Original
ISSN 2413-0516

Nitric Oxide and Hypochlorite Assessment and Screening of some β-Lactamase Encoding Genes Among Gram Negative Bacteria in Patients with Acute Leukemia

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(Submitted: 19 July 2023 – Revised version received: 21 August 2023 – Accepted: 10 September 2023 – Published Online: 26 December 2023)

Abstract

Objective: The serologic levels of both nitric oxide (NO) and hypochlorite (ClO^-) were assessed in this study for Iraqi acute leukemic patients infected with Gram negative bacteria (GNB). Alongside, the phylogenetic analysis for both bla_{SHV} and bla_{TEM} genes of GNB was performed.

Methods: The clinical samples were recovered from acute leukemic patients at hematology center in Baghdad from January 2021 to December 2021. The identification of bacterial isolates and susceptibility test were performed using Vitek 2 Compact System. Serum levels of NO and ClO^- were assayed by Enzyme Linked Immunosorbent assay and colorimetric method, respectively. Gene screening was done utilizing polymerase chain reaction. DNA sequencing, GenBank accession numbers submission, and phylogenetic analysis were also conducted.

Results: From 260 patients with acute leukemia, 485 clinical samples were collected from different sites. Of this total, 70 (15%) isolates of GNB were obtained, distributing as *Klebsiella pneumoniae* 23 (33%), *Escherichia coli* 21 (30%), *Pseudomonas aeruginosa* 18 (26%), and *Acinetobacter baumannii* 8 (11%). These isolates were mainly collected from urine 39 (55.71%), followed by blood 23 (32.85%), and the least from swabs 8 (11.42%). The infections of GNB were higher among acute myeloblastic leukemia (AML) patients 40 (57.14%) than these with acute lymphoblastic leukemia (ALL) 30 (42.85%). The levels of NO were higher among groups of patients than control groups. Additionally, ClO^- levels were observed to be slightly increased in patients above these of controls. Most isolates of *K. pneumoniae* and *E. coli* showed high resistance rates for penicillins (ampicillin and ticarcillin), cephalosporins (ceftazidime, cefotaxime, ceftriaxone, and cefepime), followed by gentamicin and ciprofloxacin. Cefoxitin, aztreonam, imipenem and meropenem were nearly more effective against GNB isolates. On the other hand, about half isolates *P. aeruginosa* and all *A. baumannii* were resistant to the tested antibiotics. Extended Spectrum β-Lactamases (ESBLs) were released in 49 (70%) of the tested isolates of GNB. The multidrug resistant (MDR) pattern was noticed among 58 (82.85%) of GNB. Interestingly, ESBLs producing MDR isolates were determined in 34 (58.62%) of the studied GNB. Genotypic screening revealed that bla_{SHV} and bla_{TEM} genes were characterized in 20 (28.57%) and 32 (45.71%) of GNB isolates, respectively, while all GNB were negative for bla_{CTX-M}. DNA sequencing of bla_{SHV} amplicons revealed the occurrence of one point mutation (111T>A) in six isolates of *K. pneumoniae* with a missense effect (p.31Q>L) on the encoded protein. Forty-five GenBank accession numbers were recorded in NCBI to represent the studied variants of ESBLs. Phylogenetic analysis of both bla_{SHV} and bla_{TEM} displayed the possible epidemiologic routes of the tested GNB by detecting the origin host, source of collection, and geographic spread as compared with the reference sequences.

Conclusion: High serum levels of both NO and ClO^- indicated the potential role of these microbicidal agents to predict the serious infections of GNB in acute leukemia population. Phylogenetic analysis represented an important tool, possibly aiding in early control and prevention of pathogenic GNB.

Keywords: Acute leukemia, gram negative bacteria, nitric oxide, hypochlorite, phylogenetic analysis

Introduction

Leukemia is a type of hematological malignancies diseases develops due to the failure of hematopoietic stem cells to normally differentiate into mature white blood cells at various stages of development within bone marrow.[1] Acute leukemia is characterized by the rapid progression of leukemic cells at early stages of their development and requires aggressive timely treatment.[1] Acute leukemia can be divided into two main subtypes namely acute myeloid leukemia (AML) when cells generate from myeloid progenitors lineage and acute lymphoid leukemia (ALL) when cells generate from lymphoid progenitors lineage.[1]

Stimulatingly, acute leukemic patients become immunocompromised due to the impairment of vital functions of leukocytes, thus they become more vulnerable for infectious diseases, specifically these caused by Gram negative bacteria (GNB).[2] Neutrophils represent the first line of innate immune system induced by patients against infections of GNB.[2] In line with these cells, macrophages both of which are phagocytes that stimulate the inflammatory response against GNB by performing phagocytosis, a professional function that leads to eliminate pathogenic bacteria in phagolysosomes.[3] During phagocytosis, the active process of respiratory burst is triggered by consumption of molecular oxygen (O_2), releasing two highly reactive microbicidal agents, reactive nitrogen species (RNS) and reactive oxygen species (ROS).[3]

Nitric oxide (NO) is an important RNS microbicidal molecule with a very short half-life that formed by the action of nitric oxide synthase (NOS) in response to bacterial infections.[2] Nitric oxide performs the killing action by interacting with the bacterial nucleic acids, proteins, and lipids, causing cellular dysfunction and even death through apoptosis by blocking the activity of caspases.[4] Furthermore, NO displays more cytotoxic effect when it combines with superoxide (O^-_2) or hydrogen beroxide (H_2O_2) in phagolysosome to produce peroxynitrite, a very reactive and toxic molecule that can eradicate pathogenic GNB.[3]

In acute leukemic patients, NO was observed to have a dual role either carcinogenesis or anticancer activity.[4] Determining which effect predominantes is complex and mainly depends on the concentrations of produced NO.[4] At low concentrations, NO causes leukemia progression by promoting proliferation of leukemic cells, migration, invasion, metastasis, and angiogenesis, preventing apoptosis of these cells.[5] Moreover, at high concentrations, NO exhibits anticancer activity by supporting necrosis and apoptosis of leukemic cells, making these cells more sensitive to chemotherapeutic treatment.[5]

In addition to NO, the bacterial killing within phagolysosomes is mediated by releasing of hypochlorite (ClO^-) which is demonstrated to be more potent microbicidal agent than NO due to its broad spectrum toxicity against different components of GNB.[3] Interestingly, ClO^- is formed as a type of ROS by reacting H_2O_2 produced by respiratory burst with chloride ion (Cl^-), in which this reaction is catalyzed by the role of myeloperoxidase, an enzyme releases from the degranulation of large granules in the cytoplasm of neutrophils.[2] Likewise, ClO^- can react with H_2O_2 to produce singlet oxygen (O) that can damage bacterial cell membrane by targeting fatty acids of membrane lipids.[2] In line with NO, the evidences suggested that ClO^- is essential in regulating inflammatory response and cellular apoptosis.[3]

