Research article

Molecular characterization and antibiotic sensitivity testing of bacteria in blood cultures of Hepatitis B virus infected subjects

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Abstract

Introduction: Hepatitis B virus is one of the most common infections worldwide. Many infected people are at risk of developing liver complications. Screening for common pathogenic bacterial infections that could contribute to complications is important for early diagnosis and appropriate management.

Methods: A cross sectional study was carried out on subjects aged 20-75 years for a period of 6 months (November 2016 to April 2017). Blood cultures and HBsAg rapid tests were performed on all 122 blood samples collected in Ilorin Metropolis. The screening was carried out on 92 HbsAg positive patients who presented with fever, and 30 apparently healthy HbsAg positive donors from the blood bank.

Results: Of 92 symptomatic patients, 44 (47.8%) had postive blood cultures and of the 30 HBV positive blood donors, 9 (30%) had positive blood cultures.

The prevalence of *Escherichia coli*, *Staphylococcus aureus*, P*seudomonas aeruginosa* and *Klebsiella pneumoniae* in Hepatitis B positive subjects was 5.7% (n=7), 5.7% (n=7), 23.8% (n=29) and 9.8% (n=12) respectively. In the apparently healthy HbsAg positive blood donor group, only 9 samples showed positive bacterial growth of *P. aeruginosa.*

All the bacterial isolates were resistant to amoxycillin-clavulanic acid, erythromycin, ceftriaxone, ceftazidime, cefuroxime, and ciprofloxacin. On PCR, *Nuc, Stx2, Pf* and *PSUE* genes were demostrated in *E. coli, S. aureus, P. aeruginosa* and *K. pneumoniae* respectively.

Conclusion: This study showed a high percentage (45.1%) of bacteraemia in HBV infection. Early screening and treatment of HBV infection and concomitant bacterial infection is recommended to prevent complications.

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Introduction

Many important questions still remain unanswered for many pathogen combinations. Co-infected individuals may be in worse health than those with single infections[1] and may also pose the biggest risk of transmission to others.[2] It is important to note that hepatitis is an occasional feature of the clinical symptoms induced by many agents of both living and non-living origin.

Hepatitis B virus (HBV) infection is an infection of major public health significance, being the 10th leading cause of death globally. HBV infection accounts for 500,000 to 1.2 million deaths globally each year.[3] HBV infection can cause acute hepatitis, acute liver failure, chronic hepatitis, or can cause an asymptomatic infection and approximately 15-40% of infected patients will develop cirrhosis, liver failure or hepatocellular carcinoma.[4]

Numerous studies have suggested that the genetic constitution of the host is a critical factor in determining the outcomes of HBV infection. Despite the effectiveness of the current vaccination policy, the prevalence of Hepatitis B infection remains high, and the burden for health services is considerable.[5]

Blood stream infection by Gram negative bacteria is a common complication in patients with cirrhosis. Patients with cirrhosis and ascites showed a higher susceptibility to bacterial infections because of their inadequate defence mechanisms.[6] Very little is known about the correlation between HBV and different bacterial infections due to lack of diagnostic capability. The most common pathogenic agents, which enter the liver by vascular routes, are *E. coli, K. pneumoniae, Salmonella* Typhi, *Proteus vulgaris*, *Streptococcus spp,* and *Staphylococcus* spp, but anaerobes may also be present.[7] *S. aureus, P. aeruginosa,* and *K. pneumoniae* have been implicated in liver abscess.[8] *P. aeruginosa* is one of the most common causes of bacteraemia in liver transplant recipients. *K. pneumoniae* of high virulence (hv*KP*) has also been reported to lead to liver abscess in apparently healthy young adults.[9] Among cases of pyogenic liver abscesses, *Staphylococcus aureus* has been established as the leading cause in most series.[10] Uropathogenic *E. coli* (UPEC) has been reported to play a significant role in development of primary biliary cirrhosis (PBS).[6]

Methods

Collection of samples and data

The study protocol was reviewed by the University Ethics Review Board of the Faculty of Life Sciences, University of Ilorin and approval to carry out research was obtained from the Ethics Review Committee of the State Ministry of Health. All subjects gave written informed consent and were assured that all information was confidential. The target population (with or without fever) were confirmed as HBsAg positive at the Kwara State Civil Service Hospital, Specialist Hospital Sobi, General Hospital Ilorin and Blood Banks Ilorin, Nigeria from November 2016 to May 2017. They were above 18 years and the subjects' socio-demographic information were collected by administering a questionnaire.

