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Molecular Characterization and in Silico Analysis of a Novel Mutation in TEM-1 Beta-Lactamase Gene among Pathogenic *E. coli* infecting a Sudanese Patient

Hisham N Altayb[1,*], Nagwa M El Amin[2], Maowia M. Mukhtar[1], Mohamed Ahmed Salih[3], Mohamed A M Siddig[4]

[1]Institute of Endemic Diseases, University of Khartoum, Khartoum, Sudan
[2]Department of microbiology, Faculty of medicine, university of Khartoum, Sudan
[3]Head department of biotechnology, Biotechnology Park, Africa city of technology, Sudan
[4]Botany department, Faculty of Science, University of Khartoum, Sudan
*Corresponding author: hishamaltayb@yahoo.com

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Abstract The presence of ESBLs in many *E. coli* strains are of serious concern, since these organisms are the most common cause of different human infections. In this study we isolate an *E. coli* bacterium with high hydrolytic activity against cefotaxime. The ESBLs production was confirmed by phenotypic confirmatory test, while the ESBLs genes were detected by polymerase chain reaction (PCR). This isolate was positive for TEM gene and negative for CTX-M and SHV genes. DNA sequencing was done for TEM gene. The nucleotide sequences and translated proteins were subjected to BLAST for sequences similarity and homology, BLASTp result revealed a substitution of aspartic acid in TEM-1(gb: AFI61435.1) to Threonine at position 262. In Silico tools was used for mutation analysis and prediction of secondary and tertiary structure of wild and mutant type genes. We conclude that our mutant gene is completely different from the wild types TEM-1 gene, within phenotypic and genotypic levels. So we conclude a novel mutant TEM gene with ESBLs activity is been detected in Sudan Phylogenetic tree revealed that the possible source of our gene is Iran.

Keywords: *E. coli, ESBLs, Novel TEM gene, Insilco analysis, Sudan*

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1. Introduction

The production of penicillinases and other β-lactamases is the principal mechanism of resistance to beta-lactam antibiotics among members of the Enterobacteriaceae family [1,2]. Before the CTX-M era, TEM-type extended-spectrum β-lactamases (TEM-ESBL) were the most prevalent mechanism of resistance to β-lactam antibiotics in Enterobacteriaceae. They emerged from the parental penicillinases TEM-1 and TEM-2 [3]. The TEM-type ESBLs are derivatives of TEM-1 and TEM-2. TEM-1 was first reported in 1965 from an *Escherichia coli* isolate from a patient in Athens, Greece, named Temoneira (hence the designation TEM) [4]. This TEM-1 producing *E. coli* hydrolyzed ampicillin, and within a few years after its first isolation, its plasmid-mediated resistance had spread over the world and into many different members of the Enterobacteriaceae family, including *Pseudomonas aeruginosa*, *Haemophilus influenzae* and *Neisseria gonorrhoeae*. Other β -lactamases were then discovered which were closely related to TEM-1 and TEM-2, but which had the ability to confer resistance to the extended-spectrum cephalosporins [5,6]. Hence these new β -lactamases were coined extended-spectrum β–lactamases (ESBLs).

TEM-1 is able to hydrolyze ampicillin at a greater rate than carbenicillin, oxacillin, or cephalothin, and has negligible activity against extended-spectrum cephalosporins. It is inhibited by clavulanic acid. TEM-2 has the same hydrolytic profile as TEM-1, but differs from TEM-1 by having a more active native promoter and by a difference in isoelectric point (5.6 compared to 5.4). TEM-13 also has a similar hydrolytic profile to TEM-1 and TEM-2 [7]. Well over 200 TEM-type β -lactamases have been described, of which the majority are ESBLs. Their isoelectric points range from 5.2 to 6.5. The amino acid changes in comparison with TEM-1 or TEM-2 are documented at http://www.lahey.org/Studies/temtable.asp.

Previously other studies were conducted to detect and characterize TEM and other ESBLs genes in Sudan [23,26]. But in this study we did DNA sequencing to know TEM gene type, and also we aimed to identify novel TEM genes types by using Insilco analysis tools.