At last decades, the prevalence of nosocomial and community acquired infections caused by extended spectrum β-lactamases (ESBLs) producing GNB is seriously increasing worldwide, posing significant challenges regarding therapeutic options, especially in the case of leukemic patients.[6] ESBLs are fundamentally caused by the production of sulfidrhyl-variable (SHV), temoneira (TEM), and cefotaximase (CTX-M) encoded respectively by the genes bla_{SHV}, bla_{TEM}, and $bla_{CTX\text{-}M}$.[7] The high risk of ESBLs in clinical settings comes from their ability to hydrolyze the β-lactam ring of β- lactams, broadening the capacity of GNB to be more resistant for various types of β-lactam antibiotics in common use, including penicillins, monobactams (aztreonam), and third generation cephalosporins, increasing life threatening infections, health care costs, and poorer treatment outcomes.[7] The continuous accumulation rate of mutations is mainly contributed in rapidly emerging of new variants of *bla* genes.[6] In addition, horizontal gene transfer contributes to global spread of *bla* mutants among GNB species in hospitals and environment, leading to limit the antimicrobial treatment choices.[7]

This study aimed to assess the serologic levels of both NO and ClO^- and phylogenetic analysis of bla_{SHV}, bla_{TEM}, and $bla_{CTX\text{-}M}$ genes from GNB isolated from acute leukemic patients.

Materials and Methods

Patients Characteristics

This study involved collecting of 458 samples from different clinical sites of 260 patients with an acute leukemia admitted to hematology center at Baghdad Teaching Hospital, Children Welfare Teaching Hospital, and Central Teaching Hospital of Pediatric from January 2021 to December 2021.

The clinical samples encompassed 198 (43.23%) blood and 198 (43.23%) urine samples these were both collected from each of 198 patients, whereas swabs were collected from 62 (13.53%) patients suffered from soft skin injuries.

The patients aged from 1 to 65 years, of them 156 (60%) were females aged from 1 to 63 years and 104 (40%) were males aged from 1 to 65 years. According to the decision of specialist hematologists, 132 (50.76%) and 128 (49.23%) of patients were diagnosed as an AML and ALL, respectively.

Inclusion criteria were obtained from the medical chart of patients and based on the detecting of type of acute leukemia, gender, and age, while exclusion criteria comprised of patients infected with viral and fungal infections, patients who took antimicrobial therapy before blood culture was made, patients who gave hemolytic blood samples, pregnant women, and those who refused to participate in this study.

The patients were arranged into three studied groups in regard to gender and age included 87 (33.46%) men group (18–65 years), 117 (45%) women group (18–63 years), and 56 (21.53%) children group (1–14 years). The control groups included hygienic persons with same gender and ages of patients groups.

This study was approved by the Medical Ethical Committee of Baghdad Medical City following the approval 300294. Written consent was obtained from the adult patients and children's parents to share the results of this study.

Samples Identification

The intravenously collected blood samples were immediately drawn in Brain Heart Infusion Broth bottle (AFCO, Jordan) and incubated (Memmert, Germany) at 37°C for 24 h.[8] Then, subculture was performed two times a day for the first two days and then daily for one week on Blood agar, Chocolate agar, and MacConkey agar media (NEOGEN, UK).[2] Besides, urine and swab samples were directly streaked on the mentioned above agar media and incubated under the same conditions.[8] Later, all pure colonies were morphologically identified by Gram stain kit (AFCO, Jordan) and some routine biochemical tests included oxidase, indole, and simmon citrate utilization test (Himedia, India).[8] Consequently, only GNB isolates were subjected to Vitek 2 Compact System (Biomerieux, France) using ID GN cards.[8]

Serologic Aspect

A part of blood samples each from patients and controls was injected into a gel and clot activator glass tube (Biozek, Holland) and centrifuged (Fisher Scientific, USA) at 2000 rpm for 10 min to yield sera these stored (Sysmix, Japan) at –80°C until NO and Hypochlorite assays were performed.[8] The levels of NO were estimated using Human Total Nitric Oxide Enzyme Linked Immunosorbent assay (ELISA) kit purchased from MyBiosource (USA). This kit applied the competitive ELISA technique. In addition, ClO^- levels were assayed using hypochlorite detection kit (Abcam, UK) following a colorimetric method according to the manufacturing company information. The absorbance was read at a wavelength 450 nm and 555 nm for NO and ClO^-, respectively using a microplate reader (HumaReader HS, Germany).

Antimicrobial Susceptibility Test

The susceptibility of all studied GNB isolates was conducted in accordance with Kirby Bauer disc diffusion method.[9] The bacterial suspension for all isolates was prepared with visually

turbidity equivalent to 0.5 McFarland standard.[9] In the next step, the dipped swab from each suspension was streaked all over the surface of the Mueller-Hinton agar (NEOGEN, UK) plate and then the antibiotic discs (Liofilchem, Italy) were placed and incubated at 37°C for 24 h.[9] The tested antibiotics included Ampicillin (10 µg), Ticarcillin (75 µg), Ampicillin/Cloxacillin (25/5 µg), Piperacillin/Tazobactam (100/10 µg), Ceftazidime (30 µg), Cefotaxime (30 µg), Ceftriaxone (30 µg), Cefoxitin (30 µg), Cefepime (30 µg), Aztreonam (30 µg), Imipenem (10 µg), Meropenem (10 µg), Gentamicin (10 µg), and Ciprofloxacin (5 µg). The interpretation of results was relied on the measurement of the diameter of the inhibition zone around each antimicrobial disc according to CLSI, 2021.[10] The isolates were considered multidrug resistant (MDR) according to exhibit resistance to at least three different antibiotic categories.[11]

Detection of Extended Spectrum β-Lactamases

Extended Spectrum β-Lactamases were phenotypically detected following modified double disc test.[12] In this test, an amoxicillin-clavulanate (20/10 µg) disc was placed in the center of 90 mm Mueller-Hinton agar plate and around it each of Cefotaxime, Ceftazidime, Cefepime, and Aztreonam discs were placed at 15 mm distance from the center disc of amoxicillin-clavulanate and from 90° to each other and incubated at 37°C for 24 h.[12] The positive result was interpreted through a clear extension of the edge of the inhibition zone created around Cefotaxime, Ceftazidime, Cefepime, or Aztreonam disc towards the central disc of amoxicillin-clavulanate.[12]

