Japanese Journal of Veterinary Research 72(1): 36–40, 2024

JJVR

SHORT COMMUNICATION Experimental Research

Comparison of enrichment media for recovery of *Salmonella enterica* after storage at various temperatures and alkaline stress exposure

Noriko Sakazume[1)], Kiwako Hara[1)] and Masatoshi Fujihara[1,*)]

[1)] Hokkaido Kushiro Livestock Hygiene Service Center, 127-1 Otanoshike, Kushiro, Hokkaido 084-0917, Japan.

Received for publication, December 14, 2023; accepted, March 17, 2024

Abstract

Rectal swabs containing *Salmonella enterica* may be exposed to desiccation stress while awaiting laboratory isolation. Growth of injured *S. enterica* is inhibited on selective media but the extent to which this varies across different media remains unclear. After storage at 25 °C, we compared growth recovery using four enrichment media and revealed that log 2.7 CFU and > log 4.0 CFU was required to confirm the growth of monophasic *S. enterica* Typhimurium K13-4-49 in buffered peptone water and selective enrichment media (Hajna tetrathionate broth, tetrathionate broth, and modified semisolid Rappaport agar for stab culture), respectively. Since < log 1.7 CFU was required for all four media, we recommend sample storage at ≤ 4 °C or the application of transport medium.

Key Words: desiccation stress, *Salmonella enterica*, selective enrichment

Salmonella enterica subsp. *enterica* is a foodborne pathogen that causes diarrhea. It is well-known that *S. enterica* infections are associated with the clinical signs of enterocolitis, septicemia, and abortion in livestock[1)]. Isolation of *S. enterica* from asymptomatic carrier animals and environmental farm samples can be used to assess the risk of microbial infection and monitor the level of contamination in domesticated animals. Although direct plating using selective media has been successful in isolating *S. enterica* strains, an enrichment step is necessary to enhance its detection and isolation[14)]. Since injured *S. enterica* is unable to grow on selective agar[8,13)], many reports suggest a non-selective pre-enrichment step to improve *S. enterica* isolation rates in the field of food hygiene; however, relatively few studies have evaluated this in the field of livestock hygiene. During the storage period, prior to livestock hygiene examination, *S. enterica* is exposed to various stresses including temperature changes and desiccation. The aim of this study was to determine the number of colony forming units (CFU) required before storage at −25 °C, 4 °C, and 25 °C to accurately confirm bacterial growth after storage in enrichment media. Since enrichment media are liquid or semi-solid, we compared the CFU before storage and most probable number (MPN) after storage. In addition, since slaked lime is widely used as an environmental disinfectant on farms, we examined the effects of alkaline stress on *Salmonella* recovery in enrichment media.

Three strains of monophasic *S. enterica* serovar Typhimurium K13-4-49, K13-29-42, and

* Corresponding author: Masatoshi Fujihara, Hokkaido Kushiro Livestock Hygiene Service Center, 127-1 Otanoshike, Kushiro, Hokkaido 084-0917, Japan.
E-mail: fujihara.masatoshi@pref.hokkaido.lg.jp
TEL: +81-154-57-8775, FAX: +81-154-57-6125
doi: 10.57494/jjvr.72.1_36

