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Original article

Investigation on virucidal activity of chlorine dioxide. Experimental data on Feline calicivirus, HAV and Coxsackie B5

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Key words

Chlorine dioxide • Virucidal activity • Disinfection treatment

Summary

Introduction. *The aim of this study was to evaluate the efficacy of ClO_2 with regard to viruses which show a particular resistance to oxidizing agent such as HAV and Norwalk and Norwalk-like viruses, and which play an important role in the epidemiology of viral foodborne diseases.*
In the food industry, disinfection of processing systems and equipment is a very important instrument to prevent secondary contamination and to guarantee food safety. Among disinfectants, chlorine dioxide (ClO_2) presents a good efficacy at wide range of pH values, its action is rapid and generates few reaction by-products if compared to hypoclorite. Experimental studies have highlighted that ClO_2 shows a good bactericidal activity and it is also active towards viruses. Furthermore, the low concentrations and low contact times required to obtain microbial load reduction are favourable elements for the application of this compound in the industrial sanitizing practices.
Methods*. As it is impossible to cultivate the Norwalk virus in vitro, we tested the resistance of Feline calicivirus (F9 strain) vs. ClO_2, in comparison with HAV (strain HM-175) and CoxsackieB5. Chlorine dioxide was used at concentrations ranging from 0.2 to 0.8 mg/l in water solution, at pH 7 and at +20 °C. Viral suspensions were added to disinfecting solution and, at pre-set times, were sampled to undergo to titration after blocking the disinfectant action with thiosulphate 0.05 M. On the basis of the data obtained, for each virus and in relation to different concentrations, mean reduction times were calculated for 99%, 99.9% and 99.99% using the regression analysis model.*
Results*. As regards Feline calicivirus, at a concentration of 0.8 mg/l of ClO_2, we obtained the complete elimination of the viral titre in 2 min while 30 min were required at concentrations of 0.2 mg/l. Coxackie B5 showed a similar behaviour, being completely inactivated in 4 min with 0.4 mg/l of ClO_2 and after 30 min at a concentration of 0.2 mg/l. Inactivation was quicker for HAV, which was eliminated after only 30 sec at a concentration of 0.8 mg/l and after 5 min at 0.4 mg/l.*
Conclusion. *Our data show that for complete inactivation of HAV and Feline calicivirus, concentrations ≥ 0.6 mg/l are required. This observation is true for Coxsackie B5 too, but this virus has shown a good sensitivity at all concentration tested according to regression analysis results. For Feline calicivirus and HAV, at low concentrations of disinfectant, prolonged contact times were needed to obtain a 99.99% reduction of viral titres (about 16 and 20 minutes respectively).*

Introduction

Although viruses, unlike bacteria, are incapable for replicating in food, they represent an important cause of foodborne infections.
Viruses with oral-faecal transmission play an important role in the etiological factors involved, and most cases of gastroenteritis are attributed to the Norwalk virus or to the Norwalk-like virus, viruses belonging to the family of caliciviruses, and to the hepatitis A virus.
Food is exposed to risks of primary contamination when the raw materials themselves are contaminated, or secondary contamination when they come into contact with surfaces/equipment which are contaminated [1].
Thus, sanitizing the processing systems and the equipment which comes into contact with the foodstuffs is extremely important in the food industry in order to reduce the risk of secondary contamination, and represents a fundamental instrument to reduce the risk of infection linked to the consumption of foodstuffs.
Hypochlorite and chlorine dioxide (ClO_2) are highly oxidizing and are thus commonly used as disinfectants, especially when disinfecting water.
Studies carried out on potential genotoxic effects of chlorination have shown that the use of sodium hypochlorite leads to the formation of a larger number of mutagen substances than the use of chlorine dioxide [2].
As compared to other oxidizing disinfectants, chlorine dioxide offers certain advantages, such as its efficacy in a wide range of pH values and its rapid action.
If compared to hypochlorite, for instance, it has a higher selectivity and generates a smaller number of reaction products; furthermore, the bactericidal efficacy of ClO_2 is greater than that of HClO since its greater oxidizing capacity has been demonstrated.
Inactivation assays carried out under controlled experimental conditions have shown a different sensitivity towards ClO_2 on the part of various enteroviruses: at a concentration of 0.32 mg/l in conditions of neutral pH and at a temperature of 15°C, the inactivation time for

Tab. I. Reduction of bacterial load.

