

Detection and Identification of Eight *Trichinella* Genotypes by Reverse Line Blot Hybridization

Y. B. ROMBOUT, S. BOSCH, AND J. W. B. VAN DER GIESSEN*

Microbiological Laboratory for Health Protection, National Institute of Public Health and the Environment (RIVM), 3720 BA Bilthoven, The Netherlands

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A reverse line blot (RLB) assay was developed to identify different *Trichinella* genotypes. The RLB assay accomplishes detection and specific identification of the different *Trichinella* genotypes and relies on hybridization of the amplified 5S ribosomal DNA intergenic spacer regions to specific, membrane-bound oligonucleotide probes. After one single amplification, we were able to detect and genetically identify six sibling species, i.e., *T. spiralis*, *T. britovi*, *T. nativa*, *T. murrelli*, *T. nelsoni*, and *T. pseudospiralis*, and two additional *Trichinella* genotypes, T6 and T8. Twenty-four *Trichinella* strains of different genotypes were unequivocally identified evaluated using one simple PCR-based assay based on single larvae. This assay allows the specific identification of *Trichinella* species without the need to passage larvae in laboratory animals.

Trichinellosis is one of the most important parasitic food-borne zoonotic diseases, caused by the consumption of insufficiently heated or raw meat infected with larvae of the genus *Trichinella*. In the European Union, monitoring is mandatory, based on the detection of *Trichinella* larvae during inspection of meat from pigs and horses. Control measures after positive findings consist of withdrawal of infected meat from the food chain. Still, recent outbreaks have been reported in France (1998; www.promedmail.org) and Germany (1998/1999; www.promedmail.org). Outbreaks can result from the consumption of imported meat from countries where the organism is endemic or of locally extensively produced meat infected with sylvatic *Trichinella* species (8, 22). Today, seven species (five with encapsulated larvae and two with nonencapsulated larvae) and three additional genotypes of undefined level have been identified (20, 21, 24, 30) by biochemical, biological, and molecular studies (5, 6, 7, 9, 10, 13, 14, 16, 25, 26, 33). Of these, *Trichinella britovi*, *T. nativa*, *T. nelsoni*, *T. murrelli*, *T. pseudospiralis*, *T. papuae*, T6, T8, and T9 are predominantly found in wildlife, whereas *T. spiralis* is able to maintain itself in domestic pigs and horses and is therefore the main source of infections in humans (4, 12). All these *Trichinella* genotypes are morphologically similar except *T. pseudospiralis* and *T. papuae*, which can be identified by the absence of a collagen capsule in the nurse cell during the larval muscle phase (25). It is important to identify single larvae to their genotype level to determine the source of infection, the importance to public health, and the clinical course in humans. Furthermore, the discovery that mixed infections can occur in the same host stresses the need for a test to identify single larvae (23, 24). Until now, many efforts have been made to identify isolated larvae to their specific genotype level. PCR-based assays with specific primer sets have been developed (1, 29, 32, 34). However, for these

assays multiple PCRs using different primer sets are sometimes needed, and the assay technique is not always applicable when one or a few single larvae are isolated, due to the lack of a sufficient amount of DNA. Random amplified polymorphic DNA analysis is based on the fast detection of genetic markers using only a single arbitrary primer and can be used without prior sequence information (31). This is one of the first PCR-based methods which is described for the genus *Trichinella* to identify a single larva at the species level (2, 3). However, in random amplified polymorphic DNA analysis there are problems with the reproducibility between different tests and among different laboratories. Also, if the DNA is partially damaged, the electrophoretic banding pattern will be hard to interpret by comparison to an undamaged specimen, especially for isolates that are closely related (2, 19). It was our aim to develop an easy and applicable method, based on a single PCR, for the identification of different *Trichinella* genotypes. Liu et al. (17, 18) reported that the 5S rRNA genes in nematodes are highly conserved, while the intergenic spacer regions between the tandemly repeated genes can vary greatly in size and nucleotide composition between closely related organisms. Recently, we used oligonucleotide primers based on the invariant regions of the conserved, rather small 5S rRNA gene to amplify the spacer region. We analyzed the amplified 5S rRNA intergenic region of the different *Trichinella* genotypes using the cleavage fragment length polymorphism assay (28). It was shown that there was prominent polymorphism within this region between *T. spiralis* and the sylvatic *Trichinella* genotypes and minor polymorphism among the sylvatic *Trichinella* genotypes.

