

## Study self-transmissibility of *Salmonella Typhimurium* plasmids virulence isolated from fresh chicken foods by conjugation

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### Abstract

The aim of the present investigation was experimentally the ability of the virulence plasmid of *Salmonella Typhimurium* isolated from fresh chicken foods in al- Najaf Al-ashraf governorate to transfer between different isolates which might be the cause of variant diseases in order to find new data about this objective that may help in study the control of infections in humans.

*S. typhimurium* Lst1, Mst3 and Gst1 isolates ability to transfer to the transconjugant then PCR results confirmed the frequency of *spvA*, *pefA*, *rck* and *traT* genes in *E.coli* transconjugant (TLst1) were (12%), (30%), (8%) and (26%) respectively where in (TMst1) transconjugant were(14%),(30%), (6%) and(28%) respectively, similar results appeared in (TGst1) transconjugant that were (6%),(17%), (4%)and (18%) respectively therefore present study revealed according to conjugation experiments results that the virulence plasmid of *S. typhimurium* Lst1, Mst3 and Gst1 isolates were transmissible and perhaps lead to rapid increase in virulence isolates among different bacterial species in Najaf governorate.

### Introduction

Non-typhoidal salmonellosis (gastroenteritis) is a food borne disease of primary concern in developed, as well as developing countries. Although this disease is self-limiting, it can also lead to life-threatening systemic infections in humans infants, small children, weakened or elderly, Human Immunodeficiency Virus (HIV) and Immuno-compromised patients (Parry and Threlfall, 2008; Graham, 2010: Feasey *et al.*, 2012) therefore, this disease become among one of the major public health problems in terms of socio-economic impact estimations. Acute gastroenteritis and diarrhea that caused by non-typhoid salmonellosis, were made in worldwide each year up to 1.3 billion cases resulting in 3 million deaths annually(UL-Hassan *et al.*, 2008; Razzaque *et al.*, 2009).

One of important virulence factors in *S. typhimurium* is accessory genome. Accessory genome is represented by virulence plasmids carries sequences that enhance the growth rate

of the bacterium and intracellular survival during the systemic phase of disease which increases disease severity (Tierrez and Garcia, 2005).

*S. typhimurium* virulence plasmid is self-transmissible perhaps contributed to rapid increase in virulence strains among bacteria because it conjugated inside cultured human cells and DNA transfer from donor to recipient bacteria was proportional to the probability that the two types of bacteria occupied the same cell therefore any alter in virulence plasmids lead to change of virulence (Gayle *et al.*, 2002, (Denisse *et al.*, 2011, Kong *et al.*, 2012) .

The aims of the present investigation were seeking the ability of virulence plasmid genes of bacteria isolated from fresh chicken foods in al- Najaf Al-ashraf governorate to transfer between isolates that may be the cause of diseases in order to find new data about this objective that may help in study the control of infections in humans.

## Material and methods

To achieve study objectives, this research include detecting the presence of *tra* operon by identification one of them *traT* gene to confirm the ability of virulence plasmid to be self-transmissible, to test the self-transmissible of virulence plasmid under certain conditions., the ability of several isolated strains to act as virulence plasmid donors in conjugation with *E. coli* HB101 in LB medium(*in vitro*) was assayed.

A strains used in this research ( which were cephalothin resistance and have positive results about *pef A*, *spv A* and *rck* virulence plasmid genes) were isolated from fresh chicken foods in al- Najaf Al-ashraf governorate during previous study (Jabur, 2014).

### Extraction of virulence plasmid:

Performed with high speed plasmid mini kit as manufacture company (Geneaid) guidelines; as follows :

a-Total volume of liquid RNase added to PD1buffer and stored at 4C°.

b- Precipitate formed in PD2 buffer, the PD1 buffer was warmed in 37 C° water bath by gentle shaking to dissolve.

c-Absolute ethanol was added to wash buffer prior to initial use .

d-Amount of 1.5 ml of broth cultured bacterial cells was transferred to microcentrifuge tube, centrifuged at 14000-16000× g for 1 minutes and the supematant was discarded.

