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Sub-growth Inhibitory Concentrations of Some Antibiotics Enhance Serum Susceptibility of *Escherichia Coli* at Elevated Temperature

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ABSTRACT

The ability of antibiotics to interact with host defenses provide justification for *in vitro* evaluation of the interaction of antibiotics, bacteria, and host defenses to help identify those factors that influence therapy for bacterial infections. This study evaluates the effect of sub-inhibitory concentrations (sub-MICs) of ceftriaxone, ciprofloxacin and gentamicin on killing of *Escherichia coli* ATCC 25922 by human serum. Saline-washed 18-h Mueller-Hinton broth culture of test strain was inoculated into 50% human serum-Hanks'Balanced Salt Solution mixture and serum mixture containing ¼ minimum inhibitory concentrations of ceftriaxone, ciprofloxacin or gentamicin and incubated at 37ºC or 41ºC. Counts at T = 0 h and T = 2 h of incubation by spread plate method and survival (%) at these temperatures were then determined under each of the conditions. Results are means of three determinations. At 37°C, serum susceptibility was inhibited significantly ($P<0.05$) by sub-MICs of ceftriaxone ($P=0.01$) and ciprofloxacin ($P=0.03$) but insignificantly ($P>0.05$) by sub-MIC of gentamicin ($P=0.09$). At 41°C however, serum susceptibility was enhanced significantly ($P<0.05$, $P=0.00$) by sub-MICs of the antimicrobial agents tested. In the presence of all the antibiotics tested, serum susceptibility was insignificantly ($P>0.05$) influenced at both 37°C ($P=0.38$) and 41°C ($P=0.49$). Sub-MICs of some antimicrobial agents can enhance susceptibility of *Escherichia coli* to human serum at elevated temperature (41°C) usually encountered in fever nd the common practice of reducing fever in many diseases before antibiotics treatment is commenced may be unnecessary.

INTRODUCTION

Bacterial pathogens have evolved several different strategies to escape host defenses [1] due to their possession of certain structural and physiological attributes that act together or independently to promote the survival and the growth of the pathogens in the host cells. One of these attributes is serum resistance [2] which is complement-mediated, lipopoly-saccharide structure dependent and plasmid-encoded [3-6].

The *in vivo* response of a host and bacteria to an antimicrobial agent is a product of interaction of many factors including: susceptibility of the bacteria to the agent; age and immune status of patient; existing disorder; and route of antibiotic administration [7,8]. Thus, an appropriate dose of an antimicrobial agent may not attain the expected desirable minimal inhibitory concentration (MIC) or minimal bactericidal concentration (MBC) at the affected tissue site, and may come down to a sub-inhibitory concentration (sub-MIC) unless the complex interrelation between these factors is favorable. Furthermore, sub-MICs can also result from inappropriate dose intake as is common with the use of sub-standard antimicrobial products. Exposure of bacteria to sub-MICs of antimicrobial agents may cause alteration in bacterial morphology [9]; change in growth and toxin production (10); change in genetic integrity and expression (11); inhibition of bacterial adhesion [12]; increased susceptibility to phagocytosis [13] and changes in sensitivity of the organism to serum.

Antibiotics interact with organic host defenses and produce neutral, inhibitory or synergistic effects [14,15]. For example, it is well established that the interaction of polymyxin B and serum, both of which have effect on the envelope of Gram negative cells, is synergistic [16] or additive [17]. A cooperative interaction between antibiotics and host defense mechanisms could explain why certain antibiotics exhibit greater efficiency *in vivo* than predicted from their *in vitro* bioactivities. Thus, greater understanding of antimicrobial activity *in vivo* might be obtained through investigation of sub-MICs and normal human serum, and this would allow a more rational application of antibiotics. The aim of this study is to evaluate the effect of sub-minimum inhibitory concentration of ceftriaxone, ciprofloxacin and gentamicin on the *in vitro* bactericidal action of normal human serum against *Escherichia coli* ATCC 25922 at normal and

elevated ("feverish") body temperature.

