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Optimization Method For Biomolecular Identification of Muscle Larva of *Trichinella* species

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Abstract. Trichinellosis is a food-borne parasitic zoonosis with a yearly incidence of about 10,000 clinical cases worldwide. It is one of the most serious zoonotic diseases in Romania with more than 28,000 human cases reported over the last 25 years. In this context Romania remains the country with the highest infestation with Trichinella from the word. The aim of the paper it is to identify the simple repetitive sequences from Trichinella genus through a biomolecular analysis of isolates. After conducting the DNA extraction from 50-100 muscle larvae of the six selective strains (jackal, wolf 1, wolf 4, 7.97, 7.42 and 7.29) using the " QIAamp DNA" (Qiagen, Germany) the amplification with ESV primers (forward and reverse) using the "mi-Taq Mix Kit "(Metabion, Germany) followed, obtaining in this manner a positive amplification for all the six strains taken into study, revealing also the method's efficiency in the biomolecular identification of Trichinella strains. The goal of this research, in perspective, is to improve the DNA extraction from one LM, in order to reduce the chemical and biological reagents. The positive amplification of the genomic DNA from one larva may have favorable repercussions on the genetic variability analysis in the population of Trichinella studied, which can be applied on various individuals belonging to the same population.

Keywords: Trichinellosis, DNA extraction, molecular analysis, amplification.

INTRODUCTION

Trichinellosis is a food-borne parasitic zoonosis with a yearly incidence of about 10,000 clinical cases worldwide (Pozio, 2007). It is one of the most serious zoonotic diseases in Romania with more than 28,000 human cases reported over the last 25 years (Blaga et al., 2007). Up to date, eight species and four genotypes of *Trichinella* have been identified worldwide (Murell et al., 2000; Pozio and Zarlenga, 2005). The *Trichinella* species and genotypes are present on all continents, from the tropical regions to the cold ones. Following the great number of epidemiological surveys undertaken in the period of time 2000-2006 within the framework of the European projects TRICHIPORSE and TRICHINET (MEDVETNET), there have been identified four *Trichinella* species in Europe*: T. spiralis, T. nativa, T. britovi* and *T. pseudospiralis. Trichinella spiralis*, with predominant hosts of domestic and sylvatic swine, synanthropic animals and a broad range of sylvatic carnivores (Pozio, 2001; Dick and Pozio, 2001). In Romania, during various epidemiological surveys, only two *Trichinella* species have been identified in domestic and wild animals: *T. spiralis* and *T. britovi.* (Blaga et al. 2009). A survey conducted in 2004 by the International Commission on trichinellosis shows that Trichinella is responsible for that major zoonosis in Romania, with 617 human cases, a much higher number of cases caused by other pathogens. With an incidence of 51 cases of trichinellosis at one million people per year and a prevalence of 8 cases of infestation with *Trichinella* in 10 000 pigs tested, 10 cases to 1,000 boar tested and 10 test cases for 100 bears, Romania remains the country with the highest infestation with

Trichinella from the world. The methods used to identify the Trichinella species were DNA extraction and PCR techniques. These techniques are based on the producing of a large number of copies of specific DNA sequences and then amplified to detectable levels. Besides it is very simple, PCR is robust, fast and most importantly, is flexible. A large number of variants of this method were described and published, with important results in the differentiation of species leading even to the obtainment of a new multiplex PCR test (Zarlenga et al., 1999), which uses ESV primers for amplification, eliminating the need to do more PCR tests. A restriction enzyme for the digestion of the genomic DNA was used and then the fragments were separated by gel electrophoresis, revealing several characteristic bands of the genome. The studies of repetitive sequences in the genome can be very useful in distinguishing species, as they show a great diversity and inter specific homogeneity within the same population (Dick et al., 1983).

MATERIALS AND METHODS

Specimens

The meat samples were collected from 230 pigs suspected of being infected with *Trichinella* and originating from several different farms, households, during the period of March 2008 - January 2009. The research was also conducted on meat samples taken from wild animals, such as 34 foxes, 34 rats, 24 wild-boars, 9 wolves, 4 jackals, 2 bears and 1 lynx. Beside them, 11 isolates of *Trichinella* were picked up randomly to be used in this study, described in detail (code, original host, country of origin) in Tab.1**.** They were maintained by serial passages in OF1 female mice under specific quality control. Muscle larvae (ML) were recovered from muscle tissue of infected mice through a standard pepsin-HCl digestion method.