DNA Extraction, Amplification, and Electrophoresis

The ABIO pure™ total DNA kit (USA) was used to extract the DNA from all identified GNB isolates from overnight Tryptone Soy broth (Oxoid, UK) culture according to the instructions of manufacturing company and froze at −20°C until further use. The concentrations and purity of the extracted DNA were examined by Nanodrop (Biogroup, UK) at a wavelength 260 and 280 nm.[13]

In this study, three ESBLs encoded genes were screened included bla_{SHV}, bla_{TEM}, and bla_{CTX-M} (Alpha DNA, Canada) with sequence and size listed in Table 1 utilized polymerase chain reaction (PCR) technique. The PCR mixture reaction was prepared at a volume of 20 µL consisting of 10 µL GoTaq® Master Mix (Promega, USA), 1 µL forward primer, 1 µL reverse primer, 2 µL template DNA, and 6 µL nuclease free water (Promega, USA). The Thermal Cycler (Fisher Scientific, USA) was employed to amplify PCR mixture according the following conditions: 95°C for 15 min for initial denaturation, 30 cycles of 94°C for 30 s for denaturation, 62°C for 90 s for annealing, 72°C for 60 s for extension, and 70°C for 10 min for final extension.[14]

Table 1. **Sequence of β-lactamases Primers**

Primer	Sequence (5′-3′)	Size (bp)	Reference
bla_{SHV}	F: CTTTATCGGCCCTCACTCAA R: AGGTGCTCATCATGGGAAAG	237	(H. Fang et al.)[14]
bla_{TEM}	F: CGCCGCATACACTATTCTCAGAATGA R: ACGCTCACCGGCTCCAGATTTAT	445	(H. Fang et al.)[14]
bla_{CTX-M}	F: ATGTGCAGYACCAGTAARGTKATGGC R:TGGGTRAARTARGTSACCAGAAYC AGCGG	593	(H. Fang et al.)[14]

bla: β-lactamases, bp; base pair, SHV; sulfidrhyl-variable, TEM; Temoneira, CTX-M; cefotaximase.

After amplification, the PCR products were electrophoresed (Thermo, USA) on 1% agarose gel stained with 0.5 µg/mL ethidium bromide and immersed in 1X TAE buffer (Promega, USA) at 100 V for 80 min. and visualized by UV transilluminator (Major Science, Taiwan).[13] The 100–1500 bp DNA ladder (Promega, USA) was utilized to estimate the molecular weight of the separated bands.[13]

DNA Sequencing

The resolved PCR amplicons were sequenced from both forward and reverse directions, following the instruction manuals of the sequencing company (Macrogen Inc. Geumchen, Seoul, South Korea). The chromatographs obtained from ABI (Applied Biosystem) sequence files were further analyzed, ensuring that the annotation and variations are not because of PCR or sequencing artifacts. The sequencing results of the targeted isolates were edited, aligned, and analyzed as long as with the reference sequences using BioEdit Sequence Alignment Editor Software Version 7.1 (DNASTAR, Madison, WI, USA).[15] Each detected variant within studied sequences was annotated by SnapGene Viewer ver. 4.0.4 (https://www.snapgene.com). The translation of observed DNA variations into amino acids corresponds to the reference amino acid sequences was performed using the Expasy online program (http://web.expasy.org/translate/).[16] All the analyzed sequences were submitted to the NCBI Bankit portal to get a unique GenBank accession number for the investigated sequences.[17]

Phylogenetic Tree Construction

Two specific phylogenetic trees for bla_{SHV} and bla_{TEM} genes were respectively constructed in this study according to the neighbor-joining protocol. The observed variants were compared with their neighbor homologous reference sequences using the NCBI-BLASTn server.[18] Then, the trees including the observed variant were built and annotated using the iTOL suit.[19] To find out the exact identity of the investigated isolates of GNB, only closely related reference sequences of each bacterial species were incorporated within the trees. These trees revealed the details of the studied isolates suited in the vicinity to the closest reference isolates, such as the host origin, isolation source, and the region from which the reference samples were deposited.[19]

Statistical Analysis

The non-parameters results were described as number and percentage. The parameters were presented as Mean ± S.E. by SPSS program (version 25) using one way ANOVA test by obtaining least significant differences (LSD). The differences were considered significant at $P \leq 0.05$.

Results and Discussion

Depending on morphology and biochemical characterization of 458 clinical samples, 70 (15%) isolates were identified as GNB and then the detection by Vitek 2 Compact System revealed that these GNB were belonged to four different species included *Klebsiella pneumoniae* 23 (33%),

Escherichia coli 21 (30%), *Pseudomonas aeruginosa* 18 (26%), and *Acinetobacter baumannii* 8 (11%), as illustrated in Table 2. Concerning the clinical source of collection, the confirmed identification of 198 (43.23%) samples each from urine and blood and 62 (13.53%) swab samples exhibited that GNB were diagnosed among 39 (55.71%) urine, 23 (32.85%) blood, and 8 (11.42%) swab samples. Furthermore, the infections of GNB were distributed in acute leukemia patients as 40 (57.14%) and 30 (42.85%) of AML and ALL patients, respectively, 42 (60%) and 28 (40%) of females and males, respectively, and 39 (55.71%), 24 (34.28%), and 7 (10%) of women, men, and children groups, respectively. The previously publications[20,21] gained similar finding when they proved that *K. pneumoniae* was the predominant species among their GNB isolates, in contrast to other studies[22,23] found that *E. coli* was more GNB species prevalent than *K. pneumoniae*, followed by *P. aeruginosa* and *A. baumannii*, respectively.

