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Bacteriology

Disinfectant resistance of *Salmonella* in *in vitro* contaminated poultry house models and investigation of efficient disinfection methods using these models

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ABSTRACT. *Salmonella enterica* subsp. *enterica* (*Salmonella*) shows disinfectant resistance by forming biofilms on solid surfaces. However, efficient disinfection methods to eliminate *Salmonella* biofilms from farms have not yet been examined in detail. In this study, more than 80% of *Salmonella* strains from farms in Yamagata prefecture, Japan, were biofilm producers. Regardless of the extent of their biofilm formation ability, their biofilms were highly resistant to hypochlorous acid on plastic surfaces. To establish efficient disinfection methods in farms, we developed *in vitro Salmonella*-contaminated poultry house models by depositing dust on ceramic and stainless-steel carriers in poultry houses for one month and culturing a representative *Salmonella* strain on the carriers. Biofilm-like structures, including *Salmonella*-like cells, were observed on the models by scanning electron microscopy. *Salmonella* was not efficiently removed from the models even by cleaning with a surfactant at 25/65°C and disinfection with quaternary ammonium compound or hypochlorous acid at 25°C; on the contrary, viable *Salmonella* cells increased in some tests under these conditions, suggesting that these models successfully simulate the highly persistent characteristics of *Salmonella* in farms. However, the persistent bacterial cells were markedly decreased by soaking in 65°C surfactant followed by rinsing with 80°C water, additional cleaning using chlorine dioxide or disinfection with dolomitic lime, suggesting the effectiveness of these methods against *Salmonella* in farms. Since many different disinfection conditions may be easily tested in laboratories, our models will be useful tools for establishing effective and practical disinfection methods in farms.

KEYWORDS: biofilm, disinfection, *in vitro Salmonella*-contaminated poultry house model, rearing environment

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Salmonella enterica subsp. *enterica* (*Salmonella*) is an important bacterial pathogen that causes salmonellosis in domestic animals and severe economic losses to the livestock industry. Since it is also a global cause of foodborne diseases [29], the elimination of *Salmonella* from farms is important for both animal and public health. However, its disinfection on farms is not easy [9].

Salmonella has the ability to form biofilms on biotic (living) as well as abiotic (inert/nonliving) surfaces such as ceramic tiles, plastic, cement, and stainless steel [13, 24], and the difficulties associated with disinfection for this bacterium are considered to be at least partly due to its biofilm formation ability. *Salmonella* strains in their biofilm forms were reportedly more resistant to disinfectants than their planktonic counterparts [7], and the majority of *Salmonella* isolates from environmental and animal sources tested in a previous study were able to form biofilms [27].

In a previous study by Chylkova *et al*., effective disinfectants against *Salmonella* were investigated under simulated poultry processing conditions, and the use of cetylpyridinium chloride was proposed as a tool for controlling *Salmonella* biofilms in poultry

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processing environments [7]. However, appropriate disinfection methods against this bacterium under field conditions such as poultry houses have yet to be adequately examined. Marin *et al*. investigated the capacity of glutaraldehyde, formaldehyde, and hydrogen peroxide to remove *Salmonella* in an artificial contamination test under field conditions; however, in the absence of cleaning steps, the use of these disinfectants at a concentration of 1.0% was inadequate for the elimination of *Salmonella* irrespective of the serotype, the biofilm development capacity, and the disinfectant contact time [18]. In a study by Martelli *et al*., the cleaning and disinfection of duck houses significantly reduced *Salmonella* contamination; however, after restocking, the level of *Salmonella* contamination significantly increased again, suggesting that limited residual contamination on farms after cleaning and disinfection represents a risk of infection and that thorough cleaning and disinfection procedures need to be implemented to reduce *Salmonella* infections [19]. Although the potential of trisodium phosphate as a sanitizer to reduce biofilm formation by *Salmonella* spp. on abiotic surfaces during poultry processing was previously suggested [23], the inherent roughness of the substratum on which the biofilm was grown was shown to influence the biocidal efficacy of trisodium phosphate [15]. Collectively, these findings suggest the difficulties associated with *Salmonella* disinfection under field conditions and the need for further investigations to establish effective disinfection methods against this bacterium on farms. However, the amount and distribution of bacteria are generally uneven in the field environment. In addition, labor for field tests on farms is more intensive than that for laboratories. Therefore, it is challenging to obtain stable and reliable test results from field tests, and the development of less labor-intensive test methods, which simulate farm conditions in laboratories and evaluate *Salmonella* disinfection efficacy with excellent reproducibility, are desired.

In ASTM E2871-19 of ASTM International (West Conshohocken, PA, USA), the standard test method for assessing disinfectant efficacy against biofilms using a single tube (https://www.astm.org/e2871-19.html [accessed on July 3, 2022]) is specified. The test method was optimized and validated for *Pseudomonas aeruginosa* or *Staphylococcus aureus* biofilms grown in the CDC Biofilm Reactor [4]. Although this method may be used to evaluate additional bacteria, it may not necessarily be suitable for assessing disinfection efficacy against *Salmonella* under simulated field conditions such as poultry houses. Even in the European standard methods EN 14885:2018 [11], test conditions and requirements to evaluate disinfection efficacy against *Salmonella* under simulated field conditions are not specified.

Therefore, to establish disinfection methods with the capacity to reduce *Salmonella* contamination in livestock farms and prevent the livestock product-to-human transmission of this bacterium, we investigated the biofilm formation ability of *Salmonella* strains isolated in Yamagata prefecture, Japan, selected ceramic tiles and stainless-steel boards for the carriers as the same or similar materials to those used in poultry houses, and developed *in vitro Salmonella*-contaminated poultry house models simulating *Salmonella* contamination in farms using the carriers and a representative strain with high biofilm formation ability. Using the developed models, we examined effective disinfection methods against the pathogen on farms.

MATERIALS AND METHODS

Salmonella strains

Salmonella strains were isolated from organs (the hearts, lungs, brains, mesenteric lymph nodes, ovaries, kidneys, and livers), spinal abscess, intestinal contents, rectal and excreted feces, and environmental samples (such as dust, swabs of floors and manure-collecting conveyers, and rodent droppings) in cattle farms, pig farms, and poultry farms in Yamagata prefecture between 2004 and 2021. Approximately 1 g of the organs, spinal abscess, intestinal contents, and fecal samples were cultured in 10 mL of Hajna Tetrathionate broth for selective enrichment at 37°C or 41.5°C for 24 hr. Regarding samples from the environment, non-selective enrichment was performed in Buffered Peptone Water (Oxoid, Thermo Fisher Scientific Inc., Waltham, MA, USA) at 37°C for 24 hr before selective enrichment. Each aliquot was then streaked on DHL agar (Pearlcore DHL Agar; Eiken Chemical Co., Ltd., Tokyo, Japan) supplemented with 20 µg/mL novobiocin (FUJIFILM Wako Pure Chemical Corp., Osaka, Japan) (N-DHL agar) and cultured at 37°C for 24 hr. *Salmonella* spp. were identified based on their colony morphology on selective media and biochemical properties, as previously described [10]. Serovar identification was performed by microtiter and slide agglutination methods according to the White-Kaufmann-Le Minor scheme [12].