2. Materials & Methods

2.1. Bacterial Isolates

An *E. coli* bacterium isolate was recovered from urine specimen of 45 years old female, from Gizera state that located in the central of Sudan, at May 2013, during a study to detect and characterize ESBLs genes in this area, this organism was show high hydrolytic activity to cefotaxime, and was positive for TEM gene and negative for CTX-M and SHV genes by PCR. So was selected for DNA sequencing. Chromogenic agar media (Liofilchem Co. Italy) and standard biochemical tests [8], were used in the isolation and identification of bacteria. *E. coli* ATCC 25922 and *Klebsiella pneumoniae* ATCC 700603 were used as controls.

2.2. Antibiotic Susceptibility Testing

ESBL screening and confirmation along with antimicrobial susceptibility test was done according to the Clinical Laboratory Standards Institute (CLSI) guidelines [9] (CLSI, 2012). We used antibiotic disks containing Ceftazidime (CAZ: 30 µg), Cefotaxime (CTX: 30 µg), Cefepime (CTX: 30 µg), Amoxyclav (AMC: 30 µg), Gentamicin (GM: 10 µg), Meropenem (MRP: 10 µg), ofloxacin (OF: 5µg), Tetracycline (TE: 10 µg), Amikacin (AK: 30µg), Chloramphnicol (C: 30µg) and Ciprofloxacin (CIP: 5 µg). An inhibition zone size of ≤ 22 mm with Ceftazidime, ≤ 27 mm with Cefotaxime and ≤ 18 mm with Cefepime were considered as potential ESBL producer.

2.3. Phenotypic detection of ESBLs

The ESBLs phenotype of the clinical isolate was confirmed by using ceftazidime (30µg), ceftazidime (30µg) plus clavulanic acid (10µg), cefotaxime (30µg), cefotaxime (30 µg) plus clavulanic acid (10µg) and cefepime (30 µg), cefepime (30 µg) plus clavulanic acid (10µg), (Liofilchem Co. Italy). Phenotypic detection of ESBLs was defined by a difference of ≥ 5 mm between the disk that contain clavulanic acid and that without clavulanic acid [9,10].

2.4. Genotypic detection of ESBLs

The DNA was extracted by guanidine chloride method as described by Alsadig *et al*., [11]. The PCR was carried out using thermal cycler (CONVERGYS® td peltier thermal cycle, Germany), the following primers (Metabion, GERMANY) were used, for *bla* TEM 971bp; TEM-F 5 ' TCG GGG AAA TGT GCG CG 3' and TEM-R 5 ' TGC TTA ATC AGT GAG GCA CC 3', for *bla* SHV 797 bp; SHV-F 5-GGTTATGCGTTATATTCGCC-3, SHV-R 5-TTAGCGTTGCCAGTGCTC-3 and for *bla* CTX-M 550 bp; CTX-M-F SCS 5-ATG TGC AGY ACC AGT AA-3, CTX-M-R 5-CCG CRA TAT GRT TGG TGG TG-3, in a total reaction volume of 25 µl (5µl Master mix of Maxime RT premix kit (iNtRON BIOTECHNOLOGY, Seongnam, Korea), 0.6 µl of forward primer, 0.6 µl of reverse primer, 2µl DNA and 16.8 µl deionized sterile water). Then the PCR mixture was subjected to initial denaturation step at 94°C for 5-min, followed by 30 cycles of denaturation at 94°C for 45 seconds, primer annealing at 57°C for 45 seconds, followed by a step of elongation at 72°C for 60 seconds, the final elongation was at 72°C for 5 min [12]. PCR products was analyzed by electrophoresis in a 2% agarose gel in TBE 1X that contain 2.5 µl of (20mg/ml) ethidium bromide at 100V for 40 min. Bands were visualized under U.V transilluminater (Uvite –UK).

2.5. Sequencing of TEM Gene

DNA purification and standard sequencing was performed for both strands of TEM genes by Macrogen Company (Seoul, Korea).