Microorganism	Concentration ClO_2 mg/l	Contact Times	% inactivation
Staphylococcus aureus	1.00	60 sec	99.999
Escherichia coli	0.15	300 sec	99.900
Escherichia coli	0.25	60 sec	> 99.999
Streptococcus	1.00	15 sec	> 99.999
Lactobacillus brevis	0.15	5 min	99.900
Lactobacillus brevis	1.00	5 min	> 99.999
Pseudomonas aeuruginosa	1.00	60 sec	> 99.999

99.99% was 5 min for *Echo* 7, 3 min for *Coxsakie* B3 and 7 min for the *polio*1 virus [3, 4].
The low concentrations generally required, low contact times and reduced microbial load are favourable elements promoting the use of ClO_2 in the food industry.
Table I illustrates the disinfecting activity of chlorine dioxide [5].
The aim of this study was to assess under experimental conditions the efficacy of chlorine dioxide when used with certain viruses which have proven to be particularly resistant to oxidizing agents, and which has not been throughly investigated in the literature.

Materials and methods

The following were used for the inactivation assays: the feline *Calicivirus* F9 strain grown on CRFK (feline kidney) cultures, the *Coxsakie* B5 virus grown on RC-37 (monkey kidney), and the *Hepatitis A virus* strain HM-175 grown on FRhK4 (monkey kidney embryonic) cultures.
Infected cell cultures were incubated at 37°C until the onset of the cytopathic effect, and subsequently frozen at -80°C to induce cell lysis; the contents of the flask were then submitted to ultra-filtration and re-suspended with sterile physiological solution, and finally aliquoted in sterile test tubes and stored in deepfreeze at -20°C.
The titre of the viral suspension was determined for the hepatitis A virus using the plate method and expressed as PFU, while for the other viruses it was calculated as $TCID_{50}$ (titre of the dose infecting 50% of the cell culture) according to the Reed-Muench method; we integrated the titres of 3 or 4 test for each virus and different concentrations.
Chlorine dioxide was used as disinfectant at the following concentrations: 0.2, 0.4, 0.6, 0.8 mg/l.
Viral inactivation assays were carried out at a constant temperature of 20°C with neutral pH kept constant by using a buffer solution at pH 7.

Performing the Assays

1 ml of viral suspension was added to the disinfecting solution, then aliquots of samples were collected at pre-set times – T_0,$T_{0.5}$, T_1, T_2, T_3, T_4, T_5, T_{15}, T_{30}, T_{45}, T_{60}, – and immediately placed in contact with 0.5 ml thiosulphate 0.05 M in order to neutralize the disinfectant activity.
For hepatitis A virus, we proceeded by setting up scalar 10-folds dilutions for each contact time and by inoculating them in cell cultures previously grown in 24-wells-plates (0.1 ml/well); the plates were then incubated for 2 hours at 37°C (5% CO_2). As soon as the contact time has expired, the inoculum was sucked up and a semi-solid medium was added (4% foetal calf serum, 43% MEM 2X, 43% carboxymethylcellulose). After a twelve-days incubation, the cell cultures were fixed with formalin (two hours-contact time), then washed with demineralised water and stained with Giemsa; the following day we washed away the exeeding stain and the cell lysis plaques were counted.
As regards the other viruses assayed, the 10-folds dilutions prepared for each contact time aliquot were placed in 96-wells plates (25 μl/well) previously prepared with 25 μl/well of medium and 50 μl of cell suspension has been added to each well. Finally, after an incubation at 37°C for 4 days, we detected the presence of the cytopathic effect induced by the virus.

