openaccess Journal

SCIENCE LETTERS 2014 | Volume 2 | Issue 1 | Pages 19-23

Antibiotic sensitivity of different fluoroquinolones and aminoglycosides against milk and beef bacterial isolates

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Abstract
Food is essential to mankind, but it becomes harmful for human beings when it is contaminated with food-borne pathogens. Antibiotics are used indiscriminately in controlling food-borne infections. A research was conducted to find out suitable antibiotics for treating food-borne infections. In this study, 200 samples of un-processed milk and raw beef (100 each) were collected from different outlets of Lahore, Pakistan. Milk and beef samples 75% and 85%, respectively, were positive for total viable count having 30-300 colonies per milliliter of milk and beef wash. Five major food-borne pathogens were isolated. The isolated bacterial species were purified and confirmed by different biochemical tests. It included both Gram positive (*Staphylococcus aureus* and *Bacillus cereus*) and Gram negative (*Escherichia coli*, *Salmonella typhi* and *Campylobacter jejuni*) bacterial strains. Antibiotic sensitivity test was applied on the isolated and purified bacterial strains with ten selected broad spectrum aminoglycoside antibiotics (amikacin, gentamycin, neomycin, streptomycin and tobramycin) and fluoroquinolone antibiotics (ciprofloxacin, levofloxacin, moxifloxacin, flumequine and pefloxacin). After 24 hrs of incubation of commercially prepared antibiotic discs on Mueller-Hinton agar plates, gram-positive cocci showed highest sensitivity to selected salts of fluoroquinolone and aminoglycoside antibiotics, while gram-negative rods were least sensitive to fluoroquinolone salts. It was concluded that among the fluoroquinolones, moxifloxacin was highly effective against *Bacillus cereus* whereas among aminoglycosides, gentamycin was the best choice of drug in therapeutics of food-borne diseases.
Key Words: Antibiotic sensitivity testing, food-borne diseases, antibiotics, infection.

Received December 12, 2013; **Revised** February 21, 2014; **Accepted** February 27, 2014
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To Cite this manuscript: Khatoon F, Fatima A, Shahzad KA, Iftikhar F, Siddique K, Qasim M, Younis M, Butt MS, Qazi MH. Antibiotic sensitivity of different fluoroquinolones and aminoglycosides against milk and beef bacterial isolates. Sci Lett 2014; 2:19-23.

Introduction

Now-a-days food-borne illness is rampant in the society. There are various reasons for that, like food adulteration using hazardous chemical preservatives, food processing in an unhygienic manner and fast food which is often not properly cooked. A new generation of antibiotics like fluoroquinolone and aminoglycoside are the classes of antibiotics, which are frequently used in treating the food-borne pathogens infected patients [2]. The fluoroquinolones interfere with DNA replication by inhibiting an enzyme complex called DNA gyrase while aminoglycoside interfere with protein synthesis by binding to the ribosome. In particular, some congeners of fluoroquinolones drug family displays high activity not only against bacterial topoisomerases, but also against eukaryotic topoisomerases and are toxic to cultured mammalian cells and *in vivo* tumor models [4]. Aminoglycoside shows adequate activity against enterococci when it is used synergistically with a cell wall-active antibiotic [1]. In contrast to the fluoroquinolone, an aminoglycoside is a class of antibiotics which shows resistance due to different types of enzymes modifications, e.g., aminoglycoside acetyltransferases (ACC), aminoglycoside nucleotidyltransferases (ANT) and aminoglycoside phosphotransferases (APH) enzymes. Owing to this enzyme modification, aminoglycoside lose their ribosome-binding ability and thus protein synthesis is no longer inhibited [2]. Quinolone induced DNA damage was first reported by Hussy *et al*. [3]. When any patient goes to any clinician with a history of food poisoning or food borne illness, clinician treats the patient with commercially available antibiotics in the market. Sometime antibiotics do not respond due to indiscriminate use of antibiotic resistant food-borne pathogen. Commonly encountered food borne bacterial species are *Bacillus cereus*, Campylobacter spp. and Salmonella spp. A major causative agent of food borne diseases is Campylobacter spp. and Salmonella spp. [4]. Salmonella is causative agent of non-typhoidal salmonellas (food born disease) in the Indian subcontinent, south–east Asia, South and Central America and Africa. The non typhoidal species of salmonella such as *Salmonella wien*, *Salmonella typhimurium*, *Salmonella johannesburg*

and *Salmonella oranienburg* [5]. This study was undertaken to isolate the major food borne pathogens from raw milk and raw beef samples and to evaluate the antibiotic sensitivity of different salts of aminoglycoside and fluoroquinolone drugs. Moreover, the effect of the drugs on different bacterial strains was compared.

