Consideration of Bacteria Inspection Methods Used to Examine Salt and Manufacturing Process Samples

Salt Production

Keywords: bacteria inspection method, viable cell count, coliform bacteria, *Vibrio parahaemolyticus*, manufacturing process samples

Abstract

The present study set out to examine the optimum bacteria inspection method for determining the viable cell count, the presence of coliform bacteria and *Vibrio parahaemolyticus* in salt and seawater. To identify the most effective inspection method, the pour plate method, membrane filter (MF) method, and surface spread plate method were compared.

No adverse effects of a NaCl mixture on a culture medium, as determined from an examination of a sample solution, were observed in terms of the viable cell count when using the pour plate method. However, the variation in the measured value increased because the number of bacteria inoculated into the sample was very small when using the pour plate and surface spread plate methods. In the examination of the coliform bacteria, the NaCl concentration in the culture medium increased when the amount of inoculated bacteria was increased when using the pour plate method, while the number of detected coliform bacteria decreased.

Based on these results, we concluded that the MF method, which can increase the amount of the sample solution without inducing an increase in the NaCl concentration in the culture medium, provides the optimum means of inspecting the viable cell count and the presence of coliform bacteria in a salt sample taken during the salt manufacturing process.

Also, the level of *Vibrio parahaemolyticus* in seawater was measured and the measured count obtained with the MF method was compared with the results obtained with the pour plate and surface spread plate methods (standard methods). The counts obtained with these methods were found to be equal. However, the MF and pour plate methods are more suitable for obtaining a count than the surface spread method when examining the manufacturing process samples. This is because the number of bacteria in the sample is low and the surface spread method cannot increase the amount inoculated in the sample.

The measured count obtained when using an MF with a large pore size was equal to that obtained with the normal MF method. Therefore, we thought that this method can be used to measure a sample with a large amount of insoluble content. We used this method to investigate the behavior of *Vibrio parahaemolyticus* in the manufacturing process samples. Then, this bacterium was separated from the seawater by a sand-filtering device. We confirmed that this bacterium was not present in the product.

E-mail: yu-nakayama@lab.shiojigyo.or.jp Tel: +81-465-47-3161,

1. Introduction

The viable cell count and number of coliform bacteria is conventionally monitored as an index of the sanitary level or retained quality of general foodstuffs. These bacteria are prevented from multiplying and the number of bacterium is small in salt manufacturing process samples, because of the very high NaCl concentration. However, to ensure the sanitary conditions of the salt manufacturing process, it is important to be aware of the existence and amount of these bacteria. On the other hand, *Vibrio parahaemolyticus*, which is a *halophilous* bacteria that can cause food poisoning, exists in seawater in large quantities and can multiply in NaCl concentrations of about 3%[1)]. As such, it is possible that the bacteria could remain in a salt manufacturing process sample given that, in Japan, salt is normally made from seawater.

Therefore, it is important to be able to determine the number of these bacteria in a salt manufacturing process sample.

As mentioned above, these bacteria are measured in accordance with the food hygiene inspection guidelines of Japan (official method). For many foods, these bacteria are cultivated by mixing a diluted sample with a culture medium (the pour culture method). However, it can be presumed that, when the pour culture method is applied to a salt manufacturing process sample, the multiplication of the bacteria could be inhibited, given that the NaCl concentration of a culture medium increases under the influence of the NaCl in a salt manufacturing process sample. Furthermore, it is possible that the pour culture method would not be able to determine the bacteria, given that the amount of the inoculation in the sample solution cannot be increased when using this method and the number of bacteria in the sample is very small. Consequently, when using a measurement method other than the pour culture method for a salt manufacturing process sample, we expected the measurement accuracy to increase.

Moreover, there is the possibility of the detection of bacteria becoming difficult since any salt manufacturing process sample could include a large amount of insoluble matter, depending on the season. However, there have been few studies regarding the optimum bacteria inspection method for samples such as those taken during the salt manufacturing process, which feature high salinity and a large amount of insoluble matter.