Table 1

The results of the artificial digestion

Nr.	Species numbers	Grame digerate	Larvae numbers	LPG	Origin	Date
1	7.03	39.41	323300	8203.50	Fierbinţi, Ialomiţa County	19.11.08
2	7.18	39.57	97500	2463.99	Fierbinţi, Ialomiţa County	24.11.08
3	7.21	38.7	165000	4263.57	Fierbinţi, Ialomiţa County	17.11.08
4	7.29	49.2	76675	1558.43	Fierbinţi, Ialomiţa County	24.11.08
5	7.30	46.8	95000	2029.91	Fierbinţi, Ialomiţa County	17.11.08
6	7.42	35.05	150000	4279.60	Fierbinţi, Ialomiţa County	20.11.08
7	7.68	43.05	121675	2826.36	Fierbinţi, Ialomiţa County	20.11.08
8	7.97	29.51	40000	1355.47	Fierbinţi, Ialomiţa County	19.11.08
9	Wolf 1	154.55	8000	51.76	Mures County	14.01.09
10	Wolf 4	95.6	2450	25.63	Cluj County	17.02.09
11	jackal	49	6817	139.12	Danube Delta	20.02.09

The DNA extraction

The genomic DNA was obtained from small pools (approximately 100 ML) of *Trichinella* isolates, using the “QIAamp DNA” kit (Qiagen, Germany), according to the manufacturer’s instructions for mouse- or rat-tail protocols.

The PCR amplification

The primers used in this study, for each locus, are specified in Tab. 2. (Zarlenga et al., 1996). The PCR reactions were performed using either EX Taq kit (Takara, France) or the Mi Taq Mix (Metabion, Germany). PCR was performed in a 25µl reaction volume containing 2,5 µl buffer; 2,00 µl dNTP; 0,125 µl Taq polymerase; 1,5 µl of each forward and reverse primer; 5 µl AND (50 ng); 12,375 µl ultrapure water for EX Taq kit (Takara, France) and 12,5 µl Mi Taq Mix; 1,5 µl of each forward and reverse primer; 5 µl AND (50 ng); 4,5 µl ultrapure water for Mi-Taq Mix (Metabion, Germany). All reactions were performed in a MyGenie96 Thermal Block (Bioneer, Korea) under the following PCR conditions: one cycle at 94°C for 5 min, 35 cycles at 94°C for 30 seconds, 53°C for 45 seconds, 72°C for 1.5 min , one extension cycle at 72°C for 10 min and storage at +4°C. The amplicons were electrophoresed on a 1% agarose gel (Sigma-Aldrich, St. Louis, Missouri, USA) containing 3.5 µl of Syber-Safe (Invitrogen, USA) and visualized under an ultraviolet transilluminator.

Table 2

Primers sequence

Primers	Sequence	Tm °C
ESV 5’	5’ –GTTCCATGTGAACAGCAGT-3’	55
ESV 3’	5’ –CGAAAACATACGACAACTGC-3’	55

RESULTS AND DISCUSSION

The results of the DNA extraction from 50-100 muscle larvae in all six selected strains (*T. spiralis* and *T. britovi*) using the “QIAamp DNA” kit (Qiagen, Germany) and the amplification with ESV primers (forward and reverse) using “mi-Taq Mix Kit ”(Metabion, Germany) are obtained through a positive amplification for all six strains, revealing the method's efficiency in the biomolecular identification of Trichinella strains. (Fig. 1)

