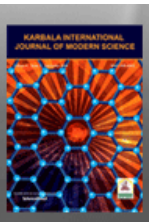

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A Successful Elimination of Indonesian SARS-CoV-2 Variants and Airborne Transmission Prevention by Cold Plasma in Fighting COVID-19 Pandemic: A Preliminary Study

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Abstract

Global infection and mortality rates have soared to millions due to SARS-CoV-2 human-to-human transmission from via droplets which then declared as pandemic. This study examined the created cold plasma equipment (CPE) effectiveness in reducing COVID-19 transmission in a confined space. CPE sucked air using a fan in a test chamber then pushed it into a cold plasma reactor. The results indicated that it was able to terminate all SARS-CoV-2 variants along with bacteria and fungi indoors by keeping it turned on for 30 minutes' minimum. CPE was proven as safe and effective to hinder virus transmission with the acceptable ozone emission as the side effect.

Keywords

bacteria; cold plasma equipment; COVID-19; fungi; SARS-CoV-2

Authors

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RESEARCH PAPER

A Successful Elimination of Indonesian SARS-CoV-2 Variants and Airborne Transmission Prevention by Cold Plasma in Fighting COVID-19 Pandemic: A Preliminary Study

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Abstract

Global infection and mortality rates have soared to millions due to SARS-CoV-2 human-to-human transmission via droplets which then declared as pandemic. This study examined the created cold plasma equipment (CPE) effectiveness in reducing COVID-19 transmission in a confined space. CPE sucked air using a fan in a test chamber then pushed it into a cold plasma reactor. The results indicated that it was able to terminate all SARS-CoV-2 variants along with bacteria and fungi indoors by keeping it turned on for 30 min' minimum. CPE was proven as safe and effective to hinder virus transmission with the acceptable ozone emission as the side effect.

Keywords: Bacteria, Cold plasma equipment, COVID-19, Fungi, SARS-CoV-2

1. Introduction

The prevalence of SARS-CoV-2 was firstly discovered in China [1] which then globally infected millions of people worldwide [2,3]. The decision to declare this massive infection as a pandemic by the World Health Organization was made in March 2020. The global health and world economy have been harmed by this extensive outbreak [4,5]. This worldwide catastrophe has forced scientists to act fast in mobilizing extensive investigations on SARS-CoV-2 centering on its clinical features, position, and its contagion procedures with an ultimate intention to prevent more disastrous consequences [6,7]. In this current global health threat, approximately 450 million people have become the victims of this recent outbreak, SARS-CoV-2 or COVID-19, which was identified as the seventh corona virus resulting in more than 6 million mortalities globally. From the data retrieved from John Hopkins University online website which keeps an eye of COVID-19 cases in actual time, the number of traced cases in Indonesia alone has reached more than 5.8 million cases, meanwhile fatalities due to this lethal virus have hit around 150,000 people [8].

The classification of the coronavirus family itself contains four different genera, from *Deltacoronavirus*, *Gammacoronavirus*, *Betacoronavirus*, and

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Alphacoronavirus. Meanwhile, SARS-CoV-2 genome is identified as a single-stranded positive-sense RNA with roughly around 30 kb. Furthermore, the genome determines four structural proteins, which are envelope (E), spike (S), membrane (M), and nucleocapsid (N) [9,10]. In the development of a vaccine for SARS-CoV-2, it is the aforementioned spike protein which becomes the primary targeted antigen [11]. Formerly, it was the constructed structural protein which became a peptide-based vaccine nominee [12]. Moreover, an infection was triggered by the virus-host interplay including a complicated response from the immune system [13]. On the other hand, Indonesian COVID-19 isolates express contradictory phenomena with the discovery of antibody-dependent enhancement (ADE) within them [14]. Therefore, ADE has become a major steppingstone in developing treatment and vaccines based on antibodies [15,16].