Results

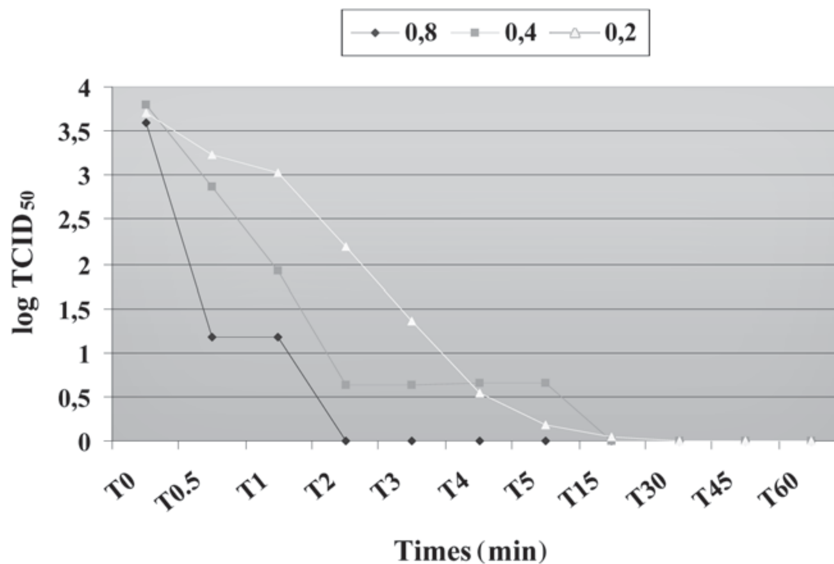
Feline calicivirus

In order to assess the trend of the inactivation assays with *Feline calicivirus*, the logarithms of the titres were calculated and the trends at various concentrations at various times are shown in Figure 1.
At a concentration of 0.8 mg/l the viral titre was eliminated after two minutes, and after thirty minutes at a concentration of 0.2 mg/l.
Mean reduction times were then calculated for 99%, 99.9% and 99.99% inactivation through analysis of regression, giving the results shown in Table II.
Table II shows that at a concentration of 0.8 mg/l a 99.99% reduction was obtained after 2.1 minutes, while 15.5 minutes were required for concentrations of 0.2 mg/l.

Coxsackie B5 virus

In this case, logarithms of the titres, as $TCID_{50}$, were calculated too, and results are shown below in Figure 2. It is possible to note that at a concentration of 0.6

Fig. 1. Trend of viral titre (log $TCID_{50}$) at different ClO_2 concentration and contact times ***Feline calicivirus***.



Tab. II. Mean reduction times for various inactivation percentages.

ClO_2 – Feline calicivirus – neutral pH			
[ClO_2] (mg/l)	Mean time (min) to obtain reduction of: 99% (CI 95%)	99.9% (CI 95%)	99.99% (CI 95%)
0.80	0.50 (0.16-0.74)	1.10 (1.05-1.26)	2.10 (2.02-2.22)
0.40	1.24 (0.98-1.52)	3.48 (3.10-3.73)	9.59 (9.16-9.81)
0.20	2.20 (1.80-2.09)	6.20 (5.90-6.90)	15.50 (15.20-15.90)

Tab. III. Mean reduction times at various inactivation times.

ClO_2 – Coxsackie Virus B5 – neutral pH			
[ClO_2] (mg/l)	Mean time (min) to obtain reduction of: 99% (CI 95%)	99.9% (CI 95%)	99.99% (CI 95%)
0.60	0.12 (0.08-0.17)	0.34 (0.27-0.45)	1.00 (0.87-1.15)
0.40	0.57 (0.41-0.79)	1.18 (0.94-1.47)	2.41 (2.20-2.73)
0.20	0.63 (0.45-0.89)	1.52 (1.18-1.95)	3.73 (3.18-4.38)

mg/l the titre was eliminated after 4 minutes, while at a concentration of 0.2 the titre was eliminated after 30 minutes.
By calculating the regression, it was possible to record the mean reduction times at 99%, 99.9% and 99.99%. Results are shown in Table III.
Results show that at concentrations of 0.6 mg/l a 99.99% reduction of the viral titre was obtained after 1 minute, while at a concentration of 0.2 mg/l it was obtained after 3.73 minutes.

Hepatitis A virus

As regards the Hepatitis A virus, the viral titre was calculated according to the PFU method. Figure 3 shows the trends of viral titres at various concentrations

At a concentration of 0.8 mg/l the titre was completely eliminated after only 30 seconds, while at concentrations of 0.4 complete elimination was obtained after 5 minutes.
Finally, mean reduction times for elimination at various percentages were calculated here, too, using the regression analysis as shown in Table IV.

Considerations and Conclusions

Chlorine dioxide is an excellent bactericidal agent, as has been reported in various studies, although its virucidal activity has not been thoroughly investigated as yet [6-12].

Fig. 2. Trend of viral titre (log $TCID_{50}$) at different ClO_2 concentration and contact times Coxsackie B5 virus.

