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Original article

A phenomenon useful for the detection of *Salmonella* implementing a device from citrus extracts

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Abstract: The effect of lemon slices, as well as ascorbic and citric acid impregnated paper discs, on the growth of ten non-typhoidal *Salmonella*, six *Citrobacter freundii* and four *Proteus mirabilis* species on Desoxycholate Hydrogen Sulfide Lactose (DHL) agar were examined in comparison to controls without fruit slices or paper discs applied. After 24 h incubation, thick black rings were observed around fruit slices and impregnated discs growing on non-typhoidal *Salmonella* serovars and not around the other species. We named this the "MY Phenomenon". We propose that the phenomenon can be used as a rapid, simple and inexpensive screening test that distinguishes non-typhoidal *Salmonella* species from other enterobacteriaceae in stool samples.

Key words: *Salmonella, Proteus, Citrobacter,* identification, hydrogen sulfide, citrus, ascorbic acid, citric acid, Laos, Japan

INTRODUCTION

Bacteria of the genus *Salmonella* contaminate chicken eggs and other foods and are the main food-poisoning pathogens globally [1]. The latest data in Japan shows that *Salmonella* is the second largest cause of food-poisoning (first among bacteria) [2]. Outbreaks of *Salmonella* food-poisoning still occur even in developed countries like Japan [3]. In Japan, when *Salmonell* strain is detected in human feces, the infected individual is barred by law from employment in food handling or processing.

In order to screen for *Salmonella*, stool samples of employees in the food industry are requested periodically for culture. However, the clinical laboratories in Japan and other developed countries rarely detect fecal *Salmonella* (≤ 0.1% of samples), [4] because the sanitation system has improved greatly. Since most fecal samples in Japan proved to be *Salmonella* negative, a cost effective method for the screening of *Salmonella* is needed. In the present study, bacteria producing hydrogen sulfide (H_2S) showed the same shape on media such as DHL agar for isolation, even when the species were different. For example, within the Enterobacteriaceae, *Salmonella, Proteus,* and *Citrobacter* produce H_2S on these culture media by reacting with the iron component in the agar and forming black colonies due to the production of ferrous sulphate [5]. Because the characteristic feature of *Salmonella*, *Proteus* and *Citrobacter* etc. is the production of H_2S, the *Salmonella* colonies are difficult to distinguish from those of other species [6,7]. A method to exclude non-*Salmonella* strains such as *Citrobacter* etc., that is a rapid high throughput screening method, is needed [8].

We developed a new method to isolate *Salmonella* from many other bacterial strains that produce H_2S [9]. In Japan, the usual method for the identification of *Salmonella* involves the initial cultivation of samples in EEM (enterbacteriaceae enrichment mannitol) broth and selenite broth medium with subsequent culture on DHL agar (Table 1) or *Salmonella Shigella* Sucrose Bromcresol purple (SS-SB) agar medium [10]. Although non- H_2S producing *Salmonella* such as typhoid *Salmonella* remain an exception [11], we describe a new technique in which non-typhoidal *Sal-*

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Table 1 Formula/Liter of DHL agar. Final pH: 7.2±0.2 at 25℃

Meat extract	3g
Peptone	20g
Lactose	10g
Sucrose	10g
Bile salt No.2	1g
Sodium thiosulfate	2.3g
Ferric Citrate	1g
Sodium Citrate	1g
Neutral Red	0.03g
Agar	15g

monella species can be differentiated from other Enterobacteriaceae by applying circular slices of lemon fruits (or citric or ascorbic acid-impregnated paper discs) to the bacterial cultures on DHL agar.

MATERIALS AND METHODS

Organisms

Non-typhoidal *Salmonella* serovar Typhimurium Thompson, Enteritidis, Braenderup, Bredeney, Oranienburg, Chester, Infantis, Litchfield and Saint-Paul were obtained from the Health Environment Research Department in Mie Prefecture, Japan. These had been isolated from *Salmonella* food-poisoning patients in Mie Prefecture, Japan. The identification of species and serotype was achieved by means of current standard methods of identifying Tri Sugar Iron (TSI, Eiken Kagaku Japan), as well as Lysin Indole Mobility semisolid agar (LIM Eiken Kagaku Japan) and serology test by serum set (Denka Seiken Japan). Six *Citrobacter freundii* and 4 *Proteus mirabilis* strains were isolated from a fresh meat market (Tarasao Markets) in Vientiane, Lao P.D.R.. These strains were screened using DHL agar and identified using not only the TSI and LIM medium but also API20E (bioMerieux, Lyon).