The serologic assay revealed that NO had elevated mean levels reached 14.33 ± 1.29, 16.59 ± 1.74, and 14.53 ± 0.88 umol/L in men, women, and children groups, respectively as compared with those in control groups, in which their means were 8.51 ± 0.37, 8.86 ± 0.33, and 9.07 ± 0.19 umol/L, respectively. The statistical differences were observed to be significant ($P \leq 0.05$) among men, women, and children groups versus their control groups, whereas no significant differences ($P > 0.05$) were observed among all patients groups. Altogether, early publications[24-26] gained higher levels of NO among their leukemic patients. Over the last decades, efforts have focused on using NO in clinical practice as an anticancer molecule.[5] This is based on utilizing NO at high dose as a co-delivery drug in combination with chemotherapeutic agents like fisetin to improve the efficacy of treatment of acute leukemia, especially to prevent the relapse of AML.[4,5]

The mean serum hypochlorite levels were slightly higher among men (0.88 ± 0.05 U/L), women (0.70 ± 0.06 U/L), and children (0.71 ± 0.05 U/L) groups than healthy men, women, and children in control groups, where mean levels of hypochlorite exceeded 0.66 ± 0.05, 0.69 ± 0.06, and 0.66 ± 0.06 U/L, respectively in their sera. The statistical differences were significant ($P \leq 0.05$) in men group, whereas no significant differences ($P > 0.05$) were shown in women and children groups compared to control groups. On the other hand, significant differences ($P \leq 0.05$) in hypochlorite levels were found among patients in three studied groups. Two previous studies also reported high mean levels when they measured serum hypochlorite among leukemia and hematological malignancies diseases compared with control persons.[27,28] Moreover, hypochlorite concentrations could be inhibited in acute leukemic patients with the progression of cancer due to impairment of functions of neutrophils.[2] This obviously leads to defective in phagocytosis activity, facilitating bacterial colonization and invasion.[2]

The antimicrobial susceptibility test showed that *K. pneumoniae* isolates were high resistant for the studied antibiotics at rates ranged between 22 (95.65%) to 16 (69.56%), as listed in Table 3. It was noticed that the most high resistant rates were 22 (95.65%) for both ampicillin and ticarcillin, respectively, 21 (91.30%) each for ampicillin/cloxacillin, cefotaxime, cefepime, and imipenem, respectively, 20 (86.95%)

Table 2. **Characteristics of bacterial isolates and patients**

Characteristic	Samples before bacterial Identification *n* (%)	Total *n* (%)	Samples after bacterial Identification *n* (%)	Total n (%)
GNB isolates				
K. pneumoniae	–		23 (33)	
E. coli	–	–	21 (30)	70 (100)
P. aeruginosa	–		18 (26)	
A. baumannii	–		8 (11)	
Sources of clinical isolates				
Urine	198 (43.23)		39 (55.71)	
Blood	198 (43.23)	458 (100)	23 (32.85)	70 (100)
Swabs	62 (13.53)		8 (11.42)	
Type of leukemia				
AML	132 (50.76)	260 (100)	40 (57.14)	70 (100)
ALL	128 (49.23)		30 (42.85)	
Gender				
Male	104 (40)	260 (100)	28 (40)	70 (100)
Female	156 (60)		42 (60)	
Age Groups				
Men group	87 (33.46)		24 (34.28)	70 (100)
Women group	117 (45)	260 (100)	39 (55.71)	
Children group	56 (21.53)		7 (10)	

GNB; Gram negative bacteria, AML; acute myeloid leukemia, ALL; acute lymphoid leukemia.

Table 3. **Resistance patterns of GNB**

Antibiotics	*K. pneumoniae* *n* (%)	*E. coli* *n* (%)	*P. aeruginosa* *n* (%)	*A. baumannii* *n* (%)
Ampicillin	22 (95.65)	21 (100)	–	8 (100)
Ticarcillin	22 (95.65)	21 (100)	–	–
Ampicillin/Cloxacillin	21 (91.30)	21 (100)	–	–
Piperacillin/Tazobactam	19 (82.60)	15 (71.42)	7 (38.88)	8 (100)
Ceftazidime	18 (78.26)	20 (95.23)	10 (55.55)	8 (100)
Cefotaxime	21 (91.30)	20 (95.23)	–	8 (100)
Ceftriaxone	20 (86.95)	19 (90.47)	–	8 (100)
Cefoxitin	16 (69.56)	7 (33.33)	–	–
Cefepime	21 (91.30)	21 (100)	11 (61.11)	8 (100)
Aztreonam	19 (82.60)	15 (71.42)	8 (44.44)	8 (100)
Imipenem	21 (91.30)	15 (71.42)	13 (72.22)	8 (100)
Meropenem	18 (78.26)	7 (33.33)	8 (44.44)	8 (100)
Gentamicin	19 (82.60)	19 (90.47)	8 (44.44)	8 (100)
Ciprofloxacin	18 (78.26)	20 (95.23)	7 (38.88)	8 (100)

ESBL; Extended Spectrum β-Lactamase.

for ceftriaxone, 19 (82.60%) for piperacillin/tazobactam, aztreonam, and gentamicin, respectively. The subsequent rates were 18 (78.26%) against each ceftazidime, meropenem, and ciprofloxacin, respectively, and 16 (69.56%) to cefoxitin.

All *E. coli* isolates 21 (100%) were resistant for ampicillin, ticarcillin, ampicillin/cloxacillin, and cefepime, respectively, followed by a slight decrease of resistance reached 20 (95.23%) for ceftazidime, cefotaxime, and ciprofloxacin, respectively, and 19 (90.47%) for both ceftriaxone and gentamicin, respectively. Moreover, 15 (71.42%) of isolates were observed to be resistant each to piperacillin/tazobactam, aztreonam, and imipenem, respectively. Cefoxitin and meropenem showed more antimicrobial activity, in which 7 (33.33%) isolates were resistant to both agents. Regarding to *P. aeruginosa* isolates, it was found that approximately 40% to 70% of the isolates were resistant to the studied antibiotics, in which the resistance rates 13 (72.22%), 11 (61.11%), and 10 (55.55%) were respectively obtained with imipenem, cefepime, and ceftazidime. The reduced resistance rates were recorded at 8 (44.44%) for aztreonam, gentamicin and meropenem, respectively, and 7 (38.88%) to both piperacillin/tazobactam and ciprofloxacin. The total of *A. baumannii* isolates 8 (100%) exhibited resistance against all used antibiotics.

Another studies reported relatively similar with slightly variations of resistance rates due to difference in the number of the collected isolates, source of collection, geographic scale, and variety of environmental factors.[29-34] Overall, GNB of these studies showed the highest resistance to penicillins and third generation cephalosporins, and relatively graduated by aztreonam, amikacin, and ciprofloxacin. Though, their GNB were more susceptible to carbapenems and colistin.[35,36] Recently, the rate of resistance in GNB increases periodically worldwide, as a result of acquisition of ESBLs determinants by horizontal gene transfer.[7] Alongside, genomic mutations cause reducing in β-lactams uptake by modifications in target site of drug action, overexpression of efflux pumps, decreasing porins expression, or may provide alternative metabolic pathways.[6,7]

Table 4. **Distribution of ESBLs, MDR, and ESBLs producing MDR GNB isolates**

GNB sp.	ESBL *n* (%)	MDR *n* (%)	ESBL + MDR *n* (%)
K. pneumoniae	11 (47.82)	21	9 (81.81)
E. coli	18 (85.71)	21	15 (83.33)
P. aeruginosa	17 (94.44)	8	7 (41.17)
A. baumannii	3 (37.5)	8	3 (100)
Total	49	58	34

GNB; Gram negative bacteria, ESBL; Extended Spectrum β-Lactamase.

