International Research Journal of Microbiology (IRJM) (ISSN: 2141-5463) Vol. 2(9) pp. 333-337, October 2011
Available online http://www.interesjournals.org/IRJM

Full Length Research Paper

Sub-growth inhibitory concentrations of ceftriaxone and gentamicin induce changes in phenotypes of *Escherichia coli*

Ngwai YB*[1], Garasin UM[1], Ngbede FE[1], Nkene IH[1], Akpotu MO[2]

[1]Microbiology Unit, Department of Biological Sciences, Nasarawa State University, P.M.B. 1022 Keffi, Nasarawa State, Nigeria;
[2]Pharmacy Department, Federal Medical Center, Keffi, Nasarawa State, Nigeria

Accepted 07 September, 2011

The susceptibility of infecting bacteria to antibiotics is among the many factors that influence the *in vivo* response of the host and the bacteria to treatment with such antibiotics. Exposure of bacteria to sub-growth inhibitory concentrations of antibiotics does not only cause reduced susceptibility (that is, resistance), but may also modify the physicochemical characteristics and the architecture of the bacterial outermost surface, and may interfere with some bacterial functions. This study compares some phenotypes of *Escherichia coli* ATCC 25922 (Type Strain) with those of its ceftriaxone- and gentamicin-induced resistance mutants. Resistant mutants generated by growing the type strain for 7 days in sub-growth inhibitory concentrations of ceftriaxone or gentamicin were compared with the type strain in respect of the size and morphology of colonies, growth, motility and biochemical characteristics. It was observed that the type strain grew more with larger colonies than the mutant. In addition, the elevation of the colonies changed from flat (in type organism) to convex (mutant strain). Indole reaction of the ceftriaxone resistant mutant was negative as opposed to the type organism which was positive. Delay in time of colony formation, smaller and fewer colonies resulted following the exposure of *E. coli* to ceftriaxone and gentamicin sub-MICs. In addition, a change in the indole reaction of ceftriaxone-induced mutants to negative was also observed. The significance of these observations is the subject of further study by our group.

Keywords: Phenotypes, antibiotics, *Escherichia coli*, ceftriaxone, gentamicin

INTRODUCTION

Escherichia coli is a facultative anaerobic gastro-intestinal tract bacteria (Todar, 2008) found in the large intestine of humans and other warm-blooded animals (Campbell and Reece, 2002) where it benefits its host (by synthesizing Vitamin K or Preventing colonization by other pathogens) or as a pathogen causing diseases of intestinal and extra-intestinal sites (Todar, 2008; Bailey et al., 2006; Salyers and Whitt, 2002; Hudault et al., 2001; Bentley and Meganathan, 1982). *E. coli* is globally the most frequent pathogen isolated from uncomplicated urinary tract infections (UTI) responsible for 70-75% of UTIs; and in bacteremia of nosocomial or community origin, it represents about the 15.5% and 42.1% of aetiologies respectively (Luzzaro et al., 2002).

The susceptibility of infecting bacteria to antibiotics is among the many factors that influence the *in vivo* response of the host and the bacteria to treatment with such antibiotics (Howard, 2004; Mandell, 2002). Reduced susceptibility (that is, resistance) to antibiotics in bacteria can arise from a number of mechanisms involving chromosomal genes (Martinez and Baquero, 2000; Russell and Chopra, 1996). Resistance usually evolves naturally via natural selection acting upon random mutation, but it could also be engineered by applying an evolutionary stress on a population; once such a gene is

*Corresponding Author E-mail: ngwaiyb@yahoo.com; Tel: +234-80-52991889

generated, bacteria can then transfer the genetic information in a horizontal fashion (between individuals) by plasmid exchange (Ochiai et al., 1959; Woodford and Ellington, 2007).

Exposure of bacteria to sub-inhibitory concentrations (sub-MICs) of antibiotics not only provides for selection of resistant clones, but may also modify the physicochemical characteristics and the architecture of the bacterial outermost surface, and may interfere with some bacterial functions (Vidya et al., 2005). The aim of this study is to compare the well-characterized *E. coli* ATCC 25922 strain and its ceftriaxone- and gentamicin-induced resistance mutants with respect to their growth, colony characteristics (e.g. size, morphology), motility and certain biochemical characteristics.

MATERIALS AND METHODS

Test Strain and Culture Media

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 include: Mueller-Hinton agar (MHA: Oxoid Ltd, Hampshire, England), Simmons Citrate agar (SCA: LAB M Ltd, Lancashire, UK) and peptone water (BIOTEC Lab Ltd, Ipswick, UK).

