NOTE *Bacteriology*

Growth kinetics of *Salmonella enterica* in Hajna tetrathionate broth, Rappaport broth and modified semisolid Rappaport agar

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ABSTRACT. To determine the appropriate method for isolating *Salmonella enterica*, we compared the growth of *S. enterica* serovars using three selective enrichment media. *S. enterica* was more successfully isolated from artificially contaminated fecal samples after enrichment in Hajna tetrathionate broth or modified semisolid Rappaport agar than in Rappaport broth. Since most bacteria (other than motile *S. enterica*) do not migrate on modified semisolid Rappaport agar, the growth characteristics of *S. enterica* can be interpreted easily and quickly. Two *S. enterica* isolates did not migrate on modified semisolid Rappaport agar, but did grow in Hajna tetrathionate broth, which suggests that the combined use of these selective enrichment media is appropriate for isolating *S. enterica*.

KEY WORDS: modified semisolid Rappaport agar, *Salmonella*, selective enrichment medium

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Salmonella enterica is the most common foodborne disease worldwide as well as a diarrheal pathogen in livestock. Isolation of *S. enterica* from asymptomatic carrier animals can be used to assess microbial risk and to monitor the level of contamination in domestic animals. Although direct plating using selective media has been successful in isolating *S. enterica* strains, an enrichment step was necessary for enhancing the detection and isolation of the target pathogen [11]. A number of selective enrichment broth media are available, but relatively few studies have evaluated methods of bacterial isolation from fecal samples compared with food samples. Highly selective broth media with an enrichment step have been used to isolate target strains from contaminated samples [4]. Some reports have suggested that the high specificity and sensitivity of semisolid agar enhance *S. enterica* isolation [2, 6, 8, 13]. In this study, we investigated the growth kinetics of several *S. enterica* strains in two commercially available selective enrichment broth media and modified semisolid Rappaport agar (MSRA). MSRA is simple and inexpensive to prepare, and is similar in composition to commercially available modified semisolid Rappaport Vassiliadis agar. The present study determined the growth kinetics of twenty *S. enterica* serovars in three selective enrichment media to propose a rational method for isolation of *S. enterica*.

First, we determined bacterial growth ability in buffered peptone water (BPW), Hajna tetrathionate broth (HTTB) (Eiken, Tokyo, Japan), Rappaport broth (RB) (Eiken) and on MSRA. MSRA consists of casamino acid (5 g) and agar (3 g) in a 50% concentration of RB (2.5 g of peptone, 4.0 g of NaCl, 0.8 g of KH_2PO_4, 10.15 g of $MgCl_2$ and 0.06 g of malachite green) (1 *l*). As summarized in Table 1, *Enterobacter cloacae*, *Citrobacter freundii* and 20 *S. enterica* serovars (25 strains) were used in this study. All bacterial strains were isolated from livestock in the Hokkaido prefecture from 2004 to 2013 and were identified using biochemical tests and/or *S. enterica* serotyping. Approximately 15,000 CFU of bacterial cells grown in BPW were inoculated in 5 m*l* of each broth and were incubated at 37°C. The culture was serially diluted and spread on MacConkey agar (Nissui Pharmaceutical, Tokyo, Japan) plates to enumerate bacterial cell counts after 5 or 8 hr (logarithmic phase) and 24 hr (stationary phase). In addition, 3,000 CFU/10 μl of *S. enterica* were spotted on the MSRA. After a 24 hr incubation at 40°C, the migration zone diameters were measured. The results are shown in Table 1. Bacterial cell counts after the 5 hr incubation in BPW revealed a similar growth rate for each bacteria, except for the slow-growing *S*. Abortusequi. In contrast, after an 8 hr incubation in HTTB or RB, the bacteria showed varying periods of lag phase. After a 24 hr incubation, *S.* Abortusequi, *S*. Dublin and *C. freundii* had not grown in HTTB, RB or MSRA. *S*. Choleraesuis and *E. cloacae* did not grow in HTTB and RB, respectively. *S*. Uganda, *S.* Abortusequi, *S*. Dublin and *C. freundii* had decreased cell numbers after incubation in RB (less than 10^3 CFU/m*l*). Generally, host-adapted and host-restricted *S. enterica* were not well isolated after selective enrichment [1, 13]. Most of these serovars cause systemic infection and have lost some beneficial genes needed to grow in the intestinal lumen, such as tetrathionate utilization [3, 5, 12]. As a result, these serovars may have lost the ability to grow in HTTB. However, since Baggesen *et al*. reported that isolation rates of *S*. Dublin (at a concentration of 10 CFU/g feces) were 89.3% and 54.0% after selective enrichment on modified semisolid Rappaport Vassiliadis agar and in Mueller Kauffman tetrathionate broth, respectively [2], other

