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Isolation and Diagnosis of Gram-Negative Bacteria Causing Urinary Tract Infection from Transfusion Dependent Thalassemia and Study of Iron Acquisition Systems in Wasit, Iraq

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ABSTRACT
Enterobacter cloacae complex, Klebsiella pneumoniae and E. coli belong to the family Enterobacteriaceae, and it is the most clinically pertinent species, also it causes both nosocomial and communities-acquired infections.Enterobacteriaceae are the main cause of these infections, but managing them has become more challenging due to the emergence of antibiotic resistance. The current study aimed to detect and diagnosed the causative agents of urinary tract infection UTI and their virulence genes for iron acquisition systems fepAandsitA genes in transfusion dependent thalassemia TDT in Wasit province Iraq 2024. One hundred seventy-three samples of urine were collected from Center of Blood Hereditary Diseases in Al-Kut Hospital from November 2023 to April 2024. Participants ranged from 6 months to 50 years. The results showed the diagnosis of 38 isolates of bacteria with an 7.9% n=3/38 prevalence ofE. cloacae complex, 13.3% n=5/38 ofKlebsiella pneumoniae and 23.7% n=9/38 for E. coli other bacteria were gram positive with prevalence 55.1% n=21/38 and the virulence genes for iron acquisition systems fepAandsitA were 64.7% n=11/17and 94.1% n=16/17 respectively

Keywords: Blood, system, dependent, pneumoniae

INTRODUCTION

The word "thalassemia" is derived based on the Greek "-Thalassa," which means "the sea," (it refers to the Mediterranean Sea), and "-Emia," which means "related to blood,"(Abood Yasir OKAB & B SALIH, 2020). Erythrocyte precursors, inefficient erythropoiesis in the bone marrow, hypochromia, and microcytosis of circulating red blood corpuscles are the hallmarks of thalassemia syndrome (Kumar, 2019).Once there is insufficient haemoglobin in red blood corpuscles, oxygen cannot reach all areas of the body, and organs become oxygen-starved and have no ability to operate correctly (Prathyusha et al., 2019).

Thalassemia is linked to several consequences, such as frequent infections. Numerous studies have shown a significant occurrence of infections in patients with thalassemia, accounting for approximately 10% of cases (Obed et al., 2024b). Bacterial infection is a prevalent cause of mortality in individuals with thalassemia (Joudah & Hamim, 2023). Infections are the second most frequent cause of death and the primary cause of illness in patients with transfusion-dependent thalassemia (Rathnayake et al., 2019).

Urinary tract infections occur when a uropathogenic microorganism reaches the urinary tract, evades the immune system, and begins to colonize and infect the host, resulting in harm to the host (Abell-King et al., 2022).

The factors contributing to the heightened vulnerability to infection in these individuals encompass: Possible causes of anemia include dysfunction of the reticuloendothelial system due to iron and hemolyzed erythroblasts, iron excess, and altered immunological response. Other potential reasons include splenectomy, transfusion-related infections, and iron chelation (Obed et al., 2024b; Teawtrakul et al., 2015).

The aim of thisresearch to detect and diagnosed gram negative bacteria and their virulence gene for iron acquisition systems causative agents of urinary tract infections in transfusion dependent thalassemia.

MATERIALS AND METHODS

Study design

This study was structured as a case-control investigation involving a minimum of 100 samples, which were gathered from November 2023 to April 2024. Participants ranged from 6 months to 50 years, with mean age among the patients (1.473+-0.687) In the Alkut hospitals, specifically the Thalassemia Center in Wasit and Al-Numaniyah General Hospital located in Wasit province, Iraq.

Inclusion Criteria of the Patients

Patients of both genders, ranging from 6 months to 50 years, participated in the study. They were diagnosed with urinary tract infections based on clinical signs and confirmed through urine sample analysis. All the patients were transfusion dependent thalassemic patients and control group was UTI patient free of chronic illness. Verbal consent was obtained directly from the patients or patient's relative for participation in the study.

