Antimicrobial efficacy of argon cold atmospheric pressure plasma jet on clinical isolates of multidrug-resistant ESKAPE bacteria

Sarthak Das [1], G. Veda Prakash [2], Sarita Mohapatra [2], Satyananda Kar [2], Satyendra Bhatt [2], Hitender Gautam [2], Gagandeep Singh [2], Arti Kapil [2], Bimal Kumar Das [2], Seema Sood [2], Immaculata Xess [2], Sudhir Chandra Sarangi [2], and Saumya Ranjan Mallick [2]

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Abstract

The rise in multidrug-resistant (MDR) ESKAPE bacteria have become a major therapeutic challenge globally. Recently, novel cold atmospheric pressure plasma (CAP) as an antimicrobial is becoming popular. In this study, an indigenously developed AC cold atmospheric pressure plasma jet (CAPJ) fed with argon gas was used to evaluate its antimicrobial efficacy on these bacteria isolated from clinical specimens such as urine, blood, and sputum in a tertiary care hospital in India. The difference in CAP's antimicrobial activity on Gram-negative bacilli (MDR E. coli) and Grampositive cocci (MDR S. aureus) was observed with various input parameters, such as microbial concentration, CAP exposure time, and exposure distance. It was observed that oxidative stress induced by reactive oxygen and nitrogen species (O_2^-, NO^+, $OH^\cdot$, H_2O_2, $ONOO^-$, $NO^\cdot$, $NO_2^\cdot$, $HO_2^\cdot$, O_3^-, etc.) and electrostatic stress by ions (Ar^+, O^+, O_2^-, OH^-, NO^+, OH^+, NO_3^-, NO_2^-, O_3^-, etc.) might play a crucial role in microbial inactivation. In addition to this, a decrease in adenosine triphosphate concentration post-CAP exposure in a liquid media suggested an efficient microbial inactivation effect. The outcome of this research would be extremely beneficial to multidisciplinary researchers in this field.

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Abstract: **The rise in multidrug-resistant (MDR) ESKAPE bacteria have become a major therapeutic challenge globally. Recently, novel cold atmospheric pressure plasma (CAP) as an antimicrobial is becoming popular. In this study, an indigenously developed AC cold atmospheric pressure plasma jet (CAPJ) fed with argon gas was used to evaluate its antimicrobial efficacy on these bacteria isolated from clinical specimens such as urine, blood, and sputum in a tertiary care hospital in India. The difference in CAP's antimicrobial activity on Gram-negative bacilli (MDR *E. coli)* and Gram-positive cocci (MDR *S. aureus)* was observed with various input parameters, such as microbial concentration, CAP exposure time, and exposure distance. It was observed that oxidative stress induced by reactive oxygen and nitrogen species (O_2^-, NO^+, OH^-, H_2O_2, $ONOO^-$, NO^-, NO_2^-, HO_2^-, O_3^-, etc.) and electrostatic stress by ions (Ar^+, O^+, O_2^-, OH^-, NO^+, OH^+, NO_3^-, NO_2^-, O_3^-, etc.) might play a crucial role in microbial inactivation. In addition to this, a decrease in adenosine triphosphate concentration post-CAP exposure in a liquid media suggested an efficient microbial inactivation effect. The outcome of this research would be extremely beneficial to multidisciplinary researchers in this field.**

Index terms: **Antimicrobial, cold atmospheric pressure plasma jet, multidrug-resistant ESKAPE bacteria, clinical isolates**

Abbreviations:

CAP: Cold atmospheric pressure plasma; **CAPJ:** Cold atmospheric pressure plasma jet; **RONS:** Reactive oxygen and nitrogen species; **MDR:** Multidrug-resistant; **MHA:** Mueller-Hinton Agar; **AST:** Antimicrobial susceptibility testing; **ATP:** Adenosine-triphosphate; **RLU:** Relative light unit; **CLSI:** Clinical and Laboratory Standards Institute

List of microorganisms:

E. coli: *Escherichia coli*; ***E. faecium:*** *Enterococcus faecium*; ***S. aureus:*** *Staphylococcus aureus*; ***K. pneumoniae:*** *Klebsiella pneumoniae*; ***A. baumannii:*** *Acinetobacter baumannii*; ***P. aeruginosa:*** *Pseudomonas aeruginosa*

This work did not involve human subjects or animals in its research. All authors declare that they have no known conflicts of interests in terms of competing financial interests or personal relationships that could have an influence or are relevant to the work reported in this paper. This work is ethically approved by ethics committee of All India Institute of Medical Sciences, New Delhi with IAEC No: 206/IAEC-1/2019. This work was funded by IIT Delhi – AIIMS MI-FIRP program (MI02150).
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I. INTRODUCTION

The discovery of antibiotics has been a boon for humanity against infectious diseases caused by bacteria [1]–[4]. However, the recent emergence of antimicrobial resistance (AMR) in ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *and Enterobacteria spp.*) bacteria have emerged as a major therapeutic challenge [5]–[9]. In India, there has been a recent trend of an increase in the prevalence of multidrug-resistant (MDR) ESKAPE bacteria in healthcare facilities, which has raised concerns [10]–[16]. The current increase in MDR bacteria necessitates the development of novel antimicrobial strategies. In this regard, the cold atmospheric pressure plasma (CAP) technique has recently gained popularity for its antimicrobial efficacy.

