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ORIGINAL ARTICLE

Verification of Bact/Alert 3D System Results for Detection of Mycobacterium Tuberculosis

[1]Safaa Amer, [1]Azza El Hefnawy, [2]Mahmoud Mahmoud, [1]Hadir Okasha, [1]Mayada Gad*

[1]Department of Medical Microbiology and Immunology, Faculty of Medicine, Alexandria University, Egypt
[2]Department of Chest Diseases, Faculty of Medicine, Alexandria University, Egypt

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ABSTRACT

Background: *The rapid diagnosis of mycobacterial infections is essential to implement the adequate antimicrobial therapy.* ***Objective****: To verify BacT/ALERT 3D system results by PCR and evaluate its performance against Löwenstein Jensen medium as regards the recovery rate, the time to detection, and contamination rate.* ***Methodology:*** *Sixty sputum specimens were inoculated in BacT/ALERT MP bottles and on LJ slants. Ziehl-Neelsen [ZN], subculture and PCR was done to confirm the positive signal MP bottles.* ***Results****: Thirty two [53.3%] mycobacterial isolates [31 M. tuberculosis and one non-tuberculous mycobacterium] were detected. The recovery rate of 32 mycobacterial isolates for the BacT/ALERT 3D system was 100% and that of LJ medium was 71.9%. Of the 32 signal positive / PCR positive bottles, 27[84.4%] bottles were ZN smear positive [from the bottle] and 5[15.6%] were ZN negative. The mean times to detection of mycobacteria by BacT/ALERT 3D system and LJ medium were 14.2 and 24.3 days, respectively, while overall contamination rates were 6.7% and 8.3%, respectively.* ***Conclusion:*** *Sensitivity and time to detection were significantly better with BacT/ALERT 3D system than with solid LJ medium. The PCR assay allows the fast and exact identification of Mycobacterium tuberculosis directly from positive liquid medium. LJ culture still plays an important role in isolation of mycobacteria from clinical samples, it provide visible colonies that allow identification and susceptibility testing of the isolate. Signal positive /ZN negative bottles should be confirmed by other methods [PCR and/or subculture].*

INTRODUCTION

Tuberculosis is one of the leading infectious causes of morbidity and mortality worldwide, causing 1.5 million deaths and 9 million new cases annually.[1,2] The WHO recently set the End TB Strategy to eradicate TB globally, with accurate and rapid detection of TB and drug resistance as critical components of this strategy. [3,4] Although several new diagnostics are now available for TB, sputum smear microscopy continues to be widely used, despite its limited sensitivity.[5] In contrast to culture has high sensitivity but takes weeks or months to yield results.[6,7]

Since the introduction of automated liquid culture systems, the time required for both the detection and identification of mycobacteria has not only been significantly shortened, but also it increased the overall mycobacterial recovery, however unconfirmed signals hinder the release of result. Thus the application of molecular techniques directly on positive signal liquid cultures for the confirmation of mycobacterial isolates presence and its identification will lead to faster and more accurate diagnosis of tuberculosis.[8]

The aim of this study was to evaluate the performance of the BacT/ALERT 3D system [Biomerieux, France] in comparison to LJ medium and verify the BacT/ALERT results by PCR to prove the specificity of the method.

METHODOLOGY

A total of 60 sputum samples obtained from 60 patients clinically and radiologically suspected as pulmonary tuberculosis, from Alexandria Main University Hospital and El Maamora Chest Hospital, were included in this study after approval of the ethics committee of Alexandria Faculty of Medicine and obtaining patients consent.

1. Smear preparation

A direct smear from each sputum sample collected was stained by Ziehl-Neelsen [ZN] technique and examined microscopically for acid fast bacilli [AFB]. The results were reported quantitatively according to the recommendations of the International Union Against Tuberculosis and Lung Disease.[9]

Corresponding Author:
Hadir Ahmed Said Okasha
Lecturer of Medical Microbiology and Immunology, Faculty of Medicine, Alexandria University, Egypt
E-mail: Hadir.okasha@alexmed.edu.eg; Tel.: +2 03 4862506

2. Specimen processing

Sputum specimens were digested and decontaminated with equal volumes of N-acetyl-L-cysteine [NALC] - Sodium Hydroxide [NaOH] [final concentration 1%] solution according to the standard decontamination procedures.[10] After neutralization with phosphate buffered saline [0.067 M, pH 6.8] and centrifugation at 3000 g for 15 minutes, the pellet was suspended in phosphate-buffered saline to a final volume of 2 ml, and cultured on BacT/ALERT MP bottles and LJ media.

