Jpn. J. Infect. Dis., 72, 19–22, 2019

Original Article

Selective Detection of DNA from Viable *Mycobacterium tuberculosis* Complex Strains Using the EMA-PCR Method

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SUMMARY: In this study, the ability to discriminate viable from dead cells of *Mycobacterium tuberculosis* complex (MTC), using an ethidium monoazide (EMA) treatment-dependent viable bacteria selection PCR kit was examined. Detection of dead bacteria was possible for bacterial concentrations in the range 1.5×10^7–3.0×10^7 CFU/mL, which was equivalent to McFarland No. 0.05–0.10. There was a significant difference between the results for viable and dead bacteria, and the sensitivity and specificity of this method for culture-negative samples from patients were 83% and 100%, respectively. To the best of our knowledge, this is the first successful selective detection of DNA from viable cells of MTC by EMA-PCR, using the viable bacteria selection kit for PCR (gram-positive), an EMA treatment kit. We believe that application of this method could promote earlier discharge of patients undergoing tuberculosis treatment by discriminating dead from viable cells.

INTRODUCTION

Mycobacterium tuberculosis (MTB) causes a systemic, mainly respiratory, infectious disease in humans. Definitive diagnosis of the infection typically requires isolation and identification of the MTB complex (MTC); however, these strains grow slowly, making rapid diagnosis difficult (1,2). A rapid, highly sensitive, and specific diagnostic method is required for effective control of the tuberculosis infection. Currently, the main test method that fulfils these requirements is the nucleic acid amplification test (NAT). However, it cannot discriminate viable from dead cells (2). To resolve this problem, the efficacy of discrimination of viable from dead bacteria using propidium monoazide (PMA) or ethidium monoazide (EMA) has been examined, leading to the development of PMA-PCR and EMA-PCR as viable bacteria-selective detection methods using PCR and/or real-time PCR (3–5). These methods enable the selective detection of viable cell-derived DNA, using membrane-permeable dyes (PMA or EMA) that modify dead cell-derived DNA, thereby preventing their PCR amplification (4,6,7). In this study, the ability to discriminate viable from dead cells using the viable bacteria selection kit for PCR (gram-positive), an EMA treatment kit designed exclusively for gram-positive bacteria, was examined.

Received March 30, 2018. Accepted August 28, 2018.
J-STAGE Advance Publication September 28, 2018.
DOI: 10.7883/yoken.JJID.2018.111

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MATERIALS AND METHODS

Ethics statement: This research was approved after full committee review by the Ethical Review Board at Hokkaido Cancer Center (Japan).

Examination of EMA treatment conditions: The viable bacteria selection kit for PCR (gram-positive) (TaKaRa Bio, Inc., Shiga, Japan) was used as an EMA treatment kit. This kit consists of 3 reagents—Solution A-gp, Solution B-gp, and dilution buffer—and exclusively targets gram-positive bacteria, including those belonging to the genera *Bacillus*, *Listeria*, *Staphylococcus*, and *Bifidobacterium*. The package insert does not describe conditions for the EMA treatment of MTC, requiring us to determine the appropriate conditions. An MTC colony grown on 2% Ogawa medium (Kyokuto Pharmaceutical Industrial Co., Ltd., Tokyo, Japan) was collected using a 1 µL platinum loop, suspended in 500 µL sterile distilled water, and left to stand for 2 min. The supernatant was used to examine the optimum conditions for the EMA treatment of MTC. The COBAS® TaqMan® 48 (Roche Diagnostics K.K., Tokyo, Japan) (TaqMan) was used to confirm MTC colonies. We inoculated all specimens in 2% Ogawa medium to determine whether the MTB was dead or alive. The protocol used in this study was as follows (i~vii): (i) Pipette 40 µL of prepared sample into a 1.5-mL microcentrifuge tube. (ii) Add 10 µL Solution A-gp, mix, and spin down briefly using a small benchtop centrifuge. (iii) Add 5 µL Solution B-gp, mix, and spin down briefly using a small benchtop centrifuge. (iv) Leave the tube on ice for 10 min under dark conditions. (v) Take sufficient amount of ice in an icebox and adjust the height of an LED lamp such that the distance between the light source and the ice surface is 2 cm. Place the microcentrifuge tube on the ice horizontally and direct the LED light (EVERLEDS LED light bulb, 6.9 W [Panasonic, Osaka, Japan]) downward. Expose the sample to light for 5 min, then repeat steps (ii) through (v) with a 15-min exposure. (vi) After the

