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# Evaluation of the efficiency of nested q-PCR in the detection of Mycobacterium tuberculosis complex directly from tuberculosis-suspected lesions in post-mortem macroscopic inspections of bovine carcasses slaughtered in the state of Mato Grosso, Brazil

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a b s t r a c t

Bovine tuberculosis (BTB) is a zoonotic disease caused by Mycobacterium bovis, a member of the Mycobacterium tuberculosis complex (MTC). The quick and specific detection of this species is of extreme importance, since BTB may cause economic impacts, in addition to presenting imminent risks to human health. In the present study a nested real-time PCR test (nested q-PCR) was used in post-mortem evaluations to assess cattle carcasses with BTB-suspected lesions. A total of 41,193 cattle slaughtered in slaughterhouses located in the state of Mato Grosso, were examined. Of the examined animals, 198 (0.48%) showed BTB-suspected lesions. M. bovis was isolated in 1.5%(3/198) of the samples. Multiplex-PCR detected MTC in 7%(14/198) of the samples. The nested q-PCR test detected MTC in 28%(56/198) of the BTB-suspected lesions, demonstrating higher efficiency when compared to the multiplex-PCR and conventional microbiology. Nested q-PCR can therefore be used as a complementary test in the national program for control and eradication of bovine tuberculosis.



## 1. Introduction

Tuberculosis (TB) is a zoonotic disease characterized by chronic infection and debilitating effects, caused by members of the Mycobacterium tuberculosis complex (MTC). This group includes Mycobacterium bovis, the causative agent of bovine tuberculosis (BTB), which, besides affecting cattle, can infect different mammal species, including humans (Medeiros, Marassi, Figueiredo, & Lilenbaum, 2010). This important zoonosis is distributed worldwide, with a marked prevalence in developing countries. It may be regarded as a socioeconomic disease, affecting agricultural activities and causing severe impacts on livestock productivity, leading to significant economic losses (Furlanetto et al., 2012). Cattle infection by M. bovis translates into imminent risk to human health, either in the form of an occupational disease or through the consumption of animal products contaminated with the bacillus (Brazil, 2004).

Detection of the pathogen responsible for BTB is required for the control and eradication of the disease. This is performed by intradermal tuberculin testing and/or monitoring in slaughterhouses, as well as animal tracking and sanitation (OIE, 2009). With the aim of reducing the prevalence and incidence of new disease outbreaks, in 2001 the National Program for the Control and Eradication of Animal Brucellosis and Tuberculosis (Programa Nacional de Controle e Erradicação da Brucelose e Tuberculose Animal — PNCEBT) was established in Brazil, based on tuberculin testing and slaughter of infected animals, associated with surveillance of animals from certified or monitored herds in slaughterhouses, held by the official inspection service (Brazil, 2004).

However, the tuberculin test may present sensitivity problems (or lack thereof). It may present false positive results, causing disease-free animals to be slaughtered, consequently leading to economic livestock losses, or false negative results, leading to infected animals remaining in the herd and spreading the disease. These failures in diagnosis are worrying, since the reference microbiological methods considered for confirmatory diagnosis have low sensitivity, requiring large amounts of viable bacilli (≥ 100 bacilli $mL^{-1}$) for detection. In addition, they

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are also laborious and time-consuming procedures, which take between 1 and 3 months for bacterial isolation and an additional two to three weeks for biochemical identification of the isolates (Corner, 1994).

The state of Mato Grosso has emerged in Brazil's national scene as the largest beef cattle producer and second largest beef exporter in the country (Brazil, 2014), leading to annual increases in the amount of meat exported to the EU countries. Consequently, the pressure on Brazil by countries that buy Brazilian products to implant an effective, rapid and definitive diagnosis of BTB in tuberculosis-suspected lesions has also increased.

In 2012, the Ministry of Agriculture, Livestock and Supply (Ministério da Agricultura, Pecuária e Abastecimento — MAPA), determined that farms where BTB cases have been detected can no longer export beef to the Customs Union of Belarus, Kazakhstan and Russia, recalling all lots from these animal farms until the diagnosis of suspicious lesions in samples collected after slaughter could be conducted at an official MAPA laboratory (Araújo et al., 2014b; Brazil, 2012).

