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Modification of semisolid medium for stab culture to isolate *Salmonella enterica* from rectal swabs

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Abstract

Semisolid agar has shown good performance for selective isolation of *Salmonella enterica*. To control the occurrence salmonellosis within a herd, identification of asymptomatic carriers is necessary; however, rectal swab samples are not suitable for inoculation onto semisolid agar. In this study, we lowered the agar concentration of modified semisolid Rappaport agar for stab culture. *S. enterica* exhibited growth across the entirely of the medium. Other bacteria in bovine fecal samples were not observed to migrate, and the sensitivity for *S. enterica* isolation was greater than that of commercially available Hajna tetrathionate broth. As inoculation onto isolation agar plates is not necessary if no migration is observed, this stab culture method would drastically simplify the process of *S. enterica* isolation.

Key Words: modified semisolid Rappaport agar, *Salmonella enterica*, stab culture

Salmonella enterica, a rod-shaped gram-negative bacterium belonging to the family *Enterobacteriaceae*, is the causative agent of salmonellosis. It affects most animal species as well as humans and is a major public health concern. Its clinical presentation can range from a healthy chronic carrier state to patients with acute or chronic enteritis to patients with septicemia. The most common type of presentation is the carrier state, in which infected animals carry the pathogen for a variable period without showing any clinical symptoms[7]. Carriers of salmonellosis, contaminated feedstuffs, and contaminated environments are major challenges. To limit the spread within a herd, infected animals should be identified and either culled or treated. Treated animals must be rechecked several times to confirm that they are not carriers. Asymptomatic animals identified as carriers through repeated positive fecal cultures over a course of several months should be removed from the herd.

With respect to *S. enterica* isolation, several reports have shown that semi-solid agar has the highest sensitivity and specificity[1,2,3,6,9,10]. We previously reported an easy-to-prepare modified semi-solid Rappaport agar (MSRA), the composition of which is similar to that of

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Table 1. Composition of Rappaport broth (Eiken, Tokyo, Japan), modified semisolid Rappaport agar (MSRA) and MSRA for stab culture (MSRAsc) (per liter).

Rappaport broth		MSRA		MSRAsc	
		50% Rappaport broth		50% Rappaport broth	
Peptone	5.00 g	Peptone	2.50 g	Peptone	2.50 g
NaCl	8.00 g	NaCl	4.00 g	NaCl	4.00 g
KH_2PO_4	1.60 g	KH_2PO_4	0.80 g	KH_2PO_4	0.80 g
$MgCl_2$	20.30 g	$MgCl_2$	10.15 g	$MgCl_2$	10.15 g
Malachite green	0.12 g	Malachite green	0.06 g	Malachite green	0.06 g
		Casamino acid	5.00 g	Casamino acid	5.00 g
		Agar	3.00 g	Agar	1.20 g

Table 2. Migration of *Enterobacter* and *Salmonella* strains on modified semisolid Rappaport agar (MSRA) and MSRA for stab culture (MSRAsc) after 24 hr of incubation.

Organism	Strain	Migration zone diameter on MSRA (mm)		Migration in MSRAsc	
		at 40°C	at 42°C	at 40°C	at 42°C
E. cloacae	K13ec1	No migration	No migration	-	-
E. cloacae	K13ec2	61	No migration	+	-
S. Dublin	K13-R1-3	No growth	No growth	-	-
S. Livingstone	K13-H29-21	>90	68	+	+
S. Nima	K13-R3-8	>90	>90	+	+
S. Typhimurium	K13-H30-27	41	30	+	+
S. O4:i:-	K13-R1-9	55	35	+	+
S. Umblio	K13-R1-29	>90	>90	+	+

Table 3. Isolation of *S. enterica* from artificially contaminated swabs.

Organism	Strain	Inoculated CFU[a]	Number of isolations positive for S. enterica out of three samples			
			HTTB[b] →ES2[c]	MSRAsc[d] →ES2	HTTB →MLCB	MSRAsc →MLCB
S. Livingstone	K13-H29-21	4	0	2	1	2
		44	3	3	3	3
		440	3	3	3	3
S. Nima	K13-R3-8	11	2	3	2	3
		108	2	3	3	3
		1,080	2	3	3	3
S. Typhimurium	K13-H30-27	4	0	2	0	2
		40	2	3	3	3
		400	2	3	3	3
S. O4:i:-	K13-R1-9	2	0	2	2	2
		20	2	3	3	3
		200	3	3	3	3
S. Umblio	K13-R1-29	8	3	3	3	3
		76	3	3	3	3
		760	3	3	3	3

[a]CFU, colony forming unit
[b]HTTB, Hajna tetrathionate broth
[c]ES2, ES salmonellae agar II
[d]MSRAsc, modified semisolid Rapappor agar for stab culture

commercially available modified semisolid Rappaport Vassiliadis agar; MSRA exhibits advantages such as isolation sensitivity for fecal samples, earlier colony detection for serotyping and support for the growth of stressed *S. enterica*[2]. However, as *S. enterica* must be in contact with the semi-solid agar in plates to grow, ensuring an adequate amount of inoculum is essential for reliable isolation, and thus, rectal swabs are considered unfavorable for semi-solid agar plates. In this study, we modified MSRA for stab culture (MSRAsc) and lowered the agar concentration of MSRA, to allow *S. enterica* to swim in the medium.

