

**DETECTION OF VIRULENCE GENES(EAE,BLACMY-2,BOX) IN
ESCHERICHIA COLI ISOLATES FROM BETA-THALASEMIC
AND NON THALASEMIC PATIENTS BY USING PCR TECHINQUE.**

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ABSTRACT

Background: Thalassemia is a hereditary anemia resulting from defects in hemoglobin production. Beta thalassemia, caused by a decrease in the production of β -globin chains, affects multiple organs and associated with considerable morbidity and mortality. This study aimed to comparsion between thalasemic and non thalasemic by used specific primers. And identifying different microorganisms (aerobicand anaerobic) from urine and blood to detect. **Materials & methods:** The study samples consist of (41) thalasemic and (18)non thalasemic patients respectly.isolation from mid urine and (25) thalassmic and (25) non thalasemic patients respectively. Isolation

from Blood. **Results:** The main bacterial isolates were identified as47 isolates of *E.coli* (43.1%), 9 isolates (8.3%) of *Sterptococcus pneumonia.*, was 8 isolates of *Chalymidia pneumonia* (7.3 %), 3 isolates of *porteus spp* and *Klebsiella pneumonia*(11.9%). Whereas 6 isolates of *Enterobacter colaca* represented (5.5%), While *Pseudomonas aeruginosa* represente 9 (8.3 %) and lastly *Salmonella typhi* and *pseudomonas .spp* (3.7%) for each In addition, *Pantoea spp* represents (2.8%). A highly significant difference was found between thalassaemic patients and non thalasemic found virulence genes. Bacterial in urine and blood of were found to be higher in the study compared to control group and the difference was statistically highly significant ($P<0.01$). Molecular methods (PCR analysis) of virulence genes ,resistance,genotyping of *E.coli* by using primers specific to intimin(eae),cefatriaxone(blaCMY2) the PCR assay results eae (25%), blaCMY2 (83%).while (7.5%) appeared allof genes *E.coli*.finally box-pcr genotyping was 80%.

Conclusion: Patients with Beta thalassemic major had more infections severity compared to non thalassemic patients.

KEYWORDS: β -thalassemia, *Escherichia coli*.

INTRODUCTION

Beta thalassemias are a group of inherited blood disorders caused by reduced or absent synthesis of the beta chains of hemoglobin.^[1, 2] From the few studies regarding bacterial infections isolation from urine and blood among patients with bacteremia and UTI it was concluded that the prevalence and severity of this disease were higher in those patient than non thalassemic subjects.^[3] There are not genetics studies of bacteria isolation from thalassemia and non thalassemia patients.). This first study was designed to compared between thalassemic and non thalassemic patients by used specific primers.

MATERIAL AND METHODES

The study group included 109, with an age they were already diagnosed with β - thalassemia major, attending the thalassemic center in Ibn Al-Baladi Hospital for their regular checkup and blood transfusion.; Each individual was collected blood and urine samples were suffering UTI and bacteremia respectively. And DNA extraction *E. coli* isolates were grown in Luria broth (LB, Himedia, India) then at 37 °C overnight bacterial pelleted from broth and DNA extraction was done for 40 isolates.^[4,5]

PCR detection of *eae* specific gene sequence (384bp product) was performed with primers *eae* forward (-5GACCCGGCACAAGCATAAGC-3) and *eae* primer reverse (5 CCACCTGCAGCAACAAGAGG -3) in total volume 25 μ l containing 2 μ l of template DNA (50 μ g/ml), 2.5 μ l of PCR buffer (1X), 1.15 μ l of MgCl₂ (1.5mM), 0.5 μ l dNTPs(200 mM), 3 μ l of each primer (30 picomole) and 0.2 μ l of Taq polymerase(1U/reaction). (Midland / USA).

