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Journal of Microbiology & Experimentation

Research Article

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Antibiogram and plasmid profiling of resistance bacteria isolated from the blood of Hepatitis C Virus positive individuals

Abstract

In this investigation, the types of bacteria present in the blood of Hepatitis C Virus (HCV) positive individuals attending State General Hospital, Akure, Ondo State, their antibiogram and plasmid profiling. The blood was collected and subjected to standard microbiological techniques to isolate and identify the types of bacteria present. These were subjected to antibiotics disk to determine the antibiogram profile and multiple antibiotics resistance (MAR) pattern was determined. Plasmid profiling and curing was done to determine the genetic basis for resistance of the bacterial isolates. The bacteria species identified are Staphylococcus aureus, *Klebsiella pneumoniae, Pseudomonas aeruginosa, Escherichia coli, Streptococcus pneumoniae, Proteus mirabilis and Salmonella* species and most of the isolates showed multiple antibiotics resistance (MAR). The antibiogram profile of isolates revealed Ciprofloxacin as the most effective antibiotic against most isolated bacterial species while they are resistance to most of other antibiotics especially Amoxycillin, Cotrimozazole and Ceftriazone. The plasmid analysis shows that all multiple resistant bacterial isolates harbor one or more plasmids with different molecular weights and plasmid curing converts all the isolates (MAR) which were initially resistant to the conventional antibiotics to susceptible form thereby indicating that, the resistance was plasmid mediated.

Keywords: antibiogram, plasmid, antibiotics, resistance, susceptibility

Volume 7 Issue 3 - 2019

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Received: May 30, 2019 | **Published:** June 11, 2019

Introduction

Hepatitis C is an inflammatory process in the liver which is characterized by diffuse hepatocellular necrosis. In addition to viral, bacterial and fungal agents, hepatitis can also be caused by drugs, chemicals and toxins.[1] Hepatitis C is an infectious disease caused by the hepatitis C virus (HCV) that primarily affects the liver.[2] During the initial infection people often show mild or no symptoms. Occasionally a fever, dark urine, abdominal pain, and yellow tinged skin occur. The virus persists in the liver in about 75% to 85% of those initially infected. Early chronic infection typically has no symptoms. Over many years however, it often leads to liver disease and occasionally cirrhosis.[3] In some cases, those with cirrhosis will develop complications such as liver failure, liver cancer, or esophageal and gastric varices.[2] HCV is spread primarily by blood-to-blood contact associated with intravenous drug use, poorly sterilized medical equipment, needle stick injuries in healthcare, and transfusions.[3,4] With blood screening for HCV, the risk from a transfusion is less than one per two million.[3] It can also be spread from an infected mother to her baby during birth.[3] It is not spread by superficial contact.[5] It is one of five known hepatitis viruses: A, B, C, D, and E.[6] Diagnosis is by blood test to check for either antibodies to the virus or its RNA. Test is recommended for all people who are at risk.[3]

The genome of HCV is highly mutable. Because HCV is an RNA virus and lacks efficient proofreading ability as it replicates, virions infecting humans undergo evolution with time, giving rise to the notion that HCV persists as a collection of virus quasi-species. By constant mutation, HCV may be able to escape host immunologic detection and elimination.[7-9] HCV undergoes rapid mutation in a hyper variable region of the genome coding for the envelope proteins and escapes immune surveillance of the host. As a consequence, most HCV infected people develop chronic infection. HCV also knocks out the host's Innate Immunity.[10] Scar tissue in the liver restricts the flow of blood and leads to portal hypertension resulting in complications such as ascites, spontaneous bacterial peritonitis, varices and other potentially life-threatening complications. Spontaneous bacterial peritonitis is a condition caused when the body's natural bacteria enters the ascites fluid causing severe infection. This study therefore is to screen the blood of HCV positive individuals in the studied community for the types of bacterial pathogens present, their resistance basis and to know the antibiogram profile of these microorganisms.

Materials and methods

The study was carried out at State General Hospital, Akure and Don Bosco Diagnostic Medical Center, Akure, where the participants were recruited from the different departments. The blood samples were collected after approval by the ethical review committee of the healthcare institution. The sampling and isolation processes with suitable bacteriological media were commenced in October, 2016.

Isolation and identification of associated bacteria

A total of One hundred and twenty one (121) confirmed HCV blood samples were used for the research. In the Department of Microbiology laboratory, Federal University of Technology, Akure, Nigeria. About two milliliters (2ml) of the HCV positive sample was introduced into 5ml Brain-Heart infusion broth and were incubated at 37°C for 24 h before culturing on suitable agar plates (Nutrient agar, MacConkey agar, Chocolate agar and Blood agar plates) for bacterial

growth.[11] The bacterial isolates from each plate were sub cultured for pure colonies and incubated at 37°C for 18-24 h. Identification of bacterial pathogens was carried out by biochemical tests. Gram staining was performed to differentiate the Gram positive and Gram negative bacteria.[12]

Biochemical tests

For identification of the bacterial isolates, biochemical tests were performed. These include Gram-staining, catalase production, motility test, coagulase, oxidase test, indole production and sugar fermentation using the methods of Olutiola et al.,[13] and Cheesbrough.[14]

