Canine coronavirus inactivation with physical and chemical agents

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Abstract

Canine coronavirus (CCoV) is responsible for mild or moderate enteritis in puppies. The virus is highly contagious and avoiding contact with infected dogs and their excretions is the only way to ensure disease prevention. Since no studies have yet focused on the sensitivity of CCoV to chemical biocides the present investigation examined the efficiency of physical and chemical methods of viral inactivation. CCoV infectivity was stable at +56 °C for up to 30 min, but tended to decrease rapidly at +65 °C and +75 °C. Germicidal ultra-violet (UV–C) light exposure demonstrated no significant effects on virus inactivation for up to 3 days. CCoV was observed to be more stable at pH 6.0–6.5 while extreme acidic conditions inactivated the virus. Two tested aldehydes inactivated the virus but their action was temperature- and time-dependent. The methods for CCoV inactivation could be applied as animal models to study human coronavirus infection, reducing the risk of accidental exposure of researchers to pathogens during routine laboratory procedures.
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Introduction

Canine coronavirus (CCoV), a member of the family *Coronaviridae*, is an enveloped, single-stranded, positive-sense RNA virus, responsible for enteric disease in young puppies. About two-thirds of the CCoV genomic RNA is occupied by two large, partially overlapping open reading frames (ORFs), ORF*1a* and ORF*1b*, which encode two polyproteins involved in viral replicase formation. The 3′ one-third of the genome consists of other ORFs encoding the structural proteins S, E, M and N (ORF2, ORF4, ORF5 and ORF6, respectively) as well as non-structural proteins (Enjuanes et al., 2000).

Based on phylogenetic analysis and antigenic cross reactivity, CCoV is clustered into antigenic group I together with transmissible gastroenteritis virus (TGEV) of swine, porcine epidemic diarrhoea virus (PEDV), porcine respiratory coronavirus (PRCoV), feline coronaviruses (FCoVs types I and II) and human coronavirus strain 229E

(HCoV-229E). Recently, a new genotype of CCoV was detected by polymerase chain reaction (PCR) in faecal specimens of naturally infected puppies. We designated the classical strains as CCoV type II and the new strains as CCoV type I. This new designation is not based on the chronological order of discovery of the viruses, but on the genetic identity between CCoV type II and FCoV type II and between CCoV type I and FCoV type I (nt identity 73% and aa identity 74–75%) (Pratelli, 2006; Pratelli et al., 2003a,b).

CCoV is responsible for mild or moderate enteritis. The infected dogs generally recover rapidly. Fatal outcomes are infrequent unless mixed infections by canine parvovirus type 2 (CPV2), canine adenovirus type 1 (CAdV-1) or canine distemper virus (CDV), occur (Decaro et al., 2004; Pratelli et al., 1999, 2001b; Yasoshima et al., 1983). Clinical signs in young puppies may be severe and include diarrhoea, vomiting, dehydration, loss of appetite and occasionally death. Faecal shedding of CCoV has been reported to occur for 6–9 days post-infection (Keenan et al., 1976), although PCR and nested-PCR assays have detected long-term viral shedding in infected puppies (Pratelli et al., 2001a, 2002).

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Recent investigations using differential PCR assays and sequence analysis of the M and S genes to study CCoV detected in faecal samples of puppies showed that 14.5% of CCoV infections are caused by type I, 8.7% by type II and 76.8% by both genotypes, simultaneously (Pratelli et al., 2004). CCoV is highly contagious, and once the virus has become established in the environment the spread of the infection is difficult to control. 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. Like other enveloped viruses, CCoV is inactivated by most germicidal agents such as lipid solvents, formalin, phenol, hypochlorite solution and β-propiolactone, but these substances do not prevent dog to dog transmission (Appel, 1987). The virus is acid-stable and is not inactivated at pH 3.0 and +20–22 °C (Binn et al., 1974; Appel, 1987). Like other coronaviruses, CCoV is relatively heat-stable; it can be stored for years frozen at −70 °C or lyophilized at +4 °C (Tennant et al., 1994).

In 2002, a novel human coronavirus was discovered in association with outbreaks of severe acute respiratory syndrome (SARS) in Asia and elsewhere. Complete genomic sequence analysis of several SARS-CoV isolates revealed that this coronavirus is not closely related to any of the previously characterised animal or human coronaviruses (Marra et al., 2003) and it has been demonstrated that SARS-CoV might be the first member of a newly recognized group IV (Rota et al., 2003; Snijder et al., 2003) or a distinct member of the group II coronaviruses (Gibbs et al., 2004). The spread of SARS-CoV and the severity of the highly contagious disease it causes have aroused scientific interest in coronaviruses. In order to prevent the circle of infection from widening, antiviral chemotherapy and prophylaxis have been developed (Oxford et al., 2005; Shigeta and Yamase, 2005; Tan et al., 2004).