Materials and methods

Isolation and identification of microbial strains

Two hundred raw beef and raw milk samples were collected aseptically by using sterile disposable gloves and zipper bags to avoid from external contamination, from different outlets of Lahore, Pakistan. Each sample was comprised of 250 grams of beef and 250 ml of raw unpacked milk. Clean disposable zipper bags were used to collect samples, placed in ice cooler and transported to the Microbiology laboratory, Institute of Molecular Biology and Biotechnology, The University of Lahore. Various types of media such as nutrient agar, blood agar, MacConkey agar, Simmon's citrate agar, eosin methylene blue agar, Muller Hilton agar, menitol salt agar, Salmonella Shigella agar and starch agar were re-hydrated in accordance with instructions of the manufacturers and used for isolation, purification, observation of growth characteristics and identification of organisms according to standard Buxton and Fraser techniques [6]. Each of the media was examined for sterility by incubation at 37^{o}C for 24 hours. All the media were sterilized before use. Nine ml of the Phosphate Buffer Saline (PBS) was added to each of the 5 labeled test tubes and the tubes were autoclaved at 121^{o}C for 15 minutes. One ml of each of the raw milk and raw beef wash sample was added in 1st tube and tenfold dilution up to 10^{-5} for milk and 10^{-3} for raw beef wash samples were prepared. On the basis of color and morphology, different colonies were selected from plates and streaked on new agar medium plates to get pure cultures. A slide smear was prepared from a pure colony of each of the isolate and stained using Gram's techniques [7]. Stained slides were examined under oil immersion objective (100 X) of the bright field compound microscope. Staining characteristics and microscopic morphological appearance of individual bacteria were recorded. Moreover, biochemical tests were employed using brain heart infusion (BHI) medium. Indole production test, Vogues Proskauer's test, methyl red test, citrate utilization test, catalase test, starch hydrolysis test and oxidase test were performed to identify the isolated cultures to species level. Each of the pure isolate was biochemically characterized and identified by using already described standard protocols [8].

Antibiotic sensitivity analysis

Antimicrobial susceptibility testing (AST) was performed by the agar disk diffusion method to check the sensitivity of antibiotics (fluoroquinolone and aminoglycoside) against the isolated and identified microorganisms using commercially available sensitivity disks (Cat # CT025B, Oxoid, Hampshire, UK). Disk diffusion method, standardized by the National Committee for Clinical Laboratory Standards (NCCLS) to perform antibiotic sensitivity test, was employed [15]. The turbidity of the inoculums was adjusted by McFarland standard solution for the susceptibilities. Each culture was tested by streaking onto a non-inhibitory agar *i.e.*, nutrient agar to obtain isolated colonies. After incubation at 37°C overnight, 4 or 5 well-isolated colonies of each bacterial strain were selected with an inoculating loop and transferred to a tube containing sterile saline. The bacterial suspension was compared to the 0.5 McFarland standard solutions. The turbidity standard was first agitated on a vortex mixer immediately prior to use and inoculum suspensions were adjusted. A sterile cotton swab was dipped into the suspension, firmly pressed against the inside wall of the tube just above the fluid level and was rotated to remove excess liquid. The swab was streaked over the entire surface of the medium three times, rotating the plate approximately at 60 degrees. Finally, swabbing was done all around the edge of the agar surface. Fluoroquinolone antibiotic,; ciprofloxacin (5 µg), pefloxacin (5 µg), levofloxacin (5 µg), flumequine (30 µg) and foxifloxacin (5 µg) salts and aimoglycosides antibiotic: amekacin (30 µg), gentamycin (10 µg), neomycin (10 µg), streptomycin (10 µg) and tobramycin (10 µg) were used. Disks were placed individually with sterile forceps into 4 inch agar plate and were gently pressed down onto the agar. Reasonable distance between all disks was kept to avoid the overlapping of sensitivity zones. Plates were inverted and incubated at 35°C for 24 hr. After incubation, the diameters of the inhibition zones were measured

and comparison of results was statistically analyzed by applying analysis of variance (ANOVA).

Results

Among different samples, 75% of milk samples and 85% of raw beef wash were shown positive for total viable count having 30-300 colonies per ml of milk and beef wash. Samples which were announced as negative were having microbial load less than acceptable count (30-300 cfu/ml).

