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# Antiviral Efficacy of Citra-kill®, Disinfectant Solution Against Avian Influenza Virus

Chun-Nam Cha[1], Yeo-Eun Lee[2], In-Jin Kang[2], Chang-Yeul Yoo[3], Eun-Kee Park[4], Sunjeong An[5], Suk Kim, and Hu-Jang Lee*

*Research Institute of Live Sciences, College of Veterinary Medicine, Gyeongsang National University, Chinju 600-701, Korea*
*[1]Engineering Research Institute, Department of Industrial Systems Engineering, Gyeongsang National University, Chinju 600-701, Korea*
*[2]Department of Environmental Health, Graduate School of Public Health, Gyeongsang National University, Chinju 660-751, Korea*
*[3]Department of Computer Information, Gyeongnam Provincial Namhae College, Namhae 668-801, Korea*
*[4]Department of Medical Humanities and Social Medicine, College of Medicine, Kosin University, Busan 602-703, Korea*
*[5]Department of Occupational Therapy, Inje University, Gimhae 621-749, Korea*
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**ABSTRACT** - Highly pathogenic avian influenza virus (HPAIV) is already panzootic in poultry and caused a considerable economic loss in poultry industry. In addition, HPAIV continues to cross species barriers to infect humans and other mammals, often with fatal outcomes. In this study, the virucidal efficacy of Citra-Kill® composed to quaternary ammonium chloride and citric acid was investigated against avian influenza H9N2 virus (AIV). A virucidal efficacy was determined with the viability of AIV contacted with the disinfectant in the allantoic membrane of chicken embryos. Citra-Kill® and AIV was reacted on the distilled water (DW), hard water (HW) or organic matter suspension (OM) condition. On DW condition, AIV was inactivated with 2,000 fold dilutions of Citra-Kill®. When the antiviral effect on HW condition was evaluated, the antiviral activity of the disinfectant showed on 1,500 fold dilutions against AIV. With the investigation of the antiviral effect of the disinfectant on OM condition, AIV was inactivated on 500 fold dilutions of Citra-Kill®. As Citra-Kill® possesses virucidal efficacy against AIV, the disinfectant solution can be used to limit the spread of animal viral diseases.

**Key words:** Citra-Kill®, Avian influenza virus, Disinfectant efficacy

## Introduction

In 1996-1997, highly pathogenic avian influenza virus H5N1 (HPAIV) emerged in Hong Kong, and by July 2007, it had caused 191 human deaths and 300 outbreaks in domestic poultry, water fowl, or wild birds in Asia, central Europe, Africa, and parts of the Middle East[1)].

In Korea, the low and high pathogenic avian influenza (LPAI, HPAI) firstly occurred at GyeongGi-do in 1996 and at Chungcheongbuk-do in 2003, respectively[2)]. According to the epidemiological survey of high pathogenic avian influenza during 2010-2012[3)], HPAI started off at Iksan city, Jeollabuk-do, and spread into Chungcheongnam-do, Chungcheongbuk-do, Jeollanam-do, and Gyeongsangnam-do, in which an outbreak of HPAI resulted in the destruction of about 5.4 million birds at a cost of about 3,500 hundred million.

Avian influenza virus is a lipid-enveloped virus of the family *Orthomyxoviridae* and genus Influenza virus A[4)]. The virus occurs in low pathogenic and high pathogenic form in domestic poultry. Low pathogenic avian influenza virus (LPAIV) is less severe and causes mild respiratory disease. However, HPAIV causes rapid increases in mortality. Two genes are in the AIV single-stranded sequence code for envelope proteins (hemagglutinin [H] and neuraminidase [N]). Type A influenza viruses can be divided into 16 hemagglutinin (HA, H1-H16) and nine neuraminidase (N1-N9) subtypes based on the genetic sequence for the envelope proteins[5)]. H5 and H7 are the most virulent strains to cause HPAIV in domestic poultry. Although most H5 or H7 viruses are low pathogenic, H5 has been demonstrated to mutate from LPAIV to HPAIV[6)]. Pandemic influenza virus has its origins in avian influenza viruses. HPAIV subtype H5N1 is

*Correspondence to: Hu-Jang Lee, College of Veterinary Medicine, Gyeongsang National University, 900 Gajwa-dong, Chinju 660-701, Korea
Tel: 82-55-772-2352, Fax: 82-55-772-2308
E-mail: hujang@gnu.ac.kr

already panzootic in poultry, with attendant economic consequences. It continues to cross species barriers to infect humans and other mammals, often with fatal outcomes[7].