Samples were subjected to 35 PCR cycle involve the following steps: 1 cycle at 95 °C for 5 min, 40 cycles at 95 °C (denaturation), 50 °C (primer 72 °C annealing) (extension) for 45 sec. for each temperature and final extension step for min at 72 °C. The resulted after this amplification reaction condition was electrophoresis on agarose 2% for the reaction product.^[7]

PCR detection of bla-CMY2 specific gene sequence (364bp product) was performed with Primers bla-CMY2forward (/5 GGC GTG TTG GGC GGC GAT G 3\') and bla-CMY2Reverse (/5 CAG CGG AAC CGT AAT CCA 3\') in total volume 25 µl containing 2 µl of template DNA (50 µg/ml), 2.5 µl of PCR buffer (1X), 1.15 µl of MgCl₂ (1.5mM), 0.5 µl dNTPs(200 mM), 3 µl of each primer (30 picomole) and 0.2 µl of Taq polymerase(1U/reaction). (Midland / USA). Samples were subjected to 35 PCR cycle each one consists of 35 cycles include denaturation at 94 °C for 1 min., annealing of primers at 65.2 °C for 1 min. and the elongation at 72 °C for 1 min. when this reaction is complete the samples were electrophoresed on the 2% agarose gel.

Genotyping

Primer selection and preparation

Oligonucleotide primer was prepared depending on the manufacturer's instructions. The BOX primer (Alpha DNA, Canada) was provided in lyophilized form, dissolved in sterile deionized distilled water to give a final concentration of 100 picomole/µl as recommended by the provider and stored in a deep freezer until use.^[17]

5' - CTA CGG CAAGGC GAC GCT GAC G - 3

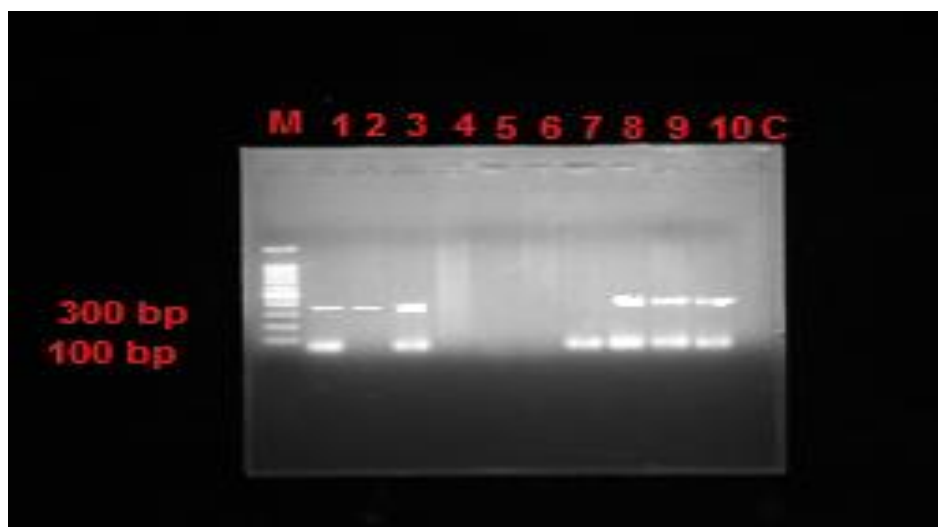
Amplification reaction

The extracted DNA, primers and Master Mix (Promega (USA)) contains: Taq DNA polymerase, MgCl₂, deoxynucleotides dNTPs, KCl, stabilizer and tracking dye and Tris-HCl (pH 9.0) was thawed at 4°C, vortexed and centrifuged briefly to bring the contents to the bottom of the tubes. Optimization of polymerase chain reaction was accomplished after several trials, PCR mixture was set up in a total volume of 50 µl included 25 µl of Master Mix, 5 µl of BOX primer (10 picomole/ µl) and 5 µl of template DNA have been used. The rest volume was completed with sterile D.W. Negative control contained all material except DNA, D.W. was added instead of template DNA. PCR reaction tubes were vortexed and finally placed into thermocycler PCR instrument. steps: 1 cycle at 95 °C for 5 min, 40 cycles at 95 °C (denaturation), ° 48 °C (annealing) (extension) for 1 min. for each temperature and final extension step for min at 72 °C. The result after this amplification reaction condition was electrophoresis on agarose 2% for the reaction product.