MATERIALS AND METHODS

Test Strain, Culture Media, Antibiotics and Chemicals

The test strain (*Escherichia coli* ATCC 25922) was provided by Mr. Adebola Onanuga (Department of Pharmaceutical Microbiology and Biotechnology, Niger Delta University, Wiberforce Island, Bayelsa State, Nigeria). The strain was maintained on nutrient agar (NA: Merck KGaA, Darmstadt, Germany) slants in a 4ºC refrigerator and re-constituted in Mueller-Hinton broth (MHB: BIOTEC Lab Ltd, Ipswick, UK) before use. Other culture media used includes: Mueller-Hinton agar (MHA: Oxoid Ltd, Hampshire, England), The antibiotics used namely: ceftriaxone (May & Baker Nigeria Plc, Ikeja), ciprofloxacin (DANA Pharmaceutical Ltd, Minna, Nigeria) and gentamicin (Yikang Pharmaceutical Co. Ltd., China) were all purchased from the Pharmacy Department, Federal Medical Center, Keffi, Nasarawa State, Nigeria. All chemicals used were from BDH Chemical Ltd, Poole, England.

Serum Preparation

Five milliliters of blood was collected from a vein of each of normal healthy volunteer students of Nasarawa State University Keffi, Nasarawa State, Nigeria using a sterile syringe. Informed consent was obtained from these volunteers in line with University Guidelines for Research using Human Subjects. The blood was then allowed to coagulate at room temperature and serum separated.

Determination of antibiotics minimum inhibitory concentration (MIC)

The MICs of ceftriaxone, ciprofloxacin and gentamicin were determined by using Macro-broth dilution method as described Clinical and Laboratory Standards Institute (CLSI) [18].

Serum susceptibility assay

Serum susceptibility was assayed as previously described [19,20]. Briefly, 2 ml of 24-h MHB culture of *Escherichia coli* ATCC 25922 was centrifuge and washed three times with 0.85% NaCl (normal saline) and re-suspended in 2 ml sterile MHB. 0.1 ml of the re-suspended culture was added to 2 ml of 50% serum in single strength Hanks' Balanced Salt Solution (HBSS: 137 mM NaCl, 5.4 mM KCl, 0.25 mM Na_2HPO_4, 0.44 mM KH_2PO_4 , 1.3 mM $CaCl_2$, 1.0 mM $MgSO_4$, 4.2 mM $NaHCO_3$, 5.6 mM D-glucose, 0.02% phenol red, distilled water to 1000 ml; membrane-filter [pore size: 0.45 µm]). Samples were each taken at T = 0 h and T = 2 h after incubation of the culture 37ºC or 41ºC, diluted in normal saline and spread on MHA and viable counts were obtained after incubating at 37ºC for 24 h. Bacterial survival (%) in antibiotic-free serum-1XHBSS mixture at T = 2 h was determined from the relation: CFU_2 /CFU_1 x 100 (where CFU_1 = CFU at T = 0 h; and CFU_2 = CFU at T = 2 h). Results are means of three independent determinations.

To determine the effect of antibiotics sub-MICs on serum lysis of test bacteria, saline-washed culture of the strain was inoculated into 50% serum-1XHBSS mixture containing ¼ MIC of ceftriaxone (2 µg/ml), ciprofloxacin (0.03125 µg/ml) or gentamicin (0.625 µg/ml). Samples were taken, counts were determined and bacterial survival (%) at T = 2 h was calculated as in the antibiotic-free serum-1XHBSS mixture above. Results are means of three independent determinations.

Statistical Analysis

Data from this study were analyzed by one-way analysis of variance (ANOVA) using Smith Statistical Package (SSP), version 2.80. Significance or otherwise, of result was determined at the 5% probability level (that is, at P = 0.05).

RESULTS

Antibiotic MICs

The obtained MIC values of the antibiotics for *E. coli* ATCC 25922 were ceftriaxone (8 µg/ml), ciprofloxacin (0.125 µg/ml) and gentamicin (0.25 µg/ml) as shown in Table 1.

