ORIGINAL ARTICLE

Action of Disinfectants on Canine Coronavirus Replication *In Vitro*

A. Pratelli

Department of Animal Health and Well-being, Strada Provinciale per Casamassima Km 3, 70010 Valenzano, Bari, Italy

Impacts

- Investigate the sensitivity of CCoV to different disinfectants that may be useful for the application of prophylaxis programme controls in kennels and dog breeding premises.
- Use in the study of human diseases associated to coronavirus infection. An animal model *in vitro*, such as dog coronavirus, may serve to enhance these studies by circumventing the safety problems related to the high pathogenicity of SARS-CoV.
- Inactivated cell culture-derived viral stocks may also be useful for the development of vaccines and the study of their safety and immunogenicity.

Keywords:
Coronavirus; dog; prophylaxis; disinfectants

Correspondence:
Annamaria Pratelli. Department of Animal Health and Well-being, Strada Provinciale per Casamassima Km 3, 70010 Valenzano, Bari, Italy. Tel.: +39 080 4679833;
Fax: +39 080 4679843;
E-mail: a.pratelli@veterinaria.uniba.it

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Summary

Canine coronavirus (CCoV) is responsible for enteric disease in pups. Infected dogs generally have a rapid recovery, so the virus is highly contagious and the spread of infection is difficult to control. Chemical disinfectants have been widely used in human disease-control programmes to prevent viral infectious diseases from spreading, but to date, there are no studies in the literature on the sensitivity of CCoV to chemical biocides. The present study investigated the sensitivity of CCoV to disinfectants currently used for prophylaxis in kennel and dog breeding locations. The effects of three agents: alkyl-dimethyl-benzyl-ammonium chloride, benzalkonium chloride and didecyl-dimethyl-ammonium chloride, on the infectivity titre of CCoV in A72 cell lines, were studied at different concentrations. Although they may regard a small number of agents, the findings showed that the sensitivity of CCoV to disinfectants varies and the differences are dose correlated. In general, virus inactivation implies a permanent loss of infectivity which can be evaluated in suspensions and hand disinfection tests.

Canine coronavirus (CCoV), a member of the family *Coronaviridae*, is an enveloped, positive-stranded RNA virus, clustered into antigenic group I. CCoV is responsible for mild enteric disease in pups. In young pups, or when mixed infections occur, the clinical signs may be severe and include diarrhoea, vomiting, dehydration and occasional death. CCoV is highly contagious and once the virus has become established in the environment, the spread of infection is difficult to control (Pratelli, 2006). Avoiding contact with infected dogs and their excretions is the only way to ensure disease prevention. Crowded, unsanitary conditions, stress during training and other factors appear to favour the development of clinical disease. The virus is acid stable and was not inactivated at pH 3.0 and +20–22°C (Binn et al., 1974; Appel, 1987). Canine coronavirus is relatively heat stable and can be stored for years, frozen at −70°C or lyophilized at +4°C (Pensaert and Callebaut, 1978). Like other enveloped viruses, CCoV is inactivated by most germicidal agents such as lipid solvents (ether and chloroform), formalin, phenol, hypochlorite solution, chlorhexidine isopropanol and β-propiolactone, but they are not effective in preventing dog-to-dog transmission.

Chemical disinfectants have been widely used in human disease-control programmes to prevent viral infectious diseases from spreading. Potential viral targets are the viral envelope, which contains lipids, the capsid and the genome. An important hypothesis was put forward in 1963 and then modified in 1983 (Klein and Deforest, 1983) which suggested that viral susceptibility to disinfectants may be based on whether viruses were 'lipophilic' in nature, because they possessed a lipid envelope, or

'hydrophilic' because they did not (McDonnell and Russell, 1999). Klein and Deforest (1983) further classified viruses into three groups, A (lipid containing), B (non-lipid picornaviruses) and C (other non-lipid viruses larger than those in group B) and categorized disinfectants into two groups, namely the broad-spectrum agents inactivating all viruses and lipophilic agents that failed to inactivate picornoviruses and parvoviruses. Unfortunately, the penetration of antiseptics and disinfectants into different types of viruses and their interaction with viral components have not been extensively studied. Some information has been provided by investigations with bacteriophages (Maillard and Russell, 1997), which are considered as 'indicator species' for assessing the virucidal activity of disinfectants (Davies, 1994).

