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SEROLOGICAL AND MOLECULAR EVALUATION OF MIGRATORY *TRICHINELLA SPIRALIS* LARVAE IN BLOOD OF HUMANS IN KADUNA METROPOLIS, NIGERIA

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ABSTRACT
Trichinellosis is an important food-borne zoonotic disease with public health implications and a worldwide distribution. In this study, Polymerase Chain Reaction (PCR) procedure using species specific ATP6 primers was used to detect the presence of migratory *Trichinella spiralis* larval mitochondrial ATP6 synthase F0 subunit (ATP6) gene, after detection of antibodies to *Trichinella* excretory-secretory (E/S) antigen using Enzyme-linked Immunosorbent Assay (ELISA), in blood of humans in Kaduna metropolis, Nigeria. The sera of 210 participants were tested for antibodies to *Trichinella* E/S antigen. Overall seroprevalence rate of 39% (82/210) was recorded using ELISA. Out of the 9 ELISA samples selected randomly, PCR detected migratory *Trichinella spiralis* larval ATP6 gene in 4 (44.4%) at the amplicon size of 250 base pairs using the whole blood of the participants. The 9 samples comprised 7 seropositive and 2 seronegative. The bands at lanes 1, 2, 3 and 4 were positive for ATP6 while lanes 5,6,7,8 and 9 were negative for ATP6. Lanes 4 and 5 were ELISA negative for anti-*Trichinella* antibodies. One in 5 of the 128 ELISA negative samples was positive for ATP6 representing a 25.6% prevalence rate by extrapolation. PCR using ATP6 gene as a genetic marker is valuable for the detection of *T. spiralis* migratory larvae in blood samples of humans and consequently the early diagnosis of trichinellosis in humans.
Keywords: Trichinellosis; PCR; ELISA; *Trichinella spiralis*; ATP6; Migratory; Antibodies; Larvae.

INTRODUCTION

Trichinellosis is an important food-borne zoonotic disease with public health implications and a worldwide distribution. It is caused by the ingestion of raw or undercooked meat containing *Trichinella* larvae. The parasites can infect many warm and cold blooded carnivores and omnivores especially mammalian species (Pozio and Murrell, 2006; Atterby *et al*., 2009; Ribicich *et al*., 2010). *Trichinella spiralis* is the most widely distributed species and the most common cause of human trichinellosis which causes death in severe cases (Dupouy-Camet, 2009; Pozio *et al*., 2009). Although raw pork is the most common source of infection for humans, other important sources include undercooked or improperly cooked meat and meat-derived products from carnivores, omnivores and herbivores (Gottstein *et al*., 2009).

Trichinella worms are characterized by two generations in the same host: the enteral and parenteral. Female adult *T. spiralis* parasite produces live offspring at 5 days post infection (dpi) and one female worm produces about 1500 larvae in 5-10 days with the potential of larval production continuing as long as the female worm remains in the intestine. The duration of production of *T. spiralis* larvae differs in different hosts persisting for 10-20 days in mice and rats and 4-6 weeks in humans (Bell, 1998). The newly formed larvae migrate to the bloodstream via the lymphatics and veins and then invade skeletal muscle cells to give rise to the complex infective larvae and the nurse cells that evolve from invasion of these muscle cells by the larvae (Wang and Bell, 1986; Gottstein *et al*., 2009).

Serologic and molecular tests are important in the diagnosis of trichinellosis (Dupouy-Camet *et al*., 2002; Yera *et al*., 2003). Serological methods are useful for the presumptive or retrospective diagnosis and surveillance of human infection (Gottstein *et al*., 2009; Ojodale *et al*., 2015a). Most ELISA tests presently available do not yield 100% specificity and therefore a complementary test is usually needed to confirm the diagnosis of any initial ELISA seropositivity (Gamble, *et al*., 2004; Ojodale *et al*., 2015a).

Polymerase chain reaction (PCR) is useful for species identification of *Trichinella* especially as their morphological characteristics useful for identifying taxa are lacking (Zhifei, *et al*., 2011). It has been widely applied to the amplification of DNA from muscle larvae of *T. spiralis* (Pozio and Murrell, 2006) and the detection through PCR of larvae of the organism in blood or muscle of some hosts has been reported (Uparanukram and Morakote, 1997; Caballero- Garcia and Jimenez-Cardoso, 2001; Atterby *et al*., 2009; Attia *et al*., 2016). The target gene fragment of this study, ATP6 ATP synthase F0 subunit gene (GeneID: 802674; GU33913), is conserved in *Trichinella* and easily amplified (Golab *et al*., 2009; Attia *et al*., 2016).

