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Bactericidal Efficacy of a Fumigation Disinfectant Containing Paraformaldehyde Against *Salmonella* Typhimurium

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ABSTRACT - This study was performed to evaluate the bactericidal efficacy of a fumigation disinfectant containing 35% paraformaldehyde against *Salmonella* Typhimurium (*S.* Typhimurium). In this study, the efficacy test of a fumigant against *S.* Typhimurium was carried out according to French standard NF T 72-281. The *S.* Typhimurium working culture suspension number (N value), all bacteria numbers on the carriers exposed to the fumigant (n1, n2, and n3), the number of bacterial suspensions by the pour plate method (N1), the number of bacterial suspensions by the filter membrane method (N2), and the mean number of bacteria recovered on the control carriers (T value), were obtained from the preliminary test. In addition, the reduction number of *S.* Typhimurium exposed to the fumigant (d value) was calculated using the T value, the mean number of bacteria in the recovery solution (n'1) and the mean number of bacteria on carriers plated in agar (n'2). The N value was 5.5×10^8 colony forming units (CFU)/mL, and n1, n2, and n3 were higher than 0.5N1, 0.5N2 and 0.5N1, respectively. Additionally, the T value was 3.5×10^6 CFU/carrier. In terms of the bactericidal effect of the fumigant, the d value was 5.25. According to the French standard for fumigants, the d value for an effective bactericidal fumigant should be greater than 5. The results indicated that the fumigant containing 35% paraformaldehyde had an efficient bactericidal activity against *S.* Typhimurium, and, therefore, can be used to disinfect food materials and kitchen appliances contaminated with foodborne bacteria.

Key words: Fumigation, Paraformaldehyde, *S.* Typhimurium, Disinfectant efficacy

Foodborne diseases comprise various acute syndromes that result from ingestion of foods contaminated with either bacteria or their toxins, viruses, or inorganic chemical substances and poisons derived from plants and animals[1)]. Outbreaks of foodborne disease among humans caused by *Salmonella* spp. are due to *Salmonella* Typhimurium (*S.* Typhimurium), *Salmonella* Cholerasuis, *Salmonella* Enteritidis and many other similar species, and common presenting symptoms include fever, headache, nausea, vomiting, abdominal pain and diarrhea[2)].

Reservoirs and sources of *Salmonella* infection include poultry, rats, pigs, cattle, and pets as well as human patients and convalescent carriers. The main route of transmission is by ingestion of organisms in food such as milk, poultry, meat and eggs derived from infected food animals. Food can also be contaminated by infected food handlers, by cross-contamination as a result of poor hygiene and from feces of an infected animal or person[3)]. The etiologic agents of salmonellosis are *Salmonella* spp. characterized by motile, Gram-negative, rod-shaped bacteria and facultative intracellular pathogens that can multiply within professional and nonprofessional phagocytes[4)]. *Salmonella* can survive for weeks outside a living body and are not destroyed by freezing[5)].

The global burden of non-typhoidal *Salmonella* gastroenteritis has been estimated to be 93.8 million cases of gastroenteritis with 155,000 deaths annually[6)]. Food of animal origin especially poultry meat is considered to be one of the major vehicles of *Salmonella* infections in humans and has been implicated in outbreaks of human salmonellosis[7)]. In the United States, an estimated 1 million incident cases of human salmonellosis occur annually[8)]. *Salmonella* infections account for approximately 19,336 hospitalizations, 17,000 quality adjusted life years lost, and

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$3.3 billion in total medical expenditures and lost productivity each year[9].

In Korea, the estimated annual occurrences of food borne illness are 336,138 cases, with inpatient stays (hospitalizations), outpatient visits (foodborne disease infections), and patient experiences (without visiting physicians) accounting for 2.3, 14.4 and 83.3%, respectively. In addition, 93.4% of the estimated annual foodborne illness outbreaks were caused by bacterial infections, 6.2% by viral infections, and 0.4% by parasites. *Escherichia coli* (*E. coli*), including enterohemorrhagic *E. coli*, was responsible for most illnesses, followed by non-typhoidal *Salmonella* spp.[10].