First, we determined bacterial growth and motility on MSRA and in MSRAsc. The composition of MSRA and MSRAsc is shown in Table 1. Six *S. enterica* strains were used in this study. All bacterial strains were isolated from cattle in Hokkaido prefecture from 2019 to 2021 and were identified using biochemical tests and *S. enterica* serotyping. Moreover, since *Enterobacter cloacae* is known to migrate on MSRA at 37°C in our previous study[2], two strains were isolated from healthy cattle feces by MSRA at 37°C incubation and investigated as the control. Other bacteria such as *Escherichia* and *Proteus* in cattle feces did not migrate on MSRA at 37°C. Approximately 2,000 CFU/10 µl of each bacterial suspension was directly inoculated on MSRA or instilled on cotton swabs, stabbed into MSRAsc and then incubated at 40°C or 42°C for 24 hr.

The results are presented in Table 2. In agreement with our previous study[2], *S.* Dublin did not grow on MSRA and MSRAsc. In MSRAsc, motile bacteria grew in the whole medium, especially around the aerobic region. This can be explained by oxygen taxis[4] and aerobic respiration[5]. *E. cloacae* K13ec1 and Hi-ec[2] strains did not migrate at 40°C, while K13ec2 migrated at 40°C (but not at 42°C). In contrast, all the *S. enterica* strains migrated at 42°C. However, as *S.*

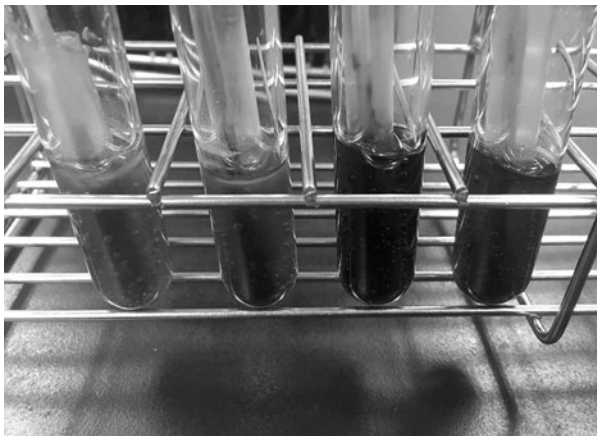

Fig. Migration of *S. enterica* from artificially contaminated swabs to MSRAsc.
S. enterica migrated from the inside of plastic straw and proliferated, exhibiting growth especially around the aerobic region, as observed in the two tubes on the left. The two tubes on the right were negative for motile bacteria. The diameters of test tubes and plastic straws are 16 and 8 mm, respectively.

Paratyphi B Ne-PB had migrated at 40°C, but not at 42°C, in our previous study (data not shown), the incubation temperature should be verified by culturing target *S. enterica* strains. Comparing MSRA with MSRAsc, if the tested bacterial strain spread over 30 mm in diameter on MSRA, migration was visible in MSRAsc.

Next, the efficacy of each selective enrichment medium for the detection of *S. enterica* strains in fecal samples was investigated. Each *S. enterica* strain was instilled on cotton swabs. After stabbing into bovine fecal samples, swabs were inoculated into 5 ml of Hajna tetrathionate broth (HTTB) (Eiken, Tokyo, Japan) or MSRAsc. In MSRAsc inoculation, swabs were passed through plastic straws to distinguish between bacterial migration and solely proliferation. After 24 hr of incubation at 37°C (HTTB) or 42°C (MSRAsc), each culture was plated onto ES salmonellae agar II (ES2) (Eiken, Tokyo, Japan) and MLCB agar (Nissui, Tokyo, Japan) for *S. enterica* isolation. As shown in Table 3, MSRAsc was superior to HTTB in sensitivity to isolate *S. enterica*. Among the isolation agars, MLCB showed a higher sensitivity of isolation than that of ES2 after selective enrichment via culturing on HTTB. Agar media containing chromogenic substrates for salmonella-specific enzymes are known to be less sensitive but more specific, when compared to conventional media[8]. On the other hand, after selective enrichment via culturing on MSRAsc, there was no difference in sensitivity between the two isolation agars because the migrated zone is pure *S. enterica* culture condition. Furthermore, it was easier determine the culture as "motile *S. enterica* negative", as the bacteria in the fecal samples had not migrated (Fig.).

In this study, we prepared MSRAsc for the selective enrichment of *S. enterica* from swab samples. The composition of MSRAsc is the same as that of MSRA, except for a lowered agar concentration. *S. enterica* exhibited growth across the entirety of the medium, with localization especially around the aerobic region. Other bacteria from bovine fecal samples did not migrate, and given that no migration was observed, inoculation onto isolation agar plates was not necessary. Therefore, the use of MSRAsc would drastically simplify the process of *S. enterica* isolation in clinical laboratories.

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