Therefore, we set out to identify the optimum bacteria inspection method for determining the viable cell count, the presence of coliform bacteria and *Vibrio parahaemolyticus* in a sample taken from the salt manufacturing process. To identify the optimum inspection method, the pour culture method, membrane filter (MF) method, and surface spread plate method were compared. Furthermore, the application of the MF method to a sample incorporating a large amount of insoluble content was also investigated.

2. Experimental Procedure

2.1 Reagents

The viable cell count and presence of coliform bacteria were determined using a standard method agar and a lactose bouillon culture medium, respectively, both made by Nissui Pharmaceutical Co., Ltd. The presence of *Vibrio parahaemolyticus* was determined using X-VP agar, T.C.B.S. cholera medium, and alkaline peptone water made by Nissui Pharmaceutical and OXOID Co., Ltd.

A sterilized phosphate buffered saline solution (pH 7.4) was used to dilute the sample (henceforth referred to as “dilution water”). The sterilized membrane filter (MF) had a diameter of 47 mm and a pore size of 0.45 or 5 μm (Merck Millipore Corporation).

2.2 Test samples

When attempting to determine the viable cell count and the presence of coliform bacteria, suitable edible salt samples were chosen from among those listed in Table 1. The commercial edible salts were categorized as solar salt (Nos. 1–4), rock salt (5, 6) and evaporated salt (7, 8). Furthermore, products 1 to 6 were produced without heating, while 7 and 8 used heat in their manufacturing processes.

On the other hand, in the case of *Vibrio parahaemolyticus,* the samples were selected to represent the salt manufacturing processes listed in Table 2.

No.	Class
1	Solar salt
2	
3	
4	
5	Rock salt
6	
7	Evaporated salt
8	

Table 1 Samples used for test

No.	Class
A	Sea Water
B	After sand filter
C	After electrodialytic separation
D	Mother liquid
E	Bittern
F	Product

Table 2 Samples used for test

2.3 Consideration of viable cell count optimum inspection method

Figure 1 shows the operation flows of the pour plate, MF, and surface spread plate methods. The samples for the test were selected from Table 1 (Nos.1, 2 and 5, 6). The sample solutions were prepared by diluting with dilution water at a ratio of 1:9. The NaCl concentration in the sample

solution was approximately 10%. For the pour plate and surface spread plate methods, the sample solution was inoculated with 1 and 0.1 mL, according to the official method[1)]. For the MF method, the amount of the inoculation was 10, 1, and 0.1 mL. The subsequently inoculated culture medium was cultivated for 48 h at 35 ºC, after which the detected colonies were counted.

