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Comparative study from a chemical perspective of two- and three-step disinfection techniques to control *Clostridium difficile* spores

Richard Massicotte[1], Pauline Ginestet[2], L'Hocine Yahia[2], Gilbert Pichette[1], and Mafu Akier Assanta[3]

[1]Laboratoire d'études sur le contrôle des infections nosocomiales, Hôpital du Sacré-Cœur de Montréal, Montreal, QC, Canada.

[2]Bioperformance Analysis and Innovation Laboratory, Département du génie mécanique/ Institut de génie biomédical, École Polytechnique de Montréal, Montreal, QC, Canada.

[3]Food Research and Development Centre, Agriculture and Agri-Food Canada, 3600 Casavant Boulevard West, St-Hyacinthe, QC, Canada

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Abstract

Around the world, the presence of *Clostridium difficile* in hospital environments remains a great concern. To control the spread of this Gram-positive bacterium requires a better understanding of the various chemical interactions between the components of the bacterium and the surfaces to be cleaned. The objective of the present study was to compare, from a chemical perspective, two surface disinfection techniques and the use of two dilutions (1/20 and 1/10) of a 5% sodium hypochlorite solution. The following two cleaning-disinfection procedures were used: first, a two-step technique consisting of the use of a detergent-disinfectant (third-generation quaternary ammonium) followed by 5% household sodium hypochlorite; and second, a three-step technique consisting of the above steps plus a water rinse step between the detergent-disinfectant step and the disinfection product step. The results of the study show that the three-step technique with a 1/10 dilution of 5% sodium hypochlorite solution creates a chemical environment that provides greater disinfection potential compared to the two-step technique.

Key words

Clostridium difficile, spores, nosocomial infections, disinfection, cleaning, sodium hypochlorite, quaternary ammonium, surfaces, TOF-SIMS, hospital, sanitation.

Corresponding author

Dr Mafu Akier Assant
Food Research and Development Centre, Agriculture and Agri-Food Canada, 3600 Casavant Boulevard West, St-Hyacinthe, QC, CANADA
Email: Mafuaa@agr.gc.ca

Introduction

Controlling nosocomial infections in health-care institutions requires a better understanding of the chemical interactions between disinfection products, inert surfaces and microorganisms. This knowledge is particularly important in efforts to control bacteria such as *Clostridium difficile*. In hospital environments, *C. difficile* control requires an adequate strategy owing to the ability of this bacterium to form resistant spores in environments that are hostile to it.[1] This resistance is the result of the organism's dehydrated state and the presence of various protein layers protecting the bacterium's genetic material.[2]

The method used to eliminate this bacterium must therefore promote the reduction of the environmental load as well as the use of products that can inhibit the survival of the organism in either vegetative or spore form.

In Quebec's health system, we found two main methods for terminal disinfection (disinfection following the departure or bed transfer of a patient symptomatic for *C. difficile*): a two-step method and a three-step method. The two-step method consists of the use of a detergent-disinfectant (a quaternary ammonium [QA]–based product) followed by a 1/20 dilution of a 5% sodium hypochlorite (SH) solution.

The three-step method is characterized by the addition of a water rinse step between QA application and SH application.[3] For Quebec's health system, this rinse step represents some $15 million in annual labour costs.[4]

There is thus cause to wonder whether this rinse step truly promotes the creation of a chemical environment that provides superior disinfectant potential against *C. difficile* spores in comparison with the two-step method.