Attia *et al*. (2016) had carried out PCR assay with DNA extraction using ATP6 primers to detect *T. spiralis* migratory larval in blood of

infected mice and came out with the expected band size of 250bp and opined that further validation of such assays on clinical samples would propose a promising tool for the timely diagnosis of human and animal trichinellosis.

The aim of this study therefore was to detect and evaluate migratory *Trichinella spiralis* larvae in blood of humans using ATP6 gene as a genetic marker which we believe is valuable for the early diagnosis of trichinellosis in humans. This will facilitate the diagnosis of infection earlier than when using microscopy or serology especially for patients whom serology (ELISA) for specific anti-*Trichinella* antibodies yielded negative results or for immunosuppressed patients.

MATERIALS AND METHODS

Study area

The study was carried out in Kaduna Metropolis, Kaduna State, Nigeria. Kaduna State occupies part of the central position of the Northern part of Nigeria (with Kaduna as its capital City). It is located in the North West Geo-Political Zone of Nigeria and shares common borders with Zamfara, Katsina, Niger, Kano, Bauchi, Plateau and Nassarawa states. To the Southwest it shares a border with the Federal Capital Territory. The population of the state, according to the 2006 census, is 6,113,503 people with an annual projected growth rate of 2.47%. Kaduna city along with Zaria and Kafanchan are the main urban areas of the state. It is on the Kaduna River and has a total area of 1,190 sq mi (3,080 km^2) and its coordinates are 10°31'23'N'7°26'25''E. Over 60 ethnic groups namely, the Gbagyi, Hausa, Fulani, Gwong, Atuku, Bajju, Atyab, Gure and Ninkyop among others populate the state. It is a major economic hub in the region, a trade centre and transportation axis to nearby agricultural areas and states {2006 census} https://kdsg.gov.ng/demographics/ (accessed 7th July, 2020)