In this study, we analyzed the DNA sequences of the ribosomal intergenic spacer regions of the eight different *Trichinella* genotypes. Differences in nucleotide composition which were consistent for each genotype were found. The differences in DNA sequence of the 5S rRNA intergenic spacer region were used to develop a reverse line blot (RLB) assay (11, 27) with the goal of detecting and identifying the eight different genotypes of *Trichinella* based on single larvae. With this new method we were able to simultaneously detect and differenti-

* Corresponding author. Mailing address: Microbiological Laboratory for Health Protection, National Institute of Public Health and the Environment, Antonie van Leeuwenhoeklaan 9, P.O. Box 1, 3720 BA Bilthoven, The Netherlands. Phone: 3130-2743926. Fax: 3130-2744434. E-mail: joke.van.der.giessen@rivm.nl.

TABLE 1. *Trichinella* strains used in this study (28)

No.	Isolate ^a	Species
1	ISS 3	<i>T. spiralis</i> (T1)
2	RIVM (ISS 14)	<i>T. spiralis</i>
3	MAD83 (ISS 92)	<i>T. spiralis</i>
4	CO77 (ISS 88)	<i>T. spiralis</i>
5	ISS 2	<i>T. britovi</i> (T3)
6	ISS 324	<i>T. britovi</i>
7	ISS 384	<i>T. britovi</i>
8	Monegrillo (ISS 89)	<i>T. britovi</i>
9	Z191	<i>T. britovi</i>
10	ISS 10	<i>T. nativa</i> (T2)
11	ISS 296	<i>T. nativa</i>
12	ISS 335	<i>T. nativa</i>
13	ISS 29	<i>T. nelsoni</i> (T7)
14	ISS 232	<i>T. nelsoni</i>
15	ISS 103	<i>T. murrelli</i>
16	ISS 346	<i>T. murrelli</i>
17	ISS 456	<i>Trichinella</i> T6
18	ISS 34	<i>Trichinella</i> T6
19	ISS 334	<i>Trichinella</i> T6
20	ISS 124	<i>Trichinella</i> T8
21	ISS 148	<i>Trichinella</i> T8
22	ISS 149	<i>Trichinella</i> T8
23	ISS 13	<i>T. pseudospiralis</i> (T4)
24	ISS 470	<i>T. pseudospiralis</i>

^a ISS, Istituto Superiore di Sanità, Rome, Italy; RIVM, National Institute of Public Health and the Environment, Bilthoven, The Netherlands.

ate six *Trichinella* species, i.e., *Trichinella spiralis*, *T. britovi*, *T. nativa*, *T. murrelli*, *T. nelsoni*, and *T. pseudospiralis*, and two additional *Trichinella* genotypes, T6 and T8, on the basis of differences in the 5S rRNA intergenic nontranscribed region. In this paper we show that the RLB assay provides a highly sensitive and specific tool for the unequivocal identification of *Trichinella* genotypes based on differences in the spacer region of the 5S rRNA genes and can be carried out on single larvae.

MATERIALS AND METHODS

Isolates. From each of six identified *Trichinella* species and two additional genotypes, different isolates were used in this study (Table 1). The genotypes of all isolates studied, except one (code Z191), were previously identified at the International *Trichinella* Reference Center in Rome, Italy (20). All isolates were maintained in laboratory mice by serial passages. Muscle larvae were isolated by artificial digestion in 5 g of pepsin and 30 ml of 2.4 N HCl in 400 ml of water using Trichomatic35 (Foss Electric, Hillerød, Denmark) for 12 min.

DNA isolation from individual larvae. For isolation of genomic DNA from single larvae, each larva was heated in Tris-HCl (pH 7.6) at 90°C for 10 min and treated with proteinase K (100 µg/ml in a total volume of 10 µl) at 48°C

for at least 3 h (3). After inactivation of the proteinase K, 5 µl was amplified by PCR.

Preparation of genomic DNA from *Trichinella* larvae. Total genomic DNA was extracted from a pool of 2,000 muscle larvae with the Puregene DNA isolation kit (Gentra Systems, Minneapolis, Minn.), using the plant tissue protocol according to the manufacturers' instructions. Briefly, larvae were homogenized in 600 µl of cell lysis solution for 60 min at 65°C. After RNase treatment proteins were precipitated with 200 µl of protein precipitation solution, followed by DNA precipitation with isopropanol. After a washing with 70% ethanol, DNA was dissolved in 100 µl of distilled water.

