Enhanced inactivation of avian influenza virus at −20°C by disinfectants supplemented with calcium chloride or other antifreeze agents

Jiewen Guan, Maria Chan, Brian W. Brooks, Elizabeth Rohonczy

Abstract

Avian influenza outbreaks have occurred during winter months, and effective disinfection of poultry premises at freezing temperatures is needed. The commercial disinfectants Virkon and Accel, supplemented with an antifreeze agent [propylene glycol (PG), methanol (MeOH), or calcium chloride ($CaCl_2$)], were evaluated for their effectiveness in killing avian influenza virus (AIV) at −20°C or 21°C. An AIV suspension was applied to stainless steel disks, air-dried, and covered with a disinfectant or antifreeze agent for 5 to 30 min. Virkon (2%) and Accel (6.25%) with 30% PG, 20% MeOH, or 20% $CaCl_2$ inactivated 6 $\log_{10}$ AIV within 5 min at −20°C and 21°C. At these temperatures PG and MeOH alone did not kill AIV, but the 20% $CaCl_2$ solution alone inactivated 5 $\log_{10}$ AIV within 10 min. The results suggested that $CaCl_2$ is potentially useful to enhance the effectiveness of disinfection of poultry facilities after outbreaks of AIV infection in warm and cold seasons.

Résumé

Les poussées de cas d'influenza aviaire se sont produites durant les mois d'hiver, et une désinfection efficace des sites d'élevage à des températures de congélation est nécessaire. Les désinfectants commerciaux Virkon et Accel, auxquels on ajouta un antigel [propylène glycol (PG), méthanol (MeOH), ou chlorure de calcium ($CaCl_2$)], furent évalués pour leur efficacité à tuer le virus de l'influenza aviaire (VIA) à −20 °C ou 21 °C. Une suspension de VIA fut appliquée à des disques d'acier inoxydable, séchés à l'air, et recouverts avec un désinfectant ou d'antigel pour une durée de 5 à 30 minutes. Le Virkon (2 %) et l'Accel (6,25 %) avec 30 % PG, 20 MeOH, ou 20 % $CaCl_2$ ont inactivé 6 $\log_{10}$ de VIA en-dedans de 5 min à −20 °C et 21 °C. À ces températures le PG et le MeOH seuls n'ont pas réussi à tuer le VIA, mais la solution de $CaCl_2$ à 20 % seule a inactivé 5 $\log_{10}$ de VIA en-dedans de 10 min. Les résultats suggèrent que le $CaCl_2$ est potentiellement utile pour augmenter l'efficacité de la désinfection des exploitations avicoles suite à des poussées de cas d'infection par le VIA en saison chaude ou froide.

(Traduit par Docteur Serge Messier)

Avian influenza viruses (AIVs) continue to be a threat to the Canadian poultry industry. Since 2004, there have been at least 6 outbreaks of AIV infection in Canada that have caused substantial economic loss (1–3). Several of these outbreaks have occurred in regions where winter temperatures as low as −20°C are common. Canada's eradication policy to control these outbreaks requires killing of all birds on infected premises, safe disposal of the carcasses and wastes, and then cleaning and decontamination of buildings, vehicles, and equipment.

Avian influenza viruses are enveloped and under warm conditions are readily inactivated by most chemical disinfectants, including those in laundry detergents (4–6). However, at temperatures below the freezing point, disinfectants may not be effective in killing viruses (7). For that reason, the use of disinfectants in high concentrations was evaluated for the capacity to kill virus within 5 min before freezing occurred at −10°C (8). However, such a strategy may be of limited value, as freezing may be almost instantaneous when disinfectants are applied to cold metal surfaces. Furthermore, prolonged contact may be required to kill viruses in organic matter (9).

Davison et al (10) were the first to dilute disinfectants with propylene glycol (PG) or methanol (MeOH) to prevent freezing and then to demonstrate the effectiveness of these preparations in killing AIV at −15°C. The efficacy of this approach to cold-weather disinfection was supported by the studies of Guan et al (9), which showed that Virkon (2%), domestic bleach (5.25%), and surface decontamination foam, supplemented with PG, could be effective in killing Newcastle disease and infectious bursal disease viruses at −10°C and −25°C. The objective of the present study was to extend those findings by evaluating the effectiveness of Virkon and Accel, supplemented with PG, MeOH, or calcium chloride ($CaCl_2$) as antifreeze agents, at inactivating a representative AIV strain at −20°C. Virkon is widely used as a virucidal disinfectant in various environments (8,9), and Accel is an environmentally sustainable disinfectant because hydrogen peroxide (HP) breaks down into water and oxygen (according to the manufacturer).