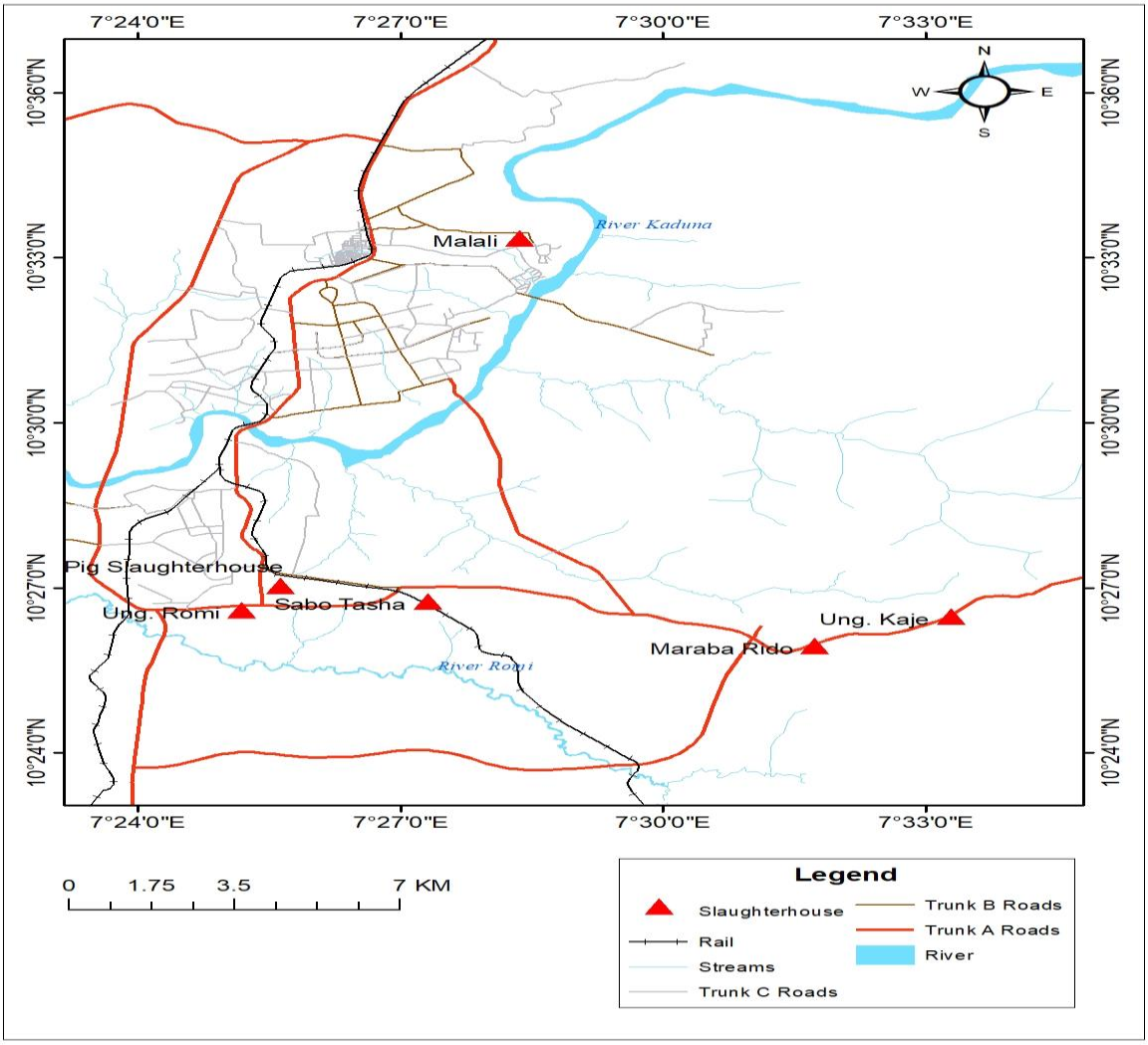

Figure 1. Map of Kaduna Metropolis showing study area and sampling sites

Ethical Consent

The ethical approval for this work was sought and obtained from 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 study is a cross-sectional community based survey to detect and evaluate anti-*Trichinella* antibodies and ATP6 gene of *Trichinella spiralis*.

Study Population

The study population included 100 respondents who are pig owners, farmers, butchers or handlers, who were surveyed by questionnaires. It also included 210 pork eaters living in pig rearing communities: Sabo Tasha, Angwa Television, Malali, Angwa Romi (see Figure 1) in Kaduna Metropolis.

Sample Collection and Processing

Two hundred and ten blood samples were obtained from respondents after their informed consent had been sought. Five milliliter of blood samples were obtained into labeled sample bottles (Vacutainer). The blood samples were divided into two portions and transported to the DNA Research Laboratory, Kaduna, in an ice-packed box. Centrifugation was done for the first portion at 3000rpm for 10 minutes to obtain the sera. The sera were dispensed into clean labeled sample bottles and stored at -20^{o}C and the other portion which was whole blood was refrigerated at -20^{o}C and kept for molecular analysis.

Procedure for Detection of anti-*Trichinella* Antibodies in Human Sera

This was carried out following the *Trichinella* ELISA kit manufacturer's protocol (Diagonostic Automation, Incorporated, USA)

Test procedure

The plates were labeled accordingly with wells A_1^1 and A_2^1 left as blanks. Exactly 100µl of the negative control was added to wells A_1^2 and A_1^3each and same quantity of the positive control to wells A_1^4 and A_1^5 each. Exactly 100µl of the diluted (1:64) test samples was added to the remaining wells. The dilution was done by diluting 5µl of sera in 315µl dilution buffer. Care was taken not to dilute the positive and negative controls since they are usually supplied pre-diluted. The plates were incubated at room temperature ($28^0C \pm 2^0C$) for 10 minutes. The contents of the plates were shaken out and washed three times with the diluted wash buffer (Wash Concentrate 20x [25ml] in 475ml of distilled water). Two drops of Enzyme Conjugate was added to each well and again incubated for 5 minutes at room temperature. The content of each well was emptied and the wells washed 3 times. Care was taken to remove the entire wash buffer by slapping plates against paper towels. Two drops of the Chromogen were added to each well and incubated for 5 minutes at room temperature. Two drops of the stop solution were added and mixing was done by tapping the strip holders. Reading was carried out using an ELISA reader at 450 nm (Ojodale *et al*., 2019).

Interpretation of ELISA Tests Results

The ELISA test results was calculated based on the manufacturers' protocols and recommendations as follows: The results was interpreted as Positive for absorbance reading greater than 0.3 OD (optical density) units and Negative for absorbance reading less than 0.3 OD units.