PCR. Primers to amplify the 5S ribosomal DNA (rDNA) intergenic spacer region of *Trichinella* have been described by Liu et al. (17). A biotin-labeled sense primer (5'-GCGAATTCCTTGGATCGGAGACGGCCTG-3') and anti-sense primer (5'-GCTCTAGACGAGATGTCGTGCTTTCAACG-3') were used in the PCR. PCR was optimized for pH and MgCl₂ concentration. PCR amplification was carried out on 5 µl of DNA from one individual larva in a total volume of 50 µl on a Perkin Elmer 4800 cyler (28). Briefly, the reaction mixtures contained 1.5 mM MgCl₂, 200 µM concentrations of each deoxynucleotide triphosphate, 1.5 U of *Taq* DNA polymerase (Perkin Elmer), and 25 pmol of each primer and were covered with mineral oil. Thermal cycling conditions were 94°C for 90 s for 1 cycle, 94°C for 30 s, 48°C for 1 min, and 72°C for 1 min for 40 cycles followed by a 10-min extension at 72°C.

DNA sequence analysis. The PCR products were purified with a QIAquick PCR purification kit (Qiagen, GmbH, Hilden, Germany) according to the manufacturer's instructions. The purified products were directly sequenced using 15 pmol of the PCR primers (without the biotin label) with an ABI Prism BigDye terminator cycle sequencing ready reaction kit (Perkin Elmer, Applied Biosystems, Foster City, Calif.). All reactions were run on an Applied Biosystems (ABI) 373 DNA sequencing apparatus (Perkin Elmer). The DNA sequences of the amplicons were analyzed using DNAsis software, version 2.5 (Hitachi Software Engineering Co., Ltd.).

RLB hybridization. For each *Trichinella* genotype, a specific oligonucleotide probe with an N-terminal *N*-(trifluoroacetamido)hexyl-cyanoethyl, *N,N*-diisopropyl phosphoramidite)-C₆ amino linker (Isogen, Maarsen, The Netherlands) had been designed (Table 2). The oligonucleotides were diluted in 150 µl of 0.5 mM Na₂CO₃ (pH 8.4) to a final concentration of 50 to 1,000 pmol/µl. The probes were covalently linked to an activated Biotinyne C blotting membrane (Pall Biosupport, Ann Arbor, Mich.). The membrane was activated by incubation in 16% 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (Sigma) for 10 min at room temperature, rinsed with distilled water, and applied in a MN45 miniblotted system (Immunetics, Cambridge, Mass.). Each slot of the miniblotted was filled with one of the specific oligonucleotide dilutions and incubated for 1 min at room temperature. After the dilutions were aspirated, the membrane was inactivated in 100 mM NaOH for 10 min at room temperature, followed by washing with 2× SSPE (2× SSPE is 36 mM NaCl, 2 mM NaH₂PO₄, and 0.2 mM EDTA, pH 7.2)-0.1% sodium dodecyl sulfate (SDS) for 5 min at 60°C. The membrane was replaced in the miniblotted, perpendicular to the previously applied specific oligonucleotides. In 150 µl of 2× SSPE-0.1% SDS, 10 µl of PCR product was heat denatured for 10 min and placed on ice immediately. The products were applied to the miniblotted and incubated for 60 min at 45°C. After aspiration the membrane was washed in 2× SSPE-0.5% SDS twice, for 10 min each time, at 57°C. The membrane was subsequently incubated in a 1:4,000 dilution of streptavidin-peroxidase (Boehringer Mannheim, Mannheim, Germany) in 2× SSPE-0.5% SDS at 42°C for 60 min. The membrane was washed twice, for 10 min each time, in 2× SSPE-0.5% SDS at 42°C, followed by two rinses, 5 min each time,

TABLE 2. Sequences of the oligonucleotide probes used in the RLB assay

Oligonucleotide	Sequence	Probe location	T _m (°C)	Specificity	Oligonucleotide concn (pmol)
T	CCAATATCATCGGTGCAGTT	605-663	58.2	<i>Trichinella</i> spp. ^a	100
Ts	TCCTTTTAAGACCACAGTGG	641-659	58.2	<i>T. spiralis</i>	1,000
Tbr	TGGTGCATTTTTTCCAATTGTC	582-603	57.7	<i>T. britovi</i>	100
Tna	GACATTTGCTAAACGAATCAAC	662-683	57.7	<i>T. nativa</i>	100
Tne	GTTTTAGCGAATGTTTAATTTTCATA	99-123	57.8	<i>T. nelsoni</i>	25
Tmu	TTTTATAACCAGACTGGCCG	637-656	58.2	<i>T. murrelli</i>	200
T6	TTATAACCAAAGTGGCCTAAAATAT	630-654	58.0	T6	400
T8	ATATCAATTTATGACACTGAACAAG	418-442	58.0	T8	12.5
Tps	TAGTGTTGTAGAAGGATCATCA	316-336	57.7	<i>T. pseudospiralis</i>	200