Citrus fruit

Japanese lemons (*Citrus limon*) were bought in Yamanaka Super Market in Suzuka, Mie, Japan.

Ascorbic acid and citric acid paper discs

Paper disks 10mm in diameter and 1 mm thickness (Toyo filter, Japan) were soaked in 100μL of ascorbic acid (0.89 mol) (Wako, Japan) and 100μL citric acid (1.41 mol Nakarai Japan) as an artificial citrus extract. Both of these acids are found in citrus fruits [12].

The effect of the application of lemon and lime slices on bacterial growth

Bacterial suspensions were adjusted to McFarland Turbidity No. 0.5 in 1-5ml of the sterilized normal saline solution from the overnight cultured nutrient agar medium (DIFCO). Sterilized swabs (Eiken, Kizai, Japan) were soaked in the bacterial suspension and then applied on 85 mm diameter Pertri dishes of DHL agar (Eiken Chemical, Tokyo, Japan), which contains ferric citrate (1g/L). One lemon slice (～5mm thick and 5cm in diameter) was applied on the medium surface over the inoculated bacteria. Control Petri dishes with bacterial suspensions on the DHL agar, but without the lemon slice, were also prepared. Control petri dishes of DHL agar with the lemon slice and without bacterial suspension were also prepared. Ten non-typhoidal *Salmonella* serotypes, 6 isolates of *Citrobacter freundii* and 4 isolates of *Proteus mirabilis* were applied using this technique. Subsequently, the above organisms were also inoculated on DHL agar, and paper disks impregnated with ascorbic acid and citric acid were placed tightly over the center of the agar medium. Control petri dishes of DHL agar with both acid discs but without bacterial suspensions were also prepared. After overnight culture, the growth of bacteria on the DHL agar was observed and examined for the formation of black rings.

RESULTS

Formation of black rings by Salmonella

After overnight incubation, black rings were observed on the DHL agar growing *Salmonella enterica* serovar Typhimurium with lemon applied, but not on those without lemon or those bacteria-free (Fig.1). Under the lemon, the color of the culture medium became pink and the *Salmonella* could not be subcultured. *Salmonella* could be subcultured from both the black ring and the outside of the black ring on the DHL agar. All of the (100%) ten different serotypes of non-typhoidal *Salmonella* tested formed the black ring (Fig. 2). Adopting the initials of the first author, we called this the “Midorikawa Yutaka (MY) Phenomenon”. However, of the 4 *Proteu*s strains, two did not turn the DHL agar black, while the other two strains changed the entire DHL agar between the lemon slice and the edge of the Petri dish black and did not form a discrete black ring(Fig. 3). Similarly, none of the 6 *Citrobacter* strains formed a discrete black ring. Three of them did not change the DHL agar to color black, and the other 3 strains changed the entire DHL agar between the lemon slice and the edge of the Petri dish to black. The latter 3 did not form a black ring (Fig. 3). All of the *Citrobacter* and *Proteus* species tested in the present study showed H_2S positive in the result of TSI medium (Table.2). Therefore, none of the *Ctrobactor* and *Proteus* species (0%) formed the black ring.

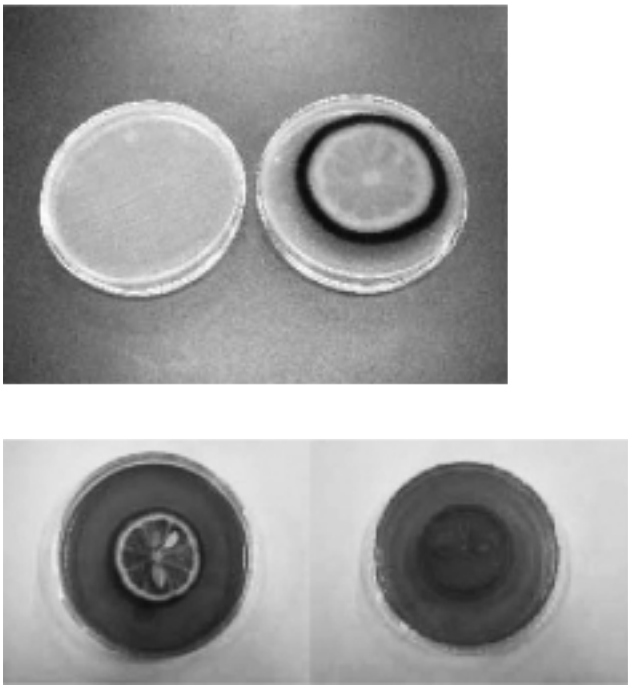

Figure 1. *Salmonella* serotype Typhimurium on DHL agar.
Upper Left: control Right: with lemon slice
Lower: With lemon and no bacteria inoculated on the agar
Left: From the front Right: From the back