Extended spectrum β-lactamases were produced in 49 (70%) of the tested isolates of GNB (Table 4), distributing as 18 (85.71%) *E. coli*, 17 (94.44%) *P. aeruginosa*, 11 (47.82%) *K. pneumoniae*, and 3 (37.5%) *A. baumannii*. Alongside, the antimicrobial resistance patterns for ESBLs producing isolates of GNB were elucidated in Table 5. Other reports gained results were nearly in accordance with the current study when they found that 66.02%, 55%, 51.55%, and 47.5%, of their GNB isolates were respectively ESBLs positive.[22,23,37,38] The MDR pattern was presented among 58 (82.85%) of isolated GNB, including all isolates of both *E. coli* 21 (100%) and *A. baumannii* 8 (100%), as illustrated in Table 4. On the other hand, this pattern was determined in most isolates of *K. pneumoniae* 21 (91.30%) and much less in *P. aeruginosa* isolates 8 (44.44%) (Table 4). Alongside, ESBLs were noticed in 34 (58.62%) of MDR isolates of GNB, of which 15 (83.33%) *E. coli*, 9 (81.81%) *K. pneumoniae*, 7 (41.17%) *P. aeruginosa*, and 3 (100%) *A. baumannii* (Table 4). The investigation of Patil *et. al*[39] recorded relatively less MDR incidence among their GNB reached 71.76%, whereas the least MDR values 33.33% and 28.7% were obtained by,[40,41] respectively.

The frequency of ESBLs producing clinical isolates of GNB increases worldwide and it is well noted that the major

Table 5. **Resistance patterns of ESBLs producing GNB**

Antibiotics	*K. pneumoniae* n (%)	*E. coli* n (%)	*P. aeruginosa* n (%)	*A. baumannii* n (%)
Ampicillin	10 (90.90)	18 (100)	–	3 (100)
Ticarcillin	10 (90.90)	18 (100)	–	–
Ampicillin/Cloxacillin	9 (81.81)	18 (100)	–	–
Piperacillin/Tazobactam	7 (63.63)	12 (66.66)	6 (35.29)	3 (100)
Ceftazidime	6 (54.45)	17 (94.44)	9 (52.94)	3 (100)
Cefotaxime	9 (81.81)	17 (94.44)	–	3 (100)
Ceftriaxone	8 (72.72)	16 (88.88)	–	3 (100)
Cefoxitin	4 (36.36)	5 (27.77)	–	–
Cefepime	9 (81.81)	18 (100)	10 (58.82)	3 (100)
Aztreonam	7 (63.63)	12 (66.66)	7 (41.17)	3 (100)
Imipenem	9 (81.81)	13 (72.22)	12 (70.58)	3 (100)
Meropenem	6 (54.45)	5 (27.77)	7 (41.17)	3 (100)
Gentamicin	7 (63.63)	16 (88.88)	7 (41.17)	3 (100)
Ciprofloxacin	6 (54.45)	17 (94.44)	6 (35.29)	3 (100)

GNB; Gram negative bacteria, ESBL; Extended Spectrum β-Lactamase.

microbial resistance is attributed to these clinical important enzymes.[6] Interestingly, ESBLs producing GNB are mainly responsible for the excessive use of other cost antimicrobials, such as carbapenems, prolonged period of hospitalization, and increasing rates of morbidity and mortality.[6,7] So far, the emerging of MDR isolates limits the efficacy of the current antibiotics and the risk is more complicated when ESBL producing MDR isolates spread worldwide, leading to a public health challenge and failure of treatment.[6,7]

Genotypic detection of ESBLs encoding genes revealed that bla_{SHV} gene was screened in 20 (28.57%) of GNB isolates with the highest prevalence rate in *K. pneumoniae* isolates 19 (82.60%), as viewed in Figure 1. Contrastingly, only one (4.76%) *E. coli* isolate was harbored bla_{SHV} gene. Out of the total 20 (28.57%) isolates, 10 (50%) were ESBLs producing isolates, 18 (90%) were MDR isolates, and 8 (40%) were ESBLs producing MDR isolates. Whilst, neither *P. aeruginosa* nor *A. baumannii* isolates were displayed bla_{SHV} gene (Table 6). A higher bla_{SHV} prevalence of 38.8% and 34.70% was respectively determined in GNB of both El Aila *et. al*[23] and Patil *et. al.*[39] Otherwise, the results of Leal *et. al*[41] and Abrar *et. al*[22] were respectively in line and less than of this study, since they detected bla_{SHV} in 29.37% and 21% of their GNB.

Besides, 32 (45.71%) of GNB were positive for bla_{TEM} distributed as 15 (65.21%) *K. pneumoniae*, 13 (61.90%) *E. coli*, 3 (37.5%) *A. baumannii*, and 1 (5.55%) *P. aeruginosa* (Figure 2). Of these, 21 (65.62%) were ESBLs producing isolates, 30 (93.75%) were MDR isolates, and 19 (59.37%) were ESBLs producing MDR isolates. Most of these isolates were belonged to *K. pneumoniae* and *E. coli*, but isolates of *P. aeruginosa* and *A. baumannii* displayed much low prevalence of the above mentioned characteristics (Table 7). Contrary to the current finding, El Aila et. al[23] characterized bla_{TEM} in 57.6% of their GNB. On the other hand, Abrar et. al[22] and Leal et. al[41] reported bla_{TEM} at lower rates of 25.87% and 28%, respectively.