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Table 1. Growth in the enrichment media used in this study

Organism	Strain	Source	Log CFU/m*l*							Migration zone diameter on MSRA (mm)
			at 0 hr	in BPW at 5 hr	in HTTB at 8 hr	in RB at 8hr	in BPW at 24 hr	in HTTB at 24 hr	in RB at 24 hr	
C. freundii	Ab-cf	Pig	3.44 ± 0.05	7.30 ± 0.19	3.20 ± 0.14	3.21 ± 0.24	8.37 ± 0.08	<3	<3	NG*
E. cloacae	Hi-ec	Poultry	3.34 ± 0.13	7.25 ± 0.12	3.69 ± 0.38	3.57 ± 0.82	8.35 ± 0.03	7.26 ± 0.25	4.04 ± 0.28	NM**
S. Abony	To-ab	Cattle	3.58 ± 0.09	7.46 ± 0.03	3.57 ± 0.03	4.58 ± 0.48	8.63 ± 0.10	8.39 ± 0.09	6.80 ± 0.26	43.3 ± 7.69
S. Abortusequi	Ne-ae	Horse	3.60 ± 0.15	5.85 ± 0.39	<3	<3	7.91 ± 0.20	3.16 ± 0.09	<3	NG
S. Abortusequi	Ab-ae	Horse	3.61 ± 0.07	5.82 ± 0.20	<3	<3	7.72 ± 0.07	3.53 ± 0.31	<3	NG
S. Choleraesuis	Ab-ch	Pig	3.46 ± 0.02	6.58 ± 0.21	3.48 ± 0.11	4.98 ± 0.51	8.81 ± 0.31	3.52 ± 0.10	7.48 ± 0.04	48.3 ± 6.44
S. Derby	To-de	Pig	3.60 ± 0.07	7.20 ± 0.12	4.49 ± 0.44	4.07 ± 0.23	8.46 ± 0.02	8.35 ± 0.06	7.54 ± 0.03	NM
S. Dublin	Ab-du	Cattle	3.39 ± 0.04	6.59 ± 0.06	3.15 ± 0.19	<3	8.45 ± 0.11	3.37 ± 0.19	<3	NG
S. Dublin	To-du	Cattle	3.41 ± 0.04	7.23 ± 0.26	<3	<3	8.47 ± 0.06	3.58 ± 0.26	<3	NG
S. Enteritidis	Ab-en	Cattle	3.56 ± 0.14	7.42 ± 0.21	3.70 ± 0.10	3.76 ± 0.16	8.57 ± 0.10	8.46 ± 0.09	6.92 ± 0.19	60.3 ± 2.33
S. Enteritidis	To-en	Cattle	3.41 ± 0.06	7.43 ± 0.10	5.90 ± 0.18	4.38 ± 0.4	8.44 ± 0.05	8.42 ± 0.06	7.50 ± 0.07	22.7 ± 0.33
S. Isangi	To-is	Poultry	3.61 ± 0.12	7.65 ± 0.07	4.68 ± 0.29	5.06 ± 0.09	8.58 ± 0.05	8.52 ± 0.11	7.61 ± 0.03	54.7 ± 6.06
S. Kedougou	Ne-ke	Cattle	3.46 ± 0.03	7.36 ± 0.10	4.88 ± 0.36	4.08 ± 0.25	8.47 ± 0.02	8.17 ± 0.14	7.21 ± 0.15	66.7 ± 6.69
