

Methods and Applications

Development of Cryogenic Disinfectants Using in $-18\text{ }^{\circ}\text{C}$ and $-40\text{ }^{\circ}\text{C}$ Environments — Worldwide, 2021

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ABSTRACT

Introduction: Since the outbreak of coronavirus disease 2019 (COVID-19) in Beijing Xinfadi Wholesale Market, a series of COVID-19 outbreaks has indicated the threat of transmission due to outer surfaces of objects under low temperatures. Therefore, 2 kinds of chlorine-containing disinfectants for $-18\text{ }^{\circ}\text{C}$ and $-40\text{ }^{\circ}\text{C}$ were developed in order to solve the problem of low temperature disinfection.

Methods: The properties of two cryogenic disinfectants were evaluated by low-temperature test. *Staphylococcus aureus* and *Escherichia coli* were used as two indicator microorganisms to evaluate the effects of the two cryogenic disinfectants in the laboratory and field tests according to *Technical Specifications for Disinfection (2002 version)* and *Test method for bactericidal effect of disinfectants in laboratory (GB/T 38502-2020)*.

Results: Two disinfectants could remain in the liquid state at $-18\text{ }^{\circ}\text{C}$ or $-40\text{ }^{\circ}\text{C}$ in 72 hours without crystallization and precipitation, and the average log values of microbial reduction before and after disinfection (the average killing log value) both on *Staphylococcus aureus* and *Escherichia coli* were >3 in the laboratory and field tests.

Discussion: The results showed that these two disinfectants remained functional at low temperature to disinfect the packaging of imported cold chain food and surrounding packaging to prevent transmission of COVID-19 from material to people.

inanimate objects and subsequent contact with those objects (2). A previous study showed that COVID-19 virus survived for 21 days on refrigerated ($4\text{ }^{\circ}\text{C}$) and frozen ($-20\text{ }^{\circ}\text{C}$ and $-80\text{ }^{\circ}\text{C}$) salmon and chicken (3) but was inactivated after 5 min at $70\text{ }^{\circ}\text{C}$ (4), indicating that cold temperature extended the survival time of the virus on surfaces.

COVID-19 virus was detected on a cutting board used for processing imported salmon in a food contamination incident that occurred on June 12, 2020 in Xinfadi Agricultural Produce Wholesale Market in Beijing (5), and subsequent outbreaks occurred in Xinjiang and Shanghai (6). These incidents demonstrated that COVID-19 virus could survive on the surface of objects in cold environments for long times and could cause cluster outbreaks.

The temperature of Xinjiang, Inner Mongolia, and other places in China has dropped below $-20\text{ }^{\circ}\text{C}$ in 2020 winter, as it has in Russia, Canada, and some northern regions of the United States. Temperature is an important factor affecting the effectiveness of disinfection as low temperature will make microbicidal efficiency decrease and there are no appropriate disinfection measures available for use at such cold temperatures. Therefore, the prevention and control of COVID-19 virus in these regions has become more difficult.

In this study, 2 chlorine-containing cryogenic disinfectant were developed to kill *Staphylococcus aureus* and *Escherichia coli* at $-18\text{ }^{\circ}\text{C}$ and $-40\text{ }^{\circ}\text{C}$, and these effects were verified.

INTRODUCTION

Since the onset of the coronavirus disease 2019 (COVID-19) pandemic, there have been more than 10 million confirmed cases and 2,320,497 deaths (1). According to the current understanding of COVID-19 virus, the virus can be transmitted by air droplets and aerosols or through deposit on the surfaces of

METHODS

Bacterial Strains, Bacterial Carriers, and media

Staphylococcus aureus and *Escherichia coli* bacteria were cultured on Tryptone soy agar (TSA) medium; Tryptone soy broth (TSB) medium was used to dilute

the bacteria cultured overnight to the required concentration; 10 μL of fresh *Staphylococcus aureus* and *Escherichia coli* were applied to cloth carriers, which were left at room temperature to dry and dropped to $-18\text{ }^{\circ}\text{C}$ or $-40\text{ }^{\circ}\text{C}$ before disinfection.