Viruses can be grouped in terms of resistance to disinfection strategies. Avian influenza is relatively easy to disinfect due to the lipid envelope that increases its sensitivity to dehydration, detergents, and surfactants[8]. For example, citric acid is effective at inactivating acid-sensitive viruses, then it can be combined with detergents, and it is typically safe for use on personnel and clothing. The quaternary ammonium compounds (QAC), lipophilic disinfectants have a broad-spectrum virucidal activity against many viruses containing AIV except for nonenveloped viruses[9,10].

Highly hygienic measure including the use of disinfectant is very effective for successful control of bacterial and viral diseases in farmed animals[11]. Several disinfectants including quaternary ammonium compounds, organic acids, glutaraldehyde, aldehydes, sodium hydroxide, and chlorohexadine are used for decontamination after outbreaks of farmed animal diseases[11,12]. However, there is not the efficacy test for the disinfectant composed of quaternary ammonium chloride and citric acid against viral animal diseases. Therefore, this study was carried out to examine virucidal efficacy of the disinfectant solution against the avian influenza virus.

## Materials and methods

### Disinfectant

The active ingredients for Citra-Kill®, the tested disinfectant solution, are quaternary ammonium chloride (10% v/v) and citric acid (30% w/v). Citra-kill® was provided by Dae Han New Pharm Co. (Seoul, Korea) The disinfectant solution was stored in the dark in room temperature and prepared for dilution on the day of evaluation. Determination of the antiviral efficacy of the disinfectant was based on Animal, Plant and Fisheries Quarantine and Inspection Agency (APFQIA) Regulation No. 2008-14, Korea[13].

### Avian influenza virus and culture

Avian influenza virus H9N2 (MS96 strain, AIV) obtained from National Veterinary Research and Quarantine Service (NVRQS). The AIV was inoculated in 10-day-old chicken embryos through the allantoic membrane route. The inoculated eggs were then incubated at 37°C at 85% humidity for 3 days. When the level of viral particles propagated in allantoic fluid was $3.0 \times 10^9$/ml, allantoic fluids were harvested and used to test the antiviral activity of disinfectant.

### Diluents and treatment condition

Testing was based on virucidal effects of disinfectant diluents in three treatment conditions (distilled water (DW) condition, standard hard water (HW) condition, and organic matter (OM) condition), pathogen control (disinfectant negative control) and DW control (both disinfectant and pathogen negative control) in Table 1. HW, an ingredient of HW treatment condition, was made by adding anhydrous $CaCl_2$ 0.305 g and $MgCl_2 \cdot 6H_2O$ 0.139 g into 1 liter distilled water. Organic suspension, an ingredient of OM treatment condition, is a solution of 1% (w/v) fetal bovine serum (FBS, Sigma-Aldrich Korea, Seoul) in HW.

### Virus-disinfectant contact reaction

Citra-Kill® was diluted 1,200, 1,500, 2,000, 2,500, 3,000, and 3,500 times with DW, and diluted 1,000, 1,200, 1,500, 2,000, 2,500, and 3,000 times with HW, and diluted 100, 200, 300, 400, 500, and 600 times with OM, respectively. After dilution of disinfectant, 2.5 ml of disinfectant diluents was added into each test tube.

One ml of the allantoic fluid containing AIV was diluted with 19 ml DW, HW, and OM, respectively. After dilution of the allantoic fluid, 2.5 ml of the diluents was inserted into each test tube containing disinfectant diluents, and incubated at 4°C for 30 min.