Exclusion Criteria of the Patients

Patients under 6 months and over 50 years, those who were not dependent on transfusions for thalassemia, individuals with other significant chronic medical conditions, and patients with other hemoglobinopathy excluded from the study.

Inclusion Criteria of the Control Group

Participants included individuals aged 6 months to 50 years, all of whom were free from any acute or chronic illnesses. There is no known family history of thalassemia. Verbal consent was obtained for the collection of urine samples and participation in the study.

Exclusion Criteria of Control Group

Individuals who are younger than 6 months and older than 50 years. Individuals with recent acute and chronic medical conditions, as well as a family history of chronic medical issues.

The Specimens Collection

The Urine Specimens Collection

A sterile container was used to collect approximately 15 mL of midstream urine . Utilizing a sterile standard loop (0.001ml) for the culturing of urine samples. All urine samples were cultured using MacConkey agar, Blood Agar, CHROMagar medium andCysteine–Lactose–Electrolyte-Deficient (C.E.L.D) agar. The culture media were incubated under aerobic conditions at 37°C for a duration of 24 hours. Conventional biochemical assays, the MA120 Automated ID&AST System, and the VITEK2 system (ID-GN, ID-GP) were employed to validate the diagnosis, along with Determination (AST-GN, and AST-GP). via the clean catch method for microscopic examination and bacterial culture.The isolations were transported in a container with ice to the laboratory at the College of Medicine in Wasit University for confirmation of all the isolations through 16s rRNA analysis and partial 16s rRNA sequencing, as well as for the detection of the bacteria's ability to produce certain virulence factors using PCR.

Molecular Study kits

PCR Bacterial DNA Extraction

The PCR diagnostic kits utilized in this study Presto™ Mini gDNA Bacteria Kit (gram positive and gram negative bacteria) Taiwan

Polymerase Chain Reaction kit

GoTaq® Green PCR master Mix Promega USA is a pre-prepared formulation including premium Taq DNA Polymerase, deoxynucleotides, and reaction buffer at a 2X concentration. It includes all essential reagents for DNA amplification. The GoTaq® G2 Green Master Mix includes an inactive green dye and a stabilizer, facilitating the direct loading of the end product onto a gel for examination.

Polymerase Chain Reaction Primers

In the following tables (2-5) and (2-7) the lists of genes and primers sequence that used in this study.

Table 2-5: List of Primers used in this study A: Universal Primers for gene 16S rRNA

Target gene		Sequence (5'-3')	Product size	Ta (°C)	Reference
16S rRNA	**F**	**AACTGGAGGAAGGTGGGGAT**	**372 bp**	**54**	(Greisen et al., 1994)
	R	**AGGAGGTGATCCAGCCGCA**			

Table 2-6: List of Primers used in this study B: Iron acquisition virulence factors genes

Gene	Sequence 5'-3'		Product size	Ta (°C)	Accession number	Reference
fepA	**F**	CGACGTCTCGGAGATCATT	**94 bp**	**54**	AJ292298.1	(S. et al., 2007)
	R	GATATCAATCTGGCGGTTGTT				
	R	CGTCGGGAACGGGTAGAATCG				
sitA	**F**	CGCAGGGGCACAACTGAT	**658 bp**	**54**	CP022609.1	(Sabri et al., 2006)
	R	CCCTGTACCAGCGTACTGG				
	R	TTAGTGAATTTGTTCACTGTGTCGA				

2.2.9.6. PCR conditions

Phase	Tm (°C)	Time	Cycles	Reference
Initial denaturation	94°C	5 min	1X	This study after optimization
Denaturation	94°C	30 sec.	35X	
Annealing	54°C	30 sec.		
Extension	72°C	1 min		
Final extension	72°C	5 min	1X	

RESULTS AND DISCUSSION

3.1.1. Results of Bacterial Culture on Artificial media.

The initial identification of bacterial isolations was conducted using bacteriological methods, such as the Gram stain. The characteristics of Gram stain isolations were subjected to biochemical tests for identification. A Gram stain was performed on all positive cultures, encompassing both Gram positive and Gram-negative bacteria. The identification of all bacterial species was achieved through biochemical tests, both manual and automated. Urine samples were cultured overnight on Blood agar, MacConkey agar, and CLED agar in an incubator set at 37°C, followed by microscopic examination at a magnification of 100X. Subcultures were performed on Blood and Mannitol salt agar, Eosin Methylene Blue (EMB), and HiCrome™ UTI Agar (Chromogenic Agar).

