Detection of *Vibrio cholerae* in street food (satar and otak-otak) by Loop-Mediated Isothermal Amplification (LAMP), multiplex Polymerase Chain Reaction (mPCR) and plating methods


1Faculty of Bioresources and Food Industry, Universiti Sultan Zainal Abidin, 22200 Besut, Terengganu
2Center for Southeast Asian Studies, Kyoto University, Kyoto, Japan
3Department of Food Science, Faculty of Bioresources and Environmental Sciences, Ishikawa Prefectural University, 1-308, Suematsu, Nonoiichi-shi, Ishikawa-ken, 921-8836 Japan
4Institute of Biological Sciences, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia
5Department of Food Science, Faculty of Food Science and Technology, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia
6Food Safety and Food Integrity, Institute of Tropical Agriculture and Food Security, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia

**Abstract**

This goal of this study was to investigate the presence of *Vibrio cholerae* in street food, namely satar and otak-otak, using Loop-Mediated Isothermal Amplification (LAMP), multiplex Polymerase Chain Reaction (mPCR) and conventional plating on Thiosulphate Citrate Bile-Salt Sucrose (TCBS) agar methods. A total of 78 satar and 35 otak-otak were purchased from different districts of Terengganu (Besut, Setiu, Kuala Terengganu and Kemaman). *V. cholerae* was found in satar with LAMP (10.3%), mPCR (10.3%) and plating (0%). No V. cholerae was found in otak-otak using the three methods. This might be due to *V. cholerae* able to survive in satar after grilling due to its thickness which may contribute to undercooking. This study concluded that low presence of *V. cholerae* in satar and otak-otak can be detected by molecular methods but not the conventional plating method. LAMP assay is a useful tool for rapid detection of pathogens in food due to its simplicity, highly sensitive and visual interpretation capability. Though the prevalence of *V. cholerae* was low in the samples, proper handling of this food will help in reducing the risk of acquiring infection from *V. cholerae* in contaminated samples.

1. **Introduction**

*Vibrio cholerae*, a member of the family *Vibriionaceae*, is a facultatively anaerobic, Gram-negative, and non-spore-forming curved rod about 1.4–2.6 mm long (Baumann *et al.*, 1984). *V. cholerae* live naturally in both marine and freshwater habitats and in association with aquatic animals (Feldhusen, 2000). The genus *Vibrio* of the family *Vibriionaceae* embraces more than 60 species, mostly marine in origin and its taxonomy is continuously being revised due to the addition of new species (Etinosa *et al.*, 2008). The natural biota of fish might contain some *Vibrio* species (Colwell, 1996; Supungul *et al.*, 2004).

*Satar* and *otak-otak* are popular street food in Terengganu that is usually consumed as snack or side dish. It is easy to find these foods in East Coast of Peninsular Malaysia, namely Terengganu and Kelantan due to the abundance of fish in these states. *Satar* and *otak-otak* normally used round scad fish (*selayang*) or chub mackerel fish (*kembung*) as their main ingredient because these types of fish are cheaper than other fish. In Malaysia, *satar* and *otak-otak* are fish-based street food products that are usually prepared and grilled in a large amount before being served to customers. They are exposed to the ambient temperature for a long period of time which might cause multiplication of microorganism. The grilling process for *satar* and *otak-otak* using charcoal require skills and doneness of the products are difficult to be determine. This is because if the grilling process is not handled carefully will result in the *satar* and *otak-otak* burnt on the outside but not cooked or undercooked inside. Thus, the microorganism or pathogen present in the sample will grow and cause
foodborne illness.

Oliver (2003) reported that, foods implicated in the spread of *Vibrio cholerae* include seafood (raw fish, crabs, shrimp, mussels, cockles, squid, oysters, clams), rice, raw pork, street vendor food, frozen coconut milk, and raw fruits and vegetables, with greater survival occurring in cooked foods. *V. cholerae* is the etiological agent of cholera which is transmitted to human by ingestion of contaminated food or water, eating raw or improperly cooked seafood or seafood products, and also direct fecal contact with food handlers. *V. cholerae* serogroups O1 and O139 are usually found as the main causes of this disease (Kaper *et al.*, 1995; Fraga *et al.*, 2007; Suzita *et al.*, 2009). This is due to more than 95% of these strains produce the cholera toxin (CT). The symptoms of cholera disease are characterized by abdominal cramps, nausea, vomiting, as well as massive acute diarrhoea. These symptoms cause the depletion of body fluids and electrolytes which are essential for life, causing dehydration to the infected person which might lead to death in severe and untreated cases (Rabbani and Greenough, 1999; Weinke *et al.*, 2008; Shrestha *et al.*, 2010). The aim of this paper is, therefore, to determine the presence of *V. cholera* in local popular street food, *satar*, and *otak-otak*, using novel molecular method LAMP, multiplex PCR and the conventional plating method.