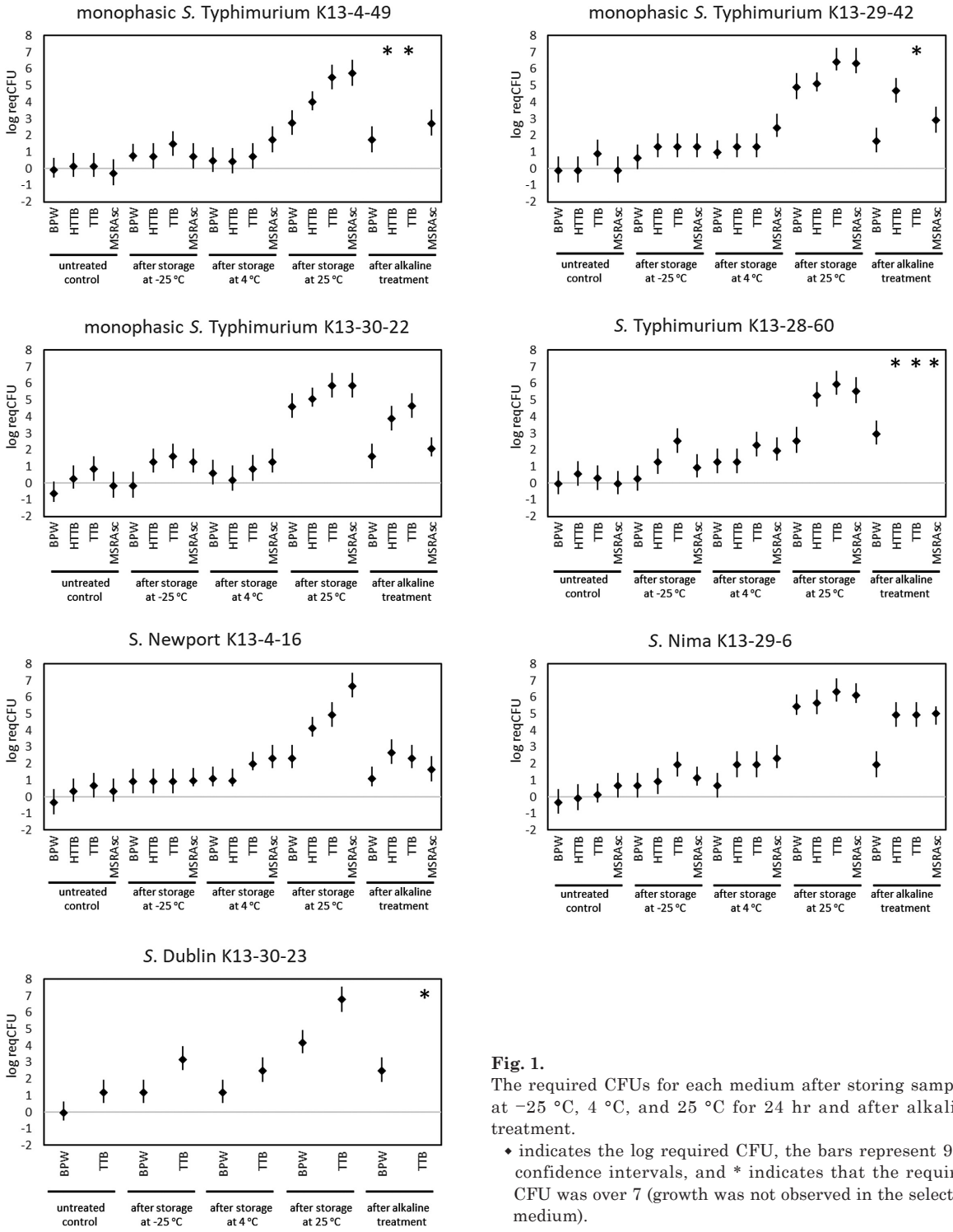

Fig. 1.
The required CFUs for each medium after storing samples at −25 °C, 4 °C, and 25 °C for 24 hr and after alkaline treatment.
◆ indicates the log required CFU, the bars represent 95% confidence intervals, and * indicates that the required CFU was over 7 (growth was not observed in the selective medium).

K13-30-22 and one strain of serovar Typhimurium K13-28-60, serovar Newport K13-4-16, serovar Nima K13-29-6 and serovar Dublin K13-30-23 were used for this study. All strains were isolated from cattle feces during the period 2016–2023 in the Hokkaido prefecture, Japan. The strains were identified using biochemical tests and *S. enterica* serotyping, and monophasic *S. enterica*