The concentration of SH solution that should be used was also questioned. The scientific literature states that the use of disinfection products that are ineffective against spores, or of subinhibitory concentrations, can be a potential source of increased sporulation.[5] According to Perez and colleagues, an oxidizing agent–based solution such as a chlorine solution at a concentration greater than 3,000 ppm is effective against the spores of *C. difficile*.[6] Other authors recommend a concentration of 5,000 ppm, such as a 1/10 dilution of 5% SH solution.[7] It should be noted that the use of QA or a detergent product as the first cleaning step is necessary owing to the poor cleansing ability of SH solution.[7]

Quaternary ammoniums are cationic surface-active agents with detergent action.[8] Once dry, however, QAs leave a film of residue on surfaces. When these ammonium residues come into contact with SH solution, they can chemically react with the solution. This reaction between the QA and the SH produces chloramine molecules (monochloramines, dichloramines and trichloramines) as well as hypochlorous acid (HOCl) molecules and hypochlorite ions (ClO^-).[9] Of these compounds, hypochlorous acid is considered to be the most effective germicidal product, followed by monochloramines (NH_2Cl). For that reason, we will use the concentration of hypochlorous acid and the concentration of monochloramines present on a surface as baseline criteria for defining an environment that could have superior germicidal potential.

The objective of the present study was therefore to chemically compare two surface disinfection techniques and the use of two dilutions of a 5% SH solution.

Materials and Methods

Test surfaces

Three common surface types found in hospital environments, namely glass (borosilicate cover slips for optical microscopy, VWR International Inc., West Chester, PA), melamine (Bel-Trim, Canada) and Arborite, also known as Formica (Compagnie Bélanger, Quebec, Canada) were used. Each surface measured 22 × 30 mm length.

Disinfection products

The commercial disinfection products used in the present study including alkyl dimethyl benzyl ammonium chloride (QA) and sodium hypochlorite (SH) with an initial concentration of 5%. Those two products are commonly utilized in Quebec's health system.

Preparation of surfaces (control surfaces to which products were not applied)

Using surfaces to which products were not applied made it possible to determine the main chemical components of the surfaces and thus to make distinctions when the products were applied to each type of surface. To ensure that the surfaces were representative of hospital environments, no particular treatment was applied. Throughout the experiment, the surfaces were handled by the edges with bare hands.

Preparation of chemicals

To represent the actual situation in hospitals environment, the Quat and SH solutions were prepared with water from the municipal water supply system of the city of Joliette. That water was not analyzed. For all the trials, the water was collected in the same sampling operation and was used quickly to limit differences in the concentration of any ions that may have been present.

In all the trials, the products were applied to the surfaces with a cotton cloth (Compagnie de produits Sany, Joliette, QC, Canada).

Test procedures

Quaternary ammonium

A Quat solution (standard 1/62 dilution as recommended by the manufacturer) was applied with a cotton cloth soaked in the product to each surface under study (glass, melamine or Arborite). To insure that the test surfaces were properly wetted, the cotton cloths were wiped across the surfaces two times. Then, everything was left to air-dry for at least 10 min before analysis with time-of-flight secondary ion mass spectrometry (TOF-SIMS) on an ION-TOF SIMS IV instrument (ION-TOF GmbH, Münster, Germany).

A commercial (5%) SH solution at a concentration of 1/20 (2,500 ppm) was applied with a cotton cloth soaked in the product solution to each surface (glass, melamine or Arborite). To ensure wettability of the inert surfaces, the cloths were wiped across the surfaces two times. Everything was left to air-dry for 10 min before analysis with TOF-SIMS, as described above. Then, step 3 was repeated using a 1/10 dilution of 5% SH solution (5,000 ppm).[7] That concentration is recognized as sporicidal by manufacturers.

With both chemical products under the study, the tests of surface cleaning-disinfections were made according to methods of two-step and the method of three-step.

Two-step cleaning-disinfection

In this part, a Quat solution (1/62) was applied with cotton cloths to the glass, melamine and arborite surfaces. To ensure better wetting of the inert material, the cotton cloths soaked in the chemical solution were also wiped across the surfaces two times before the surfaces were left to air-dry for 10 min.

For the SH, solution was applied with cotton cloths to the surfaces to which the QA had been used. The SH solution at the concentrations of 1/10 (5,000 ppm) and 1/20 (2,500 ppm) were used. In all cases, the cloths were wiped across the surfaces two times to ensure proper wettability before surfaces were left to air-dry for 10 min to be analysed with TOF-SIMS.