To date, no studies in the literature have addressed the sensitivity of CCoV to chemical biocides. In the present study, the efficiency of several physical and chemical methods of viral inactivation, including those that might inhibit viral replication, was examined.

Materials and methods

Virus and cell culture

The reference CCoV strain S378 (courtesy of Professor L.E. Carmichael, Cornell University) and a canine cell line of fibroma origin, A-72, were used throughout this study. The cells were propagated in Eagle Minimal Essential Medium (EMEM) (BioWhittaker, Cambrex Bio Science) containing 10% (v/v) fetal bovine serum. Briefly, A-72 monolayer cells were infected by inoculating the cultures with 1 mL of the virus in a 175 cm^2 tissue culture flask (BD Falcon, BD Biosciences). After incubation for 1 h at +37 °C in a 5% CO$_2$ incubator, the inoculum was removed and 40 mL EMEM was added to the flask. The cells were incubated for 3 days and observed daily for cytopathic effects (CPE). After 24 h, the inoculated A-72 cells were examined by indirect immunofluorescence (IF) using CCoV monoclonal antibodies (courtesy of Dr. Gilles Chappuis, Merial) to assess viral growth.

The supernatant was then collected, clarified by centrifugation and stored at −70 °C as the viral stock. The viral titre of the A-72 cells in 96-well microtitration plates (Falcon, Becton Dickinson Labware) was determined using a 50% tissue culture infectious dose assay (TCID$_{50}$)/50 μL. Tenfold dilutions of virus were incubated at +37 °C for 4 days and examined daily for CPE. CCoV-induced CPE of infected cells was determined based on the appearance of enlarged, bizarrely shaped cells followed by focal cell detachment (Appel, 1987). The infectivity titre of the stock virus was $10^{6.75}$ TCID$_{50}$/50 μL.

Heat treatment

Physical methods for the inactivation of viruses and other microorganisms have gained widespread application. Among these techniques, heat inactivates viruses through denaturation of secondary protein structures and alteration of the viral glycoprotein involved in the attachment of the virus to the host cells (Lelie et al., 1987). To examine the ability of heat to inactivate CCoV, 500 μL aliquots of virus samples were incubated in duplicate in a 15 mL polypropylene conical tube (Falcon, Becton Dickinson Labware) for increasing periods of time at three different temperatures: +56 °C, +65 °C and +75 °C. Every 5 and/or 10 min, virus aliquots were removed and frozen at −70 °C. Each sample was then analyzed by the TCID$_{50}$ assay using CPE as an end point and titration was performed in replicates of four. All the experiments were repeated twice under the same conditions.

UVC light treatment

UV light falls within the electromagnetic spectrum with wavelengths ranging from 100 to 400 nm, which is sandwiched between X-rays and the visible light spectrum. The UV range is divided into four categories: Vacuum UV (100–200 nm), UV–A (315–400 nm) associated with sun tanning, UV–B (280–315 nm) associated with sun burning, and UV–C (200–280 nm), the range that is best absorbed by DNA and is associated with cancer, mutations and inactivation of microbes. In terms of maximum disinfection ability, the optimum UV range is between 245 and 285 nm.

To evaluate the inactivation potential of the UV–C light on CCoV, UV–C light irradiated 1 mL aliquots of virus in 24-well tissue culture plates (Falcon, Becton Dickinson Labware) on ice. The UV–C light source (Bio air instruments) was placed on the top side of the tissue culture plates, at a distance of about 4 cm from the bottom containing the virus samples. At 1 m, the UV–C light source used emitted 27.1 μW/cm^2, where μW = 10^{-6} J/s. Sample aliquots, in duplicate, were removed at fixed time points: every 1 min for 15 min, every 15 min for the next 5 h and finally every 12 h for the following 3 days. All the samples collected (a total of 40) were frozen at −70 °C and then titrated in A-72 cells using CPE as an end point. In parallel, control aliquots were kept on ice without UV–C treatment and samples aliquots were removed at the fixed time, frozen and titrated.

pH treatment

CCoV stability in the environment is extremely low, probably because of the pH levels or because of the ions and colloids present in faeces which might inactivate the virus (Pratelli et al., 2000; Tennant et al., 1994). To evaluate the effect of pH variations on the infectivity of CCoV, virus aliquots were adjusted to different pH values using alkaline solutions (1 N and 5 N NaOH) and acid solutions (1 M and 5 M HCl). Each sample exposed to alkaline (pH 7.62, pH 9.98, pH 11.09 and pH 13.2) and acid conditions (pH 2.26, pH 4.38 and pH 6.34) was divided into three aliquots and incubated in duplicate at different temperatures: +4 °C, +25 °C and +37 °C. After 1 h incubation, the samples were reversed to neutral conditions, pH 7, with a buffered solution and stored at −70 °C. Each sample was then analyzed by the TCID$_{50}$ assay using CPE as an end point.