A total of 105 *Salmonella* strains from cattle farms (23 strains), pig farms (49 strains), and poultry farms (33 strains), which consisted of 19 serovars, including Typhimurium (ST) (40.0%), Infantis (17.1%), 4,[5],12:i:- (11.4%), and others (less than 6.0% each) (Supplementary Table 1), were used for the biofilm formation assay. Twenty-six ST and ten 4,[5],12:i:- strains, which were isolated in a region of Yamagata prefecture between 2005 and 2021, were analyzed by a macrorestriction analysis of *Xba*I-digested genomic DNA using pulsed-field gel electrophoresis (PFGE) and single nucleotide polymorphism (SNP) genotyping for molecular epidemiological investigations according to the methods described by Arai *et al*. [3]. Regarding *in vitro* disinfectant susceptibility testing, two epidemiologically unrelated ST strains (18-15 and 18-38), which were isolated in different farms and had different biofilm formation abilities, were employed, and one of them (18-38) was used in disinfectant susceptibility testing with the *in vitro* contaminated poultry house models.

Biofilm formation assay

The biofilm formation abilities of *Salmonella* strains were measured using sterile 96-well flat-bottomed polystyrene microtiter plates (Micro test plate PS 96 wells Flat-form; nerbe plus GmbH & Co., KG, Winsen/Luhe, Germany). Inocula were prepared by culturing each strain in pearlcore trypto-soy broth (TSB) (Eiken Chemical Co., Ltd.) at 37°C for five hours under aerobic conditions and diluting the cultures 1,000-fold with sterile water. To investigate biofilm formation abilities, 20 µL of the inocula (approximately 10^7 colony forming unit [CFU]) and 230 µL of TSB were dispensed in each well of a 96-well plate and incubated at 37°C for one or

three days under aerobic conditions. After cultivation, unbound cells and culture media were removed by pipettes. Adhered cells in each well were then gently rinsed with 300 µL of sterile water, fixed with 250 µL of methanol for 15 min, air-dried after the removal of methanol, and stained with 250 µL of 0.5% (w/v) crystal violet for 5 min. After rinsing off excess stains with tap water, plates were air-dried, and the dye that bound to adhered cells was resolubilized with 250 µL of 99.5% ethanol by incubating the sealed plates at 25°C overnight. The optical density (OD) of each well was then measured at 590 nm (OD_{590}) using the iMark microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Each strain was tested using seven wells, and 12 negative control wells containing only 250 µL TSB/well were set in each plate. The cut-off OD (ODc) was defined as three standard deviations above the mean OD of negative control wells, and strains were classified into biofilm producers and non-biofilm producers when OD was >ODc and ≤ODc, respectively.

Cleaners, disinfectants and neutralizing solution

The cleaners and disinfectants used in the present study are listed in Table 1. These reagents were selected based on information on disinfection programs from three layer poultry farms in Yamagata prefecture, Japan. In disinfectant susceptibility testing in tubes, 200 mM sodium thiosulfate in 100 mM phosphate buffer (pH 7.5) was used as the neutralizing solution. In disinfectant susceptibility testing on *in vitro* contaminated poultry house models, 1 M Tris-HCl (pH 7.0) (for dolomite lime emulsion) and D/E Neutralizing Broth (Difco, Becton, Dickinson and Co., Franklin Lakes, NJ, USA) (for other disinfectants) were used for neutralization. All neutralizing solutions were confirmed to neutralize the disinfectants and not to interfere with *Salmonella* growth. Sterile water was used for rinsing, the dilution of disinfectants, and disinfectant control.

Disinfectant susceptibility testing in tubes

The microbicidal efficacy of disinfectants on the biofilms and planktonic cells of ST strains 18-15 and 18-38 in tubes was evaluated under conditions of organic matter contamination using 96-well microplates.

1. Preparation of wells inoculated with planktonic cells: The ST strains grown on pearlcore trypto-soy agar (TSA) (Eiken Chemical Co., Ltd.) at 37°C overnight under aerobic conditions was suspended in sterile water, and 25 µL of the bacterial suspension, the turbidity of which was adjusted to 0.5 McFarland standard, was added to each well of the microplate.

2. Preparation of wells with Salmonella biofilms: For formation of *Salmonella* biofilms, 20 µL of the bacterial suspension (0.5 McFarland) was inoculated into 180 µL TSB in each well and cultured for one or three days as described above. Biofilms that formed on the wells were rinsed with 50 µL of sterile water, and 25 µL of sterile water was added to each well.

3. Disinfectant susceptibility testing: After preparing wells with the biofilms and planktonic cells, 25 µL of 20% (w/v) sterilized porcine fecal suspension and 200 µL of chlorine-based disinfectant (Table 1) were added to each well. After a 5-min incubation at 25°C, 50 µL of neutralizing solution was added and immediately mixed to halt disinfection. Biofilms were scraped off by pipette tips. Planktonic cells and cells from the biofilms were collected from the wells by pipettes, and the number of viable bacterial cells in the collected samples was investigated by plating serial dilutions of the neutralized samples onto TSA medium and counting colonies on plates after the incubation at 37°C for 24 hr under aerobic conditions. In all tests, four or more wells were used for each condition. In addition, four or more wells were used as no organic matter and no disinfectant controls, in which 25 µL and 200 µL of sterile water were added to each well instead of the porcine fecal suspension and disinfectant, respectively. After incubation and neutralization, ST cells in the control wells were retrieved and quantified as described above. The average number of CFU from the control wells was regarded as the number of viable cells in the no organic matter and no disinfectant control, and the efficacy of disinfectants was expressed as fold changes in viable cell numbers (viable cells in samples after the treatment/viable cells in the no organic matter and no disinfectant control).

Table 1. Cleaning agents and disinfectants used in the present study

Reagent	Purpose	Disinfectant susceptibility testing		Active ingredient	Concentration of the active ingredient/dilution rate of the reagent applied to disinfectant susceptibility testing
		In tubes	On poultry house models		
Surfactant[a]	Cleaning	Not tested	Tested	Non-ionic surfactant Amphoteric surfactant Sodium hydroxide	100 times (1% [v/v] Biosolve solution)
Chlorine dioxide[b]	Cleaning	Not tested	Tested	Chlorine dioxide	Approximately 20 ppm
Quaternary ammonium compound[c]	Disinfection	Not tested	Tested	Didecyldimethylammonium chloride	0.02%
Chlorine-based disinfectant[d]	Disinfection	Tested	Tested	Hypochlorous acid	0.02% available chlorine
Dolomite lime emulsion[e]	Disinfection	Not tested	Tested	Dolomitic lime	30% (w/v)

[a] Biosolve (Antec International Ltd., Sudbury, UK), [b] AQUA HEART KYROZID (KyroChem GmbH, Wedemark, Germany), [c] Lontect (Scientific Feed Laboratory Co., Ltd., Tokyo, Japan), [d] Antec Virkon-S (Antec International Ltd.), [e] Protect V (VESL YK, Veterinary Environment Sanitation Laboratory, Sapporo, Japan).