3. Media and inoculation

BacT/ALERT MP culture bottles contain 10 ml of modified Middlebrook 7H9 broth enriched with casein, bovine serum albumin, and catalase were used, each bottle was supplemented with 0.5 ml of MB/BacT antibiotic supplement [amphotericin B, azlocillin, nalidixic acid, polymyxin B, trimethoprim, and vancomycin] according to the manufacturer's instructions to reduce the incidence of contamination from other bacteria. A volume of 0.5 ml of the digested decontaminated specimen was inoculated into the bottles. In parallel, 0.25 ml of each processed specimen was inoculated onto 2 LJ slants; one tube was plain LJ and the other tube containing p-Nitrobenzoic acid PNBA [500mg/L] for detection of atypical mycobacteria. All culture media were incubated at 37°C. Liquid cultures automatically monitored by the BacT/ALERT 3D instrument [every 10 min] were incubated for 42 days, while solid ones were discarded after 56 incubation days. LJ slants were visually inspected once a week for mycobacterial growth, and ZN smears from suspect colonies were made. Positive signal Liquid culture bottles were considered positive only when the growth of mycobacteria was verified by microscopy [ZN method] and or by PCR. Contaminants were detected by subculture on blood agar plates. Also subculture on LJ media was made from all positive signal bottles.

4. Polymerase Chain Reaction [PCR]

PCR was done for confirmation of the positive signal BacT/ALERT MP culture bottles and for further mycobacterial species identification. ATCC 25177 standard strain of *M. tuberculosis* was included as a positive control and a tube without DNA was used as negative control. DNA of mycobacterial species was extracted from the positive signal BacT/ALERT MP culture bottles by boiling method.[11] One milliliter of mycobacterial culture was heated with 60 µl Sodium dodecyl sulfate [SDS] at 100°C for 30 minutes and the supernatant obtained following centrifugation at 12,000g for 15 minutes was stored at -20°C until use.

A multiplex PCR was performed for 65-kDa antigen gene which is specific for the genus mycobacterium and IS6110 insertion sequence which identifies *M. tuberculosis* Complex [MTC]. The primers for the 65-kDa antigen encoding gene were: TB11[5'ACCAACGATGGT GTGTCCAT3'] and TB12[5'CTTGTCGAAC CGCATAC CCT3'], which resulted in 439 bp DNA fragment. The amplification of the IS6110 insertion element was performed with the primers TB284 [5'GGACAACGCCGAATTGCG3'] and TB850 [5'TAGGCGTCGGTGACAA AGGCCA C3'], which amplified a 550 bp DNA fragment. (figure 1). All of the reactions were performed in a final volume of 50 µL containing 8 µl of DNA extract, 25 µl of Maxima Hot start PCR Master Mix [2X], 20 pM of each primer. The reaction was carried out in a DNA Thermal cycler [Progene Techne, England] with an initial denaturation at 95°C for 3 min followed by 30 cycles of denaturation at 95°C for 20 s, annealing at 65°C for 30 s, and an extension at 72°C for 30 s and a final extension at 72°C for 7 min.[12]

For *Mycobacterium tuberculosis* species identification, a conventional PCR was done to amplify the mtp40 gene, which is *M. tuberculosis* species specific, using the primers: PT1 [5'CGGCAACGCGCCGTCGGTGG] and PT2 [5'CCCCCCACGGCACCGCCGGG] which result in a 396 bp DNA fragment.(figure2). The reaction was performed in a final volume of 25 µL containing 3 µl of DNA extract, 12.5 µl of Maxima Hot start PCR Master Mix [2X], and 20 pM of each primer. The reaction was carried out in a DNA Thermal cycler [Progene Techne, England] under the following conditions: an initial denaturation at 94 ºC for 5 min followed by 35 cycles of denaturation at 94 ºC for a min, annealing at 71 ºC for 2 min, and an extension at 72 ºC for 3 min and a final extension at 72 ºC for 10 min.[13]

After the amplification, 5µl of the PCR products were analyzed by 1.5% agarose gel electrophoresis in 1X TAE buffer [40 mM Tris-acetate, 1mM EDTA]. The separated DNA segments were stained with ethidium bromide and visualized on a UV-light transilluminator.