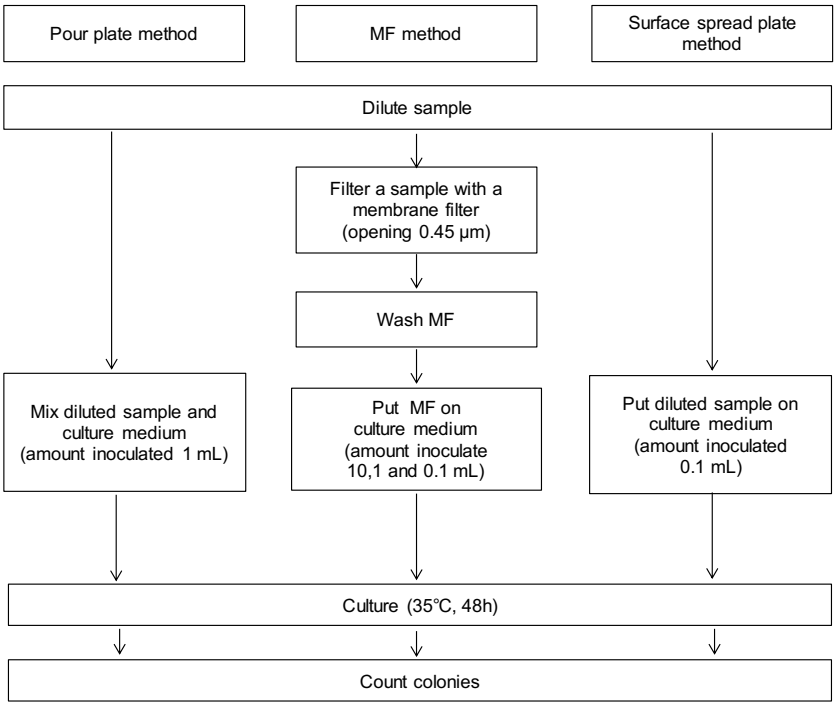

Figure 1 Flow chart for each inspection method (viable cell

2.4 Consideration of coliform bacteria optimum inspection method

Figure 2 shows the operational flow of the pour and MF fermentation tube methods for coliform bacteria. A sample solution was prepared in the same way as for the viable cell count inspection method, described in Section 2.3, above. In the pour fermentation method, a sample solution of 10 mL was inoculated into 10 mL of double-fold concentration culture medium. And sample solution of 1 and 0.1 mL were inoculated into 10 mL of one-fold concentration culture medium. On the other hand, in the MF fermentation tube method, 10, 1, and 0.1 mL of the sample solutions were filtered. This filtered MF was washed with dilution water to remove the NaCl contained in the sample solution, after which it was introduced to the culture medium. The inoculated culture medium was cultivated for 48 h at 35 ºC, and the number of test tubes in which gas was generated was counted.

2.5 Consideration of MF method application to *Vibrio parahaemolyticus* inspection of salt manufacturing process sample

2.5.1 Comparison of optimum inspection methods for salt manufacturing process sample

Figure 3 shows the operational flow of the surface spread, MF, and pour fermentation tube methods for *Vibrio parahaemolyticus*. The sample was seawater, selected from Table 2 (No. A). The sample solution was prepared in the same way as for the viable cell count inspection method, described in Section 2.3, above. In the surface spread method, the sample solution was inoculated with 0.1 mL of the TCBS culture medium when the surface was sufficiently dry. Using the MF method, the sample solution was filtered from 0.1 to 50 mL, through an MF with a pore size of 0.45 μm. Then, the filtered MF was washed with dilution water and was then placed on a TCBS culture medium. After the MF had been placed, the culture medium was cultivated for 18 h at 35 ºC, after which the detected colonies were counted. In the pour fermentation tube method, the sample solution was diluted with dilution water at a ratio of 1:10 and 1:100. These sample were inoculated with 1 mL in three fractionated test tubes with 10 mL of alkaline peptone water. Then, 1 or 0.1 mL of the sample solution that had been diluted at a ratio of 1:1000 was put into three fractionated test tubes with 10 mL of alkaline peptone water. These sample solutions were then cultivated for 18 h at 35 ºC. Subsequently, 0.1 mL of the cultivated alkaline peptone water was inoculated onto the TCBS culture medium and it was cultivated for 18 h at 35 ºC. Subsequently, the number of colonies of *Vibrio parahaemolyticus* was confirmed. The test tube in which the colony was confirmed was determined to be positive. The number of *Vibrio parahaemolyticus* was calculated from the number of positive and negative test tubes (most probable number method).

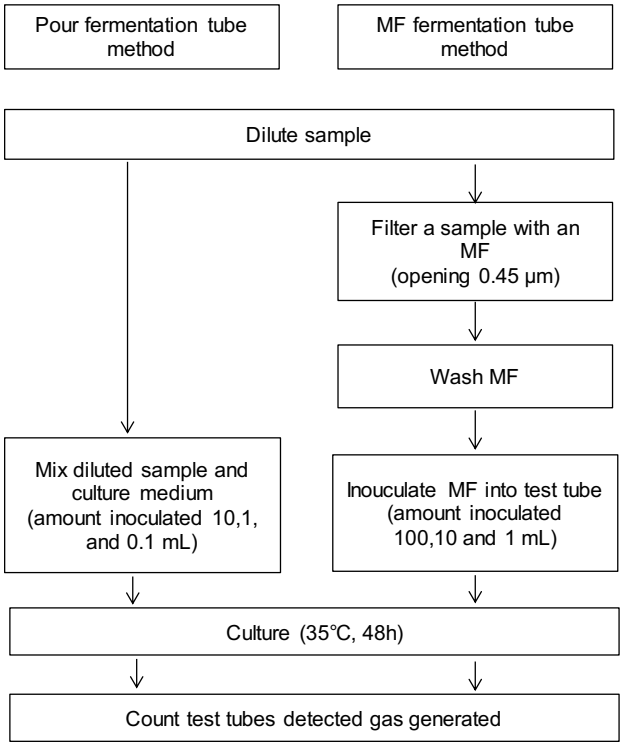

Figure 2 Flow chart for each inspection method (coliform bacteria)