Morphological characteristics of colonies, microscopic and biochemical characterization were performed to identify the bacterial cultures. Subculture technique was performed to get the purified culture of different bacterial species. Gram's staining was performed to differentiate the gram positive and gram negative bacterial species from which gram negative short rods, gram positive cocci and rods were observed. Catalase, starch hydrolysis, oxidase, indole production, methyl red (MR), Voges-Proskauer (VP) and citrate utilization tests were performed to confirm these bacterial strains (Table 1). From 75 positive milk samples 18%, 15%, 29%, 10% and 28% samples were positive for *Campylobacter jejuni*, *Salmonella typhimurium*, *Escherichia coli*, *Bacillus cereus* and *Staphylococcus aureus*, respectively. While 16%, 13%, 20%, 21% and 30% samples were positive for *Campylobacter jejuni*, *Salmonella typhimurium*, *Escherichia coli*, *Bacillus cereus* and *Staphylococcus aureus*, respectively from 85 positive beef samples.

Table 1: Biochemical Tests Performed for Confirmation of Bacterial Strains

Sr #	Biochemical tests	Bacterial species				
		S. aureus	***B. cereus***	***E. coli***	***S. typhi***	***C. jejuni***
1	Gram stain	+ve	+ve	-ve	-ve	-ve
2	Shape	cocci	bacilli	rods	rods	rods
3	Catalase Test	+ve	NA	+ve	+ve	-ve
4	VP Test	NA	NA	-ve	-ve	-ve
5	SH Test	NA	+ve	NA	NA	NA
6	Citrate Test	NA	NA	+ve	-ve	NA
7	MR Test	NA	NA	+ve	-ve	-ve
8	Indole Test	NA	NA	+ve	-ve	-ve
9	Oxidase Test	-ve	NA	-ve	-ve	+ve

Note: NA: Not Applicable, VP: Voges Proskauer, SH: Sugar Hydrolysis, MR: Methyl Red

Simon's citrate was used to confirm the *E. coli* which converts the green color agar medium in to blue color due to the production of citrate enzyme. *E. coli* was negative for VP and positive for indole test. *Salmonella typhimurium* was citrate negative and didn't change the green color of agar into blue. *Staphylococcus aureus and Campylobacter jejuni* were confirmed by catalase test shown by active bubbling. *Staphylococcus aureus and Campylobacter jejuni* both were catalase positive, but differentiated as gram positive cocci and gram negative short rods, respectively. A Positive oxidase test made *Campylobacter jejuni* differentiated from other members of Entarobacteriacae isolates. *Bacillus cereus* was confirmed by starch hydrolysis test shown by a clear zone of inhibition on starch agar plate.

Table 1 showed that among selected antimicrobial drugs of fluoroquinolone antimicrobial agent levofloxacin was highly toxic to *Staphylococcus aureus* and *Salmonella typhimurium* with 1.48±0.08 and 1.51±0.14 mm inhibitory value. *E. coli* was least sensitive with 0.90±0.11 mm value. *Staphylococcus aureus* was highly sensitive to ciprofloxacin with 1.73±0.08 mm whereas *Salmonella typhimurium* least sensitive with 0.93±0.11 mm value for ciprofloxacin. Growth of *Staphylococcus aureus* with 1.33±0.10 value was highly inhibited by pefloxacin and *E. coli* showed the least sensitivity zone with 0.83±0.10 value. Flumequine showed 1.29±0.09 mm zone of inhibition as the highest sensitivity for *Staphylococcus aurous* while *E. coli* was least inhibited with 0.60±0.11 mm value. Moxifloxacin was effectively inhibited by giving 2.25±0.08 for *Staphylococcus aurous*. On the other hand *E. coli* was least inhibited with 0.75±0.09 mm value.

The sensitivity zones of aminoglycosides are presented in Table 3. *Bacillus cereus* was highly sensitive to tobramycin having 0.75±0.12 mm value and *Campylobacter jejuni* was least inhibited as 0.55±0.10 mm zone of inhibition. Growth of *Bacillus cereus* was highly inhibited by neomycin with 0.68±0.11 mm zone whereas *Staphylococcus aureus* and *Campylobacter jejuni* were least inhibited with 0.36±0.12 mm zone of inhibition. *Bacillus cereus* showed the highest sensitivity to amekacin with 1.25±0.12 mm zone *Campylobacter jejuni* with 0.56±0.15 mm value was least sensitive. *Bacillus cereus* showed 1.09±0.07 value against gentamycin while *Salmonella typhimurium* 0.64±0.19 value with least zone of inhibition. Streptomycin showed highest sensitivity to B*acillus cereus* with0.97±0.10 and *Salmonella typhimurium* with lowest sensitivity with 0.52±0.08 value for gentamycin.

