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Original article

Bactericidal effect of direct and indirect plasma for medical instruments: Comparative experimental study

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ABSTRACT

Background: Medical equipment must be thoroughly sterilized before use. A potentially useful method for sterilizing medical equipment is non-thermal atmospheric pressure plasma technology. Plasma can be applied directly using cold atmospheric pressure plasma jet (CAPPJ) or indirectly using a plasma-activated mist (PAMi). Aim was to assess the bactericidal effect of direct plasma (CAPPJ) and indirect plasma (PAMi) as new methods of sterilizing stainless steel compared to autoclaving, which is considered the gold standard for sterilizing heat-stable material. **Method:** This comparative experimental study included 36 *E. coli* and 30 *S. aureus*, strains which were isolated from various clinical specimens and identified by conventional methods. They were exposed to three different treatment methods: direct CAPPJ, indirect PAMi, and autoclaving. The time needed to reach medical sterility assurance level (SAL) was recorded for each method. **Results**: There was a high statistically significant difference between *E. coli* and *S. aureus* bacterial counts using direct and indirect plasma at different exposure time intervals. There was a high statistically significant relation between decreasing bacterial counts and the exposure to direct plasma, indirect plasma and autoclave at different time intervals. The shortest time needed to reach SAL was recorded for direct plasma as 1.9 and 3.1 minutes for *E. coli* and *S. aureus*, respectively. **Conclusion**: CAPPJ and PAMi are rapid effective methods not only in eradication of the tested *E.coli* and *S.aureus* isolates but also in reaching SAL for stainless-steal medical instruments. These promising methods can save long time consumed by conventional methods for sterilization especially in emergencies.

Introduction

All invasive procedures involve the use of surgical instruments or medical devices to come into contact with the sterile tissues or mucous membranes of patients. Any such procedure carries a significant risk of introducing pathogenic microbes that could cause infection [1]. It is imperative to disinfect and sterilize various types of equipment to prevent avoidable secondary infections caused by pathogens. Effective sterilization methods are imperative to ensure the sterility of medical devices and consequently reduce the burden healthcare associated infections (HAIs). [2]. Medical equipment can be sterilized via a variety of methods, including autoclaving, treatment with gamma-ray, exposure to UV and the

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application of peracetic acid, formaldehyde, hydrogen peroxide, ethylene oxide [3].

Autoclaving (steam sterilization) is the most widely used method for sterilization worldwide and is considered the most robust and cost-effective method for sterilization of steam compatible, heat-stable medical devices [4,5]. Steam sterilization is nontoxic, inexpensive, rapidly microbicidal, and sporicidal and rapidly heats and penetrates fabrics. The basic principle of steam sterilization, as accomplished in an autoclave, is to expose each item to direct steam contact at the required temperature and pressure for the specified time [6]. The effectiveness of autoclaving can be monitored using chemical or biological indicators - monitoring each autoclave cycle is recommended by most guidelines and standards [7, 8].

Recently, hydrogen peroxide gas plasma sterilizers for medical instrument are commercially available. In this process, free radicals (hydroxyl and hydroproxyl free radicals) are produced during the cycle's plasma phase, which together with hydrogen peroxide gas inactivate bacteria. Hydrogen peroxide gas plasma sterilization is an effective method for treating materials and equipment that are sensitive to high temperatures and humidity, such as endoscopes, electrical devices, and corrosion-prone metal alloys [9].

Cold atmospheric plasma (CAP) is identified as non-equilibrated plasma produced from air at near-atmospheric temperatures and pressure. It is made up of molecules, such as free electrons, radicals, and positive and negative ions. Owing to its broad range of inactivation effects against microbes (bacteria, fungi, viruses), it has intriguing properties in a variety of fields, including medicine, agriculture, food, and wastewater treatment, primarily through the production of reactive species that are lethal to cells, including charged particles, UV rays, energetic ions, and reactive oxygen and nitrogen species (ROS and RNS) [10]. Since ROS have a long half-life and potent antimicrobial properties, they are frequently identified as the main affecting species. They are produced when plasma discharges in air or oxygen-containing mixtures. Due to their ability to damage DNA and RNA, oxidize amino acids, disrupt cell membranes, and erode cell walls, they are essential to the pathogen inactivation mechanism [10,11].

Cold atmospheric plasma is a potent and versatile sterilization method that can combat a wide range of bacterial species (Gram-positive and Gram-negative), including vancomycin-resistant *enterococci* and methicillin-resistant *Staphylococcus aureus*. With subsequent treatments, CAP did not exhibit any bacterial adaptation [12,13].

In order to decontaminate materials surfaces from bacteria using CAP, plasma can be applied directly using a cold atmospheric pressure plasma jet (CAPPJ) or indirectly using a plasma-activated mist (PAMi) [14]. Both kinds of CAP systems (direct and indirect plasma sources) are leading in plasma medicine because of their many advantages in the biomedical field. They are now widely used to sterilize medical devices without causing any changes to the devices and leaving no chemical residues behind [2,15]. The treatment provided by traditional methods, as chemical treatments, is not proportionate to that provided by new techniques, such as cold plasma, since cold plasma technology involves a thorough disinfection and sterilization process in a very short exposure time for contaminated surfaces [16].