Generally, to be able to facilitate any early diagnosis and regulating the virus, SARS-CoV-2 pattern of transmission needs to be acknowledged and developed. During this present time, a pattern of transmission has been well recognized, which is the claim of SARS-CoV-2 airborne infectious transfer between humans. This rapid contagion pattern of this virus through the flying droplets secreted from the respiratory tract in the form of coughing and sneezing. Besides airborne scheme, SARS-CoV-2 water transmission should get precautionary attention with the risk of SARS-CoV-2 transmission to people and animals via water is possible as well.

Furthermore, scholars these days aim to derive diverse approaches in tackling the fast spread SARS-CoV-2. However, no effective medication (drugs) yet is found to fight the virus [17–24]. Thus, this investigation offered a preliminary study of cold plasma to prevent airborne transmission to fight COVID-19 pandemic in Indonesia.

Recently, the use of cold plasma in research is quite common. This cold plasma is commonly and increasingly applied in biomedical and industrial fields at particular atmospheric pressure and room temperature because the cold atmospheric plasma (CAP) contains components of reactive oxygen and nitrogen species (RONS) displaying its high compatibility [1,18,19]. Many preliminary studies exhibited that cold plasma equipment (CPE) turns out to have great potential to be utilized as efficient approach for disinfection [18,19], especially with the termination of microorganism which related to nosocomial infections which are *Escherichia coli*, *Pseudomonas alcaligenes*, *Staphylococcus epidermidis*, *Micrococcus luteus*, and *Serratia marcescens* with the level of inactivation is up to 70% [25].

An investigations in virus immobilization using plasma as the mediator only began 20 years ago [26]. However, the publication on this had been double in number over the last few years and cold plasma-virus research also had expanded fast from the definition of virucidal property to its ability to immobilize viruses. In addition, previous studies also showed that there are only a few successful researches in using cold plasma technology in inactivating virus by creating equipment for indoors which is very crucial in this pandemic period like these days along with its acceptable ozone emission as the result of cold plasma reactors, both based on corona discharge and dielectric barrier plasma [17,25,26]. Meanwhile, the permissible concentration of ozone in a room with an occupant is only 0.08 ppm. Significantly, based on previous study, plasma reactors can eliminate microorganisms, on the other hand the ozone released into the room is still much smaller than the threshold, for example EPA 2012, Minister of Health Regulation of the Republic of Indonesia (Number 70), 2016.

Besides, it is very crucial to stick to an environmentally friendly technology in killing the virus efficiently with easily and safely application without creating any waste or other products as new pollution and containing any toxic chemicals. The idea of utilizing cold plasma exposure for terminating and inactivating the viruses intends to offer a solution with all of these features [26]. Therefore, this investigation has developed an equipment that can be placed indoors including in public spaces. This paper also discusses the results of testing an equipment called CPE.

2. Methods

2.1. Virus isolates

The virus isolate used for this study was derived from the Professor Nidom Foundation (PNF), Surabaya, Indonesia. This laboratory was one of the approved diagnostic laboratories for COVID-19 by the Ministry of Health of the Republic of Indonesia (HK.01.07/MENKES/4642/2021). Meanwhile, the virus genome was already deposited in the GISAID database (hCoV-19/Indonesia/JI-PNF-211373/2021; Accession ID: EPI_ISL_6425649).

2.2. Cells and chemicals reagents

This investigation applied ethanol (≥99.8%, Sigma–Aldrich, USA), fetal bovine serum (FBS) (Sigma–Aldrich, USA), RNA extraction kit (Geneaid, Taiwan R.O.C.), Vero cell (African green

monkey kidney) (ATCC, USA), penicillin-streptomycin (Sigma–Aldrich, USA), Minimum Essential Medium Eagle (DMEM) (Sigma–Aldrich, USA), fungizone (Sigma–Aldrich, USA) and dimethyl sulfoxide (Merck, Germany) as the chemical reagents. The cultivation of Vero cells was accomplished in DMEM at 37 °C in a 5% CO_2 incubator involving 10% FBS and penicillin-streptomycin (Gibco, USA). Then, the detachment of confluent monolayer from Vero cells was conducted using trypsin–EDTA with the cells being incubated at 37 °C for 5 min. Next, the addition of the medium was performed then it was pipetted gently and enumerated by applying a hemocytometer (Paul Marienfeld, Germany). Lastly, the addition of the cells into 96-well plates was conducted with 1×10^6 cells/10 mL and then the incubation process in 5% CO_2 was completed at 37 °C.

