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Antimicrobial susceptibility testing on isolated gram positive and gram negative bacteria from local unpasteurized milk (*Fura de nunu*)

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Abstract
The availability of antibiotics to treat infectious diseases has radically improved human and animal well-being, and also to a minor degree in plant health, they are often used in rearing animals for food and this use among others leads to the creation of resistant strains of bacteria. As the genomes of bacteria, especially pathogens, have become increasingly available, the prospect of using them to identify new targets for antibiotic discovery has renewed interest in such investigations between the public sector and large pharmaceutical and biotechnology companies. In this study, antimicrobial susceptibility testing was carried out on the local unpasteurized milk popularly called *Fura de nunu* after isolating bacteria (both gram +ve and gram -ve) from it, the resistant ability and sensitivity of the isolates were determined upon testing with some standard available antibiotics. In the present study, nineteen isolates of *E.coli* were isolated from *Fura de nunu* tested from which six (31.6%) were resistant to Sulphamethoxazole/Trimethoprim, one (5.3%) resistance to Ciprofloxacin and none was resistant to Cefpodoxime and none was resistant to two or three at a time. It was found out that some were resistant to the testing while few isolates were sensitive.

Keywords: Antibiotics, *Staphylococcus aureus, Escherichia coli,* β-lactamase-resistant.

Introduction

The widespread of use of antibiotics both inside and outside medicine is playing a significant role in the emergence of resistant of bacteria [1]. Antibiotics are often used in rearing animals for food and this use among others leads to the creation of resistant strains of bacteria. The presence of these antimicrobial compounds in the environment, waste-water from animal, Agricultural facilities, human sewage treatment plants, hospitals, pharmaceutical plants has been associated with increased levels of zoonotic pathogens as well as increasingly resistant and virulent organisms [2]. The availability of antibiotics to treat infectious diseases has radically improved human and animal well-being, and also to a minor degree in plant health, the term antibiotic which was coined by Selman Waksman in 1942 to describe any substance produced by a microorganism that is antagonistic to the growth of other microorganisms in high dilution [3]. Also, antibiotics are chemicals produced by or derived from microorganisms (i.e. bugs or germs such as bacteria and fungi), that kill or prevent the growth of other bugs and germs. Antibiotics are a specific type of antimicrobial drugs. However, the term antibiotic is now widely used to refer to all drugs that selectively target bacteria [4]. Paradoxically, this very success threatens the future utility of antibiotics. The discovery of penicillin in 1940 ushered in the era of "modern medicine." Numerous antimicrobials, including most structural classes of antibiotics were discovered during 1920 to 1970. Chemical modification of many of these compounds led to new entities with superior activities. Because of the great success in antibiotic discovery, by the late 1970s, many proclaimed that the war on infectious diseases had been won, leading ultimately to de-emphasis of antibiotic discovery during the 1980s and a decline in the 1990s. At the same time, however, widespread antibiotic resistance was emerging and resulting in impaired treatment of human diseases [5]. As the genomes of bacteria, especially pathogens, have become increasingly available, the prospect of using them to identify new targets for antibiotic discovery has renewed interest in such investigations between the public sector and large pharmaceutical and biotechnology companies.

If antibiotics are overused or incorrectly used, there is a chance that the bacteria will become resistant (the antibiotics become less effective against that type of bacterium). According to a report by Wagner, (2002) [6] in the FDA Veterinarian Newsletter, widespread dissemination of resistance to antibiotics resulting from the selective effect of drug use in food animals may have important ramifications for both human and animal health. Animal feeds and feed commodities may serve as vectors for the dissemination and maintenance of resistance determinants in the animal production environment and thereby in the food supply. Feed antibiotics include antibiotics used as growth promotants and those used for sub therapeutic; including prophylactic and growth - promotant use and

therapeutic purposes [7]. Feed antibiotics are licensed for specific uses as for meat chickens or young pigs or calves or feedlot cattle.

