International Refereed Journal of Engineering and Science (IRJES)
ISSN (Online) 2319-183X, (Print) 2319-1821
Volume 5, Issue 10 (October 2016), PP.28-35

First Study on the Diagnosis of Cysticercosis by PCR Method In Cote D'ivoire.

Yapo- Crezoit Antoinette [1] Coulibaly Ngolo David [2], Kpan Eudes [1] Ronan Jambou[3], Djaman Allico Joseph [4]

[1]*Immunology Lab- Institut Pasteur Of Côte d'Ivoire*
[2]*Department Of Molecular Biology- Institut Pasteur Of Côte d'Ivoire*
[3]*Department Of Parasitology And Mycology - Institut Pasteur Of Côte d'Ivoire*
[4]*Department Of Biochemistry- Institut Pasteur Of Côte d'Ivoire*

Abstract : Cysticercosis is a parasitic disease caused by cysticerci larvae development in an organism. This parasite infects both pigs and that man is reported endemic in several countries in South and Central America, Asia and Africa. These larvae can localize to the central nervous system, eyes, muscles and subcutaneous tissues. Its location is more common in the central nervous system and is the severity of cysticercosis. In sub-Saharan Africa it is known as the most common cause of epilepsy. The Ivory Coast is immune to these diseases morbidity is high. To set up a monitoring program for these diseases, several research teams have examined the question of the place of cysticercosis in the occurrence of epilepsy. Molecular methods based on DNA detection cysticercus are in place for the detection of Neurocysticercosis. This is the real-time PCR and conventional PCR using the pair of primers COXI-B / S-COXI producing amplicons of 113pb.
Keywords : Cysticercosis , PCR ,Côte d'Ivoire.

I. INTRODUCTION

Cysticercosis is a cosmopolitan and endemic zoonosis in many countries. It particularly affects rural areas of intensive pig breeding where sanitary conditions are defective, sanitation facilities are rare, the consumption of meat or pork preparations are frequent and where the animal-man promiscuity is very important (Sciutto, 2000 ; Raether and Hanel, 2003).

It is caused by the development of cysticerci in an organism, especially Cysticercus cellulosae, a larva of Taenia solium (Botero D ,1993; Flisser A ,2003;Delamare, 2004). It is due to the ingestion of parasite eggs by humans or pigs. These eggs are present in the water or food contaminated by human waste. This disease is called stinginess when the pig is infested.

Today, cysticercosis is reported endemic in several countries. Endemic foci recognized by WHO are Central and South America, Asia and Africa (Sub-Saharan Africa) (Roman et al., 2000; Sciutto et al., 2000). Latin America has the highest prevalence (5-10%) (Craig, 1997). In these foci, the conditions of emergence and perpetuation of this zoonosis are generally: the bad hygienic conditions, the almost total absence of toilets especially in rural areas, permanent or seasonal straying of pigs and lack of inspection carcasses in slaughterhouses (Garcia , 1998). Cysticercosis is an economic problem (Avecedo-Hernandez,1982) and also a public health problem in non-Islamic developing countries where it concerns more than 50 million people (Esterre , 1994). In Africa, the prevalence of cysticercosis is underestimated due to the lack of medical facilities and appropriate diagnosis
(Nguekam , 2003).

In Ivory Coast, there is no data on this parasitosis that infests pork as much as human. One of the most serious forms of the disease in human would be neurocysticercosis known to be a major cause of epilepsy (Camara ,2006), with an average prevalence of 8.93 ‰ in the world; the average of Côte d'Ivoire is between (7.6 to 74 ‰) (Chin, 2012). Epilepsy is an illness striking both sexes at any age and especially children. The infected people are rejected and excluded from school life, social life and professional life. The mortality rate caused by this disease is important in Sub-Saharan Africa (Preux ,2003).. To identify the risk factors of cysticercosis, a study was conducted to develop a diagnosis of cysticercosis by PCR method of DNA extraction from cysticerci larvae and detection of the cysticercus DNA by two PCR methods

II Materials And Methods

Study area

The study is experimental .The different steps are realized in two Labs of Pasteur Institute in Côte d'Ivoire, the department of molecular biology and the department of Biochemistry.

