accurate and efficient detection of *Trichinella* infections.

Materials and Methods

Ethical Consent

Ethical approval was obtained from the Health Research Ethics Committee (HREC) of the Ministry of Health and Human Services, Kaduna State, Nigeria (MOH/ADM/744/VOL.1/503).

Study Design

This cross-sectional community-based survey aimed to detect the ATP6 gene of *Trichinella spiralis*. Using systematic sampling, a pig slaughterhouse in Angwa Television, Kaduna Metropolis (Latitude 10.609319°N, Longitude 7.429504°E), was visited every other day over an 18-day period. Blood samples were collected by random sampling from pigs slaughtered on each visit.

Genomic DNA Isolation/Extraction

Genomic DNA was extracted from whole blood using the AccuPrep® Genomic DNA Extraction Kit (Bioneer Corporation, South Korea) following the manufacturer's protocol. Briefly: Proteinase K was dissolved in 1,250 µl of nuclease-free water. Then, 20 20 µl of Proteinase K was added to a 1.5 ml tube, followed by 200 µl of whole blood. Next, 200 µl of binding buffer (GB) was added and mixed by vortexing. The mixture was incubated at 60°C for 10 minutes. Subsequently, 100 µl of isopropanol was added and mixed by pipetting. The lysate was transferred to the upper reservoir of a Binding column tube fitted with a 2 ml tube, centrifuged at 8,000 rpm for 1 minute, and the Binding column tube was moved to a new 2 ml tube. Then, 500 µl of wash buffer 1 (W1) was added, centrifuged at 8,000 rpm for 1 minute, and the solution was discarded. This was followed by adding 500 µl of wash buffer 2 (W2), centrifuging at 8,000 rpm for 1 minute, and an additional centrifugation at 13,000 rpm for 1 minute to remove residual ethanol. The Binding column tube was transferred to a new 1.5 ml tube, 200 µl of Elution buffer (EL) was added, incubated at 25°C for 5 minutes, and centrifuged at 8,000 rpm for 1 minute to elute the DNA.

Polymerase Chain Reaction (PCR) Amplification

PCR Mixture

The PCR mixture, in a 50 µl reaction volume, contained 500 ng of template DNA, 10 mM Tris-HCl, 1.5 mM $MgCl_2$, 5 mM KCl, 0.1% Triton X-100, 0.1% gelatin, 200 µM each dNTP, 0.5 µg of pPRA primers, and 1.25 U of Taq polymerase (Viveros et al., 2000). Additionally, 15 µl of PCR MasterMix (composition: dATP 400 µM, dCTP 400 µM, dGTP 400 µM, $MgCl_2$ 3 mM, Taq DNA polymerase 50 µ/m), 4 µl of water, and 1 µl of oligonucleotides (primer sequence) were included. Nuclease-free water served as the negative control to verify PCR system efficacy (Viveros et al., 2000; EURLP, 2016).

Table 1 Summary of PCR mixture

Component	Quantity
Template DNA	500 ng
Tris-HCl	10 mM
$MgCl_2$	1.5 mM
KCl	5 mM
Triton X-100	0.1%
Gelatin	0.1%
dNTP (each)	200 µM
pPRA primers	0.5 µg
Taq polymerase	1.25 U
PCR MasterMix	15 µl
Water	4 µl
Oligonucleotides	1 µl

PCR amplification products were separated by electrophoresis on a 2% agarose gel stained with ethidium bromide and visualised using a UV transilluminator (Bio-Rad Cycler, Peltier-Effect Cycling, MJ Research Incorporated, USA) (Viveros et al., 2000; Godaratollah and Saberivand, 2009; EURLP, 2016).

Determination of Prevalence (%) of Trichinella spiralis Infection in Pigs Using PCR

Prevalence was calculated using the formula:

$$\text{Prevalence in pigs (\%)} = \frac{\text{Number of positive samples (bands)}}{\text{Total number of samples}} \times 100$$

Result

$$\text{Prevalence in pigs (\%)} = \frac{3}{9} \times 100 = 33.3\%$$

Table 2 PCR conditions for *Trichinella spiralis* detection

Pre-denat.	Denat.	Annealing	Ext.	Final ext.	Cycles	Hold temp.
94°C, 5 min	94°C, 30 s	48°C, 30 s	72°C, 1 min	72°C, 5 min	35	10°C, ∞

Table 3 Primer sequence for mitochondrial ATP6 synthase F0 subunit gene

Primer	Sequence	Amplicon size
ATP6-F	5'-CACACTAACCAAAGCCAAACCATC-3'	250 bp
ATP6-R	5'-GGAGTATGTTAGATGTTATTGTGTAGGAG-3'	