In the current study, we aimed to assess the bactericidal effect of direct plasma (CAPPJ) and indirect plasma (PAMi) as new methods of sterilizing stainless steel compared to autoclaving, which is considered the gold standard for sterilizing heat-stable material.

Material and Methods

This comparative experimental study was conducted in Medical Microbiology and Immunology Department, Faculty of Medicine, Zagazig University and Plasma & Energy Applications Research Laboratory, Department of Engineering Physics and Mathematics, Faculty of Engineering, Zagazig University. It included 36 *E. coli*, and 30 *S. aureus* strains isolated from randomly collected samples from hospitalized patients have bacterial infections at any site of the body at General surgery department, Zagazig University Hospitals during study time from January to April 2024.

Ethical consideration

This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by Institutional Review Board, Faculty of Medicine, Zagazig University (No:11251-9-1-2024). Written informed consent was taken from all patients participating in our study after illustrating the nature and aim of the study.

Bacterial strains isolation and identification:

Bacterial strains were isolated from different clinical specimens and identified by conventional methods. All samples were subjected to direct smear microscopic examination, cultivation on the suitable culture media; Nutrient agar (Oxoid, UK), blood agar (Oxoid, UK) and MacConkey's agar (Oxoid, UK) then were identified by their colonial morphology, microscopic examination of Gram-stained films and conventional biochemical reactions [17].

Antimicrobial susceptibility testing (AST):

All isolates were subjected to antibiotics susceptibility test using disc diffusion method. The interpretation of results was according to Clinical and Laboratory Standards Institute guidelines (CLSI) [18]. Multidrug resistant (MDR) strains were identified as non-susceptibility to at least one agent in three or more antimicrobial categories [19].

Preparation of bacterial cell suspensions:

A master suspension was prepared from each strain to be used in preparation of all samples exposed to different treatment methods. In addition to clinical isolates, we used quality control strains; *E. coli* ATCC 25922 and *S. aureus* ATCC 25923. Bacterial density was visually adjustment to 0.5 McFarland (Bio-Merieux, France) turbidity standard. Then the bacterial suspension was diluted (1/10) [20].

Preparation of samples:

A calibrated loop was used to distribute 10µl of this master suspension on identically sized sterile medical grade stainless steel AISI 316 coupons of cubic shape of 2.0 × 2.0 cm^2 surface area and 0.2 cm thickness (obtained from a local supplier), then air dried for 30 min [20]. Instantly following the drying procedure, the samples were exposed to different treatment methods, one of them not exposed to any treatment method and considered as untreated control. Medical grade stainless steel coupons with each test strain were subjected to 3 different treatment methods (CAPPJ, PAMi and autoclave) for different exposure time intervals, one coupon for each time interval. All the steps were performed in duplicates.

1-CAPPJ device: was designed and constructed by staff members of the physical and mathematical engineering department, Faculty of Engineering, Zagazig University, in which the plasma extends outward due to injection of compressed air between anode and cathode.

A copper cylinder with a 30 mm height and 54 mm outer diameter with a 3.2 mm diameter exit nozzle in the middle serves as the anode. The cathode is a 150 mm long and 3.2 mm diameter tungsten rod that is protected from outside by a ceramic insulator. The space between the cathode tip and the existing nozzle is where the discharge happens inside the torch **(Figure 1a)**. Electrical plasma characteristics were measured to ensure device performance validity [14, 21, 22]. At a distance of 3.5 cm from the nozzle, the sample surfaces were located and subjected to a plasma jet, exposure time intervals were as follow: 15, 30, 60, 120, 180, 240 and 300 seconds [14].

2- PAMi: a commercial mist maker with 10 ultrasonic transducers (DNYSYSJ Ult, China) immersed in a water-filled tank to create the mist generator. A ceramic disk with a diameter of 16 mm and a piezoelectric crystal makes up the ultrasonic transducer. When the transducer is submerged in water, it can transform high frequency electrical impulses into high frequency mechanical vibrations on the disc that result in a thin mist with droplet sizes of a few tens of microns. A Teflon tube is used to inject water mist into the area between the two electrodes, ensuring that the mist and the compressed air used as the working gas are well mixed **(Figure 1b)**. Samples were treated at a distance of 5 cm from electrodes, exposure time intervals were as follow: 15, 30, 60, 120, 180, 240 and 300 seconds [14].

3- Steam sterilization using autoclave (WiseClave, DAIHAN Scientific, South Korea): Samples of each strain were exposed to moist heat at 121ºC, 15 psi**,** holding time intervals were as follow; 60, 120, 180, 240 and 300 seconds.