Preparation of cysticerci larvae of Taenia solium

Biological material used consists of samples of cysticerci larvae of Taenia solium collected after ante mortem and postmortem examination on pig carcasses infected in a farm in Madagascar **(Assana, 2007, Jambou ,2010)**

These collected larvae were placed in a 15 mL vial tube containing a phosphate buffered saline buffer (PBS) and stored at under 20°C for the DNA extraction .The sonication of the samples was made before DNA extraction .

The sonication of the sample

It consisted of breaking the cells of the membrane larvae contained in an eppendorf tube and the samples were placed in an ice bath using an ultrasonic probe device (Branson Sonifer 250).After 2 minutes, the proteins, RNA and DNA were released into the medium. Centrifugation at 3000 rpm for 5 min at 4 ° C was conducted, and the supernatant was collected in 2ml eppendorf tubes.

Extraction of Cysticercus DNA by solution phenol / Chloroform

The Cysticercus DNA extraction by the phenol / chloroform was made from the modified method of Maxwell. This extraction method by the phenol chloroform which consists of treating the cell lysate with a mixture of phenol / chloroform / isoamyl alcohol (**Ausubel, 1999)**. Phenol is a deproteinizing agent wherein the nucleic acids are not soluble. Chloroform is able to cause denaturation of proteins. The defoaming activity of isoamyl alcohol favors the separation of the deproteinized aqueous phase. The recovery of the genomic DNA is achieved after precipitation steps with ethanol and centrifugation steps.
It includes several steps:

(i)Cell lysis

200 µL of this sample is collected in a 2 mL eppendorf tube to which 50 L of Proteinase Kwere added (20 mg / mL) and 200 µL of lysis buffer constituted of (2 µl of 1M Tris-HCl, 0.4 µl 5 M NaCl, 0, 0004 mL of 0.5 M EDTA, 10 µl 10% SDS and 0.1876 mL of water Biomol). The mixture is runned at the vortex is then carried to a dry water bath for 1200 rpm at 62 ° C under steady stirring overnig

(ii)ProperExtraction: After cell lysis, 450 ul of phenol saturated Tris (pH8) were added and mixed gently by inversion and then centrifuged at 13,000 rpm for 10 min at 4°C.The upper aqueous phase was collected in a sterile eppendorf tube. Then 450 .mul of chloroform-isopropanol (24: 1) were added to the aqueous phase, and mixed gently and then centrifuged at 13,000 rpm for 10 min at 4°C.The formed supernatant was collected in a sterile eppendorf tube.

(iii) Precipitation of nucleic acids: 200 L of the solution to which was added 1 / 10th (20µ L) of sodium acetate and 500 µl l of ice-cold absolute ethanol. The tube containing the solution is stored at -20 ° C overnight.

(iiii) Washing of nucleic acids: After storage, the solution was centrifuged at 13,000 rpm for 40 min at 4 ° C, the supernatant was removed by inverting the tube, and 1 mL of ethanol at 70 ° C iced base was added. The mixture was centrifuged at 13000 rpm for 1 min at 4 ° C. The supernatant must be eliminated again by inverting the tube.

(iiiii) Recovery of nucleic acids: The pellet containing the DNA was dried in a thermoblock at 70 ° C for 30 min and recovered in 200 L of elution buffer. The extracted DNA was subsequently stored at -20 ° C for later use or at 4 ° C for immediate use.

