strains with concentrations ranging from 97.656 μg/ml to ≥ 50,000 μg/ml. Low MIC value indicates better antibacterial activity (Andrews, 2001). The lowest MIC_{90} which is 90% of Detergent 1 can inhibit the bacterial was observed at 1562.5 μg/ml in Table 3. While, D1 showed the lowest MIC among the others detergents which can inhibit *V. parahaemolyticus* isolate VP002 at 97.656 μg/ml. D2 showed the lowest MIC was 390.25 μg/ml to inhibit the growth of isolate VP002 while D3 showed the lowest MIC which is at 781.25 μg/ml to inhibit the isolate VP002 as well. Findings of this research indicated that detergents had high anti-*V. parahaemolyticus* potential and can inhibit the growth of antibacterial-resistant *V. parahaemolyticus* isolates. Minimum inhibitory concentration is an important factor to be considered while choosing a detergent as MIC shows the effectiveness of detergents toward pathogenic microorganism (Andrews, 2001). MBC values are defined as the lowest concentration of detergents required to kill a particular bacteria. After the end point of 24 h, D1 showed the lowest concentration can killed the resistant isolates which is in the range of 781.25–3125 μg/ml (Tables 3 and 4). MBC_{90} which can killed 90% of the resistant isolates for D1 was observed at 3125 μg/ml while D2 and D3 was able to kill 90% of *V. parahaemolyticus* isolates at the concentration level of 6250 μg/ml respectively (Table 4).

As showed in Table 4, all MBCs values were higher than MICs values (less than 4 times) meaning that the tested detergents were able to kill and inhibit growth of antibacterial-resistant *V. parahaemolyticus* isolates. This is compatible with French (2006) who stated that antimicrobial agents can be regarded as bactericidal if the MBC value is not more than four times higher than MIC value. Therefore, D1 (alkyl benzene based) was the most effective in inhibition of the antibacterial resistant *V. parahaemolyticus* growth. (Table 5).

Table 7
Summary of time-kill curve test using Fisher's exact test.

V. parahaemolyticus isolates (as referred to Fig. 1)	Time-kill (h)	Concentration (μg/ml)	Contingency (Fisher's exact test) (R. A. Fisher, 1992)
ATCC 17802	1.0	8 × MIC	3.96
	4.0	4 × MIC	3.52
	6.0	2 × MIC	3.52
VP 003	0.5	8 × MIC	3.96
	4.0	4 × MIC	3.52
	6.0	2 × MIC	3.52

3.4. Time–kill assays

Results of time kill assays showed that D1 (alkyl benzene base) significantly inhibited bacterial growth compared to the control culture (Fig. 1A). The bactericidal activity of detergents was fast-acting against *V. parahaemolyticus* (ATCC 17802) which killed at 8 × MIC within 1 h and the reduction in CFU/ml was 3 log units (99.9%) with P value < 0.05 was considered statistically significant. The bacterial end point for VP003 was reached after 0.5 h of incubation (Fig. 1B) at 8 × MIC. The result indicated a fast acting bactericidal activity of D1 against *V. parahaemolyticus*. According to

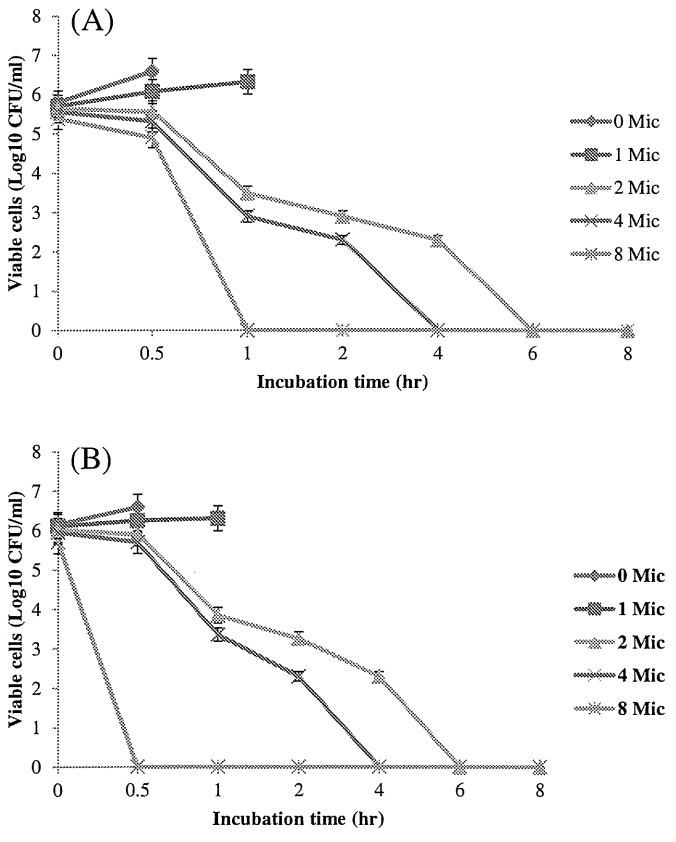

Fig. 1. Antibacterial activity of D1 against resistant *V. parahaemolyticus* strains (n = 16). (A) Activity of D1 (alkyl benzene base) against *V. parahaemolyticus* control strain 17802. Figure (B) Activity of resistant #VP003 against D1. Results obtained from three experiments performed with four replicates.

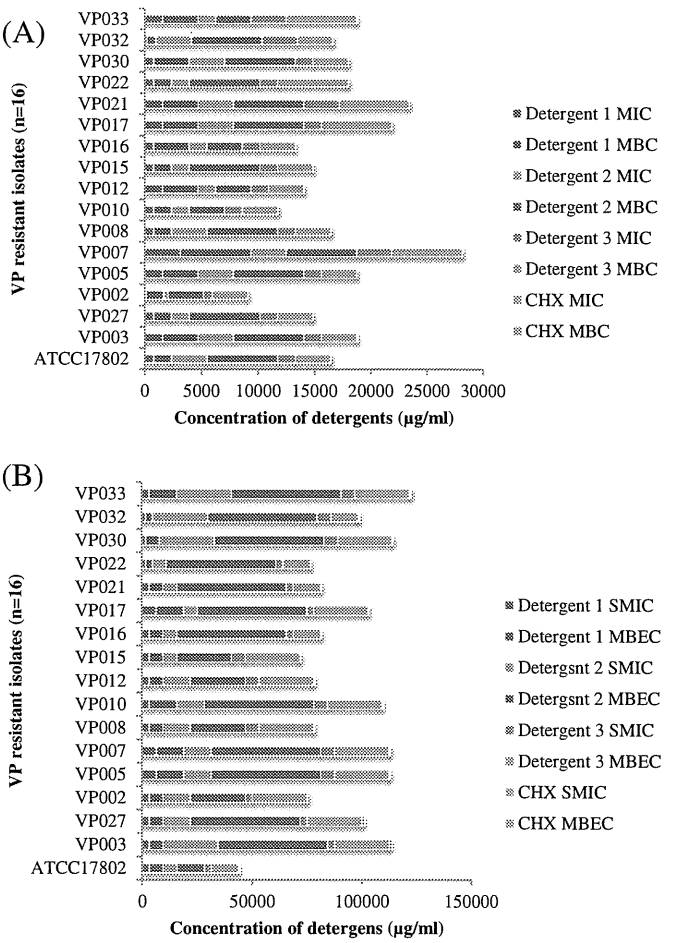

Fig. 2. Comparison between (A) MICs and MBCs of planktonic cells of *V. parahaemolyticus* with the (B) SMICs and MBECs of the biofilms production as referring to Tables 3 and 5.

Table 6
Comparative *in vitro* activities of detergents against resistant *V. parahaemolyticus* strains forming biofilms.

V. parahaemolyticus isolates or antibacterial agent	SMIC (μg/ml)			MBEC (μg/ml)		
All isolates (n = 16)	Range	50%	90%	Range	50%	90%
Detergent 1 (D1)	1562.5–6250	3125	6250	3125–12500	6250	12500
Detergent 2 (D2)	6250–25,000	12,500	25,000	25,000–≥50,000	≥50,000	≥50,000
Detergent 3 (D3)	3125–6250	3125	6250	12,500–25,000	25,000	25,000
CHX (Standard conrol)	78.125–312.50	78.125	312.50	312.5–12,500	312.5	12500

the Fisher's exact test (Table 7), the contingency values for *V. parahaemolyticus* ATCC 17802 was killed is at 4 h of incubation at 4 × MIC with *P*-value obtained 3.52 compared to 4 h at concentration of 4 × MIC with *P*-value; 3.82 for VP003. The total probability with smaller values were selected.