In view of these difficulties in tuberculosis diagnosis, some alternatives for the quick and specific identification of BTB in clinical samples or in isolated colonies have emerged, such as molecular methods based on PCR and its variants (simplex PCR, multiplex PCR, nested PCR, real-time PCR and nested real-time PCR) (Araújo et al., 2014a, 2014b; Soini & Musser, 2001).

In this context, the purpose of the present study was to evaluate the performance of nested real-time PCR (nested q-PCR) in the detection of MTC species directly from suspected BTB lesions, in addition to comparing nested q-PCR, the "gold standard methodology" of bacterial cultures and multiplex-PCR (m-PCR).

## 2. Methodology

### 2.1. Cattle carcass inspections at slaughterhouses

The slaughter and sanitary inspection of 41,193 cattle carcasses were monitored in slaughterhouses inspected by the Federal Inspection Service (Serviço de Inspeção Federal — SIF), located in the state of Mato Grosso. From May to October 2009, the rulings awarded by the SIF regarding tuberculosis and lymphadenitis located in the head, neck or chest cavity of the carcasses (BTB-suspected lesions) were recorded and the samples were collected. Suspicious lesions were examined macroscopically according to the post-mortem SIF official standards of inspection (Brazil, 1952), photographed, divided and properly packed for bacteriological culturing and m-PCR and nested q-PCR testing.

### 2.2. Isolation of M. bovis colonies

Approximately 3 g of each sample were crushed and macerated with ground glass and decontaminated by hexadecylpyridinium chloride (HPC 0.75%) and by the adapted Petroff method (NaOH 4%) (Ambrosio, Oliveira, Rodriguez, Ferreira Neto, & Amaku, 2008). After decontamination, the samples were plated, in duplicate, into tubes containing Stonebrink and Lowenstein–Jensen culture media and incubated at 37 °C for 90 days. After the growth of the isolated M. bovis colonies, the colonies were stained by the Ziehl–Neelsen method, to highlight the presence of acid fast bacilli (AFB) in accordance with the recommendations by the Brazilian Ministry of Health, dictated in the national surveillance manual laboratory of tuberculosis and other mycobacteria (Brazil, 2008).

### 2.3. Identification of the isolated colonies by m-PCR

The DNA from the isolated colonies from the lesion samples was extracted by thermal lysis at 100 °C for 10 min and amplified by m-PCR according to conditions previously set by Figueiredo et al. (2009), using primers for the amplification of genomic sequences IS6110 (245 bp) INS1: (5′-CGTGAGGGCATCGAGGTGGC-3′) and INS2: (5′-GCGTAGGCGTCGGTGACAAA-3′) (Hermans et al., 1990), present only in MTC members, and RvD1Rv2031c (500 bp) Jb21: (5′-TCGTCCGCTGATGCAAGTGC-3′) and Jb22: (5′-CGTGAACGTAGTCGCCTGC-3′) (Rodriguez et al., 1999) present only in M. bovis.

### 2.4. DNA extraction from the BTB-suspected tissue fragments

The DNA from the BTB-suspected tissue fragments was extracted using a Qiagen extraction and purification commercial kit (DNeasy® Blood & Tissue Kit), applying the protocol modifications as described by Figueiredo et al. (2012). A 1 g tissue fragment was removed per animal. The tissue was macerated with a scalpel and then suspended in 180 μL of lysis buffer (20 mg $mL^{-1}$ lysozyme, 20 mM Tris–HCl (pH 8.0), 2 mM EDTA and 1.2% Triton) and incubated for 1 h at 37 °C. After this step, DNA extraction followed the recommendations of the extraction kit manufacturer. DNA was quantified in a Nanodrop ND1000 (Thermo Scientific, USA).