Formaldehyde and glutaraldehyde treatments

Two aldehydes, glutaraldehyde and formaldehyde (methanol, CH_2O), are very important disinfectants. Glutaraldehyde, a dialdehyde, is supplied at an acid pH which makes it more stable but less active; it is activated by making the solution alkaline before use. As the external pH is altered from acidic to alkaline, more reactive sites will be formed on the cell surface, leading to a more rapid bactericidal effect. The cross-links thus obtained make the cell unable to undertake most, if not all, of its essential functions (McDonnell and Russell, 1999). A 2% solution is active against bacteria, spores and viruses. Glutaraldehyde is mainly used to disinfect medical instruments and, occasionally, as an inactivating agent for vaccines.

Formaldehyde is a monoaldehyde that exists as a freely water-soluble gas. Formaldehyde solution (formalin) is an aqueous solution containing ca. 34–38% (wt/wt) CH_2O with methanol to delay polymerization. Like glutaraldehyde, formaldehyde has bactericidal, sporicidal, and virucidal activity, but it works more slowly and is less effective than glutaraldehyde (Power, 1995; McDonnell and Russell, 1999), especially in the presence of protein organic matter. The reactivity of proteins with formaldehyde has been well-documented. It involves both stable and labile covalent formaldehyde linkages which occur primarily with lysine, tyrosine, histidine, and cysteine residues to form hydroxymethylamine (Jiang and Schwendeman, 2000). It has been also suggested that formalin binds to RNA, blocking RNA polymerase reading of the genome (Fraenkel-Conrat, 1954).

Formaldehyde solution, 36.5% (Riedel-de Haën) and glutaraldehyde solution, 2% (Carlo Erba Reagenti) were primarily diluted 1:10 in sterile phosphate buffered saline (PBS). Both aldehydes were then diluted to virus stock to achieve final dilutions of 1:1000 (final concentration 0.036%) and 1:4000 (final concentration 0.009%) of formaldehyde and final dilutions of 1:1000 (final concentration 0.002%) and 1:2000 (final concentration 0.001%) of glutaraldehyde. To examine formaldehyde and glutaraldehyde inactivation of CCoV, aliquots of virus and aldehyde samples, each at two different dilutions, were incubated at +4 °C, +25 °C and +37 °C, respectively, for up to 3 days. The test was repeated twice. All the samples were gently vortexed every day and an aliquot from each sample was collected and frozen at −70 °C. The samples were then titrated in A-72 cells using CPE as an end point.

Results

The effect of heat treatment on the infectivity of CCoV was evaluated at three different temperatures, +56 °C, +65 °C and +75 °C. At +56 °C a significant decrease in infectivity was achieved after 30 min, but the virus maintained its infectivity close to the limit of detection for 60 min (Fig. 1a). Treatment at +65 °C inactivated the virus after only 5 min ($10^{2.0}$ TCID$_{50}$/50 µL) and complete inactivation was reached after 40 min (Fig. 1b). At +75 °C infectivity was quite reduced after 5 min ($10^{1.0}$ TCID$_{50}$/50 µL). Some infectious virus could still be detected close to the limit of detection for the end point test after 15 min, and after 30 min the virus was completely inactivated (Fig. 1c).