3.5. Sessile minimum inhibitory concentration (SMICs) and minimum eradication bactericidal concentration (MBECs)

The emergence of biofilm with factors such as numbers of organisms (Eng et al., 1985), their metabolic states (Tuimen et al., 1986), and their protection by the exopolysaccharide glycocalyx (Nickel et al., 1985) in seafood can affect the ability of detergents to penetrate and killed the resistant *V. parahaemolyticus* strain forming biofilm in seafood. These adherent cells are frequently embedded within a self-produced matrix of extracellular polymeric substance (EPS) will cause the microorganism to be more resistant toward the antimicrobial compound that can regenerate the strong colonies.

As shown in Table 6, the SMIC values were between 2 and 3 times higher than MIC values of planktonic cells in Fig. 2. As showed in Fig. 2(A) and (B), MICs and MBCs of planktonic cells of antibiotic resistant *V. parahaemolyticus* isolates were lower than SMICs or MBECs of biofilm. The SMICs for D1 ranged from 1562.5 to 6250 μg/ml while the MBECs ranged from 3125 to 6250 μg/ml (Table 6). D1 (alkyl benzene base) was an effective detergent to inhibit the growth of antibiotic resistant *V. parahaemolyticus* isolates due to its linear alkylbenzene (LAB) base structure. Linear alkylbenzene sulphonate was reported to be able to remove biofilm in river system (Boeije, Schowanek, & Vanrolleghem, 2000). In a similar case, Krupesha Sharma et al. (2010), stated that formation of biofilm by *Vibrio alginolyticus* on shrimps and biofilm forms showed better antibiotic resistance compared to free cell of *V. alginolyticus*. D3 (sodium hydroxide base) is a very strong base that can break most of the chemical bonds and dissolve tissue and cells. SMICs and MBECs of D3 ranged from 3125 to 6250 μg/ml and 12500–25000 μg/ml, respectively. While D2 contained quaternary ammonium compounds which are widely used as disinfectants. Use of quaternary ammonium compounds as antiseptics is contraindicated. The SMICs and MBECs values ranged from 6250 to 25000 μg/ml and 25000–≥50,000 μg/ml, respectively. Based on the results, the eradication concentration for removing biofilm is much higher than the concentration required for killing single cell of antibacterial resistant *V. parahaemolyticus*.

As shown in Fig. 2(B), biofilm formation and development can occurred within 1 h. During biofilm development process, almost 10% of the *V. parahaemolyticus* population irreversibly adhered to the surface and after 8 h more than 90% of the *V. parahaemolyticus* have been irreversibly attached.

4. Conclusion

Nowdays, increase in antibiotic-resistant *V. parahaemolyticus* is a food safety issue in seafood industries. Due to excess usage of detergents, alter in microorganism's genetic makeup was observed and new adapted species have emerged. Detergents-resistant bacteria can eventually multiply and become more frequent in environment. Hence, proper cleaning is an important factor in food-poisoning prevention. Cross-contamination of final products usually occur at food processing units du to improper hygienic conditions. As *V. parahaemolyticus* is able to survive at low temperatures, proper cleaning of surfaces and equipments with an effective detergent is essential to obviate the risk of contamination. This research was the first reported assessment of biofilm formation by *V. parahaemolyticus* in Malaysia. Tested detergents showed antibacterial activity against antibiotics resistant *V. parahaemolyticus* isolates. This study provided a deeper insight on the effectiveness of detergents (especially detergents as antibacterial agents) as growth inhibitors for antibiotic-resistant *V. parahaemolyticus* strains at the beginning of cleaning process. Type of disinfectant, concentration of disinfectant, exposure time, age of biofilm and mechanical damage are the factors affecting eradication of biofilm by disinfectants. Future studies are suggested to investigate the effect of mentioned factors to find the best formulation and method for elucidation of *V. parahaemolyticus* biofilms. Support of regulatory agencies for application of anti-biofilm detergents is highly needed.

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RESEARCH NOTE

Transfer of *Listeria monocytogenes* between Abiotic Surfaces under Different Weights

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Abstract Cross contamination of *Listeria monocytogenes* from two different surfaces (plastic wrappers and stainless steel coupons) was simulated using a series of different weights. Enumeration of transfer was based on surface bacterial counts of biofilm stained surfaces. Direct contact between 2 surfaces has a constant rate of transfer that is independent of the pressure applied. This is the first report on the study of cross contamination between surfaces using pressure to illustrate transfer of bacteria in a food processing line.

Keywords: *Listeria monocytogenes*, biofilm formation, stainless steel, plastic wrappers, pressure

Introduction

Listeria monocytogenes is a recognized foodborne pathogen with the ability to cause severe health problems, particularly for high risk individuals. Some of the diseases caused by *L. monocytogenes* are bacteremia, meningitis, and may lead to abortion. Human listeriosis is reportedly increasing in several countries, including England and Wales (1), and in Europe (2). Reports from 2000-2004 showed an upsurge in the disease incidence in England and Wales among the elderly population with an unexplainable cause, due to a limitation of knowledge in the epidemiology of listeriosis. The dispersed form of the disease suggests that the increase in human listeriosis may not be due to common exposure to a single food source (1). In Denmark, a report for 2009 also showed a rise in the number of cases of listeriosis (3). The dire health consequences caused by *L. monocytogenes* are known to be 99% related to consumption of contaminated foods (4). The common isolated serotypes of *L. monocytogenes* are 1/2a and 4b; however, serotype 1/2a is usually found in food isolates while 4b is usually found in outbreaks (5).

Food contamination with *L. monocytogenes* has been reported frequently in food industries and generally occurs after thermal processes, and may be related to re-contamination of a food product (6). It has been acknowledged that *L. monocytogenes* cannot completely be eradicated because it is commonly found in the environment (6.1%) and in food processing factories (12.8%) (7). Higher incidences of *L. monocytogenes* in food processing facilities may be attributed to the ability of the bacterium to form a biofilm (8) that increases survival and persistence (9,10).

Biofilms are commonly a source of food contamination in industrial facilities because food residues that accumulate on surfaces can act as a continuous culture system for propagation of microorganisms on the surface (11). A biofilm is initiated when attachment of a bacterium takes place, followed by formation of microcolonies and subsequent maturity of the microcolonies into a biofilm structure constructed of extrapolymeric substances. It was found that sessile cells in a biofilm are more resistant to disinfectants than planktonic cells, especially after attachment to a surface (12). Formation of biofilms on different surfaces has been widely documented over the years, including stainless steel, glass, polypropylene, and rubber in food processing plants (13). Surface roughness of a stainless steel material can have an effect on the degree of bacterial

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adherence. Arnold and Bailey (14) reported 3 different types of finishing treatments on stainless steel for poultry processing tested for an affinity of bacterial attachment wherein it was proposed that food safety can be improved by using materials that are resistant to bacterial contamination. Electropolished stainless steel with the least rough surface has fewer cell attachments and biofilm formations than other surfaces tested. However, Rodriguez *et al.* (15) reported that a cocktail *L. monocytogenes* strains showed no difference in adhesion values between electropolished stainless steel and mechanically polished stainless steel, and reported that electropolishing stainless steel does not increase the degree of food sanitation over mechanically finished stainless steel.

Food contact surfaces are of great concern because contamination of food processing surfaces, such as conveyor belts and packaging surfaces, with high levels of microbes, such as *L. monocytogenes* (16) increases the risk of cross contamination. Contact surfaces have been recognized as critical control points for food safety assessments. Reports regarding survival of *L. monocytogenes* on surfaces such as conveyor belt materials (17) highlight the importance of investigating the nature of cross contamination.

Factors affecting biofilm dispersal that lead to cross contamination include temperature, oxygen levels, pH, specific compounds, food residues, and antimicrobial additives on the surfaces (18-20). Transfer of contaminants to food products occurs through contact and via pressure on a biofilm. No studies have reported the transferrable amounts of cells when food products come into contact with biofilm-contaminated surfaces with consideration of applied weight. Therefore, this study focuses on the effect of weight in transfer of biofilm bacteria from stainless steel coupons. Transfer of bacteria from the *L. monocytogenes* ATCC 19112 biofilm (serotype 1/2 a-common in food) formed on stainless steel was enumerated for polypropylene plastic wrappers under different weights.