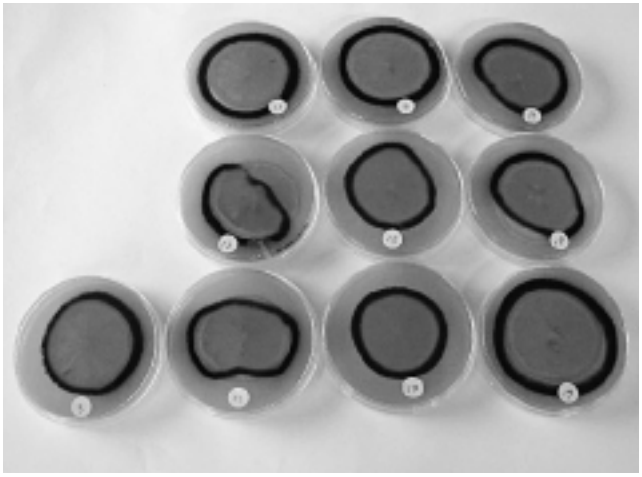

Figure 2. *Salmonella* of different serotypes on DHL agar with lemon slices.
From left to right:
Top: serotypes Typhimurium, Thompson, Enteritides,
Middle: Braenderup, Bredeney, Oranienburg
Lower: Chester, Infantis, Litchfield and Saint-Paul

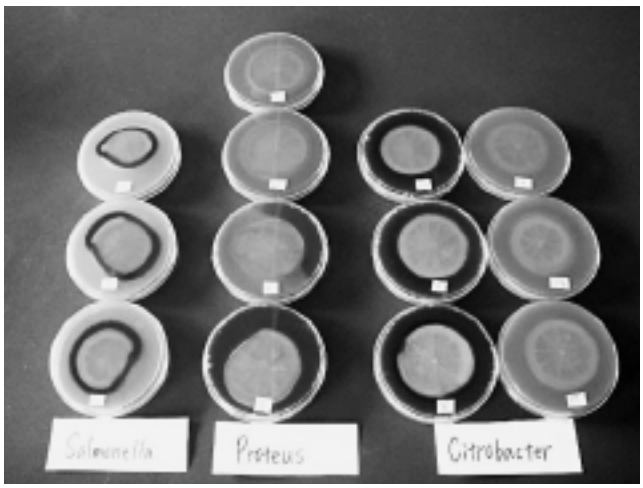

Figure 3. DHL agar color differences between non-typhoidal *Salmonella*, *Citrobacter* and *Proteus* cultures
Left: non-typhoidal *Salmonella* Center: *Proteus mirabilis*
Right: *Citrobacter* freundii.

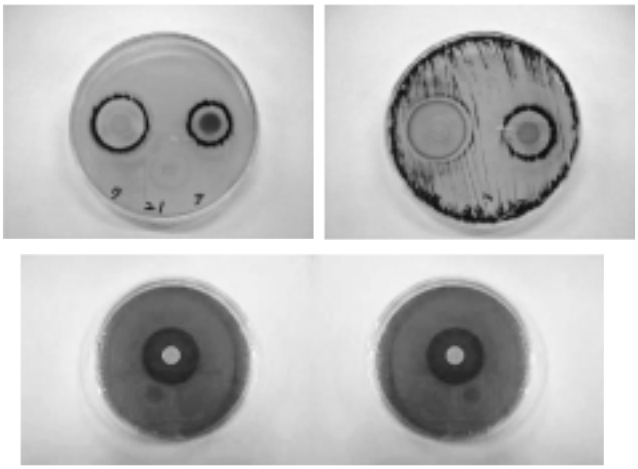

Fig 4. Effect of the ascorbic acid (left in dish) and citric acid (right in dish) on the color change of the growth of *Salmonella* Typhimurium (upper left) and *Citrobacter freundii* (upper right). Effect of the ascorbic acid (left in dish) and citric acid (right in dish) on the color change without bacterial growth (Lower).