second light exposure step, spin down briefly and heat the tube for 10 min at 95°C for sterilization on a heat block. (vii) Perform PCR using TaqMan. Ice is essential when using a halogen lamp as a light source to avoid increase in the sample temperature, but it is not essential when using LED light because the latter does not generate heat. However, EMA may permeate not only dead bacteria, but also viable bacteria in a non-specific manner, and this phenomenon is more likely to occur in gram-positive bacteria than in gram-negative bacteria (4). Since cold treatment can inhibit non-specific staining, it is recommended that steps (ii) through (v) be performed on ice for clearer discrimination of viable from dead bacteria (4).

Bacterial suspension prepared with colonies grown on Ogawa medium: A basic examination was conducted in which concentration-unadjusted bacterial suspensions were used to determine conditions for EMA treatment that limit dead-bacteria detection. Next, the limit of detection of bacteria by this method was examined by preparing bacterial suspensions adjusted to known concentrations. A colony grown on Ogawa medium was identified as MTC by TaqMan. The concentration-unadjusted bacterial suspensions were prepared according to the method described in subsection "Examination of EMA treatment conditions" of the MATERIALS AND METHODS section. The concentration-adjusted bacterial suspensions were prepared to obtain a turbidity of McFarland No. 0.05–3.0, measured using a BD PhoenixSpec™ nephelometer (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). To compare the results for viable and dead bacteria, the prepared bacterial suspensions were divided into sterilized and unsterilized groups, and subjected to EMA-PCR. For sterilization, the samples were autoclaved at 121°C for 30 min (8). A total of 28 strains, including 7 viable and 21 dead strains of the bacterium, were used for the preparation of concentration-unadjusted and -adjusted bacterial suspensions.

Patient samples: Sputum samples from 22 patients diagnosed with tuberculosis or suspected for active tuberculosis were used. All the sputum samples were treated by CC-E (Japan BCG Laboratory, Tokyo, Japan) prior to use (8). For comparative analyses, all samples were divided into EMA-treated and untreated groups before they were subjected to PCR. Among the samples from 21 patients that were MTC-positive by PCR, 7 samples were culture-positive and 14 samples were culture-negative. Five sputum samples from the remaining patients submitted during the 4-week period after hospitalization were subjected to EMA-PCR and culture. Smear grades were described according to the World Health Organization guidelines for AFB smear positivity (9).

Data analysis: A chi-square (χ^2) test was conducted to examine whether EMA treatment discriminates viable from dead bacteria in the bacterial suspensions prepared from colony (prepared samples) as well as patient samples. Inter- and intra-day reproducibility (5 days) was also examined using bacterial suspensions at 2 concentrations (0.01 and 3.0 McFarland standards) to evaluate the PCR accuracy in this study. The statistical analyses were implemented in StatFlex (Artech Co. Ltd., Osaka, Japan).

RESULTS

Prepared samples: Dead bacteria were detected by EMA-PCR from bacterial concentrations in the range 1.5×10^7–3.0×10^7 CFU/mL, which is equivalent to McFarland No. 0.05–0.10. These bacterial concentrations were estimated based on the predictive concentrations in the package insert of the nephelometer (Table 1). The results of discrimination of viable bacteria from dead bacteria by EMA treatment are summarized in Table 2; χ^2 and *P*-values of 9.333 and 0.0023, respectively, were obtained. The sensitivity and specificity of EMA-PCR for prepared samples that were culture-negative were 67% and 100%, respectively. Regarding technical variation in the measurements over 5 days, the mean C_T value, SD, and CV of the bacterial suspension at McFarland No. 0.01 were 39.44, 0.58, and 1.46, whereas those at McFarland No. 3.0 were 33.42, 0.76, and 2.28, respectively, indicating good consistency.