Disinfectant susceptibility testing on in vitro Salmonella-contaminated poultry house models

1. Preparation of in vitro Salmonella-contaminated poultry house models: Clean unglazed ceramic tiles (10 × 10 cm, non-glazed) (LIXIL Corp., Tokyo, Japan) and stainless-steel boards (2.5 × 3 cm, SUS430 [Japanese Industrial Standards]) (HIKARI Co., Ltd., Osaka, Japan), which were used as carriers, were placed in four different windowless cage layer houses with chickens of different ages (i.e., 4–17 months old) for one month in different seasons (i.e., in August–December, 2020 and January–May, 2021), and dust was left to be deposited on the carriers (Fig. 1). The absence of viable ST cells in dust-deposited carriers was confirmed by both quantitative investigations and delated secondary enrichment methods as described below. Other bacteria present in dust-deposited carriers were investigated by culturing the samples on Trypticase™ Soy Agar with 5% Sheep Blood (TSA II) (BBL; Becton, Dickinson and Co.) under air plus 5% CO_2 conditions and ABCM agar (Eiken Chemical Co., Ltd.) supplemented with 5% horse blood, 0.1% L-cysteine monohydrochloride, and additional agar (to a final concentration of 3%) under anaerobic conditions at 37°C for 48 hr. The species of the isolated bacteria were identified using API 20 E, API 20 Strep, and API Staph (bioMérieux Japan Ltd., Tokyo, Japan) and/or 16S rRNA gene sequencing. 16S rRNA genes were amplified, purified, and sequenced as described in Supplementary Table 2. Sequencing was performed by FASMAC Co., Ltd. (Atsugi, Japan), and data were analyzed using the EzBioCloud server (https://www.ezbiocloud.net) [30].

In vitro contaminated poultry house models were prepared in the laboratory by inoculating strain 18-38 on dust-deposited carriers and culturing them at 25°C for three days. Bacterial suspensions for inoculation were prepared by culturing the strain on TSA at 37°C for 24 hr under aerobic conditions and suspending the grown bacterial cells in sterile water at a final concentration of approximately

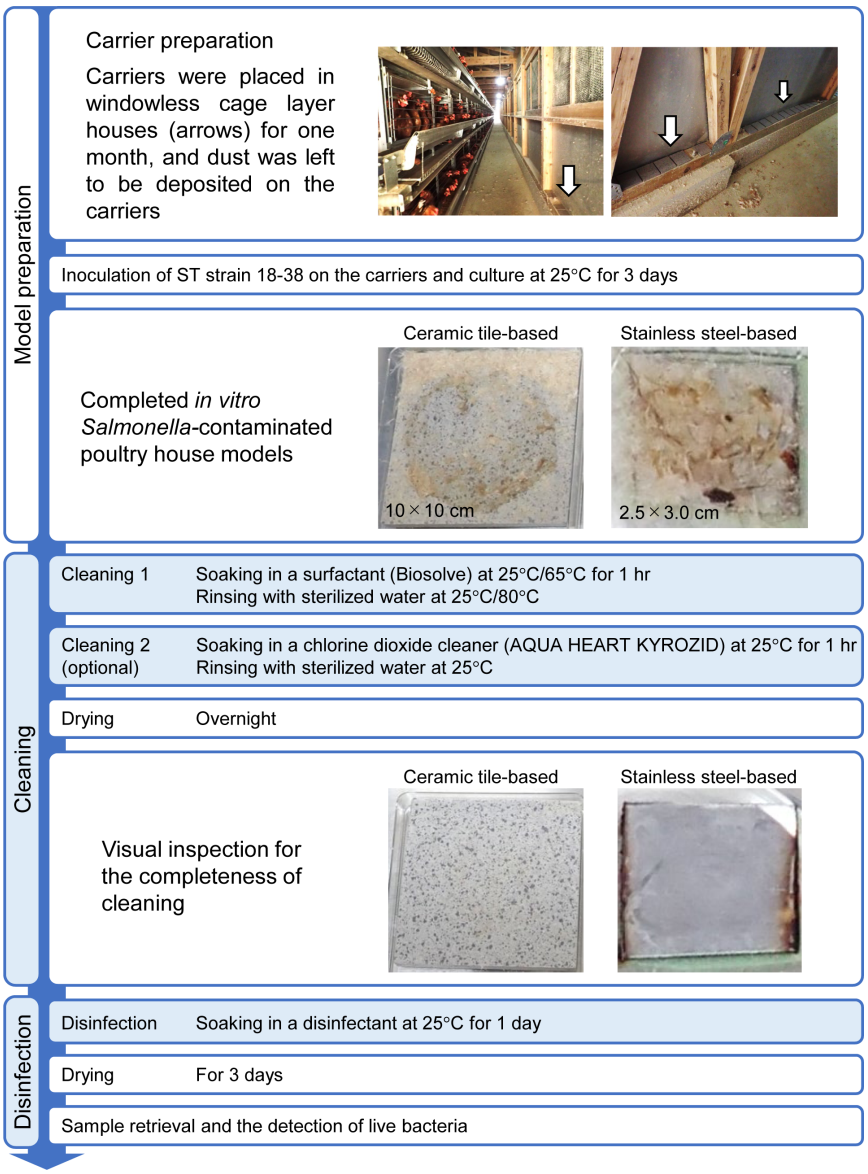

Fig. 1. An outline of procedures for *in vitro Salmonella*-contaminated poultry house model preparation and disinfectant susceptibility testing using these models.

10^8–10^9 CFU/mL. Final bacterial concentration in each inocula was determined by plating serial dilutions of the inocula onto TSA and counting colonies on the plates after incubating them at 37°C for 24 hr under aerobic conditions. One milliliter of the inocula was inoculated onto each dust-deposited carrier, and the dust and bacteria were mixed using disposable inoculating loops to spread the bacterial cells over the entire carriers before culturing them at 25°C. Dust-deposited carriers, which were inoculated with the same amount of ST and air-dried, but not cultured for three days, were used as "the dust-deposited but non-cultured ST control (DD-ST-NC control)". We also prepared "the artificial dust and ST mixture control (AD-ST-NC control)" by artificially and simultaneously loading ST and dust onto the carriers, but not culturing them for three days. Briefly, 1 mL of the ST inocula prepared as described above was inoculated onto clean autoclaved carriers. Approximately 0.3 g of dust collected from poultry houses was then put on the carriers. After spreading the dust and inocula mixture over the entire carriers using disposable inoculating loops, the carriers were dried at room temperature and used as AD-ST-NC control.

2. Scanning Electron Microscopy (SEM) analysis: The surface of a stainless steel-based contaminated poultry house model prepared as described above using ST strain 18–38 was observed by SEM. The same strain grown at 25°C for three days on a clean stainless-steel board and the strain on the AD-ST-NC control were also analyzed for comparison. After the air-drying of samples, unattached dust on them was removed by spraying air. Samples were then fixed for 15 min by fumigation with 2% (w/w) osmium tetroxide, and the surfaces were coated with a 5-nm layer of platinum using JEC-3000FC AUTO FINE COATER (JEOL Ltd., Tokyo, Japan). Images were taken at × 6,000 magnification in the low-vacuum mode with the SEM system (FEI Quanta 400; Thermo Fisher Scientific Inc.).