2.6. Bioinformatics Analysis

The sequences chromatogram was viewed by FinchTV program, (http://www.geospiza.com/Products/finchtv.shtml). Then the nucleotides sequences of the TEM beta-lactamases gene were searched for sequences similarity using nucleotide BLAST (http: //blast.ncbi.nlm.nih.gov/Blast.cgi.) [13]. Highly similar sequences were retrieved from NCBI and subjected to multiple sequence alignment using BioEdit software [14]. In GeneMarkS version 4.25 (http://exon.gatech.edu/genemark/genemarks.cgi), the gene sequences were translated into amino acid sequence [15]. Protein sequences similarity were searched with BLASTP (http://blast.ncbi.nlm.nih.gov/ Blast.cgi CMD =Web&PAGE_TYPEBlastDocs), highly similar sequences were achieved from NCBI and subjected to multiple sequence alignment and evolutionary analysis using BioEdit software. Phylogenetic tree of TEM beta-lactamase gene and their evolutionary relationship with those obtained from database was done online by Clustal W2 http://www.ebi.ac.uk/Tools/msa/clustalw2/) [16]. The secondary structures of predicted amino acids were carried out by phyre2 [17]. Chimera version 1.9 software was used to predict the tertiary model of protein [18]. Mutation analysis was done online by project hope software (http://www.cmbi.ru.nl/hope/report/2064?10) [19], and Expasy (http://web.expasy.org/compute_pi/) [20].

3. Results

3.1. Phenotypic Detection of ESBLs

After the initial CLSI screening tests, the isolate was found resistant to cefotaxime, amoxyclav, tetracycline, nalidixic acid, chloramphnicol and ciprofloxacin, and sensitive to meropenem, cefepime, ceftazidime and amikacin, see Table 1. Then ESBLs production was confirmed by combination disc diffusion test, cefotaxime/cefotaxime +clavulanic acid gave a positive result (showed a difference of > 5 mm), while ceftazidime/ceftazidime+clavulanic and cefepime/cefepime+clavulanic were negative (showed a difference of < 5 mm), as presented in Figure 1.

Table 1. The results of screening tests using different antibiotics

Isolate	Antibiotics inhibition zone diameter (mm)										
	CAZ	CTX	NA	C	MRP	CPM	AMC	CIP	AK	TE	OF
E. coli	30	12	8	8	30	34	0	10	20	0	0

Abbreviations: CAZ= ceftazidime, CTX= cefotaxime, NA= nalidixic acid, C= chloramphnicol, MRP= meropenem, CPM= cefepime, AMC= amoxiclave, CIP= ciprofloxacin, AK= amikacin, TE= tetracycline, OF= ofloxacin.

Figure 1. Phenotypic confirmatory test with CAZ= ceftazidime, CAL= ceftazidime+clavulanic acid, CTX= cefotaxime, CTL= cefotaxime+clavulanic acid, CPM= cefepime, FEL= cefepime+clavulanic acid, on Muller Hinton agar

3.2. Genotypic Detection of ESBLs

The selected isolate showed a band typical in size to TEM beta-lactamase gene (971bp), see Figure 2A, and was negative for both CTX-M (550bp) and SHV (797bp) beta-lactamase gene.

3.3. Bioinformatics Analysis

Analysis of TEM gene sequences was done to determine their relationship to other TEM gene sequences available in Gen-Bank database using BLAST nucleotide algorithm (http://www.ncbi.nlm.nih.gov/). BLAST analysis of TEM gene (971-bp) showed 99% identity to nucleotide sequence of TEM-1 of different bacterial species, that obtained from Gen-Bank database; *E. coli* TEM-1 from China gb: AFI61435.1|, *S. enterica* TEM-1 from Island gb|AB571794.1, TEM-1B from Malaysia gb|HQ625489.1, *L. adecarboxylata* TEM-1 from China gb|JF910132.1 and *K. pneumoniae* TEM-1 from China gb|KF268357.1). Our mutant TEM gene was different by an inserted G at position 880 and deleted G at position 931 see Figure 2B. This mutation resulted in a substitution of amino acid Aspartic acid to Threonine (D-T) at position 262, when compared to TEM-1 gene (gb: AFI61435.1|) and TEM-169 (gb|AFI61433.1|) as presented in Figure 3.

3.4. Multiple Sequence Alignment

The multiple sequence alignment of the mutant isolate with similar nucleotide sequences that obtained from BLASTn was carried out to find the homology and evolutionary relation between these sequences. As shown by BioEdit software there is an inserted and deleted G and at very conserved region, see Figure 2C.