Statistical analysis

A sample was considered true negative when no mycobacterial growth took place in any of the two culture methods. A sample was considered true positive when the presence of AFB was confirmed in at least one of the culture methods. The false positive rate for BacT/ALERT 3D system was calculated as the number of bottles that were instrument-positive but after ZN smear and PCR were found to be ZN-negative and PCR-negative for mycobacteria. The finding of bacterial growth on LJ medium with no presence of AFB by ZN staining was considered to be due to bacterial contamination and a negative result for the presence of mycobacteria. This was considered a true negative when no growth was observed in BacT/ALERT and a false negative if mycobacteria were isolated by BacT/ALERT 3D system. A combination of results of solid and liquid media was regarded as the "gold standard" to measure sensitivity, specificity, and predictive values.[14]

Data were fed to the computer and analyzed using IBM SPSS software package version 20.0. Qualitative data were described using number and percent. Quantitative data were described using range [minimum and maximum], mean, standard deviation and median. The significance of differences in recovery and contamination rates was determined by Fisher's Exact correction for the chi-square test. The comparison of isolation times was performed by the Student t test. A. P value ≤0.05 was considered significant.

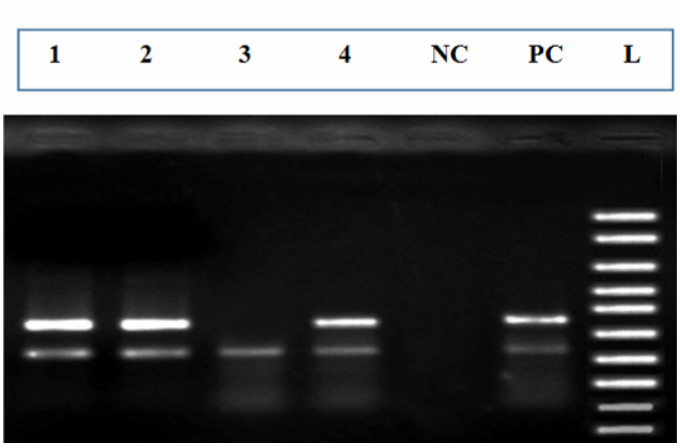

Fig. (1): Multiplex PCR-based detection of *M. tuberculosis* complex targeting 65-kDa antigen gene and IS6110. Lanes 1, 2,4 show 2 bands of 439 bp and 550 bp in the indicated the presence of the two target genes (MTC), while in Lane 3 shows the presence of 65-kDa antigen gene only (atypical mycobacterial strain). NC (negative control) PC (positive control). Ladder 100 bp was used in Lane L.

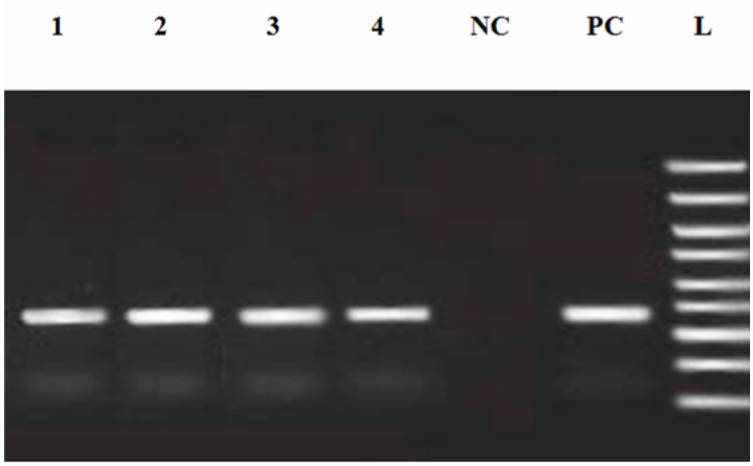

Fig. (2): Conventional PCR-based detection of *M. tuberculosis* species targeting mtp40 gene. The presence of a 396 bp amplicon in the Lanes 1,2,3,4 indicated the presence of the target. Lane NC was negative control and Lane PC was positive control. Ladder 100 bp was used in Lane L

RESULTS

Sixty sputum specimens were examined microscopically, of which 21[35%] were smear positive and 39[65%] were smear negative. Out of the 21 smear positive sputum specimens, 5[8.3%] specimens were grade 1[+] on smear microscopy, while 7[11.7%] were grade 2[+] and the remaining 9[15%] were grade 3[+].