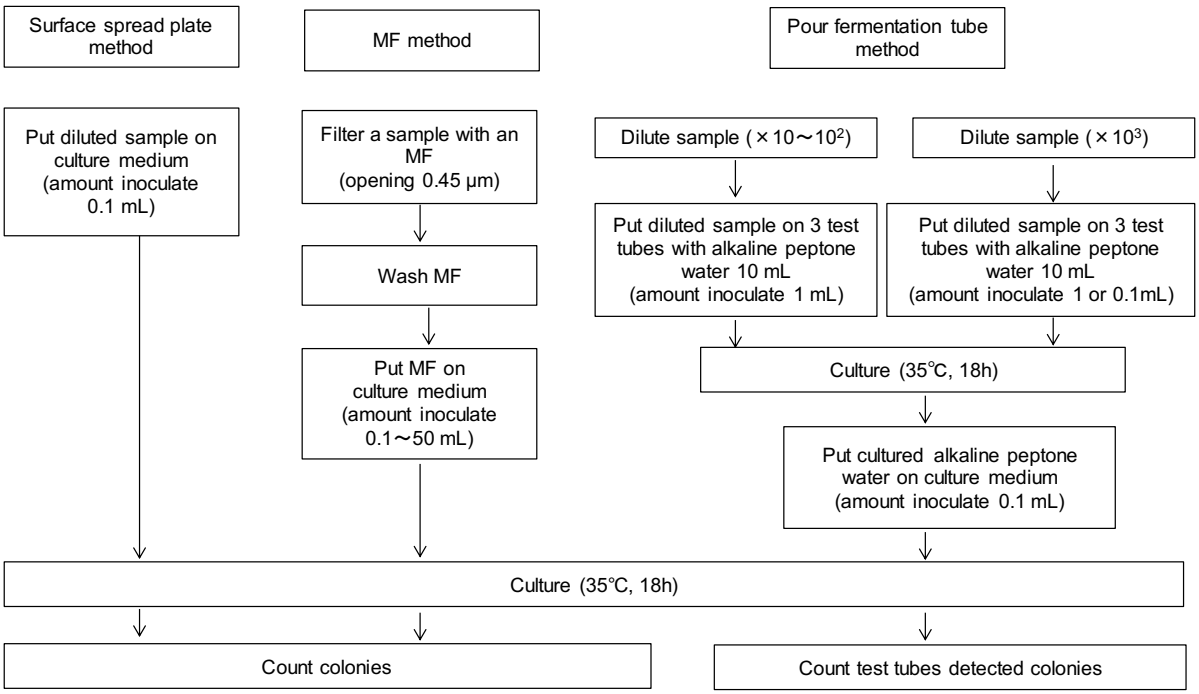

Figure 3 Flow chart for each inspection method (*Vibrio parahaemolyticus*)

2.5.2 Consideration of inspection method for *Vibrio parahaemolyticus* in sample with large amount of insoluble matter

Figure 4 shows the operational flow of the MF method using an MF with a 0.45 and 5 μm pore size. The MF with the 5 μm pore size was used to remove insoluble matter from a sample taken from the salt manufacturing process. This method is abbreviated to “alteration MF method” in this paper. On the other hand, the MF method we described in Section 2.5.1, above, is called the normal MF method. With the normal MF method, the MF through which the sample solution has been filtered is placed on a TCBS culture medium. On the other hand, with the alteration MF method, the sample solution is filtered through an MF with 5 μm pores. Subsequently, 5 mL of the sample solution is passed through an MF with a 0.45 μm pore size. This 0.45 μm MF was then placed on the TCBS culture medium. The 5 μm MF through which the sample solution was filtered was placed in 10 mL of dilution water and ultrasonicated for 10 min to extract the bacteria. We then inoculated 0.1 mL of this extracted solution onto a TCBS culture medium. These culture mediums after sample inoculation were cultivated for 18 h at 35 ºC.