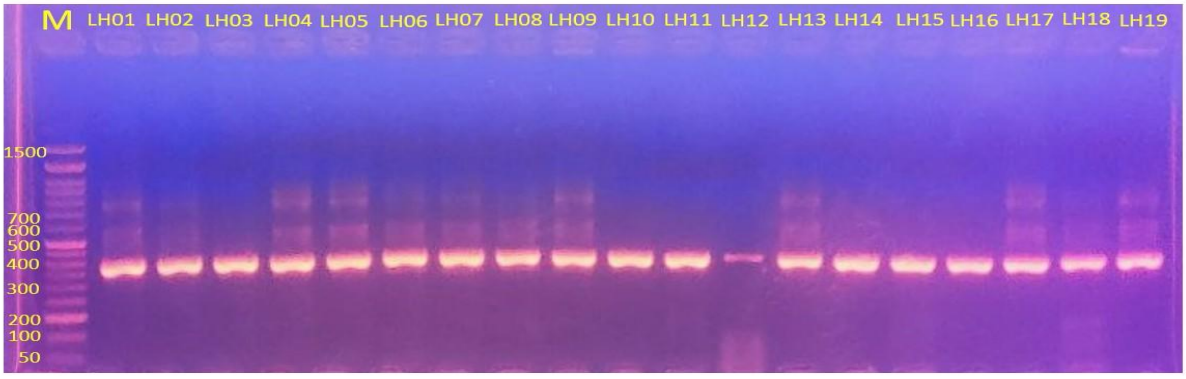
In this study It were observed only (21.96% n=38) were positive for bacterial culture, this result is similar to (Obed et al., 2024) they have same positive growth percentage of UTI sample was 20% n=12/50.

Gram positive bacteria represent 55.3% n=21/38 and gram negative bacteria represent 44.7% n=17/38 our results agreed with (Al Badry et al., 2016) since their result showed gram positive bacteria Staphylococcus genus has the highest prevalence 38.88% n=7/18 while other gram negative bacteria showed less prevalences that's my due to the iron overload in TDT so the Gram-positive bacteria thrive in this patients and to their compromised immune system.

Molecular detection of 16s rRNA of pathogen isolates

In this study depending on Vitek2 and MA120 techniques we used a specific primer (listed in Table (2-7) in chapter two) used to identify bacterial species that were diagnosed by previous mentioned techniques. Some of isolated bacteria didn't show any band in agarose gel electrophoresis, so the idea took a shape of misdiagnosis of bacteria by early mentioned techniques. For that reason, we used universal 16s rRNA primer (Table (2-5) in chapter two), and confirmatory partial sequencing for certain diagnosis of the all isolates.

All isolates (thalassemic patient n=38 and control group n=20) showed positive band with size 372bp in 1.5% agarose gel as showed below in Fig (3-1) other isolates figures are in appendix.



Fig(3-1):PCR products of the amplification of partial region of gene 16s rRNA of Bacteria

The size of the PCR product is 372bp. The gel was 1.5% and the DNA dye is RedSafe (Intron, Korea). V: 90, Time: 45 minutes. M: DNA ladder
The frequency and distribution of bacterial species for the thalassemic patients according to the results of PCR and 16s rRNA partial sequencing as showed in the chart below in table (3-xx) and Fig (3-2).