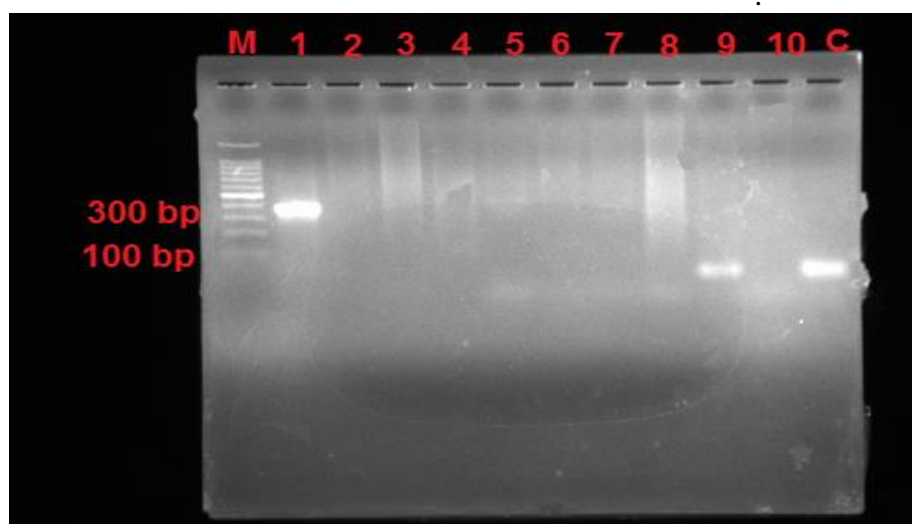
RESULTS AND DISCUSSION

41 and 18 bacterial isolates collected from thalassemic and non thalassemic patients as midstream urine samples of complicated UTIs and 25,25 blood samples were suffering

bacteremia and were identified by using cultural, morphological and biochemical tests Results showed 6 samples thelssamia (E1, E2, E3,E8,E9,E10) and non thalassemiae samples(E1,E12) gave positive results. As shown in Fig(1,2).



Figure(1): Agarose gel electrophoresis of PCR amplification products of *E.coli eae* gene from thalassemia (agarose gel 1.2 %, 60 volt, 2 hours) 1-Line M : DNA marker (100-1500bp). 2-Line C : is control negative.



Figure(2): Agarose gel electrophoresis of PCR amplification products of *E.coli eae* gene from non thalassmia (agarose gel 1.2 %, 60 volt, 2 hours) 1-Line M : DNA marker (100-1500bp). 2-Line C is control negative.

Measurement of amplified bands size according to the size of limiting band of DNA marker which found in the first line, the results explain that the bands occur between (300-500bp) the results were closed to study performed by the scientists design the primer Intimin is an outer

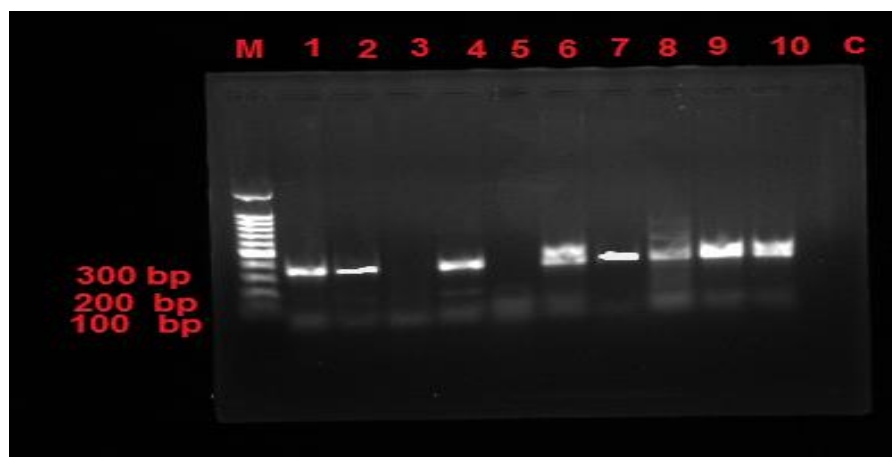
membrane protein encoded by a gene (*eae*) that is part of LEE (locus of enterocyte effacement) pathogenicity island in the chromosome, the expression of intimin associated with production of A/E lesion. It is responsible for the intimate adherence between bacteria and enterocyte membrane. Categories of *E.coli* that differs in their virulence factors have *eae* gene encode for intimin as part of pathogenicity island EPEC and EHEC. Evolutionary analysis has shown that *E.coli* strains with the virulence properties and serotypes of EPEC and EHEC they carry distinct intimin alleles. There are three variants of intimin α , β and γ intimin.^[7,8]

Table(1): Distribution of sample study in Thalassemia and healthy according to *eae* gene.

