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Detection of Tn*917* Conferring Erythromycin Resistance in Clinical Isolates of *Streptococcus pneumonoiae*

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Abstract: This study was conducted to investigate the genetic organization of *erm*-gene carrying Tn*917* in clinical isolates of *Streptococcus pneumonia.* Swab samples were collected from pharyngitis, tonsillitis, sputum and cerebrospinal fluid samples were collected from patients suffering from respiratory tract infections who attended Al-Yarmouk Teaching Hospital, Central Child Teaching Hospital and The Baghdad Teaching Hospital in Baghdad during the period from November/2017 to April/2018. A total of 15 isolates of *S. pneumoniae* isolates were examined and was found to contain Tn*917* element. Susceptibility of these isolates to different antibiotics was also examined, results showed that these isolates are resistant to penicillin in percentage of 93%, then to streptomycin (87%), clindamycin (73%), kanamycin (50%), erythromycin and azithromycin (40%), tetracycline and trimethoprim (80%), ciproflaxin and levofloxacin (20%). Genomic DNA was extracted from *S. pneumoniae* isolates for detection Tn*917* by using specific primers to amplify *erm* gene carried by this transposable element. Results showed that five of *S. pneumoniae* isolates were found to contain Tn*917* element giving them erythromycin resistance. *erm* gene encodes this antibiotic but does not mediate resistance to other antimicrobial agents. On the other hand, nucleotide sequence for *erm* gene was determined, and compared by alignment with the *erm* gene sequence located on the same transposable elements in standard strains of *S. pneumoniae* recorded in NCBI data base. Results of alignment showed 100% identity between these sequences.

Keywords: *S. pneumonia*, Tn*917*, erythromycin resistance.

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Introduction:

Streptococcus pneumoniae, the pneumococcus, is a commensal of the human nasopharynx and an opportunistic pathogen that is a leading worldwide cause of death for children under the age of 5 years (1). Suspected pneumococcal upper respiratory infections and pneumonia are often treated with macrolide antibiotics (2). In addition to localized infections such as otitis media and pneumonia, the pneumococcus may cause severe invasive disease (IPD) including bacteremia and meningitis. Widespread macrolide use, however, is associated with increased macrolide resistance in *S. pneumoniae* (3,4). Clinical failures of macrolide treatment of pneumococcal infections have been reported for lower respiratory tract infections (5) and bacteremia (6,7). Widespread macrolide use is a strong selective pressure contributing to the expansion of macrolide-resistant *S. pneumoniae* (8,9). Globally, macrolide resistance among *S. pneumoniae* is geographically variable but ranges from <10% to >90% of isolates (10,11,12). Erythromycin is one of macrolides bind reversibly to the 23S rRNA at a site near the peptidyl

transferase center of the 50S ribosomal subunit (13). The *mef* (E)/*mel*-containing genetic element Mega is found in at least six distinct chromosomal sites within the pneumococcal genome (14), while *mef* (A) is found on Tn*1207.1* (15). The most common Tn*916*-like elements in *S. pneumoniae* containing erythromycin resistance cassettes include Tn*2009*, Tn*6002*, and Tn*2010* (16). Tn*917*, an *erm*(B)-containing transposon insertion into *orf9* of Tn*916* creates Tn*3872* (17). A similar recombination event likely occurred with Tn*2009* and Tn*3872* to create Tn*2017*, which is a Tn*916*-like element with a Mega insertion in *orf6* and Tn*917* in *orf9* of Tn*916* (18). According to the importance of Tn*917* in spreading erythromycin resistance between bacterial isolates, this study was aimed to explore the presence of Tn*917* in *S. pneumonia* isolates.

Materials and methods:

Bacterial Isolates:

Clinical isolates of *S. pneumonia* were obtained from pharyngitis, tonsillitis, sputum and cerebrospinal fluid samples were collected from patients suffering from respiratory tract infections who attended Al-Yarmouk Teaching Hospital, Central Child Teaching Hospital and The Baghdad Teaching Hospital in Baghdad during the period from November/2017 to April/2018. Those fifteen isolates were maintained on chocolate agar medium, and then fresh cultures of each bacterial isolate were obtained by inoculating Brain-Heart infusion broth medium and incubated at 37 °C with 5% CO_2 for 16 hrs. (19).

Susceptibility testing:

Susceptibility of *S. pneumonia* isolates against different antibiotics (penicillin, streptomycin, trimethoprim, tetracycline, trimethoprim, clindamycin, kanamycin, erythromycin, azithromycin, ciproflaxin and levofloxacin) was examined on Muller-Hinton agar medium according to the standard disc diffusion method (20). These antibiotics were supplied by Bioanalyse/Turkey.

Detection of Tn*917*:

Tn*917* transposable element was detected in the fifteenth isolates of *S. pneumonia* was detected by amplification of erythromycin resistance gene carried by this transposon using specific primers (21); APHA1: 5'-GCCGATGTGGATTGCGAAAA -3' and APHA2: 5'-GCTTGATCCCCAGTAAGTCA -3', provided in lyophilized form, and were dissolved in sterilized distilled water to give a final concentration of 10 picomole/µl. PCR master mix supplied by promega\USA was prepared to be consisting of the following components:

Material	Concentration
PCR buffer (pH=8.5)	2X
$MgCl_2$	3 mM
dNTPs	400 mM
Taq DNA polymerase	5 units

Optimum conditions for amplification of Tn*917* were described in Table (1).