Gram staining

A small drop of distilled water was placed on a clean grease free slide and with the help of an inoculating needle; the isolate was picked from the surface of agar plate and gently emulsified in the water dropped, and spread to make a thin smear. The smear was air dried and passed through a Bunsen flame to fix. It was then flooded with crystal violet for about 30 seconds and rinsed off with water. Lugol's iodine solution was then applied for 30 seconds and washed off. Ninety five (95%) ethyl alcohol was applied for 30 seconds. It was again washed with water and safranin was added and left for 30 seconds. The safranin was then washed off and the smear dried in air. The slide was examined under oil immersion microscope.[14]

Catalase production

A drop of sterile water was placed on a clean glass slide; the bacterial isolate was picked with a flamed wire loop and emulsified on the glass slide. Several drops of 3% hydrogen peroxide were added and were observed for the presence of bubbles. An effervescence bubble formation was observed which indicated a positive catalase test.[14]

Triple sugar iron (TSI) test

The Triple Sugar Iron (TSI) test is a microbiological test roughly named for its ability to test a microorganism's ability to ferment sugars and to produce acid, hydrogen sulfide (H_2S), and/or gas. Ten milliliters (10ml) of TSI medium was prepared into each test tube. The test tubes were properly covered and sterilized in an autoclave at 121°C for 15 minutes. After sterilization, a sterilized inoculation loop was used to pick an isolated colony and inoculated on TSI agar by firstly stabbed through the center of the medium to the bottom of the tube and then streaked on the surface of the agar slant. A test tube without inoculated organism served as control. The test tubes were incubated for 24 h at 37°C and observed for acid, H_2S and gas production. Yellow coloration indicated acid production, H_2S production was indicated by black colour while gas production was indicated by bubbles/cracks on the medium.[15]

Motility test

A drop of bacterial suspension was placed on the center of cover slip, soft paraffin was applied over the corners of the cover slip. A glass slide was gently placed over the cover slip and held upside down. It was in such a manner that bacterial was hanging between the cover slip and glass slide. The bacterial isolate was first examined under the microscope with 10x magnification and later with 40x magnification.[14]

Oxidase test

A piece of a filter paper was placed on a clean petri dish; 3 drops of oxidase reagent was added on the filter. Using a piece of sterile wooden stick, the organisms were collected and smear on the filter paper. The appearance of a blue-purple colour within 10 seconds indicates a positive reaction.[14]

Indole test

The test isolate was inoculated into a bijou bottle containing 3 ml of sterile peptone water. This was incubated for 48 hours at 37°C. About 0.5 ml of Kovac's reagent was added and shaken gently. Appearance of red colour indicates the presence of indole.[14]

Coagulase test

A drop of physiological saline was placed on each end of a slide. A colony of the test organism in each of the drop was emulsified to make a thick suspension. A drop of plasma to one of the suspension was gently mixed. Clumping of the organisms within 10 seconds indicated a positive coagulase test.[14]

Methyl red test

Exactly five millimeters of glucose phosphate broth (1 g glucose, 0.5% KH_2PO_4, 0.5% peptone and 100 mL distilled water) were dispensed in clean test tubes and sterilized. The tubes were then inoculated with the test organisms and incubated at 37°C for 48 h. At the end of incubation, few drops of methyl red solution were added to each test and colour change was observed. A red colour indicates a positive reaction.[13]

Voges-Proskaeur test

Exactly five millimeter of glucose phosphate broth were dispensed in clean test tubes and sterilized. The tubes were then inoculated with the test organisms and incubated at 37°C for 48 h. After incubation, 6% α-naphthol and 6% Sodium hydroxide were added to about 1 mL of the broth culture. A strong red coloration formed within 30 min indicates positive reaction.[13]

Optochin test

Streptococcus pneumoniae strains are sensitive to the chemical optochin (ethylhydrocupreine hydrochloride). Optochin sensitivity allows for the identification of alpha-hemolytic streptococci such as *S. pneumoniae*, other alpha-hemolytic *Streptococcal* species are optochin-resistant. Commercially made Optochin (P) disks (6 mm, 5µg) were obtained. Optochin disks are often called "P disks" and are labeled with a capital "P". The tested strain was grown for 24 h on a blood agar plate at 37°C in a candle-jar. A loop was used to remove isolated colony from the overnight culture on the blood agar plate and streaked onto fresh blood agar plate. 'P' disk was placed within the streaked area of the plate and was incubated for 24 h at 37°C in a candle-jar. The growth on the plate near the 'P' disk was observed and the zones of inhibition were measured. A zones of inhibition of 14 mm or greater indicates sensitivity and allows for identification of pneumococci.

Preparation of media

All the media used were prepared according to the manufacturer's

specification and sterilized using autoclave at 121°C for 15minutes. The media used were nutrient agar, MacConkey agar, blood agar, chocolate agar for the isolation of pathogens from blood samples and Mueller Hinton (MH) agar for antibiotics susceptibility test.