### 2.5. m-PCR testing on the DNA extracted from the animal tissue fragments

Five microliters of template DNA (about 100 ng) were used for the m-PCR test based on the method described by Figueiredo et al. (2009) using 5 μL of buffer (Invitrogen, USA), 0.2 mM dNTPs (Fermentas, USA), 1.5 U of recombinant Taq polymerase (Platinum®Taq — Invitrogen, USA), 5 mM $MgCl_2$ (Invitrogen, USA) and 20 pmol of each primer (Invitrogen, USA) in the reaction for the amplification of genomic sequences IS6110 (245 bp) INS1: (5′-CGTGAGGGCATCGAGGTGGC-3′) and INS2: (5′-GCGTAGGCGTCGGTGACAAA-3′) (Hermans et al., 1990) present only in MTC members, and RvD1Rv2031c (500 bp) Jb21: (5′-TCGTCCGCTGATGCAAGTGC-3′) and Jb22: (5′-CGTGAACGTAGTCGCCTGC-3′) (Rodriguez et al., 1999) present only in M. bovis. The final volume was 50 μL. Amplification of the target sequence was performed according to the following parameters: 94 °C for 5 min, followed by 37 cycles for 1 min at 94 °C, 1 min at 68 °C and 1 min at 72 °C with a final extension at 72 °C for 7 min. The resulting PCR products were analyzed by ultrapure agarose gel (1.5%) (Invitrogen, USA), stained with ethidium bromide (10 mg $mL^{-1}$) and visualized/documented on a MiniBISpro system (DNR Bio-imaging Systems, USA).

### 2.6. Nested q-PCR testing on the DNA extracted from the animal tissue fragments

Primers for the Rv2807 gene present only in the genome of members of the MTC (Table 1) were used for the nested q-PCR testing, which was performed in two steps, according to the method described by Araújo et al. (2014a, 2014b), with some modifications.

During the first step, conventional PCR was performed using 10 pmol of each primer (Applied Biosystems, USA), 10 mM dNTPs (Invitrogen, USA), 2.5 μL of 10× buffer (Sigma, USA), 1.25 U of Taq DNA polymerase (Sigma, USA), 100 ng of template DNA, in a final reaction volume of 25 μL. The PCR amplification conditions were 95 °C for 4 min, 35 cycles at 95 °C for 90 s, 65 °C for 30 s, 72 °C for 45 s with a final extension at 72 °C for 3 min (Araújo et al., 2014b).

Table 1
Target genes, primer sequences and probes of nested q-PCR for the identification of species from the M. tuberculosis complex (MTC).

| Target gene | Sequence primers 5′–3′ | Reference |
|---|---|---|
| Rv2807 | Forward outer — GGC GGT GGC GGA GTT GAA GGC GAT GAG<br>Forward internal — CAT TGC TGC GTA ATT CGA TCA<br>Probe — 6FAM CAT CCA CAC CTG TTC G MGBNFQ<br>Reverse internal — GAC CTT GGG CGC CTC AT<br>Reverse outer — GCC GCG AG GAG TCT GGG CGA TGT C | Araújo et al. (2014b) |

Target gene, GenBank, NCBI Reference Sequence ID: 888907.

The real-time PCR (q-PCR) tests were performed during the second step, using 5 pmol of each primer (Applied Biosystems, USA), 5 pmol of TaqMan probe (Applied Biosystems, USA), 6.25 μL of TaqMan PCR Master Mix (Applied Biosystems, USA), 3 μL of conventional PCR amplicon generated by the conventional PCR conducted during the first stage, in a final reaction volume of 12.5 μL. Amplification conditions of the q-PCR were 95 °C for 10 min for initial denaturation, 35 denaturation cycles at 95 °C for 15 s and annealing/extension at 62 °C for 30 s (Araújo et al., 2014a, 2014b). All reactions were performed on a StepOne Plus thermocycler (Applied Biosystems, USA), in duplicate, used template DNA from M. bovis AN5 and M. tuberculosis H37Rv as positive reaction controls.

### 2.7. Determination of the limit of detection of the m-PCR and nested q-PCR assays

The target sequences IS6110, and RvD1Rv2031c and Rv2807 used in the m-PCR and nested q-PCR assays, respectively, were cloned separately in the plasmid pGEM®-T Easy vector (Promega, USA). The recombinant plasmids obtained were propagated in Escherichia coli DH5α and purified with Wizard Plus Miniprep Kits (Promega, USA). Each plasmid DNA was quantified by Qubit® (Invitrogen, USA) and the number of DNA copies was estimated as described previously by Ke et al. (2006). Plasmid DNA was serially diluted ($10^5$; $10^4$; $10^3$, $10^2$; $10^1$ and $10^0$ copies/mL) in milli-q water, and was used to spike 1 g of lymph node tissue free of the M. bovis bacillus. Samples of lymph node tissue were spiked with the six concentrations of each plasmid DNA. DNA templates were prepared from the spiked samples using a Qiagen extraction kit (DNeasy® Blood & Tissue Kit), as described by Figueiredo et al. (2012).

