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Evaluation of the Impact of Hydrocarbon-Generated Soot on Antibiotics Susceptibility of *Staphylococcus aureus* and *Escherichia coli* Isolates

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Abstract

Background: The antibiotic susceptibility of bacterial interaction with soot, a by-product of incomplete combustion of fossil fuel, has not been established. **Aim:** The study aimed to establish the antibiotics susceptibility of *Staphylococcus aureus* and *Escherichia coli* exposed to soot. **Method:** The bacteria were exposed to 12.5%, 25%, and 50% concentrations of soot at different time intervals. Control bacterial cultures without exposure to soot were also carried out. These cultures were incubated for 24 hrs. The numbers of surviving bacteria were determined by analyzing 10 µL of the incubated cultures at 6 hrs and 24 hrs on tryptone soy agar. Again, the bacteria were inoculated on Mueller Hinton agar and subjected to antibiotics susceptibility testing using the disk diffusion method. **Results:** After 6 hrs of exposure, the number of *E. coli* in the absence of soot was $102.50 \pm 3.54 \times 10^3$ CFU/mL while at 12.5%, 25%, and 50% of soot, the surviving *E. coli* were 26.00 ± 1.41 ($p = 0.0012$), 21.00 ± 1.41 ($p = 0.0011$) and 5.50 ± 2.12 ($p = 0.0009$) $\times 10^3$ CFU/mL respectively. Similarly, the population of *S. aureus* without soot was $122.5 \pm 3.53 \times 10^4$ CFU/mL while at 12.5%, 25.0% and 50.0% of soot, the surviving *S. aureus* 46.00 ± 2.83 ($p = 0.0017$), 23.00 ± 1.41 ($p = 0.0007$) and 11.50 ± 2.12 ($p = 0.0007$) $\times 10^4$ CFU/mL respectively. Similar results were obtained after 24 hrs of exposure. The soot shows some level of potency in reducing the number of *E. coli* and *S. aureus* significantly ($p < 0.05$). After 24 hrs, almost all treatment conditions (except for the Gentamicin for *S. aureus*), there was resistance to all the antibiotics while at 0 hr there was sensitivity to these drugs. **Conclu-**

sion: These results suggest that while soot has some potency on *E. coli* and *S. aureus*, their exposure to soot could induce resistance.

Keywords

Soot, Drug Resistance, Virulence, Biofilm, Antibiotics

1. Introduction

Black carbon commonly called soot has detrimental effects on human health and the environment [1]. Soot can affect the ecosystem because it spreads in the atmosphere and settles on soil, plants, and water. A study had shown that inhalation of soot can cause inflammation, cardiovascular function alteration, oxidative stress, macrophage function impairment, and also host damage [2]. However, there is a paucity of data on the effect of soot on bacteria response. Bacteria are the major cause of respiratory infection. They also play an important role in the functioning and diversity of the normal microbiome, which is essential for the maintenance of human health. Soot production has increased because of the introduction of illegal refineries producing petroleum products locally known as "Asari" or "Kpo-fire" in the Niger Delta region especially Rivers State. It is necessary to understand the role of soot on bacteria. The effects of soot are observed using a Gram-positive bacterium, *Staphylococcus aureus* and a Gram-negative bacterium, *Escherichia coli*.

Air pollution constitutes the largest among all the environmental risks according to WHO. A principal source of air pollution is industrial activities. Niger Delta including Port Harcourt in several studies has emphasized to have poor air quality [3]. Fumes from diesel exhaust are the major source of soot in developed countries while indoor burning of biomass for heat and fuel is the major source in developing countries [4]. Across the United Kingdom in 2014, soot level ranged from 1 to 7 μg/m^3 and average of the country-wide was 1.6 μg/m^3 [5]. Furthermore, North America and Europe account for about only 13% of global black carbon emissions whereas developing countries such as Africa accounts for about 80% with China and India being the biggest global contributors.

