BIOLOGICAL AND GENETIC CHARACTERISTICS OF TWO *TRICHINELLA* ISOLATES IN CHINA; COMPARISON WITH EUROPEAN SPECIES

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Summary :

The most recent taxonomic revision in *Trichinella* genus included 10 taxa. *Trichinella spiralis* (Ts) is a thoroughly studied species but most of the isolates came from the Europe or American continents. Few information is available from China about the *Trichinella* isolates and their diversity. In this report two Chinese isolates were characterized and compared with European strains. The *in vitro* release of newborn larvae (NBL) was determined for the two species. NBL is observed in supernatant of cell culture after adults purification on day 4 post infection (pi) with Ts, and on day 5 pi with *Trichinella nativa* (Tna). A new parameter was thus proposed to characterize *Trichinella* strains. Restriction fragment length polymorphism (RFLP) of genomic DNA and random amplified polymorphic cDNA (RAPD) were used to define genetic variability among different isolates. Species specific pattern could be obtained with RAPD but it was far more difficult to get geographical markers for *Trichinella* using these methods. A low genetic variability in *Trichinella* species (i.e a strong "clonality") is suggested.

KEY WORDS : China, cDNA, PCR, sequencing, *Trichinella*.

Up to now, the genus *Trichinella* has been partitioned into seven species including *T. spiralis* (Ts), *T. nativa* (Tna), *T. britovi* (Tb), *T. pseudospiralis* (Tps), *T. murrelli* (Tm), *T. nelsoni* (Tn), *T. papuae* (Tp) (Pozio *et al.*, 1999), and three unclassified gene pools, T6, T8 and T9. Each species was characterized by biological, epidemiological, biochemical, and molecular properties in the genus *Trichinella* (Pozio *et al.*, 1992; La Rosa *et al.*, 1992, Pozio & La Rosa, 2000). Genetic variations were found in various geographic isolates of the same species. Zarlenga *et al.* (1996) analyzed the ribosomal DNAs of three isolates of Tps from a raccoon dog in Russia, a black vulture in North America, and a quoll in Australia; differences were observed in the PCR amplification patterns and in the nucleo-

tidic sequence of ribosomal genes. Minchella *et al.* (1994) analyzed the genomic DNA variation of Tm isolates coming from various hosts (opossum, coyotes, raccoons and red foxes) and found a variation in the polymorphic repetitive DNA profiles. La Rosa & Pozio (2000) performed a molecular investigation of three African isolates of Tn, and the species specific probe (pT7.3) hybridized Kenya and Tanzania isolates but failed to recognize South African isolates.

Ts is characterized by its high prolificity and infectivity in human but few information exist on the Ts Chinese strains. The present study analyzed some biological and genetic characteristics of two Chinese *Trichinella* isolates. Comparison with European strains was performed using restriction fragment length polymorphism (RFLP) of genomic DNA and random amplified polymorphic cDNA (RAPD). An easy to use new parameter was defined to identify strain with high prolificity (Ts).

MATERIALS AND METHODS

PARASITE

The following isolates were maintained in OF1 female mice by serial passages: Chinese Ts ISS534 (C1), Chinese Tna ISS531 (C2), European Tna ISS10 (E2) and two European Ts ISS406 (E3) and ISS3 (E1). Muscle larvae (ML) were collected by artificial digestion 35 days post infection (pi) as described by Gamble *et al.* (1983). Adult worms were purified from the intestinal tract on days 3 (Ad3), four, five and six post infection (pi) as previously described (Vayssier *et al.*, 1999).

THE INITIAL TIME

OF NEW BORN LARVAE (NBL) RELEASE;

DETERMINATION OF *IN VITRO* ADULT WORM FECUNDITY

Four groups of mice were infected with four isolates (C1, C2, E2 and E3) respectively. Each group was constituted of five mice and each mouse was infected *per os* with 2,000 ML. One mouse of each isolate was

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killed on days 3, 4, 5 and 6 pi. A total 96 adults of each isolate was selected every day and cultured individually in 96-microwell culture plates in RPMI 1640 (Gibco) containing 1 % pyruvate, 15 % foetal calf serum, 100 i.u./mL penicilline and 100 µg/mL streptomycine, 1 % L-glutamine for three days, at 37° C in 5 % CO₂ atmosphere. NBL released were checked by microscopy and a quantification was performed in each well every day.