Interestingly, bla_{CTX-M} gene was not confirmed among the investigated isolates. Otherwise, previous studies detected

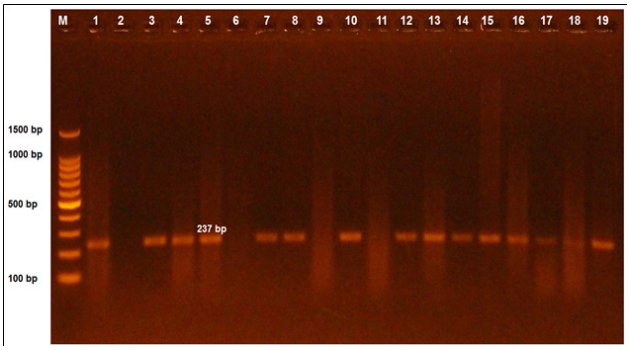

Fig. 1 **Amplification of bla_{SHV} gene for *Klebsiella pneumoniae* isolates showed PCR fragments at 237 bp run on 2% agarose gel at 100 V. for 80 min. Lane M: DNA ladder (100–1500 bp). Lanes 1, 3, 4, 5, 7, 8, 10, 12, 13, 14, 15, 16, 17, 18, 19: PCR positive products.**

bla_{CTX-M} at various rates among their isolates of GNB.[22,23,41] The co-existence of bla_{TEM} and bla_{SHV} genes was only found in the isolates of the species *K. pneumoniae* 14 (60.86%), in which 7 (50%), 13 (92.85%), and 6 (42.85%) isolates were ESBL producing, MDR, and ESBL producing MDR isolates, respectively, as showed in Table 8.

With regard to bla_{SHV} amplicons, the cuurent results indicated the presence of only one DNA substitution of the transversion (111T>A), replacing thymine (T) with adenine (A) at the position 111 in six of the investigated *K. pneumoniae* isolates (S13, S14, S15, S16, S17, and S18), as noted in Figure 3. Thus, this mutation was of missense effect (p.31Q>L) in the encoded protein, replacing glutamine (Q) with leucine (L) at the position 31, as shown in Figure 4. Over the time, the frequently accumulation of mutations in *bla* genes can greatly be led to inefficacy of common antibiotics against serious infections of GNB due to the structural alternations within the active site of the encoded protein of β-lactamase resulted from the effect of mutations. Concerning bla_{TEM} amplicons, our results indicated the absence of any detectable DNA variations

Table 6. **Distribution of bla$_{SHV}$ positive isolates**

GNB sp.	*bla*$_{SHV}$ positive isolates *n* (%)	ESBL + *bla*$_{SHV}$ positive isolates *n* (%)	MDR + *bla*$_{SHV}$ positive isolates *n* (%)	ESBL + MDR + *bla*$_{SHV}$ positive isolates *n* (%)
K. pneumoniae	19 (82. 60)	9 (47.36)	17 (89.47)	7 (36.84)
E. coli	1 (4.76)	1 (100)	1 (100)	1 (100)
P. aeruginosa	0	0	0	0
A. baumannii	0	0	0	0
Total	20	10	18	8

bla; β-lactamases, SHV; sulfidrhyl-variable, MDR; multidrug resistant, ESBL; Extended Spectrum β-Lactamase, GNB; Gram negative bacteria.

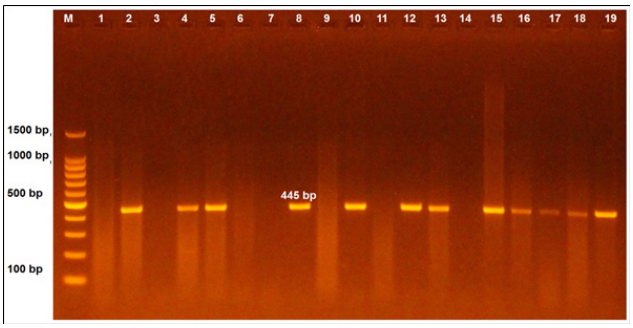

Fig. 2 **Amplification of *bla*$_{TEM}$ gene for *Klebsiella pneumoniae* isolates showed PCR fragments at 445 bp run on 2% agarose gel at 100 V. for 80 min. Lane M: DNA ladder (100–1500 bp). Lanes 2, 4, 5, 8, 10, 12, 13, 15, 16, 17, 18, 19: PCR positive products.**

in all investigated isolates. Thus, no effect was found in the encoded protein.

All the investigated *bla*$_{SHV}$ PCR amplicons sequences were deposited in the NCBI web server, and unique accession numbers were obtained for the analyzed sequences. Sixteen GenBank accession numbers OP999053, OP999054, OP999055, OP999056, OP999057, OP999058, OP999059, OP999060, OP999061, OP999062, OP999063, OP999064, OP999065, OP999066, OP999067, and OP999068 were obtained to represent the S4, S5, S7, S8, S10, S12, S13, S14, S15, S16, S17, S18, S20, S21, S22, and S23 *K. pneumoniae* isolates, respectively. Concerning, *bla*$_{TEM}$ sequences, Fifteen GenBank accession numbers of OQ077522, OQ077523, OQ077524, OQ077525, OQ077526, OQ077527, OQ077528, OQ077529, OQ077530, OQ077531, OQ077532, OQ077533, OQ077534, OQ077535, and OQ077536 were obtained to respectively represent the S2, S4, S5, S8, S10, S12, S13, S15, S16, S17, S18, S19, S20, S21, and S23 samples of *K. pneumoniae*. Thirteen GenBank accession numbers of OQ077537, OQ077538, OQ077539, OQ077540, OQ077541, OQ077542, OQ077543, OQ077544, OQ077545, OQ077546, OQ077547, OQ077548, and OQ077549, were obtained to respectively represent the S24, S27, S29, S30, S32, S33, S36, S38, S39, S40, S41, S43, and S44 samples of *E. coli*. One GenBank accession number of OQ077550 was obtained to represent the S56 sample of *P. aeruginosa*. In addition, three GenBank accession numbers of OQ077551, OQ077552, and OQ077553 were obtained to respectively represent the S64, S66, and S68 samples of *A. baumannii*.

The phylogenetic tree of *bla*$_{SHV}$ gene (Figure 5) contained 16 *K. pneumoniae* isolates (S4, S5, S7, S8, S10, S12, S13, S14, S15, S16, S17, S18, S20, S21, S22, and S23) alongside with other relative reference DNA sequences, respectively, thus the total number of aligned sequences in this tree was thirty-six. Within this tree, five bacterial isolates belonged to *E. coli* were added as an out-group to assess the extent of the *K. pneumoniae* variations. However, the investigated isolates were clustered into only one phylogenetic clade of extremely close distances between the incorporated clades of *K. pneumoniae*. This aggregation was found away from the out-group of the *E. coli* clade, since there was a considerable phylogenetic distance between both clades, depicting the ability of the *bla*$_{SHV}$ sequences to differentiate between both closely related species.