Exposure of CCoV to UV–C light resulted in a weak inactivation after 6 min ($10^{6.0}$ TCID$_{50}$/50 µL) with a moderate efficiency boost after 15 min ($10^{5.5}$ TCID$_{50}$/50 µL). No additional inactivation was observed from 15 to 24 min. UV–C light at a distance of 4 cm for 24 min had no significant effects on CCoV inactivation. Prolonged exposure time for up to 3 days modified the virus titre. A substantial decrease in CCoV infectivity was observed after the first 24 h of exposure, when an infectivity titre of $10^{5.0}$ TCID$_{50}$/50 µL was registered. After further 24 h, a 100-
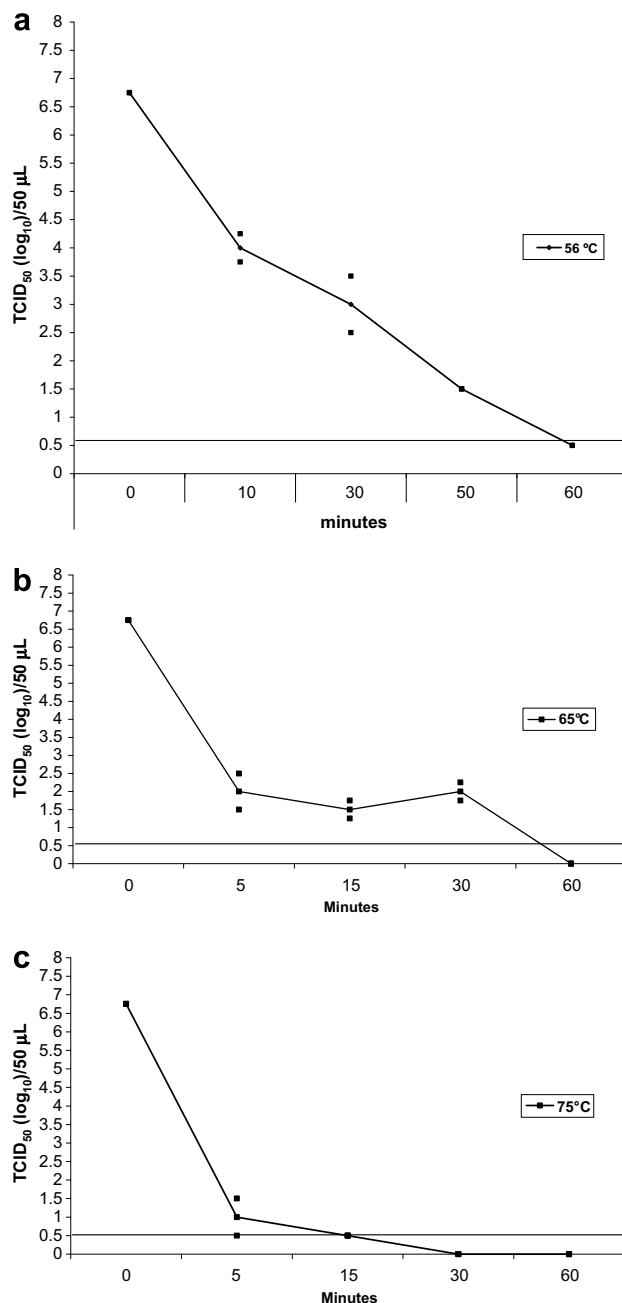

Fig. 1. Effect of heat treatment on CCoV infectivity *in vitro*. Virus aliquots were incubated at three different temperatures, +56 °C (a), +65 °C (b) and +75 °C (c), and were removed and titrated by the TCID$_{50}$ assay at fixed time points. The straight line delineates the detection limit of the test.

fold decrease was observed and on the third observation day (72 h) the virus had an infectivity titre of $10^{2.0}$ TCID$_{50}$/50 µL (Fig. 2). The control aliquots retained infectivity during the observation period and after 72 h the infectivity titre was $10^{6.0}$ TCID$_{50}$/50 µL.