The aim of the present study was to investigate the sensitivity of CCoV to different disinfectants that may be used for prophylaxis in kennels and dog breeding premises. The experiments were carried out on a canine cell line of fibroma origin, A-72, and the reference CCoV strain S378, courtesy of Professor L. E. Carmichael (Cornell University, Ithaca, NY, USA) was used throughout this study. The infectivity titre of the stock virus was $10^{6.75}$ tissue culture infectious dose$_{50}$ ($TCID_{50}$)/50 μl. The disinfectants used in the present study were three chlorides: (i) alkyl-dimethyl-benzyl-ammonium chloride (ADMBA), 2.5 mg/ml, which has a specific weight of 1.000–1.040, a pH value ranging from 4.9 to 7.0 and smells like glutaraldehyde. (ii) Benzalkonium chloride (BKC), 1.75 mg/ml, a quaternary ammonium compound (Rutala, 1996), effective against Gram-positive, Gram-negative, pleuro-pneumonia-like organisms, viruses and protozoa and is an inhibitor of algal and fungal growth. BKC is characterized by a fast action and a high penetrating power. It is neither irritating nor caustic, has no colouring or oxidizing effects and is atoxic. (iii) Didecyl-dimethyl-ammonium chloride (DDA), 5 mg/ml, with a density of 900 kg/m^3, a pH value ranging from 6 to 9 and smells like 2-propanol. To determine whether the action of the disinfectants on CCoV replication *in vitro* was related to alterations in the number of viable A72 cells, cytotoxicity assays were performed twice for each of three concentrations for each anti-viral drug. The A72 cells were cultured in a 25-cm^2 flask (Falcon; Becton Dickinson Labware, Franklin Lakes, NJ, USA) containing Eagle's minimal essential medium (EMEM) supplemented with 10% fetal bovine serum (FBS) and no drug, control flasks or a specific drug concentration. Concentrations of drugs were as follows: ADMBA: 2.5, 0.25 and 0.025 mg/ml; BKC: 1.75, 0.175 and 0.0175 mg/ml; DDA: 5, 0.5 and 0.05 mg/ml. Cells from the control flask and cells from flasks each containing one drug concentration were examined to detect morphological changes and their confluence. The cells were then harvested at different points in time (24, 48 and 72 h). The cells cultured in each drug concentration were counted twice at each point in time, and the total number of cells in each flask was calculated. The cell number for each drug concentration was expressed as the per cent reduction relative to the control flasks containing no drug. Further experiments were carried out both to estimate the toxic concentration (tc) defined as the drug concentration at which the A72 morphology changed after 1 h of absorption using a range of concentrations (starting from $1 : 10^{-1}$ to $1 : 10^{-2}$) and to estimate the effect of the drugs on CCoV infectivity *in vitro* using the $TCID_{50}$ assay. Briefly, A72 cells were cultured in a growth medium consisting of EMEM supplemented with 10% FBS in 12-well culture plates (Falcon, Becton Dickinson Labware), until approximately 75% confluence was reached. The growth medium was thoroughly decanted from each well and each disinfectant at different dilutions were left to absorb for 1 h at 37°C with gentle rocking at 10-min intervals. The disinfectants were removed and the cell monolayers were gently washed three times with EMEM. The viral suspension, 100 $TCID_{50}$/50 μl, was then dispensed on the cell monolayers. Two wells were used as controls. The well-cultured plates were incubated for 3 days and then frozen at −70°C. The samples were later analysed by $TCID_{50}$ assay using cytophatic effect as the end point. Each drug concentration and the non-treated control solution were assessed in duplicate wells.

Obvious changes in A72 morphology confluence or viability (rounding, detaching, clumbing) were observed when all the drugs were used pure and 1/10. At 1/100, decreased confluence and changes in shape were evident for DDA. For all the three agents tested, the number of A72 cells decreased as the disinfectant concentration increased. Over the 72 h, the number of A72 observed daily in the medium containing ADMBA and BKC generally ranged from 15% to 20%. The greatest mean daily reduction in the number of A72 cells during the 72-h culture period was of about 30% with DDA (data not shown). The tc was evaluated after 1-h adsorption with a concentration ranging from $1 : 10^{-1}$ to $1 : 10^{-2}$ (Table 1). Concentrations up to 1/70 of ADMBA exhibited toxic effects after about 30 min. Concentrations up to 1/90 of BKC and up to 1/100 of DDA exhibited toxic effects after 10 and 40 min, respectively. ADMBA 1/100 and DDA 1/200 were the lowest concentrations able to completely inhibit virus growth in the cell cultures. ADMBA 1/200 and 1/400 were still effective in reducing viral titres ($10^{2.50}$ $TCID_{50}$ and $10^{4.50}$ $TCID_{50}$, respectively), while no evident decrease in the infectivity titre was observed starting from a 1/600 dilution. DDA 1/600 significantly reduced the CCoV titre ($10^{4.50}$ $TCID_{50}$), but the 1/800