Antibiotic disc sensitivity testing

The Kirby-Bauer disc diffusion method of Bauer et al.,[16] and Cheesbrough[14] were used to determine antibiotic susceptibility on Mueller-Hinton (MH) agar (Oxoid Ltd., England). The bacterial isolates were tested against antibiotic discs (Fondiscs, Nigeria) which comprised of Augmentin-Amoxicillin/Clavulanic Acid (30µg), Ceftriaxone (30µg), Nitrofurantoin (300µg), Gentamicin (10µg), Cotrimoxazole (25µg), Ofloxacin (5µg), Amoxycillin (25µg), Ciprofloxacin (5µg), Tetracycline (30µg), Pefloxacin (5µg), Streptomycin (10µg), chloramphenicol (30µg) and erythromycin (15µg). They were grouped accordingly into Gram stain reaction categories (gram positives and negatives) and tested against corresponding Gram category of bacteria. Briefly, for the Kirby-Bauer test, bacterial suspension was evenly spread onto MH agar and allowed to dry. Antibiotics discs were then placed on the surface of the agar and incubated at 37°C for 24 h. Thereafter, growth inhibition zones were measured with a ruler, and values obtained interpreted as sensitive or resistant according to the Clinical and Laboratory Standards Institute CLSI,[11] guidelines.

Determination of multiple antibiotic resistance (MAR) index

Multiple Antibiotic Resistance index is a useful differentiation tool in bacterial source tracking and risks analysis by analyzing resistance profile of bacteria against antibiotics tested.[17] MAR was calculated as reported by Christopher and Ali, (2013) as follows:

$$\text{MAR index} = \frac{\text{Number of antibiotics to which the isolate showed resistance}}{\text{Number of total antibiotics exposed to the isolate}}$$

Results were interpreted according to the criteria of Nandi & Mandal:[18]

MAR index ≤ 0.2 was considered low risk, while ≥ 0.2 indicated a high risk of antibiotic contamination.

Extraction of Plasmid

Plasmids were extracted following the method described by Zhou et al.[19] Briefly, cell pellets were obtained from 5 ml of an overnight bacteria broth by centrifugation at 13,000 rpm for 1 min in a centrifuge (Eppendorf 5418, Germany). Pellets were re-suspended in residual supernatant (≈50µl) by vortexing, and 300µl of TENS (25 mM Tris, 10 mM EDTA, 0.1 N NaOH and 0.5% SDS) added and mixed by inverting tubes about 3-5 times. Subsequently, 150µl of 3 M sodium acetate (pH 5.2) was added, vortexed thoroughly, and centrifuged for 5 min to pellet cell debris and chromosomal DNA. Clear supernatant was then carefully transferred into a new 1.5 ml micro centrifuge tube, mixed thoroughly with 900µl of ice cold absolute ethanol, and centrifuged for 10 min to pellet down. Pellets were further washed with 1ml of cold 70% ethanol, air dried and re-suspended in 20µl of TE buffer (10mM Tris-CL, pH 7.5; 1 mM EDTA). The integrity of the re-suspended pellets (plasmids) was verified on 1% agarose gel electrophoresis.

Plasmid curing and re-assessment of antibiotic susceptibility

Bacteria isolates containing plasmids and those not containing plasmid (in order to nullify false-negative results that might be associated with plasmid extraction protocol) were used for a plasmid curing procedure using acridine orange by following the method described by Akortha & Filgona[20] with slight modification. A 5 ml aliquot of overnight suspension cultures of bacteria were sub-cultured into tubes containing 5 ml of nutrient broth supplemented with 0.1 mg/ml acridine orange. Tubes were then incubated at 37°C for 48 h. Subsequently, these bacterial cultures were then plated out on Mueller-Hinton agar and used in the re-assessment of antibiotic susceptibility pattern as described above.

Statistical analysis

The data was subjected to one way analysis of variance (ANOVA) and differences between samples were determined by Duncan's Multiple Range test using SPSS 16.0 version. P values<0.05 was regarded as significant and P values<0.01 as very significant.

Results

Anti-HCV positive blood samples cultured

Out of 121 HCV positive blood sample cultured, exactly 63 showed bacterial growths while 58 positive blood samples did not shown any growth. Of the 63 bacteremic blood samples, 25 blood samples showed mixed bacteria growth as shown in Table 3. The total number of pure isolates obtained was 148 bacteria as shown in Table 2.

Isolation and identification of microorganisms associated with blood samples

Table 1 shows the morphological and biochemical characterization of the isolated Bacteria from both HCV infected and non-infected blood samples. Microorganisms isolated shown different form of colony which include circular, round, raised, spiral and flat. The shape ranges from short-rod, rod to cocci. Biochemical tests showed that five out of the seven isolated organisms were Gram-negative, while two were Gram-positive. *Escherichia coli, S. aureus, P. mirabilis, P. aeruginosa, Salmonella* sp. were catalase positive, whereas, *S. pneumoniae* and *K. pneumoniae* were catalase negative. *E. coli, P. mirabilis, S. pneumoniae, P. aeruginosa* were coagulate negative, *K. pneumoniae* and *S.aureus* were coagulase positive. *E. coli, P. mirabilis* are indole positive whereas others were negative. *P. aeruginosa* and *K. pneumoniae* were citrate positive while *E. coli* and *S. pneumoniae* were negative. Only *P. aeruginosa* and *Salmonella* sp. were oxidase positive, while all others were negative. Only *P. aeruginosa* was not utilized the sugars in the TSI agar while others did, Gas production was only by *E. coli*, but *P. mirabilis and Salmonella* sp. were H_2S positive while others were negative, *S. pneumoniae* are optochin positive while *S. aureus* negative.