Bacterial resistance can be intrinsic or acquired. An example of intrinsic resistance is a gram -ve bacterium that has an outer membrane that is impermeable to the antibiotic. Acquired resistance occurs when a previously susceptible bacterium becomes resistant through mutation (vertical evolution) or acquisition of new DNA (horizontal evolution). Mutation is the result of a random event that occurs spontaneously. The presence of antibiotic will subsequently select for the resistant mutants [7]. *Staphylococcus aureus* was one of the earlier bacteria in which penicillin resistance was found in 1947. Half of all *S. aureus* infections in the US are resistant to penicillin, methicillin, and tetracycline. The β-lactamase-resistant penicillins (Methicillin, oxacillin, cloxacillin, and flucloxacillin) were developed to treat penicillin-resistant *S. aureus*, and are still used as first-line treatment. Methicillin was the antibiotic of choice, but has since been replaced by Oxacillin due to significant kidney toxicity. Methicillin-resistant *Staphylococcus aureus* (MRSA) was first detected in Britain in 1961, and is now "quite common" in hospitals. MRSA was responsible for 37% of fatal cases of sepsis in the UK in 1999, up from 4% in 1991. Half of all *S. aureus* infections in the US are resistant to penicillin, methicillin, tetracycline and erythromycin [8]. This left vancomycin as the only effective agent available at the time. *Escherichia coli* is a Gram-negative, rod-shaped bacterium that is commonly found in the lower intestine of warm-blooded organisms. Most *E. coli* strains are harmless, but some serotypes can cause serious food poisoning in humans, and are occasionally responsible for product recalls [9]. The harmless strains are part of the normal flora of the gut, and can benefit their hosts by producing vitamin K and by preventing the establishment of pathogenic bacteria within the intestine [10]. *E. coli* shed into the environment can survive for significant periods [11]. Their cells are able to survive outside the body for a limited amount of time, which makes them ideal indicator organisms to test environmental sample for feacal contamination [12].

Mechanisms of action of some selected antimicrobial Drugs

Ciprofloxacin is a synthetic chemotherapeutic antibiotic of the fluoroquinolone drug class [13]. It is a second-generation fluoroquinolone antibacterial. It is a broad- spectrum antibiotic active against Gram positive and Gram negative bacteria. It kills bacteria by interfering with the enzymes that cause DNA to rewind after being copied, which stops synthesis of DNA and of protein. This mechanism can also affect mammalian cell replication. In particular, some congeners of this drug family (for example those that contain the C-8 fluorine) display high activity not only against bacteria but also against eukaryotic and are toxic to cultured mammalian cells and *in vivo* tumor models [14]. As such the pediatric use of ciprofloxacin is restricted to proven complicated urinary tract infections and pyelonephritis due to *E. coli* and inhalation anthrax [15]. Also, some fluoroquinolones may cause injury to the chromosome of eukaryotic cells. Vancomycin is an antibiotic used to treat intestinal diseases. This antibiotic treats only bacterial infections of the intestines. It will not work for other bacterial infections or viral infections (e.g., common cold, flu). The original indication for vancomycin was for the treatment of penicillin resistant *S. aureus* [16]. Vancomycin never became the first line treatment for *S. aureus* for several reasons; because of it poor oral bioavailability, it must be given intravenously for most infections. Secondly, beta-lactamase-resistant semi synthetic penicillin's such as methicillin were subsequently developed, which have better activity against non MRSA *Staphylococci.* These findings led to vancomycin are being relegated to the position of last resort.

Sulphamethoxazole/Trimethoprim (SXT) sometimes referred to as co-trimoxazole. Co-trimoxazole was claimed to be more effective than either of its components individual in treating bacterial infections, although this was later disputed [17]. The global problem of advancing antimicrobial resistance has led to a renewed interest in the use of co-trimoxazole in various settings more recently [18]. Specific indications for its use include; susceptible strains of *E. coli*, shigellosis among other bacterial infection. Cefpodoxime is an oral third- generation cephalosporin antibiotics. It is active against both Gram positive and Gram negative bacteria. It is commonly used to treat acute otitis media, pharyngitis, and sinusitis. It also finds use as oral continuation therapy when intravenous cephalosporin (such as ceftriaxone) is no longer necessary for continued treatment.

