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SARS-CoV-2 RNA viability on high-touch surfaces and evaluation of a continuous-flow ozonation treatment

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Key words: SARS-CoV-2, ozone, surfaces, virus, decontamination, disinfection
Parole chiave: SARS-CoV-2, ozono, superfici, virus, decontaminazione, disinfezione

Abstract

Background. *The COVID-19 emergency has highlighted the importance of prevention systems and environmental microbiological monitoring as fundamental elements in the response to epidemics and other such threats to individual and collective health. The use of automated "No-touch" room disinfection systems eliminates or reduces the dependence on operators, thus allowing an improvement in the effectiveness of terminal disinfection.*
Study design. *In the present study, we focused on possible SARS-CoV-2 contamination of surfaces of commercial services, and the effectiveness of ozone treatment on the virus.*
Methods. *Analyses were conducted on 4-7 October and 27-30 December 2021 in four supermarkets in an Apulian city; supermarkets A and B were equipped with an ozonisation system, while C and D were without any environmental remediation.*
Results. *SARS-CoV-2 RNA was detected by real-time RT-PCR only in December, in 6% of the surfaces tested, and all examined samples were found to be negative after viral culture, since no cytopathic effect was observed. A statistically significant difference emerged from the comparison of October vs. December ($p = 0.0289$), but no statistically significant difference ($p = 0.6777$) emerged from the comparison between supermarkets with and without the ozonisation system.*
Conclusions. *Although no important changes were observed by treating the environments with ozonisation systems, further studies are needed to validate the effectiveness of environmental treatments with airborne disinfectants.*

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Introduction

Interest in the degree of microbial contamination of surfaces has increased significantly during the COVID-19 pandemic, compared with the past. The COVID-19 emergency has highlighted the importance of prevention systems and of environmental microbiological monitoring as fundamental elements in the response to epidemics and other such threats to individual and collective health (1-3). Although the risk of infection from contact with a contaminated surface has been estimated to be relatively low (4-6), virus-contaminated surfaces may play a role in indirect transmission (7-9).

The SARS-CoV-2 outbreak has highlighted the need to develop methods for environmental decontamination, particularly those applicable to healthcare settings. The use of automated "No-touch" room disinfection systems eliminates or reduces the dependence on operators, thus allowing an improvement in the effectiveness of terminal disinfection (3). Among the different systems currently used, the most common are hydrogen peroxide aerosol systems, H_2O_2 vapour systems and ultraviolet C radiation systems (3).

Ozone generators have recently assumed an important role due to the efficient penetration of ozone into inaccessible places (1) and their effectiveness on bacteria, fungi and viruses (10). In particular, ozone is known for its viricidal activity, as it affects the proteins of the viral envelope, inhibiting their entry into host cells (11).

Ozone gas derives from an unstable triatomic oxygen molecule (O_3) which rapidly degrades to a stable state (O_2), carrying hydroxyl radicals as secondary oxidants with high reactivity and short reaction time. However, the application of this gas is limited by its toxicity; therefore, treatments with high ozone concentrations can only be applied in the absence of operators (12-14).

Studies prior to the COVID-19 pandemic demonstrated the efficacy of ozone against viruses that can be considered surrogates for SARS-CoV-2, such as MHV (mouse hepatitis virus) and Phi6 (1, 15-18).

We have conducted other studies on environmental contamination by SARS-CoV-2 during the COVID-19 pandemic (4, 19, 20). We checked the surfaces of various community environments, particularly in periods of reduced mobility due to the COVID-19 restrictions, finding viral RNA in almost all of the environments examined. In addition, we previously evaluated the effectiveness of an ozonisation system on the presence of bacteria and fungi in the air and on the surfaces of supermarkets (21). Therefore, in the present study, we focused on the possible SARS-CoV-2 environmental contamination and its viability particularly on surfaces touched by the users of commercial services, and the virucidal effectiveness of a continuous ozonation treatment.

