Susceptibility to Antibiotics and Reactive Oxygen Species in *Escherichia coli*: A Survey of Clinical and Environmental Isolates

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Authors’ contributions
This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Aims: Some bacterial responses to oxidative stress also diminish antibiotic susceptibility; also, some antibiotics do increase oxidative stress within bacterial cells. Linkage or cross-resistance to prooxidants and antibiotics could facilitate the selection of antibiotic resistance and/or virulence. We made this survey in order to detect this possible linkage in *Escherichia coli* isolates.

Methodology: The susceptibility of 102 *E. coli* clinical (causative of urinary or gastrointestinal infections) and environmental (rural or urban dust) isolates towards paraquat, H₂O₂, and antibiotics was measured using disc assays. Catalase and superoxide-dismutase (SOD) activities were measured.

Results: Susceptibility to prooxidants was similar across isolates of all four sources, but urinary and urban dust isolates were more resistant to antibiotics. H₂O₂ “resistant” organisms had more antibiotic resistance phenotypes, particularly towards sulfadiazine and tetracycline. Paraquat “resistance” seems associated to beta-lactam resistance; but paraquat “susceptibility” seems associated to resistance towards chloramphenicol, gentamicin, ciprofloxacin and nitrofurantoin.

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1. INTRODUCTION

The linkage between bacterial susceptibility towards antibiotics, and towards some reactive oxygen species (ROS; particularly superoxide, \(O_2^-\), and hydrogen peroxide, \(H_2O_2\)), have been explored extensively. On the one hand, some responses to oxidative stress, such as the one governed by the soxRS genes in \(E. coli\) and other gram-negative bacteria, include mechanisms that reduce the activity of several antibiotics. This is accomplished mainly by a diminished cytoplasmic accumulation of the drugs both, by reduced permeability and by increased efflux. Strains lacking these regulatory genes are more susceptible to several antibiotics, while mutants constitutively expressing the soxRS regulon are less susceptible to antibiotics than their wild-type counterparts [1,2]. This has been documented in other bacterial species (e.g., [3]). Similarly, the bacterial response governed by OxyR towards \(H_2O_2\) and related oxidative stress, also regulates antibiotic resistance in \(E. coli\) and other gram-negative bacteria [4]. On the other hand, a number of papers have reported that some antibiotics, especially those deemed "bactericidal", increase the intracellular production of ROS [5], up to the point that this has been proposed to be the actual mechanism of their antimicrobial action [6]. Although this does not seem to be the case [7], some antibiotics are likely to increase the levels of ROS within the bacterial cell, perhaps contributing to the overall stress during antibiotic exposure [8]. It is therefore possible that increased resistance towards ROS might confer some protection against antibiotics that produce oxidative stress. As bacteria, both as human commensal/pathogens (e.g., [9]), or in the open environment (e.g., [10,11]), commonly face prooxidants and antibiotics, mechanisms that protect both may have potential repercussions in the efficacy of antibiotic treatments. Most of these observations, however, come from laboratory strains and conditions, but little is known about the occurrence of such phenomena in clinical or environmental settings. Here, the activity of representative antibiotics, and known sources of ROS (\(H_2O_2\) and paraquat, a known generator of intracellular \(O_2^-\)), was tested against a group of clinical and environmental \(E. coli\) isolates. This is, to our knowledge, the first attempt at co-relating the susceptibility towards prooxidants and antibiotics, in a set of isolates from very diverse origin.

2. MATERIALS AND METHODS

2.1 Isolates

A total of 102 \(E. coli\) isolates were included: 24 causative of community-acquired urinary tract infection (Uri); 21 from fecal samples from patients with diarrhea, where the isolate was deemed causative of the illness (Fec); 27 from outdoor rural dust (RD), collected at the Mezquital Valley, close to irrigation canals receiving raw wastewater from Mexico City; and 30 from urban dust (UD) collected at Mexico City. All organisms were identified using standard biochemical methods, and kept in glycerol-containing (25%) liquid media, under liquid nitrogen.

2.2 Antibiotic and Prooxidant Susceptibility Assays

Susceptibility towards ampicillin, amoxicillin-clavulanate, cefotaxime, sulfadiazine, chloramphenicol, tetracycline, gentamicin, ciprofloxacin and nitrofurantoin, was assessed by the method of disc diffusion on Mueller-Hinton agar plates [12] and using commercially-available antibiotic discs (BBL, 10, 20/10, 30, 250, 30, 30, 10, 5 and 300 µg, respectively). Susceptibility to paraquat and hydrogen peroxide was also assessed by disc diffusion, plating \(\sim 5x10^8\) CFU of each strain on LB agar plates, and then filter paper discs containing either 400 µg of paraquat (PQ, Sigma), or 8.8 µmol of \(H_2O_2\) (freshly prepared by dispensing concentrated solution, Sigma) were placed on top [13];
inhibitory halos around each disc were measured after a 35 °C/overnight incubation.