- e- Amount of 200 $\mu$ l of PD1buffer was added to the tube and the cell pellets were re-suspended by vortex .
- f- Amount of 200 $\mu$ l of PD2 buffer was added to the tube and mixed gently by inverting the tube 10 times, then stand at room temperature for at least 2 minutes to ensure the lysate was homologous.
- g- Amount of 300 $\mu$ l of PD3 buffer was added to the tube and mixed by inverting the tube 10 times, then tube was centrifuged at 14000-16000 $\times$  g for 3 minutes.
- h- PD column was placed in 2 collection tube and then the supematant from step (g) was added, centrifuged at 14000-16000 $\times$  g for 30 sec. then the flow- through was discard and a PD column was placed back in 2collection tube.
- i- Amount of 400 $\mu$ l of W1 buffer was added to a PD column, centrifuged at 14000-16000 $\times$  g for 30 sec. and then the flow- through was discard and a PD column placed back in 2collection tube (optional step)
- j- Amount of 600 $\mu$ l of wash buffer was added to a PD column, centrifuged at 14000-16000 $\times$  g for 30 sec. and then the flow- through was discard and a PD column was placed back in 2collection tube, centrifuged at 14000-16000 $\times$  g again for 3 minutes to dry the column matrix .
- k-The dried PD column was transferred to new microcentrifuge tube and 50  $\mu$ l of elution buffer was added in the center of column matrix then it was stand at for at least 2 minutes to allow elution buffer completely absorbed, centrifuged at 14000-16000 $\times$  g again for 2minutes to elute the DNA.

### Polymerase Chain Reaction Assay:

The primers used to identify the *tra T* gene on virulence plasmid of *s. typhimurium* were (BioNeer) are given in Table (1).

**Table (1):**Primer sequence of the genes for detection the *tra T* gene on virulence plasmid of *s. typhimurium* using PCR assay.

| Gene Name   | Primer Orient-ation | DNA Sequences (5'-3')         | Product size bp | Reference                     |
|-------------|---------------------|-------------------------------|-----------------|-------------------------------|
| <i>traT</i> | F                   | 5- GAT GGT TAC ACT GGT CAG-3' | 500             | (Guerra <i>et al.</i> , 2002) |
|             | R                   | 5- TCT GAG ATC TGT ACG TCG -3 |                 |                               |

The Bioneer primers were prepared depending on manufacturer instruction The final picomoles depended on the procedure of each primer (Amini *et al.*, 2010; Wondwossen *et al.*,2009; Cerro *et al.*, 2003).

**PCR Cycling Profiles:** Polymerase chain reaction assays were carried out in a 25  $\mu$ l reaction volume, and the PCR amplification conditions performed with a thermal cycler were specific to primer set (Table 2) depending on their reference procedure, as follows:

**Table (2):** The PCR amplification conditions of tra T gene that related to virulence plasmid.

| All PCR programs consisted of 35 cycles |                              |                       |                   |                   |                     |
|---|------------------------------|-----------------------|-------------------|-------------------|---------------------|
| Gene Name                               | Initial denaturatio.<br>Time | Denaturation.<br>Time | annealing<br>Time | Extension<br>Time | elongation.<br>Time |
| Tra                                     | 95 / 5min                    | 95 / 1min             | 56 / 1min         | 72 / 1min         | 72 / 7min           |

### Preparation of Agarose Gel:

Agarose gel was prepared by adding 1 gm of agarose powder to 100 ml of TBE buffer previously prepared (90 ml D.W were added to 10 ml TBE buffer 10X) in conical flask, the final concentration was 1 X and pH 8. The conical flask was placed in boiling water bath until it become clear and then allowed to cool to 50°C, and 1.5  $\mu$ l ethidium bromide at concentration of 0.5  $\mu$ l/ml was added. The agarose poured kindly in equilibrated gel tray earlier set with two combs fixed in the end and in the middle, and the two ends of gel tray were sealed. The agarose allowed solidifying at room temperature for 30 minutes. The comb was removed gently from the tray and the seal was removed from the ends of the tray. The comb made wells used for loading DNA samples(Tennant *et al.*, 2010).