2. Materials and methods

2.1 Sample collection

![Fish-based product sold as street food. A) Satar and B) Otak-otak](image)

Satar and *otak-otak* were chosen as popular fish-based street food products as shown in Figure 1. A total of 113 samples which consist of seventy-eight *satar* and thirty-five *otak-otak* were purchased from different stalls in districts of Terengganu such as Besut, Setiu, Kuala Terengganu, Marang and Kemaman. Each sample was labeled with an identification number to differentiate their place of origin.

2.2 Enrichment of *Vibrio cholerae* in *satar* and *otak-otak*

For enrichment method, 10 g of sample was weighed into a sterile stomacher bag and added with 90 mL of alkaline peptone water (APW) (Merck, Germany). The mixture was homogenized in the stomacher (Seward, UK) for 1 min at 250 rpm and incubated at 37°C for 24 h.

2.3 Isolation of *Vibrio cholerae* from *satar* and *otak-otak*

A loopful of the incubated samples was taken from the top pellicle and streaked onto duplicate selective medium TCBS agar plates (Merck, Germany). The agar plates were then incubated for 18–24 h at 37°C. The presumptive colonies of *V. cholerae* (yellow colonies with 2-3 mm diameter) on TCBS agar plate were selected for further confirmation using PCR.

2.4 DNA extraction

*V. cholerae* DNA from vegetable samples will be extracted using boiled-cell method as described in Tang *et al.* (2014) with slight modification. A portion of 500 µL enriched sample was centrifuged at 10,000 x g to pellet the cells. The supernatant was discarded, and the pellet will be re-suspended with 500 µL of sterile deionised water. The tube containing the re-suspended cells pellet was boiled at 100°C for 10 mins using digital dry bath (Corning, Japan). The boiled mixture was cooled at -20°C for 10 mins before undergone centrifugation for 10 mins at 10,000 x g. The supernatant containing DNA was used for *V. cholerae* detection by multiplex PCR or LAMP.

2.5 Molecular detection method

2.5.1 Multiplex PCR assay

A multiplex PCR was used to detect the presence and virulence gene (*hlyA*, *tcpI*, *ctxB*) of *Vibrio cholerae* in *satar* and *otak-otak*. Table 1 shows the sequence of specific primers used for detection of *V. cholerae*.

<table>
<thead>
<tr>
<th>Target genes</th>
<th>Nucleotide sequences 5′−3′</th>
<th>Tm (°C)</th>
<th>Amplicon size (bp)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>ctxB</em>−<em>F</em></td>
<td>ATG AGG CGT TTT ATT CCA TAC AC</td>
<td>57.5</td>
<td>128</td>
<td>Imani <em>et al.</em> (2013)</td>
</tr>
<tr>
<td><em>ctxB</em>−<em>R</em></td>
<td>TAC CAG GTA GTC AAC ATA TAG ATT CA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>tcpI</em>−<em>F</em></td>
<td>TGC GTG ATG CTA ATT GGA CT</td>
<td>60.4</td>
<td>444</td>
<td>Imani <em>et al.</em> (2013)</td>
</tr>
<tr>
<td><em>tcpI</em>−<em>R</em></td>
<td>TTC GGT TTG TTT GCT TGA TG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>hlyA</em>−<em>F</em></td>
<td>GGC AAA CAG CGA AAC AAA TAC C</td>
<td>59.0</td>
<td>738</td>
<td>Panicker <em>et al.</em> (2004)</td>
</tr>
<tr>
<td><em>hlyA</em>−<em>R</em></td>
<td>CTC AGC GGG CTA ATA CGG TTT A</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>
Multiplex PCR was performed in a 25 µL reaction mixture containing 12.1 µL of sterile ultra-pure water, 5 µL of 5X Green Go Taq Flexi Buffer (Promega, USA), 2 µL of 25 mM MgCl$_2$ solution (Promega, USA), 0.5 µL of 10 mM deoxynucleotide triphosphate (dNTP) (Promega, USA), 0.5 µL of 10 µM ctxB forward and reverse primers (IDT, Singapore), 0.5 µL of 10 µM tcpI forward and reverse primers (IDT, Singapore), 0.5 µL of 10 µM hlyA forward and reverse primers (IDT, Singapore), 0.4 µL of 5U/µL Go Taq DNA polymerase (Promega, USA), and 2 µL of DNA template.