Materials and Methods

1. Study design

During 2021, Italian regions were classified into one of four levels - white, yellow, orange, and red - as a function of the growing risk of SARS-CoV-2 spread, according the directive of the Istituto Superiore di Sanità, based on symptomatic COVID-19 patients, hospitalizations, new outbreaks, occupied hospital beds, and deaths owing to COVID-19.

The present study was conducted on October 4th, 5th, 6th and 7th, 2021 and December 27th, 28th, 29th and 30th, 2021, when the Apulia region in Southern Italy was at a white level, so there were no restrictions on mobility. In addition, the number of subjects positive for COVID-19 (molecular survey on nose-pharyngeal swab) was calculated for the sampling days.

Within an Apulian city, 16 supermarkets had similar characteristics in terms of brand, size of the structure (about 200 square meters) and maintenance methods. Of these 16, 2 were equipped with an ozonisation system (hereinafter referred to as A and B). Two additional supermarkets (hereinafter referred as C and D) were randomly selected from the 14 remaining supermarkets, without an ozonation system or other environmental remediation intervention (disinfection / sanitization systems). In supermarkets not equipped with an ozonation system, the sanitizing activities adopted by the Food Business Operator (FBO) were manual, included in the procedures provided for by the FBO's own checks on the principles of the HACCP system. Therefore, specifically types of retail outlets, cleaning and sanitizing activities were carried out daily directly by employees who work in the retail trade or by an employee of any external company enrolled for this duty.

Moreover, all the analysed supermarkets (A-B and C-D) were among the most popular in the Apulian city.

It was planned to sample the surfaces that are the object of greatest contact by users.

Therefore, the most frequented areas were verified within each supermarket and all points within these areas were sampled. In particular, 2 scales for self-service, 6 refrigerator handles, 7 shopping trolley handles and 10 cash register keyboards used by cashiers and POS keyboards were sampled. Overall, in accordance with previous studies (4), 25 surfaces were sample in each supermarket, for a total of 200 samples (100 in October and 100 in December).

The ozonisation systems produced 2.64 g of ozone/hour through two or three generators (model GX, Ozotek®, Taranto, Italy), with closed corona discharge reactors, placed in the suspended ceiling of the sales area. Overnight, in the absence of staff or customers, ozonisation cycles were carried out for 2 hours and 30 minutes. During the opening hours, microcycles of 10 minutes were carried out every 2 hours, within the period from 8:00 am to 6:00 pm.

The sampling was carried out during periods of high turnout (between 10:00 a.m. and 12:00 p.m.).

2. Environmental Sampling

Sampling was carried out using sterile swabs (Easy Surface Checking (ESC)–Neutralizing Rinse Solution (NRS); Liofilchem Srl, Roseto degli Abruzzi, Italy) inserted into a plastic tube containing 10 mL of transport medium. Flat and wide surfaces were buffered on a 10×10 cm area, using a delimiter, while smaller and curve surfaces were buffered on the available area. The swabs were transported to the laboratory at a controlled temperature (+4 °C) in an isothermal refrigerator for immediate processing.

3. Molecular Analysis

Swabs were vortexed for 20 s and transferred under sterile conditions to a new 15 mL tube for the detection of SARS-CoV-2 by real-time reverse-transcription polymerase chain reaction (RT-PCR), according to previous studies (4, 20, 22-24).

Nucleic acids were extracted from 5 mL of NRS medium using the NucliSENS miniMAG semi-automatic extraction system with magnetic silica, according to the manufacturer's instructions (bioMérieux, Marcy-l'Etoile, Lyon France), the RNA was resuspended in 100 µL of elution buffer, and the extracts were kept at −20 °C. ORF-1ab gene (nsp14) was amplificated using a 25 µL mixture composed of 12.5 µL of 2 × reaction buffer supplied with AgPath-ID™ One-Step RT-PCR Reagents (Applied Biosystems ™, Thermo Fisher, Waltham, MA, USA); 1 µL of 25× RT-PCR enzyme mix; 1 µL of forward primer (12.5 µM); 1 µL of reverse primer (22.5 µM); 1 mL of probe (6.25 µM); 1.83 µL of nuclease-free water (not DEPC-