A study carried out to assess the level of air pollutions in a Niger Delta city in Nigeria had provided a correlation of the association of air-borne diseases and soot [3]. This study concluded after an air quality survey in four different locations, from emission sources at varying distances (60, 100, and 500 m) that all the samples complied with the guidelines by the department of petroleum resources (DPR) for annual average except one location whose annual means exceeded specification. Plumes of soot which constitute major air pollution have affected residents within the environment [4] [5] [6] [7].

This study was aimed at studying the antibiotic susceptibility of *E. coli* and *S. aureus* exposed to soot.

2. Methods

2.1. Materials

Incubator, autoclave, distilled water, conical flask, ruler, Bunsen flame, weighing balance, petri dish, bijou bottles, sterile universal containers, wire loop, tryptic soya broth medium, nutrient agar medium, alcohol, micropipette, measuring cylinder, sensitivity disks (gentamycin (CN), ampicillin (APX), ciprofloxacin (CPX), streptomycin (S) and septrin (SXT)) purchased from Oxoid, UK.

2.2. Collection of Hydrocarbon-Generated Soot

The soot was collected from a bunker (illegal refinery) in Isaka, Port Harcourt, River State, Nigeria.

2.3. Test Organisms

The organisms used for the study were *E. coli* ATCC 252922 and *S. aureus* from Lahor Research Laboratories, Benin, Nigeria after identification using specific 16S rRNA gene.

2.4. Media Preparation

Thirty grams (30.0 g) of tryptone soy broth and 11.2 g of nutrient agar were weighed out and dissolved in 400 mL of distilled water. The media were sterilized at 121˚C for 15 minutes at 15 psi using an autoclave. The agar was allowed to cool to about 47˚C and 20 mL was aseptically poured into each sterile petri dish. The plates were allowed to set and solidify at room temperature and placed upside down to avoid excessive moisture on the surface of the medium. They were stored in the refrigerator for subsequent uses.

2.5. Preparation of Stock Solution of Soot

The stock soot was prepared by dissolving 5.0 g of the soot in 100 mL of absolute alcohol and diluted with 100 mL of water making 5.0 g of soot in 50% alcohol.

2.6. Soot Exposure Studies

The organisms were exposed to soot at 3 different concentrations and time intervals. The first concentration (12.5%) was 1.5 mL of stock and 4.5 mL of distilled water with 10^2 CFU/mL of each bacterium. The second concentration (25%) was 3 mL of stock and 3 mL of distilled water with 10^2 CFU/mL of bacteria. The third concentration (50%) was 6 mL of stock with 10^2 CFU/mL of bacteria in a bijou bottle. The different concentrations were incubated at 0, 6, 12, and 24 hrs. 50 µL of the incubated cultures from different conditions were inoculated on already dried nutrient agar plates for enumeration.

2.7. Antimicrobial Susceptibility Assay

A volume of 100 µL of the different concentrations of soot exposed to both organisms was mixed with 5 mL of broth and incubated. Susceptibility testing was

done with commercially prepared antibiotics disk using the agar-disk diffusion method according to the following procedures. They were carried out at different time intervals which were 0, 6, 12, and 24 hrs. Discrete colonies from a pure culture were inoculated into peptone water and incubated at 37˚C for 16 hrs. The turbidity of the incubated cultures was standardized against 0.5 McFarland's standard using sterile peptone water to obtain comparable turbidities. Antibiotics susceptibility testing was done using the conventional gold standard disc diffusion method by Clinical Laboratory Standard Institute [8]. Mueller-Hinton agar (MHA) plate was inoculated with the aid of a sterile cotton swab stick dipped into the standardized bacterial culture and allowed to dry. Antibiotic discs were aseptically placed on the surface of the MHA. The cultured MHA plate was incubated at 37˚C for 16 hrs and the zone of antibiotic clearance was measured.

2.8. Data Analyses

All experiments were performed in duplicate and on two independent occasions. Results were presented as mean ± SD where necessary. Where appropriate, statistical analyses were performed using an unpaired t-test in which a two-tailed *p*-value was calculated (GraphPad Prism Software Version 5.03, San Diego, CA). Statistical significance was defined as a *p*-value of less than 0.05 at a 95% confidence interval.