Table No.1: Minimum inhibitory concentrations of antibiotics for *Escherichia coli* ATCC 25922

Antibiotics	Antibiotics MIC (µg/ml)
Ceftriaxone	8
Ciprofloxacin	0.125
Gentamicin	0.25

Effect of antibiotics sub-MICs on bacterial survival in human serum

The survival of the test strain in antibiotic-free and antibiotic-containing human serum mixture at 37ºC and 41ºC is shown in Table 2. At 37ºC, serum killing was inhibited significantly (P<0.05) by sub-MICs of ceftriaxone (P=0.01) and ciprofloxacin

Table No.2: Survival of *Escherichia coli* ATCC 25922 in 50% human sera

Serum mixture	Antibiotics MIC (µg/ml)	¼ antibiotics sub-MIC (µg/ml)	Survival (%) (mean ± SD*)	
			37ºC	41ºC
Antibiotic-free	-	-	85.39 ± 4.22	114.13 ± 1.24
Antibiotic-containing:				
Ceftriaxone	8	2	97.29 ± 1.29	92.99 ± 4.74
Ciprofloxacin	0.125	0.03125	94.25 ± 1.36	88.75 ± 4.15
Gentamicin	0.25	0.0625	93.79 ± 5.03	90.46 ± 3.48

(P=0.03) but insignificantly (P>0.05) by sub-MIC of gentamicin (P=0.09). At 41ºC however, antibiotics sub-MICs enhanced serum susceptibility having produced a significantly (P<0.05, P=0.00) lower bacterial survival than obtained in antibiotic-free serum. Irrespective of the antimicrobial agent, serum susceptibility was insignificantly (P>0.05) influenced at both 37°C (P=0.38) and 41°C (P=0.49).

DISCUSSION

The ability of antibiotics to interact with host defense mechanisms [14,15,21,22] provide justification for *in vitro* evaluation of the interaction of antibiotics, bacteria, and host defense to help identify those factors that determine the success or failure of therapy for bacterial infections.

Serum is an environment in which bacteria cells should not exist. The serum complement provide innate defense against microbial infection [23,24]. The lysozymes present in the body fluid cooperate with the complement system in the bactericidal action of serum where they catalyze hydrolysis of β-1,4 glycosidic bonds linkages between N-acetyl muramic acid and N-acetyl glucosamine [23,25]. Bacterial susceptibility to serum depends on the structure and organization of the bacterial outer membrane [23], which itself can be influenced by antibiotics when applied at subinhibitory concentrations [26,27].

The observed antibacterial activity of human serum at 37ºC in this study is consistent with earlier reports [19,23,28,29]. The results reported in this study are actually a balance between the effect of temperature on the growth of the organism and its effect on the serum killing system. For instance, population of *Escherichia coli* ATCC 25922 were killed by the serum at 37ºC, but the strain grew and proliferated at 41ºC, an indication that the increase in temperature did not inhibit serum action as well as the growth of the bacteria. This is opposed to the fact that complements responsible for bacterial killing by serum are proteins [23] and were expected to be destroyed by increasing temperature.

The inhibition of serum killing action by antibiotics sub-MIC at 37ºC is not surprising. It is well established that antibiotics and serum have effect on the envelope of gram negative bacteria which can either be synergistic [14,16] or additive [17] when used in combination. However, the enhanced action of serum in the presence of antibiotics sub-MIC at 41ºC such as obtained in fever, cannot be explained from the standpoint of denaturing of serum complements. This is because it has been established that the bactericidal and bacteriolytic properties of serum are destroyed only at 56ºC [5]. This is in contradiction of the common practice of reducing fever in many diseases before antibiotics treatment is commenced. Further investigation would be required to clarify the mechanism through which high temperature enhances serum killing of bacteria.

CONCLUSION

The results reported in this study provide further evidence that exposure of bacteria to antibiotics sub-MICs can influence their susceptibility to host defenses; and preliminary evidence that the common practice of reducing fever in many diseases before antibiotics treatment is commenced may be unnecessary.

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