Virkon solution (2%, w/v) containing 0.43% (w/v) potassium monopersulfate (MPS) as an active ingredient was prepared from Virkon tablets (Antec International, Sudbury, England). Accel solution (5% or 6.25%, v/v) containing 0.35% or 0.44% (w/w) HP as an active ingredient was prepared from Accel Prevention Concentrate (Virox Technologies, Oakville, Ontario). Standard hard water used in the study was prepared to have a standard hardness of

Ottawa Laboratory (Fallowfield), Canadian Food Inspection Agency, 3851 Fallowfield Road, Ottawa, Ontario K2H 8P9.
Address all correspondence to Dr. Jiewen Guan; telephone: 343-212-0450; fax: 343-212-0206; e-mail: Jiewen.Guan@inspection.gc.ca
Received March 19, 2015. Accepted May 6, 2015.

Table I. State of disinfectant solutions with addition of antifreeze agent after 1 h at −20°C

Antifreeze agent, concentration	Disinfectant solution; state[a]		
	Virkon (2%)	Accel (5%)	Accel (6.25%)
Propylene glycol (PG)			
10%	S	S	S
20%	L	G	G
30%	L	L	L
40%	L	L	L
Methanol (MeOH)			
10%	S	S	G
20%	L	L	L
30%	L	L	L
40%	L	L	L
Calcium chloride ($CaCl_2$)			
15%	S	G	G
20%	L	L	L
25%	L	L	L
30%	L	L	L

[a] S — solid; L — liquid; G — gel.

400 parts per million of calcium carbonate (11). The antifreeze agents, PG (1,2-propanediol; Sigma-Aldrich, Oakville, Ontario), MeOH (Caledon, Georgetown, Ontario), and anhydrous $CaCl_2$ (Sigma-Aldrich), were added during preparation of the disinfectant solutions to final concentrations of 10% to 40% (v/v) for PG and MeOH and 15% to 30% (w/v) for $CaCl_2$. The solutions were used immediately after preparation. Aliquots (1 mL) of the disinfectant solutions containing 1 of the antifreeze agents were dispensed into 2-mL Nalgene cryogenic vials (Thermo Fisher Scientific, Ottawa, Ontario), and the vials were placed in a freezer with the temperature set at −20°C. After incubation for 1 h the solutions were observed to determine if they remained liquid or were frozen. The lowest concentrations at which the disinfectant solutions remained liquid (30% PG, 20% MeOH, and 20% $CaCl_2$) were selected for further testing.

An H6N2 strain of AIV (A/Turkey/Mass/3740/65) was used for this study. Virus stock was prepared with 10-day-old embryonated chicken eggs (ECEs) from a specific-pathogen-free flock of White Leghorn chickens maintained by the Canadian Food Inspection Agency and testing negative for AIV. The amount of virus was determined by limiting dilutions in ECEs (12) and expressed as the 50% embryo infectious dose (EID_{50}).

The 2nd-tier quantitative carrier test (13) was used to evaluate the virucidal activity of the disinfectant solutions. Disks (1 cm in diameter; 0.75 mm thick) of brushed stainless steel (AISI no. 430; Muzeen & Blythe, Winnipeg, Manitoba) were washed 3 times with distilled water and sterilized at 121°C for 25 min before use. Contact time course experiments containing time points of 10, 20, and 30 min and single time point experiments using a contact time of 5 min were carried out. For each time point, triplicate sample disks and control disks were prepared: 10 µL of the virus inoculum (containing approximately 5 to 6 $\log_{10}$ EID_{50} of AIV according to virus recovered from the control disks) was applied to the surface of each disk, the disk was air-dried in a biosafety cabinet for 1 h and then, with the inoculum side up, was placed in a 30-mL polypropylene straight-side vial, and the vials were placed into wells of custom-made metal blocks preconditioned to maintain the test temperatures. For the sample disks, disinfectant solution (50 µL) preconditioned to the test temperatures was added to cover the dried inoculum. For the control disks, phosphate-buffered saline (PBS, pH 7.0) with or without antifreeze agent (50 µL) was added. The vials containing the disks were incubated at −20°C or 21°C, simulating cold and warm conditions for specific periods of up to 30 min. At the end of each contact time a neutralizer solution (950 µL) was immediately added to each vial (including vials with control disks) to stop the activity of the disinfectant. The neutralizer was a mixture of 9 volumes of Difco D/E neutralizing broth (Thermo Fisher Scientific) and 1 volume of antibiotic–antimycotic (Invitrogen Canada, Burlington, Ontario). The suspension from each vial was then serially diluted 10-fold and tested for AIV infectivity in ECEs (14). Before testing of the disinfectant solutions, preliminary experiments were conducted to test the effect of the antifreeze agents and the neutralizer on survival of the virus and the ECEs and the effect of the neutralizer on the activity of the disinfectants.