3. Results

3.1. Effect of Soot on Bacteria Growth at 6 Hours

Figure 1 shows the number of surviving *E. coli* and *S. aureus* after exposure to different concentrations of soot in 6 hrs. The number of *E. coli* in the absence of soot (control) was $102.50 \pm 3.54 \times 10^3$ CFU/mL while in the presence of different concentrations of soot at 12.5% soot, 25.0%, and 50.0%, the surviving *E. coli* were 26.00 ± 1.41 ($p = 0.0012$), 21.00 ± 1.41 ($p = 0.0011$) and 5.50 ± 2.12 ($p = 0.0009$) × 10^3 CFU/mL respectively in comparison with the control. Similarly, the population of *S. aureus* without soot was $122.5 \pm 3.53 \times 10^4$ CFU/mL while in the presence of different concentrations of soot at 12.5% soot, 25.0%, and 50.0%, the surviving *S. aureus* were 46.00 ± 2.83 ($p = 0.0017$), 23.00 ± 1.41 ($p = 0.0007$) and 11.50 ± 2.12 ($p = 0.0007$) × 10^4 CFU/mL respectively in comparison with the control. These results show an overall significant decline (p-value of <0.05) in the population of the bacteria in the presence of soot compared to the unexposed control.

3.2. Effect of Soot on Bacteria Growth at 24 Hours

Figure 2 shows the number of surviving *E. coli* and *S. aureus* after exposure to different concentrations of soot in 24 hrs. The level of *E. coli* without soot was $247.50 \pm 3.54 \times 10^3$ CFU/mL while in the presence of different concentrations of soot at 12.5% soot, 25.0%, and 50.0%, the surviving *E. coli* were 126.00 ± 1.41 ($p = 0.0005$), 23.50 ± 2.12 ($p = 0.0002$), and 3.00 ± 1.41 ($p = 0.0001$) × 10^3 CFU/mL

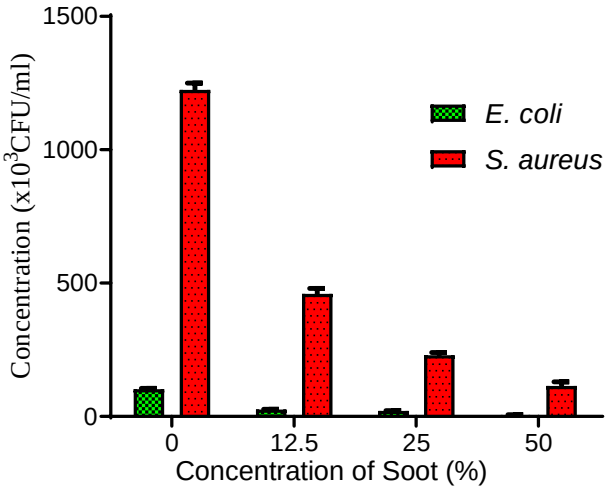

Figure 1. The population of *E. coli* and *S. aureus* after exposure to different concentrations of soot for 6 hrs. The error bars represent the standard deviation of measurements for 2 separate experiments.

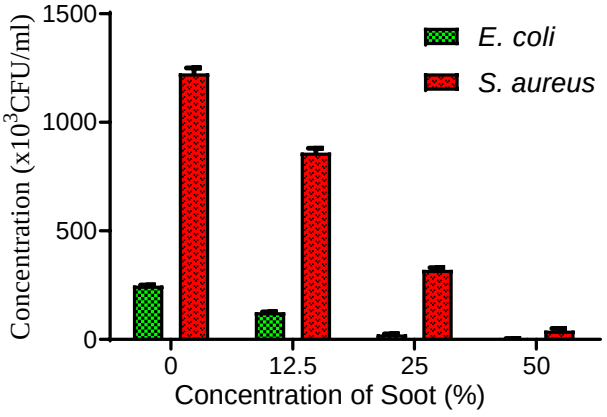

Figure 2. The number of *E. coli* and *S. aureus* after exposure to different concentrations of soot for 24 hrs. The error bars represent the standard deviation of measurements for 2 separate experiments.