Table 3-xx: Bacterial distribution for thalassemic patients

Thalassemia		
Item	**Frequency**	**Percentage**
Gram positive speciese		
Enterococcus faecalis	3	7.9%
Enterococcus faecium	1	2.6%
Staphylococcus epidermidis	3	7.9%
Staphylococcus aureus	1	2.6%
Staphylococcus hemolyticus	5	13.2%
Staphylococcus saprophyticus	4	10.5%
Staphylococcus sciuri	4	10.5%
Gram negative speciese		
Escherichia coli	9	23.7%
Enterobacter cloacae	3	7.9%
Klebsiella pneumoniae	5	13.2%

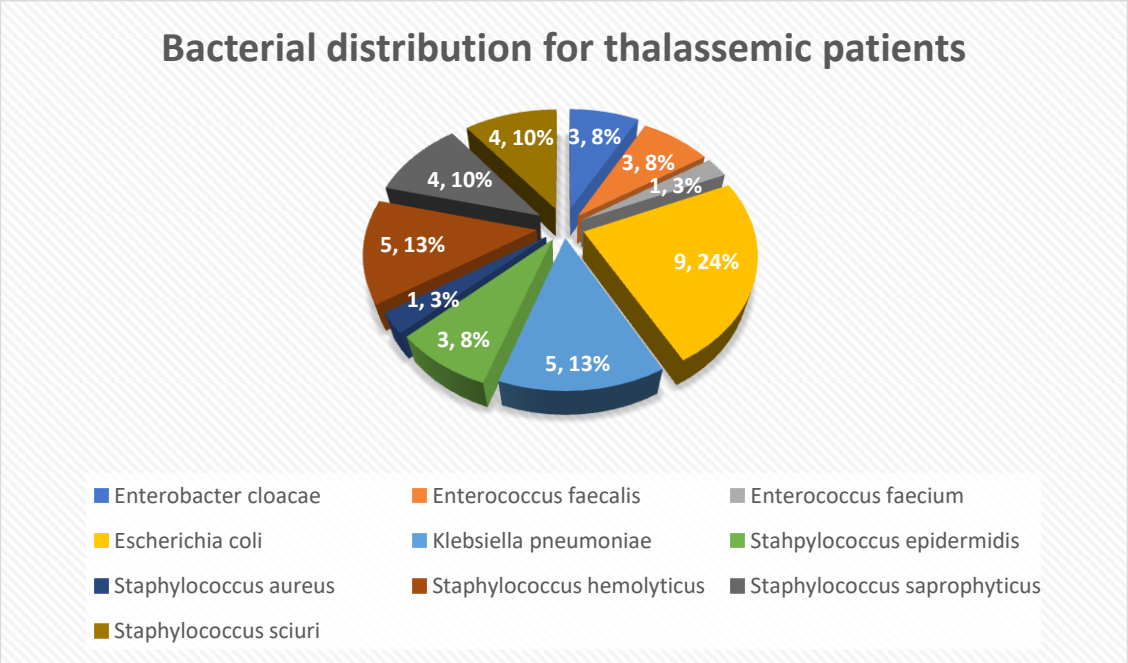


Fig 3-2: Bacterial distribution for thalassemic patients

Molecular detection of virulence factors genes
All isolates of TDT and 20 isolates of predominant bacteria of control group were molecularly diagnosed for virulence factors of iron acquisition systems, the genes were enterobactin (**febA)**andiron transport protein **(sitA)** for gram negative bacteria.The highest prevalent genes in gram negative bacteria in TDT was sitA 94.1% n=16/17. The lowest prevalence was fepA 64.7% n=11/17.
The control group isolates were all gram-negative bacteria since it the predominant bacteria. The highest prevalence was also for sitA those showed higher prevalence than TDT with 100% n=20/20. The lowest prevalence was also for fepA gene 35% n=15/20. The table (3-xx) show the details of gene prevalence

