

# Sub-lethal exposure to cold atmospheric plasma *in vitro* induces changes in bacterial antibiotic resistance profiles. A pilot study

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## Abstract

To evaluate the effect of sub-lethal exposure to cold atmospheric plasma (CAP) on their antibiotic resistance, meticillin-resistant *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Streptococcus mutans*, and *Candida albicans* were exposed *in vitro* to a commercially available CAP. This antimicrobial CAP inhibited growth but changed survivors' antibiotic resistance.

**Keywords:** cold atmospheric plasma, antimicrobial drug resistance.

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## Introduction

Passing an impedance-controlled electric current through atmospheric air produces a glow discharge between the electrodes. The resulting ionized air is referred to as cold atmospheric plasma (CAP) that contains reactive nitrogen and oxygen species, including ozone. Reportedly, CAP has efficacy against diverse pathogens, including *Staphylococcus aureus*,<sup>1,2</sup> *Pseudomonas aeruginosa*,<sup>3</sup> and *Candida albicans*.<sup>4</sup> Killing is attributed to microbial cell-membrane damage.<sup>5</sup> There are diverse CAP generation and delivery systems, including single or multiple plasma jets<sup>6</sup> and dielectric barrier discharges.<sup>7</sup>

Professionals involved in the prevention of healthcare associated infections must be aware of the commercial availability and use of CAP devices both in healthcare facilities and at home, with potential applications ranging from disinfection of inert surfaces to the treatment of chronic wounds.<sup>6-8</sup>

At a time when antibiotic resistance poses a growing challenge, it has been observed that a sub-lethal exposure of *S. aureus* to argon plasma may change the antibiotic resistance profile in the surviving colonies.<sup>9</sup> The aim of this pilot investigation was to assess *in vitro* the antimicrobial efficacy of a commercially available battery-powered CAP delivery system and to test the surviving colonies for possible changes in their antibiotic resistance profiles.

## Materials and methods

### Plasma generation system

We tested a commercially available US-patented CAP device. This portable battery-powered generator has a dielectric plate array with multiple plasma emitters, collectively intended to produce a 3.5 X 6 cm corona discharge. This CAP generator operates in atmospheric air to release reactive nitrogen and oxygen species, including ozone, with negligible ultraviolet (UV)-C emission. Manufacturer's instructions indicate that the array is to be handheld  $\leq 1$  cm directly above the patient's skin.

### Test microorganisms

The following strains with known antibiotic resistance profiles were tested. Two methicillin-resistant *S. aureus* (MRSA) ATCC 43300; and USA300 NRS643; *Staphylococcus epidermidis* NRS 101; *P. aeruginosa* ATCC

25619; *Escherichia coli* ATCC 10586; *Streptococcus mutans* ATCC 25175; and *C. albicans* ATCC 10231.

### Microbiological procedures

Each test strain was suspended in tryptic soy broth (Bioxon, Mexico) and its concentration adjusted using a MacFarland Standard, serial dilutions were tested to optimize the inoculum to  $10^5$  cfu/mL. Each bacterial strain was seeded in triplicate onto blood agar (Bioxon) and Mueller-Hinton agar (Bioxon). *Candida* spp. was seeded in triplicate onto dextrose agar with chloramphenicol (Bioxon), and onto Sabourad agar (Bioxon).

Immediately after seeding, using a plastic spacer the array was held 4 mm above the agar, allowing the microorganisms' uniform immersion into the glow of the CAP-corona at the maximum power setting (3176 Hertz) for 10 minutes. After aerobic incubation at 37°C for 24 hours, the plates exposed to CAP were examined and from each culture, a surviving colony was retrieved and seeded in tryptic soy broth (Bioxon). The survivor's antibiotic or antifungal susceptibility or resistance was evaluated after 24 hours' incubation at 37°C.

### Antibiotic and Antifungal susceptibility testing

To screen for changes in the antibiotic resistance profile, the kit API ATB-G5 (BioMérieux, France) was used as per manufacturer's instructions. This kit challenges bacteria with 21 antibiotics, some of them in two concentrations, allowing a preliminary minimal inhibitory concentration (MIC) determination: amoxicillin, amoxicillin+clavulanic acid, piperacillin, piperacillin+tazobactam, ticarcillin, ticarcillin+clavulanic acid, cephalothin, cefoxitin, cefotaxime, ceftazidime, cefepime, cefuroxime, meropenem, imipenem, ceftazidime 1, cotrimoxazole, tobramycin, amikacin, gentamicin, netilmicin, and ciprofloxacin. Antibiotic concentrations in ATB-G5 correspond with Clinical Laboratory Standards Institute (CLSI) standards. For comparison against CAP exposed survivors, the unexposed strain was included as control.

For MRSA strains ATCC-43300 and USA300, antimicrobial disk-susceptibility tests were performed, as indicated in the CLSI method,<sup>10</sup> against nine additional antibiotics; fosfomycin, trimethoprim-

sulfamethoxazole, penicillin G, vancomycin, tetracycline, erythromycin, oxacillin, clindamycin, and cephalothin (Polidiscos. Productos Biológicos de México, Mexico). Fluconazole and nystatin discs (Oxoid. Hants, UK) were used for *C. albicans* ATCC 10231 on Sabourad agar.

## Results

For all type strains tested, exposure of seeded plates to a sub-lethal dose of CAP consistently inhibited colony growth in the area directly under the array's corona discharge. The typical rectangle-shaped inhibition of growth is shown in Figure 1.