Group	Positive		Negative		Chi-square
	No.	%	No.	%	
Thalassemia (No. =20)	6	31.58	13	68.42	9.741 **
Healthy (No. =20)	2	10.00	18	90.00	

The Statistical Analysis show thalassaemic patients is high significant than healthy Patients who are splenectomised are often referred to as being immunocompromised.^[12,13] This is because splenic macrophages are responsible for the filter and phagocytosis of bacterial and blood borne pathogens. Regular blood transfusion also results in immunomodulation. hypersplenism is an absolute indication for splenectomy in thalassaemia major patients.^[14] that infections are more frequent or severe in patients with iron overload either related to genetic haemochromatosis or to transfusions, as in thalassaemiae (151,6). The bla CMY2 family of the ampC beta- lactamase genes confer broad- spectrum resistance to beta-lactam antimicrobials, including ceftriaxone and ceftiofur, as well as to beta-lactamase inhibitors such as clavulanic acid. Organisms with the blaCMY2 phenotype have been recovered from the environment and from retail meat products, posing a potential public health risk. The objectives of this study were to sequence the blaCMY2 gene from *Escherichia coli* and *Salmonella enterica* from multiple sources that had a reduced susceptibility to ceftriaxone and to determine the effect of observed mutations in the blaCMY2 gene on the antimicrobial resistance phenotype of the isolates (10). Results showed 17 samples (E1,E2,E4,E6,E7,E8,E9,E10,E11,E12,E13,E14,E15,E16,E17,E18,E20) gave positive results from thalassaemiae and (E1,E2,E4,E5,E6,E7,E8,E9,E10, E11,E12,E13,E15, E16, E19,E20) from healthy size of amplified band occur between (369bp) as shown in Fig (3). The our

results of this study demonstrate that a simulated patient treatment with ceftriaxone can alter the expression of antimicrobial resistance genes, including blaCMY-2 and percentage 83 % because the blaCMY-2 genes are commonly located on large, multidrug-resistant plasmids. Increased expression of the blaCMY-2 gene may be associated with increased expression of other drug resistance genes. located on the plasmid.^[11]



Figure(3): Agarose gel electrophoresis of PCR amplification products of *E.coli* bla CMY 2 gene from thalassaemia (agarose gel 2 %, 60 volt, 2 hours) Line M : DNA marker (100-1500bp). Line C: is control negative

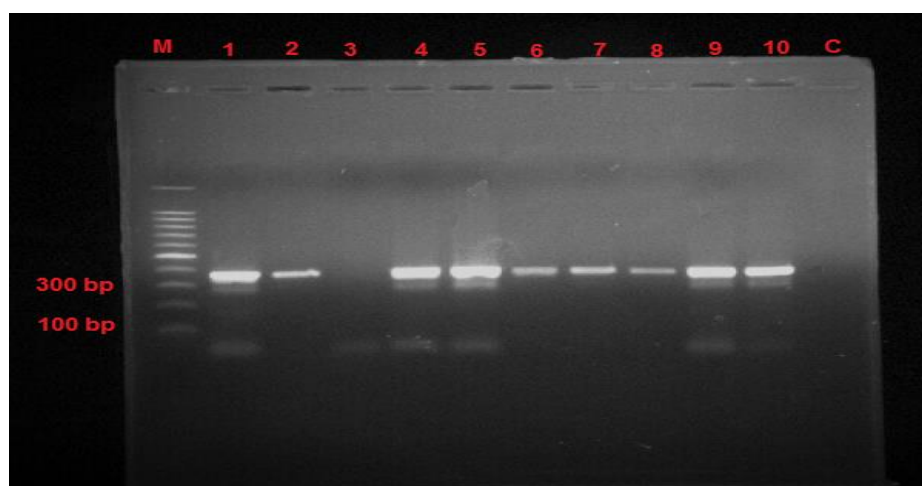


Figure (4): Agarose gel electrophoresis of PCR amplification products of *E.coli* bla CMY2 gene from non- thalassaemia (agarose gel 2 %, 60 volt, 2 hours) Line M : DNA marker (100-1500bp). Line C: is control negative .