(iiiiii) Determination of DNA purity: 30 µl of the DNA extract was sampled to determine the concentration and optical density in a spectrophotometer (Biophotometer Plus), the apparatus using light transmission through a solution to determine the concentration of a solute within the solution. The sample was placed in a plastic tank, a further tank containing water was used as a reference. The nucleic acid concentration is usually determined by a measurement made at 260 nm against a blank. Interference by contaminants can be recognized by a calcul "ratio". Absorbing proteins at 280 nm, A260 / A280 ratio was used to estimate the purity of the nucleic acid. A ratio, measured by spectrophotometer was set to a value between 1.8 and 2 indicating that the extracted DNA is pure whereas if the value is less than 1.7 a protein contamination may be considered.

Detection of the cysticercus DNA by conventional PCR

PCR was done in a thermal cycler (Gene AMP PCR system 9700, Applied Biosystems)and the amplification was carried out under the following conditions;

- Initial denaturation at 95 ° C for 5 minutes
- Denaturation at 95 ° C for 15 seconds
- Hybridization at 60 ° C for 20 seconds
- Elongation at 72 ° C for 20 seconds
- final elongation at 72 ° C for 10 minutes

} 40 cycles

The final reaction volume is of 50 L containing 23.8 µl of water biomol, 5x 0.2 µl of Taq polymerase, 3 . µl of MgCl2, 1 µl of dNTPs, 1 µl of 10 µl M COXI-B, 1 µl of 10 µM COXI S-, 10 µl of 5X GoTaq Buffer and 10 µl of DNA extract. To each series a negative PCR control (10 µl water for injections) and standard (a dilution of 1/10, 1/100, 1/1000, 1/10000 of the DNA extract) were introduced.The primers COXI-B / COXI-S used for the detection of cysticercus DNA produce amplicons of 113pb (Tab 1et 2)

Separation of DNA fragments by electrophoresis on agarose gel

The electrophoresis apparatus consists of a plexiglass plate placed horizontally on a flat support. A comb for forming the wells is aligned parallel to the top of the plate. The assembly is connected by electrodes to a current generator. Agarose gel was prepared at a concentration of 2% with a TBE 1X buffer. The agarose solution is melted in the microwave; after cooling 3 mul of BET is added. The solution is then poured onto the plate. After total drying of the gel, the comb is removed and the cured gel covered with 1x TBE buffer. 20 µL of each amplification product was collected and placed in the different wells. In parallel with the amplification products, a molecular weight marker is placed in a well. The migration lasts 15 to 20 minutes and is performed under a voltage of 120V.

Revelation of the DNA fragments

After migration, the separated fragments are visualized under ultraviolet lights and the gels are scanned using the Gel Doc EZ imager Bio-RAD.

Detection of cysticercus DNA by the real-time PCR

A real-time PCR was developed to detect the presence of cysticerci DNA larvae. The sub unit COXI DNA gene that code for enzymes of the respiratory chain was amplified by a pair of primers: Primers direction (COXI-S) and antisense primers (COXI-S). PCR was done on 7500 Fast Real-Time PCR system Applied Biosystems and the amplification was carried out under the following conditions;

- Initial denaturation at 95 ° C for 5 minutes
- Denaturation at 95 ° C for 15 seconds
- Hybridization at 60 ° C for 20 seconds.

} 40 cycles

Melting curve 95 ° C for 15 seconds, 70 ° C for 1 minute, 85 ° C for 1 minute.
The transition from 70°C to 80 °C increments of 0.5°C so the reading is between 70 and 85°C. 60 ° C for 1 minute.

The final reaction volume is 20 . µl containing 2.9 µl of water biomol, 0.5 µl roxdye, 10 µ l Maxima SybrGreen, 0.8 µl of 10 µM COXI-B, 0.8 . µl of 10 . µM COXI-S and 5 µl of DNA extract. To each PCR series a negative control (5 µl of water PPi) and 3 standards (containing 10-1, 10-2 and 10-3 of extracted DNA) were introduced.

Tableau 1: Sequences of amorces.