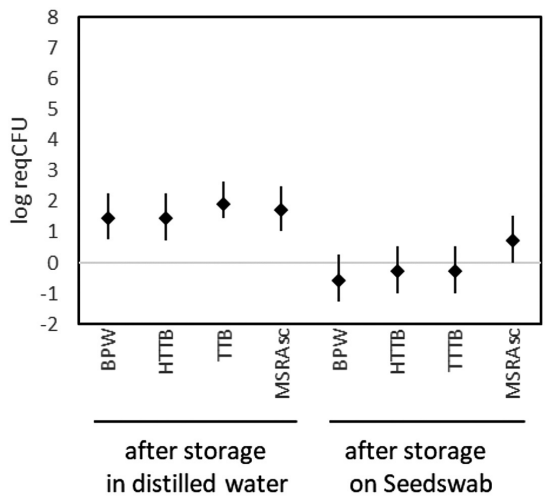

Fig. 2.
The required CFUs of monophasic *S. enterica* Typhimurium K13-4-49 after storage in distilled water and on Seedswabs at 25 °C.
◆ indicates the log required CFU and the bars represent 95% confidence intervals.

Typhimurium was identified by PCR[10]. To examine the effects of storage, *S. enterica* grown in buffered peptone water (BPW; Nissui, Tokyo, Japan) for 24 hr at 37 °C was serially diluted (ten-fold) in distilled water. Subsequently, each bacterial suspension (50 µL) was pipetted onto cotton swabs and Muller-Hinton agar (Eiken, Tokyo, Japan) to determine the CFU after incubation for 24 hr at 37 °C. After storage at −25 °C, 4 °C, or 25 °C for 24 hr, each cotton swab was used to inoculate three tubes (3 ml each) of BPW, Hajna tetrathionate broth (HTTB; Eiken), tetrathionate broth (TTB; Nissui), and modified semisolid Rappaport agar for stab culture (MSRAsc)[5]. After 24 hr of incubation at 37 °C (BPW and HTTB) and 42 °C (TTB and MSRAsc), cell growth was determined by plating the cultures onto mannitol lysine crystal violet brilliant green (MLCB) agar (Nissui). From the tubes displaying growth positive numbers (more than 10^5 CFU/ml in BPW, HTTB, and TTB, and observation of migration in MSRAsc), the MPN was calculated[17]. We defined the required CFU (reqCFU) as the CFU numbers before treatment that was equivalent to 1 MPN (minimal number expected to confirm *S. enterica* growth) in each enrichment medium after treatment. The reqCFU was calculated using the equation: reqCFU = CFU before treatment/MPN after treatment. To examine the effects of alkaline stress, the *S. enterica* cultures in BPW (100 µL) were diluted with supernatant (900 µL) including slaked lime solution (1%). After 60 sec at room temperature, bacterial suspensions were serially diluted (ten-fold) with distilled water and the suspensions (50 µL) were inoculated into three tubes of each medium. The reqCFUs were calculated as described above.

The results are presented in Fig. 1. *S. enterica* Dublin did not grow in HTTB and MSRAsc. These results are consistent with our previous study[4-6]. Regarding the untreated controls, there were little differences between the CFU and MPN in all media, and the log reqCFUs ranged from −0.3 to 1.2. After storage at −25 °C, 4 °C, and 25 °C for 24 hr, the log reqCFUs were −0.1 to 3.2, 0.6 to 2.5, and 2.3 to 6.8, respectively. In selective enrichment media, especially TTB and MSRAsc, after storage at 25 °C the reqCFUs increased to a higher level than those observed in BPW (the log reqCFUs in BPW, HTTB, TTB, and MSRAsc were 2.3 to 5.4, 4.0 to 5.7, 4.9 to 6.8, and 5.5 to 6.7, respectively). To clarify whether desiccation causes an increase in the reqCFUs, monophasic *S. enterica* Typhimurium K13-4-49 was stored for 24 hr at 25 °C in distilled water and on the Seedswab γ No. 1 (Eiken), which contains Cary-Blair medium (the reqCFUs were calculated as described above). After storage in distilled water and on Seedswabs, the log reqCFUs were less than 2.0 in all media (Fig. 2). This suggested that desiccation is the main factor that increases the reqCFUs.