Table(2): Distribution of sample study in Thalassemia and healthy according to bla CMY 2 gene.

Group	Positive		Negative		Chi-square
	No.	%	No.	%	
Thalassemia (No. =20)	16	84.21	4	20.00	13.733 **
Healthy (No. =20)	16	80.00	4	20.00	
** (P<0.01) significant.					

The Statistical Analysis show thalassaemic patients is high significant than healthy Patients. A patient with thalassaemia major must not be considered as immunocompromised, particularly if the disease is well compensated by treatment. On the other hand, many alterations to the body's immune system have been described in thalassaemia, including reduction in neutrophil numbers, changes in number and function of natural killer cells, increase in number and function of CD8 suppress cells, occurrence of macrophages, chemotaxis and phagocytosis and interferon gamma production.^[10,14]

Genotyping

BOX-PCR typing was carried out to precisely differentiate among the Fourty of *Ecoli* isolates divided into two groups, the first included 20 thalassemic and 20 non thalassemic patients. BOX-PCR fingerprinting is applicable for typing of *Ecoli* isolates that can be grouped according to the BOX-PCR to 5 different genotypes; being their similarity (cut off point) were 20% as it depicted in (figure9), named clusters. BOX PCR fingerprinting revealed 5 main allele as it is shown in (table3). allele (A) members shared 20% similarity including 3 thalassamia isolates and 4 non thalassemia isolates. Nevertheless, 2 thalassemic isolates were categorized under allele 2(a) with similarity of 2.5%. On the other hand, 2thalassemia isolates isolates were categorized under allele 3(b) with similarity of 10% allele 3 (B) while 4 thalasemia isolates and 2 healthy isolates were categorized under allele 4(c) similarity of 10%, 2 thalassemia and 2 healthy isolates were categorized under allele 5(d) similarityof 10%.^[17]

Table 3:-Genotyping alleles and source of isolates of *Ecoli*.

Genotyping	isolates	sourse
Allele A	E1,E5,E6,E10 thalassemia and E2,E7,E8,E9,E10,E11,E12 healthy	Urine and blood

Allele a	E6 thalassemia	Urine
Allele B	E8,E9 thalassemia	Urine
Allele C	E11,E12,E13,E14 thalassemia and E13,E14 healthy	blood
Allele D	E15,E16 thalassemia and E15,E16 healthy	blood

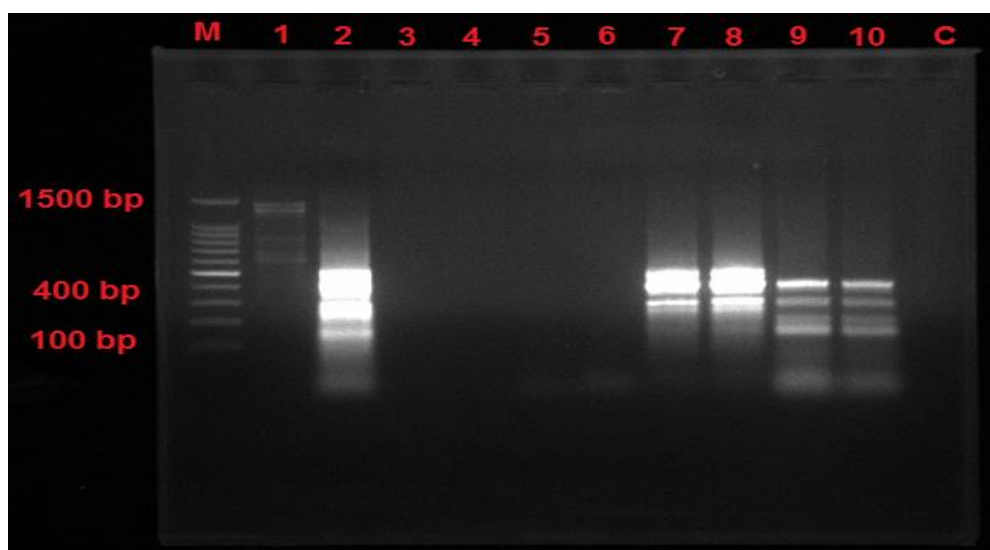


Figure (5) : BOX-PCR fingerprinting of *E.coli* isolates from thalassemia. Lane M: Molecular weight marker (MW100-1500bp) Lane C: Control negative