## 3. Results

According to the criteria adopted in this study, of the 41,193 examined cattle carcasses, 198 (0.48%) showed BTB-suspected lesions. According to the SIF, the suspicious lesions were not classified as tuberculosis, being ruled as common or non-specific lymphadenitis. The criteria adopted for this study were for the collection of all lymphadenitis samples from the head, neck and chest cavity lymph nodes of the carcasses. The distribution and classification of the BTB-suspected lesions are described in Table 2.

M. bovis was isolated in 03 samples (03/198), or 1.5% of the 198 samples tested by conventional bacteriological methods, while the m-PCR detected, in addition to the previously mentioned 03 samples, another 11 BTB-positive samples (14/198), confirming the presence of bovine tuberculosis in 7.0% of all the examined cattle carcasses. The nested q-PCR test detected MTC in 28% (56/198) of the analyzed lesions, including the same 14 samples detected by m-PCR.

When compared to the other methods tested in the present study, the nested q-PCR assay is 18 times more effective than bacterial culture and 4 times more effective than the m-PCR assay in detecting MTC species in BTB-suspected lesions. The high efficiency of the nested q-PCR technique is due to the two PCRs performed during the method, where an initial amplification of DNA by standard PCR is conducted, followed by amplification of the amplicon by the q-PCR technique. Nested q-PCR assay showed a better performance than m-PCR since he minimum limit of detection for DNA from MTC members in this assay was 100-fold higher than that of the m-PCR assay. Nested q-PCR assay was able to quantify genomic MTC-DNA as few as 133 copies per mL of spiked bovine macerated tissue (Fig. 1). The specificity of nested q-PCR was 100%, since the negative controls were not amplified and all positive controls, including all MTC DNA templates tested were.

## 4. Discussion

The reason for studying suspicious lesions localized in the lymph nodes of the front portion of the carcass is due to reports that up to 86% of BTB lesions occur in the head or chest cavity lymph nodes of the animals (Asseged, Woldesenbet, Yimer, & Lemma, 2004; Corner et al., 1990; Whipple, Bolin, & Miller, 1996). The criterion used for sample collection was for the consideration of all lesions characterized by the SIF as tuberculosis or lymphadenitis located on the front portion of the carcass as BTB-suspicious lesions (Brazil, 1952), due to the frequency with which these lesions are involved in the disease diagnosis. We, thus, attempted to increase the chance of BTB detection in the inspected carcasses, in the case of any errors in the macroscopic evaluation and interpretation of the lesions (Asseged et al., 2004).

Table 2
Confirmed and suspected tuberculosis lesions in 41.193 cattle carcasses slaughtered in the state of Mato Grosso, Brazil.

| Animal body parts | Member and percentage of lesions | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Macroscopic examination | | Culture | | m-PCR | | Nested q-PCR | |
| Respiratory apparatus | 29 | (14.6%) | 0 | (0%) | 4 | (2%) | 6 | (3%) |
| Lung | 6 | 3 | 0 | 0 | 1 | 0.5 | 1 | 0.5 |
| Apical lymph node | 4 | 2 | 0 | 0 | 1 | 0.5 | 2 | 1 |
| Esophageal lymph node | 7 | 3.5 | 0 | 0 | 0 | 0 | 0 | 0 |
| Mediastinal lymph node | 4 | 2 | 0 | 0 | 0 | 0 | 1 | 0.5 |
| Tracheo-bronchial lymph node | 6 | 3 | 0 | 0 | 2 | 1 | 2 | 1 |
| Thoracic cavity | 2 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| Head | 22 | (11.1%) | 3 | (1.5%) | 5 | (2.5%) | 7 | (3.5%) |
| Retropharyngeal lymph node | 19 | 9.5 | 3 | 1.5 | 5 | 2.5 | 7 | 3.5 |
| Parotid lymph node | 1 | 0.5 | 0 | 0 | 0 | 0 | 0 | 0 |
| Sublingual lymph node | 2 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| Carcass | 147 | (74.2%) | 0 | (0%) | 5 | (2.5%) | 43 | (21.5%) |
| Pre-pectoral lymph node | 108 | 54.5 | 0 | 0 | 4 | 2 | 32 | 16 |
| Pre-scapular lymph node | 37 | 18.6 | 0 | 0 | 1 | 0.5 | 10 | 5 |
| Ischiatic lymph node | 2 | 1 | 0 | 0 | 0 | 0 | 1 | 0.5 |
| Total | 198 | 100 | 3 | 1.5 | 14 | 7.0 | 56 | 28 |