Table 3-xx: Prevalnce of fepAand sitA genes**.**

Genes	Study groups					
	TDT n=17			Control n=20		
	p	N	percentage	p	N	percentage
fepA	11	6	64.7%	15	5	75%
sitA	16	1	94.1%	20	0	100%

Statistical analysis of virulence genes

Item		Control	Thalassemia	P-Valeu
fepA	Negative	5	6	0.495
	positive	15	11	
sitA	Negative	0	1	0.272
	positive	20	16	

Enterobactinreceptorgene (**febA)**

The ferric enterobactin receptor (fepA) serves as a multifunctional outer membrane protein, identified across various genera within the Enterobacteriaceae family(Page, 2019). It is important to highlight that fepA encodes the binding protein(receptor) for iron-enterobactin complexes, thus playing a crucial role in the process of iron acquisition(Flores et al., 2024). The gene fepA was found in multiple copies in K. pneumonia 3 in chromosome and one carried on plasmid(Kumar et al., 2019). The figure (3-xx) showed the fepA bands in agarose gel detected by conventional PCR in some isolates. In this study fepA represented in 64.7% n=11/17 (Fig (3-xx) showed the the prevalence of fepA gene in TDT isolates) from TDT isolated gram negative bacteria, E. cloacaefepA prevalence was 33.33% n=1/3 this results disagreed with (Mosaffa et al., 2024) there study conducted in Kerman-Iran they found FepA prevalence was 60% n=9/15 and (Ghanavati et al., 2018) their study conducted in Iran also they found fepA represent 50.9% n=29/57 for E. cloacae, although fepA is represent the lowest prevalence of all Enterobacter spp., this low prevalence and our small specimens number may explain the discrepancy in the result of this study and them study. E. colifepA prevalence was 55.56% n=5/9 this result contradictory to (Krawczyk et al., 2023) in their study conducted in Poland the fepA show high prevalence 96% n=82/83 this difference may due to the environmental and phylogenetical reasons of bacteria in their study. and K. pneumoniaefepA prevalence was 100% n=5/5 this result is similar to (Daoud et al., 2022) their study conducted in United Arabic Emarat they found fepA prevalence 100% n=38/38 of UTI specimens. The control group in this study represented by two predominant bacteria E. coli and K. pneumoniae, E. coli shows higher prevalence than study group for fepA was 75% n=9/12 this results almost near to(Krawczyk et al., 2023) previously mentioned, but K. pneumonia showed lower prevalence of fepA than study group was 75% n=6/8. statistically non-significant differences were found in the distribution of fepA between TDT and control group (p=0.495).