In contrast, the reqCFUs of alkaline stressed *S. enterica* tended to increase, particularly those of HTTB and TTB; however, these observations vary among strains (Fig. 1). In the case of monophasic *S. enterica* Typhimurium K13-4-49, the reqCFUs in BPW, HTTB, TTB, and MSRAsc were 1.7, > 7, > 7, and 2.7, respectively. The bile acid components and brilliant green are known to inhibit the recovery of injured cells[2,9]; therefore, the selective components in HTTB and TTB are considered to be lethal for alkaline injured *S. enterica*. Since the four alkaline stressed *S. enterica* strains

(monophasic *S. enterica* Typhimurium K13-4-49, K13-29-42, and K13-30-22 and *S. enterica* Newport K13-4-16) showed little difference in the reqCFU between MSRAsc and BPW, malachite green in MSRAsc is considered to cause less damage to injured *S. enterica*.

When compared to that of HTTB, a larger increase in the reqCFUs was confirmed in MSRAsc after storage at 25 °C on cotton swabs; however, this increase was smaller after alkaline stress. When lowering the incubation temperature to 37 °C, the log reqCFUs in TTB and MSRAsc (after storage at 25 °C on cotton swabs) decreased to 2.8 (95% confidence intervals [CI]: 2.4–3.6) and 4.4 (95% CI: 3.7–5.3), respectively. Further, by elongating the incubation period to 40 hr, the log reqCFU in MSRAsc became 2.8 (95% CI: 2.4–3.6). Therefore, we hypothesize that most of the desiccated *S. enterica* turns to a dormant state[3] but not sublethal state. Since stressed bacteria elongate in the lag phase, especially in a highly selective medium[6,11], cell growth may not be observed in TTB and MSRAsc after 24 hr of incubation at 42 °C.

Stressed *S. enterica* was recovered by BPW more effectively than selective enrichment media. It is unclear whether the pre-enrichment step improves *S. enterica* isolation from fecal samples. Rigby and Pettit[15] reported that pre-enrichment in BPW increases the *Salmonella* isolation rates from chicken feces; however, Kim *et al.*[12] reported that the pre-enrichment step did not increase the isolation rate in pig feces. Hundreds of livestock animals are examined on farms to identify the carriers when *S. enterica* is isolated; therefore, cotton swabs with small amounts of feces attached are the main form of specimen used in the livestock hygiene service center and these sample swabs can easily dry out. In such cases, pre-enrichment will improve the isolation rates of *S. enterica*.

In conclusion, we revealed that the reqCFUs, especially in selective enrichment media, were increased by storage and alkaline stresses. Although some differences were observed among strains, the increases in the reqCFUs showed similar tendencies. We demonstrated that incubation in selective enrichment medium at 42 °C is unsuitable for desiccated samples; however, alkaline injured *S. enterica* is recovered more effectively in MSRAsc than in HTTB and TTB. Our results indicate a need for storing rectal swab samples in transport media in situations where the culture tests will only commence on the day after sampling. If this method is impossible, these samples should be stored at ≤ 4 °C and protected from desiccation until they can be cultured in a laboratory. If *S. enterica* inspection samples are subjected to stress conditions, the pre-enrichment step will improve the isolation sensitivity. Stress tolerance is known to be influenced by prior growth conditions and cell density[7,16]; therefore, further studies investigating the relevance of these results to bacterial conditions and recovery in selective media are required.

Acknowledgements

We would like to thank Editage (www.editage.com) for English language editing.

References

1) Adem J, Bushra E. Bovine salmonellosis and its public health importance: a review. Advances in life science and technology 44, 62–71, 2016.
2) Blackburn CW, McCarthy JD. Modifications to methods for the enumeration and detection of injured *Escherichia coli* O157:H7 in foods. Int J Food Microbiol 55, 285–290, 2000. doi: 10.1016/s0168-1605(00)00205-1.
3) Deng X, Li Z, Zhang W. Transcriptome sequencing of *Salmonella enterica* serovar Enteritidis under desiccation and starvation stress in peanut oil. Food Microbiol 30, 311–315, 2012. doi: 10.1016/j.fm.2011.11.001.
4) Fujihara M, Hayashi M, Hara K, Sakazume N, Tsukuda T, Tagaino Y. Verification of different methods used for isolating *Salmonella enterica* serovar Dublin from cattle feces. J Vet Med Sci 85, 1077–1082, 2023. doi: 10.1292/jvms.23-