Determination of Prevalence rate (%) of trichinellosis

Percentage prevalence was calculated using the formula:

$$\text{Prevalence (\%)} = \frac{\text{Number of positive serum samples for trichinellosis}}{\text{Total number of samples}} \times 100$$

$$\text{Overall prevalence for human trichinellosis (\%)} = \frac{82}{210} \times 100 = 39.0\%$$

DNA extraction

DNA was extracted from whole blood using *AccuPrep*® Genomic DNA Exraction Kit (Bioneer Corporation, South Korea) according to the manufacturer's protocol. Briefly:

Before the extraction process began, Proteinase K was completely dissolved in 1,250µl of nuclease-free water. Exactly 20µl of Proteinase K was added to a clean 1.5 ml tube and then 200µl of whole blood to the tube containing the Proteinase K. It was followed by the addition of 200µl of binding buffer (GB) to the sample and mixed immediately by a vortex mixer. Incubation at 60°C for 10 minutes was carried out. One hundred microliter of Isopropanol was added and mixed well by pipetting. The resulting lysate was carefully transferred into the upper reservoir of the Binding column tube with a 2ml tube carefully fitted without wetting the rim. The tube was closed and centrifugation was done at 8,000 rpm for a minute. The tube was then opened and the Binding column tube was transferred to a new 2ml tube for filtration. There was addition of 500µl of wash buffer 1 (W1) to the tube without wetting the rim; the tube was closed and centrifuged at 8000 rpm for 1 minute. The 2ml collection tube was opened and the solution was dispensed into a disposal bottle. An addition of 500µl of wash buffer 2 (W2) was carefully transferred into the collection tube to prevent wetting of the rim. The tube was closed and again centrifugation was done at 8000 rpm for 1 min. Centrifugation at 13,000 rpm for 1 min was carried out again to completely remove ethanol, and to make sure there is no droplet clinging to the bottom of the Binding column tube. The Binding column tube was transferred to a new 1.5ml tube for elution and then 200µl of the Elution buffer (EL) was added onto the Binding Column tube and kept for 5min at room temperature (25°C) until the Elution buffer was completely absorbed into the glass fiber of the Binding column tube. Once again centrifugation was done at 8,000 rpm for 1min to elute.

Polymerase Chain Reaction (PCR) amplification

The negative control was nuclease free water; which is usually used in the amplification session to verify the efficacy of the PCR system (Viveros *et al*., 2001).

PCR Mixture

PCR mixture included in 50µl reaction volume containing 500ng of template DNA, 10mM Tris-HCl, 1.5mM $MgCl_2$, 5mM KCl, 0.1 % Triton X – 100, 0.1 % gelatin, 200µM each dNTP, 0.5µg of pPRA primers and 1.25 U of Taq polymerase (Viveros *et al*., 2001). Fifteen microlitres of PCR MasterMix (composition: dATP 400µM, dCTP 400µM, dGTP 400µM, $MgCl_2$ 3mM, Taq DNA polymerase 50µ/m), 4µl of water, 1µl of oligonucleotides (primer sequence).

Table 1: PCR condition used for the detection of *Trichinella spiralis*

Pre- denaturation	Denaturation	Annealing temperature	Extension	Final extension	Number of Cycles	Hold temperature
94°C	94°C	48°C	72°C	72°C	35	10°C
5min	30sec	30sec	1min	5min		∞

The PCR amplification products were separated by electrophoresis on a 2% agarose gel, stained with ethidium bromide. The resulting DNA fragments were visualized by UV transilluminator. Bio-Rad Cycler, Peltier-Effect Cycling (MJ Research Incorporated, USA). (Viveros *et al*., 2001; Godaratollah and Saberivand, 2009; EURLP, 2016).

Determination of Prevalence (%) of *Trichinella spiralis* infection in Humans and Pigs Using PCR

Percentage prevalence was calculated using the formula:

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

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

$$\text{Prevalence in humans (\%)} = \frac{4}{9} \times 100 = 44.4\%$$

$$\text{Prevalence in humans negative for ELISA (\%)} = \frac{1}{5} \times 100 = 20\%$$

$$\text{Total prevalence in humans negative for ELISA} = \frac{20}{100} \times 128 = 25.6\%$$

Or $\frac{1}{5} \times 128 = 25.6\%$

Table 2: Primer sequence used to detect mitochondrial ATP6 synthase F0 subunit gene