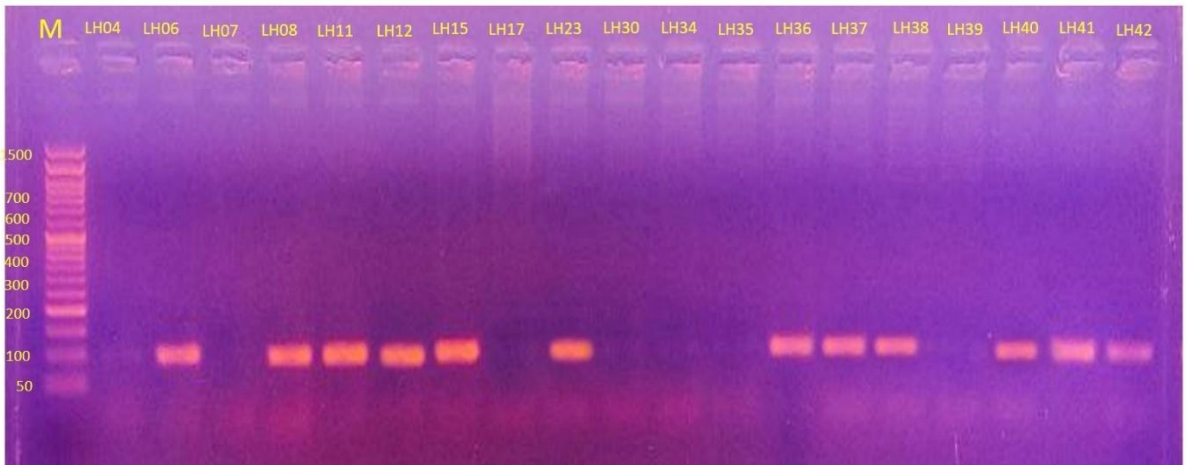


Fig 3-xx: Bands of PCR products of the amplification of partial region of gene fepA the size of the PCR product is 94 bp. The gel was 1.5% and the DNA dye is RedSafe. Tracks from LH04 to LH30 for TDT isolates and from LH34 to LH42 for control group.

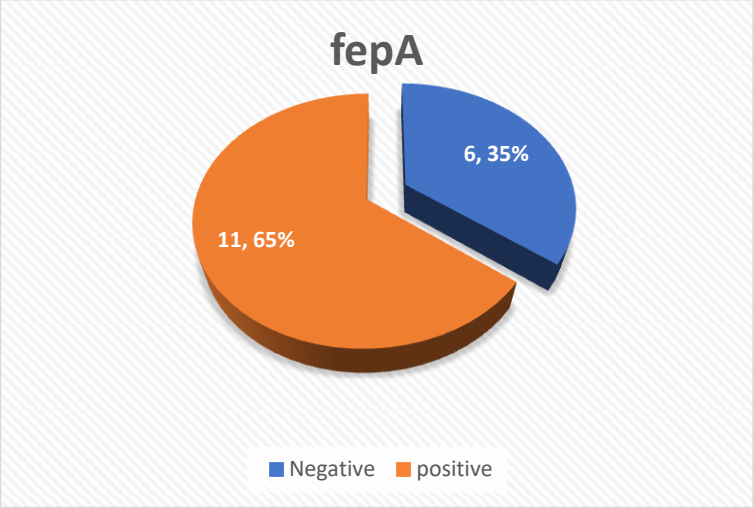


Fig 3-xx: Prevalence of fepA gene in TDT isolates

Iron transport protein (sitA):

The iron acquisition mechanisms employed under low-iron circumstances are regulated by the sitA gene(Kocúreková et al., 2021). SitABCD constitutes an efficient ferrous iron absorption mechanism previously characterized in Salmonella enterica and Shigella. SitA is a periplasmic binding protein(Monárrez et al., 2019). Is among the members of the periplasmic binding protein-dependent ATP-binding cassette (ABC) family of metal transporters(Shea et al., 2024). Previous phylogenetic analyses of the sit operon from E. coli and Shigella flexneri indicated that they were likely acquired through multiple distinct genetic events involving horizontal gene transfer(Lozica et al., 2022). The figure (3-xx) showed the sitA bands in agarose gel detected by conventional PCR in some isolates the. In this study sitA prevalence was 94.1% n=16/17 in TDT gram negative isolated bacteria as showed in Fig (3-xx) below. The lowest prevalence of sitA in this study was in E. cloacae it was 66.67% n=2/3 there is no previous study showed the presence of sitA in E. cloacae and we investigate about it because sitA could transmitted horizontally by plasmid.The highest prevalence was in appeared equally in E. coli and K. pneumoniae both showed 100% n=9/9 and n=5/5 respectively these results concerning E. coli agreed with (Li et al., 2020) in their study conducted in Australia were they found sitA represented in 91% n=61/67 of specimens of women with UTI, slight differences in the prevalence of sitA was found in study of (Zeng et al., 2021) in their study that conducted in Shanghai-China where they found sitA represents 82% n=124/151 of UTI isolates. The sitA gene considered as pathogenicity island marker (Royer et al., 2022; Zeng et al., 2021) depending on this criteria all our isolates of E. coli from TDT considered pathogenic strains. In concern of K. pneumoniae we couldn't find any similar studies that investigated about sitA gene and we investigate about it because sitA could transmitted horizontally by plasmid. statistically non-significant differences were found in the distribution of sitA between TDT and control group (p= 0.272). control group of this study showed similar results of sitA prevalence both E. coli and K. pneumoniae exhibit 100% presence of sitA in all isolates.