Amorces	**Sequences (5' □ 3')**
COXI-B	**ACAACGAAGATGATAAAGGTGGGTAA**
COXI-S	**GAATGCTTTAAGGTGCATGGTTGTTAGT**

Tableau 2 : Composition of reactionnel medium - conventional PCR and real-time PCR

	rate / sample	
Reagent	**conventional PCR**	**real-time PCR**
Molécular Biology water	21,8 µL	2,9 µL
Tampon 5x color and no color	10 µL	
$MgCl_2$(25mM)	3µL	
DNTPs (10mM)	1 µL	

Amorce CoxI-B (10 µM)	1 µL	0,8µL
Amorce CoxI-S (10 µM)	1 µL	0,8µL
Roxdye		0,5µL
DNA Taq Polymerase	0,2 µL	
Maxyma SYBRGREEN		10µL
Mix Volume	40 µL	15 µL
AND	10 µL	5 µL
Total Volume	**50 µL**	**20 µL**

II. RESULTS AND DISCUSSION

The step of sonicating

It was the most decisive sonication. The destruction of rigid membranes of cysticerci larvae released DNA in the medium, facilitating the action of the other elements for its recovery.

Cysticercus DNA extraction

The method of phenol / chloroform used for cysticercus DNA extraction allowed to observe a DNA ball, confirming the success of the method. The purity and concentration of the extract were determined from optical densities obtained with the spectrophotometer. After reading, a concentration of 32.8 ug / ml and a ratio of 1.78 were obtained. The A260 / A280 ratio is above 1.7. It shows that the extracted DNA was not contaminated by proteins.

Cysticercus DNA detection after migration on agarose gel

The presence of cysticercus DNA was detected in the extract with the pair of primers :COXI-S or forward primers and COXI-B or backward primers.

Fig 1 represents the gel samples performed after electrophoresis on agarose gel of the DNA fragments amplified by conventional PCR. These samples are represented by:

Pc: positive control (cysticercus DNA extracted) certifying the sample quality.
Pc-^{1}: positive control (cysticercus DNA extracted diluted 1/10) certifying the sample quality.
Pc-^{2}: positive control (cysticercus DNA extracted diluted 1/100) certifying the sample quality.
Pc-^{3}: positive control (cysticercus DNA extracted diluted 1/1000) certifying the sample quality.
Pc-4: positive control (cysticercus DNA extracted diluted 1/10000) certifying the sample quality.
nC: Negative control (water used to adjust the reaction mixture volume to 50 uL) attesting to the absence of contaminants.
M: molecular weight marker (1500 to 100 bp).

At the positive control (Pc) and the diluted 1/10 control (Pc-^{1}), a band was observed.

The observed amplicons band indicates the presence of a large amount of DNA in our extract. The amplicon size is 113pb, corresponding to the DNA gene of the COXI subregion which encodes the enzymes of the respiratory chain (sequence of the gene determined on genebank).

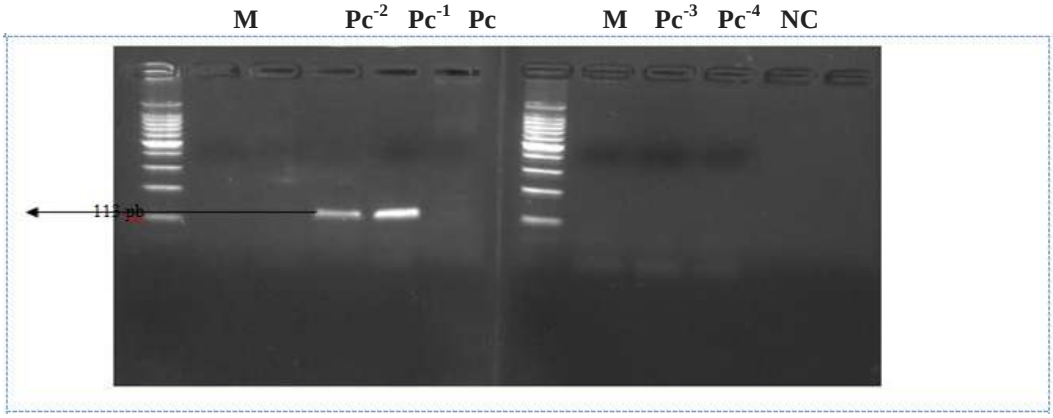

Figure 1: Migration of the amplification products of the COXI gene cysticercus larva of Taenia solium in the agarose gel at 2%.