A total of 122 HBsAg positive blood samples from patients attending Kwara State Civil Service Hospital, Sobi Specialist Hospital, Ilorin General Hospital, and the Blood Bank, Ilorin, Nigeria were confirmed between November 2016 – May 2017

Ninety-two (92) of the blood samples were collected from HBsAg positive patients of Kwara State Civil Service Hospital, Sobi Specialist Hospital and Ilorin General Hospital. Thirty blood samples (30) were collected from apparently healthy HBsAg positive donors from the blood bank.

Seven ml of venous blood was taken aseptically by needle and syringe with 2ml dispensed in 18ml of Thioglycollate medium and 5ml into a plain bottle. The samples in plain bottles were left at room temperature for 20-30 minutes to clot, centrifuged at 3000rpm for 5 minutes, and the resultant clear serum samples were packaged and transported to the laboratory in the Department of Microbiology, University of Ilorin for serological analysis of HBV using HBsAg and HBcAg (LifeSpan BioSciences, inc) rapid testing kits. All samples were tansported in a cold box at +2 °C to +8 °C and kept at -20 °C in the laboratory for 18-24 hours.

Assay procedure for concomitant bacteria

Thioglycollate media samples were incubated for 7 days with intermittent sub-culturing on Blood Agar at 37 °C for 24hrs and examined for growth. Positive blood cultures were identified and sub-cultured on Blood Agar, MacConkey, Salmonella-Shigella Agar and Mannitol Salt Agar. Colonial and cellular morphology of the culture plates were observed, and necessary biochemical tests were carried out.[11]

Antibiotics susceptibility test and ESBL screening

This was done according to Clinical Laboratory Standard Institute (CLSI) Guidelines.[12] Standard antimicrobial discs for Gram negative and Gram positive organisms was placed onto the surface of the inoculated agar plates accordingly and incubated for 24 hours. The zones of inhibition were measured and interpreted using the CLSI guidelines. ESBL screening was also performed by the disk synergy test.[13]

DNA extraction and amplification of genes

Crude method was used for DNA extraction of the bacterial isolates and polymerase chain reaction (PCR) was carried out to detect Universal genes *Nuc, Stx2, Pf* and *PSUE* in isolated *S. aureus, E.coli, Klebsiella spp. and Pseudomonas spp.* respectively using the primers and cycling parameters listed in Table 1. PCR amplification of genes was carried out for each gene singly using geneAmp PCR system 9700 thermal cycler (Applied Biosystems). All PCR assays were performed directly from bacterial suspensions obtained after rapid DNA extraction method. For the amplification master mix, an aliquot of 2µl of the bacterial suspension was added to 23µl of PCR mixture containing 50mM KCl, 10mM Tris-HCl (pH 8.6), 1.5mM $MgCl_2$, 5% glycerol, 0.08% NP-40, 0.05% Tween-20, 0.2mM of each deoxynucleoside triphosphate (dATP, dTTP, dGTP, and

dCTP), 10µM of respective primers and 25 units/ml of Taq DNA polymerase. All PCR assay runs incorporated a reagent control (without template DNA).

Table 1: Summary of the primers and PCR operating conditions

Gene	Primer Sequence 5'→ 3'	Size	Cycle	Cycling Parameters
Nuc	GCGATTGATGGTGATACGGTT AGCCAAGCCTTGACGAACTAAAGC	274Bp	35	94 °C–30s; 5 5°C–30s; 72 °C–1 min
Stx1	GTT ACG GGA AGG AAT CAG GGT AAA CGC GGA AGG AAT CAG GGT	300Bp	35	94 °C–30s; 60 °C–30s; 72 °C–1 min
Psue	AGCGTTCGTCCTGCACAAGT TCCACCATGCTCAGGGAGAT	98Bp	35	94 °C–30s; 58 °C–30s; 72 °C–1 min
Pf	ATT TGA AGA GGT TGC AAA CGA T TTC ACT CTG AAG TTT TCT TGT GTT C	615Bp	35	94 °C–30s; 57 °C–30s; 72 °C–1 min;

