Monitoring of Legionella pneumophila populations in environmental water is very important for public health, because the bacterium is the primary cause of Legionnaires’ disease. The conventional plate culture method, similar to ISO 11731 (2017), is generally used to enumerate Legionella spp. in water samples. The method, however, is highly complicated and requires sample handling and colony identification skills due to the growth of non-target bacteria and fungi on the agar plates. Thus, the development of a simpler method that could deliver accurate enumeration results is urgently needed.

The Legiolert®/Quanti-Tray® most probable number (MPN) method (IDEXX Laboratories, USA) is a novel method to enumerate L. pneumophila in potable (e.g., tap water) and non-potable (e.g., cooling tower water) water samples. According to the manufacturer’s instructions, the principle of this method is based on a bacterial enzyme detection technology that signals the presence of L. pneumophila through utilization of a substrate present in the Legiolert reagent. L. pneumophila cells grow rapidly and reproduce using the rich supply of nutrients present in the Legiolert reagent. Actively growing strains of L. pneumophila use the added substrate to produce a brown color indicator. There are four different protocols in the manufacturer’s instructions, two for potable water and the other two for non-potable water. Recent papers have reported method comparison studies for the enumeration of L. pneumophila between Legiolert and the conventional plate culture method from potable water, such as hot water taps, showers, and ice machines (Sartory et al., 2017; Petrisek and Hall, 2017; Spies et al., 2018) and non-potable water, such as cooling tower water (Petrisek and Hall, 2017; Rech et al., 2018). In this paper, we report the first comparison of the enumeration of L. pneumophila from bath water samples using the two Legiolert potable water methods and the conventional plate culture method.

All water samples were collected from natural hot springs (53 samples) and public baths using tap water (79 samples) between May 2018 and March 2019. These samples were collected in sterile 500-mL polypropylene bottles with sodium thiosulfate, and were examined within 3 d after sample collection.

The Legiolert/Quanti-Tray MPN method was carried out according to manufacturer’s instructions. We compared the two potable water protocols (10 mL and 100 mL protocols) described in the instructions. In the 10 mL protocol (Legiolert-10 mL), 10 mL of each water sample was transferred into a sterile 120 mL vessel and diluted with 90 mL of sterile deionized water. In the 100
mL protocol (Legiolert-100 mL), 100 mL of each water sample was transferred into a sterile 120 mL vessel, and total water hardness was measured by using Aquadur hardness test strips (Macherey-Nagel, Germany). One milliliter or 0.33 mL of Legiolert Supplement was then added to all samples with hardness more than 249.2 mg/L (3-4 pads positive on the test strip) or 71.2 to 249.2 mg/L (0-2 pads positive on the test strip), respectively, to neutralize hardness in the water samples. Legiolert blister pack was added to each sample, and the vessel was shaken gently until the reagent was dissolved. The mixture was poured into a Legiolert/Quanti-Tray and immediately sealed with a Quanti-Tray Sealer PLUS (IDEXX Laboratories). Sealed trays were incubated (paper side downward) at 39±0.5°C in a humidified environment. To create the environment, moist paper towels were placed at the bottom of a large plastic food container, and a metal rack was placed between the paper towels and trays to prevent the latter from becoming wet. Legiolert/Quanti-Tray samples were analyzed after 7 d to see the presence of brown pigments and/or turbidity. The positive wells were enumerated and *L. pneumophila* populations were calculated based on the MPN table. (Detection limits are 10 MPN/100 mL and 1 MPN/100 mL for Legiolert-10 mL and Legiolert-100 mL, respectively.)

The specificity of Legiolert/Quanti-Tray was examined by performing secondary confirmations by culturing and/or gene detection on selected wells. Those wells chosen for analysis were all positive ones from large wells, and up to 10 positive ones from small wells. Secondary confirmations were then performed through the following procedure. For each chosen well, the sampling area on the paper side of the Legiolert/Quanti-Tray was disinfected with 75% ethanol. Each culture broth was collected with a 1 mL disposable syringe and transferred into a sterile 1.5 mL micro tube. The broth was inoculated onto BCYE (Nikken Bio Medical Laboratory, Japan), GVPC (Nikken Bio Medical Laboratory) and blood agar (nutrient agar supplemented with 5% horse blood) plates by using an inoculating loop, and incubated at 37°C for 2 d. *Legionella* strains were identified by using the immune serum aggregation assay (Denka Seiken, Japan) and the *Legionella* latex test (Oxoid, UK). *Legionella* strains that could not be identified by the above assay were identified by evaluating the 16S rRNA gene partial sequences using *Legionella* specific primers (Miyamoto et al., 1997). *Legionella* were detected by qPCR using a Cycleave PCR *Legionella* (16S rRNA) Detection Kit (Takara Bio, Japan) according to manufacturer's instructions, if could not detect by the plate culture.