Antibiotics and Chemicals

The antibiotics used in this study namely: ceftriaxone (May and Baker Nigeria Plc, Ikeja) and gentamicin (Yikang Pharmaceutical Co. Ltd., China) were all purchased from the Pharmacy Department, Federal Medical Center, Keffi, Nasarawa State, Nigeria. Stock solutions of the antibiotics were prepared and either used immediately or stored in the refrigerator for future use. All chemicals (such as sodium chloride, paradimethylaminobenzaldehyde, amyl alcohol, and hydrochloric acid, methyl red dye, potassium hydroxide, napthol) used

Generation of antibiotic-resistant mutants

MICs of ceftriaxone and gentamicin against the type strain were determined by macro-broth dilution method using Mueller-Hinton broth (MHB) in accordance with Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2002).

Tubes of MHB containing ½ MIC of ceftriaxone (4 µg/ml) or gentamicin (0. 25 µg/ml) were prepared, inoculated with 0.1 ml of 24-h MHB culture of the type strain and then incubated for 7 days under static condition at 37°C. 0.1 ml from each tube was spread on MHA plates and incubated, about 5 µl from the broth was plated out in nutrient agar and incubated at 37°C for 24-48 h to recover the resistant clones. MICs of the antibiotic-exposed bacteria were determined to confirm acquisition of resistance.

Growth of Type Organism and Resistance mutants

Two milliliters of MHB in tubes were separately inoculated with 5µl of 24-h MHB culture of type strain or the resistance mutants. 0.1 ml samples were each taken immediately (T = 0 h) and at T = 5 h after incubation of the culture at 37ºC, diluted (10^{-2} to 10^{-4}) in 0.85% NaCl (normal saline), spread in triplicate on MHA and incubated at 37°C to count viable colonies after 24 h incubation. Increase in growth (%) at T = 5 h was determined from the relation: $CFU_2 - CFU_1 / CFU_1 \times 100$ (where CFU_1 = CFU at T = 0 h; and CFU_2 = CFU at T = 5 h). Results are means of three independent determinations.

Colony Characteristics of type strain and mutants

Samples (0.1 ml) of 24-h MHB culture of type strain and resistant mutants were sub-cultured on MHA and incubated at 37°C for 24 h. Colonies of both strain types were compared with respect to size, morphology and number of colonies formed on the plates. The time for formation of visible colonies was determined for both type strain and resistant mutants by inspecting the incubated culture periodically after the first 10 h of incubation.

Biochemical tests of type strain and mutants

The “IMViC (indole, methyl red, Voges-Proskauer and citrate) characteristics and motility of 24-h MHA grown type strain and mutants were compared as described by Cheesbrough (Cheesbrough, 2005).

Statistical analysis

Growth 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).

Table 1: Minimum inhibitory concentrations of antibiotics for *Escherichia coli* ATCC 25922 and antibiotic-induced mutants

Antibiotics	Antibiotics MIC (µg/ml)	
	Type Strain	Resistant mutants
Ceftriaxone	8	32
Gentamicin	0.5	2

Table 2: Growth increase of *Escherichia coli* ATCC 25922 and antibiotic-induced mutants

Antibiotics	% Growth increase (± SD)		P-value	Remarks
	Type Strain	Resistant mutants		Difference is:
Ceftriaxone	60.35± 3.13	49.7± 2.63	0.01	Significant (P<0.05)
Gentamicin	64.64 ± 1.47	61.47 ± 1.28	0.04	Significant (P<0.05)

*SD = standard deviation

Table 3: Colony characteristics* of *Escherichia coli* ATCC 25922 and antibiotic-induced mutants

Feature	Type Strain	Resistant mutants	
		Ceftriaxone	Gentamicin
Time for formation of visible colonies**	18-24 h	24-48 h	24-48 h
Number of colonies at T = 0 h	182	162	143
Colony size	5 mm	3 mm	2-3 mm
Opacity	Opaque	Transparent	Transparent
Surface	Rough	Smooth	Smooth
Edge	Entire	Entire	Entire
Elevation	Flat	Convex	Raised

*Colony characteristics were observed by two independent observers; ** This was done by inspecting the incubated culture periodically after the first 10 h

RESULTS

Antibiotics MICs of type strain and resistant mutants

The obtained MIC values of the antibiotics for *E. coli* ATCC 25922 were ceftriaxone (8 µg/ml) and gentamicin (0.5 µg/ml); while those for resistance mutants were each 4X higher for ceftriaxone (32 µg/ml) and gentamicin (2 µg/ml) as shown in Table 1.