Types and frequency of occurrence of bacterial isolates from the HCV infected blood samples

A total of seven (7) bacterial species were isolated from the HCV infected blood. These isolates are *S. aureus, K. pneumoniae, P. aeruginosa, Escherichia coli, S. pneumoniae, P. mirabilis* and *Salmonella* sp. With *S. aureus* had the highest occurrence of

35(23.65%) followed by *E. coli* with 31 (20.95%), *Salmonella* sp. with 23(15.54%), *P. aeruginosa* with 20 (13.51%), *K. pneumoniae* with 16(10.81%), *S. pneumoniae* 13(8.78) and *P. mirabilis* with 10 (6.76%) the least as shown in Table 2.

Table 1 Morphological and biochemical characterization of isolate

Microorganism	Col	Gra	Sha	Mot	Cat	Oxi	Ind	MR	VP	Coa	Cit	Opt	TSI			
													S	B	G	H_2S
Staphylococcus aureus	Round	+	Cocci	_	+	_	_	+	_	+	DE	_	Y	Y	_	_
Klebsiella pneumonia	Raised	_	Rod	_	_	_	_	_	+	+	+	NA	Y	Y	_	_
Pseudomonas aeruginosa	Flat	_	Rod	+	+	+	_	_	_	_	+	NA	R	R	_	_
Escherichia	Circular	_	Short	+	+	_	+	+	_	_	_	NA	Y	Y	+	_
Coli			Rod													
Streptococcus pneumonia	Raised	+	Cocci	_	_	_	_	_	+	_	_	+	Y	Y	_	_
Proteus mirabilis	Spiral	_	Rod	+	+	_	+	+	_	_	DE	NA	Y	Y	_	+
Salmonella sp.	Circular	_	Rod	+	+	--	--	+	--	--	+	NA	R	Y	_	+

Keywords: COL, Colony; GRA, Gram reaction; SHA, Shape; CAT, Catalase; MOT, Motility; COA, Coagulase; LAC, Lactose; OXI, Oxidase; IND, Indole; MR, Methyl-red; VP, Voges Proskauer; CIT, Citrate; OPT, Optochin; TSI, Triple Sugar Iron; G, Gas; S, Slope; B, Butt; H_2S, Hydrogen Sulphide; R, Red, Y, Yellow; NA, Not Applicable; +, positive, -, negative; DE, Delayed.

Table 2 Types and frequency of occurrence of bacterial isolates from the HCV infected blood samples

S/N	Microorganism	Frequency	Percentage (%)
1	*Staphylococcus aureus*	35	23.65
2	*Klebsiella pneumoniae*	16	10.81
3	*Salmonella sp.*	23	15.54
4	*Pseudomonas aeruginosa*	20	13.51
5	*Escherichia coli*	31	20.95
6	*Streptococcus pneumoniae*	13	8.78
7	*Proteus mirabilis*	10	6.76
Total	7	148	100

Frequency of occurrence of mixed bacterial pathogens in the samples of HCV infected blood

More than one bacterial species were isolated in 25 of the individuals sampled as shown in Table 3. *Staphylococcus aureus/ Escherichia coli* has the highest frequency of 11(44%), followed by *Escherichia coli/Salmonella* sp. with frequency of 6 (24%), followed by *Salmonella* sp./*Klebsiella pneumoniae* with the frequency of 5 (20) and the least *Pseudomonas aeruginosa/Proteus mirabilis* with the frequency of 3(12%).

Percentage antibiotic resistance of gram positive isolates

The antibiotic resistance pattern of the bacteria isolated from the HCV positive blood samples were conducted. Table 4 showed the percentage resistance pattern of Gram positive isolates, all of them demonstrated high level of resistance to Erythromycin, Cotrimoxazole, Amoxycillin, Ceftriaxone, Pefloxacin and Ofloxacin. Ciprofloxacin showed to be most effective of all the antibiotics used. The antibiotics resistance pattern of the isolates showed the following ranges; Erythromycin (86-91%), Cotrimoxazole (62-71%), Amoxycillin (57-69%), Ceftriaxone (54-66%), Pefloxacin (37-39%), Ofloxacin (23-34%), Chloramphenicol (8-31%), Gentamycin (15-17%), Streptomycin (0-14%) and the least percentages of (0-6) were resistant to Ciprofloxacin.

Table 3 Frequency of occurrence of mixed bacterial pathogens in the samples of HCV infected blood

S/N	Bacterial isolates	Frequency	Percentage (%)
1	*Staphylococcus aureus* / *Escherichia coli*	11	44
2	*Pseudomonas aeruginosa* / *Proteus mirabilis*	3	12
3	*Salmonella sp.* / *Klebsiella pneumoniae*	5	20
4	*Escherichia coli* / *Salmonella sp.*	6	24
	Total	25	100

Percentage antibiotic resistance of gram negative isolates

Table 5 showed the percentage resistance pattern of Gram negative isolates from HCV positive blood samples which showed the following ranges; Amoxicillin (100-100%), Augmentin (90-100%), Cotrimoxazole (81-100%), Ceftriaxone (74-100%), Nitrofurantoin (13-36%), Tetracycline (13-30%), Gentamycin (6-30%), Ofloxaxin (9-10%), Pefloxacin (6-9%) and the least percentages of (0-5%) were resistant to Ciprofloxacin.