*Legionella* spp. were enumerated according to the conventional plate culture method (ISO11731, 2017).

Collected water samples were concentrated 100-fold by centrifugation (6400 x g, 30 min). A portion of the concentrated samples (0.5 mL) was pretreated with the acid buffer and inoculated onto GVPC selective agar plates. The plates were incubated at 37 °C for 6 to 8 d, and the colonies of *Legionella* spp. that grew on GVPC selective agar plates were enumerated. The detection limit for this method is 10 CFU/100 mL. The species of the isolated *Legionella* strains (up to 5 strains per sample) were identified as described above.

TABLE 1 shows the results for the detection of *Legionella* by Legiolert-10 mL, Legiolert-100 mL and the conventional plate culture method. And, TABLE 2 also shows the extracted results of the false negative samples by Legiolert-10 mL and Legiolert-100 mL. Legiolert had a higher detection ratio than the plate culture method. Legiolert-10 mL detected *L. pneumophila* in all 15 samples that were also identified by the conventional plate culture method. Two positive samples identified by the plate culture method alone contained *Legionella londiniensis* (Sample No. 117) and *Legionella thermalis* (No. 122). The inability to detect these species by Legiolert is expected based on the product features. We think that the cause of the other two false negative samples (No.36 and 39) by Legiolert-100 mL will be the effect of water quality. Because *L. pneumophila* was detected from these two samples by Legiolert-10 mL. These false-negative samples may have high concentrations of various components except hardness, because of the samples are natural hot springs and have high hardness. However, further examinations are needed to clarify it.

There were 20 and 25 *L. pneumophila*-positive samples that were only identified by Legiolert-10 mL and Legiolert-100 mL, respectively. In subsequent secondary confirmations, 15 and 14 samples were identified *Legionella*-positive samples, respectively. Out of 5 false-positive samples detected by Legiolert-10 mL, *Serratia marcescens* was identified in one sample. Out of the other 4 samples, one sample was a hot spring water. Meanwhile, out of 11 false-positive samples identified by Legiolert-100 mL, *Serratia marcescens*, *Pseudomonas otitidis* and *Pseudomonas mendocina* were found in 3 samples. Isolated *S. marcescens* and *P. otitidis* produced a brown pigment in the medium when these strains were inoculated into Legiolert medium. *S. marcescens* is known to produce a red pigment on agar plate. Although the components of Legiolert medium are unknown, we clarified in this study that *S. marcescens* grows in Legiolert medium and produces pigments. We think that this will be the cause of false positives. Of the other 8 samples, 5 samples were natural hot spring waters with high hardness.

Figure 1-a shows the bacterial counts produced from
TABLE 1. Comparison of the results for the detection of Legionella by Legiolert-10 mL, Legiolert-100 mL, and the conventional plate culture method.

|                      | Legiolert-10 mL |          | Legiolert-100 mL |          |          |          | Total  |
|----------------------|-----------------|----------|------------------|----------|----------|----------|--------|
|                      | Positive        | Negative | Positive         | Negative |          |          |        |
| Conventional plate   | 15              | 2        | 14               | 3        | 17       |          |        |
| culture              |                  |          |                  |          |          |          |        |
| Positive             | 20              | 95       | 25               | 90       | 115      |          |        |
| Total                | 35              | 97       | 39               | 93       | 132      |          |        |

TABLE 2. Results of the false negative samples by Legiolert-10 mL and Legiolert-100 mL.