2.3. Cold plasma equipment and study design in the test chamber

Cold plasma equipment (CPE) was developed using corona discharge reactors [18–24]. Inside the CPE, there were two cylindrical reactors with a length of 15 cm and a diameter of 5 cm. Corona discharge electrodes configuration was serrated with wire and cylinder. CPE was generated in the corona discharge implementing a DC voltage of 4 kV. CPE utilized a fan at the top and this fan could suck air from the room at a rate of 5 m^3 min^{-1}. This fan pushed the air which was already spoiled with microorganisms and chemical pollutants into the corona discharge reactor. The cold plasma in the corona discharge converted the dirty air into clean air [18]. The test chamber with dimension $90 \times 90 \times 120$ cm was also contaminated with SARS-CoV-2.

Viruses that passed through the cold plasma would die because the virus entered the areas contained with reactive oxygen species, reactive nitrogen species, electrons, ions, and electromagnetic waves [27]. After passing through the cold plasma area in the corona discharge reactor, the air was very clean and released back into the room through the window at the bottom of the CPE. The concentration of ozone in the room in which the CPE was turned on was measured at 0.04 ppm. This concentration was smaller than 0.08 ppm set by the ozone emission threshold in a contained space with an occupant according to the 2012 US EPA for Health Effects of Ozone in the General Population. Fig. 1 displays the test chamber of the CPE and the numbers 1, 2, 3, and 4 exhibiting the shelves on which Petri dishes containing serum contaminated with SARS-CoV-2 need to be placed (see Fig. 2).

Fig. 1. A CPE is put in a test chamber for experi-mental transmission of SARS-CoV-2 through airborne in the chamber. This test chamber was built as a BSL3 facility. The test chamber is free from contamination when the stained plates are inserted into the test chamber. Indications with numbers 1, 2, 3, and 4 show the shelves on which to place Petri dishes containing serum with SARS-CoV-2 contamination in the chamber test.

Two milliliters of each SARS-CoV-2 and negative control serum were placed in twelve Petri dishes with the design as shown in Table 1. Petri dishes were placed on the rack provided in the chamber of CPE with positions scattered in all parts of the chamber room. The position was as shown in Fig. 1.

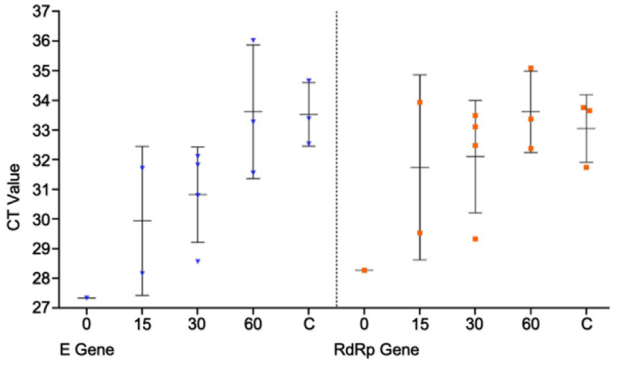

Fig. 2. CT value in post CPE test SARS-CoV-2 qRT-PCR results based on Petri dishes.

Table 1. Study design in Petri dishes.