CAP consists of electrons, positive and negative ions, atoms and molecules, UV radiation, electric field, etc. The interaction of these species with ambient air generates reactive oxygen and nitrogen species (RONS) such as $OH^{\cdot}$, $NO^{\cdot}$, $NO_2^{\cdot}$, etc. that plays a crucial role in the microbial inactivation by CAP [17]–[24]. The antimicrobial efficacy of CAP has been investigated in-vitro on various bacteria such as *E. coli* (from Family of *Enterobacteria*) [25]–[28], *S. aureus* [27]–[31], *P. aeruginosa* [28]–[33], *K. pneumoniae* [30], [31], *A. baumannii* [30], [34], etc. Further, CAP's antimicrobial efficacy has also been studied in-vivo [35]–[38], which initiates CAP's usage in clinical aspects. The outcome of these studies was quite encouraging and insights CAP to be a viable technique in the future.

Although there are numerous studies on CAP's antimicrobial property, the efficacy of CAP on clinically isolated MDR bacteria had been rarely investigated. Daeschlein et al. [39] studied the antimicrobial activity of argon CAP (Dielectric barrier discharge CAP, Pulsed CAP jet, and Non-pulsed CAP jet) on MDR and non-MDR isolated from acute or chronic wounds. The study demonstrated CAP's efficacy in inactivating all isolates, regardless of the CAP device used. Also, argon CAP was highly effective in inactivating *Methicillin-resistant* and *Methicillin-sensitive S. aureus* isolated from various clinical sites [40]. The efficacy of CAP on MDR *A. baumannii* [34], [41] and *P. aeruginosa* [33] from different health care centres have also been reported. The increasing prevalence of different MDR isolates with various drug-resistant genes such as extended-

spectrum beta-lactamase, carbapenem, etc., necessities a region-specific study on CAP activity. Hence, the study on clinical isolates with information regarding their source and drug-resistant profile would unveil CAP's clinical potency.

Thus, this in-vitro study aimed to investigate the antimicrobial efficacy of the indigenously developed AC argon cold atmospheric pressure plasma jet (CAPJ) on the MDR ESKAPE bacteria isolated from different clinical specimens such as blood, urine, and sputum received at a tertiary care hospital from New Delhi, India. To the best of our knowledge, this study on CAP's antimicrobial activity on these bacterial isolates that originated in India would be the first of its kind. The presence of species such as RONS and ions in CAP discharge was investigated qualitatively (optical emission spectroscopy) and quantitatively (mass spectroscopy). The role of these identified RONS and ions in the bacterial inactivation via inducing oxidative and electrostatic stress was analysed in detail. Further, this work also investigates the CAP's antimicrobial activity on Gram-negative bacillus (MDR *E. coli*) and Gram-positive cocci (MDR *S. aureus*) with variation in parameters, such as microbial concentration, CAP exposure distance, and exposure time. Also, a quantitative measurement of the adenosine triphosphate (ATP) pre- and post-CAP exposure at variable microbial concentrations in liquid media was performed. This study's findings would be extremely useful to multidisciplinary researchers in this field.

The following is the structure of the current manuscript. Section II provides specifics on the experimental methods and diagnostic tools that were used in the investigation. Section III presents the findings of the research. Section IV contains a discussion of various outcomes and the reasoning behind them. The summary is provided in Section V.

II. MATERIALS AND METHODS

The study was conducted in collaboration between Indian Institute of Technology Delhi (IITD), New Delhi and All India Institute of Medical Science (AIIMS), New Delhi. Ethical permission was taken prior to the study. Preliminarily, a cold atmospheric pressure plasma jet (CAPJ) was developed at IITD and transferred to AIIMS for the study. Fig. 1 depicts the image of CAPJ.

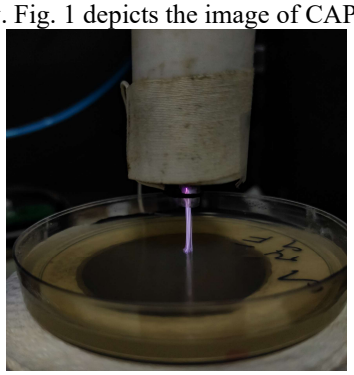

Fig. 1. Cold atmospheric pressure plasma jet.