Following treatment, 10 ml of sterile saline were added to each sample, and they were vortexed for one minute. The number of bacteria that survived was assessed by placing 10µl of the recovered suspension on nutrient agar plate using a calibrated loop, they were incubated for up to 24 hours at 37°C. After incubation, the number of colony forming units (CFU) was counted to evaluate the log reduction after each exposure time interval [23].

Sterility Assurance Level (SAL):

The Sterility Assurance Level (SAL), which measures the likelihood of a viable microorganism remaining on a sterilized medical device, is what determines how effective sterilization is. The SAL, expressed as 10^{-N}, is the expected probability of surviving organisms.

Typical SAL is 10^{-6} which means that the expected probability of any surviving microorganism after sterilization is 10^{-6}. SAL 10^{-6} is used to assure terminal sterilization of medical devices [24].

Time needed to reach medical SAL (T) for each method was calculated using the following formula: T= log (N_0-N) × D-value, where N_0= the number of microorganisms in the starting position and N=the number of microorganisms survived after each treatment of a given strain. D-value is the time required to achieve one log reduction (decrease in bacterial population by 10 times). It can be calculated by the formula: D-value =Time/ (Log a-Log b), Where a = the initial population and b = the survivors after a time interval. Average values were used for calculation. Treatment times were determined as SAL equivalent to (10^{-6}) [25].

Statistical analysis

All data were collected, tabulated and statistically analyzed using SPSS 26.0 for windows (SPSS Inc., Chicago, IL, USA). Quantitative data were expressed as the mean ± Standard Deviation (SD) while qualitative data were expressed as absolute frequencies (number) and relative frequencies (percentage). The independent sample t test was used to compare between normally distributed variables while categorical variables were compared using Chi square test. To compare quantitative data between more than two groups the one-way ANOVA test was used, Pairwise comparison post hoc test was done to identify differences between each of the two individual groups when the difference was significant. All tests were two-sided, *p*-value < 0.05 was considered statistically significant, *p*-value ≥ 0.05 was considered statistically insignificant [26].

Results

In this study, we evaluated bactericidal effect of direct plasma and indirect plasma compared to autoclave using two types of bacteria: *E. coli* and *S. aureus* representing Gram negative and Gram positive bacteria, respectively.

There was no statistically significant difference in bacterial count neither between *E. coli* ATCC: 25922 and *E. coli* clinical isolates nor *S. aureus* ATCC: 25923 and *S. aureus* clinical isolates using direct, indirect plasma and autoclave at different treatment time intervals as presented in **table (1)**, which indicated the validity of our results.

There was high statistically significant difference in bacterial count using direct and indirect plasma methods when comparing between both types of bacteria at different time intervals starting at the interval of 15 seconds till the complete sterilization. On the other hand, there was no statistically significant difference in bacterial count using autoclave when comparing both types of bacteria at different time intervals, **(Table 2)**.

It has been observed that *S. aureus* takes longer time than *E. coli* to achieve complete sterilization using direct plasma sterilization (180 seconds versus 120 seconds, respectively) and indirect plasma sterilization (300 seconds versus 240 seconds, respectively). Log reduction of bacterial count after different exposure time intervals to direct plasma, indirect plasma and autoclave is presented in **figure (2)**.

There was a high statistically significant relation between *E. coli* clinical isolates count and the exposure to direct plasma, indirect plasma and autoclave at 60 seconds interval. On doing post hoc test, we found that the difference was highly significant between autoclave and both direct and indirect plasma. Furthermore, there was a high statistically significant difference between indirect plasma and autoclave at 120 and 180 seconds intervals. Moreover, a high statistically significant relation between *S. aureus* clinical isolates counts and exposure to direct plasma, indirect plasma and autoclave at 60 and 120 seconds intervals was determined. On doing post hoc test, the difference was highly significant between autoclave and both direct and indirect plasma. Also, there was a high statistically significant difference between indirect plasma and autoclave at 180- and 240-seconds intervals, **(Table 3)**.

We detected that the time needed to reach medical SAL for both *E. coli* and *S. aureus* was shorter after exposure to direct and indirect plasma when compared to autoclave as presented in **table (4)**. The shortest time needed to reach SAL was recorded for direct plasma as 1.9 and 3.1 minutes for *E. coli* and *S. aureus*, respectively.

Results of antimicrobial susceptibility testing of clinical isolates reveal that, 24 (66.7%) of *E. coli* and 15 (50%) of *S. aureus* strains were MDR. By comparing the effect of direct, indirect plasma and autoclave on MDR and non-MDR *E. coli* and *S. aureus* clinical isolates there was no statistically significant difference between two groups at different treatment time intervals, **(Table 5).**

Table 1. Comparing bacterial count (cfu/ml) of *E. coli* ATCC: 25922 and *E. coli* clinical isolates, *S.aureus* ATCC: 25923 and *S.aureus* clinical isolates using direct, indirect plasma and autoclave at different treatment time intervals.

