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Inactivation of Avian Influenza Viruses by Alkaline Disinfectant Solution

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Avian influenza viruses cause a considerable threat to humans and animals. In this study, we investigated whether alkaline disinfectant solution can inactivate H5N1, H3N2, H6N1, and H9N2 subtypes of avian influenza virus. When H5N1, H3N2, H6N1, and H9N2 avian influenza viruses were treated with alkaline solution diluted with PBS (pH 7.2) prior to infection into MDCK cells, alkaline disinfectant solution (at dilutions up to 10^{-2}) completely inactivated all avian influenza subtypes tested. To confirm the inactivation of avian influenza viruses by alkaline disinfectant solution, we used an immunofluorescence assay with influenza A anti-nucleoprotein antibody and FITC-labeled secondary antibody to stain MDCK cells infected with avian H9N2 influenza viruses. No staining was observed in MDCK cells infected with H9N2 viruses that were pre-treated with a 10^{-2} dilution of alkaline disinfectant solution, while strong staining was observed in MDCK cells infected with H9N2 viruses without pre-treatment. Our results indicate that alkaline solution could help to control avian influenza viruses including the highly pathogenic H5N1 subtype.

Key words – Avian influenza virus, H9N2, H5N1, H6N1, H3N2

Introduction

Avian influenza viruses are the cause of considerable economic loss to the poultry industry worldwide [2]. Since their first appearance in Hong Kong in 1997, H5N1 influenza viruses are now widespread in many countries including China, Nigeria, and Thailand [1,5,7,17,21,24]. Avian H5N1 viruses are highly pathogenic to animals and humans and are regarded as potential candidates for future pandemics [23]. H9N2 subtypes of influenza viruses have become panoptic in many Asian countries including China, Korea, and Pakistan [3,4,9-11,13-15] and cause economic losses to the poultry industry through reduced egg production and about 10% mortality in broilers. H3N2 and H6N1 avian influenza viruses were reported in ducks [4].

Effective methods of controlling the outbreak of highly pathogenic avian influenza viruses in chicken farms include slaughtering of infected chickens and vaccination using antigens grown in hens' eggs. As avian influenza viruses can be transmitted among chickens via the respiratory and oral-fecal route, topical use of an effective disinfectant may help to control viral transmission. Many commercially available disinfectants are effective for inactivation of viruses such as influenza viruses and coronavirus [6,16,22]. Suarez et al. [21] showed that five disinfectants including phenolic disinfectants (Tek-trol and one-stroke environ), a quaternary ammonia compound (Lysol no-rinse sanitizer), a peroxygen compound (Virkon-S), and sodium hypochlorite (household bleach) were effective for inactivating avian influenza viruses at the recommended concentrations, but RNA of avian influenza viruses in samples inactivated with phenolic and quaternary ammonoia compounds could be detected by reverse transcriptase-polymerase chain reaction (RT-PCR). In the study using gentian violet (GV) and GV-dyed cotton to inactivate human influenza virus, human H1N1 influenza viruses could be inactivated by GV and GV-dyed cotton [16]. Previous studies showed that many other methods could be used for the inactivation of viruses [6,12,18,20]. Ozone was effective for the inactivation of enterovirus 71, and chloride dioxide could degrade poliovirus 1 genome [12,20]. It was also reported that UV light could inactivate adenovirus serotypes [18]. The study for inactivation of coronavirus showed that alkaline solution was effective in inactivation of severe acute respiratory syndrome virus (SARS-CoV) [6]. However, it has not been reported whether alkaline solution could inactivate avian influenza viruses. In this study, we investigated whether alkaline solution can inactivate H5N1, H9N2, H3N2 and H6N1 subtypes of avian influenza virus.

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Materials and methods

Preparation of alkaline disinfectant solution.

Alkaline disinfectant solution (pH 12.2) was prepared by dissolving Sodiummeta Silisilicate (1g), potassium carbonate (2g), potassium citrate (2g), borex (1g), and silver nitrate (0.1g) per litter in water. The formulation of alkaline solution was found while searching for optimal alkaline formulations that are effective for the inactivation of avian influenza viruses.

Avian Influenza Viruses and cell lines.