A. Cold atmospheric pressure plasma jet and characterization:

The cold atmospheric pressure plasma jet (CAPJ) used in this study consists of a Pyrex glass tube of 4 mm inner diameter and thickness of 1 mm. The electrodes were arranged in a cross-field configuration. For a high voltage electrode, a copper rod of diameter 1.5 mm was coaxially fed through the glass tube. A 1 mm diameter copper wire was wrapped around the glass tube as a ground electrode. Further, an indigenously developed high voltage (0 – 6.5 kV_{p-p}) and high frequency (25 kHz) AC power supply was applied to the electrodes. In the presence of the applied high voltage of 6.5 kV_{p-p} and argon gas at 3 lpm flow rate, CAP discharge was ignited with a plume length of ~ 3 cm in ambient air. Fig. 2. (a) depicts the schematic of the experimental setup. The applied voltage and discharge current (which includes both conduction and displacement current) waveform were recorded using a digital storage oscilloscope (DSOX3024T, Keysight InfiniiVision, 200 MHz bandwidth, 5 GSa/s) via a voltage probe (Tektronix P6015A) and current transformer (Model 8590C, Pearson Electronics Inc., 1.0 V/A). Fig. 2. (b) depicts the typical voltage and current profile of the CAPJ system at an applied voltage of 6.5 kV_{p-p} and a frequency of 25 kHz. The current leading the voltage signifies the capacitive nature of the CAP discharge. Further, the multiple spikes observed in the current waveform in each half cycle indicate the formation of filamentary micro discharge in the argon CAP. The average applied power to the CAPJ system over a cycle was estimated to be ~ 8.18 W (Average applied power $= \frac{1}{T}\int_0^T V(t)I(t)\mathrm{d}t$) at an applied voltage of 6.5 kV_{p-p} and 25 kHz frequency. Moreover, the CAP plume temperature was measured to be ~ 35 °C using a standard K-type thermocouple (Nicety DT 1311). The thermocouple probe tip was insulated and calibrated for the measurement of plume temperature. The details of the developed CAPJ system can be found elsewhere [22]. All the experiments in this study were performed at a fixed applied voltage of 6.5 kV_{p-p}, 25 kHz frequency, and argon gas at a flow rate of 3 lpm.

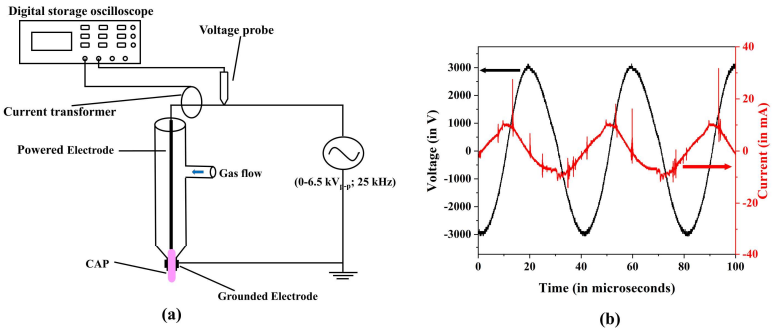

Fig. 2. (a) Schematic of the experimental setup; (b) Voltage and current waveform.

The optical emission spectra were recorded along the plume length at 1 and 2 cm from the nozzle using the 0.9 nm resolution spectrometer (Ocean optics HR 4000). The emission profile was collected in the wavelength range of 190 to 1100 nm using a 200 µm optical fibre cable arranged perpendicular to the CAP plume direction at a 5 mm distance. For the OES detection, the integration time was 100 ms. The spectroscopic data were also plotted using Origin Pro 2021, and the species were identified using the NIST Database [42].

The molecular beam mass spectrometer (MBMS) (HPR 60 MBMS, Hidden Analytics Ltd., UK) was used to study the species (positive and negative ions) composition of the CAP plume. The MBMS was operated in time-averaged mode during data acquisition in the range of 1-100 amu (m/z). To ensure

minimal discharge disturbances, the CAP plume's tip was set at a fixed distance of 10 mm from the MBMS sampling orifice, aligned centrally. The relative yield percentage of a specific ion was evaluated by the given formulae.

$$\text{Relative yield (\%)} = (Y_i / \sum_i Y_i) \times 100$$

Where Y_i = Count intensity of a specific ion

$\sum_i Y_i$ = Sum of the count intensity of all the ions (in the range 1 – 100 amu) present in the CAP plume

B. Collection and isolation of bacteria species:

Clinical samples received in the Bacteriology Lab, Department of Microbiology, All India Institute of Medical Sciences (AIIMS), New Delhi from patients at the outdoor and indoor patient department were processed following standard operating procedures. Significant bacterial growth was identified using MALDI-TOF mass spectrometer (VITEK MS 410895, BIO MERIUX). Antimicrobial susceptibility testing (AST) of all the identified isolates was performed using automated AST machine (VITEK-2 COMPACT, BIO MERIUX). The results of the AST were interpreted in accordance with the Clinical and Laboratory Standard Institute (CLSI) 2021 guidelines. Table I shows the details of bacterial isolates and their antimicrobial susceptibility profile determined by above method and used in this study.

TABLE I
BACTERIAL ISOLATES AND THEIR ANTIMICROBIAL SUSCEPTIBILITY PROFILE.

