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SHORT PAPER
Frequency of failure to isolate *Shigella* spp. by the direct plating technique and improvement of isolation by enrichment in selenite broth

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SUMMARY

In order to clarify the failure to isolate *Shigella* spp. by direct plating, we compared frequencies of *Shigella* spp. isolation by direct plating and by plating after enrichment in selenite broth. A total 67 strains were isolated in this study. The strains of 38 (56·7%) were isolated only by direct plating, and 25 (37·3%) strains were isolated by both direct plating and after enrichment. Four strains (6·0%) were isolated after enrichment but not by direct plating. Since 6% of isolated *Shigella* spp. were not isolated by direct plating, we recommend that direct plating and additional isolations from selenite broth should be performed. The significance concerning reduction of concentration of sodium selenite in enrichment broth is discussed.

Approximately 1000–1600 cases of shigellosis are reported annually in Japan [1], and the total number of shigella episodes that occur each year throughout the world is estimated to be 164·7 million [2]. The frequency of *Shigella sonnei* has increased in Japan. The symptoms of shigellosis caused by *S. sonnei* are mild in adults and some patients may be asymptomatic [1, 3]. However, extraintestinal lesions and diarrhoea caused by *Shigella* spp. disproportionately affect young children under 5 years of age [2]. The clinical diagnosis of shigellosis, as well as its differentiation from salmonellosis and other diarrhoeal diseases, has become difficult but still essential. In this situation, the laboratory examination for *Shigella* spp. is important to diagnose shigellosis.

Since Japan is located near Southeast Asia where shigellosis and salmonellosis leading to typhoid fever and paratyphoid fever are prevalent [4], we have paid special attention to those diseases. In the Japanese standard method [5, 6], the isolation of *Shigella* spp. from stool samples is performed by direct plating on agar plates such as Salmonella-Shigella (SS) agar and deoxycholate-hydrogen sulfide-lactose (DHL) agar, and *Salmonella* spp. are further isolated on the same agar plates after enrichment. Selenite broth is used as a selective enrichment medium for *Salmonella* spp. to eliminate other intestinal bacteria. Price et al. [7] reported, however, that *S. sonnei* could be isolated on agar plates after enrichment in selenite broth. We also sometimes found *Shigella* spp. on SS agar and DHL

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agar plates after the enrichment culture of samples from which direct plating could not isolate *Shigella* spp. We must determine the frequency of failure in the isolation of *Shigella* spp. in our system, and improve the enrichment culture for the bacteria. Recently, attention has been focused on the enrichment culture for the isolation of *S. sonnei* from foods [8, 9]. In clinical laboratory diagnosis, since intestinal flora such as *Escherichia coli* inhibits shigella multiplication [10], another enrichment broth for shigella is required not only for *S. sonnei* but also other *Shigella* spp., especially from stool samples.

In this study, we examined the frequency of *Shigella* spp. isolation after enrichment of stool samples that showed negative isolation by the direct plating technique. In order to determine the optimal concentration of sodium selenite for *Shigella* spp. in an enrichment broth, we examined the maximum growth allowance concentration (MAC) of sodium selenite for *Shigella* spp. using various clinical isolates.

Stool samples were collected from overseas travellers who reported a history of diarrhoea, in Osaka Airport Quarantine Station and in Kansai Airport Quarantine Station. Samples were examined by direct plating on SS agar (Eiken Chemical Co., Ltd., Tokyo, Japan) and DHL agar (Eiken Chemical Co., Ltd., Tokyo, Japan) plates at 37 °C for 18 h. The same samples were subjected to enrichment culture in standard selenite broth (Selenite Broth Base; Eiken Chemical Co., Ltd., Tokyo, Japan) containing 4 g/l sodium selenite at 37 °C for 12–18 h, followed by isolation culture on SS agar and DHL agar plates under the same conditions at the same time. Although the isolation was performed for the detection of *Salmonella* spp., shigella like colonies were screened using triple sugar iron agar (TSI; Eiken Chemical Co., Ltd., Tokyo, Japan) and lysine-indole-motility medium (LIM; Eiken Chemical Co., Ltd., Tokyo, Japan). Suspected isolates were examined by serotype testing using specific Shigella-O-antisera (Denka Seiken Co., Ltd., Tokyo, Japan). The serotyped strains were identified by biochemical tests such as amino acid and sugar assimilation tests.