Table. 2. Comparison of conventional methods implementing the MY phenomenon

Organism		MY Phenomenon: The Black Ring Formation				Not Clear Ring But Black to	Result of TSI Medium: Acid Production			Result of LIM Decarboxylase		Medium
		Lemon	Lime	Citric	Ascorbic	dish edge	Lac.or Su	Glucose	H_2S	Lysine	Indole	Motality
Salmonella	Typhimurium	+	+	+	+	−	−	+	+	+	−	+
Salmonella	Thompson	+	+	+	+	−	−	+	+	+	−	+
Salmonella	Enteritidis	+	+	+	+	−	−	+	+	+	−	+
Salmonella	Braenderup	+	+	+	+	−	−	+	+	+	−	+
Salmonella	Bredeney	+	+	+	+	−	−	+	+	+	−	+
Salmonella	Oranienburg	+	+	+	+	−	−	+	+	+	−	+
Salmonella	Chester	+	+	+	+	−	−	+	+	+	−	+
Salmonella	Infantis	+	+	+	+	−	−	+	+	+	−	+
Salmonella	Litchfield	+	+	+	+	−	−	+	+	+	−	+
Salmonella	Saint-Paul	+	+	+	+	−	−	+	+	+	−	+
Citrobactor freundii		−	−	−	−	+	−	+	+	−	−	+
Citrobactor freundii		−	−	−	−	+	−	+	+	−	−	+
Citrobactor freundii		−	−	−	−	+	−	+	+	−	−	+
Citrobactor freundii		−	−	−	−	−	−	+	+	−	−	+
Citrobactor freundii		−	−	−	−	−	−	+	+	−	−	+
Citrobactor freundii		−	−	−	−	−	−	+	+	−	−	+
Proteus mirabilis		−	−	−	−	+	−	+	+	−	+	+
Proteus mirabilis		−	−	−	−	+	−	+	+	−	+	+
Proteus mirabilis		−	−	−	−	−	−	+	+	−	+	+
Proteus mirabilis		−	−	−	−	−	−	+	+	−	+	+

Lac.: Lactose Su:Sucrose

Effect of ascorbic acid and citric acid

Both ascorbic acid and citric acid paper discs produced an MY phenomenon that exerted an effect on the growth of *Salmonella* and *Citrobacter* similar to that described above for lemon and Lao limes. Although not observed around the citrus slices, a 5 mm zone of bacterial clearing formed around the ascorbic acid and citric acid discs, demonstrating an anti-bacterial effect [13] (Fig.4 right upper). The clear black ring was observed in the case of *Salmonella,* although also observed in the case of *Citrobacter,* the black ring was not clear (Fig.4 upper left). Without bacterial growth, no black ring or black color was observed on the plate around the discs. The difference between *Salmonella* and *Citrobactor* was recognized clearly and macroscopically. All of the *Salmonella* (100%) that formed a black ring on DHL agar showed a lysin positive result. Therefore, the results of screening using both citrus extracts and current methods were the same (Tab.2).

DISCUSSION

The results suggested that the formation of a black ring at the circumference of the lemon slices is a peculiar reaction apparently limited to the non-typhoidal *Salmonella* species among the organisms tested. In view of the fact that citric acid and ascorbic acid are found in the lemon, we tested disks impregnated with these 2 components instead of the lemon and obtained the same results. Ascorbic acid and citric acid, ingredients of citrus fruits, produced a similar black ring effect around non-typhoidal *Salmonella* and in addition inhibited bacterial growth (Fig. 4). Although the inhibition of bacterial growth has already been reported [14], the black ring effect of *Salmonella* was observed for the first time in the present study. The results using the discs with citrus extract and showing no *Salmonella* growth demonstrated that the MY phenomenon is not an effect of these additives. Although other chemicals may also be responsible, these data imply that ascorbic acid and citric acid are key factors. *Salmonella*, *Proteus* and *Citrobacter* species produce H_2S in sulfur rich DHL agar that also contain ferric citrate, as do SS (Salmonella Shigella) agar SS-SB (Salmonella Shigella Sucrose Bromeresolpurple) agar medium and TSI (Tri sugar Iron) agar [15,16]. *Salmonella* species produce H_2S from sulfur-containing amino acid such as sodium thiosulfate and meat extract which are included in the DHL medium. This product reacts with ferric ammonium citrate and produces black ferrous sulfate. Without the addition of citrus slices, H_2S producing colonies turn black because of the formation of iron sulfide. With the addition of lemon, citrus or ascorbic acid, the area around the