Reference: Golab et al., 2009; Attia et al., 2016
Key: F = Forward, R = Reverse, ATP6 = ATP synthase F0 subunit 6 gene, bp = base pairs

Table 4 Detection of ATP6 gene of *T. spiralis* migratory larvae in blood of pigs

Sample No.	PCR (ATP6 gene)
2	−
3	−
4	−
5	−
6	+
7	−
8	+
9	+
10	−

Key: ATP6 = ATP6 Synthase F0 subunit gene, + = positive, − = negative

Table 5 Overall prevalence of trichinellosis in pigs by PCR

	No. Examined	No. Positive (%)
Pigs	9	3 (33.3)

Key: PCR = Polymerase Chain Reaction

Discussion

Our findings revealed that PCR detected *Trichinella spiralis* migratory larvae in pig blood samples with a prevalence rate of 33.3%. This result aligns with Attia et al. (2016), who detected *T. spiralis* migratory larval DNA in blood samples of infected experimental mice on days 4, 6, and 14 post-infection (pi), suggesting that positive samples in this study were from pigs in early infection stages with actively migrating larvae. Attia et al. (2016) reported a 50% detection rate, with 5 of 10 blood samples from infected mice testing positive. This is consistent with studies reporting varying PCR detection rates for parasitic infections in animal models (Singh et al., 2019). The lower prevalence in our study may reflect the use of naturally infected pigs with unknown infection status, unlike the controlled experimental conditions in mice studies. Larval deposition by adult female *Trichinella* occurs between 3–14 days pi and may persist for 10–20 days (Attia et al., 2016). Previous research detected circulating *T. spiralis* larvae in mouse blood from days 5–14 pi (Uparanukraw and Morakote, 1997; Li et al., 2010) and 5–17 days pi (Caballero-Garcia and Jimenez-Cardoso, 2001).

Attia et al. (2016) used PCR with ATP6 primers to detect *T. spiralis* migratory larvae in mouse blood, yielding the expected 250 bp band, and suggested that validating such assays on clinical samples could enhance timely diagnosis of trichinellosis in humans and animals. However, Li et al. (2010) noted that PCR was less effective for detecting *T. spiralis* DNA in human patients compared to mice, despite the longer larval migratory period in humans. Variable PCR results may stem from differences in DNA extraction methods or low larval burdens in blood samples (Abu-Madi et al., 2020).

Whole blood was used in this study instead of serum or plasma, as it is considered optimal for detecting pathogens, with higher detection rates compared to serum or plasma, where pathogen fractions may be reduced (Watkins-Riedel et al., 2004; Klung-Thong et al., 2007). Attia et al. (2016) confirmed that PCR with DNA extracted from plasma and serum failed to detect infections caused by *T. spiralis*, *T. pseudospiralis*, and *T. nelsoni*. While *T. spiralis* and *T. nativa* can be identified using PCR with primers targeting expansion segment V (ESV) producing 173 or 127 bp amplicons, differentiation is challenging (Fu et al., 2009). Thus, ATP6 primers yielding a 250 bp amplicon specific to *T. spiralis* were used (Attia et al., 2016). Li et al. (2010) identified a *T. spiralis* isolate from a naturally infected pig in Tibet, China, using PCR, with amplified fragments visualised by agarose gel electrophoresis.

The Enzyme-Linked Immunosorbent Assay (ELISA) has been the gold standard for presumptive or retrospective diagnosis and surveillance of trichinellosis in pigs and humans (Ojodale et al., 2015a; Ojodale et al., 2015b). Viveros et al. (2001) reported a 15% prevalence in mice positive by ELISA, with expected PCR amplification products. Some ELISA-positive results may result from cross-reactivity of TSL-1 antigens with other nematode