S. Typhimurium is one of the most important food pathogens. *S.* Typhimurium infections through the ingestion of contaminated water or foods are common, and food poisonings due to *S.* Typhimurium are usually sporadic[11]. *S.* Typhimurium is a leading cause of acute foodborne zoonoses worldwide being responsible for hundreds of millions of cases of gastroenteritis and bacteremia annually[12].

Recently, *S.* Typhimurium infections are becoming harder to treat because a high percentage of isolates are resistant to commonly used antibiotics. Due to this increasing prevalence of resistance in pathogenic isolates, there is a greater than ever need for effective cleaning and disinfection regimes to prevent infections and contain outbreaks[13].

For the disinfection of food storage facilities, many fumigants such as phosphine, chlorine dioxide, orthophenylphenol and paraformaldehyde have been widely used all over the world[14,15,16]. In particular, paraformaldehyde containing 91-95% formaldehyde is used for fumigation of buildings, rooms and in the poultry industry[17,18].

Highly hygienic measures, including the use of disinfectants, are very effective for successful control of bacterial infections in food processing and preservation facilities[19]. Several fumigants including sulfur dioxide, hydrogen peroxide and chlorine dioxide are used for decontamination against *S.* Typhimurium[20,21]. However, there is no efficacy test for a fumigant containing paraformaldehyde against bacterial foodborne pathogens. Therefore, this study was carried out to examine the bactericidal efficacy of a fumigant against *S.* Typhimurium.

Materials and Methods

Fumigant

The fumigation disinfectant was provided by Shinhan BioChem (Hwaseong, Korea). The fumigant contained 35% paraformaldehyde (1 block, 700 g) was stored at room temperature. Determination of the bactericidal efficacy of the disinfectant was based on French standard NF T 72-281[22].

Bacterial culture

Salmonella Typhimurium (G-B-14-21-62) was obtained from the Korean Veterinary Culture Collection (KVCC, Seoul, Korea). *S.* Typhimurium was cultured in Luria-Bertani (LB) broth containing 1.5% agar at 37°C for 24 h.

Dilution of bacterial suspension

According to the method of McFarland[23], the turbidity of the culture medium was measured. Continually, the number of cells in the suspension was adjusted to approximately 10^9 colony forming units (CFU)/mL using the diluent (1 L of distilled water, 1.0 g tryptone, 8.5 g sodium chloride). Then, the bacterial suspension was diluted 20-fold using reconstituted milk (1 L of distilled water, 100 g skimmed powdered milk). The final bacterial suspension adjusted to 10^8-10^9 CFU/mL was used for the bactericidal efficacy test of the fumigant.

Counting the in-milk test suspensions

After 1.0 mL of each diluted suspension was taken and added to each Petri dish, 20 mL of molten tryptic soy agar (TSA, BD, Franklin Lakes, NJ) were added to the Petri dishes. In addition, 1.0 mL of each diluted suspension was transferred into separate membrane filters (47-50 mm diameter, 0.45 μm pore size). After immediate filtration, the filter membrane was washed with the diluent. Each washed membrane was laid onto the surface of the agar medium. After all plates were incubated at 37°C for 24 h, any plates that were not countable were discarded. The remaining plates were incubated for a further 24 h, and the number of colonies on the plates was counted. The number of colonies incubated by the pour plate method and filter membrane method was represented by N1 and N2, respectively.

Application of bacterial suspension to carriers

A 0.05-mL aliquot of bacterial suspensions was deposited on two sterilized carriers made from stainless steel (3 cm diameter), and the carriers were left to air-dry in Petri dishes for no more than 45 min at 37 ± 1°C in a continuous air-exchange incubator. After the drying step, two carriers were stood vertically in their Petri dishes and left in the test room (300 m^3) at 21 ± 0.5°C and a relative humidity of 60 ± 10% for 3 h. After standing for 3 h, each carrier was removed and transferred into a conical flask containing 100 mL of sterile recovery diluent (1 L of distilled water, 1.0 g tryptone, 8.5 g NaCl). After the conical flask was shaken by hand for a few seconds, using a glass stirrer, every surface of the carrier was scraped for at least 1 min to dislodge any remaining dried suspension residues. After another quick shaking, 1 mL of recovery liquid was diluted with 9 mL of the diluent, and serial dilutions continued until a 10^{-3}