Bacterial isolates	**Isolated**	**Resistant to drugs**	**Susceptible to drugs**
E. coli	Blood	Ceftazidime, ciprofloxacin, cefoperazone sulbactam, and piperacillin-tazobactam	Meropenem, tigecycline, amikacin, cotrimoxazole, colistin, gentamicin, and imipenem
S. aureus	Blood	Cefoxitin, cotrimoxazole, ciprofloxacin, erythromycin, and penicillin	Gentamicin, linezolid, vancomycin, and teicoplanin
K. pneumoniae	Urine	Amikacin, ceftazidime, ciprofloxacin, cefoperazone sulbactam, piperacillin-tazobactam, meropenem, imipenem, nitrofurantoin, and netilmicin	-
P. aeruginosa	Sputum	Amikacin, ceftazidime, ciprofloxacin, cefoperazone sulbactam, piperacillin-tazobactam, meropenem, and imipenem	Colistin, and netilmicin
E. faecium	Urine	Amikacin, ciprofloxacin, penicillin, vancomycin, teicoplanin, rifamycin, and vancomycin	Cotrimoxazole, linezolid, and fosfomycin
A. baumannii	Sputum	Amikacin, ceftazidime, ciprofloxacin, cefoperazone sulbactam, piperacillin-tazobactam, cefoxitin, meropenem, and imipenem	Colistin

C. CAP exposure on ESKAPE isolates in solid media:

The isolated bacteria were sub-cultured on blood agar and incubated overnight at 37 ºC. Isolated colonies of the bacteria were picked and suspended in sterile 0.9 % saline with a final concentration of 0.5 McFarland standard (checked using a densitometer, DENSICHECK PLUS, BIO MERIUX) which is equivalent to 10^8 CFU/ml. The above suspension was serially diluted to 10^5, 10^4, 10^3, 10^2, and 10^1 CFU/ml. Suspension of all dilutions was streaked on Mueller-Hinton Agar (MHA) (HiMedia) in a quadruplet (three for the CAP exposure and one as control). CAP exposure was performed in triplicate for 300 s by moving the CAP plume over the streaked lines at an exposure distance of 2 cm. All the Petri dishes (including the control and CAP treated) were incubated overnight at 37 ºC. The very next day, the colonies were counted semi-quantitatively, and the inactivation percentage was calculated using the following formulae.

$$\text{Inactivation percentage (\%)} = ((N_0 - N)/N_0) \times 100$$

where, N_0: Number of colonies observed in control

N: Number of colonies observed on CAP exposure of 300 s

D. CAP exposure on *E. coli* and *S. aureus* in solid media with parametric study:

For studying the parameters such as CAP exposure distance and exposure time, we used Gram-negative bacillus *E. coli* and Gram-positive cocci *S. aureus*. The 0.5 McFarland bacterial suspension was diluted to 10^5 CFU/ml in 0.9 % saline. The 30 µl of the above diluted suspension was spread over MHA in a Petri dish via the spreading method by a sterile disposable swab. CAP exposure was performed in triplicate for 30, 60, 120, 180, 240, and 300 s at variable exposure distances of 1 and 2 cm. All the Petri dishes (including the control and CAP treated) were incubated overnight at 37 ºC. The inactivation area of the CAP treated Petri dish was measured by Image J2 software the next day. All the experiments were performed in triplicate, and then the mean inactivation area and standard deviation was calculated.

E. CAP exposure on *E. coli* and *S. aureus* in liquid media and adenosine-triphosphate (ATP) measurements:

The 0.5 McFarland bacterial suspension was serially diluted to 10^5, 10^4, and 10^3 CFU/ml in 0.9 % saline. The CAP exposure on the above suspension was performed for 300 s at an exposure distance of 2 cm. The ATP measurement of the sample to detect the viability of the isolates in pre- and post-CAP exposure was evaluated through ATP swabs using an ATP luminometer (3 M Clean Trace Luminometer LX25). The measurement was expressed in the relative light unit (RLU).

III. RESULTS

The results showed high microbial inactivation of the ESKAPE isolates at various concentrations for CAP exposure at a 2 cm distance for 300 s. While comparing the microbial inactivation due to 300 s CAP exposure, a higher inactivation was observed with lower concentrations of bacteria, i.e., 10^2 and 10^1 CFU/ml (Fig. 3). At 10^2 and 10^1 CFU/ml, 100 % inactivation was observed for all types of isolates. However, there were variations in microbial inactivation for other concentrations. Also, the percentage of inactivation among Gram-positive cocci (*S. aureus* and *E. feacium*) was observed less at a higher concentration of suspension (i.e., 10^5 – 10^3 CFU/ml) in comparison to Gram-negative bacteria (*E. coli*, *K. pneumoniae*, *P. aeruginosa*, and *A. baumannii*).

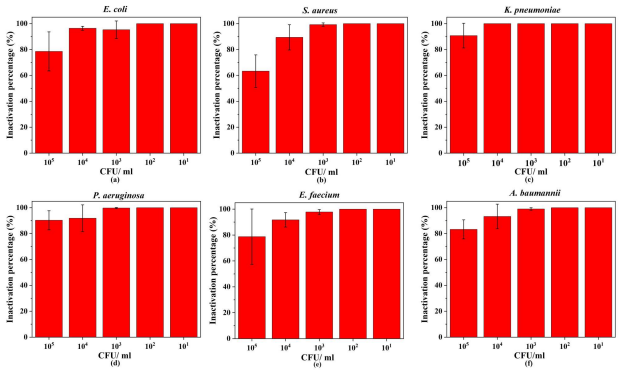

Fig. 3. Inactivation percentage of (a) *E. coli* (b) *S. aureus* (c) *K. pneumoniae* (d) *P. aeruginosa* (e) *E. faecium*, and (f) *A. baumannii* at CAP exposure time of 300 s with variation in microbial concentration (Error bars indicate standard deviation for CAP exposure in triplicate).