3. Evaluation of cleaning and disinfection programs using models: The outline and detailed conditions of the cleaning and disinfection programs tested in the present study are shown in Fig. 1 and Table 2, respectively. The poultry houses, into which the carriers were placed, basically followed the guidelines on cleaning, disinfection, and vector control for *Salmonella*-infected poultry flocks [28]; however, formalin fumigation was not performed for safety reasons. We designed the tested programs based on the cleaning and disinfection programs performed in the poultry houses. We also investigated additional reagents and conditions as shown in Table 2 to establish more efficient programs. After cleaning steps, the completeness of cleaning was visually inspected according to the recommendations of the WHO guidelines [28] (Fig. 1). Cleaners, water for rinsing, and disinfectants were poured onto the carriers using a pipette with a 2.5-mm aperture from a distance of approximately 3 cm at a constant speed (approximately 15 mL/sec). The amount of the reagents poured was 300 µL/cm^2 of the carrier. Unless otherwise mentioned, all treatments were performed at 25°C. After all treatments, bacteria on the carriers were retrieved into a tube by wiping off the surface of the carriers with a sterilized Kimwipe saturated with 20-mL appropriate neutralizing solution, and the number of viable ST cells in the samples was quantitatively investigated by plating serial dilutions of the neutralized samples onto N-DHL agar and counting black colonies on the plates after an incubation at 37°C for 24 hr under aerobic conditions (quantitative investigation). Regarding samples without the growth of black colonies, the presence or absence of viable ST cells was also investigated by the delated secondary enrichment method [20]. All black colonies randomly selected from colonies grown from ST-inoculated carriers were confirmed as O4-positive *Salmonella* by anti-O antigen sera, whereas no black colonies appeared on N-DHL agar from carriers without the ST inoculation after culturing for 24 hr. In each test, three untreated *in vitro* contaminated poultry house models were used as controls, and ST cells on the controls were retrieved and quantified as described above. The average number of CFU from the three control samples was regarded as the number of viable cells in samples before cleaning, and the efficacy of cleaning and disinfection was expressed as fold changes in viable cell numbers (viable cells in samples after all treatments/viable cells in samples before cleaning). The test under each condition was repeated three or more times.

Table 2. Conditions for disinfectant susceptibility testing on contaminated poultry house models

Conditions[a]	Cleaning 1[b]		Cleaning 2[b]		Disinfection[b,c]	Results are shown in
	Cleaner (temp)	Rinse (temp)	Cleaner (temp)	Rinse (temp)	Disinfectant (temp)	
C1	Surfactant (25°C)	Water (25°C)	ND	ND	ND	Figs. 5, 6, and 7
C2	Surfactant (65°C)	Water (25°C)	ND	ND	ND	Figs. 5 and 6
C3[d]	Surfactant (25°C)	Water (25°C)	ND	ND	QAC (25°C)	Fig. 5
C4	Surfactant (25°C)	Water (25°C)	ND	ND	HOCl (25°C)	Fig. 5
C5	Surfactant (25°C)	Water (25°C)	ND	ND	Water (25°C)	Fig. 5
C6[d]	Surfactant (65°C)	Water (25°C)	ND	ND	QAC (25°C)	Fig. 5
C7	Surfactant (65°C)	Water (25°C)	ND	ND	HOCl (25°C)	Fig. 5
C8	Surfactant (65°C)	Water (25°C)	ND	ND	Water (25°C)	Fig. 5
C9	Surfactant (65°C)	Water (80°C)	ND	ND	ND	Fig. 6
C10	ND	Water (80°C)	ND	ND	ND	Fig. 6
C11	ND	ND	Chlorine dioxide (25°C)	Water (25°C)	ND	Fig. 7
C12	Surfactant (25°C)	Water (25°C)	Chlorine dioxide (25°C)	Water (25°C)	ND	Fig. 7
C13	Surfactant (25°C)	Water (25°C)	Chlorine dioxide (25°C)	Water (25°C)	Water (25°C)	Fig. 7
C14	Surfactant (25°C)	Water (25°C)	Chlorine dioxide (25°C)	Water (25°C)	QAC (25°C)	Fig. 7
C15	Surfactant (25°C)	Water (25°C)	Chlorine dioxide (25°C)	Water (25°C)	HOCl (25°C)	Fig. 7
C16	Surfactant (25°C)	Water (25°C)	ND	ND	Dolomitic lime (25°C)	Fig. 7

[a] Both a ceramic tile and stainless steel board were used as carriers. [b] ND, not done. [c] QAC, quaternary ammonium compound; HOCl, chlorine-based disinfectant (hypochlorous acid). [d] Conditions recommended by the WHO guideline [28].

Statistical analysis

All statistical analyses were performed in EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan) [14] and R (The R Foundation for Statistical Computing, Vienna, Austria). Depending on the number of levels of each factor, logarithmic transformed fold changes in viable ST cells, which were assumed to follow a normal distribution, were analyzed by the *t*-test for two-level data or a one-way ANOVA followed by the Bonferroni *post-hoc* test for three and more-level data. In the present study, data from ceramic carriers and stainless-steel carriers were combined due to the absence of any significant differences (*t*-test, $P>0.05$), and the minimum detectable value was applied for data below the detection limit. The number of no viable ST samples (i.e., the number of delated secondary enrichment method-negative results) was analyzed by Fisher's exact test and the Bonferroni *post-hoc* test. In all tests, a value of $P<0.05$ was considered to be the threshold for significance.

RESULTS

Biofilm formation ability of Salmonella strains isolated from farms in Yamagata prefecture and its variation

Most of the *Salmonella* strains isolated in Yamagata prefecture were biofilm producers; 85% and 92% of the strains formed biofilms when cultured for one and three days, respectively, in 96-well plates. Similar to previous findings [1], the ability to form biofilms varied even among strains in the same serovar including ST, and no correlation was observed between the serovar and biofilm formation ability (Fig. 2).