Multiple antibiotic resistance (MAR) isolates from HCV infected blood according to number and combination of antibiotics

Table 6 shows the multiple antibiotics resistance of isolates from HCV positive blood samples with numbers and combination of antibiotics.

Table 4 Percentage antibiotic resistance of Gram positive isolates (HCV positive)

Bacterial isolates	No of isolates	Percentage resistance to antibiotics (%)									
		AMX	OFL	STR	CHL	CRO	GEN	PEF	COT	CIP	ERY
SA	35	57	34	14	31	66	17	37	71	6	86
SP	13	69	23	0	8	54	15	39	62	0	91

Keywords: SA, *Staphylococcus aureus*; AMX, Amoxycillin; STR, Streptomycin; COT, Cotrimoxazole; SP, *Streptococcus pneumoniae*; OFL, Ofloxacin; CHL, Chloramphenicol ; CIP, Ciprofloxacin; CRO, Ceftriaxone; GEN, Gentamycin; PEF, Pefloxacin; ERY, Erythromycin

Table 5 Percentage antibiotic resistance of Gram Negative isolates (HCV positive)

Bacterial isolates	No of isolates	Percentage resistance to antibiotics (%)									
		AUG	CRO	NIT	GEN	COT	OFL	AMX	CIP	TET	PEF
KP	16	94	88	13	6	81	0	100	0	19	6
PA	20	90	96	0	10	80	0	100	5	25	0
EC	31	90	74	36	16	87	10	100	0	30	0
PM	10	90	100	20	30	100	0	100	0	30	0
SS	23	100	74	22	22	87	9	100	0	13	9

Keywords: KP, *Klebsiella pneumoniae*; AUG, Augmentin; CRO, Ceftriaxone; COT, Cotrimoxazole; PA, *Pseudomonas aeruginosa*; NIT, Nitrofurantoin; GEN, Gentamycin; OFL, Ofloxacin; EC, *Escherichia coli*; AMX, Amoxycillin; CIP, Ciprofloxacin; TET, Tetracycline; PM, *Proteus mirabilis*; SS, *Salmonella sp.*; PEF, Pefloxacin

Table 6 Multiple antibiotic resistance of HCV positive isolates according to number and combination of antibiotics (resistotype)

Microorganisms	No of isolates	Antibiotics to which isolates were resistant	
		Number	Combinations
S. aureus	2	4	ERY, COT, PEF, GEN
	1	4	ERY, COT, PEF, CRO
	5	5	ERY, COT, CRO, CHL, AMX
	6	5	ERY, PEF, CHL, OFL, COT
	4	5	AMX, ERY, CRO, GEN, COT, CHL
	5	7	OFL, CRO, ERY, COT, AMX, STR, PEF
S. pneumoniae	2	4	PEF, AMX, ERY, COT
	2	4	ERY, PEF, AMX, CRO
	2	6	GEN, OFL, ERY, AMX, COT, CRO
	1	6	CHL, OFL, AMX, PEF, CRO, COT, ERY
K. pneumoniae	2	4	AUG, CRO, COT, AMX
	1	5	AUG, CRO, COT, AMX, GEN
	2	5	AUG, COT, AMX, CRO, TET
	3	5	GEN, AMX, CRO, COT, AUG
	1	6	AMX, TET, COT, AUG, PEF, CRO

Table Continued

Microorganisms	No of isolates	Antibiotics to which isolates were resistant	
		Number	Combinations
P. aeruginosa	8	4	AUG, CRO, COT, AMX
	1	5	AUG, CRO, COT, AMX, TET
	2	5	AMX, TET, AUG, CRO, COT
	2	6	AMX, AUG, CRO, COT, TET, GEN
E. coli	8	4	AMX, CRO, AUG, COT,
	6	5	AUG, CRO, COT, AMX, NIT
	2	6	AUG, CRO, COT, AMX, TET, NIT
	3	8	AMX, AUG, GEN, TET, COT, CRO, OFL, NIT
P. mirabilis	2	4	AMX, AUG, CRO, COT
	1	5	AMX, AUG, CRO, COT, NIT
	1	5	AMX, AUG, CRO, COT, TET
	1	5	AMX, AUG, CRO, COT, GEN
	2	6	AMX, AUG, CRO, COT, TET, GEN
Salmonella sp.	5	3	AUG, AMX, COT, CEF
	2	3	ERY, PEF, GEN, TET
	2	6	CHL, AMX, AUG, CRO, OFL
	1	8	AMX, AUG, GEN, TET, COT, CRO, OFL, NIT

Keywords: AUG, Augmentin; CRO, Ceftriaxone; COT, Cotrimoxazole; NIT, Nitrofurantoin; GEN, Gentamycin; OFL, Ofloxacin; AMX, Amoxycillin; CIP, Ciprofloxacin; TET, Tetracycline; PEF, Pefloxacin; AMX, Amoxycillin; STR, Streptomycin; CHL, Chloramphenicol; GEN, Gentamycin; ERY, Erythromycin

Multiple antibiotic resistance (MAR) pattern of bacterial isolates from HCV infected blood

A little more than half (58.11%) of the total isolates exhibited multiple resistance with the antibiotics they were tested against. The percentage incidence of multiple antibiotic resistance among the isolates include; *S. aureus* with the highest percentage of 65.71%, *P. aeruginosa* (65%), *E. coli* (61.29%), *P. mirabilis* (60%), *S. pneumoniae* (53.85%), *K. pneumoniae* (50%) and *Salmonella sp.* with the least percentage of 43.48% as shown in Table 7.