The variation in CAP antimicrobial activity was observed with variation in exposure time and distance on bacteria isolates. The images of the control and CAP exposed *E. coli* (Fig. 4) and *S. aureus* (Fig. 5) are shown. The dependency of CAP's microbial inactivation efficacy on exposure time variation showed similar trends for *E. coli* and *S. aureus* (Fig. 6). An increase in exposure time resulted in an increase in microbial inactivation area. It was also observed that the Gram-positive *S. aureus* is less susceptible to CAP exposure compared to Gram-negative *E. coli*.

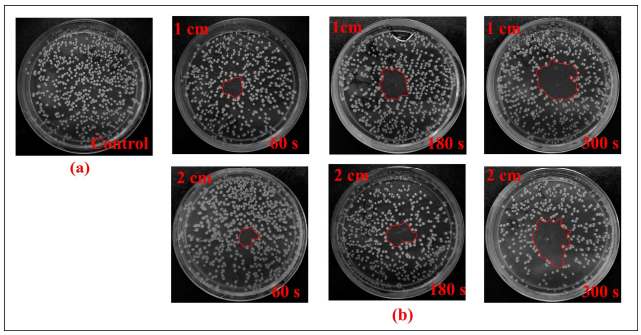

Fig. 4. Lawn culture of *E. coli* on pre- and post-CAP exposure- (a) Control (b) Top row shows CAP exposure at 1 cm distance with increase in exposure time from left to right; Bottom row shows CAP exposure at 2 cm distance with increase in exposure time from left to right.

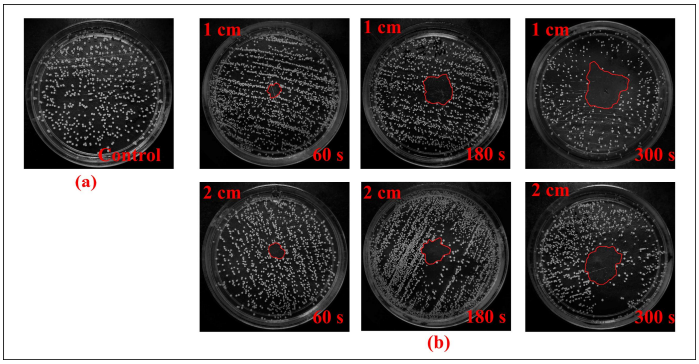

Fig. 5. Lawn culture of *S. aureus* on pre- and post-CAP exposure- (a) Control (b) Top row shows CAP exposure at 1 cm distance with increase in exposure time from left to right; Bottom row shows CAP exposure at 2 cm distance with increase in exposure time from left to right.

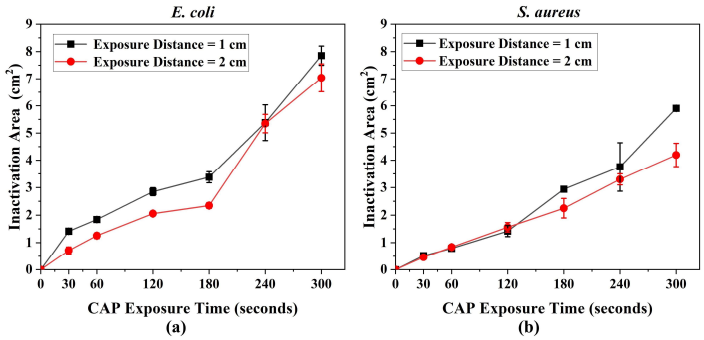

Fig. 6. Inactivation area as a function of CAP exposure time for (a) *E. coli* and (b) *S. aureus* at variable exposure distance (Error bars indicate standard deviation for CAP exposure in triplicate).

Further, as CAP exposure distance increased, the microbial inactivation area in *E. coli* decreased. The observed difference in antimicrobial activity was investigated by recording optical emission spectra at 1 and 2 cm along the length of the CAP plume (Fig. 7). Along the plume's length, there was a reduction in the intensity of CAP species, i.e., for hydroxyl (OH) (308.04 and 309.11 nm), argon (Ar I) (in the range of 696.53 to 965.59 nm) and atomic oxygen (O I) (777.19 and 844.63 nm). However, an increase in the intensity of nitrogen (N_2) (337.08, 357.52, and 380.28 nm) species was observed. These species significantly impact the formation of reactive oxygen and nitrogen species (RONS) such as hydroxyl radicals ($OH^{\cdot}$), nitric oxide radical ($NO^{\cdot}$), hydrogen peroxide (H_2O_2), etc., which play a significant role in the microbial inactivation effect, discussed in section 4. However, no significant difference in CAP activity was observed in the case of *S. aureus* up to 120 s CAP exposure time (Fig. 6. (b)). Further, an increase in CAP exposure time showed a difference in the inactivation area.