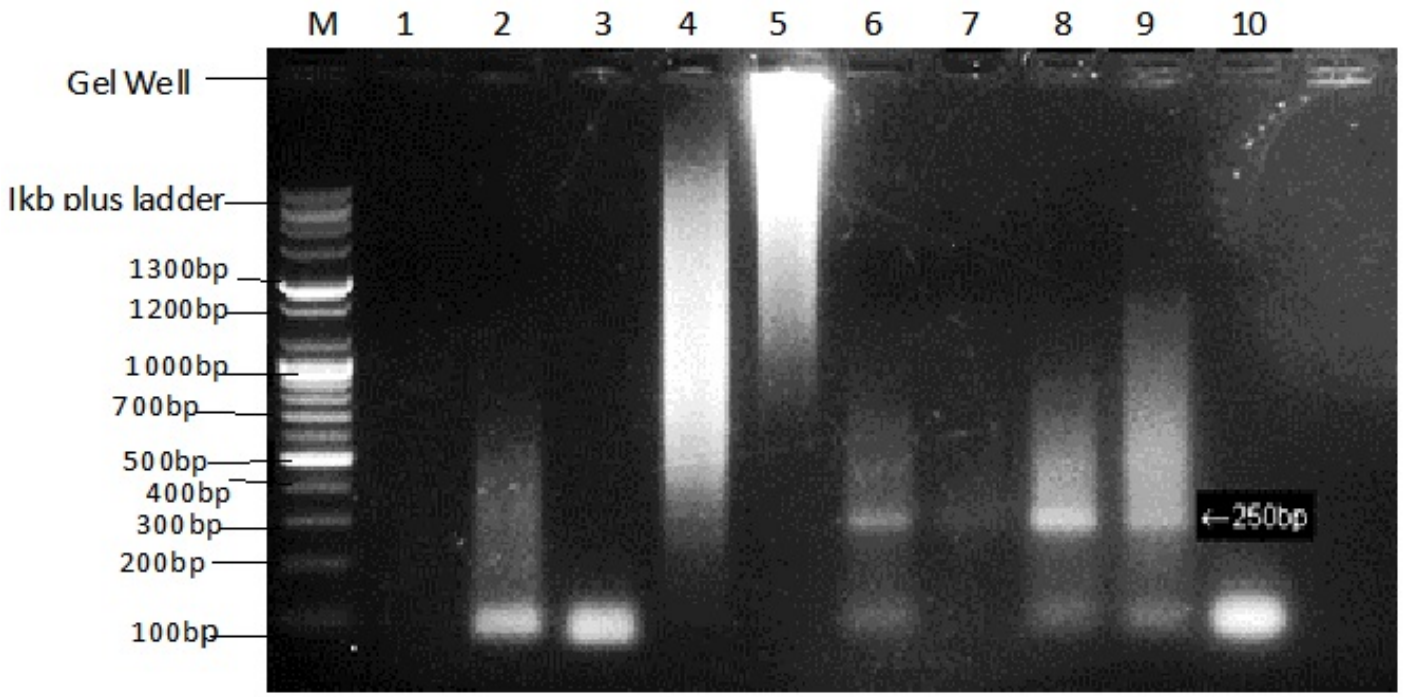

Plate 1: Agarose gel electrophoresis plate of ATP6 synthase gene from migratory larvae of *Trichinella spiralis* in pig blood using ATP6 primer. **Key:** Lane M = Molecular weight marker, Lanes 2–10 = Pig blood samples, Lane 1 = Nuclease-free water as negative control. Lanes 6, 8, and 9 = Positive for *T. spiralis* with the expected amplicon size of 250 bp. bp = base pairs

antigens, as these are prevalent in excretory/secretory products (Dea-Ayuela et al., 2000). Viveros et al. (2001) found 55% of ELISA-negative samples positive by PCR and a 25.6% prevalence for ELISA-negative samples (Ojodale et al., 2020b), suggesting that combining ELISA and PCR enhances diagnostic accuracy for trichinellosis (Ojodale et al., 2020b). Ojodale et al. (2019, 2020a) reported a 39% prevalence in humans in Kaduna Metropolis, Nigeria, indicating trichinellosis as a re-emerging zoonosis with increasing incidence compared to an earlier 16.1% (Ojodale et al., 2015b). Comparisons of pig trichinellosis prevalence rates of 16% (Ojodale et al., 2020a) and 13.4% (Ojodale et al., 2015a) using ELISA suggest a rising trend in Nigeria. Several risk factors were identified. Although serological methods are unsuitable for meat inspection, they are valuable for surveillance and epidemiological studies in high-prevalence areas like Nigeria (Ojodale et al., 2020b).

Conclusion

This study concludes that trichinellosis, a zoonotic disease, is present in pigs in Kaduna Metropolis, Nigeria. Polymerase Chain Reaction showed a prevalence of 33.3% for pigs. The PCR procedure utilising a suitable primer for ATP6 and using it as a genetic marker is valuable for early detection of *T. spiralis* migratory larvae in blood samples of pigs. The result of this study is of public health importance because meat and meat products are considered very important sources of daily protein intake and also serves to enlighten consumers and pig producers who crave for "organic" or "green" pigs that provide meat from porcine animals raised under natural situations.

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Authors' Declaration

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