Treatment time (Seconds)	Direct plasma				Indirect plasma				Autoclave			
	***E. coli* ATCC: 25922** Mean ±SD	***E. coli* (n=36)** Mean ±SD	**Test (t)**	***p*-value**	***E. coli* ATCC: 25922** Mean ±SD	***E. coli* (n=36)** Mean ±SD	**Test (t)**	***p*-value**	***E. coli* ATCC: 25922** Mean ±SD	***E. coli* (n=36)** Mean ±SD	**Test (t)**	***p*-value**
Untreated Control	100567 ±498	100183 ±630	0.864	0.427	100567 ±498	100183 ±630	0.864	0.427	100567 ±498	100183 ±630	0.864	0.427
15	4000 ±489	3816 ±462	0.599	0.575	4500 ±245	3966 ±307	2.65	0.145	---	---	---	---
30	1033 ±124	1000 ±130	0.307	0.771	2067 ±205	1766 ±200	2.157	0.083	---	---	---	---
60	200 ±84	133 ±95	0.932	0.393	1200 ±82	1233 ±162	-0.395	0.708	67467 ±3101	66683 ±3473	0.347	0.742
120	ND	ND	----	----	900 ±79	866 ±112	0.542	0.610	55767 ±2639	54833 ±2911	0.467	0.659
180	ND	ND	----	----	135 ±47	166 ±75	-1.010	0.363	30167 ±1515	26833 ±2115	2.672	0.144
240	ND	ND	----	----	ND	ND	-----	-----	14500 ±572	13833 ±1675	0.846	0.435
300	ND	ND	----	----	ND	ND	-----	-----	5567 ±1195	4967 ±1870	0.519	0.625
	***S.aureus* ATCC: 25923** Mean ±SD	***S.aureus* (n=30)** Mean ±SD	**Test (t)**	***p*-value**	***S.aureus* ATCC: 25923** Mean ±SD	***S.aureus* (n=30)** Mean ±SD	**Test (t)**	***p*-value**	***S.aureus* ATCC: 25923** Mean ±SD	***S.aureus* (n=30)** Mean ±SD	**Test (t)**	***p*-value**
Untreated Control	100317 ±609	100266 ±850	0.131	0.901	100317 ±609	100266 ±850	0.131	0.901	100317 ±609	100266 ±850	0.131	0.901
15	13000 ±1632	12833 ±1366	0.176	0.867	19333 ±1247	21000 ±2421	-1.0813	0.129	---	---	---	---
30	6300 ±9320	6500 ±973	-0.657	0.540	6500 ±1122	7500 ±1660	-1.726	0.145	---	---	---	---
60	3033 ±817	3166 ±1085	-0.343	0.745	5067 ±946	5833 ±1415	-1.521	0.188	62667 ±1882	64667 ±4346	-0.835	0.441
120	167 ±47	200 ±83	-1.581	0.174	2100 ±48973	2516 ±764	-1.338	0.238	50667 ±1247	52167 ±3625	-0.815	0.451
180	ND	ND	----	----	733 ±235	900 ±316	-1.419	0.214	25677 ±1136	27167 ±2911	-0.868	0.424
240	ND	ND	----	----	149 ±54	183 ±69	-2.236	0.075	13333 ±1700	13667 ±1972	-0.254	0.809
300	ND	ND	----	----	ND	ND	-----	-----	6967 ±772	5650 ±830	2.159	0.083

(t): Independent samples t-test, SD: Standard deviation, *Statistically significant, ND: Not detected.

Table 2. Comparing bacterial count (cfu/ml) of *E. coli* and *S. aureus* using direct, indirect plasma and autoclave at different treatment time intervals.

Treatment time (Seconds)	Direct plasma				Indirect plasma				Autoclave			
	E. coli **(n=36)** Mean ±SD	***S.aureus*** **(n=30)** Mean ±SD	**Test (t)**	***p*-value**	***E. coli*** **(n=36)** Mean ±SD	***S.aureus*** **(n=30)** Mean ±SD	**Test (t)**	***p*-value**	***E. coli*** **(n=36)** Mean ±SD	***S.aureus*** **(n=30)** Mean ±SD	**Test (t)**	**p-value**
Untreated Control	100183 ±630	100266 ±850	-0.169	0.872	100183 ±630	100266 ±850	-0.169	0.872	100183 ±630	100266 ±850	-0.169	0.872
15	3816 ±462	12833 ±1366	-37.161	**<0.001****	3966 ±307	21000 ±2421	-41.870	**<0.001****	---	---	---	---
30	1000 ±130	6500 ±973	-33.577	**<0.001****	1766 ±200	7500 ±1660	-20.565	**<0.001****	---	---	---	---
60	133 ±95	3166 ±1085	-16.716	**<0.001****	1233 ±162	5833 ±1415	-19.383	**<0.001****	66683 ±3473	64667 ±4346	0.638	0.551
120	ND	200 ±83	-14.473	**<0.001****	866 ±112	2516 ±764	-12.808	**<0.001****	54833 ±2911	52167 ±3625	1.136	0.307
180	ND	ND	---	---	166 ±75	900 ±316	-13.479	**<0.001****	26824 ±2115	27167 ±2911	-0174	0.868
240	ND	ND	----	---	ND	183 ±69	-15.763	**<0.001****	13835 ±1675	13667 ±1972	0.110	0.916
300	ND	ND	----	----	ND	ND	-----	-----	4967 ±870	5650 ±830	-0.595	0.578