GENOMIC DNAs ANALYSIS BY RFLP

Genomic DNA of each isolates (C1, C2, E1 and E2) was extracted as described by Klassen & Thiessen (1986). *Trichinella* DNA (1 µg) was digested with endonucleases *EcoRI*, *XbaI*, *DraI* using the buffer and temperature condition recommended by the manufacturer (Pharmacia). Digested DNA was analyzed after electrophoresis on a 1.2 % (w/v) agarose gel which was stained with 1 µg/mL ethidium bromide bath and photographed under UV transillumination.

cDNAs ANALYSIS BY RANDOM AMPLIFIED POLYMORPHIC SEQUENCE (RAPD)

- cDNA libraries construction: ML was collected for each isolates (C1, C2, E2 and E3). Pooled adults/new born larvae (Ad/NBL) were collected with C1, C2 and E3 isolates on day 5 pi. Total RNA of ML or Ad/NBL were extracted according to the method described by Chomczynski *et al.* (1987). λ ZAP II cDNA libraries (Short *et al.*, 1988) were established for each preparation as described (Vayssier *et al.*, 1999). Plasmids of individual cDNA libraries were excised from the phage vector under *in vivo* condition following the protocol of the manufacturer (Stratagene).

- cDNA amplification: 80 primers divided in four groups (OPA 20, OPB 20, OPC 20, OPD 20) were used in the RAPD analysis. Amplification reactions were performed in 25 µl volumes containing 10 mM Tris-HCl, pH 8.3; 50 mM KCl; 25 mM MgCl₂; 0.01 % (w/vol) gelatin; 100 mM each dATP, dCTP, dGTP and dTTP (Pharmacia); 4 µM primer (one or two or three primers); 30 ng cDNA with plasmid; and 2.5 units of Taq polymerase. The amplification was programmed for 45 cycles of one minute at 94° C, one minute at 36° C and two minutes at 72° C. Amplification products were visualized after electrophoresis on 1.4 % agarose gels.

CLONING AND SEQUENCING OF DNA AMPLICON

DNA band was purified from agarose gel using QUIAEX kit (Quiagen) and was ligated to pT-adv vector before transformation of TOP 10F *E. coli*. The insert was fully sequenced on both strands four times by Genome Express.

DNA amplified fragment was radiolabelled and used to screen the Ad/NBL Ts cDNA library by plaque hybridisation. Positive clones were selected and plaques purified two times by hybridisation. pBluescript plasmids containing cDNA were derived from the λ ZAP phages using the *in vivo* excision procedure described by Short *et al.* (1988). Several independent clones (4) were sequenced on both strands by Genome Express. Nucleotide and amino acid sequence analysis were carried out using OMIGA (Intelligenics, CA, Version 2).

RESULTS

Biological properties of Chinese and European strains were analyzed using two parameters: the initial time of NBL release by female worm and the prolificity. Chinese and European Ts isolates expelled NBL as early as day 4 pi whereas Tna did it on day 5 for both isolates. A delay in the NBL release was also observed with two European Tb strains (unpublished data). The prolificity of the female worm is reported in Table I. Ts produced always more NBL and earlier than Tna and whatever the geographic isolate the prolificity is similar for a same species. Standard deviation was important on day 4 as some female (an average of 20 %) did not expelled NBL.

In order to characterize the genome of these isolates, restriction profiles of genomic DNA using various restriction enzymes was performed (Fig. 1). The profiles between C1 and E1, and between C2 and E2, were highly similar whatever the restriction enzymes used but significant differences were identified between the two groups (Fig. 1). C1 and E1 had a unique *EcoRI* band at 1.82 kb, a *DraI* band at 0.89 kb and a *XbaI* band at 0.72 kb which were absent in C2 and E2 isolates.