S. Livingstone	Ab-li	Pig	3.54 ± 0.02	7.63 ± 0.18	5.95 ± 0.39	5.79 ± 0.26	8.59 ± 0.01	8.51 ± 0.03	7.53 ± 0.05	78.3 ± 2.73
S. Mbandaka	Ne-mb	Cattle	3.53 ± 0.13	7.59 ± 0.07	4.71 ± 0.42	5.40 ± 0.03	8.51 ± 0.09	8.52 ± 0.11	7.58 ± 0.08	65.0 ± 2.65
S. Montevideo	To-mo	Cattle	3.56 ± 0.04	7.67 ± 0.03	4.85 ± 0.45	5.60 ± 0.29	8.52 ± 0.05	8.33 ± 0.03	7.5 ± 0.06	52.7 ± 4.48
S. Newport	To-ne	Poultry	3.42 ± 0.05	7.58 ± 0.09	4.42 ± 0.81	4.50 ± 0.59	8.47 ± 0.02	8.20 ± 0.18	7.03 ± 0.06	48.0 ± 1.15
S. Paratyphi B	Ne-pb	Cattle	3.38 ± 0.03	7.24 ± 0.01	5.42 ± 0.67	4.95 ± 0.11	8.43 ± 0.09	8.41 ± 0.09	7.45 ± 0.06	26.3 ± 3.18
S. Ruiru	Ne-ru	Cattle	3.45 ± 0.03	7.52 ± 0.14	3.56 ± 0.02	5.31 ± 0.16	8.45 ± 0.04	8.36 ± 0.08	7.48 ± 0.07	62.0 ± 1.00
S. Saintpaul	Ne-sa	Cattle	3.56 ± 0.09	7.62 ± 0.19	4.37 ± 0.29	4.05 ± 0.31	8.52 ± 0.03	8.43 ± 0.07	7.10 ± 0.30	41.0 ± 3.00
S. Schwarzengrund	Ne-sc	Cattle	3.60 ± 0.12	7.24 ± 0.09	4.83 ± 0.47	5.14 ± 0.14	8.65 ± 0.10	8.58 ± 0.09	7.56 ± 0.06	>90
S. Senftenberg	Ne-se	Cattle	3.55 ± 0.06	7.65 ± 0.09	4.71 ± 0.67	5.61 ± 0.20	8.63 ± 0.01	8.51 ± 0.05	7.39 ± 0.20	84.3 ± 3.18
S. Typhimurium	Ab-ty	Pig	3.62 ± 0.09	7.52 ± 0.16	3.80 ± 0.20	3.62 ± 0.03	8.61 ± 0.03	8.40 ± 0.18	7.30 ± 0.08	57.3 ± 2.33
S. Typhimurium	Hi-ty	Pigeon	3.46 ± 0.1	7.55 ± 0.05	4.80 ± 0.53	3.52 ± 0.06	8.56 ± 0.03	8.01 ± 0.37	7.28 ± 0.09	35.7 ± 0.67
S. Uganda	To-ug	Cattle	3.39 ± 0.12	7.67 ± 0.08	4.11 ± 0.70	3.23 ± 0.58	8.55 ± 0.03	7.26 ± 0.29	<3	NG
S. 4:i:-	Ab-4i	Poultry	3.51 ± 0.03	7.57 ± 0.11	4.95 ± 0.55	4.74 ± 0.20	8.60 ± 0.10	8.15 ± 0.31	7.44 ± 0.03	54.0 ± 2.65
S. 4:i:-	Hi-4i	Cattle	3.41 ± 0.11	7.43 ± 0.03	4.27 ± 0.18	5.10 ± 0.15	8.50 ± 0.04	7.70 ± 0.18	6.91 ± 0.20	69.0 ± 2.08