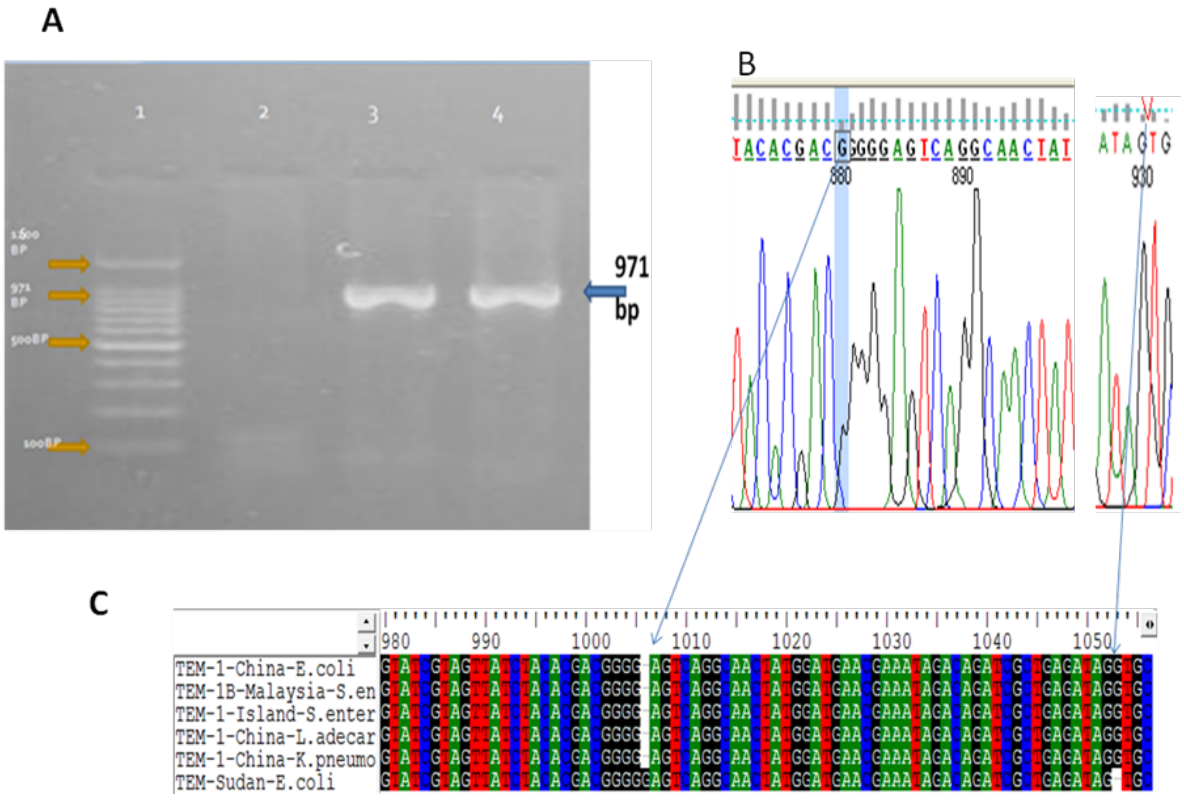

Figure 2. A. PCR amplification of TEM gene of the E. coli isolate on 2% agarose gel electrophoresis. Lane 1 DNA ladder: MW 100-1500bp. Lane 2 control negative. Lane, 3 control positive Lane 4 showing typical band size of (971bp) corresponding to the molecular size of TEM gene. B. Mutant TEM gene sequence chromatogram, shown by FinchTV software. C. BioEdit multiple sequence alignment. The inserted and deleted G in Sudan isolate illustrated by the arrows

3.5. Protein Secondary and Tertiary Structure

The prediction of secondary protein structure of wild and mutant TEM genes revealed that the wild type contain 46% alpha helix, 14% Beta strand and 11% Disordered regions, while the mutant gene contain 45%, Alpha helix, 15% Beta strand and 12% disordered regions as presented in Figure 4 A1, A2. The prediction of 3D structure of the wild and mutant TEM genes was done by Chimera software version 1.9 as presented in Figure 4 B1, B2.