After exposing CCoV to extreme alkaline conditions with pH 9.98, pH 11.09 and 13.2 for 1 h, and then suddenly neutralizing to pH 7, the virus was completely inactivated at +37 °C, while at +25 °C the aliquots exposed to pH 11.09 and pH 13.2 were inactivated and the infectivity titre
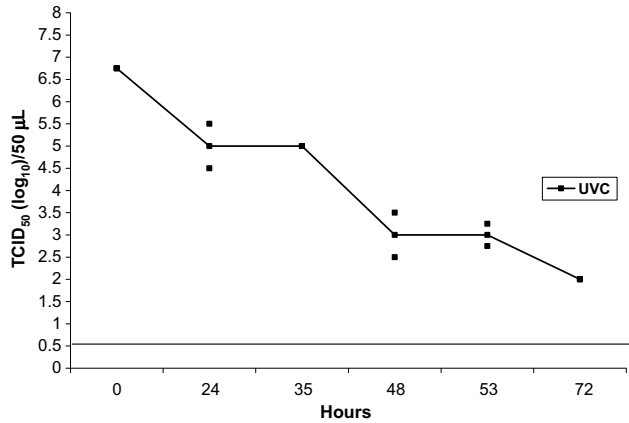
Fig. 2. Effect of UV–C radiation on the infectivity of CCoV *in vitro*. Virus aliquots were placed at a distance of about 4 cm from the UV–C source and were removed and then titrated by the $TCID_{50}$ assay at fixed time points. The straight line delineates the detection limit of the test.
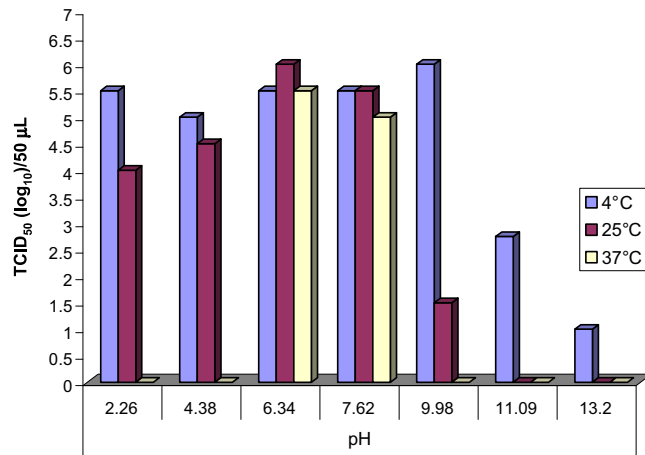
of the aliquot exposed to pH 9.98 decreased considerably to $10^{1.50}$ $TCID_{50}$/50 µL. Interestingly, at +4 °C the aliquots exposed to pH 11.09 and most of all to pH 13.2 were almost totally inactivated ($10^{2.75}TCID_{50}$/50 µL and $10^{1.0}TCID_{50}$/50 µL, respectively), while the aliquot exposed to pH 9.98 showed no significant change in infectivity titre ($10^{6.0}TCID_{50}$/50 µL). Regardless of the temperature, slight changes in the infectivity titres were observed at a pH of 6.34 and pH 7.62. Extreme acidic conditions, such as pH 2.26, and pH 4.38 completely inactivated the virus at +37 °C, but not at +25 °C or +4 °C, leaving the infectivity titres, respectively, at $10^{4.0}$ $TCID_{50}$/50 µL and $10^{5.50}$

$TCID_{50}$/50 µL (pH 2.26), and at $10^{4.5}$ $TCID_{50}$/50 µL and 10^5 $TCID_{50}$/50 µL (pH 4.38). The effects of different pH values after incubation at 4 °C, 25 °C and 37 °C on CCoV infectivity are shown in Fig. 3.

Formaldehyde, diluted 1:1000 (0.036%) and 1:4000 (0.009%), and glutaraldehyde, diluted 1:1000 (0.002%) and 1:2000 (0.001%), were incubated with CCoV at three different temperatures, +4 °C, +25 °C and +37 °C, to evaluate their virus inactivating potential. At +4 °C, formaldehyde 0.036% inactivated most of the virus after 24 h (10^2 $TCID_{50}$/50 µL) and after 72 h the virus titre was close to the limit of detection of the assay (10^1 $TCID_{50}$/50 µL). Formaldehyde 0.009% inactivated most of the virus after only 24 h (10^1 $TCID_{50}$/50 µL). By contrast, neither of the two glutaraldehyde dilutions, 0.002% and 0.001%, was able to completely inactivate the virus at +4 °C and only a reduction of the infectivity was observed (Table 1). At +25 and +37 °C, both formaldehyde dilutions inactivated most of the virus close to the limit of detection of the assay only after 24 h. Similarly, glutaraldehyde 0.002% completely inactivated the virus at +25 °C and +37 °C by day 1 and glutaraldehyde 0.001% by day 2 at +37 °C (Table 1).

Discussion

Chemical disinfectants have been widely used in human disease-control programs to prevent viral infectious diseases from spreading. The virucidal activity of chemical compounds and physical agents cannot be predicted reli-

	pH*						
	2.26	**4.38**	**6.34**	**7.62**	**9.98**	**11.09**	**13.2**
4°C	5.5/5.5	5/5	5.5/5.5	6/5	6/6	3/2.5	1/1
25°C	4.25/3.75	4.75/4.25	6/6	5.5/5.5	1/2	neg/neg	neg/neg
37°C	neg/neg	neg/neg	5.75/5.25	5/5	neg/neg	neg/neg	neg/neg

*The titres are expressed as $TCID_{50}$/50µL

Fig. 3. Effect of different pH conditions on the infectivity of CCoV *in vitro*. Virus aliquots were exposed to alkaline and acidic conditions and incubated at different temperatures: +4 °C, +25 °C and +37 °C for 1 h. After reversing to neutral conditions, the samples were titrated by the $TCID_{50}$ assay.