Growth of type strain and mutants

The percentage increase in growth of type strain and mutants is shown in Table 2. The type strain significantly (P<0.05) grew more than the ceftriaxone-induced (P = 0.01) and gentamicin-induced (P = 0.04) resistant mutants.

Colony characteristics of type and resistant mutants

The colony characteristics of the type and resistant mutant strains are as shown in Table 3. The type strain grew more and faster with larger colonies than the resistant strains. There was a change in the elevation of the colony from flat (Type strain) to convex (ceftriaxone-induced mutant) and raised (gentamicin-induced mutant).

Biochemical tests of type strain and resistant mutants

The biochemical tests of the type and mutant strains are as shown in Table 4. Ceftriaxone-induced resistance mutant showed a negative indole reaction as opposed to the Type organism which was indole-positive. All the other biochemical tests evaluated were same for both

Table 4: Biochemical tests of *Escherichia coli* ATCC 25922 and antibiotic-induced mutants

Feature	Type Strain	Resistant mutants	
		Ceftriaxone	Gentamicin
Indole	+	-	+
Methyl Red	+	+	+
Voges-Proskauer	-	-	-
Citrate	-	-	-
Motility	+	+	+

type and mutant strains.

DISCUSSION

Antibiotics sub-MICs, which can arise from overuse of antibiotics in humans or their use as growth promoters in food of animals (Johnson et al., 2006), selects for resistance to such antibiotics (Roe and Pillai, 2003). It may also modify the physicochemical characteristics and the architecture of bacterial outermost surface, and may interfere with some bacterial functions such as surface hydrophobicity, fimbriation, motility, adhesiveness, sensitivity to serum-killing and phagocytosis (Vidya et al., 2005; Braga et al., 1995; Lorian and Gemmel, 1991).

The higher MICs of antibiotics obtained for the resistance mutants compared with those for the type strain is confirmation that the antibiotic environment selects for resistant clones through mutations (Woodford and Ellington, 2007; Roe and Pillai, 2003). Perfeito *et al*. (2007) have suggested that the rate of adaptive mutation in *E. coli* is in the order of 10^{-5} per genome per generation, which is 1000 times as high as previous estimates, a finding which may have significance for the study and management of bacterial antibiotic resistance.

The significantly lower percentage increase in growth, the delay in forming colonies, the fewer number of colonies and smaller colony sizes observed in the resistant mutants could be a result of a change in the genetic integrity and expression of the bacterium following exposure to the antibiotics sub-MICs as reported previously in staphylococci exposed to sub-MICs of cerulein (Adhikari and Novick, 2005).

The change (to negative) in the indole reaction of the ceftriaxone-induced mutant is not surprising as variable changes which depend on the type of antibiotic and bacterial species have been reported in the biochemical properties of bacteria following exposure to antibiotics sub-MICs (Goudarzi et al., 2004; Gemmel and Ford, 2002; Kavamura-Sato et al., 2000; Chopra and Linton, 1986; Doss et al., 1993). It should be noted however, that lack of more sensitive and more sophisticated instruments that can detect qualitative and/or quantitative changes at the test sub-MIC concentration could account for the non-detection of biochemical changes in gentamicin-induced mutants in our study.

In conclusion, ceftriaxone-induced and gentamicin-induced resistance can result from as short as 7 days exposure of *E. coli* to sub-MICs; with corresponding changes in their colony characteristics (size and morphology). In addition, ceftriaxone-induced resistance changed the indole reaction of *E. coli* from positive to negative which may affect laboratory diagnosis.

ACKNOWLEDGEMENT

We are grateful to the staff of the Microbiology Laboratory in Nasarawa State University, Keffi for technical support and the positive attitude shown during the study.

REFERENCES

Adhikari RP, Novick RP (2005). Subinhibitory cerulein inhibits staphylococcal exoprotein production by blocking transcription rather than by blocking secretion. Microbiol. 151: 3059- 3069.

Bailey MT, Engler H, Sheridan JF (2006). Stress induces the translocation of cutaneous and gastrointestinal microflora to secondary lymphoid organs of C57BL/6 mice. J. Neuroimmunol. 171: 29-37.

Bentley R, Meganathan R (1982). Biosynthesis of vitamin K (menaquinone) in bacteria. Microbiol. Rev. 46(3): 241-280.

Braga PC, Dal Sasso M, Maci S, Reggio S, Piatti G (1995). Influence of subinhibitory concentration of brodimoprim and trimethoprim on the adhesiveness, hydrophobicity, hemagglutination and motility of *Escherichia coli*. Chemotherapy 41: 50-58.