dilution was achieved. Continually, 1-mL aliquots from two sets of the 10^{-2} and 10^{-3} recovery diluent dilutions were transferred into Petri dishes to carry out counts by agar-plating in an agar medium. After incubation of the Petri dishes at 37°C for 24 h, the number of colonies in the agar medium was counted, and the means for each of the 10^{-2} and 10^{-3} dilutions represented the number of bacteria on the carriers (T).

Screening test for an inhibitory effect of fumigant residues

Fig. 1 presents a schematic diagram of the preliminary screening test for an inhibitory effect of fumigant residues.

Three carriers with deposited bacterial suspensions were stood vertically in their Petri dishes and left in the test room (300 m^3) at a height of 1.02 m from the floor and a distance of 8.4 m from the ignited fumigant, under the same conditions mentioned above. After exposure to the fumigant for 3 h, each carrier was transferred into a conical flask containing 100 mL of the sterile recovery diluent. After the conical flask was shaken by hand for a few seconds, 1 mL of the suspension and 1 mL of each bacterial diluted suspension (10^{-7} and 10^{-8}) were added to the Petri dish. Nutrient broth was poured into the Petri dish, and the Petri dish was incubated at 37°C for 48 h. After incubation, the mean of the number of colonies was represented as n1.

The remaining recovery liquid (98 mL) was poured into a sterile filter membrane and the membrane filter was washed in triplicate with 50 mL of the diluent. The washed membrane, 1 mL of each bacterial diluted suspension (10^{-7} and 10^{-8}) and 1 mL of the recovery liquid containing the cleaning carrier were added to the Petri dish. Nutrient broth was poured into the Petri dish, and the Petri dish was incubated at 37°C for 48 h. After incubation, the mean of the number of colonies was represented as n2. 1 mL of each bacterial diluted suspension (10^{-7} and 10^{-8}) and 1 mL of the recovery liquid containing the cleaning carrier were added to the Petri dish. Nutrient broth was poured into the Petri dish, and the Petri dish was incubated at 37°C for 48h. After incubation, the mean of the number of colonies was represented as n3. n1, n2 and n3 should be greater than 0.5N1, 0.5N2 and 0.5N1, respectively.

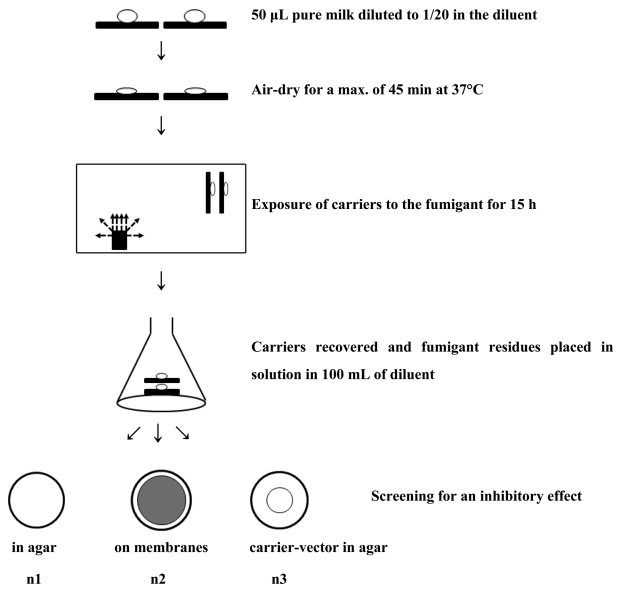

Fig. 1. Schematic diagram of the preliminary screening test for an inhibitory effect of fumigant residues.