Table 1
Effect of formaldehyde (0.036% and 0.009%) and glutaraldehyde (0.002% and 0.001%) on CCoV infectivity *in vitro*

Aldehydes	Dilution (%)	Day 1			Day 2			Day 3		
		+4 °C	+25 °C	+37 °C	+4 °C	+25 °C	+37 °C	+4 °C	+25 °C	+37 °C
Formaldehyde	0.036	10^2	10^1	10^1	10^2	10^1	10^1	10^1	10^1	10^1
	0.009	10^1	10^1	10^1	10^1	10^1	10^1	10^1	10^1	10^1
Glutaraldehyde	0.002	10^4	$<10^1$	$<10^1$	10^3	10^1	$<10^1$	10^3	$<10^1$	neg
	0.001	10^5	10^2	10^2	10^3	10^1	neg	10^3	10^1	neg

Virus aliquots were incubated at 4 °C, 25 °C and 37 °C, respectively, for up to 3 days and then titrated by the $TCID_{50}$ assay. The detection limit for the $TCID_{50}$ assay is 10^1.

ably only on the basis of their mechanism of action and the nature and morphology of the viruses to be inactivated. Virucidal testing using standardised procedures is required.

The stability of coronaviruses at various temperatures appears to be dependent on the nature of the environmental conditions. It has been demonstrated that coronaviruses are inactivated at +56 °C in 10-15 min, at +37 °C in several days and at +4 °C in several months, while virus frozen at −60 °C survives for many years without loss of infectivity (Siddell et al., 1983). More recent studies have confirmed and completed these preliminary observations. SARS-CoV is stable at +4 °C at room temperature (about +20 °C) and at +37 °C without remarkable changes in its ability to infect cells. After exposure to temperatures of +56 °C, +67 °C and +75 °C for different periods of time it is no longer able to infect cell cultures with a severe reduction of its infectivity (Duan et al., 2003; Darnell et al., 2004; Kariwa et al., 2004). Rabenau et al. (2005) have also observed that thermal inactivation of SARS-CoV at 56 °C over 30 min was highly effective in the absence of protein; while in the presence of 20% protein (FCS, fetal calf serum) the reduction factors were only 1.93 log_{10} instead of >5.01 log_{10}.

Although anecdotal reports suggested a longer survival of CCoV in the faeces of infected dogs in the winter months (Carmichael and Binn, 1981) presumably due to lower environmental temperatures, the effect of temperature on the infectivity of CCoV was first described by Tennant et al. (1994). CCoV infectivity was retained after several days at +4 °C and for at least 2 years at −20 °C and −70 °C. However, the virus tended to lose infectivity rapidly at +37 °C and over several days at room temperature. In the present study some CCoV particles were stable for up to 30 min at +56 °C, maintaining an infectivity titre of $10^{3.0}$ $TCID_{50}$/50 μL. At +56 °C the virus remained infectious after 60 min even if at a level close to the detection limit for the assay. The virus was completely inactivated at +65 °C after 40 min and at +75 °C after 30 min. This observation suggests that pasteurization temperatures are able to inactivate CCoV.

UV-irradiation of eukaryotic cells reduces the rate of DNA synthesis. Pyrimidine dimers, the predominant UV-induced DNA lesions, appear to jeopardize the replication machinery and inhibit elongation of growing DNA strands (Rommelaere and Ward, 1982). In particular, UV–A is poorly absorbed by DNA and RNA bases; it is much less effective than UV–B and UV–C in inducing pyrimidine dimers, but it may induce additional genetic damage through the production of reactive oxygen species which cause oxidization of bases (Darnell et al., 2004). UV–B can cause pyrimidine dimer induction, but less efficiently than UV–C (Perdiz et al., 2000). UV–C is absorbed by DNA and RNA bases and can cause the photochemical fusion of two adjacent pyrimidines into covalently linked dimers (Perdiz et al., 2000).

The effect of UV irradiation on SARS-CoV has been examined by different researchers to implement infection control measures against SARS and to establish effective guidelines for the prevention of SARS outbreaks. Kariwa et al. (2004) observed that irradiation with ultraviolet light at 134 μW/cm^2 for 15 min reduced the infectivity from 3.8×10^7 to 180 $TCID_{50}$/mL; however, prolonged irradiation for 60 min failed to eliminate the remaining virus, leaving 18.8 $TCID_{50}$/mL. The studies performed by Darnell et al. (2004) revealed that UV–A light exposure of SARS-CoV for a 15 min period had no significant effect on virus inactivation. In contrast, exposure to UV–C light resulted in partial inactivation after 1 min with increasing efficiency up to 6 min and, after 15 min, the virus was completely inactivated to the limit of detection of the $TCID_{50}$ assay (<1.0 $TCID_{50}$/mL). The results of the present study are not in agreement with the findings of Darnell et al. (2004) that showed SARS-CoV inactivation by UV–C light treatment for 15 min. In our study CCoV exposure to UV–C demonstrated no significant effects on virus inactivation over a 15 min period and a 3 day exposure was needed to obtain a substantial decrease. The apparent UV–C light stability of CCoV in contrast to SARS-CoV as described by Darnell et al. (2004) may be explained by the different UV–C light intensities employed during the experimental procedures.