The identification of M. bovis and, consequently, bovine tuberculosis diagnosis, has been enhanced with the aid of molecular techniques such as PCR and its variations. The q-PCR technique, besides using specific primers, also uses a fluorescent probe that increases the specificity of the amplification of target DNA fragments, allowing for real-time visualization during each cycle, eliminating the need for amplicon fractionation by electrophoresis, simplifying and speeding up the diagnosis for result visualization (Parashar, Chauhan, Sharma, & Katoch, 2006). When comparing conventional PCR to q-PCR, several advantages are observed with regard to q-PCR, such as the quicker and more specific identification of several pathogenic species (Courtney, Kostelnik, Zeidner, & Massung, 2004; Kim et al., 2007), including mycobacteria (Lim, Kim, Lee, & Kim, 2008), allowing for the detection and quantification of different microorganisms simultaneously, besides the possibility of processing a larger number of samples at a time (Kim et al., 2007; Lim et al., 2008; Monis, Giglio, Keegan, & Andrew Thompson, 2005).

It is known that the success of q-PCR directly depends on the quality of the template DNA used, the correct design of the primers and the correct choice of target genes for the unambiguous identification of the pathogen (Zanini et al., 2001). The target gene chosen for the nested q-PCR in the present study is present only in the genome of the targeted species, referenced in the NCBI-GenBank (http://www.ncbi.nlm.nih.gov), which ensures the analytical specificity of 100% of this assay for MTC, corroborating recent results from Araújo et al. (2014b).

The nested q-PCR assay was 100 times more sensitive than the m-PCR assay, since the former was able to detect 133 copies of the target gene per mL of macerated tissue ($1.33 \times 10^2$ copies/mL), while the latter detected 13,300 copies ($1.33 \times 10^4$ copies/mL), explaining the better

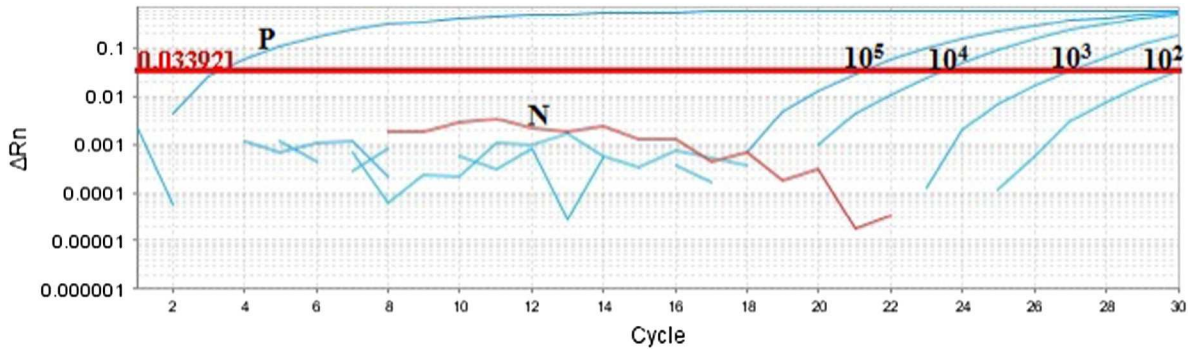


Fig. 1. Minimum detection limit of the nested q-PCR assay in artificially contaminated tissue samples. One gram of bovine lymph node tissue free of Mycobacterium bovis was spiked with $10^5$ to $10^0$ copies/mL of plasmid DNA carrying the Rv2807 sequence. P: positive control (M. bovis DNA templates); N: negative control (DNA templates from lymph node tissue free of Mycobacterium bovis); $10^5$ to $10^2$ plasmid DNA copies/mL of macerated tissue.

performance of the nested q-PCR assay in analyzing BTB-suspected lesions (Table 2). Other studies have also demonstrated that real-time PCR shows better analytical sensitivity in the detection of M. bovis in bovine tissue (Costa et al., 2013; Tacker, Harris, Palmer, & Waters, 2011).