Agarose gel electrophoresis

Agarose gel electrophoresis was carried out using 0.7% agarose gel in 0.5X Tris borate EDTA buffer (44.5mM Tris borate and 1 Mm EDTA, pH 8.3). At the end of the run, the gel was transferred to a syngene gel documentation system (Syngene, UK) for agarose gel visualization using UV light. The DNA bands were then captured and visualized with a short wave ultraviolet trans illuminator and photographed using Syngene gel bio imaging system (UV trans-illuminator).

Results

Table 2 shows the distribution of bacterial isolates among the 122 samples collected. Twenty nine (23.8%) of *P. aeruginosa*, 12 (9.8%) of *K. pneumoniae*, 7 (5.7%) of *S. aureus*, 7 (5.7%) of *E. coli* made up the 55 (45.1%) samples positive for bacterial infections. The prevalence of bacterial infections in patients with HBsAg was determined as 45.1%.

Table 2: Age distribution of the prevalence of bacterial isolates among all HBV +ve subjects

Age (years)	No Examined	Positive samples	*E. coli*	*P. aeruginosa*	*K. pneumoniae*	*S. aureus*
18-27	40	15	1	7	4	3
28-37	19	11	2	7	1	1
38-47	34	17	3	8	5	1
48-57	18	10	1	6	2	1
58-67	8	2	0	1	0	1
>68	3	0	0	0	0	0
Total	**122 (100%)**	**55 (45.1%)**	**7 (5.7%)**	**29 (23.8%)**	**12 (9.8%)**	**7 (5.7%)**

Table 3: Age distribution of the prevalence of bacterial isolates among HBsAg +ve patients

Age (years)	No Examined	Positive samples	*E. coli*	*P. aeruginosa*	*K. pneumoniae*	*S. aureus*
18-27	32	13	1	5	4	3
28-37	14	9	2	5	1	1
38-47	27	13	3	6	4	0
48-57	12	8	1	4	2	1
58-67	5	1	0	0	0	1
>68	2	0	0	0	0	0
Total	**92 (100%)**	**44 (47.8%)**	**7 (7.6%)**	**20 (21.7%)**	**11 (12.0%)**	**6 (6.5%)**

Table 4: Age distribution of the prevalence of bacterial isolates in healthy HBsAg +ve blood donors

Age (years)	No Examined	Positive samples	*E. coli*	*P. aeruginosa*	*K. pneumoniae*	*S. aureus*
18-27	8	2	0	2	0	0
28-37	5	2	0	2	0	0
38-47	7	2	0	2	0	0
48-57	6	2	0	2	0	0
58-67	3	1	0	1	0	0
>68	1	0	0	0	0	0
Total	**30 (100%)**	**9 (30.0%)**	**0 (0.0%)**	**9 (30.0%)**	**0 (0.0%)**	**0 (0.0%)**

Forty-five males (36.9%) and seventy-seven females (63.1%) were enrolled in the study. Table 5 shows the gender distribution of the bacterial isolates

Table 5: Gender distribution of the prevalence of bacterial isolates among HBV +ve subjects

Gender	Samples	*E.coli*		*S.aureus*		*P.aeruginosa*		*K. pneumonia*	
		n	**%**	**n**	**%**	**n**	**%**	**n**	**%**
Male	45	3	6.7	4	8.9	13	28.9	3	6.7%
Female	77	4	8.9	3	3.9	16	6.67	9	6.6
Total	**122**	**7**		**7**		**29**		**12**	

Figure 1 compares the distribution of bacterial isolates in the HBV positive patient group and HBV positive control group (blood donors). *P. aeruginosa* was the only isolate in the blood donor group.