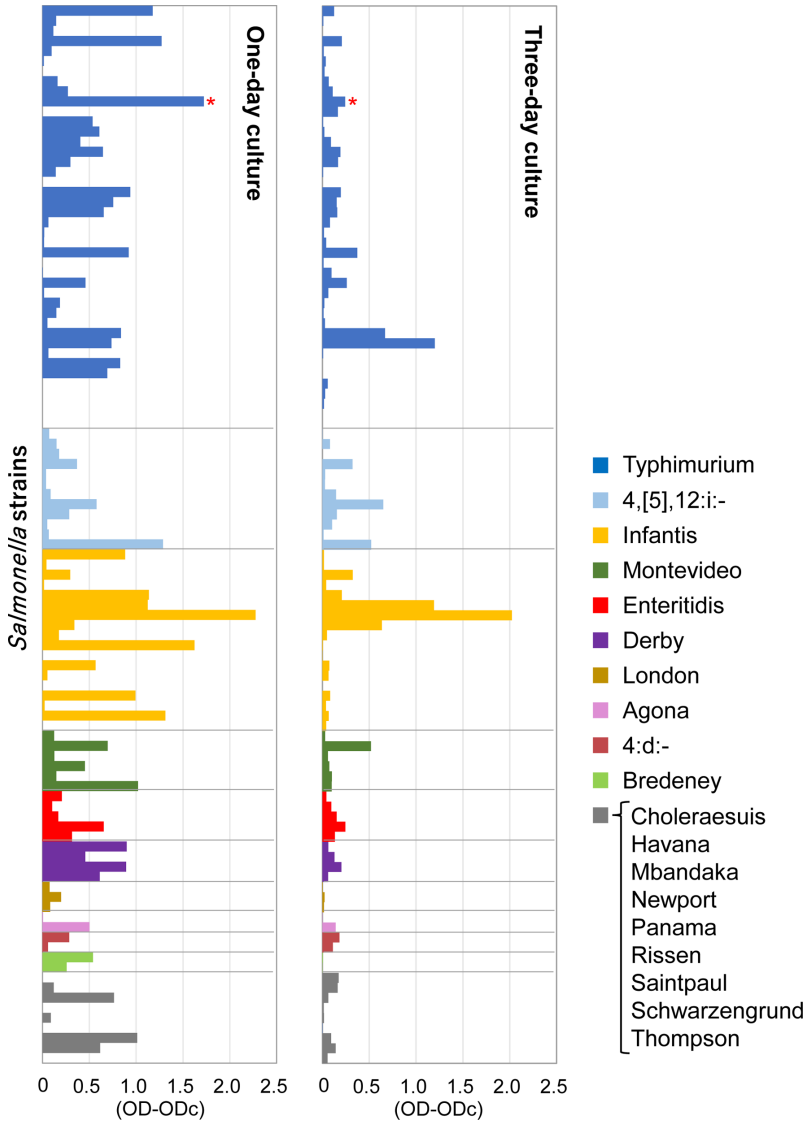

Fig. 2. Biofilm formation ability of *Salmonella* strains isolated from farms in Yamagata prefecture, Japan. Biofilm formation ability was measured as described in MATERIALS AND METHODS, and each bar represents the OD-ODc value of each strain. OD is the optical density measured at 590 nm, and ODc is the cut-off OD defined as three standard deviations above the mean OD of negative control wells. Strains were regarded as biofilm producers when the OD-ODc value was >0. Red asterisks indicate ST strain 18-38, which was used to prepare the *in vitro Salmonella*-contaminated poultry house models.

We selected 26 ST and 10 4,[5],12:i:- strains to investigate their phylogenetic relationships and differences in their ability to form biofilms among clonal strains. The 36 strains were classified into nine pulsotypes with some subtypes (Ia, Ib, II-V, VIa, VIb, VIIa, VIIb, VIII, and IX) by the PFGE analysis and six SNP genotypes (types 1, 3, 5, 6, 8, and 9) by SNP genotyping (Supplementary Fig. 1). A correlation was noted between the pulsotypes and SNP genotypes (Supplementary Fig. 1). Although the 20 strains with pulsotype I and SNP genotype 3 were considered to be clonal, the ability to form biofilms varied even in clonal strains (the OD-ODc value= −0.039–0.939 [when cultured for one day] and −0.015–1.200 [when cultured for three days]) (Supplementary Fig. 1).

Biofilm producers in Yamagata prefecture show disinfectant resistance in biofilm forms

Among the ST strains isolated in Yamagata prefecture, we selected two biofilm producers, strain 18-38 (pulsotype V, SNP genotype 1) with a relatively high biofilm formation ability and strain 18-15 (pulsotype I, SNP genotype 3) with a relatively low biofilm formation ability (Supplementary Fig. 1) and compared their susceptibility to a chlorine-based disinfectant between their biofilm and planktonic forms under conditions of organic matter (porcine feces) contamination. In 96-well plates (i.e., on plastic surfaces), both strains showed significantly higher disinfectant resistance in the biofilm forms than their planktonic counterparts (one-way ANOVA and the Bonferroni test; $P<0.05$) (Fig. 3). In addition, biofilms cultured for three days showed significantly higher resistance than those cultured for one day (one-way ANOVA and the Bonferroni test, $P<0.05$) (Fig. 3).

In vitro Salmonella-contaminated poultry house models

To simulate *Salmonella*-contaminated poultry houses, we selected ST strain 18-38 with a high biofilm formation ability (Fig. 2 and Supplementary Fig. 1) and prepared *in vitro Salmonella*-contaminated poultry house models by depositing dust on ceramic and stainless-steel carriers in poultry houses for one month and culturing the strain on dust-deposited carriers for three days. When the strain was grown on a clean stainless-steel board, the SEM analysis showed that biofilms purely consisted of many bacterial cells of 2 µm in length (Fig. 4A). Biofilm-like structures, including *Salmonella*-like bacterial cells, were also observed on the *in vitro Salmonella*-contaminated poultry house model; however, bacterial cells adhered to the carrier by being interwoven with the dust, suggesting the complex surface structure of these models (Fig. 4B). In contrast, no bacterial cells were observed on the surface of the AD-ST-NC control, on which ST was inoculated with artificially collected dust but not cultured (Fig. 4C). Although ST was not isolated from the dust-deposited carriers themselves, *Citrobacter youngae, Enterobacter cloacae*, *Enterococcus casseliflavus*, *Aerococcus viridans*, *Staphylococcus xylosus*, *Staphylococcus chromogenes*, *Corynebacterium freneyi*, *Corynebacterium* sp., and *Brachybacterium massiliense* were isolated.

After cleaning steps, the carriers appeared to be clean (Fig. 1). On AD-ST-NC and DD-ST-NC controls, the number of viable ST cells was greatly decreased in all tests either by the cleaning step alone or by the cleaning plus disinfection steps (a–e in Fig. 5). However, significantly more ST cells survived on the *in vitro Salmonella*-contaminated poultry house models than on the DD-ST-NC control after disinfection with quaternary ammonium compound (QAC) (*t*-test; e vs. f in Fig. 5, $P<0.05$). On the contrary, the number of viable ST cells on the model increased after the disinfection in some experiments (f in Fig. 5). Disinfection efficacy did not increase

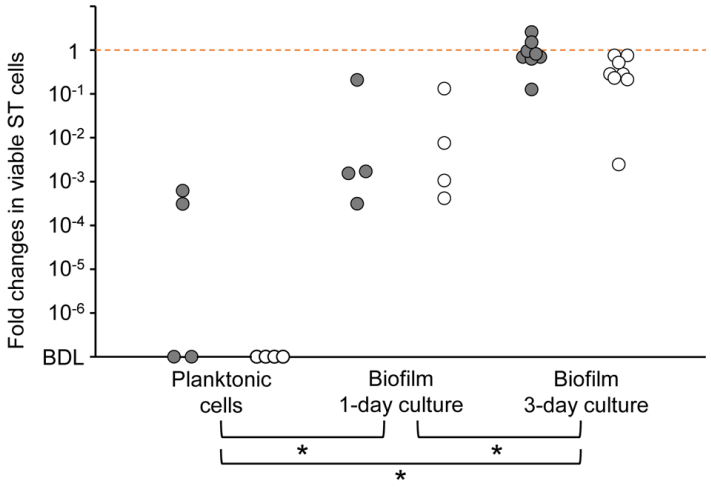

Fig. 3. Disinfectant resistance of *Salmonella* Typhimurium (ST) strains 18-38 and 18-15 in biofilm forms. The microbicidal effects of the chlorine-based disinfectant on the planktonic and biofilm forms of strains 18-38 (closed circles) and 18-15 (open circles) were investigated in 96-well plates under conditions of organic matter (porcine feces) contamination as described in MATERIALS AND METHODS. Data were collected from four or more independent tests. In each test, sterile water was added to control wells instead of the porcine fecal suspension and disinfectant, and the average number of colony forming unit (CFU) from the control wells was regarded as the number of viable cells in the no organic matter and no disinfectant control. The survival of ST strains was expressed as fold changes in viable cell numbers (viable cells in samples after the treatment/viable cells in no organic matter and no disinfectant control). BDL represents "below the detection limit" of the test. Asterisks indicate a significant difference (one-way ANOVA and the Bonferroni *post-hoc* test, $P<0.05$).