Materials and Methods

Bacterial strains and culture preparation *L. monocytogenes* ATCC 19112 (serotype 1/2a) was used. A pre-culture of *L. monocytogenes* was obtained after a sample of the organism was inoculated into tryptic soy broth (Merck, Darmstadt, Germany) and allowed to grow in an incubator shaker (MaxQ 4000; Barnstead Lab Line, Dubuque, IA, USA) for 12 h at 37°C until the stationary phase was reached, as determined using spread plating of the culture of a consistent density of 10^9 CFU/mL of cells. Next, 100 μL of the pre-culture was transferred to one side of a stainless steel coupon (hairline finish, 50×50×1 mm, Type 304; Supreme Steelmakers Sdn Bhd, Kuala Lumpur, Malaysia)) and placed in pre-sterilized plastic containers (50×50×30 mm). Stainless steel coupons and containers were previously washed using dishwashing detergent, sonicated (Powersonic 420; Hwashin Technology, Seoul, Korea) for 20 min, and soaked in 90% ethanol (Merck) for 1 h, followed by autoclaving (HVE50; Hirayama Manufacturing Corporation, Saitama, Japan) at 121°C for 15 min. The stainless steel coupons (Type 304; Supreme Steelmakers Sdn Bhd) in the plastic container were subjected to UV sterilization for 15 min prior to the transfer procedure. An amount of 100 μL of the culture was placed on the sterile stainless steel in the plastic container and covered for incubation at 30°C for 48 h.

Bacterial and biofilm transfer The stainless steel coupons in the sterile plastic container were covered with a lid and placed in an incubator (Sanyo MIR253; Sanyo Electric Biomedical Corporation Ltd., Osaka, Japan) at 30°C for 48 h. The transfer procedure was conducted in a sterile environment. Initial counts of cells attached to the stainless steel coupons were made prior to the transfer procedure using the plate spreading procedure on PALCAM agar (Merck) with 1% crystal violet (Merck) staining Conditions were designed to mimic a contaminated surface used for packing foods in plastics. Plastic wrappers that were previously sterilized with soaking in 70% ethanol (Merck) and UV for 30 min measuring 50×50 mm were placed on the surface of the stainless steel coupons under pressure using weights of 0.1, 0.2, 0.3, 0.4, and 0.5 kg for 30 s. The individual weights were prepared using plastic bags containing water to ensure full contact between surfaces during pressurization based on water flow in the plastic bags. Surfaces of the plastic bag weights were sterilized using 70% ethanol (Merck) prior to use. The simulated transfer material was then subjected to the spread plating with crystal violet (Merck) staining for enumeration after bacterial transfer from the stainless steel surface to the plastic wrap. Experiments were conducted in triplicate and repeated twice.

Calculation of compression due to weights was based on the weight applied to the plastic wrapper and the stainless steel. Determination of the effective transfer rate based on the pressure applied was performed using the following calculation (21):

$$\text{Transfer rate (\%)} = \frac{\text{Number of CFU on plastic}}{\text{Initial number of CFU on stainless steel}} \times 100$$

Enumeration of colonies Enumeration of bacterial contamination on the plastic wrappers and stainless steel coupons was conducted by putting the materials into a sterile stomacher (BagLight Model L; Interscience, St.

Nom la Breteche, France) containing 10 mL of buffered peptone water (Merck), followed by mixing the materials by scrubbing the surface against the bag to dislodge the attached cells and colonies, and vigorous shaking of the suspended colonies by hand for 10 s. Then, 1 mL of the broth from the bag was diluted using buffered peptone water (Merck) followed by plating on PALCAM agar (Merck). Plates were incubated at 30°C for 48 h prior to a colony count.

Biofilm readings The crystal violet staining procedure was carried out with slight modification of the method of Djordjevic *et al.* (22). Briefly, abiotic surfaces were rinsed with running sterile distilled water (Elga Purelab Classic UVMK2; ELGA LabWater, High Wycombe, UK) for 30 s, then stained with 1 mL of 1% crystal violet (Merck) that was filter sterilized using 0.2 μm syringe filter (Minisart; Sartorius AG, Goettingen, Germany). The crystal violet solution was allowed to stand on the contact surfaces for 20 min, followed by rinsing under running water using sterile distilled water (ELGA Option-S 78P; ELGA LabWater) The abiotic surfaces were dried in a laminar hood (Esco AVC-4A1; ESCO Technologies, Inc., Hatboro, PA, USA) for 1 h, then a total of 5 mL of 95% absolute ethanol (Merck) was added to elute the crystal violet (Merck). Reading of biofilms was carried out using a spectro-photometer (Spectrophotometer 170-6930; Bio-Rad, Hercules, CA, USA) at 570 nm with an appropriate blank and baseline. Preparation of blank was based on the elution of crystal violet using 95% absolute ethanol (Merck) and baseline was the negative control to ensure the stainless steel does not affect the absorbance reading of crystal violet. Results were obtained and presented as mean values and standard deviations of absorbance readings.

Statistical analysis An analysis of variance (ANOVA) was performed to identify significant differences between means. Analysis used the SPSS version 17.0 software package (SPSS Institute, Cary, NC, USA). Statistical significance was defined as $p<0.05$.

Results and Discussion

The initial number of cells on the stainless steel surface was a mean value of 9.51 log CFU/mL with a mean absorbance ($A_{570\,nm}$) value of 0.654 using crystal violet staining. Formation of *L. monocytogenes* biofilms can occur with various counts and under on various surfaces, such as on Buna-N rubber (23), at a level of 2.1 log CFU/cm^2 on 0.8% minimal NaCl medium (24), and on glass and steel surfaces (25). The experimental conditions of this study favored formation of a biofilm on stainless steel after 48 h. Serotype 1/2a is commonly isolated from food so emphasis on this serotype can deepen our understanding of cross contamination of foods.

Contamination of *L. monocytogenes* was found to be independent of the pressure applied to the contact surface between stainless steel and plastic. Results (mean and standard deviation values) for biofilm density (A_{570}) showed no significant ($p>0.05$) (Table 1) effect for pressure on the transferred log CFU/mL value or on absorbance values of the stainless steel or plastic surfaces between the weights applied. A cell count of approximately 2.5-3.6 log CFU/mL was obtained for the plastic after transfer from stainless steel at an original steel contamination level of (mean) 9.51 log CFU/mL (Table 1). The percentage of cells transferred to the plastic surface was not proportional to the weight applied, with a transfer rate range in the range of 24 to 35% to the plastic surface. These percentage values can be considered as a range for uniform transfer of biofilm cells (CFU/mL) for contact between two types of dry surface.

Previous studies have identified factors involved in transfer of biofilm bacterial cells from surfaces. Montville and Schaffner (26) reported a connection between the inoculum size and the percent transfer rate in cross contamination activities. The bacterial transfer rate remained constant even though there was an increase in the initial inoculum size. Different inoculum sizes were investigated in relation to the number of cells transferred and found that a constant transfer rate led to a decrease in the percent of transfer rate in the cross contamination study (26), which

Table 1. Means and standard deviations for cell and colony transfer from a biofilm to plastic wrappers based on applied weights

Weight (kg/m^3)	Plastic wrapper (mean±SD)		
	Absorbance	CFU/mL	Percentage of transfer
40	0.221±0.01	3.295±0.68	34.2±6.18
80	0.277±0.03	3.649±0.67	35.2±5.78
120	0.271±0.01	2.623±0.64	27.1±6.07
160	0.317±0.05	3.335±0.58	34.6±5.56
200	0.282±0.05	2.511±0.82	24.9±8.29
Statistical Analysis			
ANOVA (F; $p<0.05$)	0.853; 0.5	0.52; 0.721	ND[1)]

[1)]ND, not detected

was similar to findings of a constant transfer rate in this study. However, Vorst *et al.* (27), in a study of transfer of *L. monocytogenes* in mechanical slicing of turkey breast, bologna, and salami, reported significant transfer of *Listeria* when a force of 4.5 kg was placed on samples, compared with no weight. Contamination of non-spore producers can readily occur via contact between wet sponges, stainless steel surfaces, and food (21). Contamination of pathogens with pressure ranged from 30 to 100% for *Staphylococcus aureus, Salmonella enteritidis,* and *Campylobacter jejuni.* Transfer of these pathogens from stainless steel to food was affected by the pressure applied.