SD: Standard deviation, (t): Independent samples t-test, **Statistically highly significant, ND: Not detected

Table 3. Relation between *E. coli* and *S.aureus* clinical isolates count (cfu/ml) and direct plasma, indirect plasma and autoclave at different treatment time intervals**.**

Treatment time (Seconds)	***E. coli*** **(n=36)**				
	Direct plasma Mean ±SD	**Indirect plasma** Mean ±SD	**Autoclave** Mean ±SD	**Test**	**p-value**
60	133 ±95	1233 ±162	66683 ±3473	**(F)** 18.00	**<0.001****
	p1:0.669	**p2: <0.001****	**p3 :<0.001****		
120	ND	866 ±112	54833 ±2911	**(t)** -41.428	**<0.001****
180	ND	166 ±75	26833 ±2115	**(t)** -28.178	**<0.001****
240	ND	ND	13833 ±1675	--	---
300	ND	ND	4967 ±1870	--	---
	S. aureus **(n=30)**				
	Direct plasma Mean ±SD	**Indirect plasma** Mean ±SD	**Autoclave** Mean ±SD	**Test**	**p-value**
60	3166 ±1085	5833 ±1415	64667 ±4346	**(F)** 8.253	**<0.001****
	p1:0.293	**p2: <0.001****	**p3: <0.001****		
120	200 ±83	2516 ±764	52167 ±3625	**(F)** 9.429	**<0.001****
	p1:0.232	**p2: <0.001****	**p3: <0.001****		
180	ND	900 ±316	27167 ±2911	**(t)** -20.064	**<0.001****
240	ND	183 ±69	13667 ±1972	**(t)** -15.279	**<0.001****
300	ND	ND	5650 ±830	--	---

SD: Standard deviation, (F): One way ANOVA test, Pairwise post hoc test (p1: difference between direct and indirect plasma, p2: difference between direct plasma and autoclave, p3: difference between indirect plasma and autoclave), (t): Independent samples t-test, **statistically highly significant, ND: Not detected

Table 4. D-value and time needed to reach SAL (minutes) for different treatment methods.

Characteristic		*E. coli*	*S. aureus*
Direct plasma	**D-value**	0.3	0.6
	SAL time	1.9	3.1
Indirect plasma	**D-value**	0.4	0.8
	SAL time	2	4
Autoclave	**D-value**	2.2	2.6
	SAL time	11.2	12.6

SAL: Sterility assurance level, D-value: time needed to achieve one log reduction

Table 5. Comparing effect of direct, indirect plasma and autoclave at different treatment time intervals on bacterial count (cfu/ml) of MDR and non-MDR *E. coli* and *S.aureus* clinical isolates.