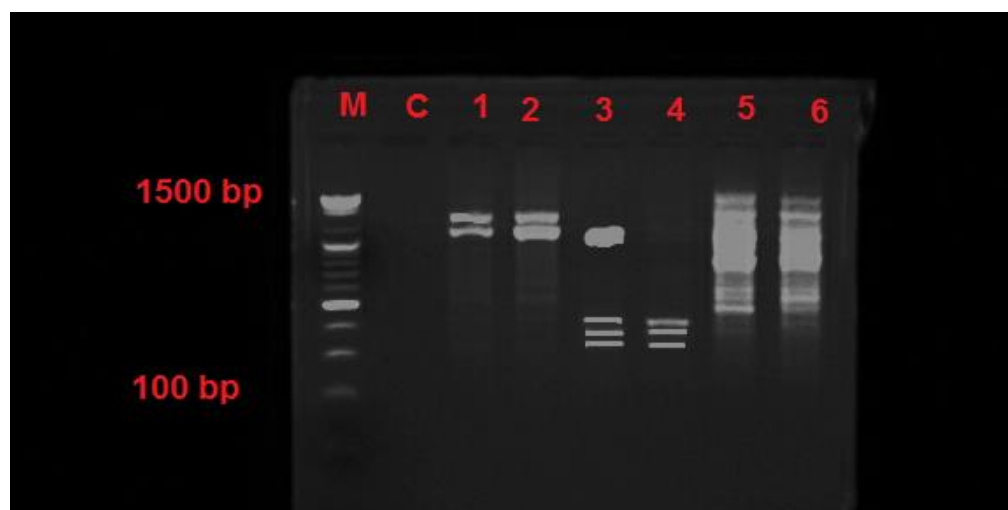


Figure (6) : BOX-PCR fingerprinting of *E.coli* isolates from thalassemia. Lane M: Molecular weight marker (MW100-1500bp) Lane C: Control negative.

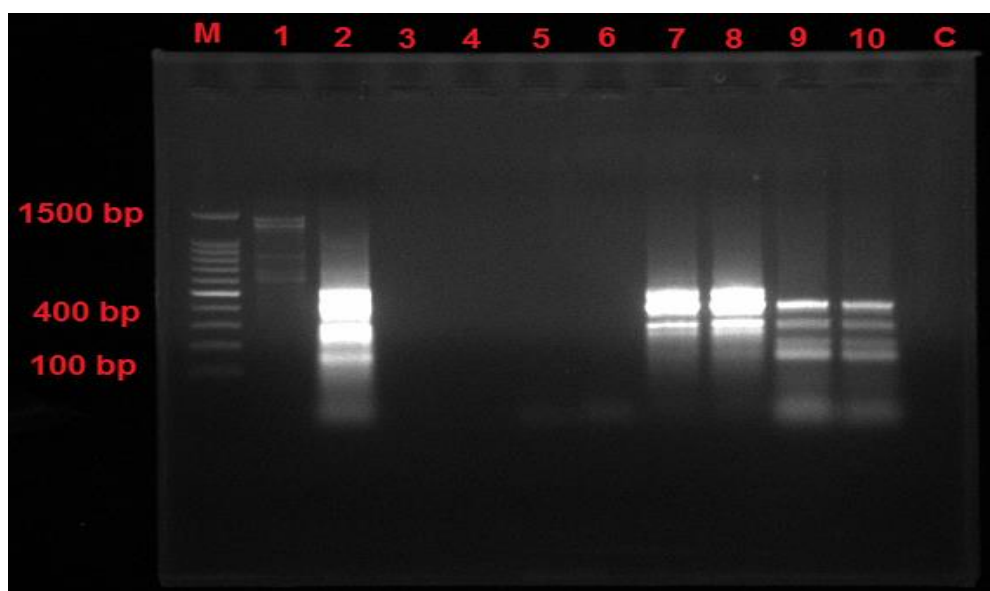
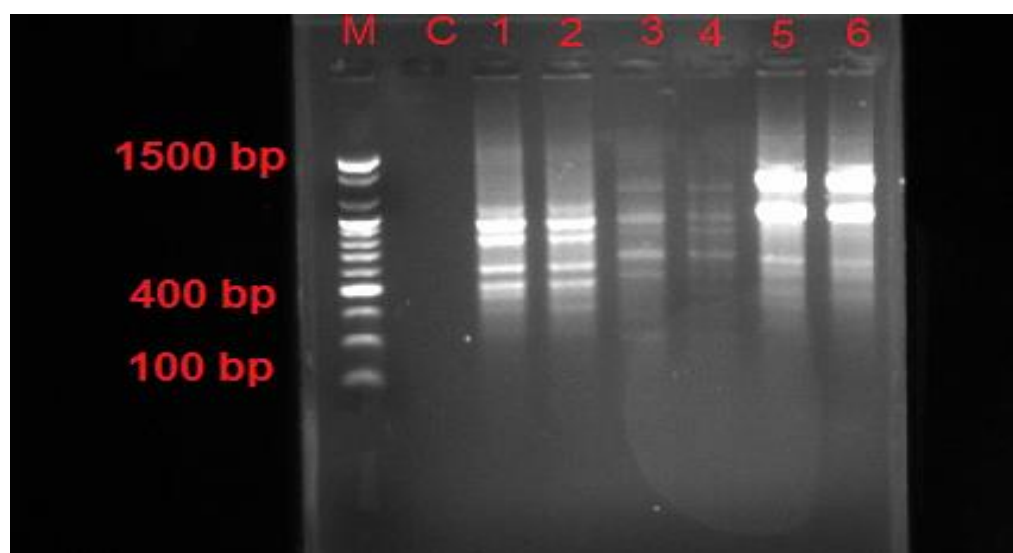