treated); 1.67 µL Real-Time PCR Detection Enhancer (Applied Biosystems ™, Thermo Fisher, Waltham, MA, USA); and 5 µL of RNA for each sample. The primer and probe sequences used were as follows: CoV-2-F/ ACA TGG CTT TGA GTT GAC ATC T; CoV-2-R/AGC AGT GGA AAA GCAT GTG G; and CoV-2-P/FAM-CAT AGA CAA CAG GTG CGC TC-MGBEQ (4, 19, 22). The thermal cycling conditions were as follows: a reverse transcription phase (50 °C for 30 min), the inactivation of the RT phase (95 °C for 10 min) and 45 amplification cycles (95 °C for 15 s and 60 °C for 45 s). Cycle threshold (Ct) cut-offs were used as indicators of the SARS-CoV-2 RNA copy number in samples and a cycle cut-off value < 40 was interpreted as positive for SARS-CoV-2 RNA.

The experiments were conducted in duplicate using the CFX96 Touch Deep Well Real-Time PCR System (Applied Biosystem ™, Thermo Fisher, Waltham, MA, USA).

4. Virus isolation

The Vero E6 cell line (African green monkey kidney cells) was used for SARS-CoV-2 isolation (25). Cells were cultured in Eagle's minimal essential medium (EMEM) (Life Technologies, Carisbad, CA, USA) supplemented with 10% (vol/ vol) foetal bovine serum (FBS) (Life Technologies, Carisbad, CA, USA), and 100 U/mL penicillin and streptomycin (Life Technologies, Carisbad, CA, USA).

The virus isolation from swabs was conducted as previously described (26).

Briefly, cells were plated into a 25 cm^2 cell culture flasks (Corning, CLS430168) at a confluency of 70–80% in 6 mL EMEM with 6% FBS and incubated overnight at 37 °C. The following day, 1500 µL of the swab medium was incubated with 500 µL of an antibiotic solution (2000 U/mL of penicillin/ streptomycin and 300 U/mL of neomycin) for 1 h at room temperature. The suspension was then inoculated on the monolayer of the VeroE6 cells. The flask was incubated at 37 °C for 1 h.

After incubation, 4 mL of EMEM with 6% foetal bovine serum (FBS) was added and incubated again at 37 °C for 72 hours. The EMEM 6% FBS was replaced every 72 hours in order to maintain the vitality of cells. The infected cell cultures were observed every day for up to one week and the result was defined on the basis of the presence/absence of a cytopathic effect by observation with an inverted microscope (Eclipse TS2-FL, Nikon, Tokyo, Japan). All of the procedures that involved handling the SARS-CoV-2 and infected cell cultures were held in a BSL-3 laboratory, following the laboratory biosafety guidelines.

5. Statistical analysis

We carried out statistical analyses using Fisher's exact test for the comparison of October vs. December, and the presence of an ozonisation system vs. the absence of an air-diffused remediation system.

In both types of analysis, p-values < 0.05 were considered statistically significant. We used R version 3.6.3 in the statistical analysis (The R Project for Statistical Computing, Vienna, Austria).

Results

SARS-CoV-2 testing was always negative for samples taken in October. In December, the virus was detected in 6% of the surfaces tested, i.e., POS keyboards used by customers (n = 4) and refrigerator handles (n = 2) (Table 1). The mean Ct was 37.76, with a median Ct of 37.72 (range 37.29–38.20). All examined samples were found to be negative after viral culture, since no cytopathic effect was observed in any sample.

A statistically significant difference emerged from the comparison of the two analysis periods (October vs. December) (p = 0.0289, Fisher's exact test).

Table 1 - Swabs performed in four supermarkets with/without ozonisation system, resulted positive for research of SARS-CoV-2. The sampling was carried out in October and in December

Examined supermarket	Positive Surface swabs/period (No)	
Positive Surface	October	December
A - B With ozonisation system		
POS keyboards	0	1
Refrigerator handles	0	1
B - C Without ozonisation system		
POS keyboards	0	3
Refrigerator handles	0	1

With reference to positive subjects for COVID-19 in the Apulian region, 427 cases were recorded on October 4-7th 2021, while 9,804 cases were recorded on 27-30 December 2021. In particular, in the province of the city involved in the analysis, in the period between the 4th and 7th of October 2021, 109 subjects were found to be new SARS-CoV-2-positive cases vs. 3,032 new SARS-CoV-2-positive cases in the period between 27th and 30th of December 2021 (27).

