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Original Article

Antibiotic Sensitivity and Evaluation of Plasmid Profile of Major Foodborne Pathogens

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Abstract

This study revealed the reason behind the antibiotics resistance of isolated food-borne pathogens through their susceptibility testing to various antibiotics of choice. The results of the study revealed that resistance of the bacteria isolates which are Streptococcus sp., Staphylococcus aureus, Proteus vulgaris, Shigella sp., Escherichia coli, Pseudomonas aeruginosa, Bacillus sp. to different antibiotics varies and differs considerably. For instance, Pseudomonas aeruginosa showed resistance to Amoxicillin, Augmentin, Gentamicin and Tetracycline. Staphylococcus sp. isolated showed multiple resistances to Cloxacillin, Erythromycin, Amoxicillin, Augmentin and Gentamicin. Proteus vulgaris showed multiple resistances to five antibiotics in-vitro which are Augmentin, Nitrofurantoin, Amoxicillin, Cotrimoxazole and Nalidixic. The seven isolates were then assayed for plasmid profiling by agarose gel electrophoresis. All the isolates has plasmid with varying sizes of between 9–21kb. Further conjugative study will reveal more reason behind the resistance.

Introduction

Food borne disease arises by the consumption of contaminated foods or beverages. Many different pathogens can contaminate foods, which have given rise to so many different food borne infections[13].

Also, poisonous chemicals, or other harmful substances can cause food borne diseases if they are present in food [24].More than 250 different food borne diseases have been described. Most of these diseases are infections, caused by a variety of bacteria, viruses, and parasites that can be food-borne[13]. Other diseases are poisonings, caused by harmful toxins or chemicals that have contaminated the food, for example, poisonous mushrooms. These different diseases have many different symptoms, so there is no one"syndrome" that is a food borne illness[22]. However, the microbe or toxin enters the body through the gastrointestinal tract, and often causes the first symptoms there, so nausea, vomiting, abdominal cramps and diarrhoea are common symptoms in many food borne diseases[19].Many microbes can spread in more than one way, so we cannot always know that a disease is food borne. However, the distinction matters, because public health authorities need to know how a particular disease spreads in order to take the appropriate steps to stop it. For example, Escherichia coli O157:H7 infections can spread through contaminated food, contaminated drinking water, contaminated swimming water, and from toddler to toddler at a day care centre[15]. Depending on which means of transmission caused a case, the measures to stop other cases from occurring could range from removing contaminated food from stores, chlorinating a swimming pool, or closing a child day care centre[25].

Materials and Methods

Antibiotics Susceptibility Test

Susceptibility of isolates to different antibiotics were tested following Kirby Bauer disc diffusion method using Muller Hinton Agar against selected antibiotics, namely Ampicillin (25µg), Chloramphenicol (50µg), Kanamycin (30µg), Streptomycin (30µg) and Tetracycline (100µg) (Hi-Media, Mumbai). Amoxicillin(30µg), Amoxicillin-Clavulanic acid (30 µg), Cefoxitin (30 µg), Cefotaxime (30 µg), Ceftriaxone (30 µg), Cefuroxime (30 µg), Ceftazidime (30 µg), Gentamicin (10 µg),), Ofloxacin (30 µg), Cloxacillin (5µg), Nitrofurantoin (30 µg) Levofloxacin (30 µg), Ciprofloxacin (30 µg) (Oxoid, UK). Inhibition zone size was interpreted using standard recommendation of National Committee for Clinical Laboratory Standards [N.C.C.L., 2000] now known as Clinical Laboratory Standard Institute (CLSI) [C.L.S.I., 2005]

Procedure For Performing The Disc Diffusion Test

The growth method was performed as follow;

(1) At least three to five well isolated colonies of the same morphological type was selected from an agar plate culture. The top of each was touched with a loop, and the growth was transferred into a tube containing 4 to 5ml of a suitable broth medium.(2) The broth culture was incubated until it achieved or exceeded the turbidity of the 0.5McFarland standards which took up to six hours.

Inoculation of Test Plates.

Optimally, within 15 minutes after adjusting the turbidity of the inoculum's suspension, a sterile cotton swab was dipped into the suspension. The swab was then rotated several times and pressed firmly on the inside wall of the tube above fluid level. This was done to remove excess inoculums from the swab.(2) The dried surface of a Mueller-Hinton agar was inoculated by streaking the swab over the entire sterile agar surface. This procedure was repeated by streaking two or more times, by rotating the plate approximately 60$^{?}$C each time to ensure an even distribution of inoculums. As a final step. the rim of the agar was swabbed.(3) The lid was left ajar for some minutes to allow for any excess surface moisture to be absorbed before applying the drug-impregnated discs.