### Agarose Gel Electrophoresis:

The amplified PCR products were detected by agarose gel electrophoresis and visualized by staining with ethidium bromide. PCR products were loaded to the agarose gel wells: 5 $\mu$ l from single product to single well in known sequencefollowed by ladder (100-1500 bp) to one of the wells in each row. The gel tray was fixed in electrophoresis chamber. IX TBE buffer was added to the chamber until covering the surface of the gel. The electric current was performed at 60-90 volt for 1.5 hour (Tennant *et al.*, 2010).

The electrophoresis result was detected by using gel documentation. The base pair of DNA bands were measured according to the ladder. Positive results were distinguished when there was DNA band equal to the target product size. Finally, the gel was photographed using gel documentation saving picture(Tennant *et al.*, 2010).

## Transconjugation experiment:

However, to determine whether the virulence plasmid genes is horizontally transferable , the *S. typhimurium* isolates Lst1, Mst3 and Gst1(appeared four study virulence genes) were tested for transferred study virulence plasmid genes by conjugation experiments carried out in Luria broth with streptomycin resistance *E. coli* HB101 as recipient strain. Because all experimented *S. typhimurium* isolates in previous study did not appeared plasmid marker(antibiotic resistance) on culture media (Jabur, 2014), the results identification of transconjugation carried out with PCR assay (after end conjugation period each conjugation broth subjected to growth on MaCconky's agar to selected only *E. coli* colonies then DNA of *E. coli* was extracted, amplified with PCR and identification each studied gene production with electrophoresis (El-sayed *et al.*, 2012).

## Bacterial conjugation experiment:

This experiment was performed according to (Chishih *et al.*, 2008) with some modifications (used PCR after conjugation to detect transconjugant isolates) as follow :

- a- In conjugation, the donor *S. typhimurium* and recipient *E. coli* HB101 strains were grown individually on LB medium to ( $4 \times 10^8$  bacteria/ml) and mixed at a ratio of 1:1.
- b-The bacterial mixture was incubated without shaking at 37C for 18 hrs. and 24 hrs. and then serial dilutions to  $10^9$  were prepared .
- c- The dilutions from  $10^5$  to  $10^9$  were plated on MacConkey's plates containing appropriate drugs (512 $\mu$ g/ml Streptomycin) for *E. coli* HB101 selection (Mami *et al.*, 2005), and then a plate containing 10-20 colony of *E. coli* HB101 was chosen.
- d- After conjugation each colony of *E. coli* was cultured on the surface of MacConkey's plates containing (512 $\mu$ g/ml Streptomycin) (Mami *et al.*, 20005)
- e-Total DNA extraction using Geneaid kit for *E. coli* HB101 transconjugant was done followed by PCR and electrophoresis to determine which colony that contain any gene of *S. typhimurium* virulence plasmid to regard as transconjugant .
- f- The efficiency of mating was calculated by dividing the number of transconjugant by the input number of the parent.
- g- Frequency of colonies that be transconjugant was calculated by the following equation :

No. of transconjugants

**Frequency of conjugation=-----×100**

Total No. of recipients cells

## Extraction of genomic DNA

It was performed with genomic DNA mini kit as manufacture company (Geneaid) guidelines; as follows :

a-Absolute ethanol was added to wash buffer prior to initial use .

b-An amount of 1.5 ml of cultured bacterial cells was transferred to micro- centrifuge tube, centrifuged at 14000-16000× g for 1 minutes and the supernatant was discarded.

c- An amount of 200 $\mu$ l of GT buffer was added to the tube and re- suspended the cell pellet by shaking vigorously and incubated at room temperature for 5 minutes .

d- An amount of 200 $\mu$ l of GB buffer was added to the tube and mixed by shaking vigorously for 5 minutes, incubated at 60 c° for 10 minutes to ensure the lysate was clear, during incubation the tube was inverted every 3 minutes and this time, pre heated the elution buffer to 60 c° for step (h) DNA elution.