Primer	Amplicon size	Reference
ATP6-F 5-CACACTAACCAAAGCCAAACCATC-3′		
ATP6-R 5-GGAGTATGTTAGATGTTATTGTGTAGGAG-3′	250 bp	Golab *et al*., 2009; Attia *et al*., 2016

Key:
F – Forward
R – Reverse
ATP6 – ATP synthase F0 subunit 6 gene
bp – base pairs

RESULTS

Table 3: Detection of anti-*Trichinella* antibodies and ATP6 gene of *T. spiralis* migratory larvae in human blood

Sample No.	ELISA (antibodies)	PCR (ATP6 gene)
1	+	+
2	+	+
3	+	+
4	-	+
5	-	-
6	+	-
7	+	-
8	+	-
9	+	-

Key: ATP6 – ATP6 Synthase F0 subunit gene
+ = positive - = negative

Agarose gel electrophoresis plate of ATP6 synthase gene from migratory larvae of *Trichinella spiralis* in human blood using ATP6 primer

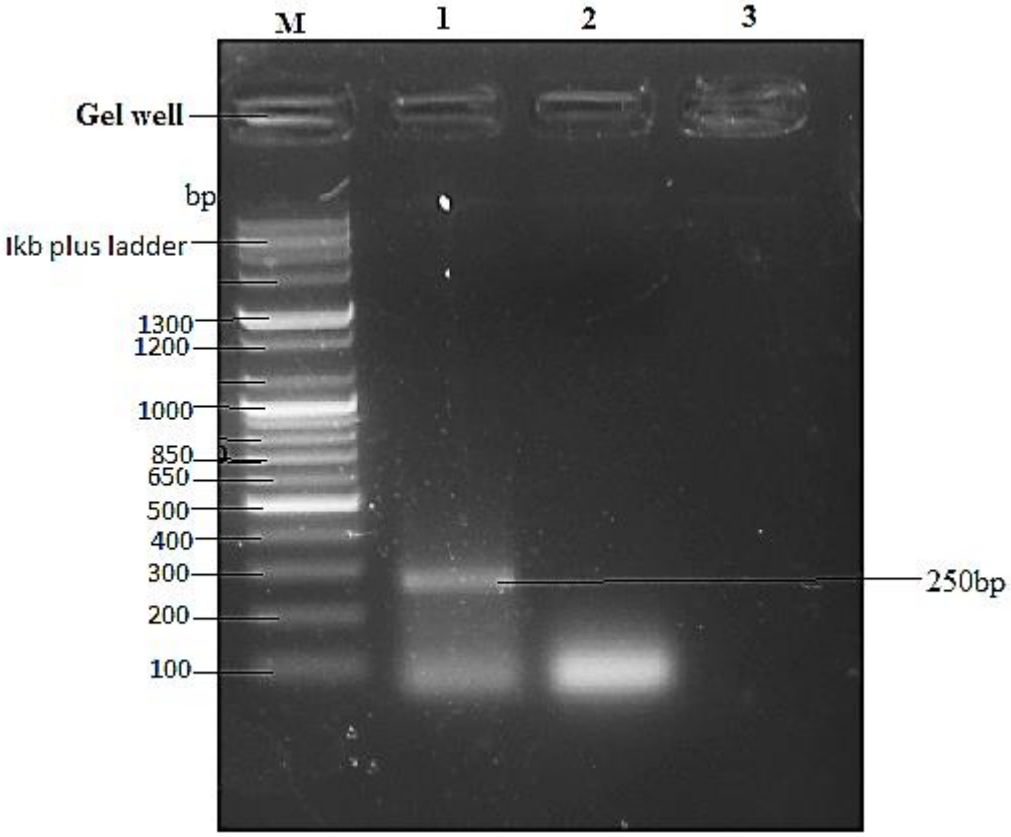

Plate I: Agarose gel electrophoresis plate of ATP6 synthase gene from migratory larvae of *Trichinella spiralis* in human blood using ATP6 primer

Key: Lane M = Molecular weight marker, Lanes 1-2 = human blood samples, lane 3 = nuclease free water as negative control. Lane 1= Positive for *T. spiralis* with the expected amplicon size of 250bp. bp = base pairs

