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Detection of non-typhoid *Salmonella* infection by citrus and citrus extracts in Lao PDRYutaka Midorikawa^{1*}, Satoshi Nakamura², Rattanaphone Phetsouvanh³, Manivanh Vongsouvaht³, Kaoru Midorikawa⁴¹Suzuka University Medical Science²Research Institute of National Center for Global Health and Medicine³Wellcome Trust–Mahosot Hospital Oxford University Tropical Medicine Research Collaboration⁴Department of Environmental and Molecular Medicine, Mie University Graduate School of Medicine

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ABSTRACT

Objective: To know the current state of non-typhoid *Salmonella* infection in Laos. To examine the usefulness of new screening methods for *Salmonella* using citrus. **Methods:** Non-typhoid *Salmonella* infection of person in Lao PDR was studied in this research (2004–2009). The site was Vientiane capital city in 2004. Research from rural villages locating suburb of Vientiane during 2005–2008 was carried out. Rural villages in Attapu province where ethnic minorities were living was searched for this study in 2009. During this research, to detect *Salmonella* strain, a new method using citrus and citrus extract named MY phenomenon that observing black ring (MIDO ring) on DHL agar was tried. The slice lemon and lime were used for this trial in 2004. After 2005, disk of ascorbic acid and citric acid were used for the device instead of citrus fruits itself. **Results:** During this research, 65 of 272 human samples (23.9%) were infected with non-typhoid *Salmonella*. **Conclusions:** During this study, the method using citrus and citrus extracts was accepted for the detection of *Salmonella*. This study shows that with citrus and citrus extract, detection of *Salmonella* is possible using only DHL media. Results suggest that infectious rate of non-typhoid *Salmonella* was high.

1. Introduction

Non-typhoid *Salmonella enterica* (*Salmonella*) is currently the most common bacteria that cause food poisoning in Japan[1]. As food hygiene, prevention of *Salmonella* food poisoning is an important issue[2]. *Salmonella* carriers are restricted to employment and engaged in food and food-service operation. Because of system improvement of the food sanitation, infectious rate of developed country as Japan are now decreased less than 0.1%[3].

However in developing country, risk of *Salmonella* seems rather high[4,5]. For example, we had done the research of fecal samples from person living in Dong Chan Island locating in Mekong River Vientiane capital city Lao PDR in 1994. As the results, 4 of 15 persons infected with the non-typhoid *Salmonella*[5]. Except this previous research, no information about non-typhoid *Salmonella* infection in Lao

PDR is available. Infection of *Salmonella typhi* is sometimes informed in the hospital in Vientiane[6]. However non-typhoid *Salmonella* is not seems to be important pathogen in the country.

Ten years after the previous research, from 2004 to until now, we have been continued the research of infectious rate of *Salmonella* in Lao. During the research, the new screening method with using citrus device was established[7].

Citrus device means sliced citrus such as lemons and limes *etc.*, or disks that are including ascorbic acid and citric acid *etc.* Those are used for isolate non-typhoid *Salmonella*.

When *Salmonella* was inoculate on DHL agar medium which include iron source with put the slice lemon *etc.*, a black ring appeared on the medium around sliced lemon *etc.* after 24 h incubation. We named MY phenomenon for this fact[8]. The black ring formed by MY phenomenon was also named MIDO ring. Among many bacteria species that produce H₂S, only non-typhoid *Salmonella* showed MIDO ring was shown. By means of MY phenomenon, non-typhoid *Salmonella* was able to detect by this screening method without using media for detection such as triple

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sugar iron(TSI), lysine indole mobility (LIM) etc. Research of *Salmonella* infection using MY phenomenon had been carried out in Lao PDR until established the method.

This study provides the detection of non-typhoid *Salmonella* in Lao by means of the method MY phenomenon using citrus extract.

2. Materials and methods

2.1. Ethical clearance

The research was carried on the ethical clearance of Ministry of Health National Ethics Committee for Health Research (No.102/NECHR). When collecting stool samples from people, it was subjected that their name, age and sex should not be published.

2.2. Used media and equipments

For the detection of *Salmonella*, these media etc. were used. Transport medium (Eiken kagaku Japan), Rappaport medium (Eiken Kagaku Japan), Rappaport–Vassiliadis Soya Peptone (RVS), Broth (OXSOID), Petri dishes (9 cm diameter equipment Eiken Kagaku Japan), DHL medium TSI (Eiken Kagaku Japan)^[9], LIM medium (Eiken Kagaku Japan), *Salmonella* O antigen serum (Denka Seiken)^[10].