From a total of 60 sputum specimens, mycobacteria were identified in 32 cultures [53.3%]. Of these 32 cultures, 31 [96.9%] grew *M. tuberculosis* and one culture [3.1%] grew non tuberculous mycobacterium [NTM]. Table 1 summarizes the recovery rate of mycobacteria by each culture system. The recovery rate of 32 mycobacterial isolates for the BacT/ALERT 3D system was significantly higher [100%] than that of LJ medium [71.9%] [^{FE} *P*=0.002]. Out of the 31 *M. tuberculosis* isolates recovered by both methods combined, the recovery rate for the BacT/ALERT 3D system was significantly higher [100%] than that of LJ medium [70.96%] [^{FE} *P* = 0.002]. Both BacT/ALERT 3D system and LJ medium recovered the NTM isolate. The BacT/ALERT 3D system in conjunction with LJ medium did not recover more isolates than the

BacT/ALERT 3D system alone. LJ medium failed to recover mycobacteria from 9 clinical specimens; five of these specimens were smear-negative and four specimens were smear-positive. In addition, non-mycobacterial contamination on LJ medium was observed in two of these smear-positive specimens.

Table 1: Rates of recovery of mycobacteria from sputum specimens by BacT/ALERT 3D system and LJ medium:

Mycobacteria [no. of isolates]	*Number [%] of isolates recovered by:*		*FE P value*
	BacT/ALERT 3D system	**LJ medium**	
***M. tuberculosis* [31]**	31 [100%]	22 [70.96%]	0.002*
NTM [1]	1 [100%]	1 [100%]	-
All Mycobacteria [32]	32 [100%]	23 [71.9%]	0.002*

FE: Fisher Exact for chi square test
*: Statistically significant at $p \leq 0.05$

Table 2 summarizes the recovery rates of mycobacteria by both culture methods according to the initial smear result. The recovery rate of mycobacteria in smear positive specimens was higher by BacT/ALERT 3D system [100%] than by LJ medium [80.95%]. This difference was not statistically significant [FE *P*=0.107]. While in smear-negative specimens, the recovery rate of mycobacteria was significantly higher by BacT/ALERT 3D system [100%] than by LJ medium [54.5%] [FE *P*= 0.035].

Table 2: Rates of recovery of mycobacteria from sputum specimens by BacT/ALERT 3D system and LJ medium:

ZN smear result [no. of isolates]	*Number [%] of isolates recovered by:*		*FE P*
	BacT/ALERT 3D system	**LJ medium**	
Smear positive [21]	21 [100%]	17 [80.95%]	0.107
Smear Negative [11]	11 [100%]	6 [54.5%]	0.035*
Total isolates [32]	32 [100%]	23 [71.9%]	0.002*

FE: Fisher Exact for chi square test
*: Statistically significant at $p \leq 0.05$

The presence of *Mycobacterium tuberculosis* in positive signal BacT/ALERT MP bottles was confirmed by ZN staining and by PCR. Multiplex and a conventional PCR were done on culture medium of MP bottles. The multiplex PCR was able to detect atypical mycobacteria and mycobacteria belonging to the *M. tuberculosis* complex followed by conventional PCR to confirm *Mycobacterium tuberculosis* species. The use of both amplifications offers an advantage when compared with the acid fast ZN staining, since this method is only able to detect AFB.

On BacT/ALERT incubation of the inoculated 60 MP bottles, 36[60%] MP bottles gave positive signals via visual and audible alerts, while 24[40%] bottles were negative. The 36 positive signal bottles were unloaded from the BacT/ALERT 3D system and the presence of mycobacteria in MP bottles was confirmed by ZN staining and by PCR. Out of the 36 positive signal bottles, 32[88.9%] were positive by PCR and LJ subculture, while 4[11.1%] were PCR negative [False positive by BacT/ALERT 3D system]. Of the 32[88.9%] signal positive / PCR positive bottles, 27[84.4%] bottles were ZN smear positive [from the bottle] and 5[15.6%] were ZN negative (false negative).