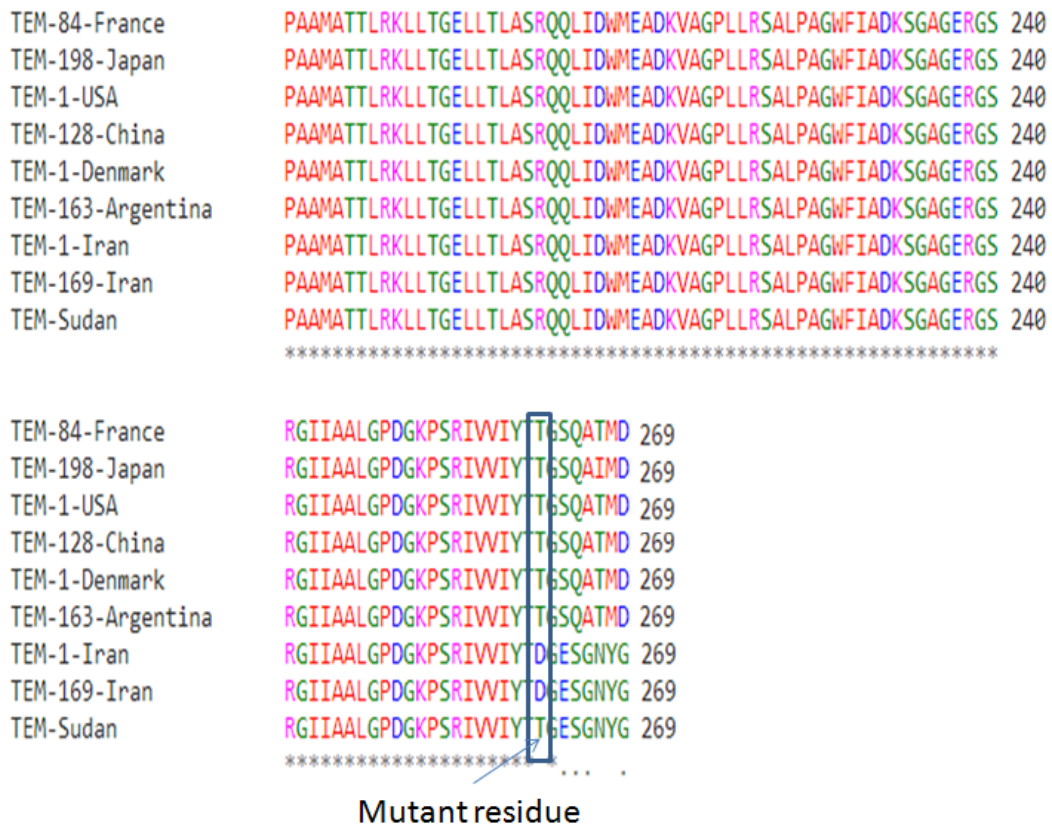

Figure 3. Amino acid multiple sequence alignment of mutant TEM-Sudan beta-lactamase gene compared to other TEM gene from database, the mutant Threonine (T) at position 262 shown by an arrow. The alignment was performed using the Clustal W2 sequence alignment