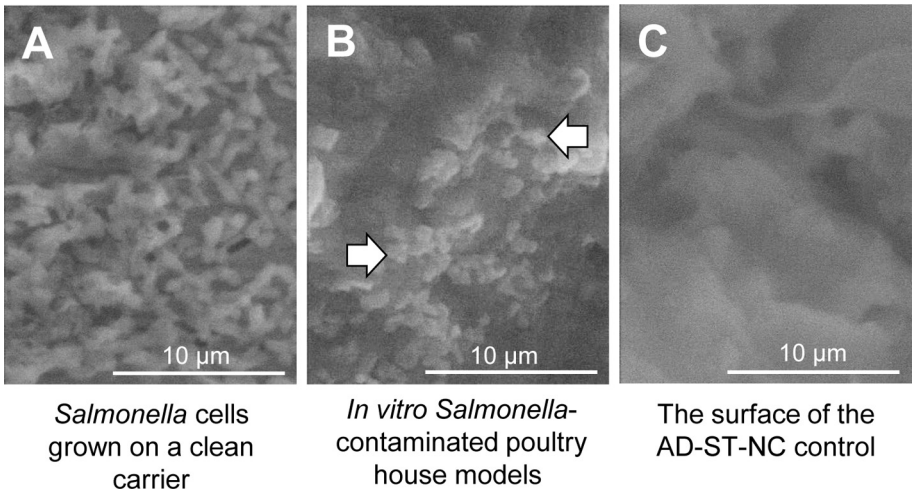

Fig. 4. Scanning electron micrographs of the surface of *Salmonella* Typhimurium (ST)-inoculated stainless-steel boards. (**A**) ST strain 18-38 cells purely grown on a clean stainless-steel board. (**B**) ST strain 18-38 cells interwoven with dust (indicated by arrows) on the *in vitro Salmonella*-contaminated poultry house model. (**C**) The surface of the artificial dust and ST mixture control (AD-ST-NC control), in which ST strain 18-38 and dust from poultry houses were artificially and simultaneously loaded onto a stainless-steel board, but not cultured for three days. No *Salmonella*-like bacterial cells were observed on the AD-ST-NC control. Images were taken at ×6,000 magnification in the low-vacuum mode with the FEI Quanta 400 SEM system (Thermo Fisher Scientific Inc.).

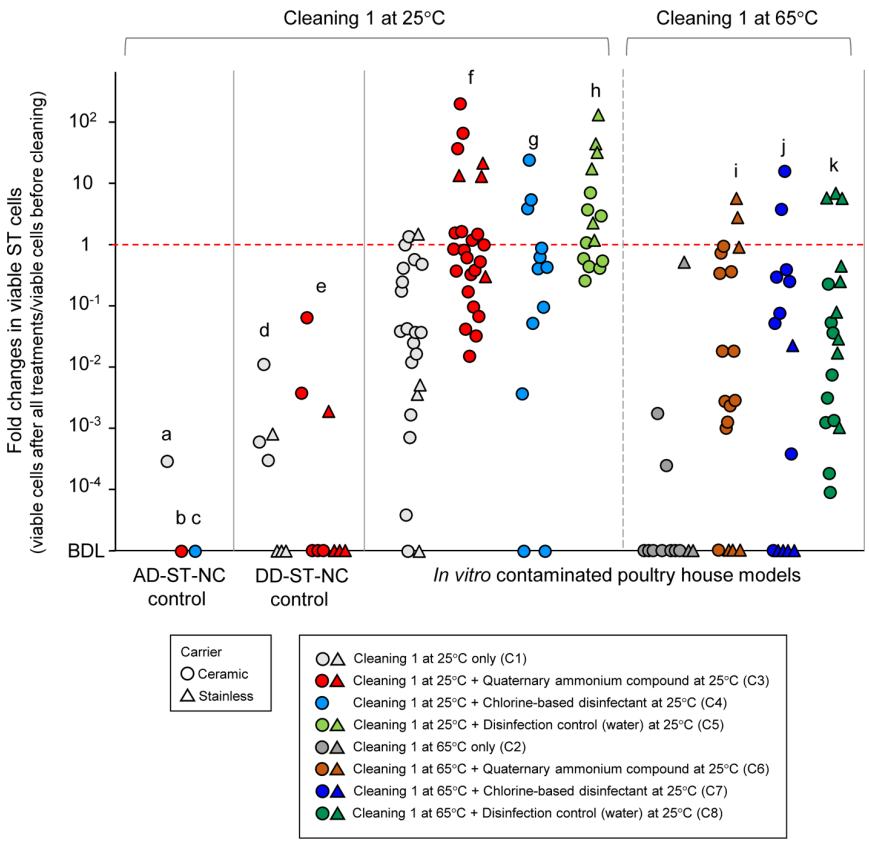

Fig. 5. Disinfectant resistance of *Salmonella* Typhimurium (ST) strain 18-38 on *in vitro Salmonella*-contaminated poultry house models. A surfactant (Biosolve) was used for cleaning 1. The survival of the ST strain was expressed as fold changes in viable cell numbers (viable cells in samples after all treatments/viable cells in samples before cleaning). AD-ST-NC control (the artificial dust and ST mixture control) is the carrier onto which artificially collected dust and ST were loaded, but not cultured for three days. DD-ST-NC control (the dust-deposited but non-cultured ST control) is the dust-deposited carrier inoculated with ST and air-dried, but not cultured for three days. BDL represents "below the detection limit" of the quantitative investigation. C1-C8 are disinfection conditions shown in Table 2. The letters a-k in the graph are the conditions under which the results are explained in the RESULTS section.

when a chlorine-based disinfectant (HOCl) was used, and fold changes in viable ST cells on the models did not significantly differ among QAC (f in Fig. 5), HOCl (g in Fig. 5), and the disinfection control (water) (h in Fig. 5) (one-way ANOVA and the Bonferroni test; P=0.123; only ceramic data was used for the test due to the lack of stainless data on HOCl). When the surfactant cleaner was treated at 65°C, QAC reduced viable ST cells significantly more than under the corresponding 25°C condition (t-test; f vs. i in Fig. 5, P<0.05). However, even under 65°C cleaning conditions, neither QAC nor HOCl had a stronger impact on disinfection efficacy than the disinfection control (water) (one-way ANOVA and the Bonferroni test; i vs. j vs. k in Fig. 5, P=0.9). These results demonstrated the strong resistance of the ST strain against cleaning and disinfection on *in vitro Salmonella*-contaminated poultry house models.

Effective disinfection methods

An increase in the surfactant temperature to 65°C markedly reduced the number of viable ST cells on *in vitro* contaminated poultry house models even without disinfection steps (one-way ANOVA and the Bonferroni test; conditions C1 vs. C2, P<0.05) (Fig. 6). In addition, the combination of 65°C surfactant and 80°C rinsing (conditions C9) further affected the viability of ST; no viable ST was detected even by the delated secondary enrichment methods in 10 of the 15 tests (indicated as ND in Fig. 6). The number of ND was significantly larger under condition C9 than under condition C1 (Fisher's exact test and the Bonferroni test; P<0.05) (Fig. 6). In contrast, viable ST was still detected in most cases when the models were treated with 80°C water only (condition C10) (Fig. 6), suggesting the importance of cleaners for effective pathogen removal from the models.