(1)Location: Medical College in capital city Vientiane. Target: student 39 people (21–22 years old Lao) stool samples (Research in 2004). (2)Location: Phailom Village, Xaithani District near the capital Vientiane Laos. Target: Villagers (Lao) 63 random (Research in September 2005). (3) Location: Phailom Village, Xaithani District suburb of Vientiane, the capital of Laos. Target: 24 Villagers (Lao) everyone having the history of detection of *Salmonella* in previous research (Research in September 2006). (4) Location: Phailom Village, Xaithani District, suburb of Vientiane. Target: Villagers (Lao) that 60 people complained of abdominal pain (Research in September 2007). (5) Location: Khoksaat Village, Saitani District suburb of Vientiane, capital of Laos. Target: Residents Village (Lao), 57 stool samples (Research in September 2008). (6) Location: Pak Pae Village, Sanxai District, Attapeu Province Laos. Target: Residents Village (Alak ethnic minorities) 29 stool samples (Research in March 2009).

Collected stool samples stored in transport medium were suspended in 5 mL of Rappaport medium, then incubated (42 °C) time 24 to 48 h. Then one drop of cultured medium was inoculated on DHL medium by loop. After 24 h incubation (35°C), colonies with containing black part of iron sulfide (Fe₂S₃) by hydrogen sulfide (H₂S) production were scraped with swab. Next the colony on swab was inoculated by coating on surface of DHL medium. Then, in year 2004, sliced lemon or lime (5–7 cm in diameter) was put on the center of the petri dish. Following 24 h incubation (35 °C), MIDO ring of MY phenomenon was observed by naked eyes. The results using screening method by using TSI, and LIM medium (conventional method) were compared. The strain which showed MIDO ring by MY phenomenon was mixed with O anti-sera of *Salmonella*. If there was agglutination, the strain identified as *Salmonella*.

From the study 2005, instead of using citrus itself, by using disk of ascorbic acid that contained in citrus, *Salmonella* was tried to detect. For the disk 100 μL of 0.89 mol ascorbic

acid solution sucked into 10 mm diameter paper (Toyo filter paper Advantech TOYO Japan) and dried, then used as citrus device.

In 2006, before put the disk of ascorbic acid, the strain was inoculated to draw a circle on DHL's medium 2/3 diameters (60 mm) of the dish. From 2006–2009, this procedure was carried out.

Conventional method of screening using TSI, LIM medium did not use after 2006.

3. Results

All results were shown in Table 1 during 2004–2008.

In the research 2004, stool sample of total 39 students was collected. Seventeen of them possessed H₂S colony. Eleven students had *Citrobacter freundii* (*C. freundii*) and 6 of them infected with *Salmonella*. *Salmonella* infected rate was 15%. All 6 *Salmonella* strains isolated in this time showed MIDO ring and positive reaction by O anti-sera test (Figure 1). One strain in 11 *C. freundii* strain showed also same MIDO ring as *Salmonella*.



Figure 1. Black ring formed by slice lemon on *Salmonella* culture.

The research in 2005, H₂S positive strain was isolated from the 25 villager specimens of total 63 stool samples. Using screening of MY phenomenon (Figure 2) method and O anti-sera test, 18 villagers possessed *Salmonella* was shown. Though 7 *C. freundii* isolated, 2 of them formed same MIDO ring. MIDO ring was formed all of the *Salmonella* strain. The *Salmonella* infectious rate was 28.6% in this year.



Figure 2. MIDO ring formed by ascorbic acid disk paper.

Research in 2006, from total 24 specimen H₂S positive bacteria was isolated from 16 specimen and 7 of them were

Table 1Research results *Salmonella* infection by MY phenomenon from 2004–2009.

Research year and place	Total sample	Number of bacteria produced H ₂ S	Number of <i>Salmonella</i> detected	<i>Salmonella</i> infectious rate (%)
2004 Vientiane City	39	17	6	15.3
2005 Phailom	63	25	18	28.6
2006 Phailom	24	16	7	29.2
2007 Phailom	60	39	22	36.7
2008 Khoksaat	57	28	8	14.0
2009 Pak Pae	29	10	4	13.8
2004–2009	272	116	65	23.9

infected with *Salmonella*. Infectious rate of *Salmonella* was 29.2%. Other than *Salmonella*, isolated bacteria were 6 *C. freundii*, 1 *Proteus vulgaris* and 1 *Enterobacter cloacae*. Three out of 6 *C. freundii* showed same MIDO ring (Figure 3). After 24 h passed from culture started, *Salmonella* formed a small black ring around the disc. The black ring was seen on day 1 then disappeared after 1 more days passed. As other bacteria of *Salmonella*, for example *C. freundii* formed not only a small black ring, but a large black ring also formed on the outside. In case of *C. freundii*, though the small black ring disappeared after the second day, the large black ring did not disappear forever (Figure 4). Using this principle, *Salmonella* screening had done in particular to distinguish from *C. freundii*.