Code	Formula	Duration
1.1	SARS-CoV-2 (2 mL)	15 min
1.2	SARS-CoV-2 (2 mL)	30 min
1.3	DMEM (2 mL)	30 min
2.1	SARS-CoV-2 (2 mL)	30 min
2.2	SARS-CoV-2 (2 mL)	60 min
2.3	DMEM (2 mL)	60 min
3.1	SARS-CoV-2 (2 mL)	30 min
3.2	SARS-CoV-2 (2 mL)	30 min
3.3	DMEM (2 mL)	60 min
4.1	SARS-CoV-2 (2 mL)	15 min
4.2	SARS-CoV-2 (2 mL)	60 min
4.3	DMEM (2 mL)	60 min

Each Petri dish was opened, then CPE was turned on by using a remote for 15, 30, and 60 min. Petri dishes were then taken according to the code and duration to be continued with a viral RNA extraction stage. After 60 min, CPE was turned off, then all the remaining Petri dishes were taken to continue with the viral RNA extraction stage (see Table 2).

2.4. RNA extraction

The extraction of viral RNA was accomplished by using Viral Nucleic Acid Extraction Kit II (Geneaid, Taiwan R.O.C.) as mentioned in the manufacturer's protocol. After this step was completed, the sample directly was processed into real-time PCR (qPCR) [28].

2.5. Real-time PCR (qRT-PCR)

The application of qRT-PCR with QuantStudio 5 Applied Biosystem (AB) PCR machine was proceeded with STANDARD M nCOV Real-Time Detection Kit (Lot #MNCO0120026) and MBioCoV-19 RT-PCR Kit (Lot #6900820) as the kits which was performed at the Coronavirus and Vaccine Formulation Research Group, Professor Nidom Foundation, Surabaya, Indonesia. The detection of positive criteria was confirmed when two genes (E and RdRP) $\leq$36. In case the results were only exhibited in E gene $\leq$36, then the results would express inconclusiveness. On the other hand, any negative criteria will be confirmed when E and RdRP were not detected.

Table 2. The results of CPE testing for Covid-19 virus in the test chamber using RT-PCR (based on Petri dishes).

Code	Description	qRT-PCR	
		E Gene	RdRp Gene
	C-19-original	27.33	28.27
1.1	C-19-15m-1	28.16	29.53
4.1	C-19-15m-2	31.71	33.94
	C-19-original	27.33	28.27
1.2	C-19-30m-1	31.82	33.11
2.1	C-19-30m-2	28.56	29.33
3.1	C-19-30m-3	30.79	32.48
3.2	C-19-30m-4	32.11	33.49
	C-19-original	27.33	28.27
2.2	C-19-60m-1	31.55	32.28
3.3	C-19-60m-2	36.02	35.09
4.2	C-19-60m-3	33.27	33.37

2.6. Median tissue culture infectious dose ($TCID_{50}$) and inhibition analysis

This study applied 315 μL of virus dilution from 10^{-1} to 10^{-7} in PBS placed in a micro tube. At this stage of study, the chemical reagent used was Vero cells that had been confluent monolayered on 48 well plates. The removal of the growth medium was completed and then the cells were cleaned by washing them three times with PBS. A total of 100 μL of virus dilution was then mixed to each well according to the sign; meanwhile, for the negative control, the virus was replaced with PBS. Next, the incubation was done in 5% CO_2 for 60 min at 37 °C. Maintenance medium (MM) was added as much as 100 μL per well which then proceeded again with incubation for 48 h in the same condition which was in 5% CO_2 at 37 °C. After 48 h, the removal of medium from each well was conducted and 10% formalin at 100 μL for each well was employed to fix the cells which then placed at room temperature for 30–60 min. Following this, the formalin was removed and then the cells were washed using running water carefully. Then the cells were stained using crystal violet and left at room temperature for 5 min. Next, the plate was washed under running water. The method from Reed and Muench was employed to read and calculate $TCID_{50}$ value [29].

2.7. Methods for bacterial and fungal reduction using cold plasma equipment

Microbiological tests were analyzed using the Microbiological Air Sampler (MAS-100 NT). MAS-100 NT employed the inspection principles when the air was sucked in and passed through a Petri dish containing nutrient agar (NA) media to grow bacteria. MAS-100 NT operating time was adjusted to the volume of the room (according to MAS-100 NT standard) to be tested. Tests were carried out before and after the CPE was turned on. Meanwhile, sampling was completed from the test room after CPE was turned on for 1 h, 2 h and 3 h. Ozone Scientific was utilized to calculate the total ozone concentration after the indoor CPE was switched on.