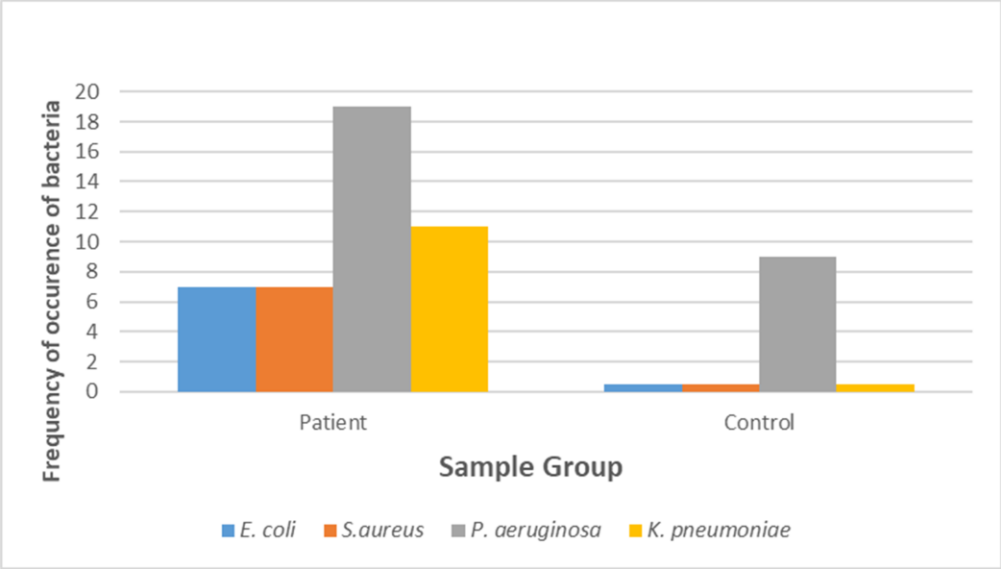


Figure 1: Distribution and comparison of bacterial isolates in HBV positive subjects (patients and control)

Antibiotic susceptibility testing of the *S. aureus* isolates showed multiple resistance to amoxycillin-clavulanic acid (100%), erythromicin (100%), cloxacillicin (100%), and ciprofloxacin (100%). High sensitivity was also recorded with ofloxacin (85.7%), and gentamicin (71.4%).

As shown in Table 5, antibiotic susceptibility testing for the Gram negative bacteria shows high resistance to ceftazidime (100%), cefuroxime (100%), ciprofloxacin (100%), amoxycillin-clavulanic acid (97.9%), and ceftriaxone(91.6%). *E. coli* and *K. pneumoniae* showed high level susceptibility to ofloxacin (83.3%), imipenem (83.3%), gentamicin (75%).

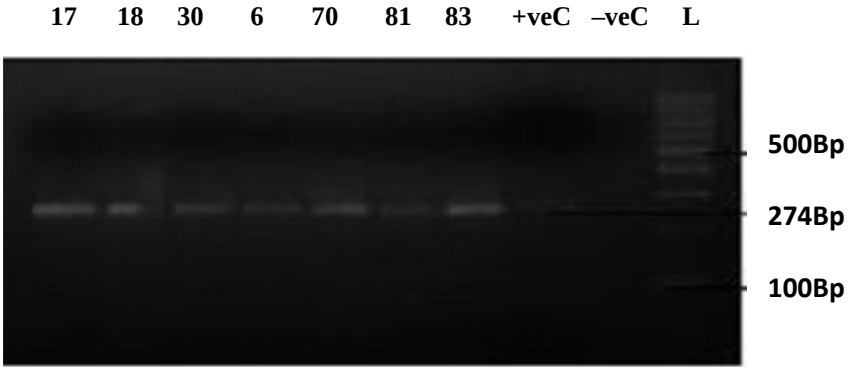
Phenotypic detection of extended spectrum β lactamase (ESBL) production showed that 7 (24.1%), 3 (50%) and 3 (25%) of the *P. aeruginosa*, *E. coli* and *K. pneumoniae* isolates were ESBL producers.