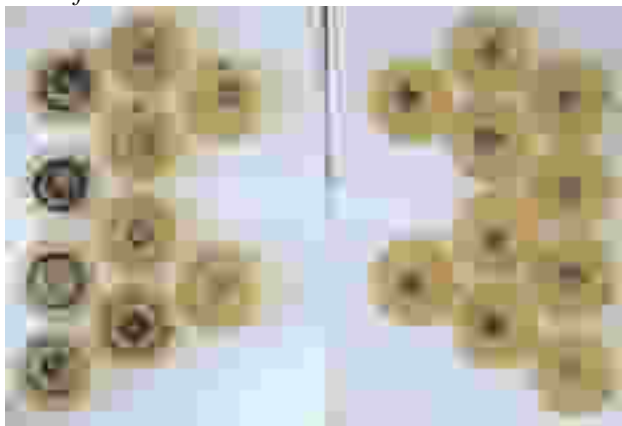


Figure 3. MIDO rings formed after 24 h incubation. Left: *C. freundii*, Right: *Salmonella*.



Figure 4. MIDO rings formed after 48 h incubation. Though small circle of *Salmonella* MIDO ring disappeared, outer black ring of *C. freundii* became clearer.

In the research 2007, total 25 H₂S positive colony was isolated, and 22 of 60 villagers were infected with *Salmonella*. Three villagers were infected with *C. freundii*. Infectious rate of *Salmonella* was 36%.

Study site of 2008 was Khoksaat Village near the Phailom Village. Twenty eight stool samples of 57 villagers possessed H₂S positive bacteria. *Salmonella* infection was shown in 8 villagers. Infectious rate or *Salmonella* was 14%. The research results of 2008 showed that people not only Phailom Village but also another village near Vientiane was highly infected by non-typhoid *Salmonella*.

In research of 2009, 10 of them possessed H₂S positive bacteria. *Salmonella* was isolated 4 out of 29 stool samples. The infectious rate of *Salmonella* was 13%. The research in March 2009 was carried not on rainy season but on dry season. The site was Attapeu Province not in Vientiane area. In past research, the survey site was near the Laos capital of Vientiane, this time, selected Attapeu Province southern Laos near the Vietnamese. Past study was carried out on September rainy season. This time the research had done in March dry and hot season. Even in dry season and in Alak ethnic person, *Salmonella* infection was high.

4. Discussion

This study had 2 aims. One of them was to establish the new method of screening *Salmonella* by using the citrus extracts such as ascorbic acid. Other was that by means of MY method, infectious rate of *Salmonella* was surveyed in Lao PDR. The results of 2004 indicate that citrus such as lemon and lime formed MIDO ring of MY phenomenon on *Salmonella* strains specifically. By using these fruits, possibility of a new screening method for *Salmonella* was shown.

Instead of using citrus itself, disk of ascorbic acid was used for device of MY phenomenon in the research 2005. The results showed that disk of citrus extracts were more useful than citrus itself.

Though very few case of *C. freundii* showed same reaction of *Salmonella* in the previous studies, distinguish *C. freundii* from *Salmonella* became possible by observing the MIDO rings by means of the method after 2006. After 2006, *Salmonella* became able to isolate from *C. freundii* by inoculating 2/3 diameters (60 mm) of the dish. Because

outer ring of *C. freundii* did not disappear by additional 24 h culture.

Without using the method of TSI, LIM medium *etc.*, and *Salmonella* were isolated with citrus device only research 2007–2009.

Using MY phenomenon was shown to be a more cost-effective screening procedure than the TSI–LIM screening. The method using MY phenomenon was improved in this study of *Salmonella* detection during five years in Laos.

In this study, an epidemiological study of *Salmonella* from 2004 to 2009, the results showed existence of 13%–36% of *Salmonella* carriers in Laos. At least 65 participants of 282 (23%) *Salmonella* carriers were detected in this research. When compared with Japan, the risk of *Salmonella* in Lao was a few hundred times more. The difference of infection rate between Lao and Japan shows disparity two countries in health status. Incidentally, the *Salmonella* infection rate in Japan is less than 0.1%.

In developing countries, non-typhoid *Salmonella* accounts for a steadily increasing proportion of human infections^[11]. Non-typhoid *Salmonella* is increasing recognized as an important pathogen associated with bacteremia especially in immune suppressed patients^[12–14].

Infectious rate of *Salmonella* was 23% in this research. This means that 1 of 5 persons were infected by *Salmonella*. Urban and rural area of Vientiane, the capital city, as well as further south Lao Attapeu Province even rainy and dry seasons, high prevalence of *Salmonella* was shown. The same results were getting even in Lao and ethnic minority. It can be concluded that the population at risk of *Salmonella* was high. Making effort of decrease infectious rate is needed.

Conflict of interest statement

We declare that we have no conflict of interest.

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