Table 3 shows sensitivity, specificity, and positive and negative predictive values of ZN smear, LJ culture and BacT ALERT 3D system for diagnosis of pulmonary mycobacterial infection. BacT/ALERT 3D system had the highest sensitivity in detecting pulmonary mycobacterial infection [100%], followed by LJ culture [71.9%] then direct ZN smear [65.6%]. Regarding specificity, both direct ZN smear and LJ culture showed the highest specificity [100%], followed by BacT/ALERT 3D system [85.7%].

Table 3: Evaluation of direct ZN smear, LJ culture and BacT ALERT 3D system for diagnosis of pulmonary mycobacterial infection:

Diagnostic method	*Sensitivity*	*Specificity*	*PPV*	*NPV*	*Overall accuracy*
Direct ZN smear	65.6%	100%	100%	71.8%	81.7%
LJ culture	71.9%	100%	100%	75.7%	85%
BacT/ALERT 3D system	100%	85.7%	88.9%	100%	93.3%

Note: PPV= Positive predictive value, **NPV**= negative predictive value

The growth detection times [mean days, standard deviation, range and median] for mycobacteria in BacT/ALERT 3D System and LJ medium are summarized in table 4. Growth detection times for mycobacteria were significantly shorter in BacT/ALERT 3D System than in LJ medium culture [*P* <0.001]. The overall mean time to detection of mycobacteria was 14.2 days for BacT/ALERT 3D system and 24.3 days for LJ medium.

According to ZN smear results, time required for the recovery of mycobacteria in both smear positive and smear negative specimens was significantly shorter in BacT/ALERT 3D System than in LJ medium [*P* <0.001 in smear positive specimens, < 0.017 in smear negative specimens]. In smear positive specimens, the mean time for detection of mycobacteria was 9.8 days for BacT/ALERT 3D system and 20.3 days for LJ medium. While in smear negative specimens, the mean time for detection of mycobacteria was 22.5 days for BacT/ALERT 3D system and 35.8 days for LJ medium.

Table 4.Time [days] to detection of mycobacteria in sputum specimens by BacT/ALERT 3D system and LJ medium:

ZN smear result	*BacT/ALERT 3D system*			*LJ medium*			*P value*
	Mean ± SD	**Range**	**Median**	**Mean ± SD**	**Range**	**Median**	
Smear positive	9.8 ± 5.08	2-20	11	20.3 ± 4.50	13-28	20	<0.001*
Smear Negative	22.5 ± 2.05	16-32	21	35.8 ± 4.50	26-49	33	<0.017*
All isolates	14.2 ± 7.93	2-32	13.5	24.3 ± 9.16	13-49	23	<0.001*

*: Statistically significant at $p \leq 0.05$

The contamination rate for LJ medium was higher [8.3 %] than that of BacT/ALERT 3D system [6.7%]. The difference was not statistically significant [*P= 1.000]*. Out of the 60 inoculated BacT/ALERT MP bottles, false alarm due to non mycobacterial overgrowth occurred in 4[6.7%] bottles. The contaminants were gram positive organisms that were detected by subculture on blood agar plates. 5[8.3%] of the 60 inoculated LJ slants were contaminated, the slants showed growth of non mycobacterial colonies that were negative by ZN staining for AFB.

DISCUSSION

Early diagnosis of tuberculosis is critical in the control of the disease. The BacT/ALERT 3D system is not radiometric and totally automated system for mycobacteria culture. This instrument was approved by the Food and Drug Administration in1996.[15]

Actually the recovery rate of mycobacteria increased when the liquid-medium methods were combined with the Löwenstein Jensen solid medium as recommended by Center for Disease Control for recovery of all mycobacteria. This combination is currently regarded as the "gold standard" for primary isolation of mycobacteria.[14,16]

In our study, the grading of the ZN stained smears was done according to the recommendations of the International Union Against Tuberculosis and Lung Disease. Where out of the 21 smear positive sputum specimens, 5 specimens were grade 1[+] on smear microscopy, while 7 were grade 2[+] and the remaining 9were grade 3[+]. Our findings were in accordance with those of Farnia P et al.[17] The grading of the sputum smears gives an idea about the bacterial load in sputum specimens. It depends upon different factors such as the time of collection, the nature of the samples, and the treatment with antituberculous drugs and its duration. Most of our sputum specimens were collected from patients prior to initiation of therapy. So more than 42% of the 21 smear positive specimens were grade 3[+]. On contrast to other studies [18,19] carried out on patients under treatment for variable time periods, reducing the bacterial load in the sputum and giving lower grades on microscopic examination.