e-Amount of 200 $\mu$ l of absolute ethanol was added to the tube and mixed by shaking vigorously .

f- GD column was placed in 2 ml collection tube and then all mixture from step (d) was added , centrifuged at 14000-16000× g for 2 minutes. Then the flow- through was discard and a PD column was placed back in 2collection tube.

g-Amount of 400 $\mu$ l of W1 buffer was added in to a GD column, centrifuged at 14000-16000× g for 30 sec. then the flow- through was discard and a GD column placed back in 2collection tube.

h- An amount of 600 $\mu$ l of washing buffer was added to a GD column, centrifuged at 14000-16000× g for 30 sec. and then the flow- through was discarded and a GD column was placed back in 2 collection tubes, then tube was centrifuged at 14000-16000× g again for 3 minutes to dry the column matrix .

i-The dried GD column was transferred to new micro-centrifuge tube and 100  $\mu$ l of elution buffer was added into the center of column matrix and leave it for at least 2 minutes to allow elution buffer completely absorbed, then it was centrifuged at 14000-16000× g again for 30 sec. to elute the purified DNA.

## Polymerase Chain Reaction Assay:

This procedure performed as above except the following:

1-The list of primers used to identify the plasmid virulence genes of *S. typhimurium* on genomic *E. coli* HB101 were (BioNeer) are given in Table (3).

2- the PCR amplification conditions performed with a thermal cycler were specific to each single primer set (Table 4) depending on their reference procedure, as follows:

**Table (3):** Primer sequence of the genes for detection the plasmidic virulence genes of *S. typhimurium* virulence by using PCR assay.

| Gene Name   | Primer Orientation | DNA Sequences (5'-3')                | Product size bp | Reference   |
|-------------|--------------------|--------------------------------------|-----------------|---|
| <i>pefA</i> | F                  | 5-TGT TTC CGG GCT TGT GCT -3'        | 500             | (Murugkar <i>et al.</i> , 2003)   |
|             | R                  | 5-CAG GGC ATT TGC TGA TTC TTC C - 3' |                 |   |
| <i>SpvA</i> | F                  | 5'-(GTC AGA CCC GTA AAC AGT) -3'     | 641             | Amini <i>et al.</i> , 2010,<br>Wondwossen <i>et al.</i> , 2009, Del Cerro <i>et al.</i> , 2003) |
|             | R                  | 5'-(GCA CGC AGA GTA CCC GCA)-3'      |                 |   |
| <i>rck</i>  | rck-F              | 5' TCG TTC TGT CCT CAC TGC-3'        | 500             | (Magdalena , <i>et al.</i> , 2009, Guerra <i>et al.</i> , 2002)                                 |
|             | R                  | 5' TCA TAG CCC AGA TCG ATG -3'       |                 |   |
| <i>traT</i> |                    | As above                             |                 |   |

**Table (4): The PCR amplification conditions of virulence factors genes that related to virulence plasmid.**

| All PCR programs consisted of 35 cycles |                               |                       |                   |                   |                     |
|---|-------------------------------|-----------------------|-------------------|-------------------|---------------------|
| Gene Name                               | Initial denaturation.<br>Time | Denaturation.<br>Time | annealing<br>time | Extension<br>Time | elongation.<br>Time |
| <i>spvA</i>                             | 95/5min                       | 95/1min               | 56/1min           | 72/1min           | 72/7min             |
| <i>PefA</i>                             | 95/5min                       | 95/1min               | 55/1min           | 72/1min           | 72/7min             |
| <i>Rck</i>                              | 95/5min                       | 95/1min               | 50/1min           | 72/1min           | 72/7min             |
| <i>Tra</i>                              | As above                      |                       |                   |                   |                     |

## Results and discussion

### Study Transfer of plasmids virulence genes by conjugation:

The conjugation process is the transfer of DNA element directly from one bacterial cell to another by mechanism that require cell-to-cell contact (Somikiate *et al.*, 2007).