The concentration of ozone in the test chamber measuring 3 × 3 × 3 m using a unit of CPE was 0.08 ppm. This ozone concentration test was very important to be qualified within the 2012 EPA standard. In growing the bacteria (NA medium), the incubation of the previously used sample cup containing the media was accomplished for 24–48 h at 37 °C, then the number of developing colonies on the medium was measured by using the colony counters [30].

3. Results and discussions

3.1. Cold plasma equipment testing for SARS-CoV-2

From the cycle threshold (CT) value data obtained from different positions and exposure times of CPE, it displayed an increased CT value from the original virus and after testing in the test chamber. CT value seems to increase linearly with the test time length. Based on this CT value, it can be concluded that CPE can reduce 10–12% (15 min), 13–14% (30 min) and 19–23% (60 min) the amount of SARS-CoV-2 (viral load) in the environment. Any confirmation of COVID-19 negative result is robust if CT value is > 36.00.

In detecting the existence of the virus, a certain method needed to be applied, which was PCR. This method multiplied the genetic materials of RNA cyclically making it easily to be spotted on. In addition, CT value is the total number of cycles required for spotting the virus. Theoretically, the higher CT value shows the lesser detected SARS-CoV-2 [6] (see Tables 3 and 4).

In media without virus (DMEM) which was placed in the test chamber of CPE during the test, it showed a CT value of 29.31–40.00 (see Table 5).

Table 6 displays the decreasing $TCID_{50}$ value after the CPE was turned on for 15 min which can also mean that the virus concentration decreases. Meanwhile, with 30 min CPE exposure, only one of the four samples displayed a lower concentration of $TCID_{50}$ compared to 15 min. Additionally, with 60 min CPE treatment duration, $TCID_{50}$ value was zero, expressing that there is no SARS-CoV-2 in the Petri dish anymore.

Table 3. Average results of SARS-CoV-2 testing in CPE chamber using qRT-PCR.

Formula	Description	qRT-PCR	
		E Gene	RdRp Gene
Viral Control	C19-original	27.33	28.27
SARS-CoV-2 2 mL	C19-15m	29.93	31.73
SARS-CoV-2 2 mL	C19-30m	30.82	32.10
SARS-CoV-2 2 mL	C19-60m	33.61	33.61

Table 4. Results of media testing without SARS-CoV-2 in the CPE chamber using qRT-PCR.

Code	Formula	Description	qRT-PCR	
			E Gene	RdRp Gene
	Viral Control	C19-original	27.66	28.61
1.3	DMEM-2 mL	Media-30m	33.38	33.65
4.3	DMEM-2 mL	Media-60m-1	32.53	33.76
2.3	DMEM-2 mL	Media-60m-2	34.66	31.74

Table 5. Significant difference values of SARS-CoV-2 testing in CPE chamber using qRT-PCR.

Formula	Description	qRT-PCR	
		E Gene	RdRp Gene
Viral Control	C19-original	27.33 ± 0.00	28.27 ± 0.0
SARS-CoV-2 2 mL	C19-15m	29.93 ± 2.51	31.73 ± 3.11
SARS-CoV-2 2 mL	C19-30m	30.82 ± 1.61	32.10 ± 1.89
SARS-CoV-2 2 mL	C19-60m	33.61 ± 2.25	33.61 ± 1.37
Control	Control	33.52 ± 1.07	33.05 ± 1.14

Based on PCR examination, E gene and RdRP gene experienced a significant difference ($p < 0.05$) between formulas. The most significant difference appeared in the leaping CT values at 0 min of treatment to 60 min of treatment.

The percentage of CPE's inhibition against SARS-CoV-2 ranged from 99 to 100% in 60 min of CPE exposure. There was a significant difference between CT value of qRT-PCR and the percentage of inhibition displaying that CT value recorded in qRT-PCR test did not indicate a live virus, but could be inactive or in the form of SARS-CoV-2 virus particles.