The concentrations of moisture and fats in food, compared with dry plastic surfaces, may be related to contrasting results. Smooth plastic surfaces may not provide a favorable medium for bacterial transfer of *Listeria.* Another transfer factor is the surface condition (dry/moist) as transfer can occur more easily on moist surfaces (26). The previous study of Vorst *et al.* (27) regarding the effect of weight used application of 4.5 kg of force for transfer of *Listeria.* This study used increments of 0.1 kg of force, which may not have been sufficient for transfer of *Listeria* between the surfaces. Transfer of cells and colonies from a bacterial biofilm is largely affected by the medium for transfer, particularly for foods with high fat and moisture contents. Therefore, when transfer between dry abiotic surfaces was studied in the present work, the effect of weight does not facilitate the transfer between the contact surfaces.

Chavant *et al.* (28) reported that *L. monocytogenes* forms biofilms more rapidly on hydrophilic surfaces than on hydrophobic surfaces, compared to other typical microorganisms, probably due to interactions between the cell surface and environmental surfaces that enable cells to overcome repulsive forces and attach irreversibly (29). Hydrophilic surfaces are common in the food preparation environment, and contamination and subsequent adherence of pathogens to surfaces can occur if a good cleaning procedure is not practiced (30). Hansen *et al.* (30) reported a significant improvement in the survival of desiccated cells in a biofilm, compared to non-biofilm cells, for transfer to salmon slices. The efficiency of transfer was significantly affected by the presence of the biofilm that aided cell survival in a desiccated form, and subsequent transfer to food. This study focused on abiotic surfaces. The potential of food contamination (CFU/mL) that is higher than the initial contamination on a surface exists due to revival of injured, non-laboratory-cultivable cells that subsequently act to increase the rate of cross-contamination along the processing line (30).

Contamination of *L. monocytogenes* can be an important consequence for food safety. Estimation of transfer rates based on contact weight may be an interesting focus for providing data for microbiological risk assessments, such as application of a uniform distribution represented by (minimum 24.9; maximum 35.2) for cross contamination modeling between two surfaces. This is the first study to report cross contamination of biofilm transfer between abiotic surfaces based on a contact pressure series. Application of findings in this study can be used for estimating the risk involved in cross contamination of foods at the factory level when biofilms are involved. A biofilm is a complex structure that plays an important role in persistence of microorganisms. Many other factors should also be included in estimation of cross contamination.

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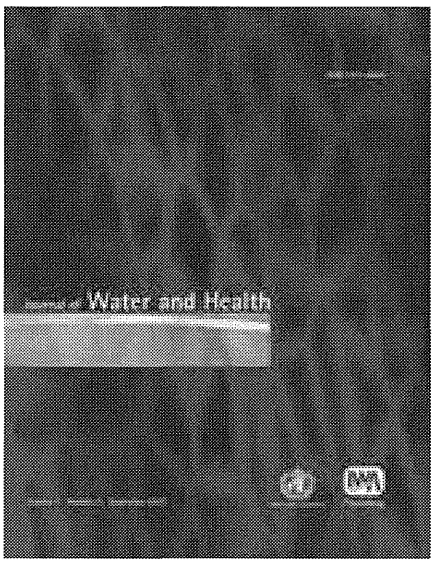

Direct detection of *Mycobacterium avium* in environmental water and scale samples by loop-mediated isothermal amplification

Yukiko Nishiuchi, Aki Tamaru, Yasuhiko Suzuki, Seigo Kitada, Ryoji Maekura, Yoshitaka Tateishi, Mamiko Niki, Hisashi Ogura and Sohkichi Matsumoto

ABSTRACT

We previously demonstrated the colonization of *Mycobacterium avium* complex in bathrooms by the conventional culture method. In the present study, we aimed to directly detect *M. avium* organisms in the environment using loop-mediated isothermal amplification (LAMP), and to demonstrate the efficacy of LAMP by comparing the results with those obtained by culture. Our data showed that LAMP analysis has detection limits of 100 fg DNA/reaction for *M. avium*. Using an FTA® elute card, DNA templates were extracted from environmental samples from bathrooms in the residences of 29 patients with pulmonary *M. avium* disease. Of the 162 environmental samples examined, 143 (88%) showed identical results by both methods; 20 (12%) and 123 (76%) samples were positive and negative, respectively, for *M. avium*. Of the remaining 19 samples (12%), seven (5%) and 12 (7%) samples were positive by the LAMP and culture methods, respectively. All samples that contained over 20 colony forming units/primary isolation plate, as measured by the culture method, were also positive by the LAMP method. Our data demonstrate that the combination of the FTA elute card and LAMP can facilitate prompt detection of *M. avium* in the environment.

Key words | bathroom, direct detection, FTA elute card, loop-mediated isothermal amplification (LAMP), *Mycobacterium avium*

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INTRODUCTION

The incidence of *Mycobacterium avium* complex (MAC) infection is gradually increasing all over the world, especially in developed countries (Falkinham 1996; Field *et al.* 2004; Griffith *et al.* 2007). MAC organisms inhabit the environment and are transferred to susceptible humans or farm animals, leading to infection and disease (Falkinham 2002; Field *et al.* 2004; Angenent *et al.* 2005). *M. avium* and other nontuberculous mycobacteria are widely distributed in natural and artificial environmental habitats, including natural water bodies, drinking water distribution systems, hot tubs, forest soils, peats, and potting soils (Falkinham 2009). We previously reported that MAC was frequently detected in

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samples from bathrooms in the residences of patients with pulmonary MAC disease, suggesting that the bathroom is the possible source of infection (Nishiuchi *et al.* 2007, 2009). Although MAC colonization in the human environment was polyclonal and displayed genetic diversity, some genotypes were identical or similar to the clinical isolates obtained from the corresponding patients (Nishiuchi *et al.* 2007, 2009). Moreover, the characteristics of MAC disease, such as multiple infections with genetically different strains (Wallace *et al.* 1998, 2002) and frequent relapse or reinfection (Kobashi & Matsushima 2003), could be attributable to the presence of a reservoir for MAC in the environment immediately surrounding the patients. It is important to break this cycle of infection by removing the infection source; identification of the source in the environment is thus the initial important step for controlling the disease.

In previous investigations, we isolated *M. avium* organisms by conventional culture. Although this method is basic and essential for the assessment of genetic diversity and drug susceptibility, the procedure is time consuming; it takes 3 weeks to obtain primary isolates and another 2 weeks to obtain pure cultures, followed by polymerase chain reaction (PCR) analysis for species identification (Nishiuchi *et al.* 2007, 2009). Thus, at least 5 weeks are usually required to detect *M. avium* organisms, underscoring the need for an alternative, rapid, and accurate method of *M. avium* detection in environmental specimens, which would in turn facilitate accelerated diagnosis. Nucleic acid amplification (NAA) tests are commonly used in hospitals to directly detect *Mycobacterium tuberculosis* and *M. avium* in clinical specimens because they require less time than culture. Several recent systematic investigations have confirmed the high specificity and sensitivity of NAA tests (Ichiyama *et al.* 1996; Soini & Musser 2001; Huggett *et al.* 2003; Park *et al.* 2006). A novel NAA method, termed loop-mediated isothermal amplification (LAMP), is commonly used to detect viruses, parasitic protozoans, and bacteria including *M. tuberculosis* complex (Iwamoto *et al.* 2003; Boehme *et al.* 2007; Pandey *et al.* 2008), *M. avium* (Iwamoto *et al.* 2003), *M. avium* subsp. *paratuberculosis* (Enosawa *et al.* 2003), *M. intracellulare* (Iwamoto *et al.* 2003), *M. kansasii* (Mukai *et al.* 2006) and *M. gastri* (Mukai *et al.* 2006). The LAMP method has been applied to detect mycobacteria in clinical samples (Iwamoto *et al.* 2003; Boehme *et al.* 2007; Pandey *et al.* 2008), but it has not been tested for environmental samples. In the present study, environmental samples obtained previously (Nishiuchi *et al.* 2009) were subjected to LAMP analysis for the direct detection of *M. avium* using novel primer sets targeting the *M. avium* 16S rRNA gene. The results were compared with those obtained previously by culture (Nishiuchi *et al.* 2009). We also employed FTA® elute cards for genomic DNA extraction; these cards allowed very easy recovery of DNA templates from the environmental samples without resorting to the use of any harmful reagent.