Patient samples: Table 3 shows results from the patient samples. After statistical analyses, χ^2 and *P*-values

Table 1. Bacterial suspension prepared with colonies grown on Ogawa medium

(A) Viable bacteria

Concentration	PCR (MTC)	Ct value	EMA-PCR (MTC)	Ct value	Culture result
Mc. 3.0	+	23.1	+	24	positive
Mc. 1.0	+	24.1	+	24.3	positive
Mc. 0.5	+	23.4	+	24	positive
Mc. 0.15	+	24.8	+	31.5	positive
Mc. 0.10	+	25.2	+	31.5	positive
Mc. 0.05	+	27	+	34.2	positive

(B) Dead bacteria

Concentration	PCR (MTC)	Ct value	EMA-PCR (MTC)	Ct value	Culture result
Mc. 3.0	+	32.7	+	40	negative
Mc. 1.0	+	33.4	+	35.5	negative
Mc. 0.5	+	33.2	+	40.5	negative
Mc. 0.15	+	34	+	44.1	negative
Mc. 0.10	+	33.6	+	44.9	negative
Mc. 0.05	+	34.6	−	−	negative

Mc.; McFarland.

Table 2. Bacterial suspension prepared with colonies grown on Ogawa medium

	EMA-PCR MTC-positive	EMA-PCR MTC-negative	total
viable bacteria	7	0	7
dead bacteria	7	14	21
total	14	14	28

All specimens before EMA treatment were PCR MTC-positive.
Dead bacteria with EMA-PCR MTC-positive were extremely high turbidity values more than McFarland No. 0.1.
χ^2-values: 9.333 (*P*-values: 0.0023).
sensitivity; 67%, specificity; 100%.

of 15.758 and 0.0001 were obtained. The sensitivity and specificity of EMA-PCR results for patient samples that were culture-negative were 83% and 100%, respectively. Table 4 summarizes the results of 14 samples that were culture-negative among the patient samples. EMA-PCR of the smear-positive, PCR-positive, culture-negative samples from a single subject, obtained 3 weeks after hospitalization, yielded negative results. (Table 5) The patient had been treated with isoniazid, rifampicin, ethambutol, and pyrazinamide during this period.

Table 3. Patient samples

	EMA-PCR MTC-positive	EMA-PCR MTC-negative	total
culture-positive	8	0	8
culture-negative	3	15	18
total	11	15	26

All specimens before EMA treatment were PCR MTC-positive.
χ^2-values: 15.758 (*P*-values: 0.0001).
sensitivity; 83%, specificity; 100%.

Table 4. Culture-negative in among the patient samples

AFB grade[1)]	PCR (MTC) Ct value	EMA-PCR (MTC) Ct value
−	40.1	−
−	45.1	−
4+	39.8	43.7
1+	40.6	−
−	41.5	−
4+	40.1	42.9
2+	39.3	−
−	41.7	−
−	41.6	−
−	40.9	−
2+	40.6	−
2+	39.7	−
2+	41.9	−
−	44.4	−
2+	40.6	−
1+	38.5	−
−	41.4	−
4+	39.2	43.8

[1)]: Smear grades described according to the World Health Organization guidelines for AFB smear positivity.
AFB; acid-fast bacilli.

DISCUSSION

To the best of our knowledge, this is the first successful selective detection of DNA from viable mycobacterial cells by EMA-PCR, using the viable bacteria selection kit for PCR (gram-positive).