What had increased the clinical specificity of *bla*$_{SHV}$ sequences was attributed to the presence of the human subjects as only one host for these closely related sequences. In contrast, more host divergent cases were observed from the closely related *E. coli* out-group strains due to the presence of one *E. coli* strain isolated from chickens.

Moreover, the isolation sources showed diverse origins in corresponding reference sequences due to the multiple portions (feces, environment, wound, respiratory tract, and other unknown clinical sources) from which the incorporated isolates were collected.

Furthermore, no specific geographical distribution was detected to represent a possible origin for the studied isolates, in which these isolates were positioned beside variable strains of *K. pneumoniae* that were deposited from Asian, European, American, Australian, and African. This indicated the influence role of this tree to determine the epidemiologic routes of pathogenic GNB. Indeed, this data indicated that the identified 111T>A variant had shown a slight phylogenetic effect on the evolutionary positioning in comparison with the reference sequences of *K. pneumoniae*.

The phylogenetic tree of *bla*$_{TEM}$ amplicons (Figure 6) consisted of the 15 *K. pneumoniae* isolates (S2, S4, S5, S8, S10, S12, S13, S15, S16, S17, S18, S19, S20, S21, and S23), 13 *E. coli* isolates (S24, S27, S29, S30, S32, S33, S36, S38, S39, S40, S41, S43, and S44), one *P. aeruginosa* isolate (S56), and three *A. baumannii* isolates (S64, S66, and S68). These isolates were respectively aligned alongside with other relative reference sequences, thus the total number of aligned sequences in this tree was fifty.

Four clades of incorporated *bla*$_{TEM}$ sequences were constructed within this tree. One of these clades belonged to *A. baumannii*, which was made of seven extremely closely related strains of the same species. Due to the absence of any genetic variation in the investigated *A. baumannii* isolates, no phylogenetic distances were observed between these isolates and the reference sequences in the same clade. Near this clade,

Table 7. **Distribution of *bla*$_{TEM}$ positive isolates**

GNB sp.	*bla*$_{TEM}$ positive isolates *n* (%)	ESBL + *bla*$_{TEM}$ positive isolates *n* (%)	MDR + *bla*$_{TEM}$ positive isolates *n* (%)	ESBL + MDR + *bla*$_{TEM}$ positive isolates *n* (%)
K. pneumoniae	15 (65.21)	7 (46.66)	14 (93.33)	6 (40)
E. coli	13 (61.90)	11 (84.61)	13 (100)	11 (84.61)
P. aeruginosa	1 (5.55)	1 (100)	0	0
A. baumannii	3 (37.5)	2 (66.66)	3 (100)	2 (66.66)
Total	32	21	30	19

bla; β-lactamases, SHV; sulfidrhyl-variable, TEM; Temoneira, MDR; multidrug resistant, ESBL; Extended Spectrum β-Lactamase, GNB; Gram negative bacteria.

Table 8. **Co-existence of *bla*$_{TEM}$ and *bla*$_{SHV}$ genes in GNB**

GNB sp.	*bla*$_{TEM}$ + *bla*$_{SHV}$ positive isolates *n* (%)	ESBL + *bla*$_{TEM}$ + blaSHV positive isolates *n* (%)	MDR + *bla*$_{TEM}$ + *bla*$_{SHV}$ positive isolates *n* (%)	ESBL + MDR + *bla*$_{TEM}$ + *bla*$_{SHV}$ positive isolates *n* (%)
K. pneumoniae	14 (60.86)	7 (50)	13 (92.85)	6 (42.85)
E. coli	0	0	0	0
P. aeruginosa	0	0	0	0
A. baumannii	0	0	0	0
Total	14	7	13	6

bla; β-lactamases, SHV; sulfidrhyl-variable, TEM; Temoneira, MDR; multidrug resistant, ESBL; Extended Spectrum β-Lactamase, GNB; Gram negative bacteria.