Three-step cleaning–disinfection

For this step, a Quat solution was applied with cotton cloths to the surfaces of glass, melamine and arborite. The cloths were wiped across the surfaces two times to ensure better wettability before everything was left to air-dry for 10 min. Then, the surfaces were cleaned with cotton cloths soaked in warm tap water. The wet cloths were wiped across the surfaces two times before everything was left to air-dry for 10 min.

For SH, a solution 1/20 dilution (2,500 ppm) and 1/10 (5,000 ppm) were applied with cotton cloths to the surfaces as described previously before air-drying the substrata for 10 min. In both cases, Spectroscopy of Mass of secondary Ion in time of Theft (TOF-SIMS) was used.

Measurement of the main chemical components found on the surfaces

The TOF-SIMS instrument available at the Bioperformance Analysis and Innovation Laboratory of the Université de Montréal's École Polytechnique de Montréal was used.[11,12] The measurements were taken with an ION-TOF SIMS IV system (ION-TOF GmbH, Münster, Germany). The system pressure was

5×10^{-9} torr. The source was monatomic and consisted of a pulsed beam of gallium 69. The mass/charge ratio of the detected ions was measured.

A voltage of 15 V and a current of 0.5 µA were used to remain in static mode. The surface was therefore not modified by the bombardment, and the lifetime of a monolayer is a few hours, so in dynamic mode the surface is broken in a few nanoseconds and the current is 1 A/cm^2. A dose less than 10^{11} ions/cm^2 had to be respected. The verified parameters were as follows: a current of 2.0 pA, a hydrogen resolution less than 0.8, and a silicon resolution greater than 8,000. The primary ions were pulsed at a width of 30 ns and a current of 2.0 pA with a primary ion dose less than 5×10^{11} ions/cm^2, which was well below the threshold of 1×10^{13} ions/cm^2 required to remain in static mode. Electrostatic charges were neutralized by a low-energy electron source. The secondary ion spectra were acquired on a surface area of 40 × 40 µm with 128 × 128 pixels (one pulse per pixel), on only one position per sample. The measurements were conducted at both polarities, i.e. positive (SIMS+) and negative (SIMS–).

The measurements were conducted in three places for each treated surfaces and the control. The spectres of three measures were compared to standard spectres. The most closer spectre to the standard spectre was considered and used as spectre for the identification and quantification. It is about a semi-quantitative measure of the concentration present.

The first step consisted of analyzing the chemical composition of the three surface types under study. In the second step, the surfaces were characterized individually, first with SH and second with QA only. With the results that were obtained, a table to distinguish between the elements present and those not present on the control sample was created. The elements that were present therefore characterized the substance or its interaction with the surface. In keeping with the objective of the present study, the emphasis was placed on products recognized for their role as bactericidal or sporicidal agents. All the ions of interest to this study were of negative polarity. For that reason, the results obtained by positive SIMS were not considered.

The ions that were retained were hypochlorite ions (ClO^-) and hypochlorous acid (HOCl) because they are characteristic of the oxidizing (ClO^-) and bactericidal (HOCl) power of SH and are thus evidence of its activity. The bactericidal effect of hypochlorous acid is due to its similarity to the water molecule, a characteristic that facilitates the penetration of hypochlorous acid inside bacterial cells. It should be noted that the method that was used did not allow us to evaluate the hypochlorous acid ion evaporation rate.

The results of the quantification of the number of ions were normalized using the number of detected Cl_2 ions as a reference. That choice is justified because this molecule is shared by the prepared SH and QA solutions. The preparation of these products generally involves dilution with water from the municipal water supply system, water that contains Cl_2 ions.

After calibration, the molecules of interest were identified from their molecular masses. For the control substrates, the same molecular masses were explained by other combinations of elements of the periodic table selected on the basis of prior knowledge of the chemical composition of the substrates. With the software supplied with the instrument, the areas under the peaks were calculated by integration of the region delimited by the curve of the peak.