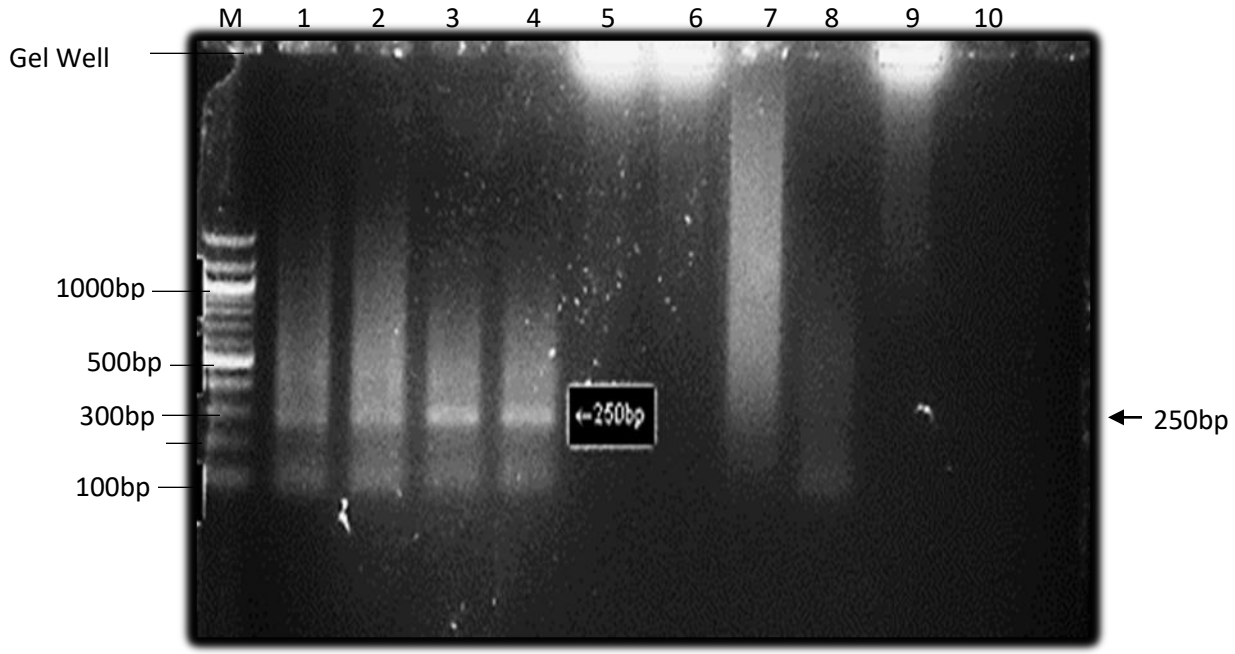

Plate I: Agarose gel electrophoresis plate of ATP6 synthase gene from migratory larvae of *Trichinella spiralis* in human blood using ATP6 primer

Key: Lane M = Molecular weight marker, Lanes 1-9 = human blood samples, lane 10 = nuclease free water as negative control. Lanes 1, 2, 3 and 4= Positive for *T. spiralis* with the expected amplicon size of 250bp. Lanes 4 and 5 were negative for ELISA. bp = base pairs

Table 4: Overall prevalence of Trichinellosis in Humans (ELISA and PCR)

	ELISA		PCR	
	No. Examined	No. positive (%)	No. Examined	No. positive (%)
Humans	210	82 (39)	9	4 (44.4)

Key: ELISA: Enzyme Linked Immunosorbent Assay, PCR: Polymerase Chain Reaction

DISCUSSION

The use of excretory/secretory (ES) antigens, as used in this study, has proven to be useful in ELISA for the sero-diagnosis of human and animal trichinellosis (Nöckler *et al*., 2005). The overall prevalence of 39% in the human population in this study indicates that trichinellosis is not only a re-emerging zoonosis but also present in Kaduna metropolis and its occurrence among the human population is high and increasing (Ojodale *et al*., 2015a). Sensitivity of ELISA accounts for the high prevalence rate since it captures both new and existing cases (Nöckler *et al*., 2000). The sensitivity of ELISA was reported to be higher with ES antigen than with tyvelose for detecting circulating antibodies early in the infection (Pozio, 2007).

In this study PCR after DNA extraction was used to detect *T. spiralis* migratory larval DNA in humans. Our findings showed that PCR detected the migratory larvae from human blood samples with prevalence of 44.4%. A 25.6% prevalence rate was recorded for human blood samples that were negative for ELISA. This result is in tandem with the findings of Attia *et al*. (2016) who detected *T. spiralis* migratory larval DNA from blood samples of infected experimental mice on days 4, 6 and 14 pi (post infection).