Placement of Discs

The predetermined battery of antimicrobial discs was dispensed onto the surface of the inoculated agar plate. Each disc was pressed down to ensure complete contact with the agar surface. Whether individually or with a dispensing apparatus, they must be distributed evenly so that they are no close than 24mm from center to center. Ordinarily, no more than 12 discs should be placed on 150-mm plate, or more than five discs on a 100m plate. Because some of the drug diffuses almost instantaneously, a disc should not be relocated once it has come into contact with the agar surface. Instead, place a new disc in another location on the agar.

The plates were then inverted and placed in an incubator set to 350c within 15 minutes after the discs are applied.

Reading Plates and interpreting results

After 16 to 18 hours of incubation, each plate was examined, the diameters of the zones of inhibition (as judged by the unaided eye) are measured, including the diameter of the disc.

Zones are measured to the nearest whole millimeter, using sliding calipers or a ruler, which is held on the back of the inverted Petri plate. The sizes of the zone of inhibition are interpreted by referring to the NCCL's table on Zone Diameter Interpretive Standards and Equivalent Minimal Inhibitory Concentration (MIC) Break points for Enterobacteriaceae.

Plasmid Profile Analysis

Plasmids are circular deoxyribonucleic acid molecules that exist in bacteria, usually independent of the chromosome. The study of plasmids is important to medical microbiology because plasmids can encode genes for antibiotic resistance or virulence factors. Plasmids can also serve as markers of various bacterial strains when a typing system referred to as plasmid profiling, or plasmid fingerprinting is used. In these methods, partially purified deoxyribonucleic acid species are separated according to molecular size by agarose gel electrophoresis. In a second procedure, plasmid deoxyribonucleic acid which has been cleared by

restriction endonucleases can be separated by agarose gel electrophoresis and the resulting pattern of fragments was used to verify the identity of bacterial isolates. Because of the many species of bacteria, plasmid profile analysis has been used to investigate outbreaks of many bacterial diseases and to trace inter-and intra species spread of antibiotic resistance.

Mechanism of plasmid profile analysis

Plasmids were detected by a modified rapid method. Cells were grown in 3ml of L-broth overnight at 30^{0}C to an optical density at 600mm of 0.8 and pelleted by centrifugation (5,700 rpm, 4^{0}C, for 7 min in a Sorvall SS34 rotor). The cell pellets were thoroughly suspended in 1ml of E buffer. The cells were then lysed by adding 2ml of lysing solution, which was mixed by brief agitation.

The solution was heated at 50 °C to 65°C for 20min in a water bath, and 2ml volumes of phenolcholoform solution (1:1, vol/vol) were added. The solution was emulsified by shaking, and the emulsion was broken by centrifugation (6,000 rpm, 15min, 4^{0}c IN A Sorvall SS34 rotor). Avoiding the precipitate at the interface, the upper aqueous phase was transferred to a screw cap tube by using a polyethylene Pasteur pipette (Ulster Scientific, Inc, Highland, N.Y). Sample was withdrawn directly for electrophoresis immediately. 35pl of sample was mixed on a square of parafilm "M" (American Can Company, Greenwich, Conn.) with 10 pl of 0.25% bromocresol purple in 50% glycerol-0.05 M Tris- acetate (pH7.9). To minimize sharing of large plasmids it is important that samples be withdrawn and transferred with Drummond 50-ud micropipettes and an Adams no 4555 suction apparatus (Becton, Dickinson & Co., Rutherford, N.J.) After centrifugation, the supernatant was subjected to agarose gel electrophoresis for the detection and sizing of plasmid DNA.

The organism were separated into two groups (i.e gram positive and gram negative) and the extraction mechanism for each was applied. Details are given below;

Extraction of plasmids from Gram positive bacteria

The plasmids were extracted by growing the bacterial cellsovernight on Luria –Bertani medium supplemented with 50µg/mL ampicillin. It was then centrifuged at 13,000 rpm for 2 minutes to pellet cells. 500µL of normal saline was added, vortex and pulse at 13,000 rpm for 10 seconds to wash cells. The supernatantwas removed and cells re-suspended in 500µL of Tris (10mM)-EDTA (1mM)-Sucrose (50mM) buffer (pH 8.0). Later 500µL Lysis buffer solution containing SDS (1%) and Lysozyme (2mg /mL) in Tris (10mM)- EDTA (1mM) buffer (pH 8.0) added. Then incubated at 37^{0}C for 2 hours. The preparation was boiled at 95°C for 3 minutes and the suspension was neutralized by adding 3M KOAC (pH 5.2). Then the suspension wascentrifuged at 13,000 rpm for 5 minutes. The upper layer was transfered into a new sterile Eppendorf tube. Twice the volume of absolute ethanol (95.5%) was added and centrifuged at 10,000 rpm for 5 minutes. The supernatant was decanted and pellet washed with 70% ethanol. Twice the volume of absolute ethanal was added and centrifuged and the pellet was dried in the air. The DNA pellet was re-dissolved in 40µL of Tris-EDTA buffer (pH 8.0). Then the purity of the DNA solution was assessed using nanodrop Spectrophotometer and the plasmid DNA samples were electrophoresed on 0.9% agarose at 70V for 40 min.