METHODS

Design of LAMP primers

Using conserved sequences of the 16S rRNA gene as a target, two inner primers, namely the forward inner primer (FIP) and backward inner primers (BIP), two outer primers (F3 and B3), and two loop primers (FL and BL) for *M. avium* were designed using PrimerExplorer V3 software (https://primerexplorer.jp; Eiken Chemical Co. Ltd, Tokyo, Japan). The primer sequences and other details are listed in Table 1.

LAMP reaction

LAMP was performed in 50 μl reaction volumes containing 4 μl of the extracted DNA template, 20 μmol l^{-1} each of FIP and BIP, 25 μmol l^{-1} each of F3 and B3, 30 μmol l^{-1} each of FL and BL, 1.4 mmol l^{-1} deoxynucleoside triphosphate mix, 0.8 mol l^{-1} betaine (Sigma-Aldrich, St Louis, MO, USA), 20 mmol l^{-1} Tris-HCl (pH 8.8), 10 mmol l^{-1} KCl, 10 mmol l^{-1} $(NH_4)_2SO_4$, 8 mmol l^{-1} $MgSO_4$, and 6.4 U of *Bst* DNA polymerase (large fragment; New England Biolabs Inc., Beverly, MA, USA). The mixture was incubated at 64 °C for 60 min in a Loopamp® real-time turbidimeter (LA-200; Teramecs Co., Kyoto, Japan) and then heated to 80 °C for 2 min to terminate the reaction.

Analysis of LAMP products

The LAMP reaction causes turbidity in the reaction tube, which is proportional to the amount of amplified DNA. The reaction was considered positive when a turbidity of ≥0.1 was observed

Table 1 | Primers used for loop-mediated isothermal amplification

Primer type		Sequence	Location of the target sequence on the complete genome sequence[a]
F3	Forward outer	5′ – CTGGCTCAGGACGAACG – 3′	1,487,551 – 1,487,563
B3	Backward outer	5′ – GCCCATCCCACACCGC – 3′	1,487,759 – 1,487,746
FIP	Forward inner primer	5′ – TGCCCACGTGTTACTCATGCAAGTCGAACGGAAAGGCCT – 3′	1,487,654 – 1,487,638 + 1,487,588–1,487,609
BIP	Backward inner primer	5′ – TCGGGATAAGCCTGGACCAGAAGACATGCGTCTTGA –3′	1,487,669 – 1,487,683 + 1,487,732–1,487,712
FL	Loop forward	5′ – GTTCGCCACTCGAGTACCTCCG – 3′	1,487,634 – 1,487,613
BL	Loop backward	5′ – GAAACTGGGTCTAATACCGG – 3′	1,487,684 – 1,487,703

[a]*M. avium* 104 (GenBank accession no. CP000479.1).

within 50 min. For further confirmation, the amplified products were examined by restriction analysis using *Taq*I enzyme, which was selected on the basis of the restriction maps of the target sequences of the LAMP product. Following overnight digestion at 37 °C, the digested products were analyzed by agarose gel electrophoresis using a 2% agarose gel, followed by staining with ethidium bromide. For further confirmation that the correct LAMP product was obtained, melting curve analysis was performed as follows. The LAMP reaction was carried out after addition of SYBR Green I (1:50,000; Molecular Probes Inc., Eugene, OR, USA), and the melting curves of LAMP amplicons were obtained over a temperatures range of from 64–95 °C using an Applied Biosystems 7500 fast real-time PCR system. The ROX reference dye was not used.

Strains and environmental samples

The specificity of the selected primer sets was examined by performing the LAMP method for DNA extracted from various bacterial strains: *M. tuberculosis* ATCC 25618, *M. bovis* Ravenel, *M. bovis* BCG Tokyo, *M. africanum* ATCC 25420, *M. microti* TC 77, *M. kansasii* ATCC 12476, *M. avium* ATCC 15769, *M. avium* subsp. *paratuberculosis* ATCC 19698, *M. intracellulare* ATCC 13950, *M. marinum* ATCC 927, *M. simiae* ATCC 12476, *M. shimoidei* ATCC 27962, *M. nonchromogenicum* ATCC 19530, *M. xenopi* ATCC 19250, *M. scrofulaceum* ATCC 19981, *M. gordonae* ATCC 14470, *M. chelonae* subsp. *abscessus* ATCC 19977, *M. fortuitum* ATCC 6841, *M. austroafricanum* ATCC 33464, *M. pulveris* ATCC 35154, *M. asiaticum* ATCC 25276, *M. tokaiense* ATCC 27282, *M. malmoense* ATCC 29571, *Achromobacter xylosoxidans*, *Acinetobacter haemolyticus*, *Bacillus cereus*, *Escherichia coli*, *Klebsiella pneumoniae*, *Legionella pneumophila*, *Pseudomonas aeruginosa*, *Shigella boydii*, *Staphylococcus aureus*, and *Streptococcus haemolyticus*. Genomic DNA was prepared from the bacterial strains by mechanical disruption, as described previously (Suzuki *et al.* 1995), and dissolved in 300 μl of TE buffer containing 10 mmol l^{-1} Tris-HCl (pH 8.0) and 1 mmol l^{-1} ethylenediaminetetraacetic acid (EDTA). All extracts were verified to contain DNA of >10 ng μl^{-1} concentration. Environmental samples collected by us previously (Nishiuchi *et al.* 2009) were also used in the present study. In brief, the samples collected on cotton swabs (scale and slime) were preincubated in 1 ml tryptic soy broth for 3 h at 25 °C and subjected to alkali treatment by the addition of 3 ml 2% sodium hydroxide solution and incubation for 10 min, followed by addition of 6 ml of phosphate buffer (PB) at pH 6.8 and centrifugation at 2270 × *g* for 15 min. The pellets were resuspended in 0.5 ml of PB, and 0.2 ml of these suspensions was used for culture while the remaining samples were frozen until DNA extraction for use in the LAMP method. The water samples (200 ml) were centrifuged and subjected to alkali treatment, as described above.

Comparison of various methods of DNA extraction for LAMP analysis

Five methods of DNA extraction were used for comparing DNA detection limits obtained by the LAMP method. The methods included the conventional phenol/chloroform/isopropanol extraction method (Suzuki *et al.* 1995), the

Puregene Yeast and Gram-positive Bacteria Kit (Gentra, Tokyo, Japan), the QIAamp DNA Micro Kit (Qiagen, GmbH, Hilden, Germany) after overnight treatment of samples with 2 mg ml^{-1} of lysozyme solution (1 mol l^{-1} NaCl, 0.1 mol l^{-1} EDTA, 10 mmol l^{-1} Tris-HCl (pH 8.0), 0.5% Brij-58, 0.2% deoxycholate, and 0.5% sarkosyl), the silica-based method, which is capable of detecting 1–10 mycobacteria in samples (Bahador *et al.* 2004), and the FTA elute card method (Whatman Inc.), for which 40 μl of cell suspension containing 1.0×10^2–10^5 colony forming units (CFU) 100 μl^{-1} was used. For the other four methods, 100 μl of the cell suspension was used. Extractions using kits were performed according to the manufacturers' instructions, and extracts were eluted with 30 μl of TE buffer.

When punching FTA elute cards for recovery of DNA templates, precautions had to be taken to exclude the risk of contamination with carryover DNA. Therefore, every time an elute card was punched, the puncher was decontaminated by subsequently punching a wet Kimwipes® containing 1000 ppm of sodium hypochlorite, which is a well-known chemical decontaminant for DNA (Prince & Andrus 1992). We confirmed the effectiveness of this hypochlorite system for *M. avium* bacilli (up to 4×10^8 cells) and *M. avium* DNA (up to 1.2 μg) on FTA elute cards (data not shown). Subsequently, the decontaminant and damaged DNA remaining on the puncher were removed by punching a clean Kimwipes® twice.

Extraction by the silica-based method was performed as described previously (Bahador *et al.* 2004). In brief, 500 μl of lysis buffer (1.2% guanidine thiocyanate (Fluka Chemie AAG, Switzerland) in 0.1 mol l^{-1} Tris-HCl (pH 6.4), 36 mmol l^{-1} EDTA, and 2% Triton X-100) was added to 100 μl of the cell suspension, followed by 20 μl of acid-washed silica. The suspension was mixed vigorously and incubated for 30 min at 60 °C, followed by centrifugation at 13,800 × *g* for 2 min. The pellet was washed twice with washing buffer containing 12% guanidine thiocyanate in 0.1 mol l^{-1} Tris-HCl (pH 6.4), twice with 70% ethanol, and once with acetone and then dissolved in 30 μl of TE buffer.