respectively in comparison with the control. Similarly, the population of *S. aureus* without soot was 122.50 ± 3.53 × 10^4 CFU/mL while in the presence of different concentrations of soot at 12.5% soot, 25.0%, and 50.0%, the surviving *S. aureus* 86.00 ± 2.83 (p = 0.0079), 32.00 ± 1.41 (p = 0.0009) and 4.00 ± 1.41 (p = 0.0005) × 10^4 CFU/mL respectively in comparison with the control. These results depict an overall significant reduction in the population of the bacteria in the presence of soot.

3.3. Effect of Antibiotics on *E. coli* and *S. aureus* Exposed to Soot

Table 1 shows the antibiotic susceptibility pattern of *E. coli* exposed to soot at different hours. There was a gradual reduction in the sensitivity to gentamycin with an increase in the duration of exposure. This further led to resistance development after 24 hrs exposure for 12.5% soot. No zone of inhibition was also

detected after 6, 12, and 24 hrs exposures for 25.0% and 12 hrs and 24 hrs for 50.0% soot. Again, Ampicillin showed complete resistance for all the concentrations of soot at all the duration of exposure. Furthermore, ciprofloxacin showed relatively moderate sensitivity for the 0.0% soot across the different duration of *E. coli* exposed to 12.5% and 50.0% soot showed resistance after 6, 12, and 24 hrs, while the 25.0% showed resistance at 24 hrs. Also, both streptomycin and septrin showed a high zone of clearance for 0.0% soot across the different duration of exposure, while the other concentrations showed no zone of clearance at 6, 12, and 24 hrs.

Table 2 shows the antibiotic susceptibility pattern of *S. aureus* exposed to soot at different durations. The *S. aureus* exposed to the 0.0% soot showed relatively the same level of sensitivity to gentamycin. All the *S. aureus* at 0 hr exposure showed a moderate level of zones of clearance. These zones of inhibition were reduced with an increase in the duration of exposure and in some cases no zone of clearance was detected. Contrary to the observation in *E. coli*, ampicillin showed zones of inhibition for *S. aureus* for all the conditions of exposure. Similar to the observations noted in *E. coli*, streptomycin and septrin showed zones of clearance for 0.0% soot across the different durations of exposure for *S. aureus*, while the other concentrations showed no zone of clearance at 6, 12, and 24 hrs.

Table 1. Antibiotic susceptibility of *E. coli*.

Soot [%]	CN				APX				CPX				S				SXT			
	Duration [hrs]				Duration [hrs]				Duration [hrs]				Duration [hrs]				Duration [hrs]			
	0	6	12	24	0	6	12	24	0	6	12	24	0	6	12	24	0	6	12	24
0.0	23	22	20	21	0	0	0	0	27	27	26	28	26	27	27	28	18	17	19	17
12.5	24	17	12	0	0	0	0	0	26	0	0	0	26	0	0	0	18	0	0	0
25.0	22	0	0	0	0	0	0	0	28	16	14	0	25	0	0	0	20	0	0	0
50.0	22	14	0	0	0	0	0	0	25	0	0	0	24	0	0	0	19	0	0	0

Keys: CN-Gentamicin, APX-Ampicillin, CPX-Ciprofloxacin S-Streptomycin, SXT-Septrin. The figures in the table represent the diameters of the zones of inhibition in mm.

Table 2. Antibiotic susceptibility of *S. aureus*.