As a cleaner, chlorine dioxide was superior to the surfactant; more ST cells were removed by chlorine dioxide (conditions C11 and C12) than the surfactant only (condition C1) (Test 1 in Fig. 7 [one-way ANOVA and the Bonferroni test], P<0.05). Moreover, after sequential cleaning by the surfactant and chlorine dioxide (condition C12), significantly more ND results were obtained over condition C1 (Test 1 in Fig. 7 [Fisher's exact test and the Bonferroni test], P<0.05). However, additional disinfection steps with QAC, HOCl, and water (control) (conditions C14, C15, and C13, respectively) did not further affect the number of viable ST cells (Test 2 in Fig. 7 [one-way ANOVA and the Bonferroni test], P=0.648). In contrast, disinfection with dolomitic lime had a significant impact on the viability of ST on the poultry house models; even without additional cleaning 2 step with chlorine dioxide or an increase in the temperature of cleaning 1, no viable ST was detected from the carriers in any test (condition C16 in Fig. 7).

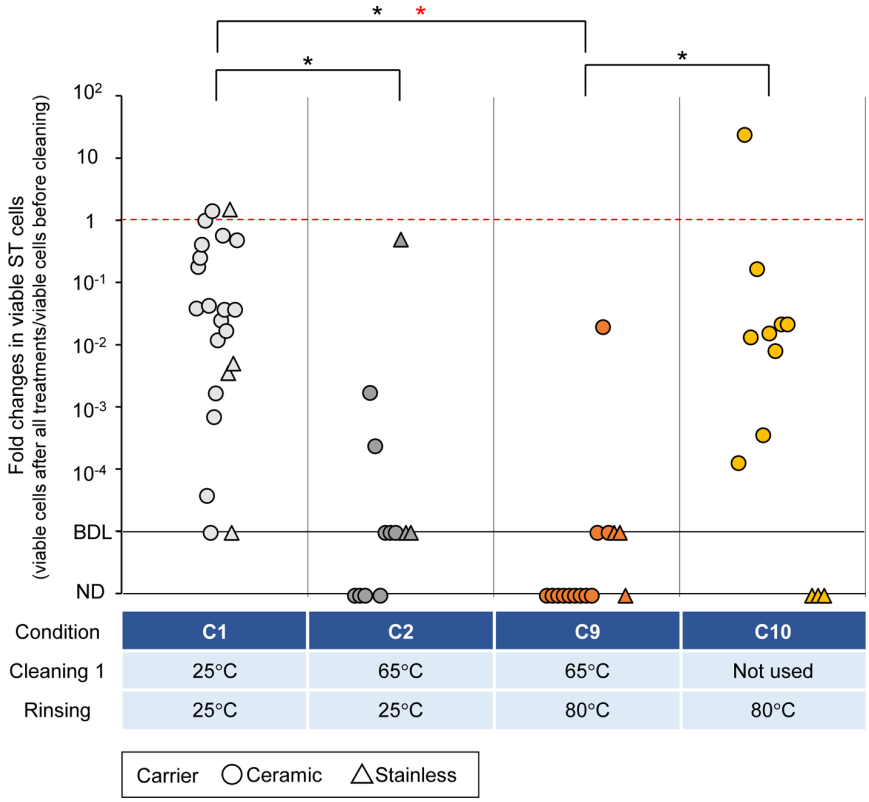

Fig. 6. Effects of temperature at cleaning and rinsing on the viability of *Salmonella* Typhimurium (ST) strain 18-38 on *in vitro Salmonella*-contaminated poultry house models. A surfactant (Biosolve) was used for cleaning 1. The survival of ST strains was expressed as fold changes in viable cell numbers (viable cells in samples after all treatments/viable cells in samples before cleaning). BDL represents "below the detection limit" of the quantitative investigation. ND represents "no ST detection" by the delated secondary enrichment methods, suggesting the complete elimination of viable ST. C1, C2, C9, and C10 are the conditions shown in Table 2. Black asterisks indicate a significant difference in fold changes in viable ST cell numbers (P<0.05 by one-way ANOVA and the Bonferroni *post-hoc* test). The red asterisk indicates a significant difference in the numbers of ND results (P<0.05 by Fisher's exact test and the Bonferroni *post-hoc* test).

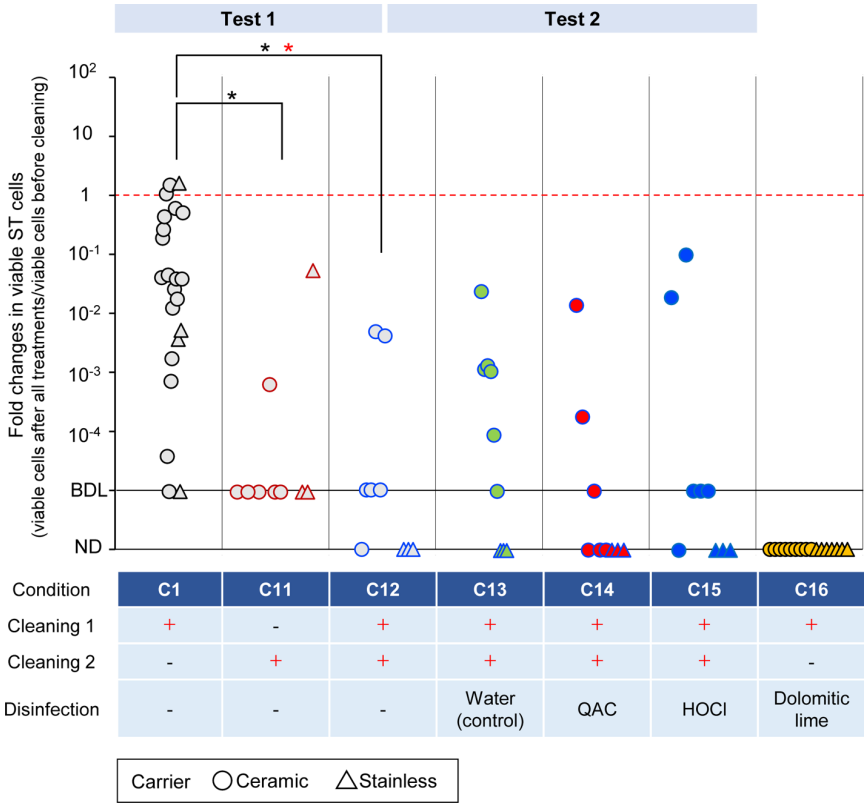

Fig. 7. Effects of chlorine dioxide at cleaning 2 step and dolomitic lime at the disinfection step on the viability of *Salmonella* Typhimurium (ST) strain 18-38 on *in vitro Salmonella*-contaminated poultry house models. A surfactant (Biosolve) and chlorine dioxide (AQUA HEART KYROZID) were used for cleaning 1 and 2, respectively. The survival of the ST strain was expressed as fold changes in viable cell numbers (viable cells in samples after all treatments/viable cells in samples before cleaning). BDL represents "below the detection limit" of the quantitative investigation. ND represents "no ST detection" by the delated secondary enrichment methods, suggesting the complete elimination of viable ST. C1 and C11–C16 are the conditions shown in Table 2. QAC, Quaternary ammonium compound; HOCl, Chlorine-based disinfectant (hypochlorous acid). Test 1 and Test 2 indicate statistical tests performed in this study using the data (see the RESULTS section). Black asterisks indicate a significant difference in fold changes in viable ST cell numbers ($P<0.05$ by one-way ANOVA and the Bonferroni *post-hoc* test). The red asterisk indicates a significant difference in the numbers of ND results ($P<0.05$ by Fisher's exact test and the Bonferroni *post-hoc* test).