The data presented are the mean amounts of virus recovered from triplicate sets of test or control disks in duplicate experiments. One-way analysis of variance (15) was used to determine significant differences in the amount of virus recovered from disks incubated at the same temperature for the same contact time. The critical level for significance was set at $P < 0.05$.

As reported in Table I, the addition of 30% PG, 20% MeOH, or 20% $CaCl_2$ to the Virkon and Accel solutions was sufficient to prevent the solutions from freezing for at least 1 h at −20°C. The antifreeze agents had no adverse effect on the ECEs after neutralization. The virus was resistant to treatment with 30% PG or 20% MeOH alone for up to 30 min (Tables II and III); thus, 30% PG or 20% MeOH did not kill AIV. In contrast, when control disks were treated with 20% $CaCl_2$ in PBS for 5 or 10 min at −20°C, a reduction of 2.0 and 4.7 $\log_{10}$ EID_{50} of AIV (compared with control disks with PBS only) was observed (Tables II and III). The killing effect may have resulted from the interactions between calcium cations and viral proteins. Such interactions could cause protein aggregation, precipitation, and denaturation, as demonstrated in cheese and tofu production (16). It was reported 70 years ago that $CaCl_2$ at concentrations of 0.05 N and 0.5 N (equivalent to 0.55% and 5.5%, w/v) produced a slight to moderate reduction of influenza virus within hours (17). In the present study, a much higher concentration of $CaCl_2$ (20%, w/v) inactivated approximately 5 $\log_{10}$ EID_{50} of influenza virus within minutes (Table II). This capacity of a 20% $CaCl_2$ solution to denature viral proteins is consistent with the report that such a solution caused coagulative necrosis of live animal tissues (18). It is noteworthy that $CaCl_2$ is readily available and widely applied to roads in Canada in winter months to melt ice. However, it is highly corrosive to metal and must be washed off to prevent damage.

In other studies a contact time of 5 to 10 min was sufficient for disinfectants to kill AIV and Newcastle disease virus at about 20°C (4,5,9). However, as the temperature drops, chemical reactions slow and the contact time required for effective disinfection increases (9). Increasing the concentration of a disinfectant may speed up the disinfection process (8), but at temperatures as low as −20°C rapid

Table II. Effect of disinfectant solutions on avian influenza virus (AIV) at various temperatures and contact times

Solution and antifreeze agent	Amount of virus (log_{10} EID_{50}) after treatment[a]					
	−20°C			21°C		
	10 min	20 min	30 min	10 min	20 min	30 min
Virkon (2%)						
PG (30%)	0	0	0	0	0	0
MeOH (20%)	0	0	0	0	0	0
$CaCl_2$ (20%)	0	0	0	0	0	0
Accel (5%)						
PG (30%)	0	0	0	0	0	0
MeOH (20%)	< 0.9	0	0	0	< 0.9	0
$CaCl_2$ (20%)	0	NT	NT	0	NT	NT
PBS						
PG (30%)	4.8	4.8	4.5	4.7	4.3	4.7
MeOH (20%)	4.7	4.7	4.6	4.5	4.6	4.7
$CaCl_2$ (20%)	**< 0.9**	**0**	**0**	**0**	**0**	**0**
None	4.7	4.7	4.7	4.7	4.7	4.7

[a] Mean from triplicate samples in duplicate experiments with a standard deviation ≤ 0.5.
0 — no virus was isolated; < 0.9 — the amount of virus isolated was below the titration limit of 0.9 log_{10} EID_{50}. The numbers in boldface are significantly different ($P < 0.05$) from those after treatment with the other agents at the same temperature and contact time.
EID_{50} — 50% embryo infectious dose; NT — not tested; PBS — phosphate-buffered saline.