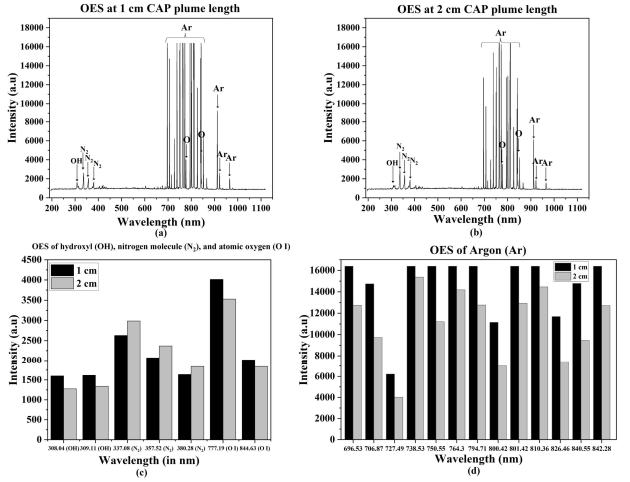

Fig. 7. Emission spectra plot at (a) 1 cm and (b) 2 cm distance, and comparison between intensity of (c) OH (308.04 and 309.11 nm), N_2 (337.08, 357.52, and 380.28 nm), and O I (777.19 and 844.63 nm) and (d) Ar I (696.53 to 842.28 nm) at exposure distance 1 and 2 cm.

The ion composition of the CAP plume was recorded by mass spectra over a range of 1 – 100 amu. Fig. 8 depicts the mass spectra of positive and negative ions. In our study, singly charged positive and negative ions were observed in relative abundance. The relative yield of O^+ (53.9 %) was the highest among the positive ions. Additionally, other positive ions O_2^+ (9.01 %), OH^+ (9.01 %), H_2O^+ (7.21 %), N^+ (7.14 %), H_3O^+ (5.47 %), and NO^+ (2.27 %) were significantly observed in the CAP plume. Also, the negative ions such as $HN_2O_3^-$ (24.17 %), $N_2O_2^-$ (10.92 %), NO_3^- (8.21 %), O_2^- (6.97 %), HO_2^- (5.13 %), O^- (4.92 %), OH^- (2.24 %), NO_2^- (2.90 %), O_3^- (1.36 %), etc. were observed in the plume. These positive and negative ions detected in the CAP plume play prominent role in microbial inactivation, which is discussed in section 4.

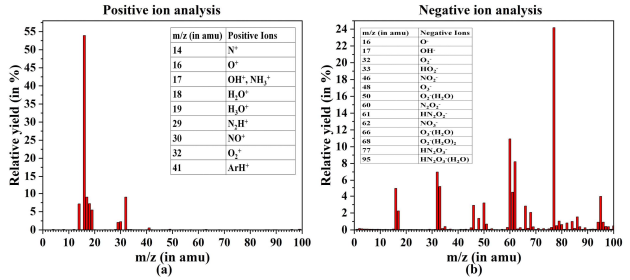

Fig. 8. Mass spectra plot of (a) positive ions and (b) negative ions in the range 1 – 100 amu (m/z).

The bacterial suspensions of different concentrations in a liquid media with pre- and post-CAP exposure were tested for their viability using an ATP luminometer. A decrease in ATP value was observed on 300 s CAP exposure in both *E. coli* and *S. aureus*, as indicated in Table II.

TABLE II
COMPARISON OF ATP MEASUREMENT AMONG THE DIFFERENT CONCENTRATIONS OF PRE- AND POST-CAP EXPOSED BACTERIAL SUSPENSION.

Bacteria	Concentration (CFU/ml)	RLU pre-CAP exposure (a.u)	RLU post-CAP exposure (a.u)
E. coli	10^5	6606	1503
	10^4	397	278
	10^3	44	27
S. aureus	10^5	351	314
	10^4	130	65
	10^3	44	29

IV. DISCUSSION

The rise in MDR bacteria has become a global issue, necessitating a sustainable solution. The CAP bacterial inactivation efficacy and its mechanism of microbial inactivation have gained attention globally. This study focussed on the effectiveness of CAP on MDR ESKAPE clinical isolates found in India, along with parametric studies and microbial inactivation mechanism.