Table 7 Multiple antibiotic resistance patterns of bacterial isolates from HCV infected blood

Organisms	Total number Isolated	Number of isolates with MAR	Percentages of MAR isolates (%)
S. aureus	35	23	65.71
S. pneumoniae	13	7	53.85
K. pneumoniae	16	8	50
P. aeruginosa	20	13	65
E. coli	31	19	61.29
P. mirabilis	10	6	60
Salmonella sp.	23	10	43.48
Total	148	86 (58.11%)	

Multiple antibiotic resistance (MAR) index

The resistance of selected isolates towards these antibiotics tested is represented as multiple antibiotics resistance (MAR) index which ranged from 0.6-0.8 (Table 8) (Figure 1) (Table 9 & 10).

Table 8 Multiple antibiotic resistance (MAR) index

Microorganisms	MAR
S. aureus	0.7
S. pneumoniae	0.7
K. pneumoniae	0.6
P. aeruginosa	0.6
E. coli	0.8
P. mirabilis	0.6
Salmonella sp.	0.8

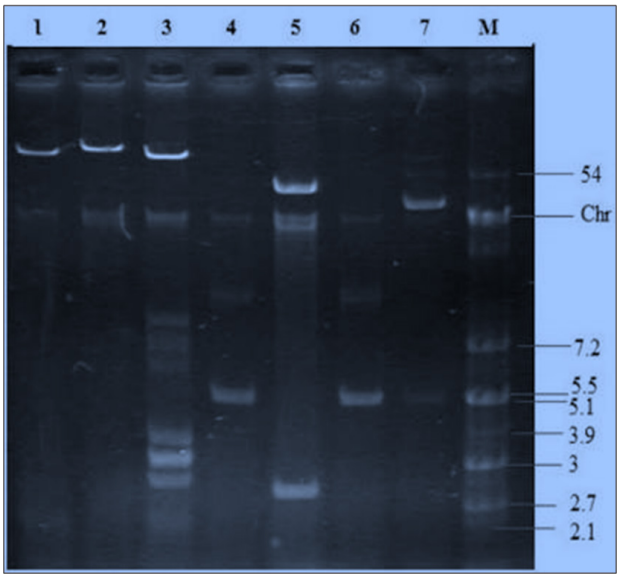

Figure 1 Plasmid profiles (1-7) of some multiple antibiotics resistant bacteria isolate.

Keywords: M, Size marker *E. coli* V517; Lanes 1, *S. aureus,* 2, *K. pneumoniae,* 3, *Salmonella sp.,* 4, *P. mirabilis,* 5, *P. aeruginosa,* 6, *S. pneumoniae,* 7, *E. coli.*

Table 9 Pre and post sensitivity curing of multiple antibiotics resistant gram positive isolates

Antibiotics	Plasmid curing	Zones of Inhibition (diameter in mm)	
		S. aureus	***S. pneumoniae***
Amoxycillin	Before	3.05±0.04^{b}	0.00±0.00^{a}
	After	5.13±0.06^{c}	12.05±0.02^{d}
Ofloxacin	Before	12.82±0.02^{a}	12.63±0.01^{a}
	After	22.27±0.18^{b}	23.13±0.01^{c}
Streptomycin	Before	10.01±0.01^{b}	2.13±0.03^{a}
	After	19.10±0.04^{d}	16.20±0.12^{c}
Chloramphenicol	Before	5.13±0.03^{b}	0.00±0.00^{a}
	After	18.11±0.10^{d}	15.07±0.07^{c}
Ceftriaxone	Before	4.54±0.02^{a}	6.28±0.02^{b}
	After	21.98±0.06^{d}	21.00±0.00^{c}
Gentamicin	Before	6.73±0.03^{a}	8.37±0.03^{b}
	After	15.10±0.06^{d}	13.00±0.06^{c}
Pefloxacin	Before	11.20±0.12^{b}	9.60±0.17^{a}
	After	18.14±0.07^{a}	16.28±0.03^{c}
Ciprofloxacin	Before	20.91±0.01^{c}	13.50±0.06^{a}
	After	20.91±0.01^{c}	15.23±0.09^{b}
Erythromycin	Before	5.00±0.15^{b}	0.33±0.24^{a}
	After	10.33±0.28^{d}	6.10±0.10^{c}
Cotrimoxazole	Before	0.00±0.00^{a}	0.00±0.00^{a}
	After	13.61±0.01^{c}	5.35±0.04^{b}

Data are presented as Mean±S.E (n=3). Values with the same superscript letter(s) along the same column are not significantly different (P<0.05)

Table 10 Pre and post sensitivity curing of multiple antibiotics resistant gram negative isolates