Results of conjugation experiments for the studied virulence plasmid genes harboring isolates are shown in (Table 5 and 6). The conjugation experiments and PCR trails confirmed that *S. typhimurium* Lst1, Mst3 and Gst1 isolates have ability to transfer the *spvA*, *pefA*, *rck* and *traT* genes to *E. coli* (Figures 1-4), the frequency of same genes in *E.coli* transconjugant (TLst1) were (12%), (30%), (8%) and (26%) respectively where in *E.coli* transconjugant(TMst1) were (14%),(30%), (6%) and (28%)respectively, similar results appeared in *E.coli* transconjugant (TGst1) that were (6%),(17%), (4%) and (18%) respectively.

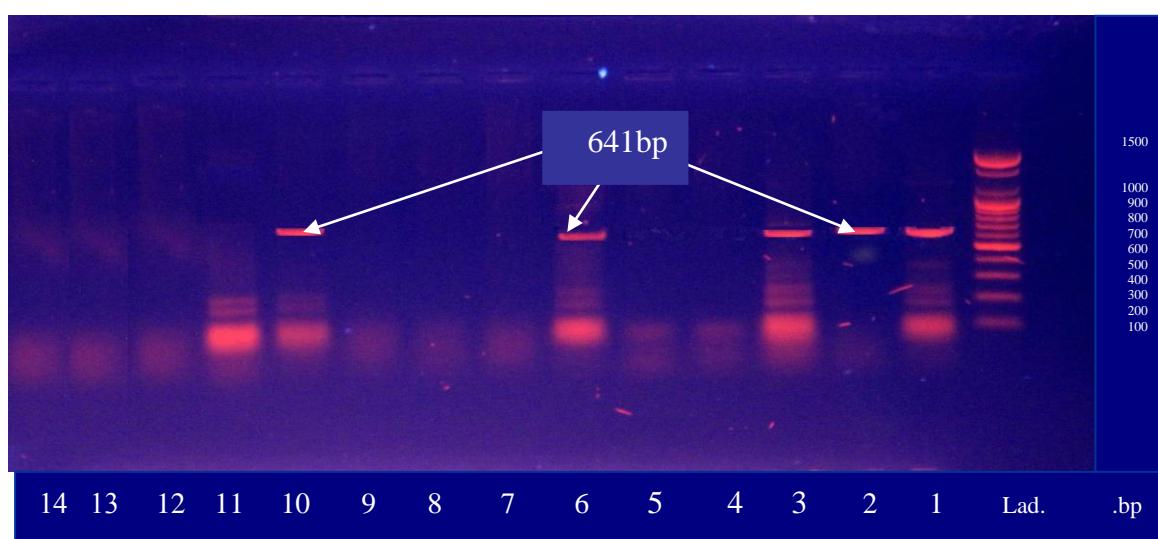
The present study revealed according to conjugation experiments results that the virulence plasmid of *S. typhimurium* Lst1, Mst3 and Gst1 isolates were transmissible.

**Table(5) :**Characterization of virulence plasmid genes of *S. typhimurium* isolates and transconjugant *E. coli* with PCR assay after conjugation

| Isolate                              | Type of gene harbored on plasmid                     | Frequency of plasmid gene in transconjugant isolates % |             |             |             |
|--------------------------------------|--|--|-------------|-------------|-------------|
|                                      |  | <i>spvA</i>  | <i>pefA</i> | <i>Rck</i>  | <i>traT</i> |
| <i>S. typhimurium</i> Lst1           | <i>spvA</i> , <i>pefA</i> , <i>rck</i> , <i>traT</i> | 61/61(100%)  | 61/61(100%) | 61/61(100%) | 61/61(100%) |
| <i>E.coli</i> transconjugant (TLst1) | <i>spvA</i> , <i>pefA</i> , <i>rck</i> , <i>traT</i> | 14/117(12%)  | 27/117(30%) | 9/117(8%)   | 30/117(26%) |
| <i>S. typhimurium</i> Mst3           | <i>spvA</i> , <i>pefA</i> , <i>rck</i> , <i>traT</i> | 68/68(100%)  | 68/68(100%) | 68/68(100%) | 68/68(100%) |
| <i>E.coli</i> transconjugant (TMst1) | <i>spvA</i> , <i>pefA</i> , <i>rck</i> , <i>traT</i> | 15/111(14%)  | 33/111(30%) | 7/111(6%)   | 31/111(28%) |
| <i>S. typhimurium</i> Gst1           | <i>spvA</i> , <i>pefA</i> , <i>rck</i> , <i>traT</i> | 73/73(100%)  | 73/73(100%) | 73/73(100%) | 73/73(100%) |
| <i>E.coli</i> transconjugant (TGst1) | <i>spvA</i> , <i>pefA</i> , <i>rck</i> , <i>traT</i> | 7/116(6%)  | 20/116(17%) | 5/116(4%)   | 21/116(18%) |
| <i>E.coli</i> HB101                  | -  | 0/45(0)  | 0/45(0)     | 0/45(0)     | 0/45(0)     |