A/chicken/korea/S1/2003 (H9N2), A/Duck/Korea/S17 /2003 (H6N1), A/Chicken/Korea/S6/2003 (H6N1) and A/ Hong Kong/212/2003 (H5N1) viruses were passaged six times in MDCK cells before use in the study. Infectious viral titers in culture supernatant were determined by log10 tissue culture infectious dose 50 (log10$TCID_{50}$) end point. To determine log 10 tissue culture infectious dose 50 ($log_{10}TCID_{50}$), viruses were serially 10-fold diluted in MEM supplemented with 1μg/ml of trypsin before the diluted viruses were infected into MDCK cells in 96-well plates in quadruplicate. The infected cells were incubated in the humidified incubator (37°C, 5%CO_2) for 72 h before the viral presence was determined by hemagglutination assay using 0.5% turkey red blood cell. Log_{10} tissue culture infectious dose 50 ($log_{10}TCID_{50}$) was calculated as described previously [19]. This work was done at BSL3+ facility.

Madin Darby canine kidney (MDCK) cells purchased from the American Type Culture Collection (ATCC, Rockville, MD) were maintained in Minimal Essential Medium (MEM) supplemented with 1% penicillin and streptomycin, 25mM Hepes buffered solution (Gibco BRL), and 10% fetal calf serum (Gibco BRL).

Treatment of avian influenza viruses with alkaline disinfectant solution.

Alkaline disinfectant solution was serially diluted 10-fold with PBS (pH 7.4). A 315 μl detergent dilution was mixed with 100$TCID_{50}$(35 μl, about 0.01 m.o.i.) virus and incubated at room temperature inside the tissue culture hood for 2 hours. The diluted viruses were infected into MDCK cells in 96 well plates in quadruplicate and incubated in the humidified incubator (37°C, 5%CO_2) for 3 days. At three days after infection, the presence of viruses was determined by log_{10} tissue culture infectious dose 50 ($log_{10}TCID_{50}$) in MDCK cells.

Treatment of avian influenza viruses with formalin solution.

Formalin solution was serially 10-fold diluted with PBS (pH 7.4) and a 315 μl of diluted formalin was mixed with 100$TCID_{50}$(35 μl, about 0.01 m.o.i.) virus. Mixed viruses were incubated at room temperature inside the tissue culture hood for 2 hours before they were infected into MDCK cells in 96 well plates in quadruplicate. The infected cells were incubated in the humidified incubator for 3 days. The presence of viruses were determined by $log_{10}TCID_{50}$ in MDCK cells

Immunofluoresence assay.

H9N2 viruses (100$TCID_{50}$) (35 μl) were treated with a 100-fold dilution of alkaline solution (315 μl) at room temperature for 2 hours. Treated or untreated H9N2 viruses were infected into MDCK cells grown on chamber slides. At 48 hours post infection, the cells were fixed with 80% cold acetone and stained with mouse anti- influenza A nucleoprotein monoclonal antibody (Serotech, Oxford, UK) and FITC-labeled secondary anti-mouse antibody. The cells were observed under fluorescence microscope (Olympus, Japan).

Results

Alkaline disinfectant solution is effective in inactivation of avian influenza viruses.

We initially tested whether alkaline disinfectant solution could inactivate virulent avian influenza viruses H9N2, H3N2, and H6N1. Of these, H9N2 influenza viruses are enzootic in poultry in many Asian countries including Korea and China [4,8]. H9N2, H3N2 and H6N1 viruses were inactivated by alkaline disinfectant solution in a concentration-dependent manner. Viruses treated with high concentrations of alkaline disinfectant solution (undiluted to 10^{-2} dilution) did not survive in MDCK cells, while viruses treated with dilutions from 10^{-4}- to10^{-6} were not inactivated. Treatment with 10^{-3} dilution of alkaline disinfectant solution reduced virus titers up to 3 fold compared with those of untreated viruses (Fig. 1A). As a positive control, we used formalin. Formalin (37%) was serially 10-fold diluted with PBS (pH 7.4) before the diluted formalin reacted with H9N2, H3N2, and H6N1 avian influenza viruses. The replication of H9N2 influenza viruses in MDCK cells was completely inhibited up to 1000-fold dilution of formalin, while that of H3N2 and H6N1 influenza viruses were completely inhibited up to 100-fold dilution