Extraction of DNA from environmental samples

The frozen samples were centrifuged at 13,800 × *g* for 10 min, and cell pellets were resuspended in 80 μl of PB (pH 6.8). Forty microliters of the concentrated environmental samples was applied to FTA elute cards, and DNA was extracted in 30 μl of TE buffer as described above.

RESULTS

Sensitivity and specificity of the LAMP method

We first examined the sensitivity of this method by monitoring the detection of serially diluted DNA extracted from *M. avium* (Figure 1). The results indicated that the DNA detection limit was 100 fg/reaction as opposed to the detection limit of 1 pg DNA/reaction obtained using the previously reported primer sets that targeted *gyr*B (Iwamoto *et al.* 2003).

The method of DNA extraction is also known to influence sensitivity because the recovery rate of DNA generally depends on both the method used and the skills of the researcher. Although many methods have been proposed, some require numerous steps and the use of corrosive reagents, such as phenol and chloroform, while others require a large number of bacilli in the starting material because of their poor recovery. Thus, we next examined how many bacilli were required in the sample for the successful detection of *M. avium*, using five different methods of DNA extraction. Among these methods (Table 2), the FTA elute card method was the most sensitive and suitable for subsequent DNA detection using the LAMP method, as it detected *M. avium* when a minimum of 400 bacilli were present in the volume (40 μl) applied to the FTA elute card. Moreover, this method only required only 2–3 h because of the simple procedure.

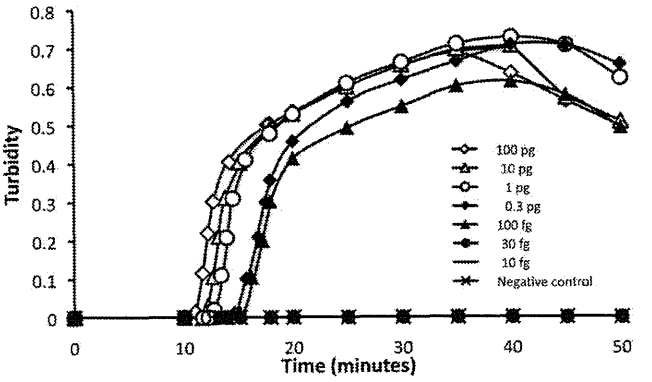

Figure 1 | Sensitivity of the LAMP method for *M. avium* detection.

Table 2 | Comparison of the DNA extraction methods[a]

Extraction method	Detection limit (CFU μl^{-1}) of sample	Processing time (h)	Use of corrosive reagent
Phenol/ chloroform/ isopropanol	$>10^5$ CFU 100 μl^{-1}	4	+
QIAamp DNA Micro Kit	10^3 CFU 100 μl^{-1}	25–26	–
Puregene yeast and Gram-Positive Bacteria Kit	10^5 CFU 100 μl^{-1}	2–3	–
Silica-based method	10^4 CFU 100 μl^{-1}	2–3	–
FTA elute card method	400 CFU 40 μl^{-1}	2–3	–

[a]DNA extracts were eluted with 30 μl of TE, and 4 μl of the extracted templates was used for the LAMP method.

The presence of contaminants such as dust, fungi, and other bacteria in the environmental samples necessitated the use of certain procedures, including pre-incubation for 3 h at 25 °C to bud fungi and spores, and the subsequent alkali-treatment to kill other microorganisms, to culture the organisms (Nishiuchi *et al.* 2009). We thus examined the effect of contaminants and the pretreatment procedures on the efficiency of DNA extraction. We used samples previously collected from the dust of air conditioners as a contaminant that contained many inorganic materials, bacteria, and fungi, but not mycobacteria (Nishiuchi *et al.* 2007). We pretreated the samples according to the previous method before extraction of DNA. Table 3 shows that both the presence of the dust and the pretreatment procedures hampered DNA extraction using the QIAamp DNA Micro Kit, but they had less effect on the efficiency of DNA extraction using the FTA elute card.

Table 3 | Effect of contaminants and pretreatment procedure on DNA extraction

	CFU of *M. avium* in sample (log_{10})								
	QIAamp DNA Micro Kit					FTA elute card method			
	6	5	4	3	2	4.6	3.6	2.6	1.6
Control	+	+	+	+	–	+	+	+	–
Added dust sample	+	+	–	–	–	+	+	–	–
Pretreatment[a]	+	+	–	–	–	+	+	+	–

[a]Pretreatment involved preculture for 3 h followed by alkali treatment.

We then evaluated the specificity of LAMP using genomic DNA from 23 different mycobacterial species and 10 other bacterial species. A successful LAMP reaction with species-specific primers caused turbidity in the reaction tubes. *M. avium* subsp. *paratuberculosis*, which causes Johne's disease, was also amplified using a *M. avium*-specific primer set and yielded a positive reaction. The specificity of amplification was further confirmed by restriction enzyme digestion of the LAMP products and melting curve analysis. As shown in Figure 2(a), restriction digestion yielded products that were in good agreement with the predicted sizes (171 and 163 bp). Furthermore, the peaks of the melting temperature curves were identical between *M. avium* genomic DNA and the environmental samples (Figure 2(b)).

Comparison between LAMP analysis and culture for the detection of *M. avium* in the environmental samples

In the present study, we used previously collected samples from bathrooms in the residences of 29 patients with pulmonary *M. avium* disease (Nishiuchi *et al.* 2009) and performed the DNA extraction followed by LAMP. The results were then compared with those obtained by culture (Table 4). Of a total

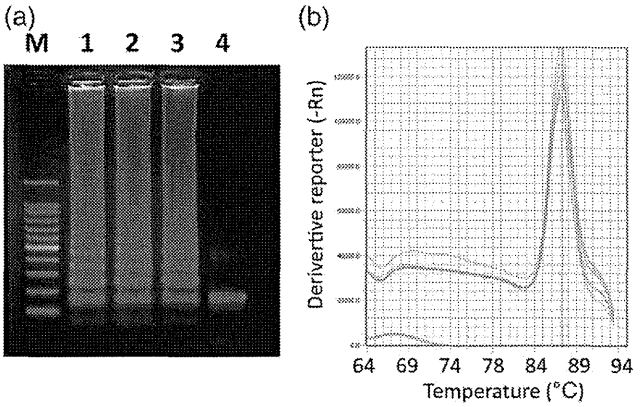

Figure 2 | The specificity of LAMP products. (a) LAMP products obtained from 10 pg of *M. avium* 104 DNA (Lane 1) and from nucleic acid extracts of pure cultures of *M. avium* 104 on the FTA elute cards (approximately 10^8 and 10^7 cells, Lanes 2 and 3, respectively), and restriction digestion of the products obtained from 10 pg of *M. avium* 104 DNA with *Taq*I (bands corresponding to 171 and 163 bp are expected, Lane 4). (b) The peaks of melting temperature curves of LAMP products amplified from control *M. avium* 104 DNA coincided with those from five isolates of environmental *M. avium*. Products from all isolates had a melting temperature of 87.3 °C.

Table 4 | Recovery and detection of *M. avium* from the residential bathrooms of patients with pulmonary *M. avium* disease using the culture and LAMP methods

	Number of samples						
Sampling site (sample type)	**Surface of the shower head (scale)**	**Inside the shower head (scale)**	**Shower (water)**	**Bathtub inlet (scale)**	**Bathtub (water)**	**Drain (slime)**	**Total no. of samples[a]**
No. of test samples	29	24	29	25	26	29	162 (29)
Culture positive	1	2	1	14	7	7	32 (15)
LAMP positive	3	0	3	13	6	6	27 (17)

[a]Numbers in parentheses represent the number of residences.
The result of the culture method is cited from the previous report (Nishiuchi *et al.* 2009).

of 162 samples *M. avium* was recovered from 32 samples (20%) by culture and detected in 28 samples (18%) by LAMP. Twenty samples (12%) were positive and 123 samples (76%) were negative for *M. avium* by both methods. The samples that tested positive by culture and/or the LAMP method are listed in Table 5. All samples that gave a *M. avium* recovery of >20 CFU/primary isolation plate by culture were also positive by LAMP.