Table 6: Antibiotic susceptibility of Gram negative isolates from patients

Antibiotic	*Pseudomonas aeruginosa* n=29				*Klebsiella pneumoniae* n=12				*Escherichia coli* n=7			
	S	%	R	%	S	%	R	%	S	%	R	%
Augmentin (30µg)	Not tested				0	0	12	100	0	0	7	100
Ceftriaxone (30µg)	Not tested				0	0	12	100	0	0	7	100
Ceftazidime (30µg)	0	0	29	100	0	0	12	100	0	0	7	100
Cefuroxime (30µg)	0	0	29	100	5	41.6	7	58.3	5	71.4	2	28.6
Gentamicin (10µg)	23	80	6	20	0	0	12	100	0	0	7	100
Ciprofloxacin (10µg)	0	0	29	100	0	0	12	100	0	0	7	100
Imipenem (10µg)	27	93.1	2	6..9	4	33.3	8	66.7	4	57.1	3	42.9
ESBL positive	22	75.9	7	24.1	9	75	3	25	4	57.1	3	42.9

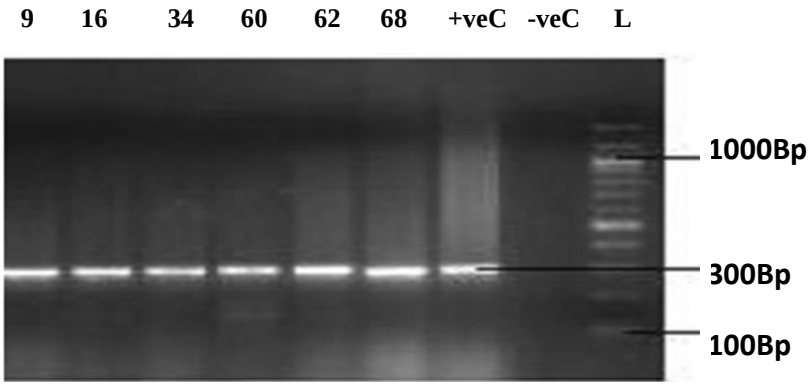
S-sensitive, R-resistance

Figures 2-5 demonstrate the relevant genes in the four species isolated from patients in the study.



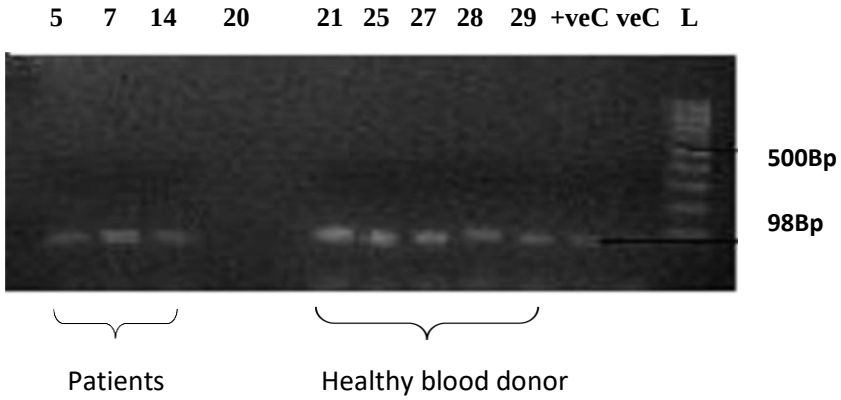
+ve- C positive control; –veC- negative control; L - Ladder

Figure 2: PCR confirmed *NUC* gene in *S. aureus* isolates



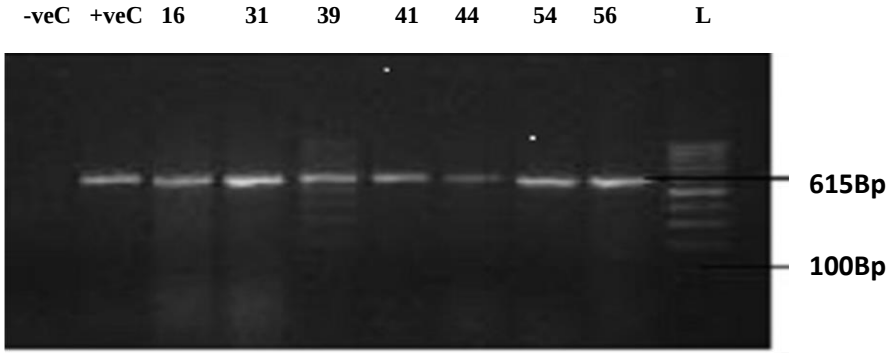
+ve C – positive control: -veC – negative control; L – ladder

Figure 3: PCR confirmed *Stx2* gene in *Escherichia coli* isolates



+ve C – positive control: -veC – negative control; L – ladder

Figure 4: PCR confirmed *PSUE* gene in *P. aeruginosa* isolates.