Figure 1: Migration of the amplification products of the COXI gene cysticercus larva of Taenia solium in the agarose gel at 2%.

Melting temperature curve of positive controls obtained after real-time PCR

Gradually increasing the temperature in the PCR tubes, the amplicons are gradually denatured at a low level until the temperature approaches the melting temperature (Tm) of COXI gene (Figure 2) . At that time, denaturation of amplicons accelerates to reach 50% of denatured amplicon when the Tm is reached (74.5). The complete denaturation of amplicons is then reached when the temperature continues to rise. Denaturation of amplicons causes the release of SYBRGreen and is therefore accompanied by a decrease in fluorescence which can be recorded.

Derived Reported

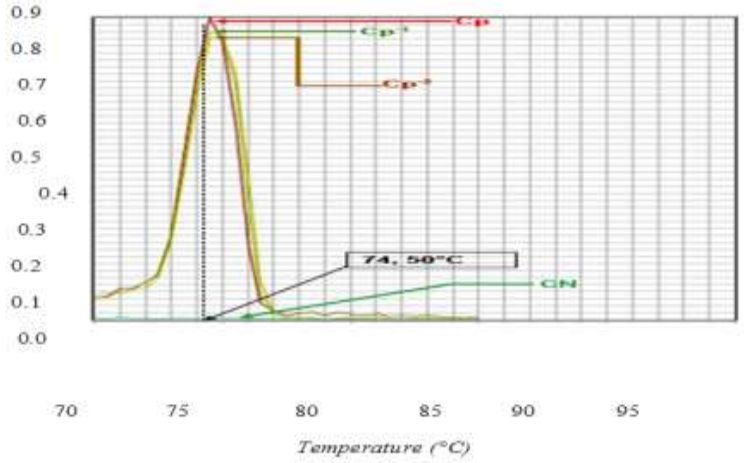

Figure 2: Diagram of melting temperature of the positive controls obtained after the real-time PCR.

The melting curve is obtained by plotting the first derivative of the fluorescence as a function of the temperature and the temperature of a double stranded corresponds to a peak on the curve.

Evolution curve of the amount of positive controls amplicon according to the number of cycle

The fluorescence values are recorded during each cycle and represent the amount of amplicons produced at a specific point in the reaction.

At the positive control, the 1/10 diluted positive control and the 1/100 diluted positive control the amount of amplicons is determined respectively at 17; 20 and 25 cycles. The exponential phase is reached at 40 cycles (Figures 3, 4 and 5).

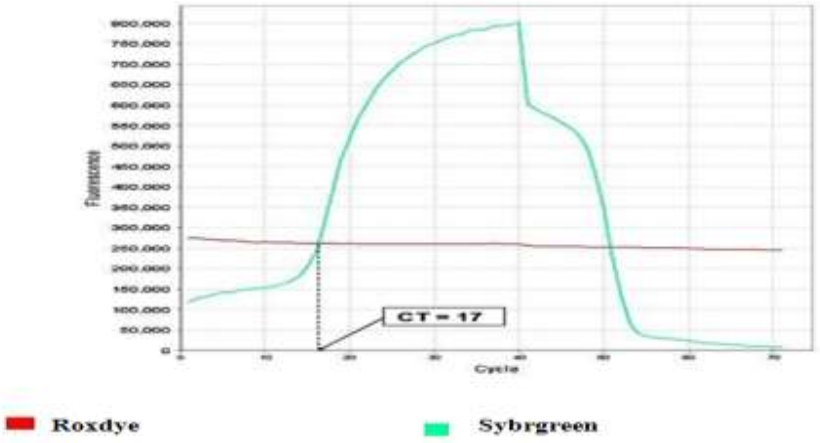

Figure 3: Curve evolution of the amount of amplicons of the positive control based on the number of cycles