2.3 Catalase and Superoxide-dismutase Activity Assays

Catalase activity was measured by a "foamometric" assay [14], where ~1x10⁸ cells from overnight cultures, in 100 µL distilled water, (with or without further incubation at 55 °C/15 min, to distinguish heat-labile HPII encoded by katG, from heat-stable HPI encoded by katE) were mixed with 100 µL 1% triton X and 100 µL 30% H₂O₂, and the height of the foam column generated by the oxygen bubbles was measured after a 10 min incubation at room temperature, and compared to a calibration curve performed using known concentrations of bovine liver catalase (Sigma). Superoxide-dismutase (SOD) activity was semi-quantified using SOD activity gels [15], running crude extracts obtained by mixing 0.1-mm zirconium beads with pelleted mid-exponential phase cells suspended in 50 mM Tris, 0.2 M NaCl, pH 7.5 buffer.

3. RESULTS

3.1 Susceptibility to Antibiotics and Prooxidants

Overall results of oxidants and antibiotics susceptibility are shown in Fig. 1. Table 1 contains average data of inhibitory halos around discs containing PQ or H₂O₂, and number of antibiotic resistance phenotypes. Fec isolates had PQ halos 11% larger than average; UD isolates had H₂O₂ halos 11% larger than average; and Uri and UD isolates were resistant to more antibiotics than Fec and RD isolates.

3.2 Prooxidant and Antibiotic Resistant and Susceptible Organisms

In order to simplify the analysis of the data, organisms were deemed "resistant" if inhibitory halos of oxidants were below one standard deviation (SD) of the global averages, or above one SD of the average number of resistances; and "susceptible" if halos were one SD above, and number of resistances were one SD below the global averages (Table 2). Both PQ₃ and PQ₅ organisms were more resistant to antibiotics (13% and 27%, respectively, measured as the difference in the average number of resistance phenotypes), while H₂O₂₃ were 20% more resistant to antibiotics, compared to the total average. Among Uri isolates, susceptibility to PQ, H₂O₂ and antibiotics are less common than average; among Fec isolates, PQ susceptibility is more common, and H₂O₂ resistance is less common than average; among RD isolates, PQ, H₂O₂ and antibiotics’ resistance are more common than average, while the opposite results were observed from UD isolates.

3.3 Individual Antibiotics and Prooxidant Susceptibility

Results for individual antibiotics are shown in Table 3. Resistance to PQ seems linked to resistance to ampicillin and amoxicillin-clavulanate (tetracycline resistance is more common among both, PQ₃ and PQ₅ subgroups); while susceptibility to PQ seems linked to resistance to chloramphenicol, gentamicin, tetracycline, nitrofurantoin and, particularly, to ciprofloxacin. Resistance to H₂O₂ seems linked to resistance to sulfadiazine and tetracycline (chloramphenicol resistance is more common among both, H₂O₂₃ and H₂O₂₅ subgroups). Uri isolates are particularly more resistant to ampicillin and amoxicillin-clavulanate, than the global average; and UD isolates are more resistant to chloramphenicol, gentamicin, nitrofurantoin, ciprofloxacin and sulfadiazine, than the average.

3.4 Catalase and SOD Activities and Prooxidant Disc Assays

Susceptibility to H₂O₂, measured as inhibitory halos in the disc assay, did correlate with production of catalase HPII encoded by katE. Average activity per 1x10⁹ cells among H₂O₂₃ isolates was 43.4 (SD 22.9) units of HPI and 44.3 (SD 18.7) units of HPII, while among H₂O₂₅ isolates was 31.4 (SD 31.4; p = 0.402, Student t test) units of HPI, and 7.7 (SD 9.7; p = 0.002) units of HPII. On the other hand, susceptibility to paraquat in the disc assay showed that, while resistant isolates have a uniform SOD activity, susceptible ones had a diversity of SOD profiles, especially affecting the inducible, Mn-SOD (Fig. 2).

4. DISCUSSION

Overall, a strong correlation between resistance to oxidative stress and antibiotics was not found. Isolates able to withstand higher concentrations of H₂O₂ seem to also be able to resist the effect of more antibiotics (a 20% increase); however,
Fig. 1. Susceptibility distribution of clinical and environmental *E. coli* isolates towards prooxidants and antibiotics. Inhibitory halos in mm around discs containing paraquat (A) or \( \text{H}_2\text{O}_2 \) (B), and number of antibiotic resistance phenotypes per isolate (C) are shown in each panel, along with the global average. Organisms with values one standard deviation above or below average were considered susceptible (S) or resistant (R), respectively (except for antibiotic resistance).