API ATB-G5 analysis of the surviving colonies, retrieved from the triplicate's inhibition areas, consistently revealed changes in acquired resistance or susceptibility to antibiotics (Table I). MRSA ATCC-43300 became susceptible (from 8 mg/L to < 2 mg/L) to cefotaxime and cefepime. MRSA strain USA300 showed no change. *Staphylococcus epidermidis* became resistant to ceftazidime (from 8 mg/L to 16 mg/L and susceptible (from 8 mg/L to < 2 mg/L) to cefotaxime. *Pseudomonas aeruginosa* became susceptible to five antibiotics and resistant to amoxicillin (from 2 mg/L to 8 mg/L) and ciprofloxacin (from <1 mg/L to 1 mg/L). *Escherichia coli* acquired resistance to five antibiotics most notably ceftazidime and amikacin (from <2 mg/L to 16 mg/L) and became susceptible to imipenem. *Streptococcus mutans* acquired resistance to five antibiotics most notably piperacillin, ticarcillin and ceftazidime (from <2 mg/L to 16 mg/L) and became susceptible to five.

Antibiotic disc testing revealed that MRSA USA300 became resistant to trimethoprim-sulfamethoxazole while the resistance profile of MRSA ATCC-43300 remained unchanged. *Candida albicans* 10231 remained resistant to fluconazole and susceptible to nystatin.

## Discussion

The results of this pilot study demonstrate that the corona discharge from a commercially available battery-powered CAP array has antimicrobial activity against MRSA, *S. epidermidis*, *P. aeruginosa*, *E. coli*, *S. mutans* and *C. albicans*.

The widely reported broad spectrum antimicrobial efficacy of CAP makes it a promising technology with diverse applications ranging from disinfection of inert surfaces to antimicrobial treatment of skin and mucosal lesions.<sup>6-8</sup> However, Lührmann *et al.* described increased resistance in MRSA surviving exposure to an argon plasma.<sup>9</sup> Our observations provide additional evidence that sub-lethal exposure to CAP may induce changes in the antibiotic resistance profile, where increased antibiotic resistance is a serious cause for concern.

Future quantitative research will address MICs and the microbial mechanisms involved in acquiring resistance or gaining susceptibility, which are beyond the scope of the present investigation.

Not all CAP generation and delivery systems are equal in power or intended applications. Other research groups can adjust their CAP systems to study the *in vitro* effects of sub-lethal exposure, determine MICs and screen surviving colonies for the expression of resistance genes.

During evaluation of CAP for wastewater treatment, it was documented that at lower plasma intensities, the wastewater itself shielded *E. coli* and MRSA cells from CAP and that the cell's components slowed the degradation of intracellular antibiotic resistance genes.<sup>11,12</sup> In a similar manner, eliminating all viable microorganisms from wounds will present a challenge in clinical use, where microbial pathogens will be embedded in mixed-species biofilms, deep in the lesion, surrounded by tissue and exudate. Moreover, observations from industry indicate that hydroxyl radicals and ozone in CAP degrade antibiotics,<sup>13</sup> which has potential relevance for patients receiving conventional antibiotic treatment and CAP therapy on their wounds.

Our results provide evidence that warrants a note of caution for manufacturers and potential users of CAP. To render this technology effective and safe for use on human patients, the exposure to CAP must be optimized, adjusting diverse variables such as the field's intensity, application time, and standardized distance between the array and the lesion. Moreover, the anti-microbial application of CAP onto skin or mucous membranes requires stringent validation.

The community of professionals dedicated to infection control must be aware of the potential risks derived from the misuse of diverse CAP devices, including those versions now available for personal use.

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### Potential conflicts of interest

All authors report no conflicts of interest relevant to this article.

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**Table 1. API ATB G-5 comparison of antibiotic (mg/L) resistance or susceptibility after sub-lethal exposure to Cold Atmospheric Plasma (CAP).**

	AM	AC	PI	TI	CF	CX	CZ	C1	AK	GE	CT	CM	FE	ME	IM	TO	NE	CI
<b>MRSA 43300</b>	CAP										<2		<2					
	C										8		8					
<b>S. epidermidis 101</b>	CAP					16					<2							
	C					8					8							
<b>P. aeruginosa 25619</b>	CAP	8	8						<2				<2	<2			<2	1
	C	<2	16						16				16	4			8	<1
<b>E. coli 10586</b>	CAP				8		16	1	16						<2			1
	C				<2		<2	<1	<2						4			<1
<b>S. mutans 25175</b>	CAP	8	16	16	<2	<2	16	<1	4			<2						<2
	C	<2	<2	8	8	8	<2	1	<2			8						4

MRSA USA300 showed no change and is not included.

The system API ATB G-5 contains 21 antibiotics only those with changes are shown.

AM-amoxicillin; AC-amoxicillin+clavulonic acid; PI-piperacillin; TI-ticarcillin; CF-cephalothin; CX-cefoxitin; CZ-ceftazidim; C1-ceftazidime 1; AK-amikacin; GE-gentamicin; CT-cefotaxim; CM-cefuroxim; FE-cefepim; ME-meropenem; IM-impipenem; TO-tobramycin; NE-netilmicin; CI-ciprofloxacin.

CAP is the exposed strain

C is the unexposed strain

Blank cell = No change



**Figure 1.** Typical area of inhibited growth after direct exposure to a sub-lethal dose of Cold atmospheric Plasma. The array was held 4 mm above the agar at 3176 Hertz for 10 min.

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