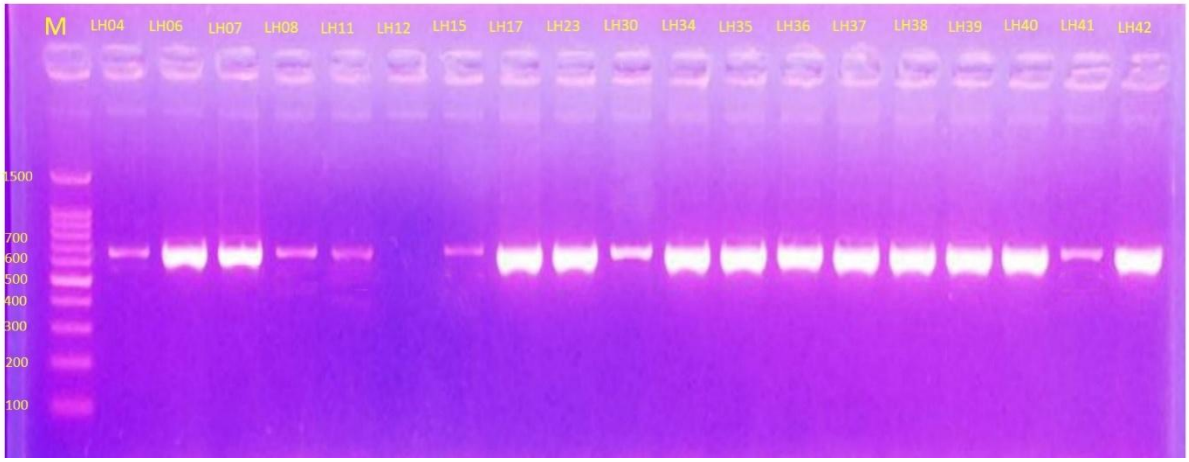


Fig 3-xx: Bands of PCR products of the amplification of partial region of gene sitA the size of the PCR product is 658bp. The gel was 1.5% and the DNA dye is RedSafe. Tracks from LH04 to LH30 for TDT isolates and from LH34 to LH42 for control group.

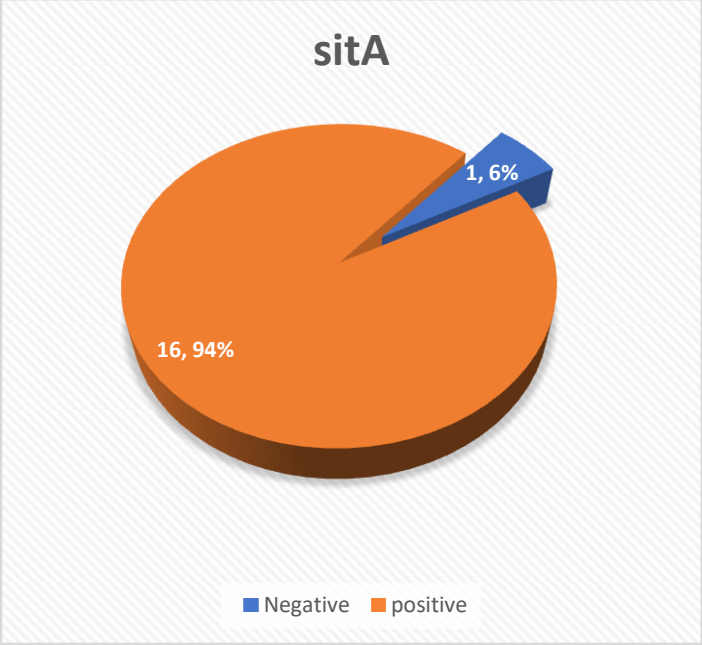


Fig 3-xx: showed the prevalence of sitA gene in TDT isolates

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