The CAP species, such as electrons, ions, excited and neutral atoms and molecules, radicals, etc., play a crucial role in microbial inactivation. In this study, we investigated the presence of species in CAP discharge qualitatively (OES) and quantitatively (MBMS). Ar I and O I, along with traces of OH and N_2 lines, were found in the optical emission spectra of the CAP plume. Dissociative and excited processes are the primary means by which these species are formed [43]. Additionally, positive (O^+, O_2^+, OH^+, H_2O^+, N^+, H_3O^+, NO^+, etc.) and negative ($HN_2O_3^-$, $N_2O_2^-$, NO_3^-, NO_2^-, O^-, HO_2^-, OH^-, O_2^-, etc.) ions in the CAP discharge were quantified through MBMS. Positive ions are generated by direct electron impact ionization, while negative ions are by dissociative electron attachment process [44]. These identified gas phase species (O I, O_2^-, OH^-, NO^+, OH^+, $HN_2O_3^-$, $N_2O_2^-$, NO_3^-, HO_2^-, NO_2^-, and O_3^-) signifies the presence of non-radical reactive oxygen and nitrogen species (RONS) in the CAP. Further, species like Ar I, N_2, N^+, H_2O^+, H_3O^+, $HN_2O_3^-$, $N_2O_2^-$, etc., would possibly play a role in the generation of radical RONS ($OH^\cdot$, $NO^\cdot$, $NO_2^\cdot$, $HO_2^\cdot$) and non-radical RONS (H_2O_2, $ONOO^-$) through reaction cascades. This involves direct ionization, dissociative ionization, photodecomposition, dissociative excitation, dissociative recombination, ion recombination, etc. [45]–[48]. From this cocktail of RONS produced in the discharge, radicals ($OH^\cdot$, $NO^\cdot$, $NO_2^\cdot$, and $HO_2^\cdot$) along with H_2O_2, O I, O_2^-, and $ONOO^-$ might directly attack the bacterial cell membrane constituting lipid bilayer and proteins. These species initiate lipid peroxidation and protein oxidation by the hydrogen atom abstraction mechanism, resulting in the loss of structural integrity in bacteria [17], [49]–[52]. Also, the interaction of gas phase species (RONS, excited species, and ions) with the embedded water in the solid media results in the formation of secondary RONS ($OH^\cdot$, H_2O_2, ONOOH, $NO^\cdot$, $NO_2^\cdot$, $HO_2^\cdot$ etc.) at the target site [45], thus inducing oxidative stress.

It is well-established that the bacterial cell wall is negatively charged due to presence of teichoic acid in Gram-positive and lipopolysaccharide in Gram-negative bacterial cell wall [53], [54]. When these negatively charged cell wall is exposed to CAP, low energy electrons and negative ions would repel from the cell wall with respect to their response times (much lesser than positive ions) [55]. Thus, a positive ion sheath (a space charge region) will be formed around the cell at the CAP-target interaction site [56]. As the cell interaction time scale approaches ion response time, the positive ions (Ar^+, O^+, O_2^+, OH^+, H_2O^+, N^+, H_3O^+, NO^+, etc.) detected in the CAP plume might bombard the cell wall inducing electrostatic stress. When this stress exceeds the tensile strength of the bacterial cell wall,

it is ruptured [51], [52], [57]. It may be noted that the high energetic electrons and negative ions could impinge the cell wall through the ion sheath. This chemical and physical invasion by RONS and ions on the bacterial cell membrane might result in membrane poration or permeabilization. Also, the transporter proteins in the cell membrane assist the movement of RONS and ions into the cell [52], [58]. Thus, cytoplasmic components such as proteins, deoxyribonucleic acid (DNA), etc. may be readily damaged by a burst of RONS and ions entering the bacterial cell through the pores or assisted by transporter proteins.

Thus, in our study, the identified excited species (Ar I, O I, OH, N_2) and ions (Ar^+, O_2^-, OH^-, NO^+, OH^+, NO_3^-, HO_2^-, NO_2^-, O_3^-, etc.) along with radicals ($OH^{\cdot}$, $NO^{\cdot}$, $NO_2^{\cdot}$, and $HO_2^{\cdot}$) in the CAP might play a significant role in bacterial inactivation. Fig. 9 depicts the schematic of the CAP-target interaction predicted from our study.

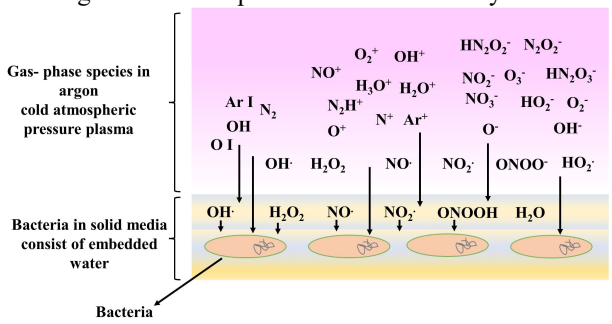

Fig. 9. Schematic of the CAP-target interaction predicted from our study.