Table (6): Frequency of transconjugant *E. coli*

| Type of donor isolate                | Total number of isolate |                  | Frequency of transconjugant isolates |
|--------------------------------------|-------------------------|------------------|--------------------------------------|
|                                      | Transconjugant          | Recipient        |                                      |
| <i>E.coli</i> transconjugant (TLst1) | 40                      | $17 \times 10^9$ | $2.3 \times 10^9$                    |
| <i>E.coli</i> transconjugant (TMst1) | 42                      | $11 \times 10^9$ | $3.8 \times 10^9$                    |
| <i>E.coli</i> transconjugant (TGst1) | 25                      | $17 \times 10^9$ | $1.4 \times 10^9$                    |

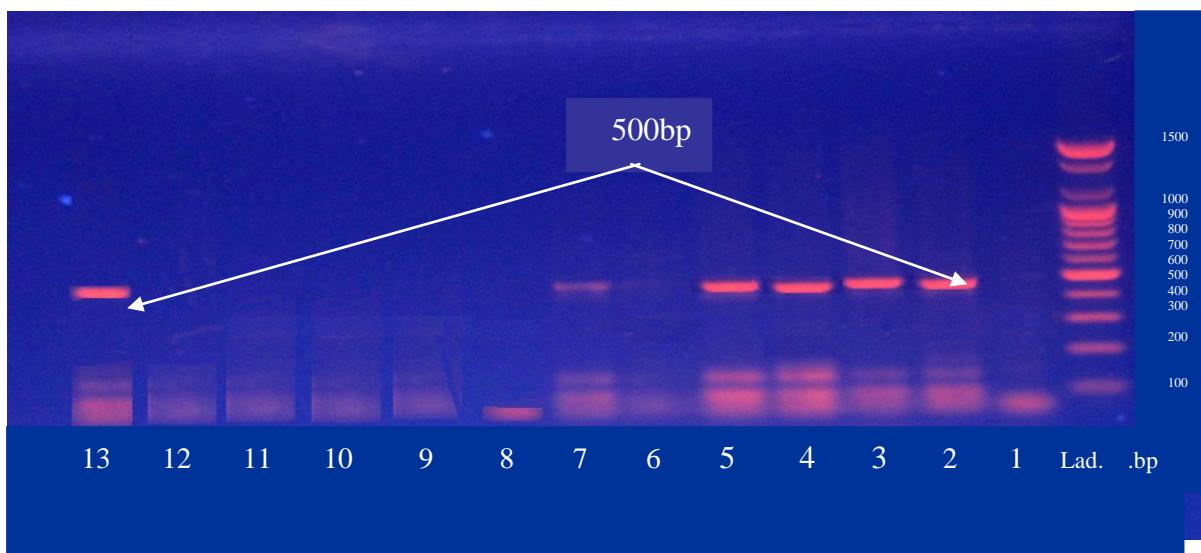


**Figure (1):** Ethidium bromide-stained agarose gel of PCR amplified products from DNA that extracted from one replicate transconjugant *E. coli* TLst1 isolates in dilution  $10^9$  (Lane 1-11), *E. coli* HB101 isolates as negative control (Lane 12) and *S. typhimurium* Lst1 isolates as positive control (Lane 13) amplified with primer *spvA* -F and *spvA* -R. Lane (lad) shows DNA marker(100 bp.). Lane(1) and (3) shows positive results with *spvA* genes as transconjugant *E. coli* TLst1 isolates. Lane(2) and (4 to 11) nagative results with *spvA* genes in standard *E. coli* HB101 isolate. Lane(13) shows positive results with *spvA* genes in *S. typhimurium* Lst1 isolate