Treatment time (Seconds)	Direct plasma				Indirect plasma				Autoclave			
	***E. coli* MDR (n=24)** Mean ±SD	***E. coli* non-MDR (n=12)** Mean ±SD	**Test (t)**	***p*-value**	***E. coli* MDR (n=24)** Mean ±SD	***E. coli* non-MDR (n=12)** Mean ±SD	**Test (t)**	***p*-value**	***E. coli* MDR (n=24)** Mean ±SD	***E. coli* non-MDR (n=12)** Mean ±SD	**Test (t)**	***p*-value**
Untreated Control	99912 ±393	100750 ±437	-1.938	0.124	99912 ±393	100750 ±437	-1.938	0.124	99912 ±393	100750 ±437	-1.938	0.124
15	3675 ±496	4100 ±103	-0.977	0.383	3850 ±229	4263 ±305	-1.294	0.265	---	---	---	---
30	950 ±111	1100 ±237	-1.309	0.260	1750 ±121	1800 ±292	-0.240	0.821	---	---	---	---
60	127 ±70	206 ±94	-1.154	0.312	1325 ±192	1250 ±67	-0.147	0.889	66275 ±3804	67502 ±276	-0.377	0.752
120	ND	ND	----	----	875 ±129	793 ±49	0.214	0.840	54214 ±3240	56504 ±589	-0.885	0.425
180	ND	ND	----	----	200 ±78	163 ±65	1.787	0.148	26250 ±1920	28641 ±2308	-0.847	0.544
240	ND	ND	----	----	ND	ND	-----	-----	14250 ±1785	13257 ±1153	0.751	0.494
300	ND	ND	----	----	ND	ND	-----	-----	5275 ±2011	4350 ±1387	0.479	0.656
	***S.aureus* MDR (n=15)** Mean ±SD	***S.aureus* non-MDR (n=15)** Mean ±SD	**Test (t)**	***p*-value**	***S.aureus* MDR (n=15)** Mean ±SD	***S.aureus* non-MDR (n=15)** Mean ±SD	**Test (t)**	***p*-value**	***S.aureus* MDR (n=15)** Mean ±SD	***S.aureus* non-MDR (n=15)** Mean ±SD	**Test (t)**	***p*-value**
Untreated Control	100267 ±776	99967 ±498	0.459	0.669	100267 ±776	99967 ±498	0.459	0.669	100267 ±776	99967 ±498	0.459	0.669
15	13598 ±1418	12667 ±1247	0250	0.814	21589 ±2943	215968 ±1632	-0.612	0.573	---	---	---	---
30	7058 ±816	9650 ±598	1.224	0.287	7333 ±1929	8667 ±1247	-0.205	0.847	---	---	---	---
60	3667 ±942	2667 ±782	1.060	0.348	5567 ±1744	6183 ±828	-0.390	0.716	66045 ±5099	63343 ±2867	0.644	0.554
120	233 ±47	167 ±94	0.894	0.421	2633 ±1007	2432 ±294	0.341	0.768	54333 ±4189	51124 ±2449	0.679	0.533
180	ND	ND	----	----	1083 ±408	853 ±269	0.679	0.534	26250 ±1920	24852 ±1247	0.707	0.518
240	ND	ND	----	----	300 ±84	167 ±47	0.500	0.643	14126 ±2054	12336 ±1428	1.176	0.304
300	ND	ND	----	----	ND	ND	-----	-----	6251 ±804	5300 ±697	0.929	0.405

Figure 1. (a) Setup of the CAPPJ device, **(b)** Setup of the PAMi device.

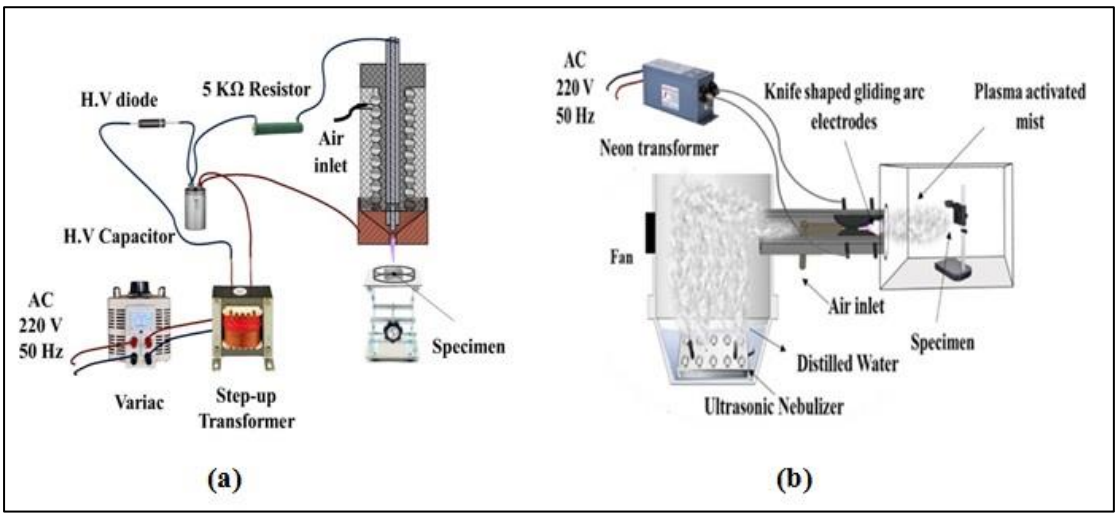
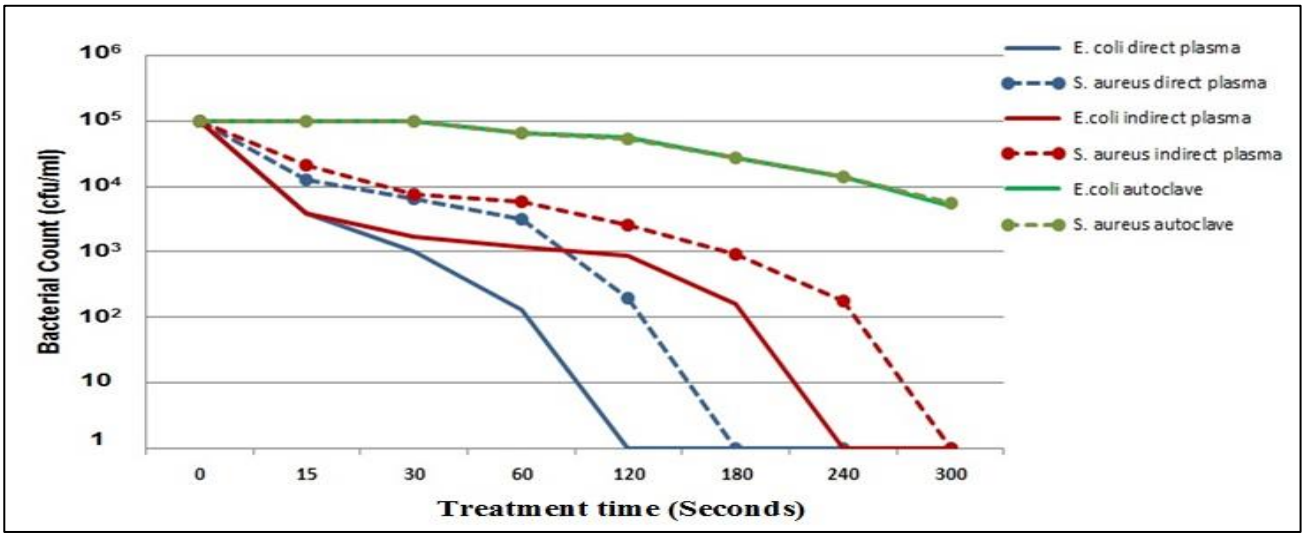