This result is a pointer to the fact that the positive participants in this study were in the early stages of the infection where the larvae were actively migrating. During early trichinellosis, there is a continuous production of newborn larvae which enter and circulate into the bloodstream until the expulsion of adult worms from the intestine. PCR detected the larvae in blood which would have been an uphill task with direct microscopic observation of the larvae in the blood because of labour intensiveness and time constraint (Li *et al*., 2010). Migrating *Trichinella* larvae and their metabolites provoke reaction, which causes immunological, pathological and metabolic disturbances and the various clinical phenomena (Gottstein *et al*., 2009).

The larval deposition by adult female *Trichinella* parasites falls between 3-14 days pi and may last between 10 and 20 days (Attia *et al*., 2016); other results revealed that PCR procedure detected circulating *T. spiralis* in the blood of mice from days 5-14 pi (Uparanukraw and Morakote, 1997; Li *et al*., 2010) and from 5-17 days pi (Caballero-Garcia and Jimenez-Cardoso, 2001).

Attia *et al*. (2016) stated that the detection rate of the PCR from blood samples of the infected mice was 50% as 5 of 10 blood samples from 10 mice were positive. The prevalence in this study is lower since the previous study was on mice experimentally infected with the parasite under laboratory controlled conditions whereas the present study was on humans for which there was no prior knowledge of infection with the disease. Also Li *et al*., 2010 revealed that PCR was not as effective for detecting *T. spiralis* DNA in human patients with trichinellosis as it was for detecting it in mice, even though the larval migratory period of the organism is longer than that in mice. In addition, the variable results of the PCR procedure could be due to the discrepancies in the DNA extraction procedures from blood samples or blood samples containing small numbers of migratory larvae (different parasitic burdens) (Uparanukraw and Morakote, 1997; Li *et al*., 2010).

In this study, whole blood was used in the PCR study instead of serum or plasma because the whole blood is adjudged ideal, this is because the detection rate of some pathogens in serum and plasma specimens can be less than that from whole blood specimens, as some pathogens fractions are maintained in the peripheral cells (Watkins-Riedel *et al*., 2004; Klung-Thong *et al*., 2007). This was confirmed by Attia *et al*. (2016), where PCR with DNA extraction from plasma and serum samples failed to detect human and animal infections caused by *T. spiralis, T. pseudospiralis* and *T. nelsoni*.

Although *T. spiralis* and *T. nativa* can be identified by PCR amplification with primers from ESV, due to the small size of 173 or 127 bp, differentiation is not easy (Fu *et al*., 2009), hence the use of ATP6 primers with a larger size of 250bp which is specific for *T. spiralis* (Attia *et al*., 2016). The predominant mode of identification of *Trichinella* species involve multiplex PCR (La Rosa, 2003) and when necessary, DNA sequencing (Pozio, *et al*., 2009). Trichinellosis caused by all species of *Trichinella* can effectively be treated with albendazole or mebendazole. This will lead to the prompt and efficacious treatment of trichinellosis at the early stages (Dupouy-Camet *et al*., 2002).

The 25.6% prevalence recorded for human blood samples that were negative for ELISA is a call for serious concern as being negative for trichinellosis using ELISA is not a guarantee of exemption from the disease but the need for a further confirmatory tests using PCR. Study by Viveros *et al*. (2001) revealed a prevalence of 15% of mice found positive by ELISA and gave the expected amplification products by PCR. Some of the positive results obtained by ELISA only, might be due to cross reactivity of TSL-1 antigens with other nematodes antigens (Dea Ayuela *et al*., 2000) as antigens are highly represented in E/S products. Viveros *et al*. (2001), recorded 55% of samples negative in ELISA gave positive results by PCR. An evidence that ELISA together with PCR will serve as a sufficient diagnostic test for trichinellosis in humans and other animals.

CONCLUSION

The PCR procedure using 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 humans. This will enhance the diagnosis of infection earlier than when using conventional diagnostic procedures such as microscopy or serology, especially for patients for whom serology (ELISA) for specific anti-*Trichinella* antibodies yielded negative results and for immunosuppressed hosts. Therefore, the result of this study is of public health importance, because meat and meat

products are considered very important sources of daily protein in-take and also serves to enlighten consumers and pig producers who prefer pork from pigs that are raised under natural situations.

CONFLICT OF INTEREST

There is no conflict of interest cited among the authors!

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