Campbell NA, Reece JB (2002). Biology. Pearson Education Inc: San Francisco.

Cheesbrough M (2005). District Laboratory Practice in Tropical Countries. Part 2. Cambridge University Press: Cambridge.

Chopra I, Linton A (1986). The antibacterial effects of low concentrations of antibiotics. Adv. Microb. Physiol. 28: 211-59.

CLSI (2002). Performance Standard for Antimicrobial Susceptibility: 14th informational supplement. M100-S14, vol.24, no.1. Clinical and Laboratory Standards Institute: Wayne, USA.

Doss SA, Tillioston GS, Amyes SG (1993). Effect of sub inhibitory concentration of antibiotics on the virulence of *Staphylococcus aureus*. J. Appl. Microbiol. 75: 123-128.

Gemmel CG, Ford CW (2002). Virulence factor expression by Gram positive cocci exposed to subMIC of linezolid. J. Antimicrob. Chemother. 50: 665-672.

Goudarzi GH, Sattari M, Bigdelli DM (2004). Evaluation of inhibitory effect of chamomile alcoholic extract on the growth and production of certain factors of *Staphylococcus aureus*. Iran J. Pharm. Res. 2: 67-68.

Howard BJ (2004). Clinical and Pathogenic Microbiology: Antimicrobial Susceptibility Testing. Mosby Company: Washington DC.

Hudault S, Guignot J, Servin AL (2001). *Escherichia coli* strains colonizing the gastro intestinal tract protect germ-free mice against *Salmonella* Typhimurium infection. Gut 49 (1): 47 - 55.

Johnson JR, Kuskowski MA, Menard M, Gajewski A, Xercavins M, Garau J (2006). Similarity between human and chicken *Escherichia coli* Isolates in relation to ciprofloxacin resistance status. J. Infect. Dis. 194 (1): 71-78.

Kavamura-Sato KY, Iinuma T, Hasegawa HT, Yamashino T, Ohta M (2000). Effect of subinhibitory concentration of macrolides on expression of flagellin in *Pseudomonas aeruginosa* and *Proteus mirabilis*. Antimicrob. Agents Chemother. 44: 2869-2872.

Lorian V, Gemmel C (1991). Effect of low antibiotic concentrations on bacteria: effects on ultrastructure, virulence and susceptibility to immunodefenses. In: Lorian V, editor. Antibiotics in Laboratory Medicine. 3rd edn. Baltimore, USA: Williams and Wilkins; 1991. pp. 493-555.

Luzzaro F, Viganò EF, Fossato D, Grossi A, Sala A, Sturla C, Saudelli M, Toniolo A, AMCLI Lombardia Hospital Infectious Study Group (2002): Prevalence and drug susceptibility of pathogens causing bloodstream infections in northern Italy: a two years study in 16 hospitals. Eur. J. Clin. Microbiol. Infect. Dis. 21: 849-855.

Mandell GI (2002). *Principles and Practice of Infectious Disease: Anti-infective therapy*. 4th ed. New York: Churchill Livingstone.

Martinez JL, Baquero F (2000). Mutation frequencies and antibiotic resistance. Antimicrob. Agents Chemother. 44: 1771-1777.

Ochiai K, Yamanaka T, Kimura K, Sawada O (1959). Inheritance of drug resistance (and its tranfer) between *Shigella* strains and Between *Shigella* and *E. coli* strains. Hihon Iji Shimpor 1861: 34 (in Japanese).

Perfeito L, Fernandes L, Mota C and Isabel Gordo (2007). Adaptive Mutations in Bacteria: ‘‘High rate and Small effects”. Science 317 (5839): 813-815.

Roe MT, Pillai SD (2003). Monitoring and identifying antibiotic resistance mechanisms in bacteria. Poult. Sci. 82(4): 622-626.

Russell AD, Chopra I (1996). Understanding Antibacterial Action and Resistance. Ed 2nd. Ellis Horwood: Herfordshire, UK.

Salyers AA, Whitt DD (2002). Bacterial pathogenesis: a molecular approach. ASM Press: Washington DC.

Todar K (2008). Online Textbook of Bacteriology. Available from: http://www.textbookofbacteriology.net/e.coli.htm. Accessed 28 December 2010.

Woodford N, Ellington MJ (2007). The emergence of antibiotic resistance by mutation. Clin. Microbiol.Infect. 13(1): 5-18.

Vidya KC, Mallya PS, Rao PS (2005). Inhibition of bacterial adhesion by subinhibitory concentrations of antibiotics. Indian J. Med. Microbiol. 23: 102-105.