The x-axis corresponds to the PCR cycles. The ordinate axis corresponds to the amount of amplified product. The intensity of the fluorescence at each cycle is proportional to the concentration of amplicons. The intersection of the curve and roxdye gives a point for the x-axis value of 17. This value corresponds to the threshold cycle (Ct) value

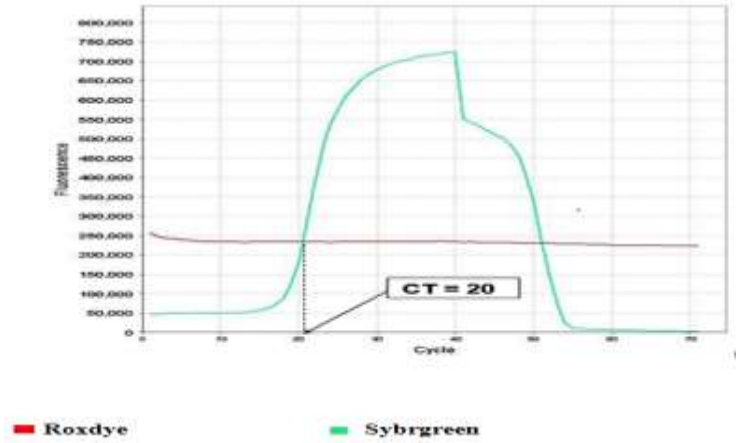

Figure 4: evolution curve of the amount of amplicons Positive control diluted to 1/10 depending on the number of cycles

The x-axis corresponds to the PCR cycles. The ordinate axis corresponds to the amount of amplified product. The intensity of the fluorescence at each cycle is proportional to the concentration of amplicons. The intersection of the curve and roxdye gives a point for the x-axis value of 20. This value corresponds to the threshold cycle (Ct) value.

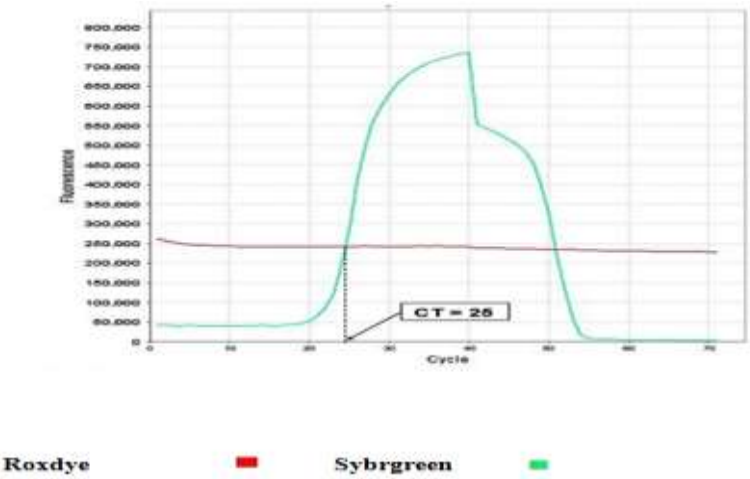

Figure 5: Courbe d'évolution de la quantité d'amplicons du Contrôle positif dilué au 1/100 en fonction du nombre de cycles.

The x-axis corresponds to the PCR cycles. The ordinate axis corresponds to the amount of amplified product. The intensity of the fluorescence at each cycle is proportional to the concentration of amplicons. The intersection of the curve and roxdye gives a point for the x-axis value of 25. This value corresponds to the threshold cycle (Ct) value.

In order to validate the PCR technique, more cysticerci DNA extraction methods have been implemented, including those using the Qiagen Kit, those using Promega Kits and those using phenol / chloroform (González , 2000).