Plasmid DNA Isolation from Gram Negative Bacteria (Alkaline Lysis method of Bimboimand Doly, 1979)

200µl of buffer 1A was added to cell pellet and vortex, and 400µl lysing solution. The test tubes were inverted20 times at room temperature. 300µl of ice cold buffer 2B added, vortex and keep on ice for 30 minutes. Suspension centrifuged at 3,000xg for 15 minutes. To the supernatant, 700µl of chloroform was added and Vortex then centrifuged at 3,000xg for 10 minutes.To 500µl aqueous layer, 1ml of absolute ethanol was added and kept in ice for 1 hour, and again centrifuged at 3000xg for 30 minutes. Pellet was then washed with 70% ethanol, decanted and dried. Then 50µl of buffer 3C was added to the pellet.

The composition of each buffer solution and lysing solution are given below;

BUFFER 1A
400mM Tris
20mM Na EDTA
Acetic acid to pH 8.0

BUFFER 2B
3MNa Acetate
Acetic acid to pH 5.5

BUFFER 3C
10mM Tris
2mM Na_2 EDTA
Acetic acid to pH 8.0

LYSING SOLUTION
4% SDS
100mM Tris

Plasmid DNA was extracted by alkaline lysis method with (Gram +ve) and without (Gram –ve) Lysozyme. Electrophoresis was done horizontally on 1% agarose gel at 70V for 40 minutes, stained with ethidium bromide, visualized under UV light and photographed thereafter.

Sizes of DNAs were extrapolated based on the relative mobilities of 10kb ladder DNA markers that were co-electrophoresed with the DNA samples.

Results

Table 1 shows the results obtained from the antibiotic sentivity test carried out, Table 2 shows the multiple resistance of bacterial isolates to antibiotics while Figure 1 shows the plasmid profiling of the isolates.

The antibiotics susceptibility testing of the isolated organism showed varied results (Table 1). Streptococcus sp. showed resistance to four antibiotics which are Cotrimoxazole, Nitrofurantoin, Tetracycline and Ofloxacin. Escherichia coli showed resistance to Amoxicillin, Tetracycline, Chloramphenicol and Augmentin. Proteus vulgaris showed multiple resistances to five antibiotics which are Augmentin, Nitrofurantoin, Amoxicillin, Cotrimoxazole and Nalidixic. The Staphylococcus sp. isolated showed multiple resistances to Cloxacillin, Erythromycin, Amoxicillin, Augmentin and Gentamicin. Shigella sp. was resistant to Amoxicillin and Augmentin. Bacillus sp. was resistant to Amoxicillin, Cotrimoxazole,

Table 1 : Antibiotics sensitivity pattern of isolated organisms

Isolated organism	Amx	Cot	Nit	Gen	Nal	Ofl	Aug	Tet	Chl	Ery	Cxc
Shigella sp	_	++	++	++	+	++	_	++	NA	NA	NA
Escherichia coli	_	++	+	++	++	++	_	_	_	NA	NA
Stretococcus sp	+	_	_	++	++	_	+	_	NA	NA	NA
Bacillus sp	_	_	_	++	++	++	++	_	NA	NA	NA
Staphylococcus	_	++	NA	_	NA	NA	_	++	++	_	_
Proteus Vulgaris	_	_	_	++	_	++	_	_	NA	NA	NA
Pseudomonas aeruginosa	_	+	+	_	+	+	_	_	NA	NA	NA

KEY : Amx = Amoxicillin, Cot= Cotrimoxazole, Nit= Nitrofurantoin, Gen= Gentamycin, Nal= Nalidixic, Ofl= Ofloxacin, Aug=Augmentin, Tet=Tetracycline, Chl=Chloramphenicol, Ery=Erythromycin, Cxc=Cloxacillin

_ stands for Resistant NA - Stands for Not Applicable
+ stands for Sensitive ++ stands for strongly sensitive