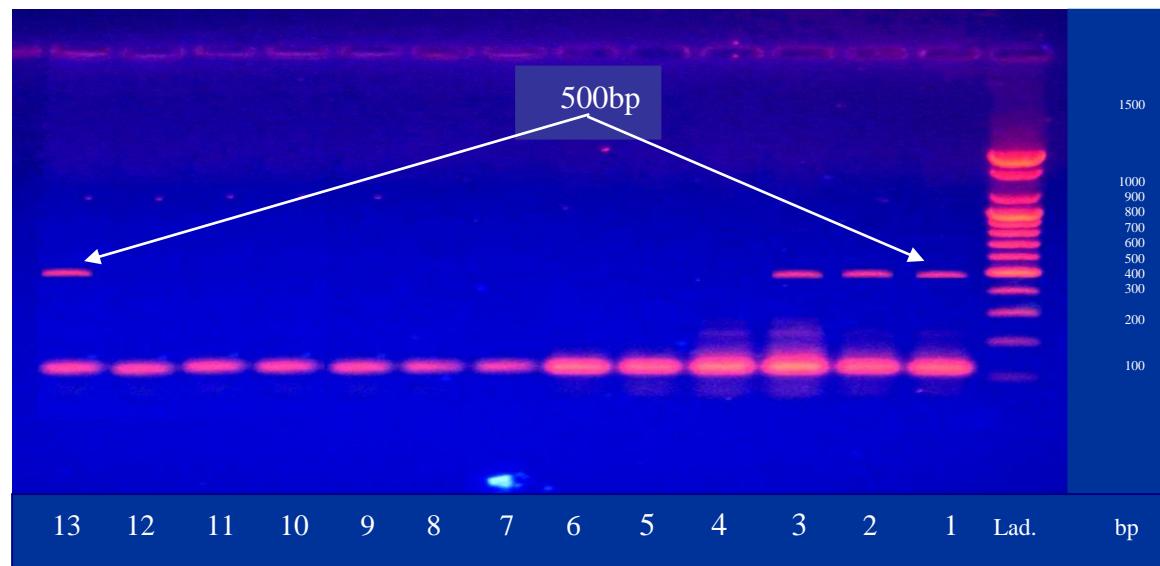
This results agreed with the results of Meritxell *et al.*, (2008) who determined that conjugal transfer of *Salmonella* virulence plasmid occurred in the ileum of mice that is infected with two *Salmonella enterica* serovar Typhimurium strains, one of which lacked the virulence plasmid. On other hand Brian *et al.*, (1999), reported that the virulence plasmid of strains LT2, 14028, and SR-11 is indeed self-transmissible but the plasmid of strain SL1344 is not.

The ability of isolates in this study to transfer its virulence plasmid perhaps contribute to rapid increase in virulence isolates among different bacterial species in Najaf governorate,