The PCR assay was performed with the Veriti 96-Well Thermal Cycler (Applied Biosystems, Singapore). The PCR reaction initiated with pre-denaturation at 95°C for 5 mins, 35 cycles of denaturation at 95°C (45 s), annealing at 55°C (1 min) and extension at 72°C (1 min 30s), followed by a final extension at 72°C for 5 mins.

PCR products were run through electrophoresis using 1.0% of an agarose gel, 70V for 60 mins. DNA-molecular ladder, 100 bp, (Promega Madison, USA) was included for each electrophoresis gel run. The result was visualized using the Fujifilm LAS-3000 Imager (Fujifilm, Japan).

2.5.2 LAMP assay

LAMP assay was carried out in a total of 25 µL of reagents mixture containing 7.2 µL of sterile ultra-pure water, 12.5 µL of 2x Reaction Mix (RM) (Loopamp, Japan), 2 µL of 30.77 uM V. cholerae Primer Mix (PM) (Loopamp, Japan), 1 µL of Fluorescent Detection Reagent (FD) (Loopamp, Japan), 1 µL of Bst DNA polymerase (Loopamp, Japan), and 2 µL of DNA template. The reagents mixture was then be heated in a thermal cycler at 63°C for 60 mins (predetermined time) and terminated at 80°C for 10 mins. Finally, the LAMP products were observed for colour changes. The changed in colour from yellowish orange to green fluorescent indicated the LAMP product was positive, but if the colour remains unchanged, it indicated negative result.

3. Results and discussion

3.1 Prevalence of Vibrio cholerae in Samples

*Vibrio* spp. infection has been found to be one of the most common foodborne infections in Asia (Sutherland and Varnam, 2000). The infections are usually initiated by consumption of raw or undercooked seafood (Ottaviani et al., 2009; Baker-Austin, 2015), which may be contaminated by the presence of the pathogen in the marine environment (Sutherland and Varnam, 2000). Poor hygiene practices by food handlers during processing and post-processing of food may cause the pathogens to multiply and re-contaminated the food under favourable conditions which might pose a significant risk of food poisoning. Table 2 and Table 3 summarizes the prevalence of *V. cholerae in satar* and *otak-otak* obtained throughout this study.

The present study showed 7.96% of 113 samples fish

<table>
<thead>
<tr>
<th>District</th>
<th>Stall</th>
<th>N</th>
<th>Multiplex PCR</th>
<th>LAMP</th>
<th>Plating</th>
</tr>
</thead>
<tbody>
<tr>
<td>Besut</td>
<td>PB (1)</td>
<td>10</td>
<td>0 0 0 0 0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>PB (2)</td>
<td>5</td>
<td>0 0 0 0 0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>PR</td>
<td>5</td>
<td>0 0 0 0 0</td>
<td>0</td>
<td>0</td>
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<tr>
<td></td>
<td>T</td>
<td>10</td>
<td>0 0 0 0 0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Setiu</td>
<td>S</td>
<td>10</td>
<td>0 0 0 0 0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Kuala Terengganu</td>
<td>BR (1)</td>
<td>16</td>
<td>4 0 4 3 4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>BR (2)</td>
<td>12</td>
<td>4 0 4 4 4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Marang</td>
<td>M</td>
<td>5</td>
<td>0 0 0 0 0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Kemaman</td>
<td>K</td>
<td>5</td>
<td>0 0 0 0 0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>Total:</strong></td>
<td></td>
<td>78</td>
<td>8 0 4 7 8</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>District</th>
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<th>N</th>
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<tbody>
<tr>
<td>Besut</td>
<td>PB (1)</td>
<td>10</td>
<td>0 0 0 0 0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>PB (2)</td>
<td>10</td>
<td>0 0 0 0 0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Setiu</td>
<td>S</td>
<td>5</td>
<td>0 0 0 0 0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Kuala Terengganu</td>
<td>BR (1)</td>
<td>10</td>
<td>0 0 0 0 0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>Total:</strong></td>
<td></td>
<td>35</td>
<td>0 0 0 0 0</td>
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</tr>
</tbody>
</table>
-based street food products including 78 satar and 35 otak-otak which obtained from different districts in Terengganu were detected for V. cholerae. From the data collected, it showed that V. cholerae was only present in satar (8 satar), and not otak-otak. This means the prevalence of V. cholerae in satar (11.5%) was higher than otak-otak (0%). All the positive samples were purchased in Kuala Terengganu from two different stalls, BR (1) and BR (2).