Referring to the results obtained in December, the comparison between supermarkets with and without an ozonisation system did not reveal a statistically significant difference ($p = 0.6777$, Fisher's exact test), although the presence of the virus was detected on two surfaces in supermarkets equipped with an ozonisation system and on four surfaces in those without a remediation system.

Discussion

Our study highlights two noteworthy aspects: i) the increased presence of SARS-CoV-2 in December; and ii) no significant difference between the environmental contamination detected in an environment treated with ozone and one without an ozonisation system.

Both the first and the second aspect are consistent with the increase in positive cases for COVID-19 documented in the province and in the entire Apulian region, and, although the number of superficial swabs examined was low, the positivity in December (6/100 samples) was statistically significant compared to October (0/100 samples).

In a previous study (4) conducted in other supermarkets during periods of reduced mobility due to COVID-19 restrictions, SARS-CoV-2 was found on 4.3% of the surfaces examined. This value appears lower than that reported in this survey. Actually, we worked in two different periods and in different circumstances: in the previous survey, the surveillance examined more supermarkets, and therefore more surface samples, and under the restrictive provisions due to the increase in COVID cases, whereas in the present study the population was not subject to any mobility restrictions, neither in October nor in December. Furthermore, although December could be considered a month of the consistent circulation of inhabitants, due to the concomitance with the Christmas holidays and because inhabitants were not subject to travel restrictions, we would have expected a greater number of positive swabs. Indeed, some authors have shown that the levels of SARS-CoV-2 RNA observed in the environment were much lower than in human nose-pharyngeal specimens, assuming that only part of the viruses are released into the environment through

Flügge droplets (28-30). Other authors have studied the stability of SARS-CoV-2 on surfaces, but these are experimental studies carried out in the laboratory and under highly controlled conditions, thus being different to the real environment, which is exposed to more variables (29). Furthermore, SARS-CoV-2 is known to have differential survival based on the type of surface. Experiments conducted under controlled conditions detected the virus for periods of less than three hours on paper, up to one day on wood and textiles, two days on glass, and for longer periods (3/4 days) on steel and plastic (2). In this regard, this study also shows that the positivity of environmental swabs by RT-PCR did not coincide with the presence of the infectious virus established with the virus culture method. It is possible that the virus detected on the surfaces was represented only by RNA residues or that the viral load was not sufficient to determine infection in vitro. In fact, there is a linear correlation between Ct values and the probability of isolating the virus in vitro; in particular, previous studies established that in the presence of high Ct values (> 35), viral growth was not observed (26, 31). However, it is possible that people's awareness of hand hygiene procedures increased, reducing the risk of transmission from fomites and therefore reducing the possibility of surface cross-contamination (19).

At the end of December 2020, the Ministry of Health introduced an immunization plan in Italy. The "green pass" certification was issued to people that were either vaccinated, recovered from COVID-19, tested with a rapid antigen test performed in the last 48 hours or tested with a molecular test performed in the last 72 hours (32). Vaccines have reduced the incidence of contagion and the number of hospitalizations and deaths, proving to be the most effective tool to combat the COVID-19 pandemic (11). The small number of positive samples was due to the wide availability and dissemination of COVID-19 vaccines during the study period, thus reducing the possibility of the spread of SARS-CoV2. In particular, the vaccination coverage of the regional population was 80.59% (4 October) and 86.33% (27 December 2021) (27).

Regarding environmental treatment, ozone, due to its strong oxidizing properties, has for years been considered an effective disinfectant, being also economical and easily accessible (10). It has viricidal activity in that it targets proteins on the viral envelope, inhibiting their entry into host cells. Studies show that this gas is promising for surface disinfection: a concentration of 20 ppm and an exposure time of 15 minutes are sufficient (1, 11).