Values are mean ± SE. *NG: No growth, **NM: No migration.

strains of these host adapted and restricted serovars may grow in HTTB, RB or on MSRA. In contrast, 16 *S. enterica* serovars grew in HTTB (10^{7-8} CFU/m*l*), RB (10^{6-7} CFU/m*l*) and MSRA (migrated 22.7–>90 mm in diameter). Although colony formation was observed, the non-motile *S.* Derby did not migrate on MSRA. Interestingly, *E. cloacae* did not migrate on MSRA at 40°C, however, migration occurred at 37°C (46.0 ± 4.2 mm). The cost of maintenance of a flagellar motility system is high for bacteria, and the expression of the flagellar system is strictly regulated under stress conditions [14]. A higher incubation temperature may be necessary for enhancing selective methods.

Next, the efficacy of each selective enrichment medium for detection of *S. enterica* strains in fecal samples was investigated. Based on their growth in the selective enrichment media, the *S.* Livingstone (short period of lag phase), *S.* Typhimurium Ab-ty strain (long period of lag phase) and *S. enterica* serovar 4:i:- (*S.* 4:i:-) Hi-4i strain (less CFU in stationary phase) were used for further examination. Three of each type of fecal sample (bovine, swine and poultry) were artificially contaminated with 8–80,000 CFU/0.1 g of *S. enterica* and inoculated into 5 m*l* of HTTB and RB or on MSRA. After a 24 hr incubation at 37°C (HTTB and RB) or 40°C (MSRA), each culture was plated onto ES salmonellae agar II (Eiken) for *S. enterica* isolation. The number of positive test results in the three fecal samples is shown in Table 2. *S. enterica* detection limits were fewer in bovine feces than in swine and poultry feces. Compared with HTTB and MSRA, RB had lower isolation rates, especially in the case of the *S.* 4:i:- Hi-4i strain. On MSRA, it was possible to make "motile *S. enterica* negative" judgment easily, since the bacteria in the fecal samples had not migrated (Fig. 1). Moreover, the migration zone was pure *S. enterica* culture conditions, and the O-antigen was determined directly from the MSRA using *S. enterica* typing antisera (Denka Seiken, Tokyo, Japan) without subculture on isolation agar plates. Although inoculum volume was relevant to successful *S. enterica* isolation, and Harvey *et al.* recommended a small inoculum size in Rappaport broth and a larger size in selenite and tetrathionate broth [7], the sensitivity of MSRA is not restricted by inoculum volume since *S. enterica* attached to MSRA will spread from the samples and grow into the sterile area.

Finally, we examined the growth kinetics of cold-stressed *S.* Livingstone and *S.* Typhimurium Ab-ty strain with consideration for enhancing stress recovery increase *S. enterica* isolation from environmental samples [15]. Stress exposure was examined according to the method of Kim *et al.* [9] with slight modification. Briefly, *S. enterica* strains in mid-log phase were incubated in PBS at 4 or 25°C for 24 hr. Cold stress conditions resulted in approximately 1.5-log reduc-

Table 2. Isolation of *S. enterica* in artificially contaminated cattle, porcine and poultry feces

Strain and inoculated CFU/0.1 g	The number of isolation positive out of three samples								
	Cattle feces			Porcine feces			Poultry feces		
	HTTB	RB	MSRA	HTTB	RB	MSRA	HTTB	RB	MSRA
S. Livingstone Ab-li									
8	3 (2)*	3 (3)	3 (3)	3 (3)	3 (2)	3 (3)	3 (1)	3 (3)	2 (2)
78	3 (3)	3 (3)	3 (3)	3 (3)	3 (2)	3 (3)	3 (2)	3 (3)	3 (3)
780	3 (3)	3 (3)	3 (3)	3 (3)	3 (3)	3 (3)	3 (2)	3 (3)	3 (3)
7,800	3 (3)	3 (3)	3 (3)	3 (3)	3 (3)	3 (3)	3 (3)	3 (3)	3 (3)
78,000	3 (3)	3 (3)	3 (3)	3 (3)	3 (3)	3 (3)	3 (3)	3 (3)	3 (3)
S. Typhimurium Ab-ty									
8	3 (3)	3 (2)	3 (3)	3 (3)	2 (0)	3 (3)	3 (2)	1 (1)	3 (3)
82	3 (3)	3 (3)	3 (3)	3 (3)	2 (1)	3 (3)	3 (3)	3 (2)	3 (3)
820	3 (3)	3 (3)	3 (3)	3 (3)	3 (1)	3 (3)	3 (3)	3 (2)	3 (3)
8,200	3 (3)	3 (3)	3 (3)	3 (3)	3 (3)	3 (3)	3 (3)	3 (3)	3 (3)
82,000	3 (3)	3 (3)	3 (3)	3 (3)	3 (3)	3 (3)	3 (3)	3 (3)	3 (3)
S. 4:i:- Hi-4i									
7	3 (3)	3 (2)	3 (3)	1 (1)	1 (1)	2 (2)	1 (0)	0	2 (2)
73	3 (3)	3 (3)	3 (3)	3 (3)	2 (0)	3 (3)	3 (2)	3 (1)	3 (3)
730	3 (3)	3 (3)	3 (3)	3 (3)	3 (2)	3 (3)	3 (3)	3 (1)	3 (3)
7,300	3 (3)	3 (3)	3 (3)	3 (3)	3 (2)	3 (3)	3 (3)	3 (1)	3 (3)
73,000	3 (3)	3 (3)	3 (3)	3 (3)	3 (2)	3 (3)	3 (3)	3 (2)	3 (3)
S. enterica additive free	0	0	0	0	0	0	0	0	0