It has been reported that PMA is more suitable than EMA for the discrimination between viable and dead bacteria in NAT because EMA influences viable bacteria more than PMA (3,5). However, our experiment revealed that EMA-PCR can discriminate dead MTC cells from viable cells as accurately as the PMA-PCR method, with high specificity. As shown in Table 1, the lower sensitivity of detecting dead bacteria by EMA-PCR in prepared samples can be explained by extremely high turbidity values compared to those for McFarland No. 0.1. This result indicates that high bacterial burdens (shown by the McFarland number) can restrict the EMA reaction. As shown in Table 4, some culture-negative sputum samples with high bacterial burden showed false-positive EMA-PCR. This result implies that high bacterial burden (shown by high AFB number) can restrict the EMA reaction. These results may suggest that specimens with high bacterial burden need a longer reaction time or a higher EMA concentration. Hence, development of this method to decrease false-positive results of samples with high bacterial burden is warranted.

As shown in Table 5, the EMA-PCR using the smear- and PCR-positive, culture-negative clinical samples obtained 3 weeks after hospitalization turned out to be negative. These results indicate that this EMA-PCR method can be used to predict that a certain sample that is MTC-positive by PCR will be culture-negative. A criterion for the discharge of patients with tuberculosis from mandatory admission by law in Japan is 3 consecutive negative sputum tests by smear or culture when examined on distinct days after the administration of standard therapies for at least 2 weeks (9). There are 2 reasons that we are convinced that this method will help physicians make decisions during the treatment of tuberculosis. Firstly, we believe that the successful prediction of negative culture results for samples that are smear-positive and PCR-positive using this method may lead to the earlier discharge of patients. Secondly, we may be able to discriminate paradoxical results from microbiological treatment failure when clinical samples are smear-positive and PCR-positive for patients receiving tuberculosis treatment. This method can be extended to the evaluation of drug susceptibility by using differences in C_T values of real-time PCR before and after EMA treatment (3–5). EMA-PCR utilizes the ability of EMA

Table 5. Comparison of culture and EMA-PCR results during the therapeutic process

patient sample	hospitalization	1 week later	2 weeks later	3 weeks later	4 weeks later
AFB	3+	2+	−	2+	2+
PCR (MTC)	+	+	+	+	−
Ct value	34.4	40.6	44.4	41.9	N/A
EMA-PCR (MTC)	+	+	+	−	−
Ct value	39.2	43.8	46.1	N/A	N/A
Culture results	positive	positive	positive	negative	negative

N/A; not available.

to permeate the damaged cytoplasmic membrane and enter cells when bacteria are maintained on ice and to subsequently bind to nucleic acids in response to light exposure. Therefore, we suggest increasing the duration of incubation of bacteria on ice as well as light exposure (4,6,7).

This study has the following limitations. First, this study was conducted in a retrospective manner with a relatively small sample size. As such, the level of evidence is limited. Prospective studies are needed to verify the usefulness of EMA-PCR for discriminating dead MTC cells from viable cells. Second, the examinations of this study were undertaken by a single researcher (S.F.). Therefore, this study may not be free from technical errors. However, our study showed that EMA-PCR can be very effective for discriminating dead MTC cells from viable cells. The EMA-PCR kit enables clinical microbiology laboratories to conduct selective PCR with lower cost than the PMA-PCR kit in Japan. Additionally, it is easier to adjust the EMA-PCR equipment for clinical microbiological laboratories in community hospitals.

In conclusions, EMA treatment using the viable bacteria selection kit for PCR (gram-positive), an EMA treatment reagent kit, was effective for the selective detection of DNA from viable mycobacterial cells. Moreover, this method can be used to predict that a particular sample identified as MTC-positive by PCR will be culture-negative. Future studies would be aimed at decreasing false-positive results of samples with high bacterial burden as well as determining the efficacy of anti-tubercular agents after starting treatment. This method can be applied in other community hospitals, as it is performed using a commercially available kit. We believe that the application of this method can lead to earlier discharge of patients undergoing tuberculosis treatment.

Conflict of interest None to declare.

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