International Journal of Sciences: Basic and Applied Research (IJSBAR)

ISSN 2307-4531
(Print & Online)

http://gssrr.org/index.php?journal=JournalOfBasicAndApplied

Detection of *Mycobacterium bovis* by Multiplex Polymerase Chain Reaction in Dairy Cattle in Bogor, West Java, Indonesia

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Abstract

Zoonotic tuberculosis (TB) could be a major threat for public health, but there is no prior information of zoonotic TB incidence in cattle in Indonesia. Definitive detection of Mycobacterium bovis and Mycobacterium tuberculosis up to species level is time consuming and difficult, due to close genetic relationship and variable biochemical patterns. Current method, such as multiplex polymerase chain reaction (PCR), could be the best alternative strategy to meet this purpose.

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The aims of this study were to determine the prevalence of bovine tuberculosis (BTB), and detect Mycobacterium bovis as a causal of Tuberculosis (TB) in dairy cattle by multiplex PCR method. This research was conducted by sampling the cattle in Kawasan Usaha Peternakan/KUNAK (dairy farm region center), in Bogor District and Kebon Pedes (Bogor City). The single intradermal tuberculin test was conducted in all sampling cattle using Bovituber®PPD. Considering proportion of cattle population, the number of sampling cattle in KUNAK was 166 cattle (50 farms) and 36 cattle (10 farms) in Kebon Pedes. The cattle prevalence of BTB was 21.78% (CI 95% 16.09-27.47%), based on tuberculin test. From 44 dairy cattle which were tuberculin positive, 44 faeces samples were tested by multiplex PCR method. Mycobacterium bovis were found in 8 faeces samples. TB in cattle was found in Indonesia, and zoonotic TB should be alerted as a potential threat to public health.

Keywords: Mycobacterium bovis; Mycobacterium tuberculosis; multiplex PCR.

1. Introduction

Tuberculosis (TB) is one of the most wide-spread infectious disease all over the world, commonly caused by infection of organism which is a member of *Mycobacterium tuberculosis complex* (MTBC). MTBC is a group of pathogens that exacts devastating tolls on human morbidity and mortality, and inflicts damaging economic losses on world agriculture [1]. MTBC includes a very closely related group of mycobacteria, i.e. *M. tuberculosis, M. bovis, M. africanum, M. canetti, M. pinnipedii, M. caprae, M. microti* [2]. MTBC also includes several variants whose taxonomy is still under debate [3]. The MTBC comprises all the mycobacteria that induce TB in mammals, except *M. avium* [4].

Mycobacterium bovis is the causative agent of the vast majority of cases of TB in cattle (Bovine TB/BTB), domesticated and wild mammal species. It causes a chronic, progressive and principally respiratory disease. Infection can potentially be spread to humans via contaminated milk or meat, or directly by inhalation/aerosols from infected animals or carcasses. In the last two decades, human infection with this bacterium has accounted for a small proportion (0.5–7.2%) of all patients with a bacteriologically confirmed diagnosis of TB in industrialized countries [3, 5-6]. By contrast, in developing countries *M. bovis* infection probably still constitutes a major threat to public health [7].

Unlike *M. tuberculosis* which (in all but exceptional circumstances) only infects humans, *M. bovis* has a broad host range and is the principal agent responsible for tuberculosis in domestic and wild mammals, including cattle. Infection can potentially be spread to humans via contaminated milk or meat, or directly by inhalation of aerosols from infected animals or carcasses [3]. TB caused by *M. bovis* is clinically indistinguishable from TB caused by *M. tuberculosis.* Little is known of the relative frequency with which *M. bovis* causes nonpulmonary TB in developing nations because of limited laboratory facilities for the culture and typing of tubercle bacilli [4, 6].

More detailed data including strain characterization have recently been presented and confirm that the occurance of *M. bovis* in the human population is a persistant,though insufficiently quantified feature in developing

countries. The isolation rate of *M. bovis* from symptomatic human patients in specific studies was 13.8% in Mexico [8], 6.9% in Uganda [9], 5% in Nigeria [10], 0.5% in Taiwan [11] and between 0 and 2.5% in 10 Latin American countries. Through the use of epidemiological tools such as genetic typing of the *M. bovis* strains or case-control studies, epidemiological links between *M. bovis* infections affecting human and cattle populations have recently been demonstrated, for example, in Mexico, Uganda and Ethiopia, respectively. Care should be taken when using individual study results in the assessment of country situations as the findings may differ significantly between regions and ethnic groupings within countries as shown, e.g. in Uganda and Taiwan [11-12].