The effectiveness of CAP's microbial inactivation was analysed in relation to bacterial concentration. A decrease in CAP activity at high bacterial concentrations might be due to the stacking effect, which restricts the interaction of the CAP species (Ar I, $OH^{\cdot}$, H_2O_2, $ONOO^-$, $NO^{\cdot}$, $NO_2^{\cdot}$, $HO_2^{\cdot}$, OH^+, NO^+, NO_3^-, NO_2^-, O_2^-, HO_2^-, etc.). Further, a variation in inactivation among all the isolates at greater than 10^2 CFU/ ml (e.g., at 10^5 CFU/ml and 300 s CAP exposure, inactivation was 78.56 % for *E. coli*, 63.30 % for *S. aureus*, etc.) might be due to the structural difference between different bacterial isolates [17], [53], [54], [59], [60], thus CAP susceptibility varies. Additionally, a significant difference in inactivation area between the *E. coli* (Gram-negative bacillus) and *S. aureus* (Gram-positive cocci) was noticed on variable CAP exposure time and distance. The peptidoglycan layer of the bacterial cell wall distinguishes Gram-positive and Gram-negative [17], [54]. The Gram-positive bacteria have a peptidoglycan thickness of 30-100 nm, whereas Gram-negative bacteria have a thickness of 1-8 nm [53], [54], [60]. The higher thickness in Gram-positive bacteria restricts the penetration of CAP species, making them less susceptible.

In this study, an increase in inactivation for both drug-resistant Gram-negative bacillus (MDR *E. coli*) and Gram-positive cocci (MDR *S. aureus*) was observed with an increase in CAP exposure time. This result is concordant with previous studies [22], [25], [26], [61]. The prolonged interaction of CAP species (Ar I, $OH^{\cdot}$, H_2O_2, $ONOO^-$, $NO^{\cdot}$, $NO_2^{\cdot}$, $HO_2^{\cdot}$, OH^+, NO^+, NO_3^-, NO_2^-, O_2^-, HO_2^-, etc.). with the target might account for the increased antimicrobial efficacy. The study on variation in CAP exposure distance showed a different trend for the isolates. The decrease in intensity variation of Ar I, OH, and O I might have contributed to the significant difference in CAP's activity against *E. coli* when exposed at different exposure distances. An increase in the intensity of N_2 species might not be enough to make up for any potential loss in RONS generation due to decrease in Ar I, OH, and O I species. However, for *S. aureus* at lower exposure time, the variation in the distance did not influence the antimicrobial activity of CAP. Until 120 s of CAP exposure time, the induced pace of chemical reaction (as discussed earlier) at the target site by the CAP reactive species might be nearly equivalent for 1 and 2 cm exposure distance for *S. aureus*. As the amount of time that CAP species interact with the target increases, a sudden rise in chemical activity over the target is expected. It might be attributed to temporal variation in the relative abundance of RONS and ions present in CAP discharge. This temporal variation might also depend on the target nature (Gram-positive and Gram-negative isolates) and exposure distance. So, the CAP–target interaction is highly dependent on the correlation between the nature of the target, CAP exposure time, and distance. Thus, with an increase in exposure time (greater than 120 s), comparatively higher RONS and ions at a 1 cm than 2 cm exposure distance might contribute to significantly higher *S. aureus* inactivation. This hypothesis on the dependency of CAP's antimicrobial activity on exposure distance would be investigated further by focusing on other bacterial isolates and recording temporal variation spectra of the discharge.

We also investigated the metabolic activity of isolated *E. coli* and *S. aureus* at variable microbial concentration (10^5 – 10^3 CFU/ ml) in liquid media pre- and post-CAP exposure. In general, the metabolic capacity in a cell is governed by the energy derived from adenosine triphosphate (ATP). A change in ATP content indicates the response of a cell to any stress, such as chemical and physical. Also, when a cell dies, ATP synthesis ceases [62][63]. Thus, cell viability or the microbial load in suspension could be estimated from the ATP measurements. In our study, the reduction in ATP values post CAP exposure on bacterial isolates indicated a decrease in metabolic activity, signifying the antimicrobial property of CAP.

V. SUMMARY

(i) ESKAPE bacteria isolated from clinical specimens were successfully inactivated using an indigenously developed AC argon cold atmospheric pressure plasma jet.

(ii) With increase in CAP exposure time, inactivation area increased for MDR *E. coli* and *S. aureus*, possibly due to prolonged interaction of CAP reactive species with the target.

(iii) In the case of *E. coli*, a 1 cm CAP exposure distance resulted in a greater inactivation area than 2 cm. It could be attributed to a decrease in the intensity of CAP species such as Ar I, OH, and O I along the length of the CAP plume.

(iv) There was a significant difference in CAP activity between *E.* coli and *S.* aureus, which instigates that CAP has a target-dependent behavior.

(v) The CAP species reacts with ambient air and the embedded water in the target, producing highly reactive oxygen species that are essential for microbial inactivation. In this study, the identified excited species (Ar I, O I, OH, N_2) and ions (Ar^+, O_2^-, OH^-, NO^+, OH^+, NO_3^-, HO_2^-, NO_2^-, O_3^-, etc.)

along with radicals ($OH^{\cdot}$, $NO^{\cdot}$, $NO_2^{\cdot}$, and $HO_2^{\cdot}$) might play a significant role in microbial inactivation via physical and chemical mechanisms.

(vi) A reduction in ATP value post CAP exposure on bacterial isolates signifies a microbial inactivation effect.

(vii) This in-vitro study's findings pave the way for future in-vivo research. Further, this type of developed CAP jet system could be used for various biomedical applications such as sterilization and disinfection of medical instruments, wound decontamination, etc.

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