Figure 2. Log reduction of bacterial count after different exposure times to direct plasma, indirect plasma and autoclave



Discussion

Currently, healthcare associated infections (HAIs) are burgeoning. To ensure the sterility of medical devices and consequently reduce the burden of HAIs, more stringent evidence of the effectiveness of sterilization method is imperative [7,27]. Non-thermal atmospheric pressure plasma technology is a promising potential sterilization method; notably, it does not suffer from the disadvantages of conventional methods [7,28]. The antimicrobial effects of plasma are mediated through reactive chemical species, UV radiation, and electric fields, based on the type of gases as well as the methods employed for plasma generation [22, 29, 30].

In the present work, we assessed the exposure time for inactivation of microbes (*E. coli* and *S. aureus*) attacking medical surfaces, using CAPP and PAMi compared to autoclave as a gold standard for sterilization. The chosen strains are well-characterized Gram-negative and -positive bacteria that are widely used as model bacteria due to their rapid growth rates. They are also regarded as

major human pathogenic bacteria in hospital settings [31].

In this research, we recorded a statistically significant difference in bacterial count using CAPP and PAMi when comparing both types of bacteria at different time intervals. The complete sterilization was achieved in a shorter time by CAPP application (120 seconds for *E. coli* and 180 seconds for *S. aureus*) when compared to PAMi (240 seconds for *E. coli* and 300 second for *S. aureus*). Similar results to our study were recorded by **Yahaya et al**., 2021 as they found out that direct plasma treatment with air as a working gas has a better bactericidal effect with shorter time than indirect plasma activated water (PAW). These findings could be explained by the generation of less ozone concentration by PAW compared with direct plasma treatment using air. Moreover, the treated water's O2 concentration decreased as a result of the longer treatment period [32]. Also, **Asghar and his colleagues** (2021) observed that the inactivation rate increases with increasing exposure time to direct atmospheric pressure plasma jet compared to exposure to indirect plasma [33]. It is anticipated that indirect plasma takes longer time for bacterial inactivation; however, it may encompass a broad sterilization surface area that makes this method more advantageous than CAPP as its bacterial inactivation is restricted to the application surface. This could be accounted for by the wide surfaces that could be dispersed by the thin droplets of plasma mist.

The mechanism through which cold plasma inactivates bacterial cells is thought to be enhanced permeability of the cell wall or membrane, allowing intracellular components to leak out. Additionally, the oxidative damage caused by the plasma compounds to intracellular proteins and DNA renders bacteria inactive [34]. The primary inactivation factor appears to be reactive chemical species in the majority of cases, though this can change based on the type of plasma generated and whether the sample is subjected to direct or indirect plasma treatment [29]. **Han et al**., 2016 observed that *S. aureus* was primarily destroyed by intracellular damage, whereas *E. coli* was rendered inactive by leakage caused by damage to the cell envelope [11].

In this work, we elucidated that inactivation of *E. coli* was achieved in a shorter time when compared to *S. aureus*. In support of this notion, several studies reported that plasma treatment is more effective against Gram-negative than Gram-positive bacteria [35-37]. **Park et al**., (2019) recorded that following exposure to a non-thermal atmospheric-pressure biocompatible plasma sterilizer, the count of *E. coli* decreased by roughly 89.43 ± 4.35% for 30 minutes and 92.33 ± 3.74% for 60 minutes, respectively, while the count of *S. aureus* decreased by roughly 76.6 ± 1.23% for 30 minutes and 88.26 ± 6.23% for 60 minutes, respectively [35]. Moreover, **Barkhade and coworkers** (2024) found that the reduction in colony forming units of the bacteria was established in 60 min and 40 min for Gram-positive (*S. aureus*) and gram-negative *Salmonella Abony* (*S. Abony*) bacteria, respectively [36]. These findings could be attributed to different structures of both bacterial strains. Gram-positive bacteria's cell walls are composed of strong, tightly structured peptidoglycan, whereas the outer membrane of lipopolysaccharide and a thin layer of peptidoglycan cover Gram-negative bacteria. Produced ROS during plasma treatment have the ability to react with peptidoglycan and lipopolysaccharide, disrupting C-O-O, C-O-N, and C-O-C bonds and shattering the molecular structure and is the leading cause of bacterial mortality. This highly efficient sterilization method renders plasma a highly promising solution for hospitals, clinics, and daily life. [34, 38, 39].