Antibiotics	Plasmid curing	Zone of Inhibition (diameter in mm)				
		K. pneumoniae	*P. aeruginosa*	*E. coli*	*P. mirabilis*	*Salmonella sp.*
Augmentin	Before	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}
	After	18.73±0.09^{d}	15.30±0.15^{c}	20.04±0.03^{e}	11.15±0.08^{a}	14.29±0.85^{b}
Ceftriaxone	Before	6.10±0.06^{b}	9.30±0.03^{d}	5.13±0.03^{a}	9.43±0.09^{d}	8.20±0.06^{c}
	After	12.00±0.00^{a}	15.63±0.03^{d}	13.90±0.06^{b}	16.52±0.01^{e}	15.00±0.00^{c}
Nitrofurantoin	Before	5.21±0.06^{c}	4.80±0.00^{b}	8.20±0.00^{d}	5.12±0.01^{c}	4.22±0.11^{a}
	After	17.20±0.06^{c}	16.40±0.25^{b}	19.07±0.03^{d}	17.00±0.06^{c}	15.63±0.27^{a}
Gentamicin	Before	8.01±0.00^{e}	2.93±0.09^{a}	4.73±0.09^{b}	6.33±0.15^{c}	7.57±0.18^{d}
	After	18.05±0.02^{d}	15.00±0.06^{a}	15.90±0.00^{b}	16.00±0.06^{b}	17.39±0.26^{c}
Cotrimoxazole	Before	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}
	After	0.00±0.00^{a}	4.67±0.12^{c}	2.00±0.00^{b}	10.47±0.18^{d}	12.03±0.03^{e}
Ofloxacin	Before	11.93±0.03^{c}	14.77±0.09^{d}	16.00±0.06^{e}	11.40±0.00^{b}	10.45±0.18^{a}
	After	20.13±0.03^{c}	24.20±0.15^{e}	17.64±0.02^{a}	21.22±0.11^{d}	20.41±0.01^{c}
Amoxycillin	Before	0.00±0.00^{a}	3.40±0.15^{b}	7.11±0.01^{c}	0.00±0.00^{a}	0.00±0.00^{a}
	After	9.31±0.01^{a}	13.90±0.06^{c}	17.80±0.00^{e}	13.11±0.01^{c}	15.17±0.09^{d}
Ciprofloxacin	Before	20.07±0.03^{a}	22.13±0.03^{b}	23.32±0.01^{c}	24.61±0.00^{e}	24.53±0.02^{d}
	After	21.20±0.15^{a}	23.24±0.00^{b}	23.43±0.03^{b}	24.68±0.01^{c}	25.24±0.07^{d}
Tetracycline	Before	14.11±0.10^{b}	0.00±0.00^{a}	8.50±0.15^{d}	10.00±0.00^{e}	5.19±0.09^{c}
	After	19.21±0.06^{d}	12.92±0.02^{a}	15.21±0.11^{c}	15.46±0.18^{c}	14.13±0.09^{b}
Pefloxacin	Before	18.50±0.21^{a}	20.10±0.06^{c}	19.10±0.06^{b}	21.01±0.00^{d}	23.21±0.00^{e}
	After	19.70±0.15^{a}	22.03±0.07^{b}	22.07±0.03^{b}	23.22±0.00^{c}	23.91±0.01^{d}

Data are presented as Mean±S.E (n=3). Values with the same superscript letter(s) along the same column are not significantly different (P<0.05)

Discussion

Nearly if not all of the bacterial isolates were of medical importance. Both gram negative and gram positive bacteria were obtained but Gram negative bacteria were more frequently involved in the blood samples than Gram positive. Most of the microbial isolates from this study are members of the Enterobacteriaceae family, which is the largest and most heterogenous group of medically significant gram-negative helix-shaped bacteria. They are most frequently isolated in clinical samples. The infections include diarrhoea, dysentery, Salmonellosis, Haemolytic-uremic syndrome (HUS), necrotizing enterocolitis and various nosocomial infections. Pathogenicity of Enterobacteriaceae as a family of gram negative bacteria is associated with the Lipopolysaccharide (LPS) situated in the outer membrane of the bacterial cell wall which is usually responsible for endotoxin production in Gram negative bacteria–a cause of septicemia. Infections from Enterobacteriaceae are regarded as one of the two leading killers of children in developing countries. This goes in agreement with the submission of Frey & Sherk.[21]

S. aureus; a leading cause of bloodstream infections throughout much of the industrialized world[22] has the highest frequency of 35(23.65%) and followed by *E. coli* with 31 (20.95%), the most common cause of community-acquired bacteremia accounting for approximately 75% of cases,[23] the trend of isolates frequency were correlated with that of Michael *et al.,* (1998), also correlated with that of Li *et al.*, (2015), where they focused on bacteria from Hepatitis B. More than one bacterial species were isolated in some blood samples, this is similar with the study carried out by Oladosu et al.,[24] on the evaluation of types of bacterial associated with HIV-1 patients blood.