In some noteworthy studies in Tanzania and Uganda, *M. bovis* accounted for 18-30% of all *M. tuberculosis complex* strains isolated from human patients, in rural settings, whereas low prevalence rates of *M. bovis* infections were found in urban populations. There are only very few studies that have investigated the prevalence of zoonotic tuberculosis in rural communities of developing countries [13]. In developing countries, the conditions for *M. bovis* transmission to humans not only exist unchanged, but the human population has a greater vulnerability due to poverty, HIV and reduced access to health care. The exact percentage of *M. bovis* in human tuberculosis cases is often difficult to determine, since generally the diagnosis of TB is made on the basis of sputum smears only [14].

The WHO reported in 1998 that 3.1% of tuberculosis cases in humans worldwide are attributable to *M. bovis* and that 0.4-10% of sputum isolates from patients in African countries could be *M. bovis*. This is despite the fact that *M. bovis* is more often associated with extrapulmonary disease in humans [6].

Although overall the proportion of *M. bovis* causing human TB is very low compared to *M. tuberculosis*, its potential impact on population groups at the highest risk should nevertheless not be underestimated. Exposure to aerosol-borne infection with *M. bovis* from cattle remains highest in farmers, veterinary staff and rural and slaughterhouse workers, while in developing countries, ethnicity, cultural and religious practices as well as socio-economic factors have been identified as additional contributors to an increased occurrence of *M. bovis* infections in humans. Differential diagnosis should take priority in control plans in order to enable the optimal use of veterinary intervention as a means to reduce the burden of human disease from an animal source. However, appropriate methods for differential diagnosis in developing countries do not exist [13].

There is no prior information of zoonotic TB prevalence in cattle due to *M. bovis* in Indonesia. It is probably happened due to no TB explosion in cattle until this recent time. Nevertheless, it should be a concern that zoonotic TB could be a major threat for public health due to direct contact between cattle and the owner or farmer, pasteurization in milk is still not well implemented, and the absence of tuberculin test as a major part of control measures in cattle in Indonesia.

The human TB cases in Indonesia is the top four in the world in 2010. However, there is no prior information of zoonotic TB prevalence due to *M. bovis* in Indonesia. Differentiation between *M. bovis* and *M. tuberculosis* is still based on culture and biochemical methods, though these methods are very laborious, time consuming and appear to be erroneous. PCR can be used as the best alternative method for the accurate differentiation of these

mycobacterial species.

The aims of this study were to determine the prevalence of bovine tuberculosis (BTB), and detect *M. bovis* as a causal of tuberculosis (TB) in dairy cattle by multiplex PCR method.

2. Materials and Methods

2.1. Study area

The cross-sectional study was carried out between January and June 2015 on dairy farms in two regions of Bogor, West Java, Indonesia (06° 34′, 06° 37′, 06° 34′S latitude, and 106° 38′, 106° 39′, 106° 47′E longitude).

2.2. Sample size and sampling technique

Sample size was calculated using the formula for cross-sectional study [15]. Assuming level of confidence 95%, TB prevalence (p) 14.31%, based on TB prevalence in India as a result of a study conducted by the authors in [16], and the precision of the estimate (L) 5%, sample size was 196. Sampling target location of this study was two dairy farm regions center in Bogor, West Java, i.e. Kawasan Usaha Peternakan/KUNAK (dairy farm region center) which gathered in a dairy farm union (Koperasi Peternak Sapi Perah/KPS) in Kabupaten Bogor (Bogor District), and public farms in Kebon Pedes (Bogor Town). Dairy cattle in KUNAK was 2 699, and 400 in Kebon Pedes. The farms tested in this study were randomly selected, the cattle from each selected farm were also randomly selected, proportionally to the size of cattle population. The specimens examined were taken and kept in sterile conditions to avoid contamination. The Multiplex PCR was performed on samples of stool from the skin-test-positive animals.