We attempted the isolation of *Shigella* spp. from overseas traveller's stool samples by means of direct plating and after enrichment. A total of 67 strains of *Shigella* spp. were isolated, including 4 *S. dysenteriae*, 16 *S. flexneri*, 8 *S. boydii* and 39 *S. sonnei*. These isolated bacteria were classified into three patterns (Table 1): (A) positive on direct plating but negative after enrichment; (B) positive both on direct plating and after enrichment, and (C) negative on direct plating but positive after enrichment. Out of the 67 *Shigella* spp. isolated, 38 strains (56·7%) were isolated only by direct plating (pattern A). By both direct plating and after enrichment, 25 strains (37·3%) were isolated (pattern B). The direct plating technique failed to isolate 4 strains (6·0%) that were isolated after enrichment (pattern C). The strains in pattern C included one *S. dysenteriae* 2 *S. boydii* and 1 *S. sonnei*. One out of 4 *S. dysenteriae* strains (25%), 4 out of 16 *S. flexneri* strains (25%), 4 out of 8 *S. boydii* strains (50%) and 20 out of 39 *S. sonnei* strains (51·3%) could be isolated after enrichment. A total of 29 strains (43·3%) were observed on plating after enrichment. We could not clarify the relationship between specific serovar of each species and isolation patterns.

The Japanese standard method for laboratory diagnosis of *Shigella* spp. [5] recommends isolation of the bacteria only by direct plating. However, at least 6·0% of *Shigella* spp. could not be isolated by this standard method. The reason for this was probably the small amount of the bacteria in stool sample, which is the possible origin of shigellosis [11].

To investigate the frequency of sodium selenite-insensitive *Shigella* spp., clinical isolates were examined in terms of their MACs as follows; By the use of stored 33 clinical isolates of *Shigella* spp., the MAC of sodium selenite was measured in broth containing various concentrations of sodium selenite (0–10 g/l). Approximately 10^6 cells were inoculated into 6 ml of selenite broth, the depth of which was adjusted to 6·5 cm in a test tube, and culture was carried out at 37 °C for 15 h. After the culture, one loop of suspension culture was plated on nutrient agar plate (Eiken Chemical Co., Ltd., Tokyo, Japan) and culture was carried out at 37 °C for 18 h. Then, the number of colonies were counted. When more than 100 colonies were counted, the bacteria were considered to be insensitive to sodium selenite (Table 2). *S. dysenteriae* could not grow in the presence of sodium selenite at concentrations equal to or greater than 4 g/l. Only two strains of *S. dysenteriae* grew in the presence of 2 g/l sodium selenite. One each of *S. flexneri* strain grew in the presence of 4 g/l and 10 g/l sodium selenite, respectively. One and five strains of *S. boydii* grew in the presence of 4 and 10 g/l sodium selenite, respectively. All 10 strains of *S. sonnei* grew in the presence of sodium selenite, and 6 out of 10 strains grew in the presence of sodium selenite at concentrations 4 g/l or greater. When sodium selenite

Table 1. *Isolation of* Shigella *spp. by direct plating and after enrichment of stool samples**

Isolation pattern†	Species	Number of isolates	Serovar
(A) direct (+); enrichment (−)	*S. dysenteriae*	3	2, 3
	S. flexneri	12	1b, 2a, 2b, 3a, UT‡
	S. boydii	4	3, 8, 14, 18
	S. sonnei	19	I
	Total	38 (56·7%)	
(B) direct (+); enrichment (+)	*S. dysenteriae*	0	–
	S. flexneri	4	2a, 2b, 6
	S. boydii	2	1, 4
	S. sonnei	19	I
	Total	25 (37·3%)	
(C) direct (−); enrichment (+)	*S. dysenteriae*	1§	9
	S. flexneri	0	–
	S. boydii	2	2, 4
	S. sonnei	1	I
	Total	4 (6·0%)	
Total		67 (100%)	

* *Shigella* spp. from direct plating and after enrichment were obtained on SS agar and/or DHL agar plate.
† Isolation pattern: isolated bacteria were divided into three patterns: successful isolation by direct plating but failure after enrichment; successful isolation by both direct plating and after enrichment; failure of isolation by direct plating but successful isolation after enrichment.
‡ UT, untypable.
§ The isolate was stored for about 6 years after the isolation and became to be sensitive for sodium selenite in the experiment of MAC.