^a Not including *T. pseudospiralis*.

with 2× SSPE at room temperature. Hybridized biotin-labeled PCR products were detected using the ECL detection system according to the procedure of the manufacturer (Amersham). The membrane was exposed to a chemiluminescent detection film (Boehringer Mannheim) for 20 min, and afterwards the film was developed in a Fuji X-ray film processor (Fuji Medical Systems Benelux).

The membrane can be reused after stripping of the PCR products by incubating the membrane in 100 ml of 1% SDS at 80°C twice for 30 min each time followed by incubation of the membrane in 100 ml of 20 mM EDTA for 15 min at room temperature. After the fluid is discarded, the membrane can be stored in Saran Wrap at 4°C.

Nucleotide sequence accession numbers. DNA sequences were submitted to GenBank under accession numbers AY009943 to AY009950.

RESULTS

PCR. The two selected primers amplified a single prominent 5S rDNA intergenic spacer region product of approximately 750 bp for all *Trichinella* genotypes tested, except for *T. pseudospiralis*. This species gave a product of 522 bp after amplification (28). The PCR can be applied both to DNA extracts of pooled and to individual larvae.

Determination of the DNA sequences of the 5S rDNA intergenic region. For each *Trichinella* genotype, PCR products of the 5S rDNA intergenic regions of two different isolates were directly sequenced from both strands. After alignment of the 5S rRNA intergenic spacer regions of the genotypes *T. spiralis*, *T. britovi*, *T. nativa*, *T. nelsoni*, *T. murrelli*, T6, and T8, several differences in the DNA sequences were found. These differences were consistently found in both isolates of each species sequenced (data not shown). The different length of the PCR product of *T. pseudospiralis* and the large number of differences in the DNA sequence made it impossible to include this species in the multiple alignment.

Development of specific oligonucleotides for RLB assay. For each genotype a specific oligonucleotide was designed (Table 2), derived from the 5S rDNA intergenic region. The locations of the different probes are shown in Table 2. For the selection of the probes, DNA sequences which showed as many mismatches as possible between the different genotypes were chosen. All of the membrane-bound oligonucleotides had a melting temperature between 57.7 and 58.2°C, which enables simultaneous hybridization. Also, a genus-specific oligonucleotide which can identify seven genotypes other than *T. pseudospiralis* was developed. The DNA sequence of *T. pseudospiralis* differed too much from sequences of the other genotypes. For this species, a specific oligonucleotide was developed.

For each oligonucleotide probe, the optimal concentration was empirically determined in such a way that all the specific hybridizations resulted in signals of approximately the same level of intensity. These concentrations are also listed in Table 2.

Specificity of the RLB assay. To test the specificity of the RLB assay, the 5S rDNA intergenic region of one reference strain of each of the eight genotypes was amplified. The forward primer TSSF was labeled with biotin to enable direct hybridization to the selected, membrane-bound oligonucleotides. The washing step after the hybridization was performed at 57.0°C. PCR products of the *Trichinella* strains hybridized with the specific selected oligonucleotides only, without cross-reaction. (Fig. 1). Also, the “catchall” *Trichinella* control oligonucleotide probe reacted with all the isolates except *T. pseudospiralis*, which had a deviant sequence. Therefore, a *T.*

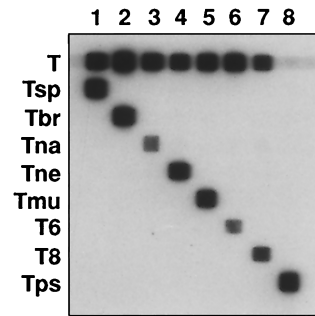


FIG. 1. RLB assay with 5S rDNA intergenic PCR products of reference strains of each *Trichinella* genotype. The oligonucleotide probes are in the horizontal lanes, and the PCR products are in the vertical lanes. The oligonucleotides are abbreviated as in Table 2. Lanes: 1, *T. spiralis* CO77; 2, *T. britovi* ISS 2; 3, *T. nativa* ISS 296; 4, *T. nelsoni* ISS 29; 5, *T. murrelli* ISS 346; 6, T6 ISS 34; 7, T8 ISS 149; 8, *T. pseudospiralis* ISS13.

pseudospiralis-specific oligonucleotide was included in the assay.