However, the surface material greatly influences the inactivation of the virus (33). Specifically, rigid inert surfaces (such as stainless steel, glass, and plastic) gave a similar inactivation of the virus when treated with ozone, while porous materials (such as floors) or copper surfaces inactivated the virus even in the absence of ozone. In particular, copper is inherently antimicrobial and is able to inactivate SARS-CoV-2 within a few minutes (34), while porous materials could trap the virus and prevent its recovery (33).

In addition, the composition of the medium is important in determining the inactivation of the virus. Some authors (33) showed that ozone flux on a liquid surface was 100 times higher than that on a dried surface, suggesting that the rehydration of the dried viral medium increases the exposure of the virus to ozone, consequently resulting in its inactivation. Therefore, ozonised water could be an alternative for environmental disinfection, as it can cause a 2.0-5.0 log10 reduction in the SARS-CoV-2 titre after only 1 minute of exposure (11). The treatment of ozonisation under real conditions usually varies from laboratory conditions. Since international standards require at least four orders of magnitude of reduction in viral titre, it is possible to assume from our results

and from previous studies (10) that the viricidal efficiency of ozone is insufficient in field conditions, although other studies (35) have confirmed its viricidal activity against coronaviruses.

Conclusions

Our study does not allow for the highlighting of significant differences between environmental treatments with and without ozone, considering probably the scarce number of swabs examined, the non-viability of virus or there were confounders that were not identified. However, the importance of observing good hygiene practices (e.g. the use of a mask and disinfection of hands) and adhering to the vaccination campaign to minimize the circulation of SARS-CoV-2 remains valid, which would explain the low frequency detected of the virus. Researchers need to conduct further studies to validate the effectiveness of environmental treatments with airborne disinfectants, including ozone, both in community facilities and in hospitals where possible, and to define the effective usefulness of this disinfection system. Moreover, it is essential to consider that in order to obtain a complete evaluation of the effectiveness of a disinfection system, the reduction of infectivity / viral viability should also be evaluated and not only the frequency of presence of viral genomes.

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Riassunto

Vitalità di SARS-CoV-2 RNA su superfici sottoposte a frequenti contatti e valutazione di un trattamento di ozonizzazione a flusso continuo

Premessa. L'emergenza COVID-19 ha evidenziato l'importanza dei sistemi di prevenzione e del monitoraggio microbiologico ambientale come elementi fondamentali nella risposta alle epidemie e ad altre minacce simili per la salute individuale e collettiva. L'utilizzo di sistemi automatizzati di disinfezione "No-touch" degli ambienti elimina o riduce la dipendenza dagli operatori, consentendo così un miglioramento dell'efficacia della disinfezione terminale.

Disegno dello studio. Nel presente studio, ci siamo concentrati sulla possibile contaminazione da SARS-CoV-2 sulle superfici dei servizi commerciali e sull'efficacia del trattamento con ozono sul virus.

Metodi. Le analisi sono state condotte il 4-7 ottobre e il 27-30 dicembre 2021 in quattro supermercati di una città pugliese; i supermercati A e B erano dotati di un sistema di ozonizzazione, mentre C e D erano privi di bonifiche ambientali.

Risultati. L'RNA di SARS-CoV-2 è stato rilevato mediante real-time RT-PCR solo a dicembre, nel 6% delle superfici testate, mentre tutti i campioni esaminati sono risultati negativi dopo la coltura virale, poiché non è stato osservato alcun effetto citopatico. Dal confronto tra ottobre e dicembre è emersa una differenza statisticamente significativa ($p = 0,0289$), ma non è emersa alcuna differenza statisticamente significativa ($p = 0,6777$) dal confronto tra supermercati con e senza sistema di ozonizzazione.

Conclusioni. Sebbene nessuna importante variazione fu osservata trattando gli ambienti con sistemi di ozonizzazione, ulteriori studi sono necessari per validare l'efficacia dei trattamenti ambientali con disinfettanti nell'aria.

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