Table 2. *Insensitivity of* Shigella *spp. isolates to sodium selenite*

Species	Number of strains	Number of strains at MAC (g/l) of										
		0	1	2	3	4	5	6	7	8	9	10
S. dysenteriae	7	5	–	2	–	–	–	–	–	–	–	–
S. flexneri	7	–	–	2	3	1	–	–	–	–	–	1
S. boydii	10	1	–	1	2	1	–	–	–	–	–	5
S. sonnei	10	–	1	–	3	3	1	2	–	–	–	–
Total	34	6	1	5	8	5	1	2	–	–	–	6
Insensitive at 4–10 g/l*	14 (41·2%)											
Insensitive at 3–10 g/l†	22 (64·7%)											

* Total number (percentage) of insensitive strains at concentrations equal to or greater than 4 g/l of sodium selenite.
† Total number (percentage) of insensitive strains at concentrations equal to or greater than 3 g/l of sodium selenite.

insensitivity was defined as that an MAC of 4 g/l or greater, the frequencies of sodium selenite insensitivity of *S. dysenteriae*, *S. flexneri*, *S. boydii* and *S. sonnei* were 0/7 (0%), 2/7 (28·5%), 6/10 (60%) and 6/10 (60%), respectively. The frequencies are similar to those of isolates with isolation patterns B and C (Table 1), that is, 25%, 25%, 50% and 51·3%, respectively. It was clarified that the insensitivity of *Shigella* spp. to sodium selenite was high not only for *S. sonnei* but also for *S. boydii*.

In order to eliminate failure in isolation, we may have to apply methods for the detection of *Shigella*

spp. other than the Japanese standard method. One possible method is the use of nucleic acid diagnosis for *shigella* spp. The nucleic acid diagnosis for shigella such as PCR is sensitive and considered to be effective in monitoring the occurrence of shigellosis [12]. However, recent studies have shown the frequent detection of drug-resistant strains of *Shigella* spp. [13, 14], and suggested the necessity of an isolation culture of such drug-resistant strains for proper treatment and epidemiology.

Another possible method is the use of a shigella-specific enrichment culture. We have used selenite broth for the enrichment culture of *Salmonella* spp. An enrichment broth for *Shigella* spp. has been reported [7, 8, 15–17]; however, further development of more effective methods for the isolation of *Shigella* spp. is needed [11]. Since enrichment culture for only shigella is not available for stool samples, an enrichment broth for both *Shigella* spp. and *Salmonella* spp. is desired. In the meantime, we may isolate more *Shigella* spp. from selenite broth. It would eliminate isolation failure for at least 6·0% of *Shigella* spp.

Commercially available selenite broth contains 4 g/l sodium selenite, and 41·2% of the strains were found to be insensitive to sodium selenite at that concentration (Table 2). This frequency of sodium selenite insensitivity at 4 g/l or greater almost agreed with the frequency of isolates after enrichment of stool samples (patterns B and C in Table 1). When we decreased the concentration of sodium selenite to 3 g/l the broth, 22 out of 34 strains (64·7%) could be recovered from the enrichment culture in the broth (Table 2). It is, however, not clear whether selenite broth containing 3 g/l sodium selenite would improve the isolation of *Shigella* spp. from a stool containing *Salmonella* spp. and other contaminating bacteria, and also whether the broth maintains the selective enrichment for *Salmonella* spp. We, therefore, recommend that suspicious colonies on SS agar and DHL agar plates should be examined for not only *Salmonella* spp. but also *Shigella* spp., when such colonies grew on these agar plates after enrichment with selenite broth, even though the improvement of isolation was only 6%.

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