DISCUSSION

Our data show that the combination of the FTA elute card (for DNA extraction) and the LAMP method is rapid and sensitive for detection of *M. avium* in environmental samples. It is advantageous over the culture method as it takes significantly less time and the entire procedure, from obtaining samples to *M. avium* detection, can be completed within 2–3 h. In general, the yields of DNA obtained from mycobacteria are low because of the presence of a robust, waxy cell wall that makes it difficult to lyse mycobacterial cells. Moreover, the samples examined in the present study, which were previously used for culturing the bacteria, had to be subjected to several procedures prior to DNA extraction, such as pre-culture to bud fungi, alkali-treatment to kill other microorganisms, freezing preservation, and concentration by centrifugation of the samples. These steps could decrease the recovery of DNA (Table 3) and the efficiency of NAA. Alkali-treatment may increase the concentration of alkali-soluble inhibitors such as humic substances that are widespread in the environment and are known to hamper PCR (Matheson *et al.* 2010). Although the presence of dust and pretreatment procedures hampered the efficiency of DNA extraction and the NAA method, they had minimal effect when the FTA elute card was employed. Therefore, use of the FTA elute card is likely to be suitable for examining the environmental samples containing mycobacteria, although it was originally developed to extract DNA from whole blood samples or buccal swabs (Tables 2 and 3).

Both the culture and LAMP methods yielded consistent results in 88% of the 162 examined environmental samples. In contrast, inconsistencies were observed with only 19 samples (12%; Table 5), which yielded <20 CFU/primary isolation plate by culture. It has been also reported that results obtained by NAA may show discrepancies with those obtained by culture (Iwamoto *et al.* 2003). This discrepancy is attributable to characteristic features of NAA, namely that it is capable of detecting DNA from dead cells. In addition, the NAA method is theoretically capable of detecting even a single copy of genomic DNA, but it is very susceptible to contamination with inhibitors and the efficiency of DNA extraction. Another possible reason for the discrepancy is the presence of viable but nonculturable (VNC) bacilli, although culture is theoretically capable of recovering a single viable bacterium. It has been recognized recently that the majority of bacteria in the environment enter a VNC state (Roszak & Colwell 1987). The pathogens in tap water (Moritz *et al.* 2010; Pawlowski *et al.* 2011) and in drinking water biofilms (Moritz *et al.* 2010) also enter the VNC state, as does *M. avium* in shower water or in the showerhead. This might also contribute to the discrepancy observed in the present study. However, whether *M. avium* can enter the VNC state remains uncertain. Further

Table 5 | List of all samples that tested positive by culture and/or LAMP methods

Participant no	Sampling site	Sample	Culture CFU/plate[a]	LAMP[b]
P-9	Bathtub inlet	Scale	>1,000	Positive
P-17	Bathtub inlet	Scale	>1,000	Positive
P-25	Bathtub inlet	Scale	>1,000	Positive
P-27	Bathtub inlet	Scale	>1,000	Positive
P-26	Bathtub inlet	Scale	>300	Positive
P-27	Bath drain	Slime	>300	Positive
P-8	Bathtub inlet	Scale	>100	Positive
P-12	Bath drain	Slime	>100	Positive
P-22	Bathtub inlet	Scale	>100	Positive
P-29	Bathtub inlet	Scale	>100	Positive
P-27	Bathtub	Water	47	Positive
P-12	Bathtub inlet	Scale	42	Positive
P-23	Bathtub inlet	Scale	20	Positive
P-29	Bath drain	Slime	13	–
P-17	Bath drain	Slime	9	Positive
P-8	Bath drain	Slime	6	Positive
P-28	Bathtub inlet	Scale	6	Positive
P-29	Bathtub	Water	6	Positive
P-9	Bathtub	Water	5	Positive
P-21	Bathtub inlet	Scale	4	Positive
P-26	Bathtub	Water	4	–
P-2	Bath drain	Slime	3	–
P-29	Showerhead inside	Scale	3	–
P-8	Bathtub	Water	1	–
P-9	Showerhead inside	Scale	1	–
P-9	Shower	Water	1	Positive
P-13	Bathtub inlet	Scale	1	–
P-15	Bathtub inlet	Scale	1	–
P-22	Bathtub	Water	1	–
P-23	Bath drain	Slime	1	–
P-25	Bathtub	Water	1	–
P-27	Showerhead surface	Scale	1	–
P-6	Shower	Water	–	Positive
P-9	Showerhead surface	Scale	–	Positive
P-9	Bath drain	Slime	–	Positive
P-11	Bathtub	Water	–	Positive
P-16	Showerhead surface	Scale	–	Positive
P-33	Showerhead surface	Scale	–	Positive
P-33	Shower	Water	–	Positive

[a]CFU/primary isolation plate where 200 μl of the sample was inoculated.
[b]The LAMP method was performed with 4 μl of the template in an assay mixture. The template was eluted from an FTA elute card with 30 μl of TE where 40 μl of the concentrated sample was originally applied to the FTA elute card.

studies are required to clarify this issue. In summary, all these facts should be taken into account when we assess environmental samples.

CONCLUSIONS

We demonstrated the utility of the LAMP method for the direct detection of *M. avium* in environmental samples by employing a novel set of six specific primers. Furthermore, we demonstrated that the FTA elute card is useful for DNA extraction from environmental *M. avium* without resorting to the use of harmful reagents. Thus, use of the LAMP method in combination with an FTA elute card for DNA extraction may facilitate the direct detection of environmental *M. avium* within a short period.

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Note

Roles of Ala-149 in the catalytic activity of diadenosine tetraphosphate phosphorylase from *Mycobacterium tuberculosis* H37Rv

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Diadenosine 5′,5‴-P^1,P^4-tetraphosphate (Ap_4A) phosphorylase from *Mycobacterium tuberculosis* H37Rv (MtAPA) belongs to the histidine triad motif (HIT) superfamily, but is the only member with an alanine residue at position 149 (Ala-149). Enzymatic analysis revealed that the Ala-149 deletion mutant displayed substrate specificity for diadenosine 5′,5″-P^1,P^5-pentaphosphate and was inactive on Ap_4A and other substrates that are utilized by the wild-type enzyme.

Key words: mycobacteria; phosphorylase; kinetics; drug target

Mycobacterium tuberculosis is the causative agent of tuberculosis (TB). In 2012, approximately 1.3 million people died from TB worldwide, and more than 8.6 million people developed TB. Furthermore, multidrug-resistant and extensively drug-resistant TB strains pose a growing public health threat and economic burden globally (World Health Organization, http://www.who.int/tb/publications/global_report/en/). This has created an urgent need to discover novel anti-TB drugs. To facilitate the structure-based design of new anti-TB drugs, our group has focused on a novel diadenosine 5′,5‴-P^1,P^4-tetraphosphate (Ap_4A) phosphorylase (EC 2.7.7.53), which converts Ap_4A to ATP and ADP in the presence of inorganic phosphate, from *M. tuberculosis* H37Rv (MtAPA).[1,2] *Rv2613c* gene, which encodes MtAPA, is an essential gene in *M. tuberculosis* H37Rv,[3] and *in silico* analyses have shown that MtAPA could be a target for new anti-TB drugs.[4] Mutations in an Ap_4A degradation enzyme lead to Ap_4A accumulation in cells, which in turn causes defects in transcription, sigmaF-mediated gene regulation, and catabolite repression.[5] Therefore, the development of specific MtAPA inhibitors could lead to production of a novel series of anti-TB drugs.

To facilitate the design of specific MtAPA inhibitors, we previously determined the three-dimensional structure of MtAPA and elucidated structure–function relationships.[2] Since the amino acid sequence of MtAPA has a histidine triad (HIT) motif (His-φ-His-φ-His-φ-φ, where φ represents a hydrophobic amino acid) (Fig. 1), the enzyme belongs to the HIT superfamily. This superfamily also includes nucleotide hydrolases and transferases, for example, *Saccharomyces cerevisiae* Ap_4A phosphorylases 1 and 2 (APA1_Yeast and APA2_Yeast, respectively) and *Homo sapiens* HIT family Ap_nA hydrolase (Fhit_Human) (Fig. 1 and [6]). In addition to MtAPA, the crystal structures of some HIT superfamily proteins have been reported.[7–11] The overall and active site structures of MtAPA are similar to those of other HIT superfamily proteins.[2] However, some amino acid residues that contribute to the active site of MtAPA are divergent from those of other HIT superfamily proteins (Fig. 1). In particular, an Ala residue at position 149 (Ala-149) is unique to MtAPA in comparison with all other HIT superfamily members (Fig. 1). Furthermore, Ala-149 is situated within a flexible loop that is located at the active site of MtAPA, suggesting that it could play a crucial role in modulating catalytic activity and substrate specificity of this enzyme.

