



Biomedical Science Research Institute
Hoseo University, 20, Hoseo-ro 79 beon-gil,
Baebang, Asan, KOREA 31499
Tel: +82-41-540-9675 Fax: +82-41-540-9829

호서대학교 바이오의과학연구소
우 31499 충남 아산시 배방읍 호서로 79번길 20
전화: 041-540-9675 전송: 041-540-9829

Final Research Report

Zoono Micro Shield Z71

Disinfectant Efficacy Study

TR19106

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Hoseo University
Biomedical Science Research Institute

Outline

Title	Zoono Micro Shield Z71 Disinfectant Efficacy Study
Client	Dongbang Cosmetic Inc. (22829) 39, Gajaeul-ro, 32beon- gil, Seo-gu, Incheon Tel : 032-571-3347 Fax : 032-571-3349
Research Institute	Hoseo University Biomedical Science Research Institute (31499) 79 beon-gil Hoseo-ro, Baebang-up, Asan-si, Chungchung namdo Tel : 041-540-9675 Fax : 041-540-9829
Principal Researcher	Sanghee Jung (DVM, PhD, Professor) Hoseo University Clinical Pathology Department Hoseo University Biomedical Science Research Institute Tel : 041-540-9675 Fax : 041-540-9829
Lead Researchers	Jungran Min (DVM, MS, Researcher) Ran Jo (Researcher), Eunhwa Cha (Researcher)
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Signature

Principal Researcher _____ **Date** 2019. 10. 25
Sanghee Jung (DVM, PhD, Professor)

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1. Summary

1.1. Summary of test result of Zoono Micro Shield Z71 disinfectant efficacy study

1) Bacteria disinfectant efficacy test

The effective dilution rates for Zoono Micro Shield Z71 against *Salmonella Typhimurium* is confirmed as 1.5 times for hard water and 1.25 times for organic matter. The recommended dilution rate is determined as 1.2 times for hard water and undiluted solution for organic matter.

2) Virus disinfectant efficacy test

The effective dilution rate for Zoono Micro Shield Z71 against avian influenza virus is confirmed as 6.25 times for hard water and 3.125 times for organic matter. The recommended dilution rate is 5 times for hard water and 2.5 times for organic matter.

<The Effective and Recommended Dilution Rates for Zoono Micro Shield Z71>

Pathogen		Effective Dilution Factor		Recommended Dilution Rate	
		hard water condition	organic matter condition	hard water condition	organic matter condition
Bacteria	<i>Salmonella typhimurium</i>	1.5	1.25	1.2	undiluted solution
Virus	Avian influenza virus H9N2	6.25	3.125	5	2.5

1.2. Overall opinion of the researcher based on the results

Disinfection Target	Targeted Disease		Recommended Dilution Rate	
			Common Bacteria	Specific Virus
			Salmonella typhimurium	AI Virus
Test Result	Hard Water		1.5	6.25
	Organic Matter		1.25	3.125
Target with less organic matter	Cattle spaces and livestock tools etc.	Common bacteria or specific virus (hard water)	1.2	5
Target with more organic matter	Cattle facility floors, soil, carcass, farm vehicles, carrying equipment etc.	Common bacteria or specific virus (organic matter)	Undiluted solution	2.5
Notation of recommended dilution rate: Highest concentration is recommended for simultaneous disinfection of Common Bacteria (Salmonella Typhimurium) and Specific Virus				

2. Purpose

In this study, an efficiency test for Zoono Micro Shield Z71 containing benzalkonium chloride, as an active ingredient, against bacteria (*Salmonella typhimurium*) and Avian influenza virus is undertaken to determine the effective dilution rate (usage method) to prevent the occurrence and spread of the disease, and for use as a data at a later time when applying for product license.

3. Materials

3.1. Test product

- 1) Name : Zoono Micro Shield Z71
- 2) Type or form : solution, colorless to pale yellow liquid
- 3) Serial number : B56700
- 4) Date of manufacture : 2019. 02. 03
- 5) Formulation (per 1L)
 - Benzalkonium chloride, USP ----- 2.8g
 - Polysorbate 80, USP ----- moderate amount
 - Purified water, KP ----- moderate amount
 - Trimethoxysilyl ----- moderate amount
propyldimethyloctadecyl ammonium chloride
(Organosilance quaternary, USP)

※The product was checked for the tightness of seal and the contents in accordance with Disinfectant Effectiveness Guidelines (No, 2018-16), Article 4-2 4.