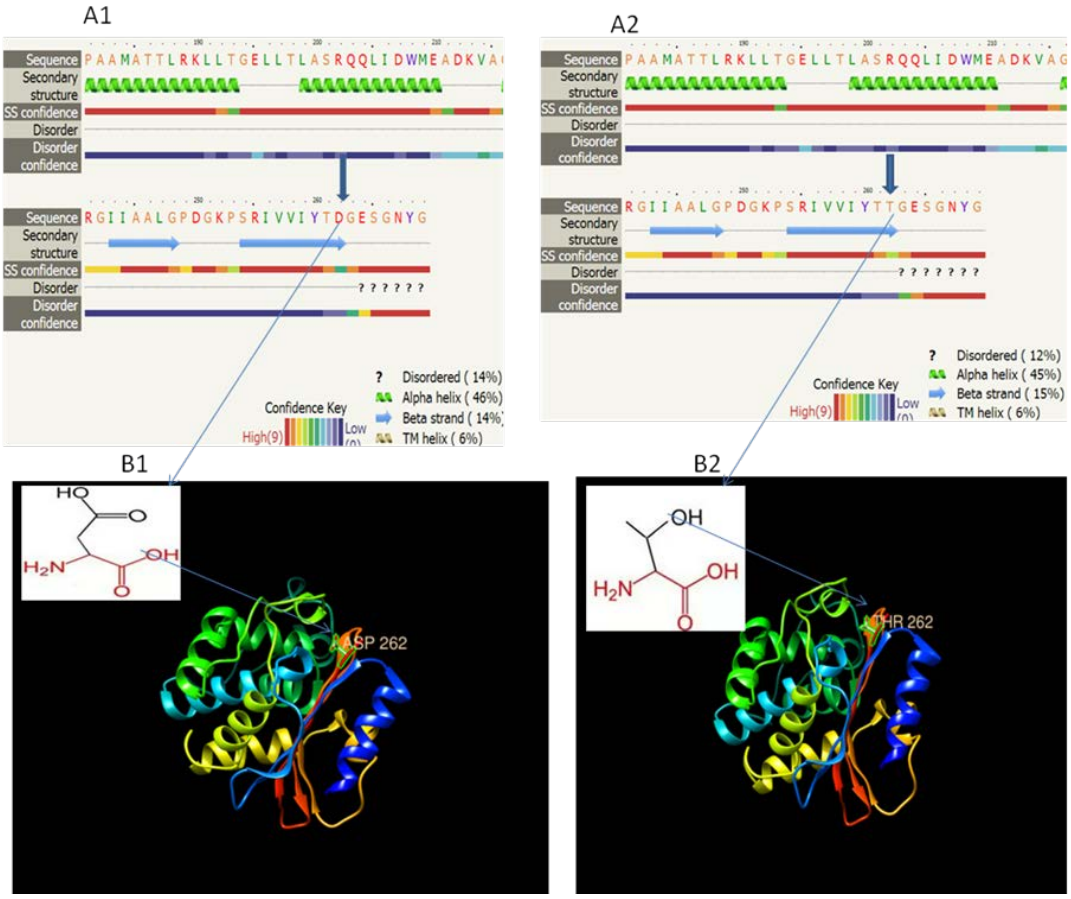

Figure 4. Left: wild type (A1, B1)of predicted amino acid Aspartic acid (ASP) at position 262 of TEM-1 gene (gb: AFI61435.1). Right: mutant type (A2, B2) of predicted amino acid Threonine (THR) at position 262. A1, A2. The secondary protein structure of wild and mutant gene that drawn by Phyre2 software. B1, B2. Tertiary protein structure of wild and mutant genes that drawn by Chimera software version 1.9

Table 2. A comparison of wild type and mutant type residues of TEM genes

Feature	Wild residue	Mutant residue
Size	Normal	Smaller than wild-type residue
Charge	Negatively charged	Neutral
Hydrophobicity-value	Hydrophobic	More hydrophobic
Structure	Located in its preferred secondary structure	Prefers to be in another secondary structure

3.6. Expasy and Project Hope

The theoretical isoelectric point of wild type gene was pI/Mw: 5.32 / 29499.79 and for mutant type was pI/Mw: 5.44 / 29485.81. Project Hope software revealed many differences between wild and mutant residues as shown in Table 2.

3.7. Phylogenetic Tree

The drawn phylogenetic tree revealed that our mutant TEM gene is so closed to Iranian TEM-1 and TEM-169, see Figure 5.

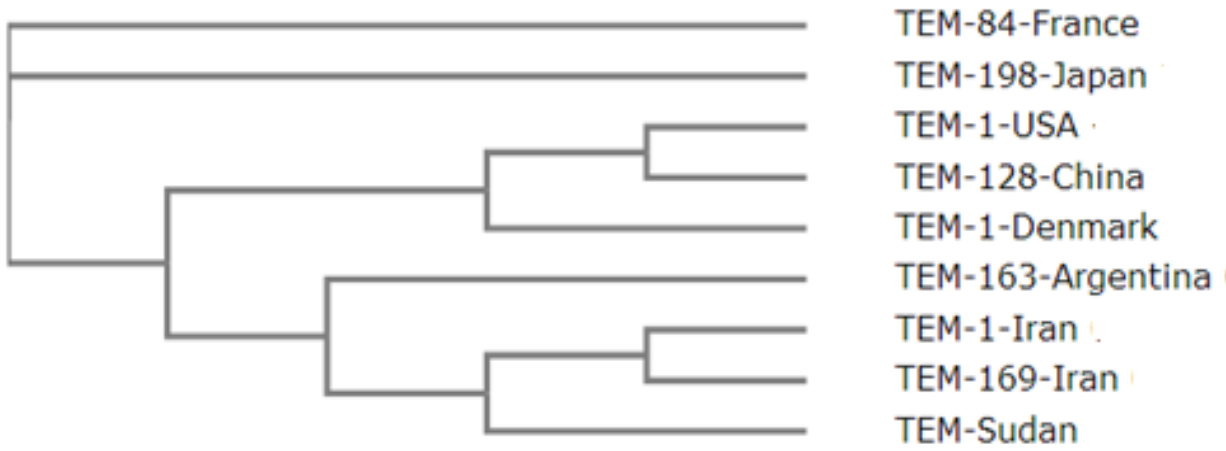

Figure 5. Phylogenetic tree of mutant Sudan TEM beta-lactamase gene and other TEM genes that obtained from database