Differential levels of antioxidant mechanisms, as reported in several reports, could be another explanation for the observed variation in *S. aureus*'s response compared to *E. coli* [38, 40,41]. Furthermore, disparities in the effectiveness of plasma treatments against different bacterial strains may be explained by the bacteria's differential production of enzymes like glutathione peroxidase, superoxide dismutase, and catalase [38].

In the current study, we tested autoclaving as the most robust, most common and cost-effective sterilization method. We found no statistically significant difference in bacterial count using autoclave when comparing both types of bacteria at different time intervals. This finding is in agreement with previous research as they reported autoclaving has same effectiveness against Gram-positive and Gram-negative bacteria [43,44].

We found a high statistically significant relation between decreasing in bacterial count and the exposure to direct plasma, indirect plasma and autoclave at 60 seconds interval for *E. coli* and 60 and 120 seconds intervals for *S. aureus*. In agreement, **Han et al**., (2016) found statistically

significant decrease in bacterial count of both *E. coli* and *S. aureus* by increasing treatment time of direct and indirect plasma from 1 to 5 minutes [11]. For our knowledge, this is the first study comparing the application of plasma and autoclave for bacterial inactivation of stainless-steel material used for medical instruments in hospital settings. However, a previous study compared the efficacy of utilization of autoclave, glutaraldehyde, and UV radiation for reduction in bacterial colonies, they observed that autoclave is the best method [45].

In the present work, we detected that the shortest time needed to reach medical SAL after application of direct plasma (1.9 min for *E. coli* and 3.1 min for *S. aureus*). Similarly, **Asghar and coworkers** (2021) found that a rapid inactivation process of *E. coli* was achieved within 80 s through the utilization of Atmospheric Pressure Plasma Jet with Dry and Wet Argon Discharges [33]. On the other hand, **Klämpfl et al.,** (2012) recorded that *Bacillus* spp. microbial load could be reduced by 6 $\log_{10}$ or 12 $\log_{10}$, respectively, in 5.3 or 10.6 minutes [25].

World Health Organization (WHO) and the US Centers for Disease Control (CDC) both have recognized the evolution of drug-resistant microorganisms as a significant public health issue in recent times [46,47]. There is unlikely to be a difference in the bactericidal action mechanism of plasma treatment between MDR and normal bacteria [29]. Our study found no significant difference between complete sterilization of MDR strains and non-MDR stains through the application of plasma and autoclave. This finding adds an advantage for plasma as a method for sterilization, as its effectiveness is not reduced or adapted by MDR organisms. In accordance with this finding, **Klämpfl et al**., (2012) demonstrated that the effect of plasma is not influenced by mechanisms of microbial resistance to antibiotics (innate or acquired). This is reasonable, since their experimental plasma system consisted of a mixture of various reactive species (UV photons, electric field, neutral reactive species, etc.) that contribute to the plasma inactivation process of microorganisms [25]. Many studies were gathered in review article showed that CAP is a highly effective bactericidal and it is difficult for bacteria to survive at appropriate exposure to it [48].

The present study has some limitations. We evaluated the efficacy of cold plasma on only two types of clinically isolated bacterial strains. Further research would be essential to elucidate the efficacy of plasma on other bacterial strains, spores, viruses and fungi. Also, we didn't test wrapped medical devices, more studies is needed to test efficacy of cold plasma in sterilization of wrapped items. This work compared the bacterial inactivation effect of cold plasma with autoclaving as a traditional method for eradicating bacteria from medical instruments. It would be beneficial to assess other conventional sterilization methods compared to plasma in future research.

Conclusion

CAPPJ and PAMi are rapid effective methods not only in eradication of the tested *E.coli* and *S.aureus* isolates but also in reaching SAL for stainless-steal medical instruments.These promising methods can save long time consumed by conventional methods for sterilization especially in emergencies.

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Competing interests

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Author contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by [Hanaa I. Abd El-Hady], [Amina A. Abdelhadi], [Mohamed El Shaer], and [Hossam Fayed]. Clinical specimens were collected by [Abd-Elrahman M. Metwalli]. The first draft of the manuscript was written by [Amina A. Abdelhadi] and [Hanaa I. Abd El-Hady]. All authors read and approved the final manuscript.

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