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Effects of ozone, ultraviolet and an organic acid-based disinfectant against porcine reproductive and respiratory syndrome virus

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Abstract

A good level of biosecurity practice is important for efficient porcine reproductive and respiratory syndrome (PRRS) control. In the current study, disinfecting ability of ozone against PRRS virus (PRRSV) was evaluated in comparison with ultraviolet (UV) and an organic acid-based disinfectant to assess the possible use of ozone for disinfecting farm vehicles, equipments, and materials to reduce the risk of new virus introduction. For *in vitro* evaluation, the levels of infectious virus and viral RNA were determined on the swabs collected from the floor surface of each room treated with either ozone, UV or the disinfectant up to 30 min after contamination with 100 mL of VR2332 (10^5 $TCID_{50}$/mL). For *in vivo* evaluation, 3, 4-week old, PRRS-free pigs were housed into those rooms right after the last swab collection. Then the pigs in each room were injected intramuscularly with the corresponding swab samples collected at the last time point and pooled per each room. Although ozone, UV, and the disinfectant significantly reduced the levels of PRRSV RNA contamination, ozone was most effective in removing the viral RNA. In addition, the virus collected after at least 10 min exposure to ozone failed to replicate in pigs while the virus collected after treatment with UV and the disinfectant for 30 min still replicated in pigs. Based on the results, it was concluded that ozone is more effective in inactivating PRRSV as compared with UV and the organic acid-based disinfectant.

Key words : PRRS, Ozone, Ultraviolet, Organic acid-based disinfectant, Disinfectant efficiency

INTRODUCTION

PRRS has been causing a great deal of economical losses worldwide due to reproductive failure, growth reduction, and high mortality of pre- and post-weaning pigs (Neumann et al, 2005; Nieuwenhuis et al, 2012; Peisak et al, 1997). Regardless of the PRRS status of farms, implementation of high grade biosecurity to prevent the introduction of a new strain of PRRSV into the farms is the number one priority for effective PRRS control. PRRSV can be introduced into farms through various routes such as infected pigs (Dee and Joo, 1997), infected semen (Christopher-Hennings et al, 1995), contaminated farm materials (boots, needles, and coveralls) (Otake et al, 2002), and contaminated transport vehicles (Dee et al, 2004). Especially, virus introduction through contaminated vehicles has long been considered as an important risk factor for PRRSV spread between farms and the long-distance (50 km) spread of PRRSV (Dee et al, 2004).

Numerous methods have been evaluated for the antimicrobial effects against various pathogens and could be applied for decontamination of vehicles which access to farms. Ozone has been applied in swine farms to reduce bacterial numbers in swine wastewater (Macauley et al, 2006) and proven its antiviral effects against murine RNA viruses using a laboratory animal model (Sato et al, 1990). Ultraviolet (UV) also has been evaluated for anti-

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microbial effects in previous studies. It has been shown that UV radiation reduced bacterial numbers in swine wastewater (Macauley et al, 2006), inactivated PRRSV contamination on various surfaces of farm materials (Dee et al, 2011), and inactivated PRRSV present in the air (Cutler et al, 2012). In addition, many disinfectants have been reported to be effective reducing PRRSV infectivity; chlorine, iodine, and quaternary ammonium compounds were effective against PRRSV (Shirai et al, 2000); glutaraldehyde and quaternary ammonium chloride were also effective in disinfecting trailers which transported contaminated pigs after washing (Dee et al, 2004). Air filtration systems implemented in large sow herds have been demonstrated to decrease the probability of a PRRSV outbreak. However, implementation of air filtration represents a considerable capital investment, and does not eliminate the risk of new virus introductions (Alonso et al, 2013).

Therefore, the current study was carried out to evaluate the virucidal effect of ozone as compared with UV and an organic acid-based disinfectant and to assess the possibility of using ozone as a disinfecting method for farm vehicles, equipments, and materials contaminated with PRRSV to reduce the risk of PRRSV introduction to swine farms.