In this study direct ZN smear had the lowest sensitivity [65.6%] in detecting pulmonary mycobacterial infection. Similar sensitivity values for direct ZN smear were reported by other authors.[20,21] Direct sputum smear is not a very sensitive tool in the diagnosis of pulmonary TB. One of the reasons for low sensitivity is reported to be due to the fact that a bacilli count of 10^4/ml are required for AFB to be seen using smear microscopy.[22]

The recovery rate of mycobacteria from sputum samples was significantly higher for BacT/ALERT 3D system than that of LJ medium, similar results were reported by other authors.[23-25] In our study both culture methods detected mycobacteria with no significant differences in smear-positive specimens. While in smear-negative specimens, the recovery rate of mycobacteria was significantly higher in BacT/ALERT 3D system than in LJ medium. This can be attributed to the reconstitution fluid that contains components necessary to ensure optimal growth of mycobacteria present in the sputum samples, increasing the capability to recover mycobacteria from tuberculous patients under treatment.

One of the disadvantages of culture in liquid medium is that it does not provide visible colonies

increasing the time required for confirmation of the result. However, the direct testing of positive BacT/ALERT MP broth medium by PCR allows for the accurate and rapid identification of *M. tuberculosis*. especially that ZN staining from the bottle failed to confirm the positive signal in 15.6% of positive samples. Martinez MR et al.[15] and Sorlozano et al[14] stated that if ZN staining form the positive signal bottles did not reveal AFB ,subculture on solid media and re-incubation of the bottles for a further 4 weeks after which ZN is repeated to confirm the results. In our study the application of PCR directly on positive signal bottles provided fast and accurate confirmation of BacT/ALERT results.

The BacT/ALERT 3D system in conjunction with solid media has been reported to yield additional 1.3% to 6.4% of mycobacterial isolates as compared to the the BacT/ALERT 3D system used alone.[26] However, in our study the BacT/ALERT 3D system in conjunction with LJ medium did not recover more isolates than the BacT/ALERT 3D system alone. Similar result was reported by Yan et al.[26] However, in other studies the recovery rate for BacT/ALERT 3D system plus LJ medium was higher than for BacT/ALERT 3D system alone.[23,27]

From the infection control point of view, the speed of mycobacterial detection in clinical specimens is very critical. In our study the time to detection of mycobacteria was significantly shorter in BacT/ALERT 3D System than in LJ medium, similar results obtained by other authors.[14,23-25, 28]

In our study the contamination rate for LJ medium was higher than that of BacT/ALERT 3D system but the difference was not statistically significant. This is also comparable with other studies that reported a higher contamination rate for LJ medium. [13-15,26,28] Many of our sputum samples were obtained from patients with chronic illnesses, including chronic obstructive pulmonary diseases and bronchiectasis who have been hospitalized for long periods of times. These patients had received multiple antibiotics previously, leading to colonization of multi-drug resistant bacteria, which often caused liquefaction and contamination of solid media. Furthermore, the liquid media used in this study were supplemented with antibiotics while the solid media were not. Gram-positive organisms were the prevalent contaminants in BacT/ALERT MP bottles even after the antibiotic supplement was revised by adding vancomycin. The additives in the reconstitution fluid of the BacT/ALERT culture medium likely enhance not only the growth of mycobacteria but also that of Gram-positive contaminants.

CONCLUSIONS

As a conclusion, BacT/ALERT 3D system was proven to be more efficient than LJ medium in isolation of mycobacteria from sputum specimens. With a shorter time to detection providing a faster initiation of treatment, especially those with AFB smear negative specimens. In addition, the application of PCR assay directly on positive liquid media of automated systems allows confirmation of the results and fast identification of *M. tuberculosis* especially that ZN staining from the bottle failed to confirm the positive signal in 15.6% of positive samples.

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