Soot [%]	CN				APX				CPX				S				SXT			
	Duration [hrs]				Duration [hrs]				Duration [hrs]				Duration [hrs]				Duration [hrs]			
	0	6	12	24	0	6	12	24	0	6	12	24	0	6	12	24	0	6	12	24
0.0	24	24	24	25	14	13	14	14	19	28	29	28	27	28	27	28	13	13	14	14
12.5	23	0	28	0	10	0	0	0	18	0	0	0	24	0	19	0	13	0	0	0
25.0	26	0	0	14	12	0	0	0	17	0	0	0	25	0	0	0	15	0	0	0
50	25	23	15	0	11	0	0	0	14	0	0	0	24	18	0	0	14	0	0	0

Keys: CN-Gentamicin, APX-Ampicillin, CPX-Ciprofloxacin S-Streptomycin, SXT-Septrin. The figures in the table represent the diameters of the zones of inhibition in mm.

4. Discussion

There is a gradual increase in the development of resistance to antibiotics globally according to World Health Organisation in 2018 [9]. This has created a lot of concerns regarding the possible factors causing this phenomenon. This study examined the growth rate during bacteria exposure to soot evaluated which is similar to a method previously used [10]. However, they used the effect of catecholamine while this current study used soot. At 6 hrs exposure of *E. coli* and *S. aureus* to 12.5% concentration of soot there was a greater number of colony counts. As the concentration was increased to 25% and 50%, the number of colonies reduced respectively. At 24 hrs, a greater number of colonies were also observed at the lower concentrations of 12.5% and 25% compared to 50%, respectively. The colonies on the controls were significantly higher than those obtained from the different concentrations.

This simply means that the colony counts of the bacteria observed in this study were indirectly proportional to the concentration of soot they were exposed to. Also, there was a reduction in the number of colonies as the concentration of soot increases. This could mean that the soot had inhibitory effects on both gram-negative and gram-positive bacteria [11]. This observation could have resulted from the ability of the alcohol to act as an adjuvant to the soot, thereby making it more antimicrobial.

When *E. coli* was not exposed to soot, it was susceptible to gentamycin (CN), ciprofloxacin (CPX), streptomycin (S), and septrin (SXT) but resistant to ampicillin (APX). Whereas, after *E. coli* was exposed to soot, it was no more susceptible to the antibiotics mentioned above after 24 hrs but still remained resistant to APX. For CN, as the concentration of soot increases, the bacteria became more resistant with increased duration of exposure. For APX, the bacterium was resistant to it with and without soot. A similar observation reported that 83% of *E. coli* isolates showed resistance to APX [12]. For CPX, as the concentrations of soot were increased at increasing time interval, *E. coli* became resistant. In S and SXT, the bacterium was susceptible without soot but at the different concentrations of soot and time interval, it became resistant.

When *S. aureus* was not exposed to soot, it was susceptible to gentamycin (CN), ciprofloxacin (CPX), streptomycin (S), ampicillin (APX), and septrin (SXT). However, when *S. aureus* was exposed to soot, it became resistant to the above antibiotics. As the concentration of the soot was increased, with longer periods of exposure, the *S. aureus* became resistant to CN, APX, CPX, S, SXT antibiotics. For *E. coli* and *S. aureus* to show resistance to APX may mean that the strains used for this study are producers of beta-lactamase enzymes. It is worthy to note that as the time of exposure was elongated the microorganism developed increasing resistance. A possible reason for this phenomenon could be that *E. coli* and *S. aureus* may have developed antibiotic resistance genes that might be responsible for the observed resistance to the antibiotics [13] [14]. This may also be as a result of mutations in the bacterial genome caused by soot, thereby mak-

ing the bacteria to alter their existing genes and was resistant to previously susceptible antibiotics. The mechanisms responsible for the phenomenon are not yet known and further research in this direction is necessary. Soot might induce bacteria to develop resistance to antibiotics as observed in *E. coli* and *S. aureus*. It also has some level of potency on these bacterial growths.

5. Conclusion

The growth response study shows an overall significant decline in the population of the bacteria in the presence of soot compared to the unexposed control. Also, the presence of the soot induces resistance with increase in exposure time and soot concentration.

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Conflicts of Interest

The authors declare that they have no conflicts of interest.

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