MATERIALS AND METHODS

Experimental design

Six rooms (2 meter each for dimension, length, and height) which have independent ventilation system were washed, disinfected, and then dried for 3 days before the experiment started. Ozone light (254 nm, 95 Watt, Dawoo, Koyang, Korea) was mounted at 20 cm from the ceiling of rooms 1-3 and ultraviolet (UV) light (254 nm, 72 Watt - 36W × 2, Dawoo, Koyang, Korea) was mounted in room 4. VR2332, North American prototype PRRSV, were prepared in 600 mL of 0.01 M phosphate buffered saline (PBS, pH 7.4) at 10^5 $TCID_{50}$/mL and dispensed equally in 6 spray bottles. One hundred mL of virus was sprayed onto the floor of each room. After virus contamination, 2 swabs were collected from each of the 4 corners and the center (10 swabs in total) of the floor in each room (0 min). Then ozone was operated in room 1, 2 or 3 for 10, 20 or 30 min, respectively, and 10 swabs were collected from each room after exposure to ozone for the specific time. In room 4, UV light was operated for 30 min and 10 swabs were collected from the floor at 10, 20, and 30 min during the UV treatment. In room 5, 1 liter of 200-time diluted disinfectant (RedCard, ELT science, Cheongwon, Korea) which contains potassium monopersulfate, citric acid, malic acid, alcohol polyoxyetylene ether, sodium hexametaphosphate, sulfamic acid, and sodium chloride was sprayed onto the floor to cover entire floor surface contaminated with virus and then 10 swabs were collected from the floor right at 10, 20, and 30 min during the disinfectant treatment. In room 6, 10 swabs were collected from the floor at 0, 10, 20, and 30 min after virus contamination with no treatment. All swab samples were collected in 1 mL of PBS, vortexed, and subjected to PRRSV real-time reverse transcription-polymerase chain reaction (RT-PCR) test and virus titration after further filtration through 0.2 μm syringe filters.

Three 4-week old, PRRS-free pigs were introduced into each room right after the last swab collection to evaluate the disinfectant efficiency of each treatment *in vivo*. In addition, the pigs in each room were injected intramuscularly with a pooled swab sample prepared by combining 10 swab samples collected at the last time point per each corresponding room. The pigs were bled before housed in the rooms (0 wk) and at 1 and 2 weeks after housing and injection. Serum samples were subjected to PRRSV real-time RT-PCR and ELISA to detect the evidence of PRRSV replication.

Viruses and cells

VR2332 (North American prototype PRRSV) was propagated in MARC-145 cells, a highly permissive clone of the African Monkey kidney cell line MA104 (Kim et al, 1993) was used for the study.

Virus titration assay

Confluent monolayers of MARC-145 prepared in 96-well plate were inoculated in quadruplicate with 100 μL

of 10-fold serial dilution for each supernatant up to eight time points and incubated for 1 hour at 37°C. After one hour incubation, inoculum was discarded and cells monolayer was replenished with RPMI growth media. Infection proceeded up to 6 days and virus titer was calculated as $TCID_{50}$ based on cytopathic effect (CPE).

PRRSV real-time RT-PCR

The level of viremia in the serum was measured by a real-time RT-PCR using TaqMan® chemistry as previously described (Kim et al, 2007). Virus-specific oligonucleotide primers and a MGB fluorescent probe were engineered to be within a highly conserved region of PRRSV ORF6. The forward primer (ORF6F: 5'-GCCATAGAAACCTGGAAATTCATC-3') and reverse primer (ORF6R: 5'-GCGGCCTAGCAAGCACAA-3') were synthesized by a commercial vendor (Bioneer, Daejeon, Korea). An TaqMan® MGB probe with a 5' reporter 6-carboxyfluorescein (FAM) and a 3' non-fluorescent quencher (ORF6P: 5'-6FAM-CCTCCAGATGCCG) was synthesized by Applied Biosystems (Foster City, CA, USA). Viral RNA was extracted using QIAamp® viral RNA mini kit (Qiagen, Valencia, CA, USA) from 140 μL of each virus material. Real-time RT-PCR was then carried out with the QuantiTect® Probe RT-PCR Kit (Qiagen) in a 25 μL reaction volume using 2.5 μL of extracted template. Primers were added at a final concentration of 0.4 μM each; the probe was at a final concentration of 0.2 μM. Cycling conditions were as follows: a) reverse transcription for 30 min at 50°C; b) an activation step for 15 min at 95°C; and c) 35 cycles of 15 sec at 94°C and 60 sec at 60°C. A set of PRRS preparations, each of which had known virus titer, was used to generate a standard curve. Samples with a threshold cycle (Ct) of 35 cycles or less were considered positive. The number of PRRSV RNA copies in each serum sample was calculated by converting Ct value to RNA copies using the standard curve made of PRRSV RNA samples with known copy numbers.

Serology

PRRSV-specific antibody response was evaluated by a commercial ELISA kit (Bionote, Hwasung, Korea) that detects PRRSV nucleocapsid-specific antibodies.