At the end of that integration, we obtained the number of molecules at the spot where the peak appeared. The parameter responsible for this quantification was designated (Int), which means integral. The molecules of interest Cl_2, ClO, ClOH, NClH, $NClH_2$ and NCl_3 were identified at positions 51.9722, 50.9620, 51.9722, 49.9717, 50.9839 and 118.9152 amu, respectively.

Results

The results of the semi-quantification of the different molecules of interest are in Table I, which show major variations in the concentrations of the main components under study. Those concentrations varied depending on the nature of the surface, the technique that was used, and the SH concentration that was applied. It can been seen that hypochlorous acid was found in higher concentrations on glass (Table I) than on the other two surfaces, particularly with the three-step technique (Table I).

For all the surfaces, we observed that the chlorinated ions were in higher concentration after using the three-steps in comparison with a two-step technique (Figure 1).

However, it is difficult to identify from this Table any clear trend showing which of the techniques and SH concentrations promote the creation of an environment that could be effective against *C. difficile* spores. To compensate for this problem, we created a figure grouping regrouping the results for the two main compounds recognized for their germicidal effect (Figure I). We can see from that table that hypochlorous acid was clearly promoted by the use of a three-step technique whatever the surface type. With respect to monochloramines, the results varied depending on the chemical nature of the surface.

Discussion

To determine which of the two techniques and the two SH concentrations would create a chemical environment that theoretically provides optimal conditions for effective impairment of *C. difficile* spores, we studied the quantities of hypochlorous acid (HOCl) and monochloramines (NH_2Cl) present on the surfaces. We used these two compounds because of their importance as bactericidal agents. In this case, the issue was achieving a situation in which the chemical equilibrium on the surface promotes a high concentration of hypochlorous acid in order to impair the protective layers composing the spore. Hypochlorous acid is a powerful oxidizing agent compared to monochloramines.

Germicidal effect of monochloramines and hypochlorous acid

The germicidal effect of products is linked in large part with the presence of negative electrical charges that occur in varying density on surfaces exposed to microorganisms and viruses.[13] In a basic environment, monochloramines are positively charged and bind to the negatively charged sites on the cell membrane, leading to tensions on the membrane and thus the death of the cell.[14] Monochloramines are recognized as having a valuable disinfection ability compared to the other two types of chloramines.[15]

The effectiveness of hypochlorous acid is linked with the absence of an electrical charge as well the molecule's chemical form, which resembles that of water. The cytoplasmic membrane allows this molecule through along with water.[16] Once inside the cell, hypochlorous acid blocks enzymatic activity, affecting the synthesis metabolism of the bacterium.[16] Hypothetically, however, the dehydrated state of the spore and the various layers that compose it could have an adverse effect on the ability of hypochlorous acid ions to penetrate the membrane to act on the genetic material. According to Fuzukaki (2006), the concentration of OH^- ions plays a role mainly in the destruction of the protein membranes of *C. difficile* spores.[16] That membrane destruction facilitates the penetration of hypochlorite $(ClO)^-$ ions, which induce lysis of the spore.[16] Without that destruction of the membranes, the negatively charged hypochlorite ions would be repelled by the anions in the membranes.[13]

Impact of the chemical nature of the surfaces

The interaction between the chemical composition of a surface and the nature of a cleaning or disinfection product in terms of the effectiveness of disinfection appears to be a neglected aspect in both hospital and agri-food environments. In general, studies have been limited to the impact of disinfection products on the reduction of the bacterial populations present on various surfaces. The chemical interactions between the surface and the disinfection product are addressed little if at all, as evidenced in the study by Snyder.[18] In the present study, we were interested mainly in that aspect.

We can see from our results (Table I) that the chemical reactions did not occur solely between SH and QA; indeed, the chemical reaction may have involved a third, not insignificant component, namely the chemical composition of the surface. The concentrations of the various products that were formed appear to be the result of the establishment of a thermodynamic equilibrium between the products that were present and the nature of the surface to which the products were applied. The cleaning and disinfection of a surface can therefore be changed by the nature of the surface.[17] For example, we see that we have more HOCl and minus of NH_2Cl on glass if we compare with melamine surface. However, the opposite effect is seen on melamine, whose chemical name is 1,3,5-triazine-2,4,6-triamine and whose empirical chemical formula is $C_3H_6N_6$.