| Sample No. (source) | MPN/100 mL species | MPN/100 mL species | CFU/100 mL species |
|---------------------|--------------------|--------------------|--------------------|
| 36                  | L. pneumophila     | <1                 | NA                 | 40                 | L. pneumophila |
| 39                  | L. pneumophila     | <1                 | NA                 | 70                 | L. pneumophila |
| 117                 | <1                 | NA                 | 10                 | L. londiniensis    |
| 122                 | <1                 | NA                 | 350.1              | L. thermalis       |

*Not applicable

*False positive

FIG. 1. Comparison of bacterial counts between the conventional plate culture method and Legiolert-10 mL (a), Legiolert-100 mL (b). Open circles in plots show that L. thermalis or L. londiniensis were detected by the plate culture method.
the results of plate culture and Legiolert-10 mL methods. The plate culture method and Legiolert-10 mL showed comparable bacterial counts for the 15 samples that were identified by both methods. Legiolert-10 mL results showed higher correlation with those of the conventional culture method than the Legiolert-100 mL results (FIG. 1-b). Additionally, the numbers of false-positives and false-negatives for Legiolert-10 mL were smaller than those for Legiolert-100 mL (TABLE 1, TABLE 2 and FIG. 1).

There are at least two possibilities why Legiolert had a higher detection ratio and bacterial counts than the plate culture method. The first one is due to the fact that Legiolert involves only simple procedures and includes no steps that might reduce the recovery like the plate culture method. Secondly, viable but nonculturable (VBNC) L. pneumophila on agar plates may be able to reproduce in Legiolert liquid medium. Nosho et al. (2018) showed the presence of Escherichia coli that cannot form colonies on agar plates but grows only in liquid medium. Thus, there may be L. pneumophila that grow well in the liquid medium but not on agar plates.

We consider that Legiolert is a very effective method for easily monitoring for the presence of L. pneumophila because it does not involve complicated procedures such as concentration or inoculation onto agar plates. The popularization of this easy-to-use method will further advance measures to guard against Legionella.

ACKNOWLEDGEMENTS

We thank Ms. Asako Morishita in our laboratory for her excellent assistance in the identification of Legionella by the molecular biological technique, and Ms. Naomi Tanaka at IDEXX Laboratories K.K. for her technical support of Legiolert testing. All Legiolert related reagents used in this study were provided by IDEXX Laboratories K.K. This work was partially supported by the Health and Labor Science Research Grants (H28-kenki-006).

REFERENCES

ISO (2017) Water quality – Enumeration of Legionella. ISO 11731 second edition, International for Standardization.

Miyamoto, H., Yamamoto, H., Arima, K., Fujii, J., Murata, K., Izu, K., Shiomori, T., and Yoshida, S. (1997) Development of a new seminested PCR method for detection of Legionella species and its application to surveillance of Legionellae in hospital cooling tower water. *Appl. Environ. Microbiol.*, 63, 2489-2494.

Nosho, K., Yasuhara, K., Ikehara, Y., Mii, T., Ishige, T., Yajima, S., Hidaka, M., Ogawa, T., and Masaki, H. (2018) Isolation of colonization-defective *Escherichia coli* mutants reveals critical requirement for fatty acids in bacterial colony formation. *Microbiology*, 164, 1122-1132.

Petrisik, R., and Hall, J. (2017) Evaluation of a most probable number method for the enumeration of *Legionella pneumophila* from North American potable and nonpotable water samples. *J. Water Health*, 16, 25-33.

Rech, M.M., Swalla, B.M., and Dobranic, J.K. (2018) Evaluation of Legiolert for quantification of *Legionella pneumophila* from non-potable water. *Curr. Microbiol.*, 75, 1282-1289.

Sartory, D.P., Spies, K., Lange, B., Schneider, S., and Langer, B. (2017) Evaluation of a most probable number method for the enumeration of *Legionella pneumophila* from potable and related water samples. *Lett. Appl. Microbiol.*, 64, 271-275.

Spies, K., Pleischl, S., Lange, B., Langer, B., Hubner, I., Jurzik, L., Luden, K., and Exner, M. (2018) Comparison of Legiolert®/Quanti-Tray® MPN test for the enumeration of *Legionella pneumophila* from potable water samples with the German regulatory requirements methods ISO 11731-2 and ISO 11731. *Int. J. Hyg. Environ. Health*, 221, 1047-1053.