The method of Phenol / Chloroform was used to extract an amount of DNA from a sonic sample with an ultrasound block. The cysticercus DNA Template amplified by conventional PCR revealed a band after migration on the gel at the positive control, and its 1/10 dilution. This band will be used as a positive control for the molecular diagnosis of cysticercosis (Lightwolers ,2003).. The extracted cysticercus DNA amplified by PCR in real time revealed the melting temperature of the COXI gene and the number of cycles to which it is detectable. These positive samples will be used as a positive control for the molecular diagnosis of cysticercosis

Conventional PCR techniques and real-time PCR were developed for the diagnosis of cysticercosis in Côte d'Ivoire. The analysis of the conventional implemented PCR method amplified a DNA fragment of 113 bp. These results are similar to those of **(Jambou ,2010).** These authors performed their work on cysticerci larvae of Madagascar using COXI-B (forward primer) and COXI-S (backward Primer). They amplified a fragment of 113 bp. The sensitivity threshold of this method is situated after the first dilution because after this dilution bands are not observed anymore.

Also the implemented real-time PCR method has determined the melting temperature (mT) at which half of the DNA extract is in double stranded form and the other half in single-stranded form. The positive PCR give a melting curve between 70 ° C and 85 ° C. The positive control, the 1/10 dilution and the 1/100 dilution have a melting curve whose peak is 74.5 ° C. At this temperature the COXI gene is detected. The values of the fluorescence recorded during each cycle represent the amount of amplicons produced at a point in the reaction. The positive control is detected at 17 cycles, the 1/10 diluted positive control is detected at 20 cycles and the 1/100 diluted positive control is detected at 25 cycles. The results obtained show that the amount of the amplicons obtained is proportional to the number of cycles. As the amount of DNA to be amplified is higher, the lower the number of threshold cycle (tc) will be. After 25 cycles, the DNA is no longer detectable and the exponential phase is obtained in 40 cycles.

The sensitivity threshold of this method is detected after the second dilution because after this dilution no pick is observed. This method is faster and easier to implement compared to the conventional PCR. The sensitivity detected at the real-time PCR is better than the one detected at the conventional PCR. The real-time PCR can prove to be a reliable technique for the diagnosis of cysticercosis.

III . CONCLUSION

This first study on the diagnosis of cysticercosis from DNA by the PCR method does not allow to draw a conclusion about the place of cysticercosis in the occurrence of epilepsy. However, preliminary results allowed the set up of two PCR methods for the diagnosis of cysticercosis. The real-time PCR is the most reliable method for the diagnosis and survey of cysticercosis in animals but also in patients suffered from neurocysticercose in Cote d'Ivoire.

REFERENCES

[1]. Acevedo-Hernandez, A., 1982. - Economic impact of porcine cysticercosis. In: Cysticercosis: present state of knowledge and perspectives. Flisser, A.; Willms, K.; Laclette, J.P.; Larralde, C. Ridaura, C. & Beltran, F. (Eds.).Academic Press, New York, pp. 63-67.

[2]. Assana E. Kanobana K., Tume C.B., Zoli P.A., Nguekam., Geerts S., Berkvens D & Dorny P., 2007. – Isolation of a 14 kDa antigen from Taenia solium cyst fluid by HPLC and its evaluation in enzyme linked immunosorbent assay for diagnosis of porcine cysticercosis. Research in Veterinary Science, 82: 370–376.

[3]. Ausubel F.M., Brent R., Kingston R. E., Moore D., Seidman J.G., Smith J.A & Struhl K., editors., 1999. - Short Protocols in Molecular Biology. Associates and wiley interscience. New York , 231:342-348. Botero D., Tanowitz H.B., Weiss L.M & Wittner M., 1993. - Taeniasis and cysticercosis. Infect Dis Clin North Am, 7:683–697.