freezing, along with the organic load on surfaces and the resistance of pathogens, may render the disinfection process ineffective (9,19). In the present study Virkon solution supplemented with 30% PG, 20% MeOH, or 20% $CaCl_2$ produced complete inactivation of 6.0 log_{10} EID_{50} of H6N2 AIV within 5 min at −20°C (Table III). These results are in agreement with those of previous studies in which Virkon and other oxidizing disinfectants supplemented with antifreeze agents remained effective in killing AIV and Newcastle disease virus at subfreezing temperatures (9,10). In the present study, Accel solution (5%) reduced the AIV by approximately 6 log_{10} EID_{50} within 5 min with the addition of 20% MeOH or 20% $CaCl_2$ but by only 4 log_{10} with the addition of 30% PG (Table III). When the Accel concentration was increased from 5% to 6.25% a 6-log_{10} reduction within 5 min was achieved with the addition of any of the 3 antifreeze agents (Table III).

Virkon and Accel are oxidizing disinfectants containing MPS and HP, respectively, as active ingredients, and their effectiveness relies on their oxidizing strength. Organic compounds including organic antifreezes (e.g., PG and MeOH) would be expected to react with these disinfectants, consume their oxidizing strength, and reduce their effectiveness (20). According to the manufacturers, HP has a lower standard electrode potential or oxidizing strength than MPS; thus, PG might have a greater impact on HP than on MPS. Nevertheless, increasing the concentration of disinfectants could help restore their effectiveness in the presence of organic antifreeze or other organic material. In comparison, $CaCl_2$ is an inert compound with its own disinfection capacity and may prove to be superior as an antifreeze agent for maintaining and even enhancing the effectiveness of oxidizing disinfectants. This could explain the complete inactivation of 6 log_{10} of AIV when we used disinfectants supplemented with $CaCl_2$, whereas with PG or MeOH as the supplement,

Table III. Effect of disinfectant solutions on AIV with 5 min of contact

Solution and antifreeze agent	Amount of virus (log_{10} EID_{50}) after treatment[a]	
	−20°C	21°C
Virkon (2%)		
PG (30%)	0	< 0.9
MeOH (20%)	0	< 0.9
$CaCl_2$ (20%)	0	0
Accel (5%)		
PG (30%)	**2.0**	0
MeOH (20%)	< 0.9	< 0.9
$CaCl_2$ (20%)	0	0
Accel (6.25%)		
PG (30%)	0	< 0.9
MeOH (20%)	0	< 0.9
$CaCl_2$ (20%)	0	0
PBS		
PG (30%)	5.9	5.7
MeOH (20%)	5.9	5.9
$CaCl_2$ (20%)	**4.0**	**< 0.9**
None	6.0	6.0

[a] Mean from triplicate samples in duplicate experiments with a standard deviation ≤ 0.5. 0 — no virus was isolated; < 0.9 — the amount of virus isolated was below the titration limit of 0.9 log_{10} EID_{50} (50% embryo infectious dose). The numbers in boldface are significantly different ($P < 0.05$) from those after treatment with the other agents at the same temperature and contact time.
PBS — phosphate-buffered saline.

there was some residual viral infectivity (Tables II and III). In the present study the disinfectant solutions were used immediately after preparation. It is not known what effect delaying their application would have on their effectiveness and on the nature of the products and the kinetics of chemical reactions derived from mixing disinfectants with the antifreeze agents.

In summary, this study showed that preparations of Virkon and Accel, supplemented with PG, MeOH, or $CaCl_2$ as antifreeze agents, could be effectively applied at temperatures as low as −20°C for disinfecting premises after an outbreak of AIV infection. Because the 20% $CaCl_2$ solution alone inactivated 5 $\log_{10}$ EID_{50} of AIV within 10 min at −20°C, this product may prove particularly useful in cleaning and disinfection operations aimed at killing AIV and possibly other enveloped RNA viruses at freezing temperatures.

Acknowledgments

This study was part of the CSSP-2013-TI-1036 project, entitled "Evaluation of readily available materials for use as antifreezing agents for subzero decontamination," which was funded by the Canadian Safety and Security Program. Sincere appreciation is expressed to Dr. Syed A. Sattar, University of Ottawa, Ottawa, Ontario, for introducing the 2nd-tier quantitative carrier test method for assessing the virucidal activity of the chemical disinfectants and to Dr. Lloyd Spencer, emeritus research scientist, Canadian Food Inspection Agency, Ottawa, Ontario, for helpful discussion and scientific advice on preparation of the manuscript.