Table I. Number of molecule by treatment and surfaces

Reactions	Molecules	Surface+Quat	Surface + SH	Two-steps	Three-steps
		Number of molecule	Number of molecule	Number of molecule	Number of molecule
Quaternary ammonium (Quat) and a 1/20 dilution of sodium hypochlorite (SH) on glass	NH_2Cl	840	60	277	122
	$NHCl_2$	164	4,86	190	22,05
	NCl_3	65,59	20,58	301	68,5
	ClOH	106	110	441	806
	ClO	600	633	744	1849
	Cl_2	100	100	100	100
Quaternary ammonium (Quat) and a 1/10 dilution of sodium hypochlorite (SH) on glass	NH_2Cl	840	142	97,85	98,83
	$NHCl_2$	164	30,93	23,16	12,75
	NCl_3	65,59	27,84	91,63	38,41
	ClOH	106	176	273	607
	ClO	600	76,29	1 107	1 035
	Cl_2	100	100	100	100
Quaternary ammonium (Quat) and a 1/20 dilution of sodium hypochlorite (SH) on melamine.	NH_2Cl	4300	204	447	453
	$NHCl_2$	100	9,78	59,38	41,18
	NCl_3	100	13,04	34,38	35,29
	ClOH	1500	117	303	335
	ClO	900	451	481	247
	Cl_2	100	100	100	100
Quaternary ammonium (Quat) and a 1/10 dilution of sodium hypochlorite (SH) on melamine.	NH_2Cl	4300	151	389	827
	$NHCl_2$	100	6,17	37,04	127
	NCl_3	100	1,23	7,41	63,64
	ClOH	1500	50,62	193	436
	ClO	900	548	478	1 127
	Cl_2	100	100	100	100
Quaternary ammonium (Quat) and a 1/20 dilution of sodium hypochlorite (SH) on Arborite.	NH_2Cl	3 667	25,85	36,86	142
	$NHCl_2$	767	1,18	2,85	30,93
	NCl_3	75	0,9217	1,74	27,84
	ClOH	592	17,24	18,36	176
	ClO	808	136	163	76,29
	Cl_2	100	100	100	100
Quaternary ammonium (Quat) and a 1/10 dilution of sodium hypochlorite (SH) on Arborite.	NH_2Cl	3667	30,32	21,15	42,81
	$NHCl_2$	767	0,977	2,12	1,68
	NCl_3	75	1,65	0,34	18,76
	ClOH	592	12,88	4,81	17,4
	ClO	808	120	83,84	185
	Cl_2	100	100	100	100

The presence and availability of nitrogen in the chemical formulation appear to be behind the different results obtained for melamine compared with those for glass. The nitrogen contained in the composition of the surface appears to participate in the formation of chloramines in the presence of chlorine. That could explain the increase in chloramines, particularly monochloramines, on the melamine and Arborite surfaces in spite of the water rinse step (see Figure I). It is possible that this concentration of monochloramines participates with SH in the creation of an environment conducive to the impairment of spore membranes on these surface types. However, these chemical reactions can also impair the physical integrity of melamine and Arborite surfaces, over time making it difficult to achieve effective cleaning and disinfection. That difficulty may be due to greater surface roughness as well as modification of the electrostatic interactions that can promote the adhesion of bacterial cells to surfaces.[19,20,21,22]

The rinse step therefore plays an important role in the creation of an environment that is potentially effective against *C. difficile* spores. Five roles could hypothetically be attributed to the rinse step with our results, as follows:

- Hydration of the spore, which could facilitate interactions with the hypochlorous acid and thus improve its effectiveness in impairing the spore's membranes.
- Through the elimination of organic residues, creation of an environment with superior disinfection potential compared with the environment created by the use of the two-step technique.
- Increase in disinfection potential and limiting of the creation of sublethal concentrations that could induce sporulation in *C. difficile* bacteria.[9]
- Application of additional mechanical action that helps eliminate spores.
- Limiting of the rate of surface deterioration.