Conventional PCR analysis and q-PCR already reported in the literature for the diagnosis of Mycobacterium spp. in cattle are not specific for pathogen identification, being restricted only to identification of members of the M. tuberculosis or Mycobacterium avium complexes (Lim et al., 2008; Shrestha et al., 2003). The definitive identification of the Mycobacterium species has been conducted only by biochemical tests after months of microbiological culture, delaying the identification of the etiologic agent in suspected cases of BTB. Williams, Ling, Jenkins, Gillespie, and McHugh (2007) standardized a q-PCR test for the identification of tuberculosis, using 194 clinical isolates. From the total isolates used in this study, 172 (89%) were identified as belonging to the M. tuberculosis complex, proving the effectiveness of the performed test, being, however unable to distinguish M. bovis from M. tuberculosis.

In the present study the nested q-PCR technique was able to amplify the Rv2807 region, specific for the MTC, in 56 of the suspected lesion samples. These 56 samples, thus confirmed as tuberculosis lesions, were from mostly pre-pectoral, pre-scapular and retropharyngeal lymph nodes (Table 2), which had previously been ruled by the SIF as "common lymphadenitis." However, the fate of the carcasses adopted by the SIF (partial condemnation of the carcass) was consistent with the "Section 196" standards of the Regulation of Industrial and Sanitary Inspection of Animal Products (Regulamento de Inspeção Industrial e Sanitária de Produtos de Origem Animal — RIISPOA) (Brazil, 1952), with regard to the presence of lesions suggestive of tuberculosis in only one part or area of the carcass.

Using the nested q-PCR technique as a method of comparison, the macroscopic post-mortem examination correctly ruled lesions in 71.7% (142/198) of the suspected BTB cases. This proves that errors do indeed occur during macroscopic lesion analyses of tuberculosis-suspected lesions in slaughterhouses, in this case, quite possibly due to the fact that sick animals are slaughtered early and with recent infection. For this reason, the use of complementary diagnostic methods and inspection personnel training are required in order to perform the final inspection. Macroscopic examinations in slaughterhouses are a crucial step in the BTB-eradication program, since, as the prevalence of the disease decreases, the identification of the remaining infected herds becomes progressively more difficult, however, even more crucial. Although there is currently no diagnosis method, ante or post-mortem, able to identify all of the tuberculosis-infected animals, detection is improved when different diagnostic methods are combined (Whipple et al., 1996).

Thus, in certain regions of Brazil and in the state of Mato Grosso, where the prevalence of this disease is very low (0.007%) (Furlanetto et al., 2012; Salazar, 2005), inspection and control programs must be alerted of the difficulties of post-mortem inspections in the macroscopic diagnosis of tuberculosis. Recommendations for the adoption of additional rapid diagnostic tests should also be made, such as nested q-PCR using template DNA obtained directly from tissue fragments presenting BTB-suspicious lesions obtained from affected lymph nodes. The implementation of effective surveillance systems adapted to local conditions may contribute to the detection of remaining BTB foci, accelerating the eradication of tuberculosis in the country.

## 5. Conclusions

The nested q-PCR test applied directly to BTB-suspicious lesions sampled from slaughterhouses was considered a sensitive, specific and quick technique, allowing for the detection of the presence of MTC species in 2 working days. The nested q-PCR assays were shown to be more sensitive than conventional m-PCR and can be considered a very robust technique, since there is low risk of contamination and no appreciable false negative or false positive results were observed.

Although the method still requires interlaboratory validation, it may be used as a complementary method in post-mortem inspections and may contribute significantly to the bovine tuberculosis control and eradication program, in disease surveillance in cattle slaughterhouses and refrigerators and in the tracking and sanitation of remaining tuberculosis foci in the country.

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