Figure (7) : BOX-PCR fingerprinting of *E.coli* isolates from nonthalassemia Lane M: Molecular weight marker (MW100-1500bp) Lane C: Control negative.



Figure(8) : BOX-PCR fingerprinting of *E.coli* isolates from non-thalassemia. Lane M: Molecular weight marker (MW100-1500bp) Lane C: Control negative.

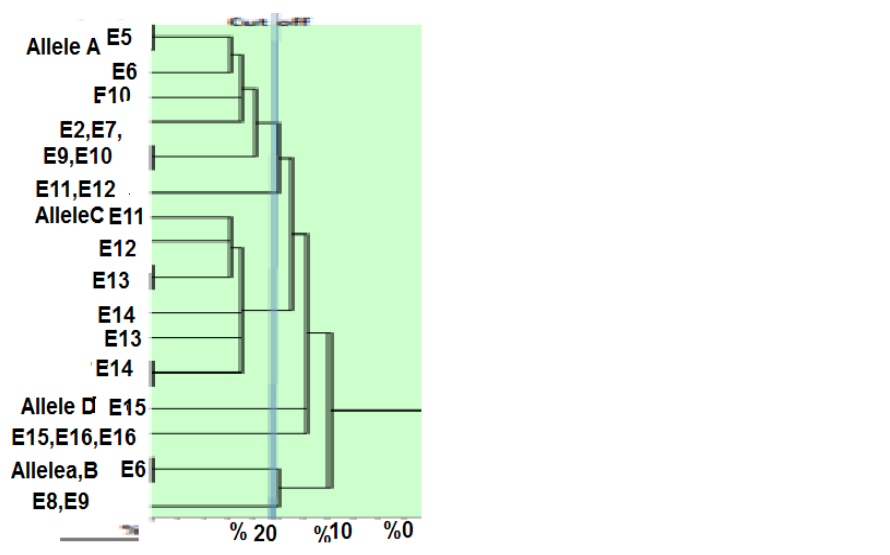


Figure (9): Dendrogram (cluster analysis) using BOX-PCR fingerprint patterns of *E.coli*.

Table(4) Distribution of sample study in Thalassemia and healthy according to Genotyping or BOX gene.

Group	Positive		Negative		Chi-square
	No.	%	No.	%	
Thalassemia (No. =20)	12	60.00	8	40.00	4.613 *
Healthy (No. =20)	11	55.00	9	45.00	
* (P<0.05) -significant.					

The Statistical Analysis show thalassaemic patients is significant. The differences in the distributions of box genes in the populations strengthen the probability that some *E.coli* strains are better adapted to the specific conditions found in specific infectious sites such as blood transfusion as thalassaemic patient.^[11]

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