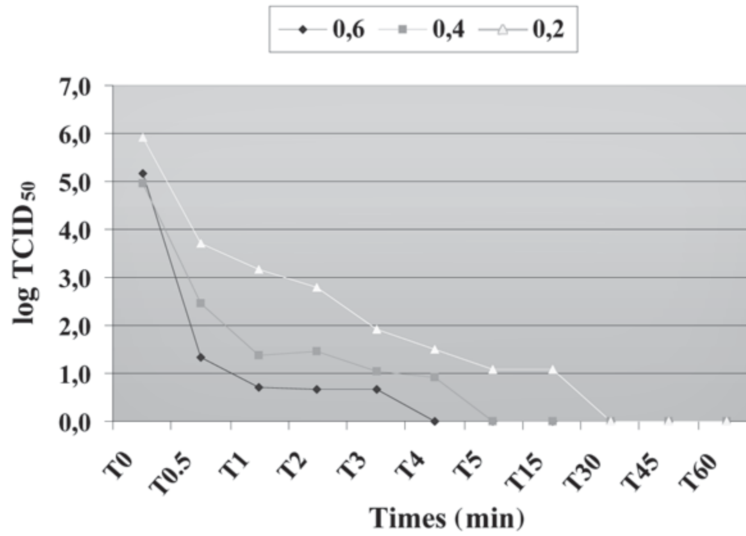
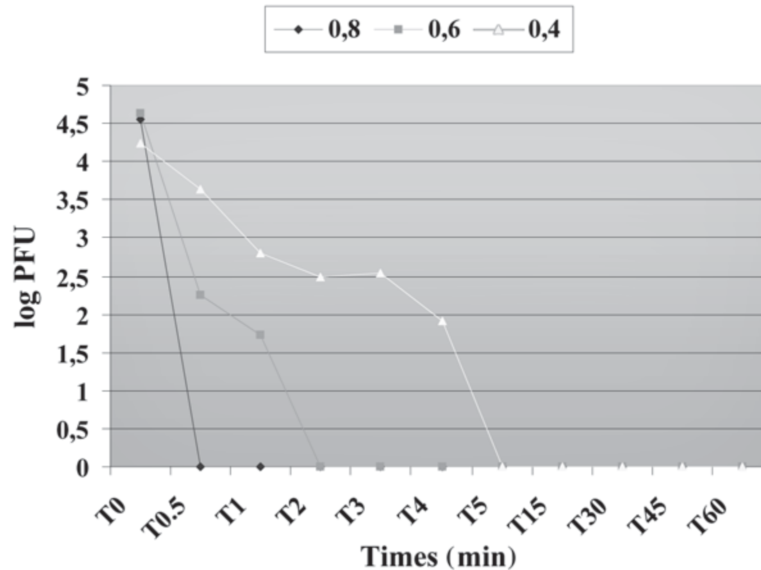


Fig. 3. Trend of viral titre (log PFU) at different ClO_2 concentration and contact times Hepatitis A virus.



The inactivation kinetics of Poliovirus 1 under experimental conditions have shown that it has a good oxidizing effect [13].

Our results have shown that it is only with concentrations greater than 0.6 mg/l that inactivation is obtained quickly for HAV and for Feline calicivirus, and only Coxsackie B5 shows great sensitivity at all concentrations assayed.

If these data are compared to the inactivation kinetics we performed on Poliovirus 1, the greater resistance of Calicivirus to Chlorine dioxide is confirmed [14]. This result is particularly important in that it confirms that the Norwalk virus, which the Feline calicivirus is a surrogate of, presents considerable resistance to hypochlorite and is also characterized by strong resistance to Chlorine dioxide. This needs to be taken into account in sanitizing procedures applied to processing systems as well as in direct disinfection treatments of foodstuffs which may be involved as carriers of this virus.

Tab. IV. Mean reduction times at various inactivation percentages.

ClO_2 – Hepatitis A virus – neutral pH			
$[ClO_2]$ (mg/l)	Mean time (min) to *obtain reduction of:*		
	99% (CI 95%)	99.9% (CI 95%)	99.99% (CI 95%)
0.80	0.26 *	0.35 *	0.43 *
0.60	0.53 (0.41-0.58)	0.85 (0.76-0.94)	1.45 (1.38-1.53)
0.40	2.35 (1.63-3.40)	6.79 (5.52-8.16)	19.58 (18.70-20.50)

* It was impossible to calculate confidence limits values because of the very short viral inactivation time.

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