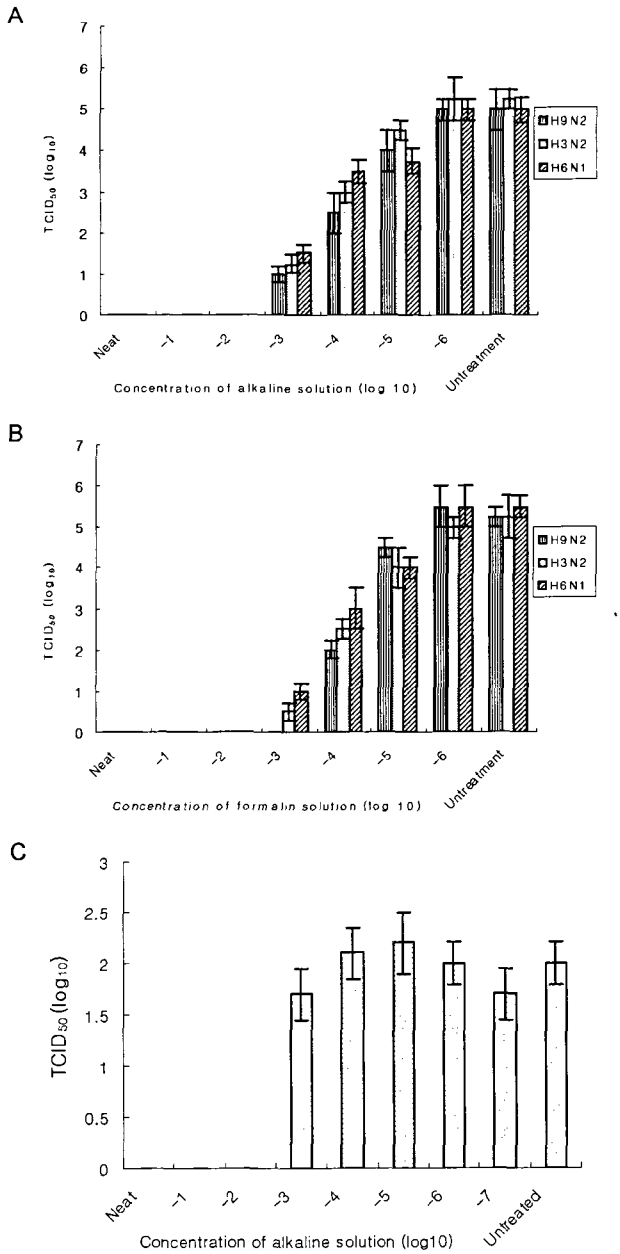

Fig. 1. Alkaline disinfectant solution inactivates H9N2, H3N2, and H6N1 subtypes of avian influenza viruses. Viruses were treated with alkaline disinfectant solution or formalin for 2 hours at room temperature before infection into MDCK cells. The infected cells with H9N2, H3N2 and H6N1 subtypes were added with MEM media containing trypsin (1 μg/ml), and were incubated for 3 days at the humidified incubator (37°C). The inactivation titers were determined by $TCID_{50}$ in MDCK cells. A. Inactivation of H9N2, H3N2, and H6N1 viruses by alkaline disinfectant solution, B. Inactivation of H9N2, H3N2, and H6N1 viruses by formalin, C. Inactivation of H5N1 viruses by alkaline disinfectant solution. Data are the mean of quadrant ± standard errors.

of formalin (Fig 1B).

The highly pathogenic avian H5N1 virus has caused considerable economic losses to the poultry industry in many countries since 1997 when this subtype first appeared in poultry in Hong Kong [15]. H5N1 influenza viruses were inactivated by alkaline disinfectant solution at dilutions up to 10^{-2}, while H5N1 viruses treated with dilutions from 10^{-3} to 10^{-7} were not inactivated (Fig. 1B). The results suggest that alkaline disinfectant solution may be used for controlling avian influenza viruses including the highly pathogenic H5N1 influenza viruses. Alkaline disinfectant solution or formalin diluted with PBS alone did not cause damage to MDCK cells used for this study.

Immunofluorescene assay further confirms that alkaline disinfectant is effective for inactivation of avian influenza viruses.

To further confirm the inactivation of avian influenza viruses by alkaline disinfectant solution, we performed immunostaining of cells. Untreated H9N2 viruses and viruses treated with alkaline disinfectant solution (10^{-2} dilution) were inoculated into MDCK cells for 48 hours before staining with anti-influenza A nucleoprotein antibody and FITC-labeled secondary antibody. Cells infected with alkaline solution-treated H9N2 viruses did not show any staining, while cells infected with untreated H9N2 viruses were clearly stained with mouse anti-influenza A nucleoprotein antibody (Fig. 2). The data indicate that alkaline disinfectant solution could inhibit the replication of avian influenza virus.