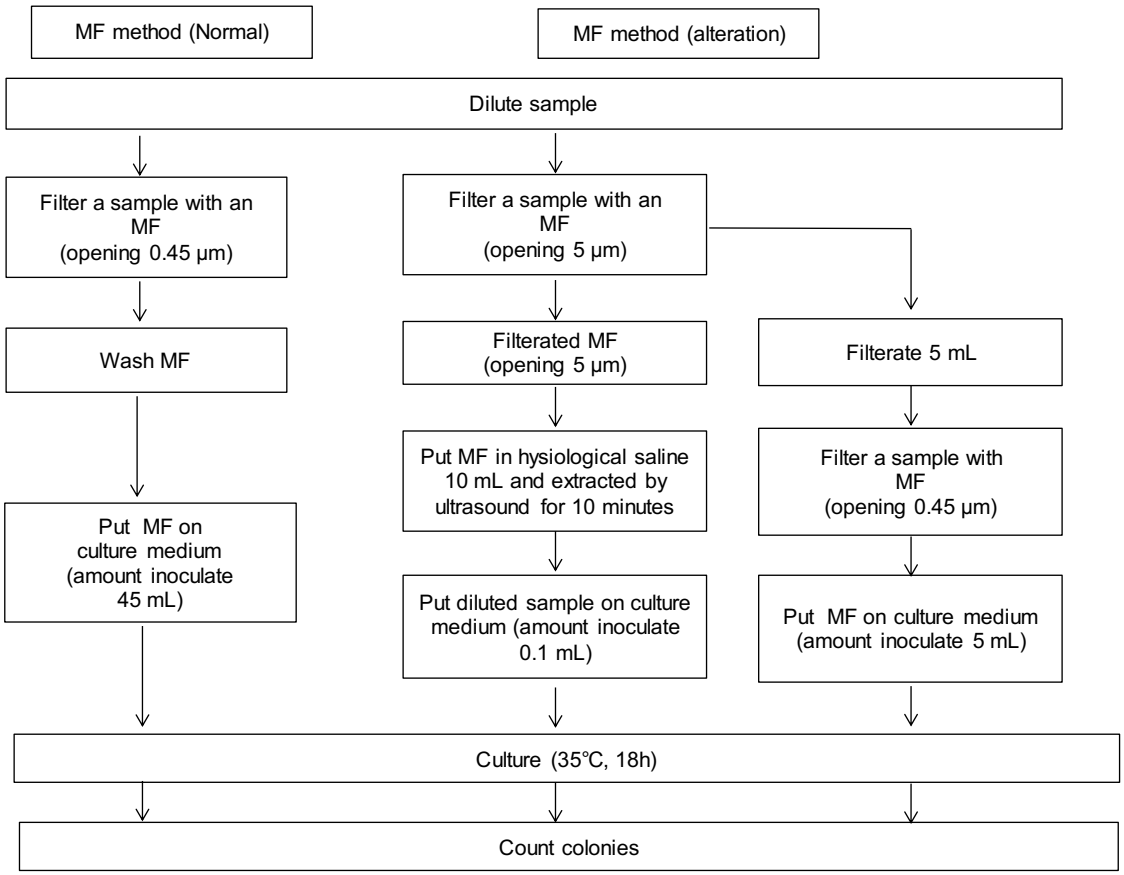

Figure 4 Flow chart for MF method (*Vibrio parahaemolyticus*)

3. Results and Discussion

3.1 Consideration of viable cell count optimum inspection method

The samples were inoculated using the three methods shown in Fig. 1. Table 3 lists the measured values. No difference between the values was observed with the pour plate and MF methods. The salinity of the culture medium was approximately 0.6% with the pour plate method, given that 1 mL of the sample solution with a salinity of approximately 10%, as mentioned above, was added to 15 mL of the culture medium. On the other hand, with the MF method, the MF is washed with dilution water after the filtering of the sample solution to remove the NaCl from the sample solution, such that the culture medium does not experience an increase in salinity during the cultivation.