Formula Determination

Calcium chloride, ethanol, and ethylene glycol were chosen as antifreeze agents. Sodium dichloroisocyanurate was selected as the main active ingredient, combined with the optimum proportion of antifreeze to assess the killing effect of microorganisms under low temperatures.

Icing Test

For this test, 5 mL disinfectant was added to 90 mm glass petri dishes, and 20 mL disinfectant was added to 50 mL centrifuge tubes; the objects were then placed in the $-18\text{ }^{\circ}\text{C}$ or $-40\text{ }^{\circ}\text{C}$ environment and observed at different timepoints.

Laboratory Bactericidal Experiment

Before each bactericidal experiment, the available chlorine concentration of the disinfectant was measured using the iodometric method described in the *Technical Specifications for Disinfection (2002 version)*(7).

For neutralizer identification test, it was divided into 4 groups according to the *Test method for bactericidal effect of disinfectant in laboratory* (GB/T 38502-2020) (8) to evaluate whether the neutralizing agent, neutralizing product, and diluent affected the bacterial tablet.

For carrier quantitative germicidal test, the effect of the disinfectant on *Staphylococcus aureus* and *Escherichia coli* was evaluated according to the *Technical Specifications for Disinfection (2002 edition)*. The infected bacterial carriers were immersed into the disinfectant (5.0 mL per carrier tablet) and positive control bacterial carriers into the diluent. The experiment was repeated three times.

Field Test

Cartons (58 cm \times 38 cm \times 34 cm) were selected as simulating container to evaluate the disinfection to the outer packaging of refrigerated articles. One piece of *Staphylococcus aureus* carrier and one piece of *Escherichia coli* carrier were fixed on each of the six sides of the carton. Then, the cartons were immediately placed in a high-speed micron spray

sterilizer for six-sided disinfection until each carrier was soaked after the bacteria carriers reached $-18\text{ }^{\circ}\text{C}$ or $-40\text{ }^{\circ}\text{C}$.

To test cold storage disinfection, a total of 10 *Staphylococcus aureus* carriers and 10 *Escherichia coli* carriers placed on each plate were evenly placed in the front, back, left, right, and middle of the $-18\text{ }^{\circ}\text{C}$ or $-40\text{ }^{\circ}\text{C}$ cold storages. A disinfectant sprayer equipped with the low-temperature disinfectant was used to spray and disinfect the cold storage, and the spraying volume is about 200–300 mL/m².

In field tests, the temperatures of the carriers were verified to reach $-18\text{ }^{\circ}\text{C}$ or $-40\text{ }^{\circ}\text{C}$ before disinfection and 3 samples for each bacterium were put into cold storage together with the experimental group but not sterilized as the positive control group.

Another 10 minutes after the carriers were completely soaked were allowed, and the bacterial carriers were placed in 10 mL neutralizer solutions while the positive control bacteria carriers were placed in 10 mL diluent. The colonies were then counted. The test was repeated three times.

RESULTS

Physical Property Tests

In this study, the disinfectant remained liquid without crystallization or precipitation in cold storage times of 72 h.

When evaluating the bactericidal effect of $-18\text{ }^{\circ}\text{C}$ disinfectant, the mean available chlorine concentration prepared in the laboratory tests was 2,919 mg/L (2,872–2,943 mg/L) while in field tests was 2,795 mg/L (2,765–2,819 mg/L). The available chlorine concentrations of $-40\text{ }^{\circ}\text{C}$ disinfectant before the 3 sets of laboratory experiments were 4,892 mg/L, 4,875 mg/L, and 4,839 mg/L, while those for field tests were 5,283 mg/L, 5,141 mg/L, and 5,247 mg/L.

Laboratory Bactericidal Experiment

For the two cryogenic disinfectants, two types of neutralizers could effectively neutralize the effect of the disinfectant remaining on the surface of bacteria in diluent on *Staphylococcus aureus*, and neither the neutralizer nor the neutralizing product had any adverse effect on *Staphylococcus aureus* or the medium (Table 1).