0190.
5) Fujihara M, Sakazume N, Tagaino Y. Modification of semisolid medium for stab culture to isolate *Salmonella enterica* from rectal swabs. Jpn J Vet Res 70, 29–32, 2022. doi: 10.14943/jjvr.70.1.29.
6) Fujihara M, Tabuchi H, Uegaki K. Growth kinetics of *Salmonella enterica* in Hajna tetrathionate broth, Rappaport broth and modified semisolid Rappaport agar. J Vet Med Sci 78, 435–438, 2016. doi: 10.1292/jvms.15-0342.
7) Gruzdev N, Pinto R, Sela (Saldinger) S. Persistence of *Salmonella enterica* during dehydration and subsequent cold storage. Food Microbiol 32, 415–422, 2012. doi: 10.1016/j.fm.2012.08.003.
8) Hsu-Ming W, Naito K, Kinoshita Y, Kobayashi H, Honjoh K, Tashiro K, Miyamoto T. Changes in transcription during recovery from heat injury in *Salmonella typhimurium* and effects of BCAA on recovery. Amino Acids 42, 2059–2066, 2012. doi: 10.1007/s00726-011-0934-y.
9) Hussong D, Enkiri NK, Burge WD. Modified agar medium for detecting environmental salmonellae by the most-probable-number method. Appl Environ Microbiol 48, 1026–1030, 1984. doi: 10.1128/aem.48.5.1026-1030.1984.
10) Ido N, Lee K, Iwabuchi K, Izumiya H, Uchida I, Kusumoto M, Iwata T, Ohnishi M, Akiba M. Characteristics of *Salmonella enterica* serovar 4,[5],12:i:- as a monophasic variant of serovar Typhimurium. PLoS One 9, e104380, 2014. doi: 10.1371/journal.pone.0104380.
11) Kawasaki S. Hosotani Y, Noviyanti F, Koseki S, Inatsu Y. Growth delay analysis of heat-injured *Salmonella* Enteritidis in ground beef by real-time PCR. Lebensm Wiss Technol 90, 499–504, 2018. doi: 10.1016/j.lwt.2017.12.066.
12) Kim JY, Bahnson PB, Kakoma I, Isaacson RE. Effect of buffered peptone water pre-enrichment on detected prevalence of Salmonella in swine feces. In: Proceedings of the Third International Symposium on the Epidemiology and Control of Salmonella in Pork. Bahnson PB. ed. University of Illinois, Urbana. pp. 69–71, 1999. doi: 10.31274/safepork-180809-971.
13) Mackey BM, Derrick CM. A comparison of solid and liquid media for measuring the sensitivity of heat-injured *Salmonella typhimurium* to selenite and tetrathionate media, and the time needed to recover resistance. J Appl Bacteriol 53, 233–242, 1982. doi: 10.1111/j.1365-2672.1982.tb04682.x.
14) Pao S, Patel D, Kalantari A, Tritschler JP, Wildeus S, Sayre BL. Detection of *Salmonella* strains and *Escherichia coli* O157:H7 in feces of small ruminants and their isolation with various media. Appl Environ Microbiol 71, 2158–2161, 2005. doi: 10.1128/AEM.71.4.2158-2161.2005.
15) Rigby CE, Pettit JR. Delayed secondary enrichment for the isolation of salmonellae from broiler chicken and their environment. Appl Environ Microbiol 40, 783–786, 1980. doi: 10.1128/aem.40.4.783-786.1980.
16) Salazar JK, Tesfaldet B, Zamperlini M, Streufert R, Fay M, Keller SE. Desiccation survival of *Salmonella enterica*, *Escherichia coli*, and *Enterococcus faecium* related to initial cell level and cellular components. J Food Prot 85, 398–405, 2022. doi: 10.4315/JFP-21-320.
17) U.S. Food and Drug Administration. Bacteriological analytical manual. Appendix 2. https://www.fda.gov/food/laboratory-methods-food/bam-appendix-2-most-probable-number-serial-dilutions [accessd on January 15, 2024].