Some bacteria contain enzymes that inactivate antibiotics, the most well-known example is β-lactamase [19]. These enzymes inactivate β-lactam antibiotcs by cleaving the β-lactam rings. Some bacteria develop resistance by preventing the antibiotic from entering the bacterial cell or by increasing the removal of the drug out of the cell (faster than it enters the cell). Reduced ability to enter the cell occurs with resistance to fluoroquinolones, aminoglycosides and penicillins and can be overcome by increasing the drug concentration [20]. Increased removal of tetracycline is encoded by genes such as tet (A) and results in resistance of many enterobacteriaceae. Similar mechanisms have been described for resistance to erythromycin, chloramphenicol and ciprofloxacin [20]. Structural changes in some bacteria can block activity of some antibiotics that successfully enter bacterial cells. For example, proteins responsible for cell wall synthesis of enterococci have low affinity for cephalosporins making these bacteria inherently resistant [19]. Elimination of the binding site by resistance genes carried on plasmids can result in resistance to macrolide, lincosamide, and streptogramin B (MLS resistance). Likewise, the tet (M) gene, produces a protein that can inhibit binding of tetracycline [20]. Bacteria can also produce alternative binding sites that are resistant to inhibition. This mechanism is observed in methicillin resistant Staphylococcus aureus (MRSA), which produce the penicillin binding protein PBP2 encoded, by mec A.

Materials and Methods

Samples Collection

Fura de nunu samples were collected between July and September, 2011 at Sabo and Star light area in Ogbomosho, Oyo State, Nigeria, into sterile plastics containers with lid, sterilized with ethanol, and then taken to the laboratory for analyses. Twenty-eight (28) samples of *Fura de nunu* were collected.

Bacteria isolation from *Fura de nunu* sample

Normal saline was prepared to carry out serial dilution of samples (0.85 g of sodium chloride $NaCl_2$ was dissolved in 100 ml of distilled water). 9 ml of normal saline was dispensed

inside a test tube each covered with cotton wool and foil paper, and they were autoclaved at 121 ^{0}C for 15 min. Serial dilution of *Fura de nunu* were carried out in order to thin out there microbial load by taking 1ml of *Fura de nunu* into 9 ml of sterile normal saline. Successive dilution was done until 10^{-10} dilutions were obtained and 10^{-3} were aseptically streaked on fresh agar plates such as Eosin methylene blue, and Mannitol salt agar with the aid of a wire loop, and the plates were incubated overnight at 37 ^{0}C. The incubated plates were examined after 24 h, and they were sub- cultured on a fresh agar plates and incubated at 37^{0}C for 24 h to obtain a pure culture. 19 *E. coli* and 23 *S. aureus* strains were isolated. Pour plating of *Fura de nunu* sample was carried out, by inoculating 1ml of 10^{-7} dilution with the aid of sterile syringe inside a sterile petri dish and a sterilized nutrient agar was poured on the inoculum and rocked, incubated at 37 ^{0}C for 24 h and the total viable counts were recorded for *Fura de nunu* sample as shown from Table 6 below.

Antimicrobial susceptibility testing

Antibiotics susceptibility testing of the isolated bacteria i.e. *Staphylococcus aureus* and *E. coli* were carried out using disc diffusion method as described by the British Society for Antimicrobial Chemotherapy (BSAC) [21]. The isolates were streaked on fresh Mannitol Salt Agar and on Eosine Methylene Blue agar plates respectively and were incubated overnight at 37 ^{0}C. The pure culture on each of the cultured medium were swabbed with swab stick and emulsified inside normal saline to obtain a bacteria suspension that correspond to the turbidity of 0.5 Mc Farland standard (McFarland was prepared by adding 0.5 ml of 0.048 M of $BaCl_2$ to 99.5 ml of 0.18 M of H_2SO_4 with constant stirring to ensure even suspension). The standard was vigorously agitated before use. The standardized suspension was used to inoculate Mannitol Salt Agar plate using sterile swab stick and this was done aseptically. Antimicrobial susceptibility discs containing Methicillin (10 µg), Oxacillin (1 µg) and Vancomycin (5µg) were used for *staphylococcus* and for *Escherichia coli* Cefpodoxime (10 µg), Ciprofloxacin (5 µg), Sulphamethoxazole/Trimethoprim (25 µg) were also used. Antimicrobial susceptibility discs were placed on the plates containing Mannitol salt agar and Eosine Methylene Blue with the aid of a forceps and this was done aseptically. The inoculated plates were incubated overnight at 37 ^{0}C. The diameters of the zones of inhibition were measured at two perpendicular planes and the average was found. The susceptibility of each isolate was determined by comparing the result with drugs from the standard charts. Bacteria were classified as either sensitive or resistant based on the zone diameter interpretative standard of the Clinical Laboratory Standards Institutes.