Consequently, we can assume that salinity does not have an adverse effect on the cultivation with the pour plate method. Furthermore, it was suggested that the pour plate method can be measured by a dilution of small magnification, in the same way as with the MF method.

In the MF method, the viable cell count was detected in all the culture media which were inoculated with 10 mL of sample solution. However, with the MF and pour plate methods, in which 1 mL of the sample solution was inoculated, a viable cell count was sometimes not detected. Moreover, in the measurement of the 1 mL of inoculated sample solution, a variation in the measurement was seen. We believed that this was the reason why the measurement count was low. Since the official method, as laid down in Japan, requires a 1 mL inoculation for the pour plate method, these results suggest that the MF method, whereby the amount of the inoculation can be increased, is suitable for the examination of samples from the salt manufacturing process.

Additionally, almost no viable cell counts were detected with the surface spread method. Since a viable cell count was not detected with the MF method, when using a 0.1 mL inoculation of the sample solution, or with the surface spread method, it was thought that these methods cannot be used for detecting the viable cell counts. Therefore, it can be assumed that the surface spread method is unsuitable for the examination of salt manufacturing process samples when the viable cell count is small.

In consequence, since the viable cell count in a salt manufacturing process sample is usually very small, the MF method, for which the inoculation amount can be increased, is suitable.

Method	Amount of inoculated (mL)	Sample number (Not-detection number n=5)			
		1	2	5	6
MF	10	9 (0/5)	14 (0/5)	16 (0/5)	14 (0/5)
MF	1	10 (1/5)	12 (2/5)	52 (0/5)	40 (0/5)
Pour-plate	1	6 (3/5)	2 (4/5)	32 (1/5)	30 (0/5)
MF	0.1	0 (5/5)	0 (5/5)	0 (5/5)	0 (5/5)
Surface spread plate	0.1	0 (5/5)	0 (5/5)	20 (4/5)	0 (5/5)

Table 3 Number of bacteria determined with different inspection methods (viable cell count)

3.2 Consideration of optimum inspection method for coliform bacteria

The samples were inoculated using the two methods illustrated in Fig. 2. Table 4 lists the measured values. With the pour fermentation tube method, none of the samples of the culture medium exhibited either a change in color or the generation of gas. On the other hand, with the MF method, a change in the color of the culture medium and the occurrence of gas were observed with inoculation amounts of 100 and 10 mL for sample Nos. 1 and 6. According to a previous report[2)], when the salinity reaches 0.2%, the multiplication of coliform bacteria is prevented.

Therefore, with the pour fermentation tube method, it is likely that the multiplication of the coliform bacteria is prevented by the salinity of the culture medium since a change in the color of the culture medium and the occurrence of gas were observed with the same 10 mL inoculation as that used with the MF method. Although the official method for coliform bacteria has never adopted the MF method using a lactose bouillon culture medium, it would appear that it is possible to measure the coliform bacteria in a salt manufacturing process sample by applying the MF method.

As a result, we can conclude that the MF method, which does not increase the salinity of the culture medium, is suitable because a salt manufacturing process sample obviously has a high salinity.

Method	MF fermentation tube			Pour fermentation tube		
Sample No.	100 mL	10 mL	1 mL	10 mL	1 mL	0.1 mL
1	1/3	1/3	0/3	0/3	0/3	0/3
2	0/3	0/3	0/3	0/3	0/3	0/3
5	0/3	0/3	0/3	0/3	0/3	0/3
6	1/3	1/3	0/3	0/3	0/3	0/3

Table 4 Number of bacteria determined with different inspection methods (coliform bacteria) (n=3)

3.3 Consideration of the application of the MF method to the inspection of *Vibrio parahaemolyticus* in a salt manufacturing process sample

3.3.1 Comparison of optimum inspection methods for the salt manufacturing process samples

The samples were inoculated using the three methods indicated in Fig. 3. Table 5 lists the measured values. We assumed that each of the three methods produced an equal count. Since the number of *Vibrio parahaemolyticus* colonies was comparatively large for this sample, it was thought that even the surface spread method, in which the amount of inoculation is small, can be applied to the measurement.