Statistical analysis

The effects of treatment with ozone, UV or an organic acid-based disinfectant as compared with non-treatment (NT) group were analyzed with Student's t-test.

RESULTS

In vitro disinfectant efficiency of ozone, UV, and a disinfectant against PRRSV

There was no difference in the number of PRRSV RNA copy detected from all of the 6 rooms contaminated with VR2332 before treatment (0 min). After exposure to ozone, UV or a disinfectant, a significantly ($P < 0.05$) lower number of RNA copy was detected from the room treated with ozone for 10 min as compared with other groups including the NT group (Fig. 1). Both

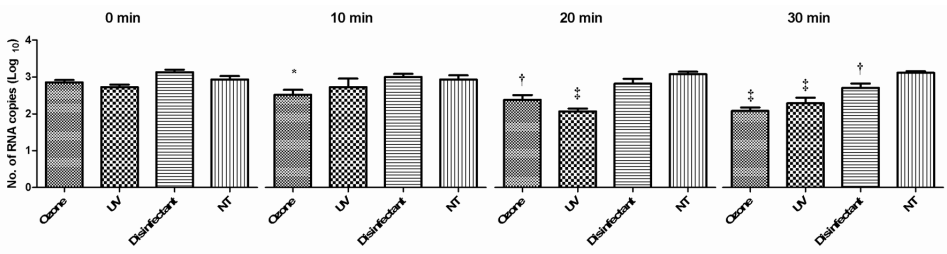

Fig. 1. Quantitative RT-PCR results of swab samples collected from rooms contaminated with PRRSV after treatment with ozone, UV or an organic acid-based disinfectant for various times. The symbols represent statistical significance (*: <0.05, †: <0.001, ‡: <0.0001) as compared with non-treated (NT) group based on Student's t-test.

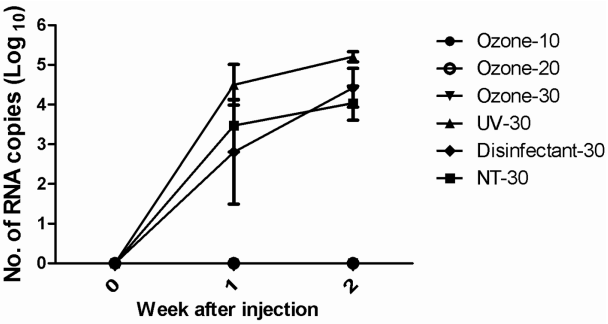

Fig. 2. Quantitative RT-PCR results of serum samples collected from pigs exposed to virus after treatment with ozone, UV or an organic acid-based disinfectant.

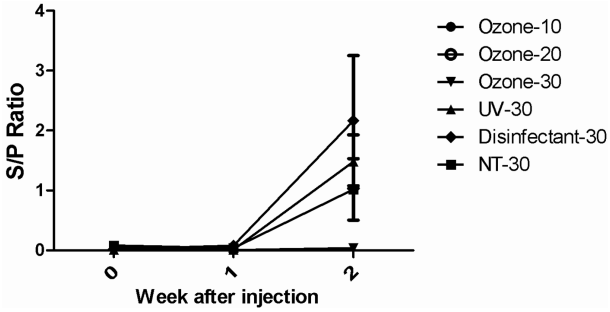

Fig. 3. The levels of PRRSV-specific antibody in serum samples collected from pigs exposed to virus after treatment with ozone, UV or an organic acid-based disinfectant.

ozone ($P<0.01$) and UV exposure ($P<0.001$) significantly reduced RNA contamination at 20 min and all treatments significantly reduced RNA copy number at 30 min as compared with the NT group. Nonetheless, the biggest reduction in the level of RNA contamination was observed in the rooms treated with ozone (Fig. 1). In addition, all of the 10 swabs collected at each time point were pooled together per each treatment (total 18 pooled samples, 2 time points×3 ozone treatment groups +4 time points×3 treatment groups). The 18 pooled samples were tittered for PRRSV after inoculation on MARC-145 cells. However, no viral growth was observed until 7 days after inoculation.