2.3. Tuberculin testing of cattle

The tuberculin test procedure in this study was following World Organization for Animal Health (OIE) Terrestrial Manual [17]. The single intradermal tuberculin test was conducted in all sampling cattle using bovine purified protein derivates (PPD), i.e. Bovituber®PPD. A site in the center of the middle third of the cattle's neck was shaved and the skin thickness was measured with a caliper. Intradermal injection of 0.1 ml (2 000 IU) was administered. The skin-fold thickness of the injection site was re-measured 72 hours after injection. The same person conducted the entire process of tuberculin testing and reading of the result, to avoid bias related to injection and reading technique. A reaction was considered to be positive if the increase of skin thickness at the site of injection was 4 mm or more.

2.4. Mycobacterium bovis and *Mycobacterium tuberculosis* isolates

The following mycobacterial reference strains were obtained from the collections of the Indonesian Research Institute for Veterinary Science (BBLITVET): *M. bovis* (Pasar Minggu strains, isolated by BBLITVET from dairy cattle), *M. tuberculosis* (BBLITVET Culture Collection).

2.5. Isolation DNA of MTBC from stool

Weigh 250 mg stool in a 2 ml microcentrifuge tube. Add 1.4 ml buffer ASL, vortex continuously for 1 min until the stool sample is thoroughly homogenized. Heat the suspension for 10 min at 90°C. Vortex for 15 sec and centrifuge sample at 14 000 rpm for 1 min to pellet stool particles. Pipet 1.2 ml of the supernatant into a new 2 ml microcentrifuge tube. Add 1 InhibitEX tablet and vortex until the tablet is completely suspended. Incubate suspension for 1 min at room temperature. Centrifuge sample at 14 000 rpm for 3 min. Pipet all the supernatant into a new 2.0 ml microcentrifuge tube. Centrifuge the sample at 14 000 rpm for 3 min. Pipet 200 µl supernatant into the 1.5 ml microcentrifuge tube containing 15 µl proteinase K. Add 200 µl buffer AL and vortex for 15 seconds. Incubate at 70°C for 10 min. Add 200 µl of ethanol 96% to the lysate, and mix by vortexing. Pipet 550 µl of the suspension into QIAamp spin column placed in a collection tube. Centrifuge at 14 000 rpm for 1 min. Place the QIAamp spin column in a new collection tube, and discard the tube containing the filtrate. Add 500 µl buffer AW1, centrifuge at 14 000 rpm for 1 min. Place the QIAamp spin column in a new collection tube, and discard the collection tube containing the filtrate. Add 500 µl buffer AW2, and centrifuge at 14 000 rpm for 3 min. Discard the collection tube containing the filtrate, place the QIAamp spin column in 1.5 ml microcentrifuge tube. Pipet 200 µl buffer AE directly onto the QIAamp membrane. Incubate for 1 min at room temperature, then centrifuge at 14 000 rpm for 1 min. Keep the eluate at –20°C.

2.6. DNA amplification using primer CSB1, CSB2, CSB3

Multiplex PCR method was applied to identify *M. bovis* and *M. tuberculosis* on the same time. Oligonucleotide sequences of the primers used in this study were: the common forward primer, CSB1 (5'-TTCCGAATCCCTTGTGA-3'), and two reverse primers, including *M. bovis*-specific, CSB2 (5'-GGAGAGCGCCGTTGTA-3'), and *M. tuberculosis*-specific, CSB3 (5'-AGTCGCGTGGCTTCTCTTTTA-3') [18].

The PCR reactions were performed in a total volume of 20 µl consisting of the following: 11.1 µl of PCR-grade water (DNAse, RNAse free), 4.0 µl of 5X KAPA2G buffer (KAPA Biosystem, USA), 0.4 µl of dNTP (KAPA Biosystem, USA), 0.8 µl of each primer (XIDT, 10 pmol), 0.1 µl of KAPA2G Fast DNA Polymerase (KAPA Biosystem, 5 U/µl) and 2 µl of template DNA. Positive and negative control should be included in every amplification.

The cycling parameters were: initial denaturation at 95 °C for 3 min, followed by 35 three-step cycles, including denaturation at 95 °C for 15 sec, annealing at 55 °C for 15 sec, and extension at 72 °C for 15 sec, and a final extension at 72 °C for 5 min. The amplification products were analyzed by electrophoresis on 2% (w/v) agarose gel and visualized by ethidium bromide fluorescence. The unique amplification product should be at 161 bp (*M. bovis*-specific) or 261 bp (*M. tuberculosis*-specific).