*The numbers in parentheses indicate the *S. enterica* dominant (the highest *S. enterica* colony count on subcultured agar plates).

tions in cell counts compared with those incubated at 37°C. Each culture was adjusted for cell counts, transferred into BPW, HTTB, RB and agar-free MSRA and incubated at 37°C (BPW, HTTB and RB) or 40°C (agar-free MSRA). After 15 hr (*S*. Livingstone) or 18 hr (*S*. Typhimurium Ab-ty strain) incubation, bacterial cell counts were determined by surface plating onto MacConkey agar plates. The migration zone diameter on MSRA was also measured after 24 hr incubation at 40°C. The data revealed that stress-exposed *S.* Livingstone showed the extended lag phase in HTTB, RB and MSRA (Table 3). *S*. Typhimurium Ab-ty strain appeared to require a longer time to enter the log phase of growth in RB. Stress exposed *S*. Typhimurium Ab-ty strain did not grow, even after 40 hr incubation, in RB (data not shown). This result, together with the faster growth in agar-free MSRA, revealed that MSRA will more effectively support growth of stressed *S. enterica* than HTTB or RB.

In conclusion, we documented the growth kinetics of *S. enterica* serovars in each selective enrichment medium and demonstrated proper culture methods for isolating *S. enterica*. Although conventional PCR, real-time PCR and enzyme immunoassays offer significant advantages over culture methods with respect to the rapidity and sensitivity of the detection of *S. enterica* strains [10, 11, 13], further investigation of bacterial isolation is necessary. MSRA showed some advantages including isolation sensitivity, earlier colony detection for serotyping and supporting the growth of stressed *S. enterica.* However, since some *S. enterica* strains did not grow or migrate on MSRA, but grew in HTTB, using multiple types of selective enrichment media, such as MSRA and HTTB together, is recommended to isolate *S. enterica* strains.

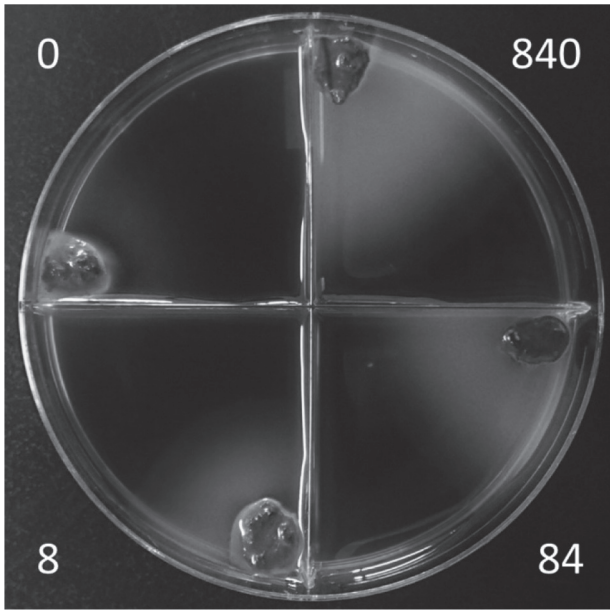

Fig. 1. Migration of *S. enterica* from artificially contaminated bovine feces on MSRA after 17 hr incubation at 40°C. Values represent the additive CFU of *S*. Livingstone.

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Table 3. Effect of cold stress on the growth of *S. enterica* in the enrichment media

	log CFU/m*l*					Migration zone diameter (mm)
	at 0 hr	BPW	HTTB	RB	Agar free MSRA	MSRA
S. Livingstone Ab-li		after 15 hr incubation				
4°C	1.62 ± 0.11	8.41 ± 0.07	5.32 ± 0.28	5.47 ± 0.21	8.40 ± 0.10	51.7 ± 3.18
25°C	1.56 ± 0.10	8.43 ± 0.06	7.17 ± 0.43	7.14 ± 0.18	8.45 ± 0.05	63.3 ± 2.85
S. Typhimurium Ab-ty		after 18 hr incubation				
4°C	1.98 ± 0.06	8.50 ± 0.03	7.60 ± 0.35	2.14 ± 0.31	8.39 ± 0.10	36.3 ± 2.03
25°C	2.23 ± 0.06	8.51 ± 0.04	8.06 ± 0.34	4.89 ± 0.10	8.26 ± 0.05	43.0 ± 1.00

Values are mean ± SE.

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