Figure 5 shows the change in the number of *Vibrio parahaemolyticus* in a seawater sample (sample A in Table 2). The number of bacteria after the middle of October was lower than that between August and the beginning of October. According to a previous report, [1)] when the seawater temperature exceeds 20 ºC, *Vibrio parahaemolyticus* proliferates. Therefore, it was suggested that the number of bacteria would be fewer in the colder seasons. Then we assumed seawater at cold season by preparing a sample with a 10 times dilution. The results of our measurements were added to Table 5 as A'. There was no difference in the measurements obtained with the pour fermentation and MF methods. However, no *Vibrio parahaemolyticus* was detected with the surface spread plate method. This is because the amount of sample inoculated

with the surface spread plate method was 0.1 mL, about 100 times fewer compared with the MF method. On the other hand, the inoculated amount of sample solution with the pour fermentation method is less than that with the surface spread plate method, as shown in Fig. 3. However, we can infer that *Vibrio parahaemolyticus* would be detected as a result of the bacteria multiplication. Therefore, both the MF and pour fermentation methods are deemed suitable for the examination of salt manufacturing process samples. However, we decided to apply the MF method in this study, because the pour plate method involves an extended process and has a complicated protocol.

	Methods		
Samples	Surface spread plate	MF	Pour fermentation tube
A	40	19	9
A' (diluted 10 times)	>10	2.6	0.9

Table 5 Number of bacteria determined with different inspection methods (*Vibrio parahaemolyticus*)

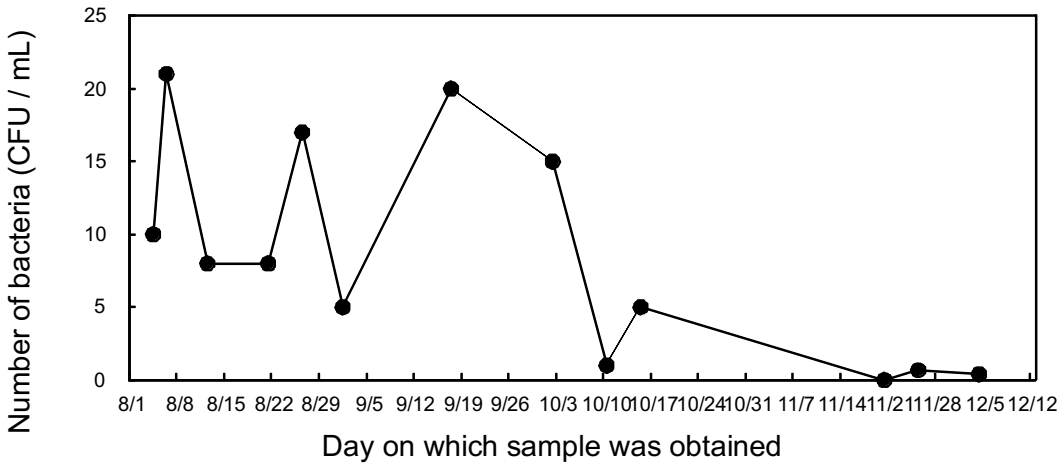

Figure 5 Change in number of *Vibrio parahaemolyticus* in sample
(MF method (Normal and alteration)