3.2. Testing bacteria and virus

Item	Strain	Agency Serial Number	
Common bacteria	<i>Salmonella typhimurium</i>	KCCM	KCCM 41038
Avian influenza virus	Avian influenza virus H9N2	APQA	VR1100013

3.3. Test organism

Pathogen	Test Organism
AI virus	Embryonated eggs without Avian influenza virus

3.4. Culture medium and conditions

Pathogen	Culture	Condition
Sal. typhimurium	Nutrient broth	37°C

3.5. Diluted solution

Diluted Solution	Formulation
Hard water	Distilled water 1 litre containing CaCl ₂ 0.305g and MgCl ₂ ·6H ₂ O 0.139g(w/v)
Organism	bacteria 5% (w/v) yeast extract: Sterilized with high pressure so yeast extract 20% (w/v) is dissolved and stored in the refrigerator. In use, dilute 4 times with hard water to make organic diluent with 5% content. Adjust pH to 7.0 with 1N sodium hydroxide solution.
	virus hard water with 5% (w/v) fetal bovine serum

3.6. Neutralized culture

Pathogen	Formulation
bacteria	Nutrient medium containing 5% inactivated horse serum
AI virus	PBS with 10% inactivated FB

4. Standards and Methods

- Disinfectant efficacy test guidelines were followed in Annex 1 "Determination test for bacteria disinfectant effective dilution rate", Annex 2 "Determination test for virus disinfectant rate", and Annex 3 "Determination test for mold disinfectant effective dilution rate " 2018-16, 2018. 5. 31 of the Ministry of Health, Labor and Welfare.
- The main test conditions are as follows.
 - Include a water-diluted solution containing the target pathogen as well as organic diluent.
 - The reaction conditions of the disinfecting agent is 30 minutes at 4 ° C.
 - Repeat three times.
- The recommended dilution rate for disinfectant is a dilution rate equivalent to 80% of the effective dilution rate (pathogen and dilution rate before reaction)

4.1. Disinfectant effective dilution rate test design for treatment groups

4.1.1. Treatment groups for disinfectant effective dilution rate determination test against bacteria

Treatment Group	Hard Water	Organic Matter	Disinfectant	Pathogen	Note
1 (Low Organic Matter)	+	-	+	+	hard water condition
2 (High Organic Matter)	+	+	+	+	organic matter / hard water condition
3 (Pathogen Control)	+	-	-	+	control group for 1 and 2

4.1.2. Treatment groups for disinfectant effective dilution rate determination test against virus

Treatment Group	Hard Water	Organic Matter	Disinfectant	Pathogen	Note
1 (Low Organic Matter)	+	-	+	+	hard water condition
2 (High Organic Matter)	+	+	+	+	organic matter / hard water condition
3 (Pathogen Control)	+	-	-	+	control group for 1 and 2
4 (Toxicity Control)	+	-	+	-	control group for 3

4.2. Test Methodology

4.2.1. Salmonella typhimurium

1) Bacteria culture

- Cultivate bacteria for 22-26 hours and maintain at 37°C until immediately before use. The concentration of the bacteria should be at least 10⁸ CFU per mL.
- The absorbance (OD_{625 nm} > 1.0) of the cultured bacteria was measured and used in the test.

2) Bacteria dilution

- For bacteria dilution, hard water and organic diluent (5% yeast extract) was used according to the conditions.
- Put 4 mL of bacteria incubated at 37°C in the 4°C hard water, and also 96mL of organic diluent.

3) Disinfectant dilution

- Disinfectant was diluted with hard water and organic diluent according to the conditions.
- Before the reaction with the test bacteria disinfectant diluent was prepared, and the disinfectant diluent was stored at 4°C for at least 30 minutes.
- As a result of performing disinfectant tests at the dilution rates specified in the test plan as shown below, the effect was not observed at a rate of 2.5, which is the minimum dilution rate for hard water condition. The test was carried out by lowering the dilution rate. In the case of the organic matter condition, the test was performed by adding the dilution rate between 1.25 and 2.5.
 - hard water conditions : x2.5, x6.25, x12.5, x18.75, x25
 - organism matter conditions : x1.25, x2.5, x6.25, x12.5, x18.75
- Therefore, the disinfectant dilution rate was changed as follows to perform the disinfectant efficacy test.
 - hard water conditions : x1.25 x1.5, x1.875, x2.5
 - organism matter conditions : x1.25, x1.5, x1.875, x2.5

4) Reaction of disinfectant

- 2.5 mL of each prepared bacterial dilution was mixed with 2.5 mL of 4°C disinfectant diluent.
- It was left to react for exactly 30 minutes at 4°C. Each test tube was treated in turn at 1 minute intervals. And mixed every 10 minutes.
- For the pathogen control group, hard water was used without disinfectant diluent.