3.2. Cold plasma equipment for bacteria and fungi

Research on the ability of CPE to reduce the number of bacteria and fungi in the room was also performed twice at different times for two months. The first experiment was in February 2020 and the second experiment was in April 2020. The time

Table 6. SARS-CoV-2 test results after CPE exposure using $TCID_{50}$ value and percentage of inhibition (inhibition test).

Code	Description	$TCID_{50}$	% Inhibition
	C19-original	5.9165×10^5	
1.1	C19-15m-1	3.574×10^3	99.40
4.1	C19-15m-2	3.987×10^2	99.93
	C19-original	5.9165×10^5	
1.2	C-19-30m-1	Zero	100.0
2.1	C-19-30m-2	Zero	100.0
3.1	C-19-30m-3	Zero	100.0
3.2	C-19-30m-4	1.163×10^1	99.99
	C19-original	5.9165×10^5	
2.2	C-19-60m-1	Zero	100.0
3.3	C-19-60m-2	Zero	100.0
4.2	C-19-60m-3	Zero	100.0

difference also caused differences in the number of colony microorganisms. Figs. 3 and 4 show the average of colony concentration and average percentage with the decrease of bacteria and fungi from five sampling positions in a test room measuring 3 × 3 × 3 cubic meters based on the exposure time of CPE.

Figs. 5 and 6 exhibit the average of colony concentration average percentage of decreasing bacteria and fungi from five sampling positions in a test chamber measuring 3 × 3 × 3 cubic meters based on the exposure time of CPE. Data collection was carried out in May 2020. From the two figures, it can be seen that the decrease in bacterial colonies is greater than the decrease in fungal colonies. After one hour of the activation of CPE in the test chamber, bacteria were reduced by about 55% while fungi were around 45%. The same thing is seen in the experiment for 2 h and 3 h' duration of activated CPE. In the April 2020 experiment, the highest bacterial reduction was 73% with CPE duration in the test chamber for 2 h, and became 65% after 3 h. This may be due to the contamination during the experiment, for a test time of 3 h. The results obtained also highlighted that CPE can eliminate microorganisms [31–33].

Furthermore, as a side effect of using cold plasma technology, CPE emits ozone as well. In this investigation, CPE has been tested for ozone released into the room to see whether this created equipment is safe to use [34]. The measured ozone in the room is still much smaller than the threshold, for example based on EPA2012, Minister of Health Regulation of the Republic of Indonesia (Number 70), 2016. This test has also shown that CPE can control pathogenic microorganisms, especially SARS-CoV-2. In addition, as CPE was developed using absolutely no chemical com-pounds, CPE uses corona discharge by suppressing the ozone released from the reactor. Thus, CPE is an equipment which is comfortable and can be placed indoors including in public spaces.

The spreading of SARS-CoV-2 is done by the exhaled respiratory droplets of infected people. There are three possibilities of transmission of

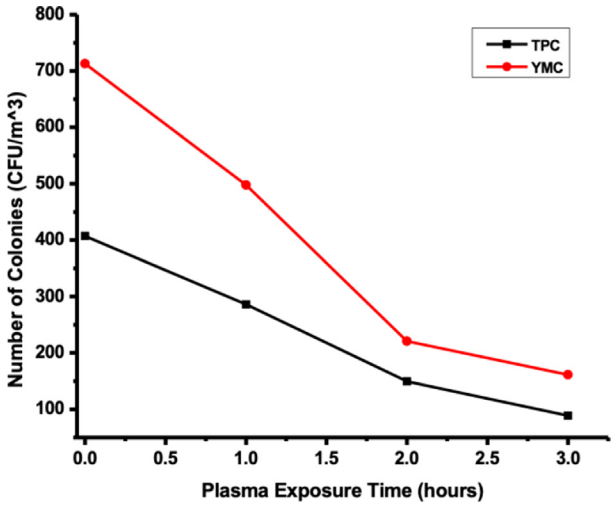

Fig. 4. Percentage of decreased (inhibited) bacteria and fungi with activated CPE in the test chamber based on plasma exposure time. Note: TPC: Total plate count; YMC: Yeast and mold counts.