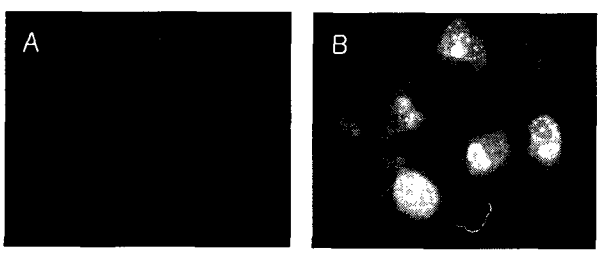

Fig. 2. Immunofluoresence staining in MDCK cells. A/chicken/korea/S1/2003 (H9N2) viruses were treated with alkaline disinfectant solution diluted in PBS (10^{-2}) and were inoculated in MDCK cells. The infected cells were incubated for 2 days before cells were stained with influenza A anti-nucleoprotein antibody and FITC -labeled secondary antibody. A. MDCK cells infected with H9N2 viruses treated with alkaline disinfectant solution. B. MDCK cells infected with untreated H9N2 virus. (X400)

Discussion

The highly pathogenic H5N1 influenza viruses are endemic in poultry in many countries and are regarded as potential candidates for influenza pandemic [5,21]. An effective disinfectant that could be sprayed over animals would help to control the spread of avian influenza viruses in poultry. Our study showed that alkaline disinfectant solution could inactivate avian influenza viruses of H5N1, H9N2, H3N2, and H6N1 subtypes.

We showed that alkaline disinfectant solution was very effective for the inactivation of avian influenza viruses including the highly pathogenic H5N1 influenza viruses. As of August 17, 2006, H5N1 influenza viruses had infected 239 humans and killed 140 (WHO report, http://www.who.int/csr/disease/avian_influenza/country/cases_table_2006_08_17/en/print. html). Most of the human infections with H5N1 were mediated by contact with poultry infected with H5N1 viruses. Effective disinfectants could be used to control the spread of H5N1 viruses among poultry. Since our preliminary data suggest that alkaline disinfectant solution in dilution of 10^{-2} may not be harmful to poultry (data not shown), it can be sprayed over the birds. Other disinfectants such as phenolic disinfectants, a quaternary ammonia compound, a peroxygen compound, and sodium hypochlorite have been shown to be effective for inactivation of avian influenza viruses [22]. It seems that these compounds may not be directly sprayed over poultry because of the possibility that these compounds may be harmful to poultry.

Since all influenza A viruses, including 16 hemagglutinin and 9 neuraminidase subtypes, are circulating in wild birds [8,24], it may be impossible to eradicate influenza viruses in humans and animals. However, use of measures such as vaccination, antiviral drugs, and efficient disinfectants may minimize damage caused by newly emerged avian influenza viruses.

The effectiveness of alkaline disinfectant solution in the inactivation of avian influenza viruses is based on *in vitro* results. In order for alkaline disinfectant solution to be used for controlling the outbreaks of avian influenza viruses on farms, further study such as field trial is needed.

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초록 : 알칼리성 소독액에 의한 조류인플루엔자바이러스 불활성화

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조류인플루엔자바이러스는 사람 및 동물에 상당한 위협을 가하고 있다. 이 연구는 알칼리성 소독용액이 H5N1, H3N2, H6N1, H9N2형 조류인플루엔자바이러스를 불활성화시킬 수 있는지를 조사했다. 알칼리성 소독용액을 생리식염수에 100배 희석한 경우 조류인플루엔자가 MDCK 세포에서 증식하는 것을 완전히 억압하였다. 형광항체법을 이용한 실험에서도 알칼리성소독액을 처리한 H9N2 조류인플루엔자바이러스는 MDCK세포에서 증식이 억제되었고, 알칼리성 소독액을 처리하지 않은 H9N2 조류인플루엔자바이러스는 증식이 억제되지 않았다. 이 결과는 알칼리성 소독액은 고병원성 H5N1을 포함을 조류인플루엔자바이러스를 방역하는데 도움이 될 수 있음을 시사한다.