The Ala-149 deletion was introduced into *Rv2613c* using the QuickChange Site-Directed Mutagenesis Kit II (Agilent Technologies, Inc., Santa Clara, CA) and the plasmid pMS2613c, which was previously constructed,[1] as the template. The Ala-149 deletion primer (5′-ggcgggtcgctggagcacctgcac-3′) and the Ala-149 deletion antisense primer (5′-gtgcaggtgctccgcgacccgcc-3′) were designed using QuickChange Primer Design Program (Agilent Technologies). The resulting plasmid was sequenced with an Applied Biosystems 3130*xl* Genetic Analyzer (Applied Biosystems, Carlsbad, CA) to confirm the mutations; verified DNAs were used to transform *Escherichia coli* BL21(DE3)pLysS (Promega, Woods Hollow Road Madison, WI) for overexpression of the Ala-149 deletion construct (Δ149A-MtAPA). The Δ149A-MtAPA was purified to homogeneity by a two-step column chromatography, as described previously.[1] Analysis of gel filtration chromatography indicated that the molecular weight of the Δ149A-MtAPA is approximately 100 kDa (data not shown), indicating that this substituent formed a homotetramer of 25-kDa subunits in solution, which was also observed with

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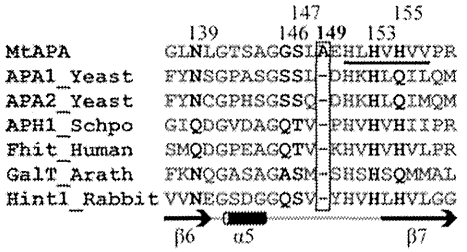

Fig. 1. Multiple sequence alignment of the active site region of MtAPA and HIT superfamily proteins (*S. cerevisiae* Ap_4A phosphorylases 1 and 2; APA1_Yeast and APA2_Yeast, *Schizosaccharomyces pombe* HIT family Ap_nA hydrolase; APH1_Schpo, *H. sapiens* HIT family Ap_nA hydrolase; Fhit_Human, *Arabidopsis thaliana* galactose-1-phosphate uridylyltransferases; GalT_Arath, and *Oryctolagus cuniculus* AMP-lysine hydrolase; Hint1_Rabbit).

Notes: Sequence alignment was performed using the ClustalW 2.1 software.[12] The HIT motif is underlined. The numbers along the top refer to the positions of amino acids in the sequence of MtAPA. The Ala-149 in MtAPA and the corresponding positions in other HIT superfamily proteins are shown in the box. In addition to Ala-149, the amino acids corresponding to Asn-139, Gly-146, Ser-147, His-153, and His-155 in MtAPA are shown in black, since these amino acids were previously presented as important residues for enzymatic activity of MtAPA,[2] and other amino acids are gray. The secondary structure of the active site based on the crystal structure of MtAPA is shown. The SWISS-PROT or TrEMBL accession numbers are as follows: MtAPA (**P9WMK9**), APA1_Yeast (**P16550**), APA2_Yeast (**P22108**), APH1_SCHPO (**P49776**), Fhit_Human (**P49789**), Galt_Arath (**Q9FK51**), and Hint1_Rabbit (**P80912**).

wild-type MtAPA. Because the tetrameric structure of wild-type MtAPA is reported to be essential for the formation of the Ap_4A-binding site,[2] we believe that the substrate-binding site of Δ149A-MtAPA is also closely linked to the tetrameric structure.

The enzyme activity of wild-type MtAPA and Δ149A-MtAPA was measured using a high-performance liquid chromatography system (Shimadzu, Kyoto, Japan) to quantify the amount of substrate remaining after the reaction as described previously.[1] The kinetic parameters of wild-type MtAPA and Δ149A-MtAPA were then compared. The K_m and k_{cat} values for Ap_4A of Δ149A-MtAPA were 0.19 ± 0.048 mM and 1.25 ± 0.099 s^{-1}, respectively. On the other hand, the K_m and k_{cat} values for Ap_4A of wild-type MtAPA were 0.10 ± 0.001 mM and 8.48 ± 0.313 s^{-1}, respectively (Supplemental Fig. 1).[1] Furthermore, the k_{cat}/K_m value for Ap_4A of Δ149A-MtAPA (6.58 mM^{-1} s^{-1}) was approximately 10% of that of wild-type MtAPA (84.8 mM^{-1} s^{-1}).

The substrate specificities of wild-type MtAPA and Δ149A-MtAPA were then compared. Although wild-type MtAPA could use a variety of substrates, vΔ149A-MtAPA showed diadenosine 5′,5‴-P^1,P^5-pentaphosphate (Ap_5A) specificity (Table 1). When wild-type MtAPA activity was further examined using various substrates, Ap_5A, Ap_4G, Gp_4G, and Gp_5G were phosphorylated with approximately the same efficiency as Ap_4A (Table 1). Furthermore, Ap_5G, Ap_4U, Ap_5U, and Ap_5dT were phosphorylated with approximately half the efficiency of Ap_4A (Table 1). On the other hand, the phosphorylation of all the dinucleotide polyphosphates by the Δ149A-MtAPA mutant was less than 50% compared to that of the Ap_5A substrate (Table 1). Moreover, the K_m and k_{cat} values for Ap_5A of Δ149A-MtAPA were 0.03 ± 0.006 mM and 2.21 ± 0.092 s^{-1}, respectively. On the other hand, the K_m and k_{cat} values for Ap_5A of wild-type MtAPA were 0.07 ± 0.007 mM and 8.29 ± 0.218 s^{-1}, respectively (Supplemental Fig. 1). Therefore, the k_{cat}/K_m value yielded by Δ149A-MtAPA with Ap_5A as a substrate (73.7 mM^{-1} s^{-1}) was approximately 10-fold higher than that for Ap_4A (6.58 mM^{-1} s^{-1}), but this difference was not observed when wild-type MtAPA was tested (118.4 mM^{-1} s^{-1} for Ap_5A and 84.8 mM^{-1} s^{-1} for Ap_4A).

Ala-149 is present on a loop that is located in the active site of MtAPA and does not appear to directly impact catalytic activity (Fig. 2(A)). The deletion of Ala-149 leads to shortening of the loop. Because other HIT superfamily proteins do not have the corresponding Ala-149 residue (Fig. 1), their loops are likely shorter, as exemplified by other HIT superfamily proteins (Fig. 2(B)). Furthermore, other HIT superfamily proteins do not show broad substrate profiles such as MtAPA; for example, the activities of APA1_Yeast and APA2_Yeast with Ap_4A are higher than those observed with Ap_3A and Ap_5A,[7] and Fhit_Human has higher activity toward Ap_3A when compared to other

Table 1. Nucleotide substrate utilization by wild-type MtAPA and Δ149A-MtAPA.

Nucleotides[a]	Relative activity (%)	
	Wild-type MtAPA[b]	Δ149A-MtAPA[c]
5′,5‴-P^1,P^3-triphosphate (Ap_3A)	19[d]	10
Ap_4A	100[d]	34
Ap_5A	106[d]	100
5′,5‴-P^1,P^6-hexaphosphate (Ap_6A)	25[d]	<1[e]
P^1-(5′-adenosyl)P^4-(5′-guanosyl) tetraphosphate (Ap_4G)	100[d]	23
P^1-(5′-adenosyl)P^5-(5′-guanosyl) pentaphosphate (Ap_5G)	76[d]	7
diguanosine 5′,5‴-P^1,P^4-tetraphosphate (Gp_4G)	107[d]	18
diguanosine 5′,5‴-P^1,P^5-pentaphosphate (Gp_5G)	106[d]	33
P^1-(5′-adenosyl)P^4-(5′-uridyl) tetraphosphate (Ap_4U)	53	12
P^1-(5′-adenosyl)P^5-(5′-uridyl) pentaphosphate (Ap_5U)	66	7
P^1-(5′-adenosyl)P^4-(5′-(2′-deoxy-thymidyl)) tetraphosphate (Ap_4dT)	6	<1
P^1-(5′-adenosyl)P^5-(5′-(2′-deoxy-thymidyl)) pentaphosphate (Ap_5dT)	41	24

[a]Substrate concentration 0.1 mM; the structures of the nucleotides are described in Supplemental Fig. 2.
[b]The relative activity in the presence of 0.1 mM Ap_4A was taken as 100%.
[c]The relative activity in the presence of 0.1 mM Ap_5A was taken as 100%.
[d]Values are from[1].
[e]Relative activity was below 1%.