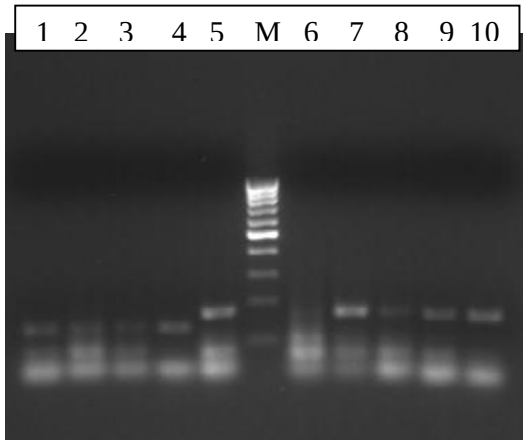

Fig.1. The electrophoresis of the amplification products obtained following the PCR with the mi-Taq Mix kit using the DNA extracted from all six strains. Line 1: jackal; Line 2: jakal; Linia 3: wolf 4; Linia 4: wolf 1; Line 5: 7.29; Line 6: 7.29; Line 7: 7.42; Line 8: 7.42; Line 9: 7.97; Linia10: 7.97

In order to compare the sensitivity of the amplification kit mi-Taq Mix with mi-Taq Only (different kinds of buffer solutions) (Metabion, Germania), an analysis of the amplification products obtained using the extracted DNA with the same type of kit “QIAamp DNA” from strain 7.21 (*T. spiralis*) was made. A positive amplification is obtained (7/7) in

the case of the amplification with mi-Taq Mix, while only one third of the samples (sample 8) were positive when using the mi-Tag Only (Metabion).

This difference in the sensitivity is due to the composition of the PCR reaction, in the case of the sample 6 (buffer solution containing NH_4 SO_4 without $MgCl_2$) and sample 7 (buffer solution containing KCl instead of NH_4 SO_4 and $MgC1_2$) as long as sample 8, a positive one, contains a buffer which includes NH_4 SO_4 and $MgC1_2$, revealing in this manner the importance of the buffer composition and respectively of the $MgC1_2$ (Fig.2).

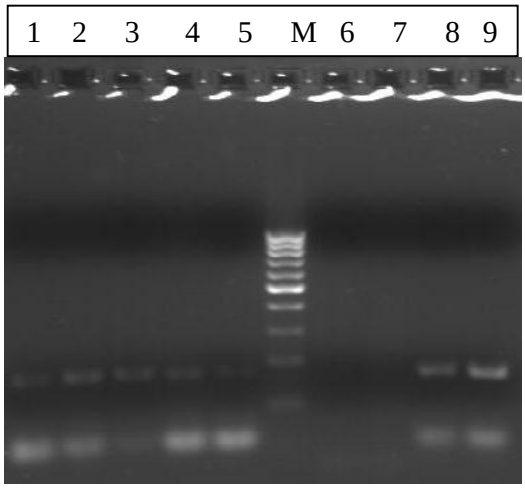

Fig. 2. The electrophoresis of the amplification products obtained following the PCR with the mi-Taq Mix and mi-Taq Only kits. Line: 1, 2, 3, 4, 5 and 9 mi-Taq Mix; Line: 6, 7, 8 mi-Taq Only.

Following the DNA extraction from 50 and 100 muscler larvas from 7.21 strain, using the same extraction "QIAamp DNA" (Qiagen, Germany), and amplification of the DNA extracted with mi-Tag Mix (Metabion), for comparative purposes, the amount of ultrapure wather used for sample elution, was changed, obtaining from the quantification, favorable results for samples 1, 2, 3, 4, 5 and 6 , that is the amount of: 44, 144, 73, 126 and 99 ng/µl of DNA using these quantities of ultrapure wather: 20µl for samples 1 and 2, 30µl for samples 3 and 4, and 40µl for smples 5 and 6. The ultrapure water amount used for the elution of samples 7 and 8 (50µl) makes the amount of DNA in these samples much lower, after obtaining the result of quantification 6ng/µl, and 8ng/µl of DNA. Although the amplification of the extraction products with specific ESV primers is positive in all 8 samples(1-8), regardless of the samples DNA concentration, it can be stated that after the DNA extraction with different amounts of water used in the elution, the amplification is pozitive and only the DNA concetration is influenced. In order to obtain higher concentrations of DNA it is recommended to use a lower quantity of water, between 20 - 40µl.(Fig. 3).