colonies becomes an intense black. We suggest that the H_2S reacts with the ferric iron that is included in the DHL agar medium, producing ferrous sulfide under the influence of sodium thiosulfate included in the DHL medium. Chemicals in the citrus fruit, including ascorbic and citric acids, form a black band of ferrous sulfide. How this occurs is unclear, but Williams and Goodfellow [17] noticed, using peptone iron agar, that the ferric sulfide was unstable in aerobic conditions. It is possible therefore that the citrus fruits and acids stabilize the ferric sulfide or form further black sulfide complexes in the relatively anaerobic conditions beneath. However, Piacentini *et al.* [18] demonstrated that H_2S production decreases as pH falls to less than 5.6. We conclude that the ascorbic acid, citric acid etc. included in lemon and other citrus fruits enhanced the production of H_2S by *Salmonella* that forms a black ring on the medium. More information on the chemistry of the black ring production is required to explain why some *Citrobacter* and *Proteus* species produce black discoloration of the entire area around the fruit slices while non-typhoidal *Salmonella* form only a ring. We look forward to the work of a specialist in chemistry to elucidate this interesting mechanism.

The present study suggests the possibility that paper disks impregnated with ascorbic or citric acid may be used for non-typhoidal *Salmonella* screening. This study overcomes the defect of DHL agar, that is, the impossibility to distinguish *Salmonella* from other strains that produce H_2S. Thus, TSI and LIM etc. agar are also needed to screen for *Salmonella.* The screening by MY phenomenon and the conventional method using TSI and LIM culture medium was compared in this study, and the result was the same (Tab. 2). The screening for *Salmonella* using TSI and LIM culture media requires sterilization at a high temperature and high pressure. Toxic chloroform is required for the indole test in the LIM medium [19]. Chloroform is a toxic chemical because it produces the harmful gas phosgene. We prefer not to use instruments that involve high temperature and pressure. If screening for *Salmonella* is possible using ascorbic acid and citric acid discs, the use of TSI and LIM media may become unnecessary. The fact that only the relatively inexpensive and available DHL culture medium, ascorbic acid and citric acid are required is an additional advantage of this method. These initial results describe a potentially new, inexpensive and simple method of screening for non-typhoidal *Salmonella* in stool samples. The method is cost effective because both ascorbic and citric acid are inexpensive and therefore may provide the food industry and hospitals with a technique to easily detect *Salmonella* in food and feces. This method may be especially useful in developing countries in which the risk of *Salmonella* infections is high [20]. Recently, new and improved methods have been developed for the detection of *Salmonella* [21, 22]. However, methods of detection involving DNA analysis are still too expensive for use in developing countries [23]. By implementing the MY Phenomenon, inexpensive and readily available fruit slices or impregnated paper discs may provide a convenient and simple Salmonella screening method, especially in developing countries where the risk of salmonellosis is high and laboratory services inadequate. We have already carried out field research in Vientiane Lao P.D.R. A total of 11 *Salmonella* strains were isolated from 23 fresh meat (chicken, pork and beef) samples collected at Tarasao, Tonkankan and Tatruan Market in Vientiane Lao. Also, *Salmonella* were isolated from 18 out of 63 human feces samples. At that time we carried out both conventional methods (TSI, LIM medium and Api20) and the MY Phenomenon method. We repeated this assay many times, and the same results were obtained between the MY method and conventional methods in this research [24]. In the future, blinded prospective assessments should be conducted by other researchers using this technique in the diagnosis of these organisms.

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REFERENCES

1. Centers for disease control and prevention. (2000): Outbreak of *Salmonella* serotype Enteritidis infection associated with eating raw or undercooked shell eggs-United States, 1996-1998. Morb. Mortal. Weekly Rep. 49: 73-79.
2. Obana M. (2001) Bacterial food poisoning. {Article in Japanese}. Nippon Rinsho 59 Suppl. 27-47.
3. Yamane, I.(2006) Epidemics of emerging animal disease and food-borne infection problems over the last 5 years in Japan. Ann. N Y Acad Sci. 1081: 30-38.
4. Kodaka H.Mizuochi S.Honda T. and Yamaguchi K. (2000) Improvement of mannitol lysine crystal violet brilliant green agar for the selective isolation of H_2S-positive Salmonella. J. Food Prot. 63: 1643-47.
5. Pathak S.P. and Gopal K. (2005) Efficiency of modified H_2 S test for detection of faecal contamination in water. Envi-