This study revealed that V. cholerae was found in certain fish-based street food products commonly found in Malaysia, even though the detection is low. In accordance to Hong Kong, Food and Environmental Hygiene Department (FEHD) (2005), indigenous microflora in the live seafood might harbor Vibrio spp. at the time of seafood capture or harvest. Meanwhile, previous studies showed positive detection of V. cholerae in raw seafood samples such as shrimp, squid, crab, cockles, mussels, mackerel, clam, prawn, and fish (Elhadi et al., 2004; Ottaviani et al., 2009; Mrityunjoy et al., 2013). Differ with raw seafood, the presence of high salt content as one of the ingredients in the seafood products may reduce the growth of V. cholerae as it can grow in the salt range of 0.1–4.0% NaCl, while optimum is 0.5% NaCl (Rabbani and Greenough, 1999).

Besides that, V. cholerae grow optimally at 37°C within the temperature range of 10°C to 43°C (FEHD, 2005) and they are very sensitive to both heat and radiation, as well as dry condition (ICMSF, 1996), hence, its prevalence in seafood products especially in satar and otak-otak may be lowered as both of these street foods used traditional grilling method in their preparation process. This type of cooking method can accelerate the reduction of moisture content of the foods. However, if the process is not properly controlled, satar and otak-otak may be burnt on the outside but undercooked inside. As such, the contaminated satar and otak-otak will pose a risk of food poisoning due to Vibrios capable of surviving for up to 6 hours (Tang et al., 2017).

Generally, microorganisms need water for their growth. The range of water activity for V. cholerae is between 0.940–0.988, with an optimum water activity of 0.984 (Rabbani and Greenough, 1999). In addition, V. cholerae may survive for several days and multiply in food that is alkaline and moist (European Commission, 1998). Heating shellfish for several minutes until the internal temperature of at least 60°C is sufficient to kill the pathogenic Vibrios (Anon, 1991). Vibrios were found to be able to grow well in a variety of cooked foods with alkaline pH at 22°C, 30°C and 37°C, such as cooked prawns, hard boiled eggs and cooked mussels (Kolvin and Roberts, 1982).

The prevalence of V. cholerae in satar was higher than those found in otak-otak might result from the undercooked thicker and moist satar in certain samples. In contrast, otak-otak is less moist than satar because of its lower in size and thickness. Therefore, otak-otak may be cooked evenly and achieved adequate heat or temperature to eliminate the growth of V. cholerae in a shorter time than satar needed.

Besides that, it has been commonly known that water is an important factor in the transmission of cholera which has been implicated with six pandemics. Generally, V. cholerae is widely distributed in tropical aquatic environments and shows the highest numbers in temperate water. According to Wong and Desmarchelier (1999), the optimum water temperatures for the isolation of V. cholerae are 20-35°C, and culturable cells may no longer be detectable when the temperature drops below 16°C.

Both molecular methods employed in this study, namely PCR and LAMP, performed equally well in detecting the presence of Vibrio cholerae in samples collected. Figure 2 shows amplification using multiplex...
PCR assay detecting ctxB, tcpI and hlyA genes of *V. cholerae*. There was no *V. cholerae* isolate obtained from conventional culture detection method using TCBS agar. This result was in agreement with previous findings that showed that the culture method lacked sensitivity for the detection of low cell numbers compared to molecular method (Harwood et al., 2004). Loop-mediated isothermal amplification (LAMP) was introduced in the year 2000 (Notomi et al., 2000) uses single temperature at 63°C and requires an hour to complete the assay. Visual observation of positive and negative reactions was possible from the colour change in the LAMP assay without gel electrophoresis step. These advantages greatly reduce the analysis time of from 4 hours in conventional PCR to 1 hour in LAMP assay. Such method is important for a small and less equipped laboratory setting or during field work. Figure 3 shows the positive and negative reaction of LAMP method.

4. Conclusion

Both multiplex PCR and LAMP method performed equally in the detection of *V. cholerae* in *satar* and *otak-otak*. The prevalence of *V. cholerae* was found to be low in *satar* and *otak-otak* sampled in Terengganu. However, proper food handling should be practised at all times to prevent foodborne diseases resulted from undercooked and contaminated products.

Conflict of interest

The authors declare that there is no conflict of interest in the present study.

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