Table 1. Range of concentrations used to estimate the toxic concentration of each disinfectant leaving A72 monolayers complete after 1-h absorption

	Drug dilution					
Drug	a	b	c	d	e	f
ADMBA	+	+	+	+	–	–
BKC	+	+	+	+	+	–
DDA	+	+	+	+	+	+

ADMBA, alkyl-dimethyl-benzyl-ammonium chloride (2.5 mg/ml); BKC, benzalkonium chloride (1.75 mg/ml); DDA, didecyl-dimethyl-ammonium chloride (5 mg/ml).
a: 1/10; b: 1/30; c: 1/50; d: 1/70; e: 1/90; f: 1/100.
+, toxic concentration; –, not toxic concentration.

dilution showed approximately only one $\log_{10}$ reduction of the infectivity titre. BKC proved to be less effective and only a 1/100 dilution reduced CCoV infectivity, while its action on CCoV replication *in vitro* was negligible starting from 1/200 (Table 2).

Anti-viral drug discovery stemmed from research implemented in the early to mid-20th century when extensive progress was achieved in the understanding of the nature of viruses and in the development of simple tissue cultures and other growth systems. The high costs involved in drug development has narrowed down the number of viral diseases of sufficient market impact to a relatively short list. The fact that anti-viral drugs are likely to be highly specific for one single infectious agent requires that accurate diagnosis of an infection be made before instituting therapy. This further limits the number of diseases of commercial interest (Littler and Oberg, 2005). The virucidal activity of chemical compounds cannot be predicted reliably only on their mechanism of action and on the nature and morphology of the viruses to be inactivated. To date, there are no studies in the literature on the sensitivity of CCoV to chemical biocides. The present study examined the effects of ADMBA, BKC and DDA on the infectivity of CCoV by detecting morphological changes, by counting the infected cells and by $TCID_{50}$ assays. Although regarding a small number of disinfectants, the results obtained showed that the sensitivity of CCoV to disinfectants varies and the differences are dose correlated. In general, inactivation of a virus implies a permanent loss of infectivity which can be evaluated in suspension and hand disinfection tests (Bellamy, 1995). It is clear that micro-organisms can adapt to a variety of environmental, physical and chemical conditions, and it is therefore not surprising that resistance to extensively used antiseptics and disinfectants has been reported. The accumulation of point mutations, small insertions and deletions in coding and non-coding sequences is the dominant force in the microevolution of (+) RNA viruses (Dolja and Carrington, 1992). When SARS-CoV emerged, the *Coronaviridae* became the focus of great attention. The first human cases of SARS were treated empirically with corticosteroids in an attempt to reduce a virus-induced immunopathology, combined with the broad-spectrum anti-viral drugs (So et al., 2003; Tsang and Zhong, 2003). Apart from all the progress made in finding effective anti-SARS agents, one of the most important factors in the prophylaxis of the SARS-CoV infection remains the identification of appropriate disinfectant drugs for the direct control of the spread of infection. Successful inactivation of the virus allows the transfer of material from a biosafety level 3 (BSL3) to a BSL2 environment and may reduce the risk of accidental infections through unsafe laboratory practices. The findings of the present study may be applied in the study of human diseases associated with coronavirus infection. An animal model *in vitro* may help to enhance these studies by circumventing the safety problems related to the high pathogenicity of SARS-CoV. Additional studies should be performed with a wide range of disinfectants to supplement and further substantiate these preliminary results.

Table 2. Effect of three drugs, ADMBA, BKC and DDA, on CCoV infectivity in cell culture (A72) after an exposure time of 3 days

	Drug dilution						
Drug	1/100*	1/200*	1/400*	1/600*	1/800*	1/1000*	1/5000*
ADMBA	neg	$10^{2.50}$	$10^{4.50}$	$10^{6.0}$	$10^{6.0}$	$10^{6.50}$	$10^{6.50}$
BKC	$10^{3.50}$	$10^{6.50}$	$10^{6.50}$	$10^{6.50}$	$10^{6.50}$	$10^{6.50}$	$10^{6.50}$
DDA	nt	neg	$10^{3.0}$	$10^{4.50}$	$10^{5.50}$	$10^{5.50}$	$10^{6.50}$

$TCID_{50}$ was used to test the samples taking cpe as the end point.
ADMBA, alkyl-dimethyl-benzyl-ammonium chloride; BKC, benzalkonium chloride; DDA, didecyl-dimethyl-ammonium chloride; neg, negative; nt, not determined; $TCID_{50}$, tissue culture infectious $dose_{50}$.
*The viral titre is expressed as $TCID_{50}$/50 μl.

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