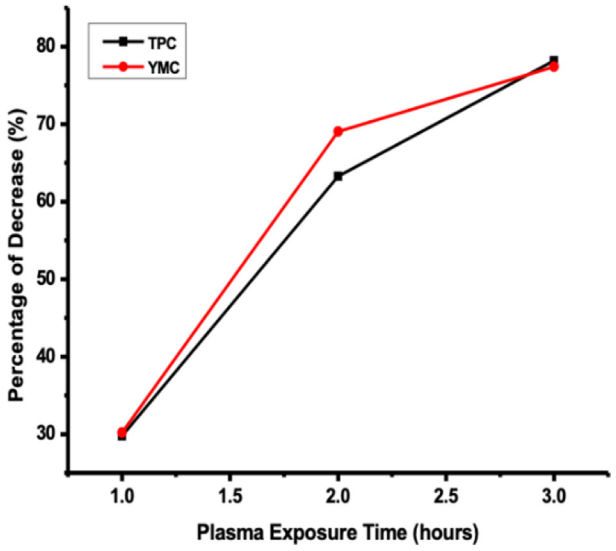

Fig. 3. Total number of bacterial and fungal colonies with CPE exposure in the test chamber based on plasma exposure time. Note: TPC: Total plate count; YMC: Yeast and mold counts.

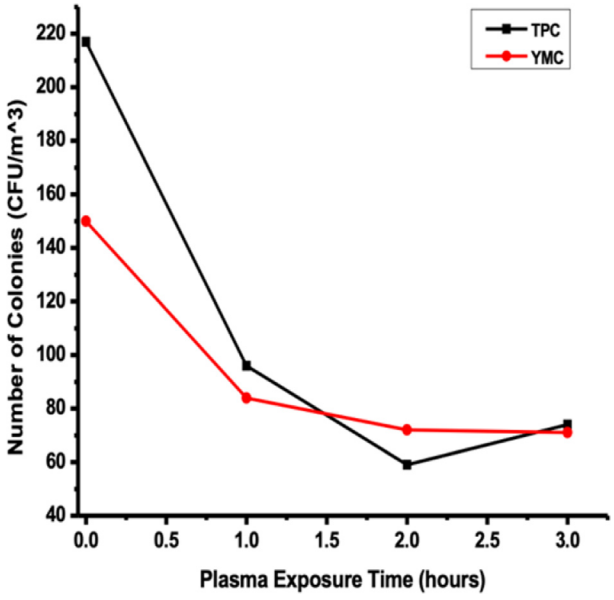

Fig. 5. Number of bacterial and fungal colonies with activated CPE in the test room as function of plasma exposure time. Note: TPC: Total plate count; YMC: Yeast and mold counts.

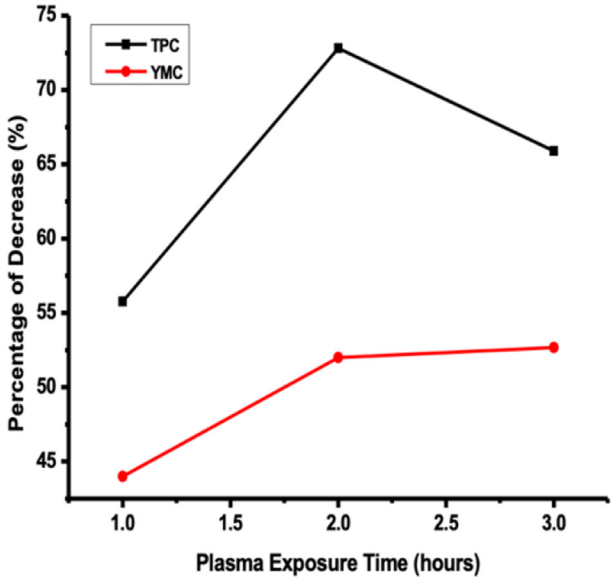

Fig. 6. Percentage of decreased (inhibited) bacteria and fungi with activated CPE in test room based on plasma exposure time. Note: TPC: Total plate count; YMC: Yeast and mold counts.