Each amplification product (10 µl) was mixed with 2 µl *loading dye* (Vivantis). The amplification products were then analyzed by electrophoresis on 2% (w/v) agarose gel (Promega) and visualized by 0.5 µg/ml ethidium bromide (Applichem) fluorescence, run on 120 V for 50 min, using DNA ladder 100 bp (Vivantis). The

electrophoresis result then visualized by UV-transilluminator.

2.7. Statistical analysis

The prevalence of TB in dairy cattle with level of confidence 95% was assumed using formula as shown below:

$$\hat{p} \pm \mathcal{Z}_{0.025} \sqrt{\frac{\hat{p}(1-\hat{p})}{\mathrm{n}}}$$

$\hat{p}$: predictive prevalence (x/n)

x : number of cattle that TB positive

n : sample size

$\mathcal{Z}_{0.025}$: 1.96

The results of Multiplex PCR method were analyzed descriptively.

3. Results

The cross-sectional study was carried out on dairy farms in two regions of Bogor, West Java, Indonesia (06° 34′, 06° 37′, 06° 34′S latitude, and 106° 38′, 106° 39′, 106° 47′E longitude). This study was conducted by sampling the cattle in KUNAK (Bogor District) and Kebon Pedes (Bogor Town).

Considering proportionality of cattle population, the number of sampling cattle in KUNAK was 166 cattle (50 farms) and 36 cattle (10 farms) in Kebon Pedes. The number of cattle from each selected farm were selected proportionally, based on the size of cattle population. All sampling cattle were tested using tuberculin test.

This study reported a representative estimate of BTB prevalence in Bogor, West Java, Indonesia. This study was an initial epidemiology research of TB in cattle in Indonesia, since there was no such prior research conducted in Indonesia. From 202 sampling cattle, 44 cattle were tuberculin positive. The BTB prevalence in dairy cattle obtained in this study was 21.78% (CI 95% 16.09-27.47%).

This prevalence was higher than TB prevalence in dairy cattle in India as a result of a study conducted by the authors in [16]. Study of BTB in India revealed that BTB prevalence at animal level was 14.31%, while BTB prevalence at farm level was 16.67%. Other cross-sectional study of TB conducted by the authors in [19] in dairy cattle in Asmara, Eritrea, Africa, resulted prevalence of positive tuberculin test similar to the result from India (14.5%).

Tuberculin test was used to determine TB cases in this study, considering that OIE recommend this test as a standard method to detect BTB in international cattle trade [17]. Faeces sample was selected in this study in

consideration of the authors in [7] statement, that infectious animals may shed *M. bovis* in a number of ways: in faeces, milk, discharging lesions, saliva and urine. From 202 sampling cattle, 44 cattle were tuberculin positive, from 26 farms. The Multiplex PCR was performed on samples of stool from the 44 skin-test-positive animals.

PCR is being used for distinguishing species of the MTBC, i.e. *M. tuberculosis* from *M. bovis* [20-21] simultaneously. These methods are particularly important when isolates of the MTBC are unlikely to be subjected to further DNA characterisation that can reveal differences both between and within species. As the range of mycobacterial sources being investigated expands, particularly in those countries where previous studies have not been done, it is becoming apparent that there are occasional situations in which animals that appear clinically affected by *M. bovis* are actually infected by *M.*

tuberculosis and, not surprisingly, this is associated with human disease [22-23]. While distinction between these tuberculosis species is important because they raise different epidemiological considerations in terms of animal and human disease. This was rarely carried out because of the long time required for cultural and phenotypic tests.

The Multiplex PCR assay was applied to DNA from 44 stool samples from tuberculin positive animals. Using one common forward primer, CSB1, and two reverse primers: CSB2 (*M. bovis*-specific), and CSB3 (*M. tuberculosis*-specific), 8 samples showed amplicon of 161 bp (Figure 1 and 2).

Sample was deemed positive when it showed the unique amplification product, i.e. at 161 bp (*M. bovis*-specific) or 261 bp (*M. tuberculosis*-specific).