Table 2 : Multiple antibiotics resistanceof isolated organisms

Bacterial isolates	Antibiotics range	Antibiotics to which multiple resistance was demonstrated
Streptococcus sp.	2-4	Cot, Nit, Ofl, Tet
Staphylococcus aureus	2-5	Amx, Gen, Aug, Ery, Cxc
Proteus vulgaris	2-6	Amx ,Cot, Nit, Nal, Ofl, Aug
Salmonella typhi	2	Amx ,Aug
Shigella sp.	2	Nal, Ery
Escherichia coli	3	Amx ,Tet, Chl
Pseudomonas aeruginosa	2-4	Amx, Gen, Aug, Tet

KEY : Amx = Amoxicillin, Cot= Cotrimoxazole, Nit= Nitrofurantoin, Gen= Gentamycin, Nal= Nalidixic, Ofl= Ofloxacin, Aug=Augmentin, Tet=Tetracycline, Chl=Chloramphenicol, Ery=Erythromycin, Cxc=Cloxacillin

Gentamycin and Tetracycline. Another organism isolated is Pseudomonas aeruginosa which showed resistance to Amoxicillin, Augmentin, Gentamicin and Tetracycline.

Discussion

Antibiotics resistance among bacteria has been a major concern and a worldwide problem, particularly hospital bacterial pathogens which led to a number of hazards as well as benefits of antimicrobial therapy [13].

All the test organisms used in this research showed multiple resistances to different antibiotics. Such response of the organism against the antibiotics indicates a possible role of plasmids in such resistance behaviour. The presence of multiple plasmids may support the high resistance profile against a range of antibiotic as plasmid borne resistance is common in many microbes. It is well reported that antibiotic resistance is often plasmid borne [46]. Proteus vulgaris exhibited the highest resistance to the antibiotics, with a value of six out of eleven antibiotics (54.5%). Resistance of Proteus vulgaris against Cotrimoxazole and Nitrofurantoin was reported by [22]. Similar results were reported by [15].

The high antibiotic resistance of Proteus vulgaris may be an indication of the resistance levels among the Enterobacteriaceae since indiscriminate ingestion of antibiotics provides selective pressure, leading to a higher prevalence of resistant bacteria [5].Not only are these species potential causes of infections but also potential reservoirs of resistance genes that could be transferred to other bacterial pathogens. The high levels of ß-lactamase production and multi-drug resistance of the isolates are indications of an increase in the resistance menace reported by many studies [22]. Staphylococcus aureus, being the second highest most resistant pathogen in this research, is mostly dependent on the presence of plasmids for resistance. Plasmids are believed to play a very important role in mediating and transferring resistance to antibacterial drugs in the Staphylococcus population. They can be vectors of resistance genes, or these genes can be localized in discrete transposable elements of DNA called transposons, which are mobile and can move from one DNA molecule to another. This can lead to the rapid spread of antibiotic resistance in a staphylococcus population, and explains the emergence of multi-resistant strains. Pseudomonas aeruginosa also exhibited a considerably high level of multiple resistances. This is in correlation with the report of[14] that Pseudomonas. Aeruginosa is frequently resistant to many commonly used antibiotics. The multiple resistances shown by Escherichia coli to Amoxicillin, Chloramphenicol and Tetracycline agrees with the work of [8] ,who reported multiple resistances of the organism to beta-lactam antibiotics. Five out of a total of seven test organisms (71.4%) were resistant to Amoxicillin, while four organisms (57.1%) were resistant to Augmentin. This agrees with the work of [1]. The high resistance observed for Amoxicillin and Tetracycline could be due to its high and prolonged usage and regular abuse in our society. Nalidixic, Chloramphenicol and Cloxacillin were the least resistant to (14.3%), with one organism each. The use of antibiotics in primary agricultural production is also considered an important cause of antimicrobial resistance selection in bacteria that may subsequently be found on foodstuffs.

This finding is in agreement with the conclusion of some researchers that high percentage of many commonly encountered gram- positive and negative food borne pathogens have at least one and frequently multiple plasmids which may be responsible for their resistance to antibiotics [15], [18] and [22]

Conclusion

The overall result of this study shows that all isolates that are resistant to more than one antibiotics possess Plasmid. Though their sizes varies but all still showed the presence of plasmid which could be said to code for their resistance to the antibiotics.

Several studies had indicated plasmid as coding for the resistance factor (R plasmid) which enables bacterial to resist antibiotics. Since presence of plasmid has been observed in all of these organisms, further molecular studies should be carried out to reveal the exact position of the plasmid and ascertain the exact plasmid responsible.

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