Table 2: Inhibition zones of different microbial strains in the presence of different fluoroquinolone antibiotics.

Bacterial species	n	Cipro	Levo	Peflo	Flumi	Moxi
Campylobacter jejuni	34	1.61±0.31	0.87±0.16	1.11±0.16	0.96±0.18	1.11±0.14
Salmonella typhimurium	28	0.93±0.11	1.51±0.14	1.01±0.15	0.80±0.23	1.08±0.13
E. coli	49	1.00±0.12	0.90±0.11	0.83±0.10	0.60±0.11	0.75±0.09
Bacillus cereus	31	1.22±0.17	1.15±0.10	0.96±0.13	1.08±0.19	1.06±0.12
Staphylococcus aureus	55	1.73±0.08	1.48±0.08	1.33±0.10	1.29±0.09	2.25±0.08
Total	197	1.33±0.37	1.18±0.30	1.07±0.23	0.96±0.30	1.33±0.60
p-value		<0.001	<0.001	<0.001	<0.001	<0.001

The *p*-value <0.05 shows that sensitivity zone is significantly different for micro-organism for a certain drug.

Table 3: Inhibition zones of different microbial strains in the presence of different aminoglycoside antibiotics.

Bacterial species	*n*	*Genta*	*Tobra*	*Neo*	*Sterp*	*Ameka*
Campylobacter Jejuni	34	0.80±0.20	0.55±0.10	0.36±0.13	0.62±0.11	0.56±0.15
Salmonella Typhimurium	28	0.64±0.19	0.65±0.10	0.36±0.12	0.52±0.08	0.58±0.09
E. coli	49	1.05±0.12	0.73±0.11	0.33±0.11	0.95±0.08	0.86±0.11
Bacillus cereus	31	1.09±0.07	0.75±0.12	0.68±0.11	0.97±0.10	1.25±0.12
Staphylococcus aureus	55	0.80±0.08	0.65±0.08	0.27±0.09	0.80±0.08	0.79±0.07
Total	197	0.89±0.20	0.67±0.12	0.38±0.17	0.79±0.18	0.81±0.24
p-value		<0.001	<0.001	<0.001	<0.001	<0.001

The *p*-value <0.05 shows that sensitivity zone is significantly different for micro-organism for a certain drug.

Discussion

In present study, 75% of milk and 85% of beef samples were positive for bacterial counts which were more than previous reports of prevalence of food-borne pathogens in 43% and 27% samples by Haimanot et al. [9] and Jayarao and Henning [10], respectively. Improper handling and unhygienic processing of food items (milk and beef) might be the reasons for such high percentage of positive samples. Milk samples were positive for *Campylobacter jejuni* (18%), *Salmonella typhimurium* (15%), *E. coli* (29%), *Bacillus cereus* (10%) and *Staphylococcus aureus* (28%). While 16%, 13%, 20%, 21% and 30% of beef samples were positive for *Campylobacter jejuni*, *Salmonella typhimurium*, *E. coli*, *Bacillus cereus* and *Staphylococcus aureus*, respectively. Whereas Smith et al. [11] reported *Staphylococcus* spp. (20.2%), Bacillus spp. (18.0%), Enterobacter spp. (1.3%) and MRSA *Staphylococcus aureus* 12 (44%) form milk samples and in another study conducted by Cuiwei et al. [12], Campylobacter spp., Salmonella spp. and *Escherichia coli* were isolated from beef samples which is lesser than our findings.

Antibiotic sensitivity testing was performed against all the isolated bacterial food pathogens. Gram positive cocci were sensitive for selected salts of fluoroquinolone while Gram positive rods were more inhibited by aminoglycoside salts. On the other hand, aminoglycoside and fluoroquinolone antibiotic disks showed intermediate zones of inhibition to Gram negative rods. Our results were in agreement with the findings of HyoBi et al. [13], Al-Allaf et al. [14] and Smith et al. [11], who used same broad spectrum antibiotics. Broad spectrum antibiotics could be more effective against Gram positive bacteria and less for Gram negative ones. It might be because of high enzyme modifications according to environmental conditions. Conclusively, among the fluoroquinolones, moxifloxacin was highly effective against *Bacillus cereus*, whereas, among aminoglycosides, gentamycin was the best choice of drug in therapeutics of food-borne diseases.

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