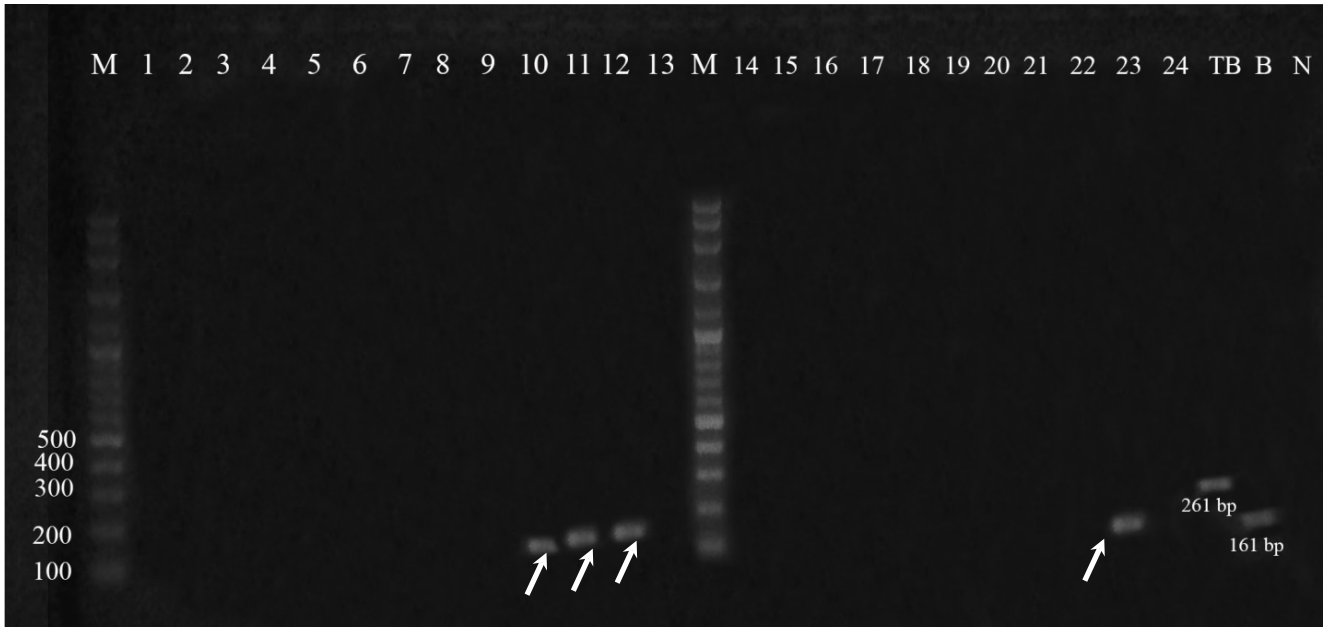

Figure 1: Ethidium bromide-stained 2% (w/v) agarose gel showing PCR products amplified from stool samples from tuberculin positive animals. Lanes: (M) DNA molecular mass marker (100 bp ladder); (1-24) Samples; (TB) positive control of *M. tuberculosis*; (B) positive control of *M. bovis*; (10, 11, 12, 23) Samples that showed amplicon of 161 bp (*M. bovis*-specific); (N) negative control.