To test the reproducibility of the RLB assay, a total of 24 *Trichinella* strains of different genotypes were tested in the RLB assay (Table 1). All *Trichinella* strains of the same genotype gave the same hybridization pattern. Only *T. nelsoni* strain ISS232 hybridized slightly with the *T. nativa* oligonucleotide (Fig. 2).

Sensitivity of the RLB assay. A 10-fold dilution of *Trichinella britovi* DNA in water was made from a pool of larvae. The 5S rDNA intergenic region was amplified and visualized after agarose gel electrophoresis to determine the detection limit compared with the RLB assay. The RLB assay was more sensitive by at least a factor of 10, as determined by comparing the visibility after gel electrophoresis (Fig. 3).

Detection of mixed infections. To test the ability to detect mixed infections, 10-pg portions of DNA of the following pairs of genotypes were mixed in equal amounts: *T. spiralis* and *T. britovi*, *T. nativa* and *T. spiralis*, *T. britovi* and *T. nativa*, and *T. nelsoni* and *T. nativa*. PCR was performed, and the products were used in an RLB assay. For every mixture, we were able to detect both *Trichinella* genotypes simultaneously without any cross-reactions (Fig. 4).

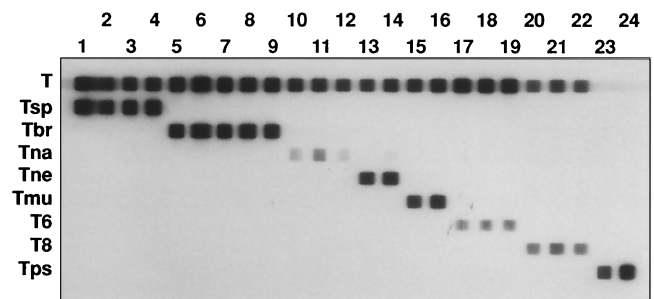


FIG. 2. RLB assay with 5S rDNA intergenic PCR products of all *Trichinella* strains, as listed in Table 1. Lane numbers correspond to numbers shown in Table 1. The oligonucleotide probes are in the horizontal lanes, and the PCR products are in the vertical lanes. The oligonucleotides are abbreviated as in Table 2.

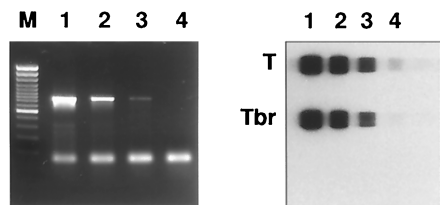


FIG. 3. Agarose gel electrophoresis and RLB assay with PCR products of serial dilutions of DNA of *T. britovi* (1–4). M, 100-bp marker (Fermentas). The RLB assay was performed with the genus-specific *Trichinella* probe (T). Tbr, species-specific *T. britovi* probe.

DISCUSSION

We developed an RLB assay (11, 27) to identify and differentiate eight different *Trichinella* genotypes based on a single PCR assay. The distribution of domestic and sylvatic *Trichinella* genotypes still constitutes a risk of human trichinellosis (22). For this reason it is important to reduce the risk of infection and to provide more information of the maintenance of different *Trichinella* genotypes in nature and in different host species. Over the last few years, analysis of different genetic, biochemical, and biological data has contributed to the identification of 10 different genotypes. Of these, eight different genotypes were used in this study. The RLB assay is based on variations in the DNA sequences between 5S rDNA genes within the genus *Trichinella*. The PCR for the amplification of the 5S rDNA intergenic spacer region, using primers within the conserved region 5S rDNA gene (17, 18), resulted in a single product for all the different genotypes studied. A prominent band of approximately 750 bp was found. Only for the isolates of *T. pseudospiralis* a smaller product of 522 bp was generated (28). This difference in length of the 5S rRNA intergenic spacer again emphasizes the genetic differences between the non-encapsulated *T. pseudospiralis* and the other seven encapsulated *Trichinella* genotypes studied. In addition, after analyses of the DNA sequence of the eight genotypes, no identical sequence in the 5S rRNA intergenic region between *T. pseudospiralis* and the other genotypes was found. The differences in nucleotide composition of the generated amplicons within the other seven genotypes showed enough variability to develop an RLB assay. Genotype-specific oligonucleotides based on this region were designed and immobilized on a membrane. The oligonucleotides can hybridize specifically with the biotin-labeled PCR products to detect single-base variations under the same hybridization conditions. In this way we were able to distinguish all the *Trichinella* genotypes tested, even T6 and *T. nativa*,