The antibiotic resistance pattern of the bacteria isolated from the HCV positive blood samples were conducted. All the isolates examined showed resistance to one or more of the ten (10) antibiotics used for the study. Gram positive isolates demonstrated high level of resistance to Erythromycin, Cotrimoxazole, Amoxycillin and Ceftriaxone. Erythromycin was the common antibiotic against which all Gram-positive organisms (*S. aureus* and *S. pneumoniae*) showed high resistance. In other studies, high level of resistance has been reported with Erythromycin and Ampicillin.[25-27] Also, Ogunlowo et al.,[28] gave a report of resistance with Erythromycin and Cefuroxime. Streptomycin, Chloramphenicol, Gentamycin was highly sensitive to both organisms. Ciprofloxacin which is a member of the Fluoroquinolones antibiotics was the most effective of all the antibiotics used in the antimicrobial susceptibility testing and this

agrees with a report from Prajna et al.,[29] on the bacteriologic and clinical efficacy of Ofloxacin and Ciprofloxacin Ophthalmic solutions in the treatment of bacterial isolates of Keratitis, stating that the antibiotics are effective and safe in the treatment of patients with culture positive bacterial keratitis.

Most of the Gram-negative organisms showed resistance to three or more groups of antibiotics. Other researchers also have reported the majority of Gram-negative isolates (*K. pneumonia, P. aeruginosa, E. coli, P. mirabilis* and *Salmonella* sp.) in their study as multidrug resistant.[30] Most of the isolates demonstrated resistance to Amoxicillin, Augmentin, Cotrimoxazole, Ceftriaxone. It can be deduced from the result that Amoxicillin and Augmentin were the least effective antibiotics, amoxicillin showing a marked resistance to all the isolates. However, Oluyege et al.,[31] also reported high level resistance of Cotrimoxazone and Ceftriaxone in bacteria isolated from HIV/AIDS. Most of the isolates were highly sensitive to Ciprofloxacin, Pefloxacin, Ofloxacin even to Gentamycin to some extent. Generally, antibiotics showed higher activities against isolates from HCV negative blood samples. Similar case was reported by Ogunshe et al.,[32] Obinna et al.,[33] and Opere et al.[34]

However, merely all the bacterial species isolated were resistant to Cotrimoxazole, Amoxycillin, Ceftriaxone, Augmentin, Ceftriaxone and Erythromycin but sensitive to Ciprofloxacin, Ofloxacin and Pefloxacin, this is similar to the work of Ernst,[35] Mahan & Escott-Stump[36] and USDHHS.[37] The incidence of antibiotic resistance to drugs by microorganisms is explained in their ability to exist even in the presence of the antibiotics having devised mechanisms to survive the effect of the antibiotics due to their genetic flexibility. In this study, the isolated organisms were resistant to most of the commonly used antibiotics. Drug resistances of bacteria to antibiotics have been attributed to the misuse (of antibiotics) as well as the possession of drug resistance plasmids.[38]

Multiple antibiotic resistance (MAR) isolates from HCV infected blood according to their number and combination of antibiotics they were resistant against, showed a combination range from 4-8 resistotype with MAR index which also ranged from 0.4–0.8. These values were higher than 0.2 suggesting that they might have originated from a high risk source of contamination where antibiotics are often used.[39] MAR pattern of bacterial isolates from HCV infected blood, where a little more than half (58.11%) of the total isolates exhibited multiple resistance with the antibiotics they were tested against, multiple drug resistances of bacteria to antibiotics might be due to misuse and overuse of antibiotics as well as the possession of drug resistance plasmids.[38]

Higher value of multiple antibiotics resistance (MAR) index of isolates ranged from 0.6-0.8 might have been accumulated in several ecological niches because of the widespread use of commercial antibiotics in hospital, agriculture and livestock. Multiple antibiotic resistances (MARs) in bacteria may be commonly associated with the presence of plasmids. The plasmid analysis shows that the multiple resistant bacteria isolates harbor one or more plasmid with different molecular weights. This finding implicates plasmids as the source of resistance genes. Uma et al.,[40] Hudson et al.[41] and Gohar et al.,[42] also reported that genes for multidrug resistance might be located on plasmid DNA or chromosomal DNA. The susceptibility pattern of antibiotics re-assessment shows that plasmid curing converted all the multiple antibiotics resistance isolates (MAR) which were initially resistant to the conventional antibiotics to susceptible form thereby indicating that, the resistance was plasmid mediated.

Conclusion

This study thus provides information regarding the types, antibiotic resistance patterns and plasmid profile of resistance bacteria isolated from HCV blood samples. In which the *S. aureus* is the most frequently encountered bacterial species in the blood of HCV positive individuals in the community studied while the least encountered pathogen was *Proteus mirabilis*. All the bacterial species isolated are resistant to Ceftriaxone, Cotrimoxazole, Amoxycillin, Augmentin and Erythromycin but sensitive to Fluoroquinolones especially Ciprofloxacin. Such data is important in the selection of empirical antimicrobial treatment of HCV secondary infection cases; in showing a path for clinical research and as an aid in educating and spreading awareness among medical personnel. This also draws our attention toward emerging trends of antibiotic resistance in pathogenic bacteria. However, as this was a single center study of limited duration, the results may not be generalized for guiding empirical therapy of HCV secondary infection in the area. It is highly recommended that more studies, involving patients from multiple medical centers are done to throw more light on the epidemiology of infectious diseases, and the resistance patterns of common pathogens; to make practicing physicians aware of the magnitude of an existing problem of antibacterial resistance, so that they join hands in fighting this deadly threat by rational prescription of drugs.

Acknowledgments

None.

Conflicts of interest

Authors declare that there is no conflict of interest.

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