DISCUSSION

We herein investigated the biofilm formation ability of *Salmonella* isolated from farms and selected an ST isolate with a high biofilm formation ability. Using the isolate, the disinfectant resistance of *Salmonella* in poultry houses was reproduced in a laboratory, and effective disinfection methods were examined using *in vitro Salmonella*-contaminated poultry house models to overcome resistance.

Regardless of animals reared in farms or the serovars of the strains, the majority of *Salmonella* strains in Yamagata prefecture produced biofilms (Fig. 2). Since the strains were strongly resistant to a common disinfectant in the biofilm form regardless of the level of their biofilm formation ability (Fig. 3), most *Salmonella* strains in this area are expected to show disinfectant resistance on farms. Biofilms are suggested to affect the host specificity and pathogenicity of *Salmonella* [17]. Moreover, biofilms are typically more tolerant to antibiotics than the corresponding strains in liquid cultures [6, 26]. Therefore, knowing the biofilm formation ability of local *Salmonella* isolates will be helpful not only for improving disinfection methods but also for understanding the ecology, pathogenicity, and antimicrobial resistance of pathogens in the area.

Although biofilm formation abilities vary among different *Salmonella* strains [16], similar variability was detected in the present study, even among clonal ST strains (Supplementary Fig. 1). *Salmonella* strains may change the quantity of biofilms depending on their culture conditions [2, 16]; however, the different levels of biofilm formation among our clonal strains were observed under the same culture conditions. Therefore, the intrinsic ability or optimum conditions for biofilm formation may easily change in *Salmonella* in the field.

In our poultry house models, a bacterial cell and dust complex adhered to the carrier surface (Fig. 4B), suggesting biofilm formation by *Salmonella*. After the cleaning step with the surfactant, carriers appeared to be clean, as shown in Fig. 1. However, viable ST cells remained and were not efficiently removed from the carriers by QAC, a disinfectant recommended by the WHO for the disinfection of smooth and clean surfaces in *Salmonella*-infected poultry farms [28] (f and i in Fig. 5). On the contrary, after the disinfection

with QAC and HClO, an increase in the number of viable ST cells was observed, similar to disinfection controls with water. In some experiments, the number of viable bacteria was more than 10- to 100-fold higher than before cleaning (Fig. 5). These results suggest that disinfection with ineffective agents increase *Salmonella* contamination levels in poultry houses possibly by supplying the water necessary for bacterial growth. Under such circumstances, ST cells remaining on the material surface or in microscopic depressions on the surface may use residual biofilm components such as extracellular polymeric substances (EPS) or organic matters trapped in the EPS as a source of nutrients for the growth, although further studies are needed to test these hypotheses.

Although *Salmonella* biofilms also showed disinfectant resistance in 96-well plates, a significant increase in the number of viable bacterial cells was not observed on the plastic surface (Fig. 3), suggesting that the microplate method is insufficient to examine disinfection conditions that are effective on farms. Stocki *et al*. also recognized the difficulties associated with recreating or evaluating "real-world" *Salmonella* contamination conditions experimentally [25]. To establish effective disinfection methods in the "real-world", it is necessary to examine conditions in practical use. However, it is challenging to repeat these tests on farms. The *in vitro Salmonella*-contaminated poultry house models developed in the present study successfully simulated the highly persistent characteristics of *Salmonella*. Since our models may be used to easily and quantitively investigate the efficacy of various disinfection conditions, they have potential as useful tools for the establishment of effective disinfection methods on farms.

By using these models, we found that the combination of a high-temperature cleaner and 80°C rinsing was effective for the elimination of viable ST (condition C9 in Fig. 6). However, a significant reduction in the number of viable ST cells was not observed when only the 80°C rinsing was applied (condition C10 in Fig. 6). Moreover, the number of viable ST cells increased by more than 10-fold in a test under condition C10 (Fig. 6), suggesting that inadequate heating by water enhanced bacterial proliferation by supplying water to the environment. Therefore, the additional use of cleaners is recommended even when hot water is used for washing.

In the present study, we also quantitively demonstrated the significant efficacy of the sequential cleaning by a surfactant and chlorine dioxide and a treatment with dolomite lime emulsion. In all or many of the tests performed under conditions C12-C16, ST was eliminated from the models (Fig. 7). Although lime wash is not listed as a recommended disinfectant for surface disinfection in poultry houses in the WHO guidelines [28], the effectiveness of lime milk on *Salmonella* Enteritidis in poultry houses was suggested by Motomura *et al*. [21]. The effects of chlorine dioxide on ST in poultry houses was also investigated [5]. However, its efficacy was not investigated quantitively. The present study not only confirmed the effectiveness of lime and chlorine dioxide suggested in the previous study, it also proposed more efficient cleaning and disinfection methods using these agents.

Since sodium hydroxide was previously shown to eradicate early *Salmonella* biofilms [8], no viable ST detection from the poultry house models treated with dolomite lime (Fig. 7) may be, at least in part, due to the destruction of biofilms under a persistent alkaline environment as well as physical containment of bacteria by dolomite lime. In Japan, the alkalinization of QAC by adding alkaline chemicals, such as slaked lime, is recommended for disinfection in farms (http://jlia.lin.gr.jp/eiseis/pdf/biosecurity.pdf [accessed on July 3, 2022]), and may also help prevent biofilm formation in *Salmonella*. However, Papakonstantinou and Efthimiou reported that although an alkaline pH hindered biofilm formation by *Salmonella*, the number of viable cells remained high even at pH 10 [22]. This suggests that the alkaline conditions alone are not sufficient to efficiently decontaminate *Salmonella*. Therefore, in order to clarify the effectiveness of alkalinized QAC on *Salmonella* in poultry houses, tests under farm environmental conditions are necessary. Our model may be used for these tests.

In the present study, we successfully investigated the effects of different disinfection methods on persistent *Salmonella* by developing *in vitro Salmonella*-contaminated poultry house models. The present results have been utilized to improve disinfection methods for poultry houses in Yamagata prefecture. Methods to develop these models may be applied to simulate other farm environments such as pig farms and cattle farms, by placing carriers in the farms of interest. Of note, although dust was deposited on the carriers under different conditions (i.e., in different seasons and different poultry houses), marked differences were not observed in the test results between the conditions. In the present study, some degree of variation was observed even in the data obtained under the same cleaning and disinfection conditions. However, the test system using our models made it possible to repeatedly obtain quantitative data and evaluate the effectiveness of disinfection statistically. Since various tests may be easily, repeatedly, and quantitively performed under the simulated farm environment, these models will be useful tools not only for investigating disinfection methods on farms, but also for research on the characteristics, survival strategy, antimicrobial resistance, and transmission of *Salmonella* in the field.

CONFLICTS OF INTEREST. The authors declare no conflicts of interest associated with this manuscript.

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