Based on neutralizer identification test, we carried out bactericidal experiments with the disinfectants. The average killing log values of the 2 disinfectants

against *Staphylococcus aureus* and *Escherichia coli* were >3.00 at $-18\text{ }^{\circ}\text{C}$ and $-40\text{ }^{\circ}\text{C}$, demonstrating the qualified effects of the disinfectants (Table 2).

Field Test

There were 1 sample of *Staphylococcus aureus* and 1 sample of *Escherichia coli* on each side of each carton, and a total of 60 samples were collected at $-18\text{ }^{\circ}\text{C}$ or $-40\text{ }^{\circ}\text{C}$. After the carton was sterilized by a high-speed micron spray sterilizer, the killing log values of *Staphylococcus aureus* and *Escherichia coli* on the carrier were all >3.00, demonstrating a qualified disinfection effect.

The killing log values of 30 *Staphylococcus aureus* tablets and 30 *Escherichia coli* tablets were all >3.00 after sterilizing the $-18\text{ }^{\circ}\text{C}$ or $-40\text{ }^{\circ}\text{C}$ storages with a sprayer (the dosage of disinfectant was 200–300 mL/m²), also meeting the requirements for qualified disinfectants (Tables 3–4).

Formulation of Disinfectant

With a combination of freezing tests, laboratory experiments and field tests, we obtained two disinfectants, the disinfectant for $-18\text{ }^{\circ}\text{C}$ contained 25% calcium chloride, 9.5% ethanol, and 3,000 mg/L sodium dichloroisocyanurate, while 30% calcium chloride, 9.5% ethanol, 9.9% ethylene glycol, 5,000 mg/L sodium dichloroisocyanurate, and 0.09% benzalkonium chloride were used in the $-40\text{ }^{\circ}\text{C}$ disinfectant.

DISCUSSION

In this study, chemical disinfectants were applied to determine effectiveness at low temperatures. Disinfectants with chlorine were considered suitable for disinfecting general object surfaces, food and drinking utensils, fabrics, fruits, vegetables, and water, etc., in public places and families (9). COVID-19 virus can be inactivated in 0.5 min at room temperature at an available chlorine level of 250 mg/L (10). Considering economic costs and convenience of application, sodium dichloroisocyanurate (available chlorine) was selected as the representative chlorine-containing disinfectant, and a small amount of benzalkonium chloride was added to synergize the disinfection effect in the $-40\text{ }^{\circ}\text{C}$ cryogenic disinfectant.

The formulation contained antifreeze agents including calcium chloride, ethanol, and glycol. Industrial calcium chloride aqueous solution was widely used in all kinds of equipment and environments that require a reduced freezing point. However, the saturation point of calcium chloride decreases with decreasing temperature, and calcium chloride is corrosive (8). Therefore, ethanol and ethylene glycol were also added as antifreeze agents. Ethanol has a antifreeze effect in chlorine dioxide compounds at $-20\text{ }^{\circ}\text{C}$ (11). Methanol and ethylene glycol have also been reported as antifreeze compounds facilitating the disinfection effect of chlorine-containing disinfectants at $0\text{ }^{\circ}\text{C}$ and $-20\text{ }^{\circ}\text{C}$ (12). Considering its toxicity and price, methanol was not

TABLE 1. Neutralizer identification test on *Staphylococcus aureus*.

Group	$-18\text{ }^{\circ}\text{C}$ Cryogenic disinfectant		$-40\text{ }^{\circ}\text{C}$ Cryogenic disinfectant	
	Average colony forming units of each group (CFU/tablet)	Error rate between groups (%)	Average colony forming units of each group (CFU/tablet)	Error rate between groups (%)
1	407		542	
2	413		592	
3	450	4.2	602	4.23
4	0		0	

Note: Group 1: soak the carrier tablet in 5.0 mL neutralizing agent for 10 minutes; Group 2: soak the carrier tablet in 5.0 mL of neutralization products for 10 minutes; Group 3: soak the carrier tablet in 5.0 mL tryptone saline solution (TPS) dilution for 10 minutes; Group 4: 1 mL of TPS diluent, 1 mL of neutralizing agent and the same culture medium as that of the first three groups was added and placed into sterile plates. The neutralizer of $-18\text{ }^{\circ}\text{C}$ Cryogenic disinfectant was 0.03 mol/L PBS containing 0.3% sodium thiosulfate, 0.1% lecithin and 1% tween-80 while 0.03 mol/L PBS containing 0.5% sodium thiosulfate, 0.5% lecithin and 2% tween-80 was for $-40\text{ }^{\circ}\text{C}$ Cryogenic disinfectant.