In vivo disinfectant efficiency of ozone, UV, and a disinfectant against PRRSV

Three pigs were introduced into each room right after the last swab collection to evaluate the disinfectant efficiency of each treatment *in vivo*. In addition, the pigs in the rooms were intramuscularly injected with pooled swab samples collected at the last collection time point and combined together per each room. Serum samples collected at 0, 1 and 2 weeks after housing and injection were subjected to PRRSV real-time RT-PCR and ELISA to detect the evidence of PRRSV replication. There was no evidence of PRRSV replication in the pigs exposed to the swab samples collected from the rooms treated with ozone (ozone-10, ozone-20, ozone-30) while the similar levels of viremia were detected in the pigs housed and injected with the swab samples collected from the rooms treated with UV and the disinfectant as compared with the NT group (Fig. 2). Similar to the PCR result, PRRSV-specific antibodies were detected from the pigs injected with the pooled swab samples collected from the rooms treated with UV or the disinfectant and the not-treated room at 2 weeks after injection (Fig. 3).

DISCUSSION

Ozone gas has a high oxidation potential and is 1.5 times more effective than chloride when used as an antimicrobial agent against bacteria, viruses, fungi, and protozoa (Mohammadi et al, 2013). Ozone has been previously applied in swine farms to reduce bacterial numbers in swine wastewater (Macauley et al, 2006) and, in the current study, the virucidal effect of ozone was compared with that of UV or an organic acid-based disinfectant to assess the possible use of ozone as a disinfecting method for farm vehicles, equipments, and materials contaminated with PRRSV to reduce the risk of PRRSV introduction. A critical component of the disinfecting method for vehicles and farm materials is the ability to inactivate PRRSV contaminated not only on the various surfaces of objects materials, but also in hidden spaces which are not directly affected by UV illumination or surface disinfectant spray. In fact, UV light or disinfectant spray is used most commonly for disinfecting vehicles that access to farms. These methods, however, cannot reach the inner space of the vehicles or the surface between stacked materials contaminated with virus. Because ozone can be produced as a gas form by

the photo dissociation of molecular oxygen (O_2) into activated oxygen atoms which then react with further oxygen molecules, it can reach almost all spaces unless it is kept air-tight (Mohammadi et al, 2013).

Under the conditions of the current study, we demonstrated that ozone effectively inactivated PRRSV contaminated at even 10 min of exposure. Ozone reduced PRRSV RNA contamination most effectively and in the fastest manner as compared with UV and an organic-acid based disinfectant (Fig. 1). In addition, there was no evidence of PRRSV replication in the pigs exposed to the swab samples collected from the rooms treated with ozone (ozone-10, ozone-20, and ozone-30). UV and the disinfectant also significantly reduced viral RNA contamination until 30 min exposure. However, both methods failed to achieve the complete inactivation of PRRSV since virus replication was observed in the pigs exposed to the swab samples after treatment with UV or the disinfectant (Fig. 2 and 3). It has been previously shown that UV radiation inactivated PRRSV contaminated on the surfaces of farm materials (Dee et al, 2011) and inactivated PRRSV present in the air (Cutler et al, 2012). The possible reasons for the discrepant result observed in the current study are the different length of exposure time to UV and different virus assay protocols. Cutler et al (2012) evaluated the disinfecting effect of UV only by virus titration on MARC-145 cells. In the current study, both virus titration on cell culture system and virus bioassay in pigs were used to detect viable virus and virus replication. Only bioassay could successfully detect virus replication while virus isolation was all negative including the non-treated group that should contained live virus particles. Consistent with this, inferior sensitivity of virus isolation compared with bioassay in pigs was demonstrated also in a previous study that compared the sensitivity of live virus detection between bioassay in boars and virus isolation in cells (Swenson et al, 1994). On the other hand, Dee et al (2011) used the bioassay for live virus detection, but demonstrated effective inactivation of PRRSV after at least 60 min exposure to UV.

In conclusion, ozone could be an effective method to disinfect farm vehicles, equipments, and materials contaminated with PRRSV and reduce the risk of PRRSV introduction as compare with UV radiation and an organic acid-based disinfectant. However, ozone inhalation for a long time can be toxic and cause epiphora, upper respiratory irritation, rhinitis, cough, headache, occasional nausea, vomiting, shortness of breath, blood vessel swelling, poor circulation, heart problems and even stroke (Bocci, 2006). Therefore, ozone should be used for non-living things and removed with sufficient ventilation with fresh air after used for disinfection as ozone is an unstable gas and has a half-life of 40 min at 20^{o}C (Bocci, 2006). In the future, further assessment of ozone may indicate its ability to inactivate other economically significant pathogens of pigs, i.e. porcine circovirus type 2 and *Mycoplasma hyopneumoniae*, thereby expanding its benefit to commercial farms.

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