[4]. Camara O., 2006. - Prise en charge et recherche de facteurs parasitaires d'épilepsie dans le cadre d'une Recherche-action en Réseau sur l'Epilepsie à Markacoungo.Université de Bamako; 112 p.

[5]. Chin J.H., 2012. - Epilepsy treatment in sub-Saharan Africa: closing the gap. Afr Health Sci, 12(2): 92-186.

[6]. Craig P.S., Rogan M.T., Allan J.C., (1997) Detection, screening and community epidemiology of taeniid cestode zoonoses: cystic echinococcosis, alveolar echinococcosis and neurocysticercosis.Advances in Parasitology, 38 :170-250.

[7]. Delamare G., 2004. - Dictionnaire illustré des termes de médecine, 28ème édit. Maloine, Editor. Paris; 215 p

[8]. Esterre P.H., Andriantsimahavandy A & Boisier P., 1994. - Relations entre pathologie et immunité dans la cysticercose. Archives de l'Institut Pasteur de Madagascar, 61 : 14-20.

[9]. Fleury A., Dessein A & Preux P.M., 2004. - Symptomatic human neurocysticercosis-age, sex and exposure factors relating with disease heterogeneity. J Neurol, 251:830-837.

[10]. Flisser A., Sarti E., Lightowlers M & Schantz P., 2003. - Neurocysticercosis: regional status, epidemiology, impact and control measures in the Americas. Acta Trop, 87: 43-51.

[11]. Garcia H.H., Araoz R., GilmanR.H., Valdez J.,Gonzalez A.E., Gavidia C Bravo M.L., Tsang V.C.W & The Cysticercosis Working Group in Peru. (1998). - Increased Prevalence of cysticercosis and taeniasis among professional fried pork vendors and the general population of a village in the Peru vianhighlands. American Journal of Tropical Medicine and Hygiene, 59(6):902-905.

[12]. González L. M., Montero E & Harrison L. J. S., 2000. - Differential diagnosis of Tænia saginata and Tænia solium infection by PCR. Journal of Clinical Microbiology, 38: 737-744.
[13]. Jambou R., Ramahefarisoa R.M., Rakotondrazaka M., Carad J.F., 2010. – Comparaison of Elisa and PCR assays for the diagnosis of porcine cysticercosis. Vet parasitol, 173: 336-339.
[14]. Lightwolers M.W., Gauci C.G., Chow C., Drew D.R., Gauci S.M., Health D.D., Jackson D.C., Dadley-Moore D.L. & Read A.J., 2003. - Molecular and genetic characterisation of the host-protective oncosphere antigens of taeniid cestode parasites. International journal for parasitology, 33: 1207-1217.
[15]. Nguekam J.P., Zoli A.P., Zogo P.O., Kamga A.C., Speybroeck N., Dorny P., Brandt J., Losson B & Geerts S.A., 2003. - Seroepidemiological study of human cysticercosis in west Cameroon. Trop Med Int Health, 8: 144–149.
[16]. Preux P.M., Tran D.S & Strobel M., 2003. - Epilepsie en zone tropicale démunie. Vientiane, pp. 11–14.
[17]. Raether W & Hanel H., 2003. - Epidemiology, clinical manifestations and diagnosis of zoonotic cestode infections: an update. Parasitol Res, 91: 412-438.
[18]. Roman G., Sotelo J., Del Brutto O., Flisser A., Dumas M., Wadia N., Botero D., Cruz M., Garcia H., De Bittencourt P.M.R., Trelles L., Arriagada C., Lorenzana P., Nash T.E & Spina-Franca A.,2000. - A proposal to declare neurocysticercosis an international reportable disease. Bull WHO, 78: 399-406.
[19]. Sciutto E., Fragoso G., Fleury A., Laclette J.P., Sotelo J., Aluja A., Vargas L & Larralde C., 2000. - Taenia solium disease in humans and pigs: an ancient parasitosis disease rooted in developing countries and emerging as a major health problem of global dimensions. Microbes Infect, 1875-1890.