COVID-19 between the infected person to the new recipients, they are through the circulation of large drop from the mouth of the infected to the recipient's mouth, nose or eyes; the physical exposure of sticked droplet in the surface (fomites) which then subsequently being transferred to the respiratory mucosae of the recipient; and at last, the microdroplets from the infected which are airborne due to the currents of ambient air and then being inhaled by recipient [17–19,33–38].

Based on a theoretical model, the prediction of airborne transmission scheme is confirmed and considered harmful as it includes the inspirations of tiny aerosol droplets which stay in the confined, well-mixed indoor place. The shear-induced or capillary instability of the mucosal linings of the lungs, respiratory tract, and saliva in the mouth leads to an array of liquid droplets to be ejected when a person coughing, sneezing, singing, talking, or breathing [17,35–38].

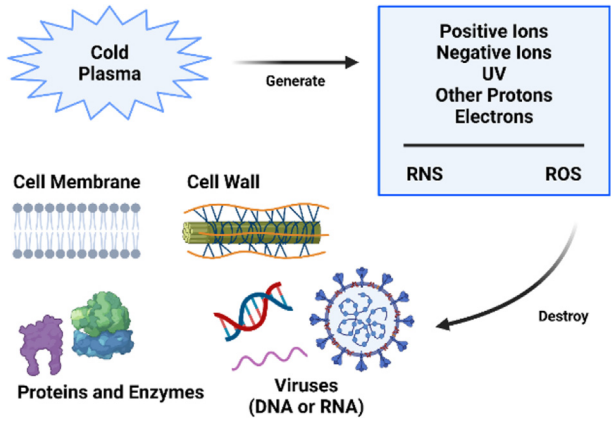

Fig. 7. A schematic mechanism of cold plasma killing bacteria, fungi, and viruses.

In this work, we designed a CPE that has demonstrated to lots of potential for being used as a disinfection method. This feature of disinfectant can be found as well in the research of Thakur et al. (2021) which outlined the effect of graphene nanoparticles in limiting the propagation of COVID-19 and having antibacterial and antiviral properties. Antimicrobial effectiveness of graphene and its derivatives is good, with both physical and chemical modes of harm. They are ideal nanomaterials for coating onto textiles such as personal protective equipment, face masks, and gloves to effectively prevent the transmission of SARS-CoV-2 due to their lightweight, excellent characteristics, and simplicity of functionalization [18].

Lastly, Fig. 7 displays a schematic illustration of cold plasma that can kill microorganisms (bacteria, fungi and viruses, including SARS-CoV-2) [39,40]. Cold plasma at atmospheric pressure always produces reactive oxygen species, positive ions, negative ions, reactive nitrogen species, electrons, photons, and ultraviolet [41,42]. However, a comprehension of the virus inactivation fundamental mechanisms by cold plasma will be essential for the fine-tuning cold plasma therapy before their distribution and application in industrial, agricultural, and medical environments along with the easier prediction of all possible consequences constituting the formation of unwanted derivation that do not support to the inactivation [26].

4. Conclusions

In summary, cold plasma was able to neutralize the new variant of Indonesian SARS-CoV-2 contained in Petri dishes or circulated in the chamber (air) seen in the increase in CT values and $TCID_{50}$ results as well as the percentage of inhibition. The application of indoor cold plasma equipment to break the chain of this virus circulation and other microorganisms such as bacteria and fungi is an important milestone in the preventive steps of COVID-19. The current research is anticipated to accelerate the development of cold plasma as extremely sensitive, precise, and cost-effective instruments for effectively regulating the spread of COVID-19 and other airborne microbes. However, the bulk of studies are based on lab-scale observations, and further research is needed to fully understand other characteristics of this material in order to build and create innovative technologies.

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