Table 1. Susceptibility towards oxidants and antibiotics among clinical and environmental isolates of *E. coli*.

|        | total     | Uri     | Fec     | RD     | UD     |
|--------|-----------|---------|---------|--------|--------|
|        | x(SD)     | x(SD)   | ratio   | x(SD)  | ratio  | x(SD)  | ratio  | x(SD)  | ratio  |
| PQ     | 14.7(3.8) | 13.5(2.4) | 0.92    | 16.3(4.4) | **1.11** | 13.5(3.0) | 0.92    | 15.7(4.3) | **1.07** |
| \( \text{H}_2\text{O}_2 \) | 25.6(3.4) | 24.3(1.5) | 0.95    | 25.8(2.8) | 1.01    | 23.3(2.6) | 0.91    | 28.4(3.4) | **1.11** |
| ATB    | 3.0(1.8)  | 3.5(1.6)  | **1.17** | 2.2(1.6)   | 0.73    | 2.5(1.7)  | **0.83** | 3.7(1.9)  | **1.23** |

Data are: averages \((x)\) with standard deviations \((SD)\) of inhibitory halos, in mm, for PQ and \( \text{H}_2\text{O}_2 \); and of number of antibiotic resistances \((\text{ATB})\). Uri, isolates from urinary infections; Fec, isolates from diarrheal samples; RD, isolates from rural dust; UD, isolates from urban dust. Ratio is the quotient between each subgroup value and the total; those with a difference \(\pm 10\%\) are in bold.
Table 2. Susceptibility towards oxidants and antibiotics among clinical and environmental isolates of *E. coli*.

|                | PQ (x(SD)) | H$_2$O$_2$ (x(SD)) | nATB (x(SD)) | Uri (x(SD)) | Fec (x(SD)) | RD (x(SD)) | UD (x(SD)) |
|----------------|------------|---------------------|--------------|-------------|-------------|------------|------------|
| **Total**      | 14.7(3.8)  | 25.6(3.4)           | 3.0(1.8)     | 24          | 21          | 27         | 30         |
| PQ*            | 10.6(0.5)  | 25.1(3.4)           | 3.4(1.7)     | 1.13        | 5(29)       | +5         | 2(12)      |
| PQ**           | 22.6(2.6)  | 27.1(4.0)           | 3.8(1.6)     | 1.27        | 1(8)        | -16        | 5(38)      |
| H$_2$O$_2$*    | 14.3(3.5)  | 21.7(0.6)           | 3.6(2.1)     | 1.20        | 2(14)       | -10        | 1(7)       |
| H$_2$O$_2$**   | 15.2(4.7)  | 31.7(2.1)           | 2.8(1.6)     | 0.93        | 0           | -24        | 3(18)      |
| ATB*           | 15.2(3.5)  | 25.2(2.5)           | 5.7(0.8)     | 1.9         | 5(23)       | -1         | 4(19)      |
| ATB**          | 14.3(3.3)  | 25.3(3.2)           | 0.99         | 0.6(0.5)    | 0.21        | 1(5)       | -19        |

Data are: averages (x) with standard deviations (SD) of diameter of inhibitory halos, in mm, for paraquat (PQ) or hydrogen peroxide (H$_2$O$_2$), or of number of antibiotic resistance phenotypes (nATB), for subpopulations deemed “susceptible” (S) or “resistant” (R; see text). Ratio is the quotient of each group’s average and total average; or the number of isolates (n) of each source, and the percentage (%) for each group; and the difference (dif) between each group’s percentage and the total percentage. Ratio or dif values with a difference ≥10% are in bold.

Table 3. Resistance towards individual antibiotics, among oxidants’ resistant and susceptible subgroups, and among each source subgroup.

|                | AM n(%) | AMC n(%) | CTX n(%) | SD n(%) | C n(%) | GM n(%) | TE n(%) | CIP n(%) | FM n(%) |
|----------------|---------|----------|----------|---------|--------|---------|---------|----------|--------|
| **Total**      | 55      | 9        | 2        | 80      | 42     | 13      | 71      | 24       | 11     |
| PQ*            | 13(76)  | +21      | 4(24)    | 0       | -2     | 14(82)  | +2      | 6(35)    | -7     |
| PQ**           | 7(54)   | -1       | 0        | -9      | -2     | 11(85)  | +5      | 7(54)    | +12    |
| H$_2$O$_2$*    | 7(50)   | -5       | 1(7)     | -2      | 1(7)   | 13(93)  | +13     | 8(57)    | +15    |
| H$_2$O$_2$**   | 8(47)   | -8       | 1(6)     | -3      | 0      | 14(82)  | -2      | 11(65)   | +23    |
| Uri            | 22(92)  | +37      | 7(29)    | +20     | 2(8)   | 20(83)  | +3      | 3(13)    | -29    |
| Fec            | 8(38)   | -17      | 1(5)     | -4      | 0      | 15(71)  | -9      | 4(19)    | -23    |
| RD             | 10(37)  | -18      | 0        | -9      | 0      | 18(67)  | -13     | 11(41)   | -1    |
| UD             | 15(50)  | -5       | 1(3)     | -6      | 0      | 27(90)  | +10     | 25(83)   | +41    |