### Evaluation of Citra-Kill® against AIV

After virus-disinfectant contact reaction, 2.5 ml of 10% inactivated fetal bovine serum was added into each test tube to neutralize efficacy of disinfectant at room temperature. The neutralized solutions were diluted 10, $10^2$, $10^3$, $10^4$, $10^5$, and $10^6$ times with phosphate buffer solution and 0.2 ml of the neutralized solution diluents was injected into the allantoic membrane of five 10 day-old-chicken per dilution time. After inoculation, the embryos were incubated for 5 days at 37°C with relative humidity at 85%. The embryos were checked for viability everyday during the incubation period. After incubation of the embryos, the allantoic fluid was collected from each embryo to examine the viability of AIV and its ability to propagate to result in a detectable

**Table 1.** Experimental design for the determination of the virucidal efficacy of Citra-kill®

| Treatment condition* | Contents according to treatment condition** | | | | |
|---|---|---|---|---|---|
| | DM | HW | OM | Disinfectant | AIV |
| DW condition | + | - | - | + | + |
| HW condition | - | + | - | + | + |
| OM condition | - | - | + | + | + |
| Bacteria control | - | + | - | - | + |
| DW control | + | - | - | - | + |

*DW, distilled water; HW, standard hard water; OM, organic matter; AIV, avian influenza virus.
**+, presence; -, absence

hemagglutination titer, using 1% chicken red blood cells[8]. Five control embryos were injected with the virus without contact with Citra-Kill® (positive controls), and five embryos did not receive any injection (negative controls).

The validity of concentration for the disinfectant was estimated with the concentration of the dilution that the viral dose in the allantoic fluid treated with Citra-Kill® was inactivated more than $10^4$ $EID_{50}$ (egg infective dose 50) compared with positive control.

$EID_{50}$ was calculated according to the method of Käber[14]. The validity of concentration for Citra-Kill® was independently examined on triplicate and determined the validity of concentration with the median of the results.

## Results and Discussion

Table 2-4 present the results of the efficacy testing of Citra-Kill® composed to quaternary ammonium chloride and citric acid against AIV. Table 5 shows the summary of the valid dilution time for Citra-Kill® against AIV. On DW condition, AIV was inactivated with 2,000 fold dilutions of the disinfectant throughout all experiments. When the antiviral effect on HW condition was evaluated, the antiviral activity of the disinfectant showed on 1,500 fold dilutions against AIV throughout all experiments. With the investigation of the antiviral effect of the disinfectant on OM condition, AIV was inactivated on 500 fold dilutions.

As organic material interferes with efficacy by either inactivating the disinfectant or blocking it from surface contact, the virucidal activity of Citra-Kill® on the OM condition lowered efficacy against AIV compared with DM or HW conditions.

Kassaify *et al*. (2007) reported that the treatment with quaternary ammonium compounds at the concentration of 10 mg/ml showed no efficacy against AIV on HW and OM condition[15]. And Patnayak *et al*. (2008) carried out the efficacy test of disinfectants and hand sanitizers against AIV. With the results of the disc test, AIV was inactivated more than 90% at the concentration of 350 ppm quaternary ammonium chloride for 10 min[16]. Lombardi *et al*. (2008) reported that citric acid at the concentration of 1% effectively inactivated AIV on hard and nonporous surfaces[17].

In the present study, the disinfectant efficacy of Citra-Kill® against AIV showed higher than the individual quaternary ammonium chloride tested by Kassaify *et al*.[15]. In addition, Citra-Kill® was higher effective than the individual citric acid