Evaluation of the fumigant in terms of bactericidal efficacy

Three carriers with deposited bacterial suspension were stood vertically in their Petri dishes and left in the test room (300 m^3) at a height of 1.02 m from the floor and a distance of 3.4 m from the fumigant under the same conditions as set out above. After exposure to the fumigant for 3 h, each carrier was transferred into a conical flask containing 100 mL of sterile recovery diluent. After the conical flask was shaken by hand for a few seconds, every surface of the carrier was treated using the same method described above. After another quick shaking, 1 mL of the recovery liquid was diluted with 9 mL of the diluents, and serial dilutions continued until a 10^{-3} dilution was achieved. Continually, 1-mL aliquots from two sets of the recovery liquid and/or 10^{-1}, 10^{-2} and 10^{-3} dilutions were transferred into Petri dishes to carry out the counts by pour-plating in an agar medium. In addition, 10 mL of the recovery liquid was poured into a sterile filter, and then, the remaining recovery liquid (87 mL) was poured in to another separate filter. After filtration, each membrane filter was transferred onto the agar medium and incubated at 37°C for 48 h. After each carrier was removed under aseptic conditions and placed in Petri dishes with the inoculum facing upward, a sufficient quantity of agar medium to cover the carrier was poured in and the dishes were incubated at 37°C for 48 h. After incubation, the number of colonies was counted and the mean of the number of colonies developed on membrane filters and carriers plated in the agar medium was expressed as n'1 and n'2, respectively.

Experimental data and calculations

On agar, plates containing over 330 or fewer than 14 colonies were not counted. The mean number of colonies obtained for two successive dilutions (10^{-6}, 10^{-7}) was calculated, and the rate of germs in suspension (N) was obtained as follows:

$$\mathbf{N} = \frac{x+y+z+w}{2.2} \times 10^6$$

(x and y at 10^{-6} dilution, z and w at 10^{-7} dilution)

The mean number of colonies obtained for two successive dilutions (10^{-2}, 10^{-3}) was calculated, and the number of germs per carrier (T) was obtained as follows:

$$\mathbf{T} = \frac{x+y+z+w}{2.2} \times 10^6$$

(x and y at 10^{-2} dilution, z and w at 10^{-3} dilution)

The log (base 10) reduction (d) was obtained by integrating the number of germs on control-carriers (T), and the number of n'1 + n'2 surviving germs. The equation to obtain the log reduction is written as:

$$\mathbf{d} = \log T - \log (n'1 + n'2) = \log [T/(n'1 + n'2)]$$

When the log reduction (d) is greater than 5.0, the fumigant has a bactericidal activity.

Results and Discussion

Screening test for an inhibitory effect of fumigant residues

Table 1 shows the number of in-milk bacterial suspensions and bacteria numbers in carriers exposed to the fumigant.

According to Association Francaise de Normalisation (AFNOR)[22], the number of germs in suspension (N) should be between 10^8 and 5×10^9, and the number of germs per carrier (T) greater than 10^6 CFU/mL. In Table 1, the N and T value were 3.9×10^8 and 3.3×10^6, respectively, which met the criteria of AFNOR[22].

According to AFNOR[22], the fumigant efficacy test can only be carried out if n1, n2 and n3 are higher than 0.5N1, 0.5N2 and 0.5N1, respectively. In Table 1, it can be seen that n1, n2 and n3 were higher than 0.5N1, 0.5N2 and 0.5N1, respectively, satisfying the criteria of AFNOR[22].

Table 2. Viable counts (CFU/mL) of *S.* Typhimurium in carriers exposed to the fumigant and bactericidal efficacy of the fumigant

Dilution time	Number of colonies in carriers[1]			n'1[2]	n'2[3]	d value (log)[4]
	C1	C2	C3			
10^2	0	0	0	23	0	
10^3	0	0	0	18	0	5.22
				19	0	

[1]C1, C2, C3, test-carriers.
[2]n'1, the mean number of bacteria in the recovery solution.
[3]n'2, the mean number of bacteria on carriers plated in agar.
[4]d, the reduction of bacterial number:
d = log T – log (n'1 + n'2) = log [T/(n'1 + n'2)]
(T, the mean number of bacteria recovered on the carriers)

Evaluation of the fumigant in terms of bactericidal efficacy

Table 2 shows the number of colonies in the carriers exposed to the disinfectant and the bactericidal effect of the fumigant.