5) Neutralization reaction and multiplication

- Immediately after the reaction, 1mL was extracted and mixed with 9 mL of 37°C neutralization medium. Then, it was inoculated with 0.1 mL into 5 nutrient medium test tubes for each disinfectant dilution step.
- It was incubated for 48 hours in a 37°C incubator.

6) Result of bacterial growth

- After 48 hours of incubation, the bacterial growth was visually checked, and then lastly placed on a solid medium to confirm.

7) Control group test

- The pathogen control group was tested without disinfectant in hard water conditions, and the neutralization reaction was checked. Also, it was confirmed that the titer was at least 2×10 CFU per mL during the growth stage.

8) Result

- Group 3 was used as a control group for groups 1 and 2.
- The test was repeated 3 times.
- The final disinfection dilution step where no more than 4 multiplications in the nutrient medium of 5 identical disinfectant diluents was determined as the effective dilution rate.
- The recommended dilution rate was 80% of the final effective dilution rate.

4.2.2. Avian influenza virus

1) Preparation of virus

- The viable virus was collected from passage culture at the maximum growth time, and stored in ice water until immediately before use.

2) Dilution of the virus

- To dilute the virus, hard water and organic diluent (5% FBS) were used accordingly
- 10 mL of virus solution at 4°C was added to both 19 mL of hard water and organic diluent at 4°C, and mixed.

3) Dilution of disinfectants

- Disinfectant was diluted with hard water and organic diluent according to the conditions.
- Diluted disinfectant was prepared before the reaction with the test bacteria, and the diluted disinfectant was stored at 4°C for at least 30 minutes.

The disinfectant test was performed at the dilution rate specified in the protocol as follows. But for hard water conditions, additional dilution rates were added between 6.25 and 12.5, and for organic matter conditions additional dilution rates were added between 2.5 and 6.25.

- hard water condition : x2.5, x6.25, x12.5, 18.75, x25
- organic matter : x1.25, x2.5, x6.25, x12.5, x18.75
- Therefore, the disinfectant dilution rate was changed as follows to perform disinfectant efficacy test.
 - hard water condition : x6.25, x6.875, x7.5, x12.5
 - organism condition : x2.5, x3.125, x3.75, x4.375, x6.25

4) Neutralization reaction and multiplication

- 2.5 mL of each prepared bacterial dilution was mixed with 2.5 mL of 4°C disinfectant diluent.
- Let it react for exactly 30 minutes at 4°C. Each test tube was treated in turn at 1 minute intervals. And mixed every 10 minutes.
- For the pathogen control group, hard water was used without disinfectant diluent.
- A disinfectant diluent diluted with hard water was used as a toxicity control group.

5) Neutralization reaction

- After the reaction of the disinfectant, in order to neutralize the efficacy of the disinfectant 1 mL of the reaction solution was taken out and put it into 1 mL of neutralization medium at 37°C, then mixed.

6) Result of bacterial growth

- Undiluted solution was diluted at $10^1, 10^2, 10^3, 10^4$ and 10^5 by using neutralizing solution PBS, and five of 9-11 day embryonated eggs were inoculated into its umbilical cavity with 0.2 mL of neutralized reaction solution at each dilution rate.
- After inoculation it was incubated at 37°C for 5 days. During those 5 days, eggs were examined daily, and embryos that died within 24 hours after inoculation were regarded dead from accidental death and excluded from test results.
- All inoculated eggs that died within 5 days but after 24 hours of inoculation were stored at 4°C.
- Allantoic fluid was collected from all embryonated eggs that survived up to 5 days after vaccination as well as dead inoculated eggs that were stored at 4°C. Then hemagglutination was conducted using 1% chicken erythrocytes to determine the status of virus and virus titer.

7) Calculation of virus content

Karber method

8) Test of control group

- The pathogen control group was tested without disinfectant in hard water conditions and during the neutralization stage the titer of the pathogen was confirmed to be $2 \times 10^5 \text{ECID}_{50}/\text{mL}$ or more.
- It was confirmed that cytotoxicity by disinfectant did not occur in the toxicity control group.

9) Results

- The disinfectant test results confirmed the effective dilution rate compared to the pathogen control group. In other words, the effective dilution rate was confirmed as a dilution rate at which the pathogen showed a reduction of 10^4 (minimum 4 \log_{10}) or more compared to the pathogen control group.
- The effective dilution rate for the product was determined as the median of the result value within an error range of 20% ($\pm 10\%$) of the arithmetic mean, with a value of 4 or more converted to the common logarithm after 3 repeated tests.
- The recommended dilution rate is the dilution rate corresponding to 80% of the final effective dilution rate.