**Table 2.** The validation of Citra-kill® against Avian Influenza Virus: first examination

| Tratment condition[1)] | Dilution time | Dilution time of neutralization solution (positive/the number of inoculation) | | | | | | $EID_{50}$[2)] | Log reduction |
|---|---|---|---|---|---|---|---|---|---|
| | | $10^{-1}$ | $10^{-2}$ | $10^{-3}$ | $10^{-4}$ | $10^{-5}$ | $10^{-6}$ | | |
| DW | 1/1,200 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | ≤ 0.5 | ≥ 5.7 |
| | 1/1,500 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | ≤ 0.5 | ≥ 5.7 |
| | 1/2,000 | 4/5 | 2/5 | 0/5 | 0/5 | 0/5 | 0/5 | 1.7 | 3.8 |
| | 1/2,500 | 5/5 | 5/5 | 3/5 | 2/5 | 0/5 | 0/5 | 3.5 | 2.5 |
| | 1/3,000 | 5/5 | 5/5 | 5/5 | 4/5 | 1/5 | 1/5 | 5.3 | 1.0 |
| | 1/3,500 | 5/5 | 5/5 | 5/5 | 5/5 | 3/5 | 2/5 | 5.5 | 0.6 |
| HW | 1/1,000 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | ≤ 0.5 | ≥ 5.7 |
| | 1/1,200 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | ≤ 0.5 | ≥ 5.7 |
| | 1/1,500 | 3/5 | 1/5 | 0/5 | 0/5 | 0/5 | 0/5 | 1.3 | 4.0 |
| | 1/2,000 | 5/5 | 4/5 | 2/5 | 1/5 | 0/5 | 0/5 | 2.9 | 3.0 |
| | 1/2,500 | 5/5 | 5/5 | 3/5 | 2/5 | 1/5 | 0/5 | 3.7 | 2.3 |
| | 1/3,000 | 5/5 | 5/5 | 5/5 | 4/5 | 3/5 | 1/5 | 5.1 | 1.2 |
| OM | 1/300 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | ≤ 0.5 | ≥ 5.7 |
| | 1/400 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | ≤ 0.5 | ≥ 5.7 |
| | 1/500 | 4/5 | 1/5 | 0/5 | 0/5 | 0/5 | 0/5 | 1.5 | 3.9 |
| | 1/600 | 5/5 | 5/5 | 1/5 | 0/5 | 0/5 | 0/5 | 2.7 | 3.2 |
| | 1/700 | 5/5 | 5/5 | 5/5 | 2/5 | 1/5 | 0/5 | 4.3 | 2.0 |
| | 1/800 | 5/5 | 5/5 | 5/5 | 5/5 | 4/5 | 3/5 | 5.9 | 0.5 |
| Positive control | | 5/5 | 5/5 | 5/5 | 5/5 | 5/5 | 3/5 | 6.1 | |
| Negative control | | 5/5 | 5/5 | 5/5 | 5/5 | 5/5 | 2/5 | 5.9 | |

[1)]DW, distilled water; HW, hard water; OM, organic matter.

[2)]$EID_{50} = -L1 - [L \times \{S/100 - 0.5\}]$

($L_1$, Log of lowest dilution tested; L, log interval between dilutions; S, sum of % mortality at each dilution)