Results and Discussion

Nineteen (19) isolates of *Escherichia coli* and Twenty-three (23) isolates of *Staphylococcus aureus* were isolated from the sample of *Fura de nunu* from Ogbomosho, Oyo State, Nigeria. Table 1 and 2 showed the 19 isolates of *E. coli* isolated from *Fura de nunu* tested, 6 (31.6%) were resistant to Sulphamethoxazole/Trimethoprim, 1 (5.3%) to Ciprofloxacin and none was resistant to Cefpodoxime, i.e all were sensitive. None was resistant to two or three at a time.

Table 1: Shows number of isolates and frequency of resistant of *E. coli* to Sulphamethoxazole /Trimethoprim (25 µg)

Samples	Number of Isolates	Frequency
Fura de nunu	19	6 (31.6%)

Table 2: Shows number of isolates and frequency of resistant of *E. coli* to Ciprofloxacin (5 µg)

Samples	Number of Isolates	Frequency
Fura de nunu	19	1 (5.3%)

Antimicrobial resistance in bacteria pathogens is a major impediment to successful therapy, and in several instances, bacteria strains have arisen that are refractory to most available antimicrobial treatments [24] (Levy, 1992). In this study, Nineteen (19) *E. coli* isolates tested from the samples collected. From the 19 isolates from *Fura de nunu*, 6 isolates were resistant to Sulphamethoxazoole/Trimethoprim, 1 (5.3%) to Ciprofloxacin, none was resistant to Cefpodoxime.

Moreover, Table 3, 4 and 5 shows the number of *Staphylococcus aureus* tested, 23 were isolated from *Fura de nunu* and 8 (34.8%) were resistant to Methicillin, 4 (17.4%) to Vancomycin and 7 (30.4%) to Oxacillin.

Table 3: Shows number of isolates and frequency of resistant of *S. aureus* to Methicillin (10 µg).

Samples	Number of isolates	Frequency
Fura de nunu	23	8 (34.8%)

Table 4: Shows number of isolates and frequency of resistant of *S. aureus* to Vancomycin (5 µg)

Samples	Number of isolates	Frequency
Fura de nunu	23	4 (17.4%)

Table 5: Shows number of isolates and frequency of resistant of *S. aureus* to Oxacillin (1 µg)

Samples	Number of isolates	Frequency
Fura de nunu	23	7 (30.4%)

Also out of Twenty-three (23) *Staphylococcus aureus* tested, 23 from *Fura de nunu*, 8 (34.8%) were resistant to Methicillin, 4 (17.4%) to Vancomycin, 7 (30.4%) to Oxacillin. A recent study by the Translational Genomics Research Institute showed that nearly half (47%) of the meat and poultry in U.S. grocery stores were contaminated with *S. aureus*, with more than half (52%) of those bacteria resistant to antibiotics [25] (Ryan, 2000).

Table 6: Total viable count for *Fura de nunu* with dilution factor of 10^{-7}

Samples Plated	Number of Colony	Dilution Factor(10^{-7})
4	2	2.0×10^7
3	3	3.0×10^7
1	5	5.0×10^7
1	6	6.0×10^7
2	7	7.0×10^7
2	8	8.0×10^7
1	13	13×10^7
1	14	14×10^7
2	18	18×10^7
1	19	19×10^7
1	25	25×10^7
1	28	28×10^7
1	36	36×10^7
1	37	37×10^7
1	38	38×10^7
1	45	45×10^7
1	60	60×10^7

The resistant of *S. aureus* to Vancomycin is high compared to that of Methicillin and Oxacillin Resistant of *E. coli* to Sulphamethoxazole/Trimethoprim is greatly high in all the samples tested than that of Ciprofloxacin and Cefpodoxime. The result from this study shows that there is prevalence of resistance in *E. coli* and *S. aureus* isolated from samples investigated in this research work. Thus, such ecosystem can serve as reservoir of clinically relevant antimicrobial drug resistance. In the Table 6, the total viable count for *Fura de nunu* with dilution factor of 10^{-7} is shown with the number of plate and number of colony formed.

Conclusion

This present study has shown that there is a good number of *E. coli* and *Staphylococcus aureus* exhibiting multi resistance to antibiotics. However the percentage, at which *E. coli* is resistant to sulphamethoxazole/trimethoprim, is higher than ciprofloxacin and cefpodoxime. So also, the percentage of vancomycin resistant *Staphylococcus aureus* is high in swine than the other samples worked upon. Hence, there is a very high chance of passing the antibiotics resistant bacteria into the human ecosystem. This portends a great danger to human health.

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