In Table 2, it can be seen that the log (base 10) reduction (d) was 5.22. According to AFNOR[22], the d value should be greater than 5.0 to have a bactericidal activity. Therefore, the fumigant used in this study has a bactericidal activity against *S.* Typhimurium.

In a previous study[24], 100 μL of *Salmonella* spp. suspensions were spot-inoculated onto the surface (5 cm^2) of lettuce (approximately 8-9 log CFU/mL), air-dried, and treated with ClO_2 gas at a concentration of 5 mg/L for 10

Table 1. Viable counts (CFU/mL) of *S.* Typhimurium in milk-to-suspensions and in carriers exposed to the fumigant

Milk-to-suspensions									Exposure to fumigant							
N1[1]				N2[2]				N[3]	Exposure-carrier				Control-carrier			T[6]
10^7		10^8		10^7		10^8				n1	n2	n3	D[5]	1	2	
33	27	15	11	32	24	15	13	3.9×10^8	1	20	18	18	10^2	277	282	3.3×10^6
									2	22	20	21				
30		13		28		14			3	21	22	22	10^3	79	83	
										21	20	20.3				

[1]N1, the number of bacterial test suspensions by the pour plate method.
[2]N2, the number of bacterial test suspensions by the filter membrane method.
[3]N, the number of bacteria in the working culture suspension:
N = {(x + y + z + w)/2.2} × 10^7 (x, y: 10^7 dilution colony; z, w: 10^8 dilution colony)
[4]n1, n2, and n3, the number of colonies on the carriers exposed to the fumigant.
[5]D, dilution time.
[6]T, the mean number of bacteria recovered on the control-carriers:
T = {(x + y + z + w)/2.2} × 10^2 × 100 (x, y: 10^2 dilution colony; z, w: 10^3 dilution colony)

min. From the study results, *Salmonella* spp. were reduced to 2.8 log CFU/5 cm^2 of lettuce. In another previous study[25], reductions of 3 and 6 log CFU/g were observed with *S.* Typhimurium associated with alfalfa seeds and mung beans, respectively, after dried alfalfa seeds and mung beans were contaminated with *S.* Typhimurium (10^8-10^9 CFU/g) and sealed in glass jars containing 180 mg of ammonia/L of air space for 22 h. In addition, in a previous study using a fumigant containing 20% ortho-phenylphenol, the reduction of *S.* Typhimurium numbers was 5.26 log CFU/mL after treatment with the fumigant for 15 h[26].

Considering target materials, space, treatment levels and time, the bactericidal activity of the fumigant used in this study was higher than that of ammonia and similar to that of ClO_2 gas and a fumigant containing 20% ortho-phenylphenol.

Based on the results of this study, the fumigant containing 35% paraformaldehyde has an effective bactericidal activity, and can be used to disinfect food materials and kitchen appliances contaminated with pathogenic bacteria.

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국문요약

본 연구는 *S.* Typhimurium을 대상으로 paraformaldehyde 35%를 함유한 훈증소독제의 살균효과를 평가하기 위해 수행되었다. 예비 시험에서, *S.* Typhimurium의 현탁액 균수는 모두 3.9×10^8 CFU/mL이었으며, 훈증소독제에 노출시킨 모든 담체의 균수는 평판배지법과 여과법으로 배양한 시험균주 현탁액의 균수의 50%보다 많았다. 또한, 대조 담체로부터 회복된 *S.* Typhimurium 균수는 모두 3.3×10^6 CFU/mL이었다. 훈증소독제의 살균효과 시험에서는, 훈증소독제를 처리한 담체의 *S.* Typhimurium의 감소 균수는 5.22 log CFU/mL로 나타났다. 이상의 결과로부터, paraformaldehyde를 주성분으로 하는 훈증소독제는 *S.* Typhimurium에 대해 효과적인 살균력을 갖는 것으로 확인되었으며, 병원성 세균에 오염된 식품재료 및 주방용품의 소독에 적용할 수 있을 것으로 사료된다.

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