TABLE 2. Killing effect of disinfectant of $-18\text{ }^{\circ}\text{C}$ – $-40\text{ }^{\circ}\text{C}$ disinfectant on *Staphylococcus aureus* and *Escherichia coli*.

Bacteria	Available chlorine (mg/L)	Logarithm of the mean colony number of the positive control group	Average killing log of bacteria at different times of disinfection		
			2.5/5 min	5/10 min	7.5/15 min
<i>Staphylococcus aureus</i>	2,919/4,869	6.18/6.07	5.81/3.91	5.88/4.12	6.18/4.10
<i>Escherichia coli</i>	2,919/4,869	6.36/6.36	5.23/4.78	5.23/5.14	6.18/5.39

TABLE 3. The disinfection effect of $-18\text{ }^{\circ}\text{C}/-40\text{ }^{\circ}\text{C}$ cryogenic disinfectant on *Staphylococcus aureus*/*Escherichia coli* on outer surface of the cartons.

Sample number	Carrier position	Colony forming units of test group (CFU/tablet)	Killing log value	Sample number	Carrier position	Colony forming units of test group (CFU/tablet)	Killing log value
1	Top side of carton 1	(265, 0)/(10, 25)	>3.00	16	Right side of carton 3	(10, 0)/(20, 5)	>3.00
2	Bottom side of carton 1	(235, 15)/(615, 0)	>3.00	17	Front side of carton 3	(5, 10)/(30, 0)	>3.00
3	Left side of carton 1	(15, 0)/(10, 15)	>3.00	18	Back side of carton 3	(0, 0)/(5, 0)	>3.00
4	Right side of carton 1	(15, 0)/(10, 0)	>3.00	19	Top side of carton 4	(0, 15)/(65, 0)	>3.00
5	Front side of carton 1	(5, 10)/(50, 30)	>3.00	20	Bottom side of carton 4	(425, 65)/(60, 950)	>3.00
6	Back side of carton 1	(0, 5)/(0, 0)	>3.00	21	Left side of carton 4	(0, 0)/(5, 0)	>3.00
7	Top side of carton 2	(0, 15)/(480, 45)	>3.00	22	Right side of carton 4	(10, 0)/(0, 0)	>3.00
8	Bottom side of carton 2	(10, 50)/(515, 20)	>3.00	23	Front side of carton 4	(5, 0)/(10, 0)	>3.00
9	Left side of carton 2	(145, 0)/(5, 0)	>3.00	24	Back side of carton 4	(10, 0)/(0, 0)	>3.00
10	Right side of carton 2	(5, 0)/(5, 25)	>3.00	25	Top side of carton 5	(0, 0)/(5, 5)	>3.00
11	Front side of carton 2	(0, 10)/(5, 55)	>3.00	26	Bottom side of carton 5	(15, 40)/(210, 5)	>3.00
12	Back side of carton 2	(0, 20)/(5, 0)	>3.00	27	Left side of carton 5	(50, 0)/(0, 5)	>3.00
13	Top side of carton 3	(5, 0)/(10, 10)	>3.00	28	Right side of carton 5	(5, 0)/(0, 15)	>3.00
14	Bottom side of carton 3	(100, 5)/(0, 10)	>3.00	29	Front side of carton 5	(0, 0)/(10, 0)	>3.00
15	Left side of carton 3	(0, 0)/(10, 0)	>3.00	30	Back side of carton 5	(230, 0)/(0, 0)	>3.00

Note: Positive control bacteria count of *Staphylococcus aureus* and *Escherichia coli*: 4.26×10^6 CFU/tablet (9.0×10^5 – 6.6×10^6 CFU/tablet), 1.91×10^6 CFU/tablet (3.15×10^5 – 9.15×10^6 CFU/tablet).