3.3.2 Consideration of inspection of *Vibrio parahaemolyticus* in a sample containing large amounts of insoluble matter

Figure 6 shows the number of *Vibrio parahaemolyticus* measured using both the alteration and normal MF methods. The number of bacteria in the filtrate when using a 5 μm pore size MF was less than that when using a 0.45 μm pore size MF. However, the number of bacteria in the solution extracted from the filter MF (pore size 5 μm) by ultrasonication was found to be greater than that

of the above-mentioned filtrate. Moreover, when the number of bacteria in the extracted solution and filtered MF were totaled, the number of bacteria was found to be equal to that obtained with the normal MF method. In general, since the size of *Vibrio parahaemolyticus* is approximately 1 μm, it is not captured by an MF with a pore size of 5 μm and is thus found in the filtrate. However, it was nevertheless detected in large quantities in the filtrate with 5 μm pore size MF. Consequently, it was thought that *Vibrio parahaemolyticus* would be detected after filtering with an MF with a pore size of 5 μm, because almost all the *Vibrio parahaemolyticus* in seawater are attached to insoluble matter. Accordingly, in a salt manufacturing process sample which includes large amounts of insoluble matter, we could measure the number of *Vibrio parahaemolyticus* by using the alteration MF method.

The behavior of *Vibrio parahaemolyticus* in the Japanese salt manufacturing process was investigated. For this investigation, we used the samples listed in Table 2. Although *Vibrio parahaemolyticus* was detected (5.0×10^2) in seawater, after the sand-filtering of the seawater, no *Vibrio parahaemolyticus* was detected in the salt manufacturing process samples. Therefore, it was thought that *Vibrio parahaemolyticus* was separated from the seawater by the sand filtration process and was not incorporated into the product.

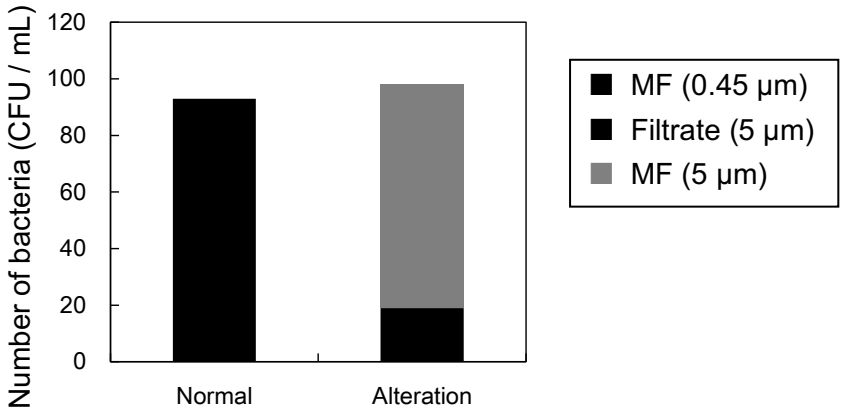

Figure 6 Number of bacteria measured using alteration and normal MF methods (*Vibrio parahaemolyticus*)

4. Conclusion

We examined the optimum means of inspecting the viable cell count, coliform bacteria, and *Vibrio parahaemolytics* in salt and seawater. To select the optimum inspection method, the pour plate, MF, and surface spread plate methods were compared.

No adverse effects of the NaCl mixture on the culture medium, as determined using a sample solution, were seen in terms of the viable cell count with the pour plate method. While the examination of the coliform bacteria used the pour plate method, the number of detected coliform bacteria decreased.

From these results, we concluded that the MF method, which can increase the amount of the sample solution without inducing an increase in the NaCl concentration in the culture medium, provides the optimum means of inspecting the viable cell count and the presence of coliform bacteria in a salt sample acquired during the salt manufacturing process.

Also, the level of *Vibrio parahaemolyticus* in the seawater was measured and the measured count obtained with the MF method was compared with the results obtained with the pour plate and surface spread plate methods. The counts obtained with these methods were found to be equal. However, the MF and pour plate methods are more suitable for obtaining a count than a surface spread method when examining a salt sample in the manufacturing process. This is because the number of bacteria in the sample is low and the surface spread method cannot increase the amount inoculated in a sample.

The count obtained when using MF with a large pore size was equal to that obtained with the normal MF method. Therefore, we thought that this method can be used to measure a sample with a large amount of insoluble matter.

References

1) Japan Food Hygiene Association, “Standard Methods of Analysis in Food Safety Regulation”, 293,Japan Food Hygiene Association, Japan(2015)
2) Katsutoshi Mise, Fujio Inoue “Syokuhintyu no biseibutukennsahou kaisetsusyo” 305, KODANSHA LTD., Japan(1996)