Several researchers have studied the influence of pH on glycoprotein conformation and the activity of some coronavirus strains. Their results differed but suggested that the S glycoprotein, which regulates several biological functions, such as attachment to cells, fusion of the viral envelope with host cell membranes and cell to cell fusion, might be sensible to pH variations (Weismiller et al., 1990; Xiao et al., 2003). Fusogenic glycoproteins of several groups of enveloped viruses, undergo pH-induced conformational changes. Alphaviruses and orthomyxoviruses infect host

cells by receptor-mediated endocytosis and penetrate the cytoplasm by fusion in acidic endosomes. This fusion appears to be mediated by an irreversible conformational change that requires a pH range of 5.0–6.0 (Kielian and Helenius, 1985; White and Wilson, 1987). Envelope glycoproteins of bunyaviruses, rhabdoviruses and murine retroviruses also exhibit conformational changes at pH 5.0–6.0 that make them fusogenic (Gonzalez-Scarano, 1985; Redmond et al., 1984). By contrast, paramyxoviruses and the human immunodeficiency virus penetrate cells at the plasma membrane directly without endocytosis and have fusogenic glycoproteins that are activated at pH 7.0 to 8.0 (McClure et al., 1988; Okada, 1962).

Mouse hepatitis virus (MHV), a member of the group II of the *Coronaviridae* family, causes cell fusion in intestinal epithelial cells during infection in the alkaline environment of the small intestine (Barthold and Smith, 1984). It therefore seems likely that the pH optimum for coronavirus induced fusion, would be neutrality or alkalinity. Pocock and Garwes (1975) analyzed the influence of pH on the growth of TGEV and its stability in adult pig thyroid cell culture. The adsorption, penetration and uncoating steps of the viral replicative cycle were shown to be unaffected by pH variations. Synthesis of TGEV RNA during the first 12 h post infection was not influenced by pH variations ranging from 6.5 to 8.0. After 12 h a breakdown of the RNA occurred in cultures kept at pH 7.2 and 8.0 but not at pH 6.5. When incubated at +37 °C for 24 h, virus infectivity was scarcely affected by pH 6.5 but when kept at +4 °C for the same length of time, it remained unchanged between pH 5.0 and 8.0. In the present study CCoV was observed to be most stable at pH 6.0–6.5 with an elevated surviving fraction in different environmental conditions. The effects of alkaline treatment were markedly different since the infectivity titre was annulled. Extreme acidic conditions, pH 2.26, inactivated the virus. It is likely that the virus maintains its infectivity only at +4 °C thanks to its considerable stability at refrigerated conditions. Taken together, these findings indicate that profound changes are induced in CCoV with variations in pH.

The biological significance of these pH-dependent changes is of great relevance, but it still needs to be correctly determined. The pH-dependent thermolability observed in MHV infectivity is the result of conformational changes in the spike glycoprotein S (Sturman et al., 1990). The virus is quite stable at pH 6.0 and +37 °C, but it is rapidly and irreversibly inactivated by a short treatment at pH 8.0 and +37 °C, during which the amino-terminal peptide S1 is released from the virions and the remaining peptide S2 remains associated with the virus envelope and forms aggregates. These modifications are presumed to be associated with virus entry and virus-induced fusion of target cells and indicate that an irreversible conformational change, i.e. a rearrangement of intramolecular disulfide bonds, has been induced in the glycoprotein by these conditions. Although it does not seem to be essential for entry and spread of the virus (Hingley et al., 1998), proteolytic cleavage of the S protein of MHV has been shown to be a prerequisite for MHV fusion but not for the Group I coronaviruses (Sturman et al., 1990).

These data suggest that different pH conditions affect the spike protein and its fusion activity (Sturman et al., 1990; Weismiller et al., 1990; Xiao et al., 2003) probably by changing the infectious nature of the viral particles. The present study provided evidence that exposure of CCoV to extreme basic or acidic conditions at +37 °C resulted in inactivation, while treatment within the neutral pH range did not. The pH of gastric secretions ranges from 1.0 to 3.5, while the secretions of the small and large intestines range from pH 7.5 to 8.0 (Guyton and Hall, 1997). All these observations suggest that entry of CCoV particles per os may result in the inactivation of most of the virions by stomach acidity. However, two important aspects need to be taken into due consideration: (1) the pH of gastric secretions might be partially neutralized by a particularly large meal or the ingestion of antacids, providing the virus with a chance to reach the slightly basic conditions of the intestines (Darnell et al., 2004); and (2) the route of CCoV infection is oro-nasal.