TABLE 4. The disinfection effect of $-18\text{ }^{\circ}\text{C}/-40\text{ }^{\circ}\text{C}$ cryogenic disinfectant on *Staphylococcus aureus*/*Escherichia coli* in $-18\text{ }^{\circ}\text{C}/-40\text{ }^{\circ}\text{C}$ storage.

Sample number	Colony forming units of test group (CFU/tablet)	Killing log value	Sample number	Colony forming units of test group (CFU/tablet)	Killing log value
1	(0, 0)/(30, 5)	>3.00	16	(5, 0)/(0, 5)	>3.00
2	(0, 0)/(0, 0)	>3.00	17	(0, 0)/(20, 10)	>3.00
3	(0, 0)/(5, 0)	>3.00	18	(0, 0)/(0, 0)	>3.00
4	(0, 0)/(0, 65)	>3.00	19	(0, 0)/(0, 0)	>3.00
5	(0, 0)/(10, 5)	>3.00	20	(0, 0)/(0, 70)	>3.00
6	(0, 0)/(10, 0)	>3.00	21	(0, 0)/(0, 65)	>3.00
7	(0, 0)/(0, 0)	>3.00	22	(0, 0)/(0, 0)	>3.00
8	(0, 0)/(0, 0)	>3.00	23	(0, 0)/(15, 135)	>3.00
9	(0, 0)/(0, 0)	>3.00	24	(0, 0)/(5, 115)	>3.00
10	(0, 0)/(0, 0)	>3.00	25	(0, 0)/(20, 10)	>3.00
11	(0, 0)/(0, 15)	>3.00	26	(0, 0)/(10, 100)	>3.00
12	(0, 0)/(5, 0)	>3.00	27	(0, 0)/(20, 0)	>3.00
13	(0, 0)/(0, 0)	>3.00	28	(0, 0)/(30, 0)	>3.00
14	(0, 0)/(5, 0)	>3.00	29	(0, 0)/(20, 340)	>3.00
15	(0, 0)/(0, 0)	>3.00	30	(0, 0)/(0, 170)	>3.00

Note: Positive control bacteria count of *Staphylococcus aureus* and *Escherichia coli*: 4.26×10^6 CFU/tablet (9.0×10^5 – 6.6×10^6 CFU/tablet), 1.91×10^6 CFU/tablet (3.15×10^5 – 9.15×10^6 CFU/tablet).

selected for this formula.

The COVID-19 virus is lipophilic and an enveloped virus (13), and it is generally believed to be more sensitive to chemical disinfection factors than

Staphylococcus aureus and other bacteria (14). So, *staphylococcus aureus* and *Escherichia coli* were considered indicators to evaluate the disinfection effect. The cryogenic disinfectant developed in this study

solved the problem of freezing and effectiveness of chemical disinfectants at low temperatures, which meant that the cryogenic disinfectants could be used for disinfection of COVID-19 virus. This study provided a new method for solving the technical problems of the disinfection of cold chain food packaging or environments, outdoor environments, and cold winter items.

This study was subject to some limitations. First, since the stability of this disinfectant has not been studied, it should be created for use immediately. Second, because there was no evaluation method for the metal corrosion of disinfectants at low temperatures, the metal corrosion test at low temperature has not been conducted for these two disinfectants.

Further studies are needed to adjust the formulation without affecting the effectiveness of disinfection to enhance stability for better application. This disinfectant should be used only when the effectiveness of disinfection reaches qualifying levels. Strict personal protection measures should be carried out in the process of disinfectant configuration and onsite disinfection because of the bleachability, corrosiveness, and skin irritation of the disinfectant as it is a chlorine-based disinfectant.

Acknowledgment: HaiYunYiJianPan-Health Techno logy Co., Ltd and Beijing Capital Agribusiness Group.

doi: 10.46234/ccdcw2021.079

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Submitted: February 12, 2021; Accepted: March 24, 2021

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