As no geographic maker could be selected with RFLP analysis, a more sensitive method was used to iden-

		NBL/female day 4 pi	NBL/female day 5 pi	NBL/female day 6 pi
Chinese isolates	Ts	5.6 ± 4.61	53.7 ± 6.8	52.4 = 3.6
European isolates	Ts	4.0 ± 2.3	50.2 ± 7.1	51.3 = 5.7
Chinese isolates	Tna	0	29.5 ± 5.3	30.1 = 2.8
European isolates	Tna	0	30.8 ± 6.2	30.5 = 5.9

Table I. – Prolificity of *Trichinella* females *in vitro*. A comparison between Ts and Tna purified on days 4, 5 and 6 pi. The quantification was done three days after purification of Ad. Ad purified on day 3 did not expelled NBL.

Fig. 1. – Genomic DNAs digested by *EcoRI*, *DraI*, *XbaI*. Lane M: Molecular weight marker. C1: Chinese Ts. E1: European Ts. C2: Chinese Tna. E2: European Tna. The fluorescence smear in each lane represents randomly cleaved non-repetitive DNA sequence; superimposed bands are visualized in each lane and represent repetitive sequences. Fluorescence intensity in each band depends on the copy number of the multi sequence family as well as the length of the repeat unit.

Fig. 2. – Random PCR with cDNA plasmidic libraries of *Trichinella*. E3a: Ad/NBL cDNA library of E3, C1: Ad/NBL cDNA library of C1, E3b: ML cDNA library of E3, C2: Ad/NBL cDNA library of C2, E4: ML cDNA library of E4. The primer used in each experiment and molecular weight markers are indicated on the right part of each gel.

tify specific nucleotidic sequence. For that purpose plasmidic cDNA libraries of C1, C2, E2 and E3 isolates were analyzed by RAPD using 80 primers under various combinations (250). Most of the amplified profiles were similar for the four isolates indicating a high stability of expressed genes. Reproducible differences were observed in the amplifications with primer OPD15 (CATCCGTGCT), OPA7 (GAAACGGGTG), OPB19 (ACCCCGAAG), OPA16 (AGCCAGCGAA) (Fig. 2), and allowed to separate Ts group from Tna one whatever the antigenic stage (ML or Adult/NBL) or the geographic isolate used (Fig. 2). Some minor differences could be obtained with primer OPD15 between the Chinese and European Ts isolates particularly one band of 520 bp appeared with less intensity in one isolate. A bright DNA band of 2,100 bp appeared highly specific of Ts group and was absent with Tna group and primer OPD15. This DNA band was purified and fully sequenced after cloning for both strains. The primer used in the amplification could be recognized on one extremity. Two third of the sequence belong to pBluscript plasmid and the last third (542 bp) was specific of *Trichinella* species. Two bases changes were found in these 542 bp sequence (Fig. 3) between Chinese Ts and French Ts isolates. This Ts DNA insert was used to screen Ts Ad/NBL cDNA library (Chinese isolate). Several independent clones were selected and sequenced. The consensus nucleotidic sequence was 1,345 bp long with a unique open reading frame of 942 nucleotides encoding a putative protein of 314 amino acids (aa) with a molecular weight of 35,56 kDa. The variation on two bases was confirmed but there was no modification of the deduced primary aa sequence for both isolates. The first modified codon encode a Thr and the second a Leu residue. Comparison of the deduced aa sequence with data base did not revealed any significant homology with known coding sequence. The full length DNA sequence hybridized the eight *Trichinella* genome by dot blot analysis (data not shown) indicating that the variability of this sequence is limited to base changes among *Trichinella* genus.