Formaldehyde is an extremely reactive chemical (Power, 1995; Russell and Hopwood, 1976) that interacts with proteins (Fraenkel-Conrat et al., 1945; Fraenkel-Conrat and Olcott, 1946), DNA and RNA (Fraenkel-Conrat, 1961) *in vitro*. The interaction with proteins results from a combination with the primary amide as well as with the amino groups, although phenol groups bind little formaldehyde (Fraenkel-Conrat, 1961). It has been proposed that formaldehyde acts as a mutagenic agent (Loveless, 1951) and as an alkylating agent by reaction with carboxyl, sulfhydryl, and hydroxyl groups (McDonnell and Russell, 1999). In the past, formalin was used to inactivate viruses for vaccines. Excessive treatment can destroy immunogenicity whereas insufficient treatment can leave infectious virus capable of causing disease. Soon after the introduction of inactivated polio vaccine, there was an outbreak of paralytic poliomyelitis in the USA due to the distribution of an inadequately inactivated vaccine. This incident led to a review of the formalin inactivation procedure and other inactivating agents are now available.

Glutaraldehyde is an important dialdehyde used as a disinfectant and sterilizer, specifically for low-temperature disinfection and sterilization of endoscopes and surgical equipment, and as a fixative in electron microscopy. Glutaraldehyde has a broad spectrum of activity against bacteria and their spores, fungi, and viruses, and a considerable amount of information is now available about the processes whereby these different organisms are inactivated. Reviews have been published regarding its mechanisms of action (McDonnell and Russell, 1999; Gorman et al., 1980; Power, 1995). The first reports in 1964 and 1965 (Gorman et al., 1980) demonstrated that glutaraldehyde possessed high antimicrobial activity. Subsequently, research was undertaken to evaluate the nature of its bactericidal (Munton and Russell, 1970a,b, 1972, 1973a,b;

McDonnell and Russell, 1999), and sporicidal (Gorman et al., 1984a,b; Thomas and Russell, 1974a,b) actions. Glutaraldehyde is a potent virucidal agent (Kobayashi et al., 1984). It reduces the activity of hepatitis B surface antigen (HBsAg) and especially hepatitis B core antigen (HBcAg) in hepatitis B virus (HBV) (Adler-Storthz et al., 1983) and interacts with lysine residues on the surface of hepatitis A virus (HAV) (Passagot et al., 1987). Low concentrations (<0.1%) of alkaline glutaraldehyde are effective against purified poliovirus, whereas poliovirus RNA is highly resistant to aldehyde concentrations up to 1% at pH 7.2 and is only slowly inactivated at pH 8.3. In the light of this, it has been inferred that glutaraldehyde-induced loss of infectivity is associated with capsid changes (Bailly et al., 1991).

In the present study both formaldehyde and glutaraldehyde inactivated the virus but in a temperature- and time-dependent fashion. At +4 °C formaldehyde significantly inactivated the virus after only 24 h, while the effect of glutaraldehyde was partially inhibited. At room temperature and at +37 °C, both aldehydes inactivated the virus after 24 h, even if with slight differences. Considering that formaldehyde and especially glutaraldehyde are often used as disinfectants for prophylaxis, care should be taken to establish the appropriate concentration, time and temperature to achieve and complete CCoV inactivation. Taken together, these findings demonstrated that treatment with heat, formalin, glutaraldehyde and extreme pH values are able to inactivate CCoV in different ways and timeframes. At the intensity tested, UV–C light was not sufficient to inactivate the virus after as long as three days.

The results of this study provide further information on the biological characteristics of CCoV. In late 2002, an unusual life-threatening respiratory disease, designated as SARS and caused by a novel coronavirus, spread in China (Rota et al., 2003). Rabenau et al. (2005) provided evidence that SARS-CoV is considerably more stable than the previously identified HCoV-229E, demonstrating that in a dried state, SARS-CoV retained residual infectivity even after 6 days, while HCoV-229E completely lost its infectivity within 24 h. Due to the severity of the disease, the contagious nature of the causative agent, and the considerably higher environmental stability of SARS-CoV compared to HCoV-229E, the WHO provided guidelines for working safely and recommended biosafety level 3 (BSL3) as the appropriate containment level for working with live virus (Darnell et al., 2004). Despite the differences in stability during heat treatment at 56 °C between SARS-CoV and CCoV, the several methods of CCoV inactivation, including inhibition of viral entry, could be applied as animal models to study human coronavirus infection, reducing the risk of accidental exposure to the virus through unsafe laboratory practices.

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