5. Test results

5.1. Salmonella typhimurium

5.1.1. Disinfectant efficacy test results

Treatment Group 1	Batch	Dilution Rate	Number of Positive / Number of Inoculations			
			1:1.25	1:1.5	1:1.875	1:2.5
Low Organic Matter (hard water)	1		0/5	0/5	3/5	5/5
	2		0/5	0/5	4/5	5/5
	3		0/5	0/5	3/5	5/5

Treatment Group 2	Batch	Dilution Rate	Number of Positive / Number of Inoculations			
			1:1.25	1:1.5	1:1.875	1:2
High Organic Matter (5% yeast extract)	1		0/5	2/5	5/5	5/5
	2		0/5	2/5	5/5	5/5
	3		0/5	3/5	5/5	5/5

Pathogen Control Group	1st time	2 nd time	3 rd time
Number of Positive / Number of Inoculations	5/5	5/5	5/5
CFU/mL	3.70 X 10 ⁶	2.50 X 10 ⁶	2.60 X 10 ⁶

5.1.2. Interpretation of the result

The disinfectant efficacy test of Salmonella Typhimurium of Zoono Micro Shield Z71 showed that the effective dilution rate was 1.5 times in hard water conditions and 1.25 times organism conditions.

Treatment Group	Hard Water	Organic Matter	Disinfectant	Note	Survey Item	Effective Dilution Rate			Final Effective Dilution Rate
						1	2	3	
1 (Low Organic Matter)	+	-	+	Hard Water Condition	Factor	1.5	1.5	1.5	1.5
2 (High Organic Matter)	+	+	+	Organism/Hard Water Condition	Factor	1.25	1.25	1.25	1.25
3 (Pathogen Control)	+	-	-	Control Group for 1 and 2	Status of Multiplication	Yes	Yes	Yes	-

5.2. Disinfectant efficacy test result against Avian Influenza H9N2 virus

5.2.1. 1st disinfectant efficacy test result

Treatment Group 1	Disinfectant Dilution Rate	Neutralization Dilution Rate (AIV positive number / inoculated embryonated eggs number)						EID ₅₀ Log ₁₀	Log ₁₀ reduction
		Undiluted Solution	10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵		
		Low	1:6.25	5/5	4/5	0/5	0/5		
Organic Matter	1:6.875	5/5	5/5	4/5	2/5	0/5	0/5	3.20	3.40
(hard water)	1:7.5	5/5	5/5	5/5	4/5	0/5	0/5	4.00	2.60
	1:12.5	5/5	5/5	5/5	5/5	2/5	1/5	4.80	1.80

Treatment Group 2	Disinfectant Dilution Rate	Neutralization Dilution Rate (AIV positive number / inoculated embryonated eggs number)						EID ₅₀ Log ₁₀	Log ₁₀ reduction
		Undiluted Solution	10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵		
		High	1:2.5	0/5	0/5	0/5	0/5		
Organic	1:3.125	4/5	2/5	0/5	0/5	0/5	0/5	1.40	5.20
Matter	1:3.75	5/5	5/5	5/5	0/5	0/5	0/5	3.20	3.40
(5% FBS)	1:4.375	5/5	5/5	4/5	2/5	0/5	0/5	3.40	3.20
	1:6.25	5/5	5/5	5/5	5/5	2/5	0/5	4.60	2.00

Treatment Group 3	Undiluted Solution	Neutralization Dilution Rate (AIV positive number / inoculated embryonated eggs number)							EID ₅₀ Log ₁₀
		10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	10 ⁻⁷	
		Pathogen Control	5/5	5/5	5/5	5/5	5/5	5/5	

5.2.2. 2nd disinfectant efficacy test result

Treatment Group 1	Disinfectant Dilution Rate	Neutralization Dilution Rate (AIV positive number / inoculated embryonated eggs number)						EID ₅₀ Log ₁₀	Log ₁₀ reduction
		Undiluted Solution	10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵		
		Low	1:6.25	4/5	3/5	3/5	1/5		
Organic Matter	1:6.875	5/5	5/5	5/5	2/5	0/5	0/5	3.60	3.20
(hard water)	1:7.5	5/5	5/5	5/5	5/5	0/5	0/5	4.20	2.60
	1:12.5	5/5	5/5	5/5	5/5	2/5	0/5	4.60	2.20