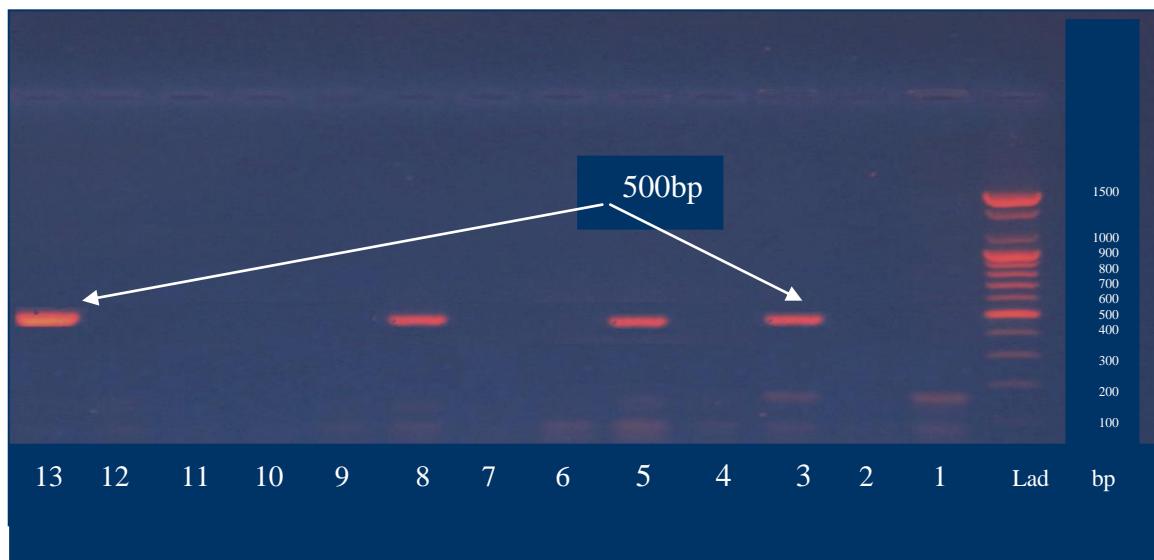
Gayle *et al* (2002) report that *Salmonella enterica* serovar Typhimurium conjugated inside cultured human cells and DNA transfer from donor to recipient bacteria was proportional to the probability that the two types of bacteria occupied the same cell.



**Figure (2):** Ethidium bromide-stained agarose gel of PCR amplified products from DNA that extracted from one replicate transconjugant *E. coli* Tlst1 isolates in dilution  $10^9$  (Lane 1-11), *E. coli* HB101 isolates as negative control (Lane 12) and *S. typhimurium* Lst1 isolates as positive control (Lane 13) amplified with primer *pefA* -F and *pefA* -R. Lane(lad) shows DNA marker(100 bp.). Lane(2-5 and 7) show positive results with *pefA* genes as transconjugant *E. coli* Tlst1 isolates. Lane (1,6,8, 9 to 11) show nagative results with *pefA* genes as transconjugant *E. coli* Tlst1 isolates. Lane(12) shows nagative results with *pefA* genes in standard *E. coli* HB101 isolate. Lane(13) shows positive results with *pefA* genes in *S. typhimurium* Lst1 isolate.



**Figure (3):** Ethidium bromide-stained agarose gel of PCR amplified products from DNA that extracted from one replicate transconjugant *E. coli* Tlst1 isolates (Lane 1-11), *E. coli* HB101 isolates as negative control (Lane 12) and *S. typhimurium* Lst1 isolates as positive control (Lane 13) amplified with primer *rck* -F and *rck* -R. Lane (lad) s DNA marker(100 bp.). Lane(1-3) show positive results with *rck* genes as transconjugant *E. coli* Tlst1 isolates. Lane (4 to 11) show nagative results with *rck* genes as transconjugant *E. coli* Tlst1 isolates. Lane(12) shows nagative results with *rck* genes in standard *E. coli* HB101 isolate. Lane(13) shows positive results with *rck* genes in *S. typhimurium* Lst1 isolate.



**Figure (4):** Ethidium bromide-stained agarose gel of PCR amplified products from DNA that extracted from one replicate transconjugant *E. coli* Tlst1 isolates (Lane 1-11), *E. coli* HB101 isolates as negative control (Lane 12) and *S. typhimurium* Lst1 isolates as positive control (Lane 13) amplified with primer traT -F and traT -R. Lane (lad) shows DNA marker(100 bp.). Lane(3,5 and 8) show positive results with traT genes as transconjugant *E. coli* Tlst1 isolates. Lane (1,2,4, 6,7,9 to 11) show nagative results with traT genes as transconjugant *E. coli* Tlst1 isolates. Lane(12) shows nagative results with traT genes in standard *E. coli* HB101 isolate. Lane(13) shows positive results with traT genes in *S. typhimurium* Lst1 isolate

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