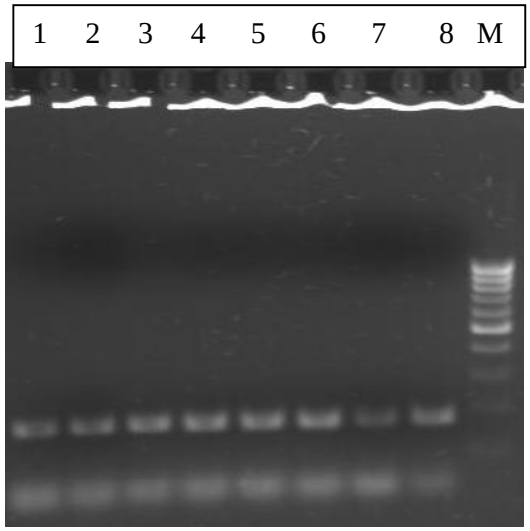

Fig. 3. The electrophoresis of the amplification products obtained following the PCR with the mi-Taq Mix using a different amount of ultrapure water from 7.21 strain.
Line 1: 20µl; Line 2: 20µl; Line 3: 30µl; Line 4: 30µl; Line 5: 40µl; Line 6: 40µl;
Line 7: 50µl; Line 8: 50µl; M-marker.

The method's sensitivity is not so suitable in identifying a single larva, fact given on one hand by to imperfect methods of extraction, indicating a high amount of impurities in the DNA extracted. The DNA extraction using proteinase K from the "QIAamp DNA" (Qiagen, Germany), from a single larva using all six strains and kit for amplifying the DNA extracted, leads to negative results. Although after quantification we obtained a high concentration of DNA (160, 201, 233, 100, 206, 221 ng/µl DNA), the amplifications results are negative, leading to the necessity of the improvement of PCR technique in order to obtain favorable results (Fig. 4).

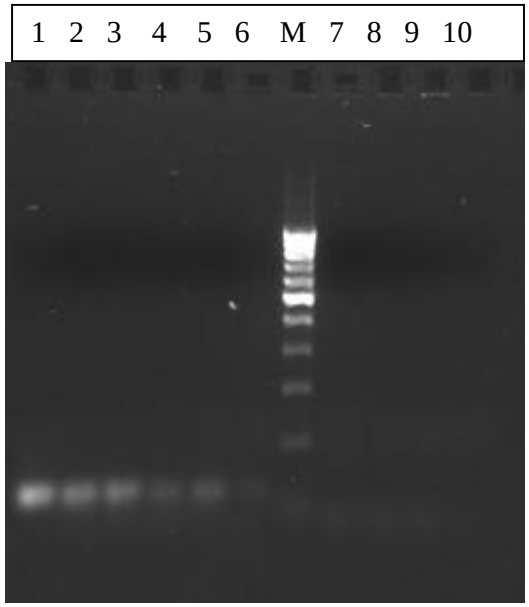

Fig. 4. The electrophoresis of the amplification products obtained following the PCR with the mi-Taq Mix and Takara kits, using the DNA extracted from a larva.
Line 1: 7.29; Line 2: 7.42; Line 3: 7.97; Line 4: jakal; Line 5: wolf 4; Line 6: wolf 1; Line 7: 7.29 Takara kit;
Line 8: 7.42 Takara kit; Line 9: 7.97 Takara kit; Linia 10: wolf 4 Takara kit.

CONCLUSIONS

This analysis reveals that following the DNA extraction from 50 to 100 muscle larvae of *Trichinella* spp. performed with "QIAamp DNA" kit (Qiagen, Germany) the results obtained are suitable, both in terms of DNA concentration and amplification with the "mi-Taq Mix Kit "(Metabion, Germany). Regarding the comparative amplification between mi-Taq Mix kit and mi-Taq Only, the importance of the $MgCI_2$ from the buffer solution's composition could be high lightened, the results of amplifications being positive.

The negative results obtained from the DNA extracted from a single muscle larva of *Trichinella* spp. challenges us to design a new protocol for DNA extraction and PCR technique improvement.

The improved DNA extraction from a larva reduces chemical and biological reagents and also saves time. The positive amplification of genomic DNA from one larvae may have favorable repercussions on the genetic variability analysis in a population of Trichinella studies which can be applied at different individuals belonging to the same population.

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