Conclusion

The objective of the present study was to chemically compare two surface disinfection techniques as well as the use of two dilutions of a 5% SH solution. By

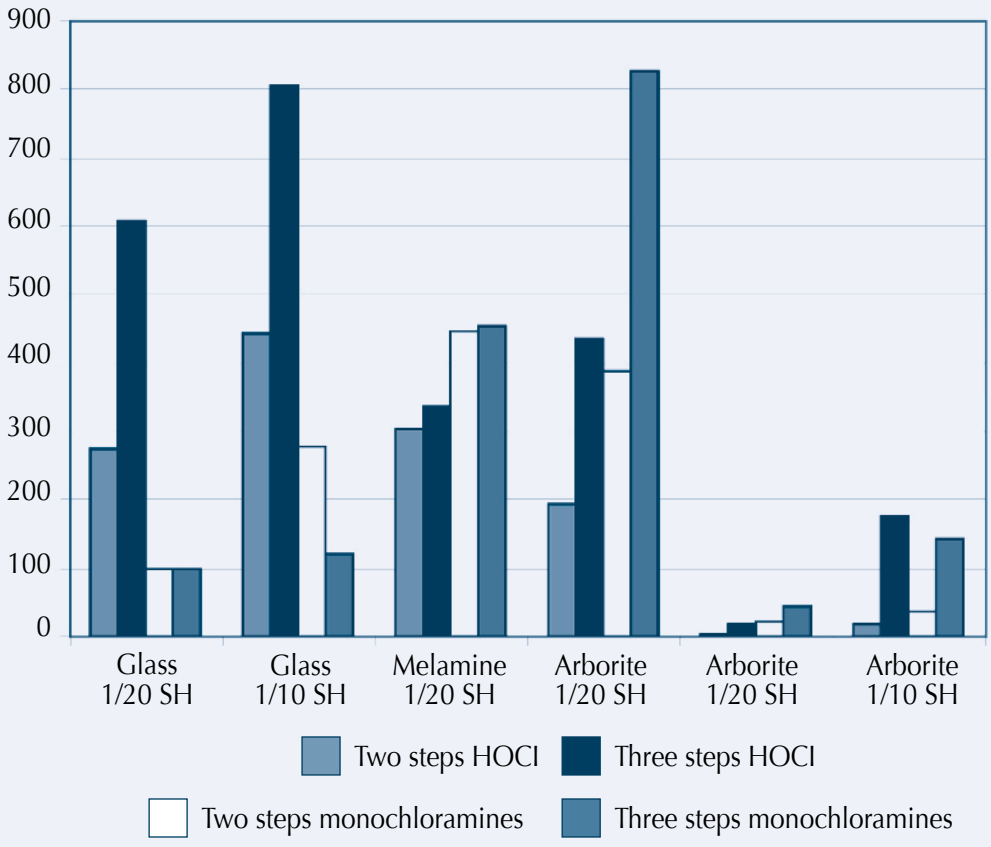

Figure 1: Quantity of molecules found on surfaces used in respect to the various applied treatments

means of TOF-SIMS, we were able to determine that the SH reacted with the QA residues. We also observed that chemical interactions with the surfaces could occur, leading to the formation of chloramines. That chloramine formation involves hypochlorous acid ions, which are thus no longer available to participate effectively in the inactivation of *C. difficile* spores. The issue is therefore the use of a technique and a SH concentration that promote an environment that theoretically provides effective sporicidal potential.

According to our results, this type of environment is obtained with SH at a concentration of 1/10 (5,000 ppm) and a three-step technique. A study is currently under way to verify whether the chemical results can be transferred to *C. difficile* spores.

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