**Table 3.** The validation of Citra-kill® against Avian Influenza Virus: second examination

| Tratment condition[1)] | Dilution time | Dilution time of neutralization solution (positive/the number of inoculation) | | | | | | $EID_{50}$[2)] | Log reduction |
|---|---|---|---|---|---|---|---|---|---|
| | | $10^{-1}$ | $10^{-2}$ | $10^{-3}$ | $10^{-4}$ | $10^{-5}$ | $10^{-6}$ | | |
| DW | 1/1,200 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | ≤ 0.5 | ≥ 5.7 |
| | 1/1,500 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | ≤ 0.5 | ≥ 5.7 |
| | 1/2,000 | 4/5 | 2/5 | 0/5 | 0/5 | 0/5 | 0/5 | 1.7 | 3.8 |
| | 1/2,500 | 5/5 | 5/5 | 3/5 | 1/5 | 1/5 | 0/5 | 3.5 | 2.5 |
| | 1/3,000 | 5/5 | 5/5 | 4/5 | 3/5 | 1/5 | 1/5 | 4.3 | 2.0 |
| | 1/3,500 | 5/5 | 5/5 | 5/5 | 5/5 | 3/5 | 2/5 | 5.5 | 0.8 |
| HW | 1/1,000 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | ≤ 0.5 | ≥ 5.7 |
| | 1/1,200 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | ≤ 0.5 | ≥ 5.7 |
| | 1/1,500 | 4/5 | 1/5 | 0/5 | 0/5 | 0/5 | 0/5 | 1.5 | 3.9 |
| | 1/2,000 | 5/5 | 5/5 | 2/5 | 1/5 | 0/5 | 0/5 | 3.1 | 2.8 |
| | 1/2,500 | 5/5 | 5/5 | 3/5 | 2/5 | 1/5 | 0/5 | 3.7 | 2.3 |
| | 1/3,000 | 5/5 | 5/5 | 5/5 | 4/5 | 2/5 | 2/5 | 5.1 | 1.2 |
| OM | 1/300 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | ≤ 0.5 | ≥ 5.7 |
| | 1/400 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | ≤ 0.5 | ≥ 5.7 |
| | 1/500 | 3/5 | 1/5 | 0/5 | 0/5 | 0/5 | 0/5 | 1.3 | 4.0 |
| | 1/600 | 5/5 | 4/5 | 1/5 | 0/5 | 0/5 | 0/5 | 2.5 | 3.5 |
| | 1/700 | 5/5 | 5/5 | 3/5 | 2/5 | 1/5 | 0/5 | 3.7 | 2.3 |
| | 1/800 | 5/5 | 5/5 | 5/5 | 5/5 | 4/5 | 3/5 | 5.9 | 0.5 |
| Positive control | | 5/5 | 5/5 | 5/5 | 5/5 | 5/5 | 4/5 | 6.3 | |
| Negative control | | 5/5 | 5/5 | 5/5 | 5/5 | 5/5 | 3/5 | 6.1 | |

[1)]DW, distilled water; HW, hard water; OM, organic matter.
[2)]$EID_{50} = -L1 - [L \times \{S/100 - 0.5\}]$
($L_1$, Log of lowest dilution tested; L, log interval between dilutions; S, sum of % mortality at each dilution)

**Table 4.** The validation of Citra-kill® against Avian Influenza Virus: third examination

| Tratment condition[1)] | Dilution time | Dilution time of neutralization solution (positive/the number of inoculation) | | | | | | $EID_{50}$[2)] | Log reduction |
|---|---|---|---|---|---|---|---|---|---|
| | | $10^{-1}$ | $10^{-2}$ | $10^{-3}$ | $10^{-4}$ | $10^{-5}$ | $10^{-6}$ | | |
| DW | 1/1,200 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | ≤ 0.5 | ≥ 5.7 |
| | 1/1,500 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | ≤ 0.5 | ≥ 5.7 |
| | 1/2,000 | 3/5 | 1/5 | 0/5 | 0/5 | 0/5 | 0/5 | 1.3 | 4.0 |
| | 1/2,500 | 5/5 | 5/5 | 3/5 | 1/5 | 0/5 | 0/5 | 3.3 | 2.7 |
| | 1/3,000 | 5/5 | 5/5 | 4/5 | 3/5 | 1/5 | 0/5 | 4.7 | 1.7 |
| | 1/3,500 | 5/5 | 5/5 | 5/5 | 5/5 | 2/5 | 1/5 | 5.1 | 1.2 |
| HW | 1/1,000 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | ≤ 0.5 | ≥ 5.7 |
| | 1/1,200 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | ≤ 0.5 | ≥ 5.7 |
| | 1/1,500 | 4/5 | 2/5 | 0/5 | 0/5 | 0/5 | 0/5 | 1.7 | 3.8 |
| | 1/2,000 | 5/5 | 5/5 | 3/5 | 2/5 | 1/5 | 0/5 | 3.7 | 2.3 |
| | 1/2,500 | 5/5 | 5/5 | 5/5 | 5/5 | 2/5 | 0/5 | 4.9 | 1.4 |
| | 1/3,000 | 5/5 | 5/5 | 5/5 | 5/5 | 3/5 | 2/5 | 5.5 | 0.8 |
| OM | 1/300 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | ≤ 0.5 | ≥ 5.7 |
| | 1/400 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | ≤ 0.5 | ≥ 5.7 |
| | 1/500 | 4/5 | 2/5 | 1/5 | 0/5 | 0/5 | 0/5 | 1.9 | 3.7 |
| | 1/600 | 5/5 | 3/5 | 1/5 | 1/5 | 0/5 | 0/5 | 2.9 | 3.0 |
| | 1/700 | 5/5 | 5/5 | 5/5 | 2/5 | 1/5 | 0/5 | 3.7 | 2.3 |
| | 1/800 | 5/5 | 5/5 | 5/5 | 5/5 | 4/5 | 2/5 | 5.7 | 0.7 |
| Positive control | | 5/5 | 5/5 | 5/5 | 5/5 | 5/5 | 4/5 | 6.3 | |
| Negative control | | 5/5 | 5/5 | 5/5 | 5/5 | 5/5 | 2/5 | 5.9 | |