4. Discussions

The reports of novel *bla*TEM enzymes that have both an extended spectrum of activity and resistance to beta-lactamase inhibitors suggest that the TEM-type enzymes are continuing to evolve in the face of current therapies [21,22]. The rapid identification of an ESBL gene would result in the initiation of the appropriate therapeutic intervention sooner and may result in fewer clinical failures. In this study we isolate an *E. coli* bacterium that produce TEM gene, from a Sudanese patient, like many other studies detect and characterize ESBLs genes in Sudan [23,24,25,26]. This isolate showed a high hydrolytic activity against cefotaxime than ceftazidime even its negative for CTX-M gene, this agree with that some TEM genes have an enhanced activity against cefotaxime like TEM-3 [27]. In addition to cefotaxime also this isolate resistant to ciprofloxacin, tetracycline, amoxyclav, ofloxacin chloramphnicol and nalidixic acid, this may indicate that the plasmid that carry the ESBL gene may carry other resistant genes, like for quinolones and aminoglycoside [28].

The BLASTn searching and BioEdit multiple sequence alignment revealed an inserted G at position 880 and deleted G at position 931, at very conserved region. The translation of amino acid sequences revealed a substitution of Aspartic acid in TEM-1 (gb: AFI61435.1) from Iran to Threonine in our mutant TEM gene at position 262. This mutation may be the cause of ESBL activity, because this mutation located at beta-lactamase/transpeptidase-like domain which is the penicillin-binding domain of PBPs, that function as DD-transpeptidases, its catalyze the final step of cell wall biosynthesis by cross-linking two strands of peptidoglycan, or DD-peptidases [29]. This agrees with that many ESBLs TEM genes evolved from one or more amino acid substitution around the active site [30]. This mutation increased protein isoelectric point from pI; 5.32 to pI; 5.44, this like in TEM-2 the first derivative of TEM-1, had a single amino acid substitution from the original β-lactamase, that is cause a shift in the isoelectric point from a pI of 5.4 to 5.6 [31].

The secondary and tertiary structure of wild and mutant type proteins also changed, the percentage of alpha and beta helixes was changed in secondary protein structure, the shape and ionic interaction also changed in tertiary protein structure. These changes may affect protein functions and folds. The affection of insertions, duplications, and deletions of sequence contribute to the structural and functional diversity of proteins has been relatively well studied in model proteins [32]. The mutant residue was found completely different from the wild type residue; in 3D-structure wild-type residue is located in its preferred secondary structure, while mutant residue prefers to be in another secondary structure, therefore the local conformation will be slightly destabilized. The wild-type residue is not conserved at this position, while the mutant residue was not among the residue types observed at this position in other, homologous sequences which might indicate that the mutation is possibly damaging to the protein because the mutation occurred near a highly conserved position. There is a difference in charge between the wild-type and mutant amino acid, the charge of the wild-type residue is lost by this mutation, this can cause loss of interactions with other molecules. The wild-type and mutant amino acids differ in size, the mutant residue is smaller than the wild-type residue, and this will

cause a possible loss of external interactions. The hydrophobicity of the wild-type and mutant residue also different. The phylogenetic tree revealed that the possible source of our new gene is Iran, this may be due that Iran is the nearest country to Sudan among these countries.

5. Conclusions

We conclude that a novel mutant TEM gene with ESBLs activity is been detected in Sudan, this is new gene is completely different from TEM-1 gene in its phonotypic and genotypic characterization. ESBLs genes are rapidly evolved among pathogenic bacteria, thus study like this to detect a new antibiotic resistant gene variants could guide the choice of optimal antibiotic therapy for successful treatment, thus improving the outcomes for patients with severe bacterial infections.

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Competing Interests

The authors declare that they have no conflict of interests.

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