References

1. Pasick J, Robinson J, Hooper-McGrevy K, et al. The roles of national and provincial diagnostic laboratories in the eradication of highly pathogenic H7N3 avian influenza virus from the Fraser Valley of British Columbia, Canada. Avian Dis 2007a;51:309–312.
2. Pasick J, Handel K, Robinson J, et al. Relationship between H5N2 avian influenza viruses isolated from wild and domestic ducks in British Columbia, Canada. Avian Dis 2007b;51:429–431.
3. Pasick J, Pedersen J, Hernandez MS. Avian influenza in North America, 2009–2011. Avian Dis 2012;56:845–848.
4. Abe M, Kaneko K, Ueda A, et al. Effects of several virucidal agents on inactivation of influenza, Newcastle disease, and avian infectious bronchitis viruses in the allantoic fluid of chicken eggs. Jpn J Infect Dis 2007;60:342–346.
5. De Benedictis P, Beato MS, Capua I. Inactivation of avian influenza viruses by chemical agents and physical conditions: A review. Zoonoses Public Health 2007;54:51–68.
6. Lombardi ME, Ladman BS, Alphin RL, Benson ER. Inactivation of avian influenza virus using common detergents and chemicals. Avian Dis 2008;52:118–123.
7. Dee S, Deen J, Burns D, Douthit G, Pijoan C. An evaluation of disinfectants for the sanitation of porcine reproductive and respiratory syndrome virus-contaminated transport vehicles at cold temperatures. Can J Vet Res 2005;69:64–70.
8. Jang Y, Lee J, So B, et al. Evaluation of changes induced by temperature, contact time, and surface in the efficacies of disinfectants against avian influenza virus. Poult Sci 2014;93:70–76.
9. Guan J, Chan M, Brooks BW, Rohonczy L. Inactivation of infectious bursal disease and Newcastle disease viruses at temperatures below 0°C using chemical disinfectants. Avian Dis 2014;58:249–254.
10. Davison S, Benson CE, Ziegler AF, Eckroade RJ. Evaluation of disinfectants with the addition of antifreezing compounds against non-pathogenic H7N2 avian influenza virus. Avian Dis 1999;43:533–537.
11. Springthorpe VS, Sattar SA. Carrier tests to assess microbicidal activities of chemical disinfectants for use on medical devices and environmental surfaces. J AOAC Int 2005;88:182–201.
12. Swayne DE, Senne DA, Beard CW. Avian influenza. In: Swayne DE, Glisson JR, Jackwood MW, Pearson JE, Reed WM, eds. A Laboratory Manual for the Isolation and Identification of Avian Pathogens. 4th ed. Philadelphia, Pennsylvania: American Association of Avian Pathologists, University of Pennsylvania, 1998:150–155.
13. Sattar SA, Springthorpe VS, Adegbunrin O, Zafer AA, Busa M. A disc-based quantitative carrier test method to assess the virucidal activity of chemical germicides. J Virol Methods 2003; 112:3–12.
14. Guan J, Chan M, Grenier C, Wilkie DC, Brooks BW, Spencer JL. Survival of avian influenza and Newcastle disease viruses in compost and at ambient temperatures based on virus isolation and real-time reverse transcriptase PCR. Avian Dis 2009;53:26–33.
15. Daniel WW, ed. Biostatistics: A Foundation for Analysis in the Health Sciences. 7th ed. New York, New York: John Wiley & Sons, 1999:606–611.
16. Ong L, Dagastine RR, Kentish SE, Gras SL. The effect of calcium chloride addition on the microstructure and composition of cheddar cheese. Int Dairy J 2013;32:135–141.
17. Knight CA, Stanley WM. The effect of some chemicals on purified influenza virus. J Exp Med 1944;79:291–300.
18. Jana K, Samanta PK. Clinical evaluation of non-surgical sterilization of male cats with single intra-testicular injection of calcium chloride. BMC Vet Res 2011;7:39.
19. Guan J, Chan M, Brooks BW, Rohonczy L. Influence of temperature and organic load on chemical disinfection of *Geobacillus stearothermophilus* spores, a surrogate for *Bacillus anthracis*. Can J Vet Res 2013;77:100–104.
20. Jones LA, Jr, Hoffman RK, Phillips CR. Sporicidal activity of peracetic acid and b-propiolactone at subzero temperatures. Appl Microbiol 1967;15:357–362.