Treatment Group 2	Disinfectant Dilution Rate	Neutralization Dilution Rate (AIV positive number / inoculated embryonated eggs number)						EID ₅₀ Log ₁₀	Log ₁₀ reduction
		Undiluted Solution	10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵		
		High	1:2.5	0/5	0/5	0/5	0/5		
Organic Matter	1:3.125	4/5	3/5	0/5	0/5	0/5	0/5	1.60	5.20
(5% FBS)	1:3.75	5/5	5/5	5/5	0/5	0/5	0/5	3.20	3.60
	1:4.375	5/5	5/5	5/5	2/5	0/5	0/5	3.60	3.20
	1:6.25	5/5	5/5	5/5	5/5	3/5	0/5	4.80	2.00

Treatment Group 3	Undiluted Solution	Neutralization Dilution Rate (AIV positive number / inoculated embryonated eggs number)							EID ₅₀ Log ₁₀
		10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	10 ⁻⁷	
		Pathogen Control	5/5	5/5	5/5	5/5	5/5	5/5	

5.2.5. Interpretation of the result

- Virus titers of Pathogen control group ranged from $3.98 \times 10^6 \sim 6.31 \times 10^6$ EID₅₀/mL.
- The effective dilution rates of Zoono Micro Shield Z71 against Avian Influenza Virus in hard water and organic matter conditions were 6.25 times and 3.125 times respectively.
- The recommended dilution rate of Zoono Micro Shield Z71 against Avian Influenza Virus in hard water and organism conditions were 5 times and 2.5 times respectively.
- No disinfectant toxicity was observed in the effective dilution rate.

<Log 10 reduction value>

Treatment Group 1	Batch	Dilution rate			
		1:6.25	1:6.875	1:7.5	1:12.5
Low Organic Matter (Compared to Treatment Group 3)	1	4.60	3.40	2.60	1.80
	2	4.40	3.20	2.60	2.20
	3	4.80	3.40	3.00	2.20

Treatment Group 2	Batch	Dilution rate			
		1:2.5	1:3.125	1:3.75	1:4.375
High Organic Matter (Compared to Treatment Plot 3)	1	6.40	5.20	3.40	3.20
	2	6.60	5.20	3.60	3.20
	3	6.40	5.40	3.60	2.80

Treatment Group	Condition	Hard Water	Organic Matter	Disinfectant	Log 10 reduction value at the effective dilution rate			
					1	2	3	Median
1 (Low Organic Matter)	Hard Water Condition	+	-	+	6.25	4.60	4.40	4.80
2 (High Organic Matter)	Organism/ Hard Water Condition	+	+	+	3.125	5.20	5.20	5.40
3 (Pathogen Control)	Control Group for 1 and 2	+	-	-	-	-	-	-

5.3. Disinfectant effective dilution rate

	Pathogen	Hard water	Organism
Bacteria	Salmonella typhimurium	1.5	1.25
Virus	Avian influenza virus H9N2	6.25	3.125

5.4. Disinfectant recommended dilution rate

	Pathogen	Hard water	Organism
Bacteria	Salmonella typhimurium	1.2	Undiluted solution
Virus	Avian influenza virus H9N2	5	2.5

6. Academic Considerations

This study uses a disinfectant containing benzalkonium chloride as an active ingredient. Benzalkonium chloride is a type of quaternary ammonium salt and its sterilization effect is closely related to the chemical properties of surfactants. In other words, the positive charges of benzalkonium chloride and didecyl dimethyl / ammonium chloride combine with negatively charged substances such as protein, which is necessary for cell structure and enzyme activity to denature and destroy proteins, results in antimicrobial effects. In addition, benzalkonium chloride and didecyl dimethyl ammonium chloride primarily bind to the phospholipids of the cell membrane to dissolve the cell membranes, causing the leakage of intracellular components, resulting in destruction of the cytoplasmic membranes, resulting in increased cell permeability, resulting in bactericidal effects. The bactericidal effect of benzalkonium chloride and didecyl dimethyl ammonium chloride is stronger against Gram (+), Gram (-) and envelop forming viruses.

From this study, it was confirmed that the bactericidal power of Zoono Micro Shield Z71 decreased about two times in organic matter conditions compared to hard water conditions. Therefore, it is necessary to increase the concentration of Zoono Micro Shield Z71 for subjects containing more organic matter.

The effective dilution rates of Zoono Micro Shield Z71 in hard water conditions for Salmonella Typhimurium and Avian influenza virus were 1.5 times and 6.25 times respectively, and in organic matter conditions the effective dilution rates were 1.25 times and 3.125 times for each pathogen.

Therefore, the recommended dilution rates of Zoono Micro Shield Z71 against common bacteria and avian influenza were determined to be 1.2 times (organic matter conditions: undiluted solution) and 5 times (organic matter condition: 2.5 times).