[1)]DW, distilled water; HW, hard water; OM, organic matter.
[2)]$EID_{50} = -L1 - [L \times \{S/100 - 0.5\}]$
($L_1$, Log of lowest dilution tested; L, log interval between dilutions; S, sum of % mortality at each dilution)

**Table 5.** The summary of the valid dilution time for Citra-Kill® against avian influenza virus

| Tretment condition[1)] | Experiment | | | Median |
|---|---|---|---|---|
| | first | second | third | |
| DW | 1/2,000 | 1/2,000 | 1/2,000 | 1/2,000 |
| HW | 1/1,500 | 1/1,500 | 1/1,500 | 1/1,500 |
| OM | 1/500 | 1/500 | 1/500 | 1/500 |
| Positive control | +[2)] | + | + | |
| Negative control | + | + | + | |

[1)]DW, distilled water; HW, hard water; OM, organic matter.
[2)]Viral activity was identified more than $10^{7.0}$ $EID_{50}$/ml.

examined by Lombardi *et al*.[16]. It might that the virucidal efficacy of Citra-Kill® was higher than the individual quaternary ammonium chloride and citric acid because the interaction of quaternary ammonium and citric acid makes the synergistic effect against AIV.

In this study, disinfectant efficacy of Citra-Kill® has a limitation that the results are based on *in vitro* test. Organic material in suspension (OM condition) could not represent all possible parameters of AIV contaminated environments.

As the efficacy of Citra-Kill® against AIV was investigated *in vitro*, a controlled field trial is required to determine whether the use of Citra-Kill® will be able to reduce AIV.

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## 요 약

본 연구에서는 4급 암모늄에 속하는 quaternary ammonium chloride와 구연산의 합제인 Citra-Kill®의 조류인플루엔자에 대한 살바이러스 효과를 확인하기 위해 국립수의과학검역원의 소독제 효력시험 중 바이러스 소독제 효력시험에 따라 수행하였다.

소독제와 조류인플루엔자바이러스를 증류수, 경수, 그리고 유기물 조건에서 반응시킨 후, 중화액을 이용하여 중화시킨 다음, 중화된 용액 0.2 ml를 10일령의 계태아 요막에 주입하여 5일 동안 배양시킨 다음, 요막액을 채취하여 바이러스의 생존여부를 혈구응집반응을 통해 확인하여 소독제의 효력배수를 결정하였다.

본 연구의 결과, Citra-Kill®은 증류수, 경수, 그리고 유기물 조건에서 각각 2,000, 1,500, 그리고 500배에서 조류인플루엔자바이러스를 불활성화시켜, 조류인플루엔자에 대해 뛰어난 소독효과를 갖고 있는 것이 확인되었다. 따라서 향후, 야외적용시험을 통해 실제적용에 따른 효과를 확인할 필요가 있을 것으로 사료된다.

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