*AM, ampicillin; AMC, amoxicillin-clavulanate; CTX, cefotaxime; SD, sulfadiazine; C, chloramphenicol; GM, gentamicin; TE, tetracycline; CIP, ciprofloxacin; and FM, nitrofurantoin.*
Superoxide dismutase (SOD) activity gels are shown for organisms considered “resistant” (A) or “susceptible” (B) to paraquat; the enzyme activity is revealed as transparent bands within a dark background, with Fe-SOD being a constitutive enzyme, Mn-SOD an inducible one, and faint hybrids between them. Strain ATCC 25922 was included as a wild-type control, and activity of some representatives of each group, labeled as the diameter of the inhibitory halo around the paraquat disc are shown. "Resistant" organisms had a very uniform activity, while a diversity of SOD profiles was observed in "susceptible" organisms.

Individual antibiotic-resistance phenotypes associated to H$_2$O$_2$-resistance, sulfadiazine and tetracycline are considered as "bacteriostatic" agents. Regarding paraquat, it was the most susceptible isolates the ones with higher number of antibiotic resistance phenotypes, including the bactericidal fluoroquinolone ciprofloxacin and, to a lesser extent, gentamicin and chloramphenicol. The activity of nitrofurantoin has been shown to depend on an enzyme which expression is governed by the soxRS genes, hence it was an expected outcome to find slightly more resistance to the drug amongst the PQ$^R$ isolates [16]. Perhaps the results from the environmental isolates can better summarize these findings: while amongst RD isolates there are more paraquat- and H$_2$O$_2$-resistant isolates, they have fewer antibiotic resistance phenotypes; on the contrary, there were more prooxidant-susceptible isolates from UD, but those have more resistance phenotypes. Whatever the conditions that are causing these environmental isolates to resist more or less to prooxidants or antibiotics, both features do not seem to be linked.

The linkage between resistance towards superoxide-generating agents, such as PQ, and diminished susceptibility to several antibiotics, have been shown in laboratory conditions, in E. coli and other gram-negative bacteria (e.g., [17]). However, such a linkage was not found, and perhaps even just the opposite. While reduced accumulation—the main mechanism of antibiotic resistance mediated by soxRS, is a successful strategy in the short term, the “trade-offs” might end up reducing fitness [18]. On the other hand, the weak correlation between H$_2$O$_2$- and antibiotic-resistance might be indicative of a more direct association of anti-oxidant defenses in protecting against the effects of antibiotics.
Nevertheless, being the two antibiotic-resistance phenotypes with the strongest linkage to H$_2$O$_2$-resistance anything but bactericidal (i.e., sulfamethoxazole and tetracycline), the role of ROS is not that clear. The exposure of *E. coli* to sulfamethoxazole was recently shown to induce a metabolic pathway that produces antioxidant pterin-phenylpyruvate conjugates [19] suggesting that sulfonamide antibiotics do exert oxidative stress. In any case, the likelihood of some antibiotics fostering or co-selecting for H$_2$O$_2$-resistance could link the use of those antibiotics to an increased bacterial virulence, as oxidative-stress responses have a known role as pathogenic determinants [20].

The method used here to assess antibiotic susceptibility is a coarse one, only capable of distinguishing between susceptibility and resistance by using clinical breakpoints. It is possible that oxidant-resistant isolates have diminished susceptibility but not up to the point of being fully-resistant. While the method lacks the resolution needed, the average size of inhibitory halos among susceptible isolates do not differ between prooxidant-susceptible and -resistant strains (not shown). The method for assessing susceptibility towards H$_2$O$_2$ and paraquat is equally coarse, unable to distinguish the underlying mechanism of purported susceptibility or resistance. While there was a correlation between the sizes of inhibitory halos, and the activities of enzymes inactivating respective ROS, very different genotypes could have been grouped under the same category, possibly confounding the associations. Further analyses, utilizing more precise and specific molecular methods to assess the existence and extent of the linkage between prooxidant- and antibiotic-resistance.

5. CONCLUSION

The proposed oxidative stress proposed as secondary “mechanism of action” for some antibiotics, do not seem to have resulted in the co-selection of oxidative stress resistance, or vice versa, in clinical and environmental isolates of *E. coli*. Weak linkage between H$_2$O$_2$-resistance and antibiotic multi-resistance was found, and resistance to sulfonamides and tetracycline seem to contribute especially to this effect. Resistance to O$_2$-generator paraquat seems only clearly related to resistance to aminopenicillins. In any case, a possible link between resistance to some antibiotics and to prooxidants could contribute to co-selection.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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