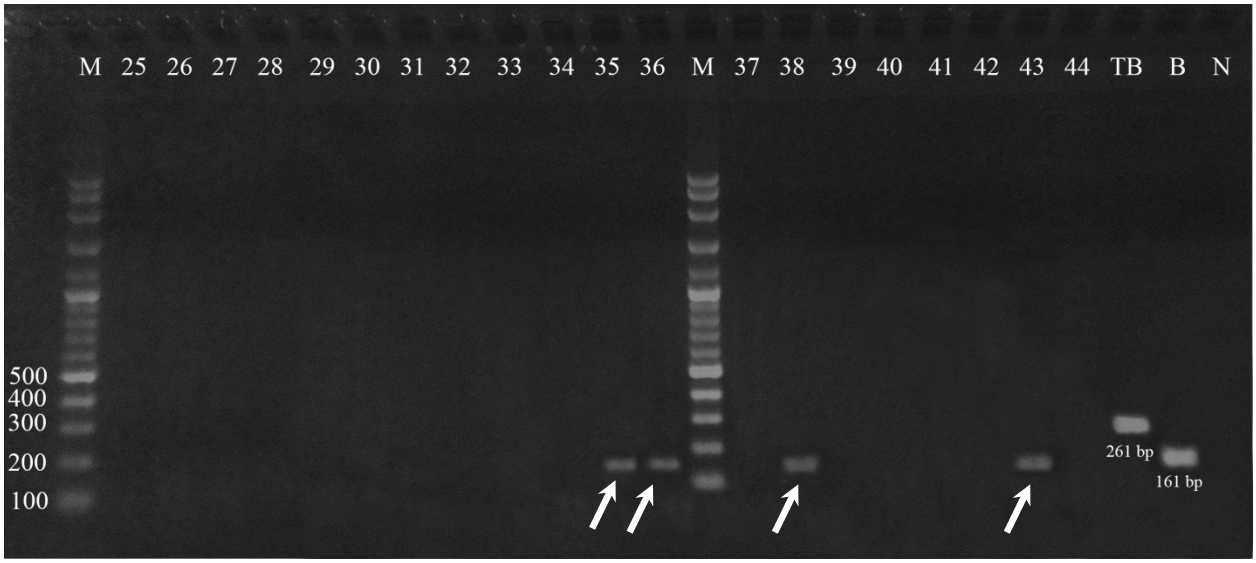

Figure 2 : Ethidium bromide-stained 2% (w/v) agarose gel showing PCR products amplified from stool samples from tuberculin positive animals. Lanes: (M) DNA molecular mass marker (100 bp ladder); (25-44) Samples; (TB) positive control of *M. tuberculosis*; (B) positive control of *M. bovis*; (35, 36, 38, 43) Samples that showed amplicon of 161 bp (*M. bovis*-specific); (N) negative control.

The results of this study showed PCR amplification products on sheer amplicon of 161 bp (*M. bovis*-specific). They were uncomparable with the dense amplicon of positive control of *M. bovis* and *M. tuberculosis*. The sheer amplicon of the positive samples were probably due to the difficulties on purifying DNA from stool sample extraction, the low total numbers of bacilli in the samples, the bacteria were not in shedding stage, or the BTB incidences were occurred long time before sampling.

The results were concordance with Hughes [24], who stated that in mycobacteriology, for obvious reasons, molecular technologies have been applied primarily to enhance detection and typing of *M. tuberculosis*. More recently, however, through international collaborative efforts, attention has been directed at *M. bovis* as a significant animal pathogen with zoonotic potential. The intracellular nature and impermeability of mycobacterial cell walls, together with the presence of polymerase chain reaction (PCR) inhibitors in clinical specimens, limit the efficiency of PCR detection. This can be problematic and impact on the potential uses of PCR detection with certain clinical specimens and in particular with specimens taken from tuberculous cattle, where low numbers of bacilli are common.

This study revealed the presence of TB in cattle in Indonesia. Therefore, zoonotic TB should be alerted as a potential threat to public health. As stated by Michel and his colleagues [13], population groups at the highest risk to *M. bovis* infection from cattle are farmers, veterinary staff, rural and slaughterhouse workers. Socio-economic factor has been identified as additional contributors to an increased occurrence of *M. bovis* infections in humans. It is important to conduct preventive and control measures for TB control, such as conducting tuberculin test program for dairy cattle, implementing a good practice of pasteurization in milk, and dissemination to the public about the importance of zoonotic threat of TB. Control measure should be intensified toward imported cattle from BTB endemic countries.

4. Conclusion

The cattle prevalence of BTB in Bogor, West Java, Indonesia was 21.78% (CI 95% 16.09-27.47%), based on tuberculin test. *M. bovis* were found in 8 faeces samples. TB in cattle was found in Indonesia, and zoonotic TB should be alerted as a potential threat to public health.

It is important to conduct preventive and control measures to control TB, such as conducting tuberculin test program for dairy cattle, applying a good practice of pasteurization in milk, and dissemination to the public about the importance of zoonotic threat of TB. For location with BTB higher risk, it is important to attempt improvement of farm management and environment modification.

Acknowledgements

The authors wish to thank the Indonesia Agricultural Quarantine Agency for funding this research; Dissemination and Agricultural Human Resource Development Agency for funding scholarship for first author's postgraduate program.

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