+ve- C positive control; –veC- negative control; L - Ladder

Figure 5: PCR confirmed *Pf* gene in *Klebsiella pneumoniae* isolates

Discussion

The severity of liver infection is likely to contribute to susceptibility to co-infection and bacterial infections can also contribute to the chronicity of disease in HBV patients. Bacterial infections are common in patients suffering with viral hepatitis and are critical for prognosis.[14] Other studies also have confirmed the association between bacterial infections and HBV and also bacterial infections in cirrhotic patients as an important cause of morbidity and mortality.[15]

It is important to note that most of the patients in the current study were in their acute stage although liver function tests were not determined. In this study, the prevalence of bacterial infections in patients with HBsAg was determined as 45.1%.

The organisms isolated were *P. aeruginosa*, *E. coli*, *K. pneumoniae*, and *S. aureus*. *P. aeruginosa* was isolated most often in both patients and blood donors in this study which was similar to a study conducted in Korea.[16] Sample cultures with no bacterial growth were 67 (54.9%) and *Salmonella* Typhi was not isolated in this study. *S. aureus* and *E. coli* were each isolated in 5.7% of the samples, in contrast with a similar study which reported 43.2% for *S. aureus*[15] and 54.5%[12] for *E. coli*. *K. pneumoniae* reported in this study was 9.8%, which was similar to a study conducted in China that reported approximately 20%.[14]

All *K. pneumoniae* isolates in the current study were resistant to ciprofloxacin as also reported by Alo et al[17] and Cheong et al[15] who reported 80% and 100% resistance respectively. It is notable that bacteraemia was significantly associated with the presence of biliary disease in cirrhotic patients and one of the most implicated bacteria was *P.aeruginosa*.[17] In this study, *P. aeruginosa* demonstrated very high resistance to ciprofloxacin and ceftazidime similar to other studies.[18] The ceftazidime resistance in this study was high (100%), in agreement with 91% that was reported in Egypt.[18]

Antibiotic susceptibility of *E. coli* isolates in the current study were similar to those previously reported. Kibret et al reported *E. coli* resistant to ciprofloxacin (79.6%) and gentamicin (71.4%) in a study in Ethiopia.[19] All *E. coli* isolates in the current study were resistant to ciprofloxacin, similar to the results of Cheong et al.[15] Ciprofloxacin resistance of *S. aureus* in the current study was 100%, similar to a study conducted in Korea which demonstrated very high resistance to ciprofloxacin and oxacillin.[11]

Of the *E. coli* isolated in the current study, 42.9% were ESBL producers. Similar results have been reported from Pakistan (56.9%)[20] and India (40%).[13]

Of *K. pneumoniae* isolated in the current study, 25% were ESBL producers. A similar result (33%) was obtained in a study in Iran[21] in contrast to an African study which reported 71.7%.[22]

Of *P. aeruginosa* isolated in the current study, 24%were ESBL producers. These results were similar to other studies which reported 22%[11] and 13.79%[23] ESBL production. The ESBL producing *P. aeruginosa* isolates exhibited co-resistance against most of the antibiotics tested, consistent with results of other recent studies.[8,18]

The high incidence of ESBL producers among the isolates in the current study has considerable health implications as shown in recent studies, where infection with ESBL producing Gram negative bacilli resulted in significantly higher fatality rates than those with non-ESBL isolates.[8]

Conclusion

Bacteraemia with or without symptoms appear to occur in those demonstrated to be HBV positive. Further studies to follow up such patients and assess the significance of these bacteraemic episodes would be useful, particularly as a significant number of the isolated organisms showed antibiotic resistance to commonly used antibiotics.

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