DISCUSSION

The analysis of biological characteristics is a common method to characterize *Trichinella* species (Belosevic & Dick, 1979; Pozio *et al.*, 1992). The initial time of NBL release and the fecundity index of adult worm after their purification from intestinal tract are two simple and discriminant biological parameters. Ts showed the highest and earliest NBL release by comparison with Tna and Tb (data not shown). Rossi *et al.* (1993) performed similar study with five *Trichinella* species (Ts, Tna, Tne, Tb, Tps), and found

Ts(C1) 5' ▲CA TCCGTGCTTG TATAGCTTTA AACCAACCGG	32
Ts(E3a) 5' ◆CA -----	
TGTGAGAATC TCTGTCTTCT GCATCCGATT GCAGCAAATG CTCAGACAAC	82

AAAATGATCA CCTTTTGCAA AAGAGTGAAG ATAACTGCTT TTTG TA ATTC	132
----- C -----	
TTGGGCAGCG GAAAGTTTGA GCTGTATCGC GCTAACCTCA GAGCTCCTCT	182

CACCATCCGA CGAAGCTGAA CTCATGGTAC CTTCTTCAGC TTGTTTCTTG	232
-G -----	
TCCAATTCTT CTTGTATTG CTTTATACTT CTTGATAATC TCGTCGTCAT	282

TGAATACACA ATCTGCCAAA CGTAGTGCCT CATCAAATTG CCTCTCATCT	332

TTTCCGAAAA AATCCACTCA ATTACAGCCA ATGATTGAAT TATCTGCATC	382

TTCAACATTT TGTCCACGAC AATCATGATT CGTTGTTCAT TTGCAAACCA	432

AACTTCGTGC AGAGTTTGAA GTAAATGAAT CTGCGCTTCT TTGCTGTCGG	482

CTGCGAATTC TTAAACACG GACATGTACT TGATCAAAAT GGCAACGGTG	532

TGACTGATAG3' 542	
----- 3'	

Fig. 3. – Sequence comparison of common DNA band (520 bp) extracted after random PCR with cDNA of C1 and E3a (primer OPB15). The sequence of the primer is identified at the starting of the DNA fragment (italic) for both species. Bases changes are in bold.

the adult worms of all species release NBL on day 6 pi. This discrepancy with our results could reflect the host influence as CD1 female mice was used in Rossi' experiment. NBL stage specific probes were used to hybridize cDNA from Ts adult worm purified on day 3 or 5 (Liu Mingyuan & P Boireau, unpublished data). Only the cDNA of Ad worm collected five days pi gave a positive result demonstrating the presence of mature NBL. *In vitro* NBL release index did not show any significant difference between Chinese and European varieties of *Trichinella* despite the fact that each strain was isolated from various hosts.

Nevertheless the highest fecundity of Ts is confirmed for Chinese isolate as previously described for other Ts strains (Pozio *et al.*, 1992). In the same order, Tna appeared with a lowest prolificity whatever the geographical variety selected.

Informative RFLP pattern were obtained with Chinese and European species, the differences could be clearly revealed between species but were difficult to repro-

duce with geographic isolates of the same species. Base changes or deletion of repetitive sequences in the intron have been a good target to distinguish various *Trichinella* phenotypes, but few studies using the encoding sequences were performed. In the present report, we attempt to select species specific probes using RAPD with cDNA. Four cDNA libraries amplified by various combinations of primers were used to analyze sequence variation among four *Trichinella* isolates. Species variation could be identified with several primers but only one primer revealed a minor geographic difference. Wu *et al.* (1998) designed two primer pairs Ts53CA and Ts43CA, based on the cDNA of 53 kDa and 43 kDa glycoproteins and used the genomic DNA as template to amplify the full length coding regions. The study revealed bases changes either in encoding sequence or in intron. Our result reinforced the interest to use cDNA sequence and PCR in taxonomic studies of *Trichinella* genus despite the fact that RAPD or directed PCR on repetitive sequence were

reported to differentiate more easily various Ts isolates (Dick *et al.*, 1992).

The nucleic acid sequence comparison between the two isolates C1(Ts) and E3(Ts) with a same amplicon revealed two muted base changes. Some punctual base changes explained the variation in RAPD pattern on cDNA.

The initial time of NBL release is a new biological parameter that can be checked to identify highly prolific *Trichinella* species (Ts). Random amplification (more than 200) on cDNA libraries proved the high stability of coding sequence for each species in *Trichinella* genus and confirmed previous results obtained with monoclonal antibodies (Boireau *et al.*, 1997): a strong clonality exist for *Trichinella* species.

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