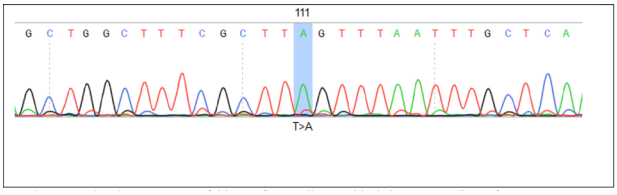

Fig. 3 **The chromatograms of *bla*$_{SHV}$ PCR amplicons with their corresponding reference sequences within the genomic sequences of *K. pneumoniae* (GenBank acc. no. CP110592.1).**

```
>UZJ01856.1 broad-spectrum class A beta-lactamase SHV-11 [Klebsiella
pneumoniae]
MRYIRLCIISLLATLPLAVHASPQPLEQIKQSESQLSGRVGMIEMDLASGRTLTAWRADERFPMMSTFKV
VLCGAVLARVDAGDEQLERKIHYRQQDLVDYSPVSEKHLADGMTVGELCAAAITMSDNSAANLLLATVGG
PAGLTAFLRQIGDNVTRLDRWETELNEALPGDARDTTTPASMAATLRKLLTSQRLSARSQRQLLQWMVDD
RVAGPLIRSVLPAGWFIADKTGAGERGARGIVALLGPNNKAERIVVIYLRDTPASMAERNQQIAGIGAAL
IEHWQR

MUTANT
MRYIRLCIISLLATLPLAVHASPQPLEQIKLSESQLSGRVGMIEMDLASGRTLTAWRADERFPMMSTFKV
VLCGAVLARVDAGDEQLERKIHYRQQDLVDYSPVSEKHLADGMTVGELCAAAITMSDNSAANLLLATVGG
PAGLTAFLRQIGDNVTRLDRWETELNEALPGDARDTTTPASMAATLRKLLTSQRLSARSQRQLLQWMVDD
RVAGPLIRSVLPAGWFIADKTGAGERGARGIVALLGPNNKAERIVVIYLRDTPASMAERNQQIAGIGAAL
IEHWQR
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Fig. 4 **Amino acid residues alignment of the investigated sequences of *bla*$_{SHV}$ PCR amplicons with their corresponding reference sequences of *K. pneumoniae* genomic sequences (GenBank acc. no. UZJ01856.1).**

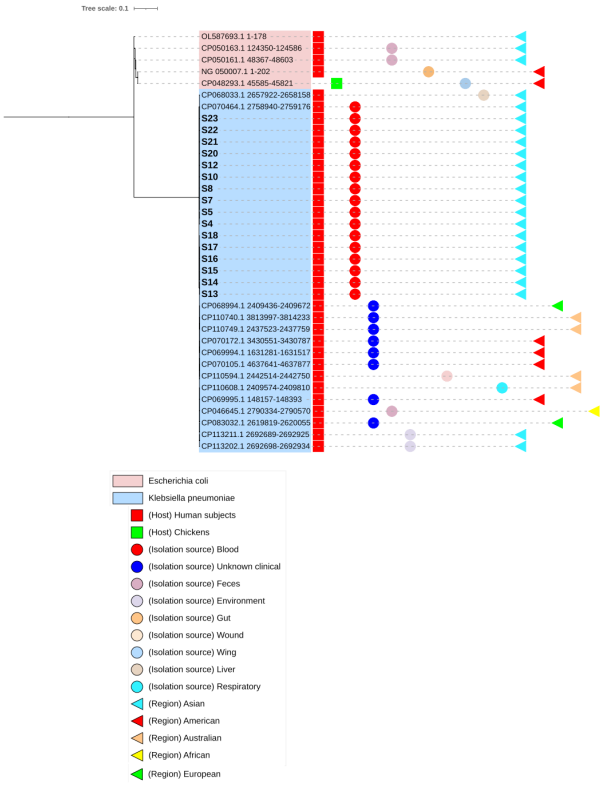

Fig. 5 **Phylogenetic tree of the *bla*$_{SHV}$ sequences of sixteen *Klebsiella pneumoniae* isolates. The black-colored rectangle refers to the analyzed variants. All the mentioned numbers refer to GenBank accession numbers of each referring species. The number "0.1" at the top portion of the tree refers to the degree of scale range among the comprehensive tree-categorized organisms. The S letter refers to the code of the investigated sample.**

the clade of *P. aeruginosa* was positioned. This clade was also made of seven samples, in which the S56 sample was incorporated. Though, both clades showed no phylogenetic distances between them. This data revealed that there were no genetic differences between the currently investigated *bla*$_{TEM}$ sequences of *A. baumannii* and *P. aeruginosa*. The same

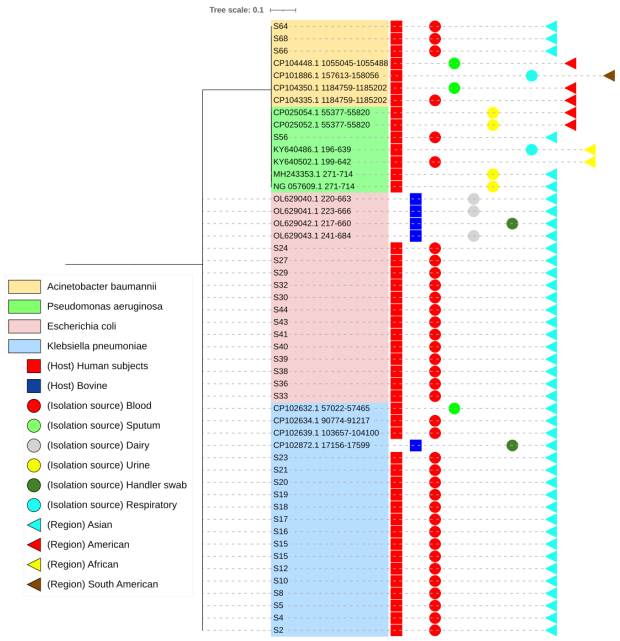

Fig. 6 **Phylogenetic tree of genetic variants of the *bla*$_{TEM}$ sequences of 32 samples of the *K. pneumoniae, E. coli, P. aeruginosa,* and *A. baumannii*. The black-colored rectangle refers to the analyzed variants. All the mentioned numbers refer to GenBank accession numbers of each referring species. The number "0.1" at the top portion of the tree refers to the degree of scale range among the comprehensive tree-categorized organisms. The letter "S" refers to the code of the investigated isolates.**

observed data were also identified in both clades of *E. coli* and *K. pneumoniae* without the presence of any noticeable phylogenetic distances between them.

The clinical specificity of *bla*$_{TEM}$ sequences came from the presence of more than one host for these closely related reference sequences, since bovine host isolated strains was observed in the clade of *K. pneumoniae* and *E. coli* sequences. while, the human subject was the only host identified in the clade of both *A. baumannii* and *P. aeruginosa* sequences.

The isolation sources had shown numerous origins due to the variable of portions from which the incorporated isolates were collected. The *P. aeruginosa* and *A. baumannii* isolates were positioned beside variable strains that were collected from sputum, respiratory tract, and urine. Similarly, due to the absence of any phylogenetic distances between *E. coli* and the *K. pneumoniae* isolates, the same diverse sources were also observed, such as sputum, dairy, and animal handler swabs.

Moreover, no specific geographical distribution was detected to represent a possible origin for the tested isolated. The *A. baumannii* and *P. aeruginosa* isolates were positioned beside variable strains that were deposited from Asian, American, African, and South American. On the other hand, a highly specific geographical distribution was identified from the clades of *E. coli* and *K. pneumoniae*. This was due to the close vicinity of the investigated isolates to the reference samples deposited from Asian regions. This observation indicated that the *bla*$_{TEM}$ amplicons of *E. coli* and *K. pneumoniae* showed high specificity in the terms of the identification of the accurate region of these isolates compared with the *bla*$_{TEM}$ amplicons of *A. baumannii* and *P. aeruginosa*.

Other authors performed sequence analysis of *bla* genes from GNB, constructed phylogenetic trees, and recorded multiple mutations among their variants, as substitutions, insertion, and deletion.[22,32,38,39,42] The phylogenetic analysis is of an influential role in the epidemiologic tracking of nosocomial origin-serious infections of GNB, providing a wide view about the host origin, isolation source, and geographical distribution of the high risk mutant of *bla* genes. Accordingly, this can be aided in the control and reducing the infections of GNB and control the horizontal gene transfer of *bla* mutants between clinical isolates or even to environmental source isolates.[22, 42]

Conclusion

The increased levels of both NO and ClO^- in acute leukemic patients could potentially refer to the microbicidal action or anticancer activity of these agents to eradicate pathogenic GNB by phagocytosis. The patterns of ESBLs, MDR, and ESBLs producing MDR GNB definitely represent a great threat to public health. This is compromised with the dissemination of GNB with the co-existence of *bla* genes, complicating clinical treatment by common antibiotics .The phylogenetic analysis of *bla* genes constituted an important tool to track the epidemiologic routes of serious infections of GNB through detect host origin, isolation source, and geographical distribution of the highly risk mutant of *bla* genes, in order to potentially improve the control and prevention manner of nosocomial and community acquired infections of GNB, especially in immunocompromised acute leukemic patients.

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