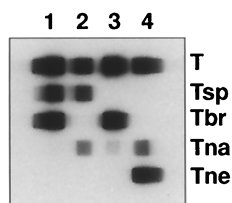


FIG. 4. RLB assay with PCR products of mixed DNA of different *Trichinella* genotypes. Lane 1, PCR products of *T. spiralis* and *T. britovi* DNAs; lane 2, PCR products of *T. spiralis* and *T. nativa* DNAs. Abbreviations are as in Table 2.

which are closely related in their allozyme grouping (1, 2), without cross-reactions. Also, a genus-specific *Trichinella* oligonucleotide, situated in a highly conserved region of the 5S rDNA, was selected. With this oligonucleotide probe, all the *Trichinella* genotypes tested could be identified, except for *T. pseudospiralis*, which could be identified only by its specific oligonucleotide probe. The reproducibility of the RLB assay was tested with 24 different strains of the different genotypes. Each strain of the different *Trichinella* genotypes reacted with its species-specific oligonucleotide probe except for one *T. nelsoni* strain, which showed some cross-reaction with the *T. nativa* oligonucleotide probe. More individual *T. nelsoni* larvae were tested in the RLB assay, and all those larvae gave the same hybridization pattern (data not shown). According to La Rosa and Pozio (15), this isolate was identified as *T. nelsoni* in the allozyme and molecular analyses. However, this *Trichinella* strain reacted differently in the Southern blot analysis that distinguishes this isolate from the others of the same species. The cross-reaction could be due to a mutation in one of the intergenic repeats, which causes this slight reaction in the RLB assay.

Overall, the intergenic spacer region of the 5S rRNA genes proved to be a stable genetic marker among the different *Trichinella* genotypes and could be used to identify these genotypes. To date, other PCR-based methods for detecting and identifying the different *Trichinella* genotypes have also been described. For the RLB assay, labeled PCR products using only one primer set are necessary, and this enables us to differentiate the genus *Trichinella* even based on individual muscle larvae. In the case of a new *Trichinella* genotype, i.e., one not included in the RLB assay, the *Trichinella* genus-specific probe will be positive while the genotype-specific probes will not give a result. Even these newly isolated *Trichinella* spp. can be analyzed by sequencing the 5S rDNA intergenic region PCR product. After this DNA sequence is compared and analyzed, a species-specific oligonucleotide probe can be determined and introduced into the RLB assay. Because the primer regions in the 5S rRNA genes are highly conserved, DNA from any possible newly identified *Trichinella* species can be amplified, sequenced, and introduced into the RLB assay.

T. spiralis and *T. britovi* have been detected in the same host species in central and southern Europe. Also, a double infection with *T. nativa* and *T. britovi* in a raccoon dog has been described (23, 24). This means that mixed infections can occur, and therefore we tested whether the RLB assay can be used to detect and to identify mixed infections. DNA samples of *Trichinella* species which can occur in the same host were mixed and were equally well amplified. We used DNA samples instead of individual larvae to be sure that the amounts of DNA for the species were equal. With the RLB assay, we were able to identify DNA of mixed genotypes. Theoretically, when different numbers of larvae of each *Trichinella* genotype are present in muscle samples, competition of the primers can occur. This stresses the importance of using assays which are based on the identification of single larvae from the original host and not using DNA of pools of isolated muscle larvae.

The sensitivity of the RLB assay, depending on the amount of intact DNA in one larva, was tested with a 10-fold serial dilution of the DNA of a single larva. With a decreasing amount of DNA, we were able to generate a specific PCR

product to detect the *Trichinella* DNA. It was clear that only a small amount of DNA was needed to obtain any PCR product. This is in contrast with other tests, where the amount of DNA is very important to get any positive results. The RLB assay was 10-fold more sensitive than agarose gel analysis.

In conclusion, the 5S rRNA intergenic spacer region is a stable marker for the identification and differentiation of *Trichinella* genotypes in the RLB assay. Only one PCR-based assay is required for the simultaneous detection and identification of eight different genotypes, starting from an individual muscle larva. The RLB assay is easy to perform and can even be used in routine laboratories. The membrane with the covalently linked oligonucleotide probes, after optimization of the concentrations, can be reused several times (11, 27). This enables us to perform the RLB assay within 1 day.

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