ronm. Monit. Assess. 108: 59-65.
6. McWhorter A.C., Haddock R.L., Nocon F.A., Stergerwalt A.G., Brenner D.J., Aleksic, S., Bockemuhl, J. and Farmer, J.J. (1991) *Trabsiella guanensis*, a new genus and species of the family Enterobacteriaceae that resembles Salmonella subgroups 4 and 5. J. Clin. Microbiol. 7: 1480-85.
7. Vogel, B.F., Venkateswaran, K.,Satomi, M.and Gram, L. (2005) Identification of *Shewanella baltica* as the most important H_2S-producing species during iced storage of Danish marine fish. Appl. Environ. Microbiol. 71: 6689-97.
8. Ruiz, J., Sempere, M.A.,Varela, M.C. and Gomez, J.(1992) Modification of the methodology of stool culture for Salmonella detection. J. Clin. Microbiol. 30: 525-26.
9. Midorikawa Y., Nakamura S., Kishimoto K., Makino A. and Takenaka Y. (2005) Device for Salmonella Detection and Method of use thereof. The World Intellectual Property Organization International Secretariat WO 2005/078120 A1.
10. Manafi M. and Rotter M.L. (1991) A new plate medium for rapid presumptive edentification and differentiation of Enterobacteriaceae. Int. J. Food Microbiol. 14: 127-34.
11. Miller R.G. and Mallinson E.T. (2000) Improved detection of nontyphoid and typhoid Salmonellae with balanced agar formulations. J.Food Prot 63: 1443-46.
12. Sinoda, Y., Murata, M., Homma, S. and Komura, H. (2004) Browning and decomposed products of model orange juice. Biosci. Biotechnol.Biochem. 68: 529-36.
13. Velazpuez, L.C., Barbini, N.B.,Escydeeri, M.E. and DE Guzman, A.M. (2005) Resistance of *Yersinia enterocolitica*, *Esherichia coli* O157: H7 and natural microflora against acidic conditions and freezing-thawing in fresh sausages. Cent. Eur. J. Public Health. 13: 89-95.
14. Derrickson-Tharrington E. Kendall P.A. and Sofos J.N. (2005) Inactivation of *Escherichia coli* O157: H7 during storage or drying of apple slices pretreated with acidic solutions. Int. J. Food Microbiol. 99: 79-89
15. Comos H.J. and Shmid T. J. (1987) Failure to detect hydrogen-sulphide production in lactose / sucrose-fermenting Enterobacteriaccae, using triple sugar iron agar. Acta. Pathol. Microbiol.Immunol Scand. 95: 85-87.
16. Kudaka J., Horikawa K., Uuyu K., Matsuyuki S., Ogata K., Kawano K., Yamaguchi Y., Yawasaki S., Watanabe H. and Iwanaga M. (2005) Symptoms of food-borne diseases and gastroenteritis in Kyushu, Japan [Article in Japanese]. Kansenshogaku Zasshi. 11: 864-70.
17. Williams S.T. and Goodfellow M. (1966) Use of peptone iron agar for the detection of hydrogen sulfide. J.Bact. 91: 907-17.
18. Piacentitni I and Fracastoro G. (1970) Effetto del pH sulla precipitazione del solfuro di ferro nei terreni de klieger e TSI di hajna. Fracastoro LXⅢ, 800-03.
19. Reller L.B. and Mirrett S. (1975) Motility-indole-lysine medium for presumptive identification of enteric pathogens of Enterobacteriaceae. J. Clin. Microbiol. 2: 247-52.
20. Midorikawa Y., Nakamura S., IwadeY., Sugiyama A., Sisavath L. and Phakhounthong R. (1996) Bacterial diarrhea in Laos, a region where cholera was endemic. South East Asian J. Trop. Med. Public Health. 27: 723-27.
21. Cui S. Jie Z. and Jianghong M. (2006) An improved method for rapid isolation of *Salmonella* against *Proteus* in chicken carcasses. J. Food Safety 26: 49-61.
22. Geimba, M.P. Tondo E.C. Oliveira., F.A.,Canal C.W. and Brandelli A. (2004) Serological characterization and prevalence of *spv*R genes in *Salmonella* isolated from foods involved in outbreaks in Brazil. J.Food Prot. 67: 1229-33.
23. Li Y. and Mustapha A. (2002) Evaluation of four template preparation methods for polymerase chain reaction-based detection of Salmonella in ground beef and chicken. Letters Appl. Microbiol. 35: 508-14.
24. Midorikawa Y. (2007) The investigation of Salmonella detection method using the citrus extraction device in Laos. Tropical Medicine and Health. 35: 131.