Table (1): PCR program for amplification of erm gene carried by Tn*917*.

Step	Temperature (°C)	Time (min:sec)	No. of cycles
Initial Denaturation	95	5 min.	1
Denaturation	95	30 sec.	30
Annealing	55	45 sec.	
Extension	72	45 sec.	
Final extension	72	7 min.	1

Results and Discussion:

Antibiotics sensitivity of *S. pneumoniae*:

Results of antibiotic susceptibility of *S. pneumoniae* illustrated in Table (2) showed that multi-drug resistant was spread in the clinical isolates of *S. pneumoniae* as these isolates gave different resistant patterns to these antibiotics. Results indicated in Table(2) showed that most of bacterial isolates (93%) were resist to penicillin antibiotic, then to streptomycin (87%), tetracycline and trimethoprim (80%), clindamycin (73%), kanamycin (50%), erythromycin and azithromycin (40%), ciproflaxin and levofloxacin (20%). In recent years, treatment for *S. pneumoniae* has become difficult owing to the global rise in the prevalence of antibiotic resistance, particularly against first-line antibiotics such as erythromycin and penicillin as it was mentioned by Xu, *et al.* (22).

Table (2): Antibiogram of *S. pneumoniae* clinical isolates.

Isolates No.	Azi	cip	CD	E	K	LEV	P	S	T	TM
D1	R	R	R	R	R	R	R	R	R	R
D2	R	S	R	R	R	R	R	R	R	R
D3	R	R	R	R	R	R	R	R	R	R
D4	S	R	R	S	S	S	R	S	S	R
D5	R	R	R	R	R	R	R	R	R	R
D6	R	R	R	R	R	R	R	R	R	R
D7	R	S	R	R	R	S	R	R	R	R
D8	S	S	S	S	R	S	R	R	R	S
D9	R	R	R	R	R	R	R	R	R	R
D10	R	S	R	R	R	R	R	R	R	R
D11	S	S	S	S	S	S	R	R	R	R
D12	S	S	S	S	S	S	R	R	R	R
D13	S	S	R	S	S	S	S	S	S	S
D14	S	R	S	S	S	S	R	R	S	S
D15	R	R	R	R	R	R	R	R	R	R

R: Resistance; **S:** Sensitive; **AZI:** Azithromycin; **CIP:** Ciproflaxin; **CD:** Clindamycin; **E:** Erythromycin; **K:** Kanamycin; **LEV:** Levofloxacin; **P:** Penicillin; **S:** Streptomycin; **T:** Tetracycline; **TM:** Trimethoprim.

On the other hand, results also showed the most resistant isolates are the isolates symbol D1, D3, D5, D6, D9 and D15 as they show resistance to all antibiotics used in this study, then D2 and D10 which was resist to nine antibiotics (90%),then D7 (80%), then D4, D8, D11 and D12 (40%), then D14 (30%), then D13 (10%). Resistance and multiresistance to a large group of antibiotics, including polypeptides, aminoglycosides and first-generation quinolones has been reported in several studies (1). Findings of this study is consistent with previous studies(23. showed an increase in antibiotic resistance rates, especially to penicillin and erythromycin (24;25).

Detection of Tn*917* in *S. pneumoniae* isolates:

Tn*917* is a nonconjugative transposon which is responsible for the spread of erythromycin resistance (26). Tn*917* was found to be inserted into a number of different Tn*916*-like elements.

Erythromycin gene of Tn*917* was amplified by using specific primers. Results illustrated in Figure (1) showed an amplified product of 292 bp appeared after electrophoresis on agarose gel (1%) represents erythromycin gene of Tn*917*, this transposable element was detected in four isolates of *S. pneumoniae* out of the total isolates (15 isolate). These isolates are D1, D5, D6 and D7. All these isolates are resistance to erythromycin and this result explain the erythromycin resistant phenotype in these four isolates that arise from the presence of Tn*917*.

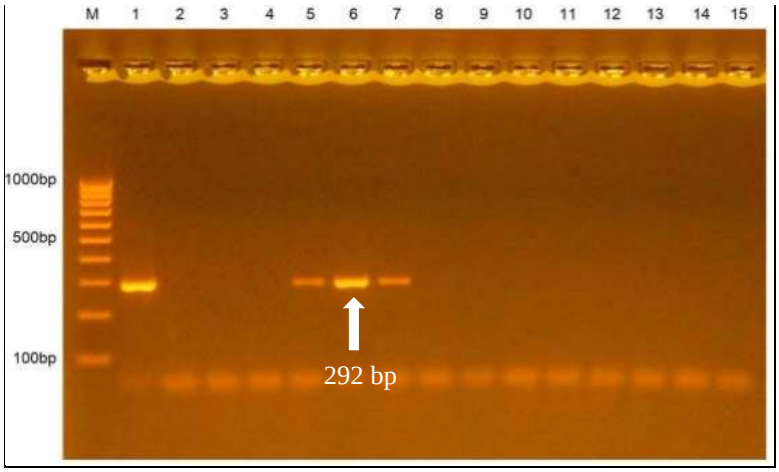

Figure (1): Erythromycin gene of Tn*917* detected after amplification of genomic DNA for *S.pneumoniae* isolates and electrophoresis on agarose gel (1%) for one hour. Lane (M): Ladder marker; Lane (1-15): Bacterial isolates of *S.pneumoniae*.

On the other hand, results indicated in Table (2) showed that there are other five erythromycin resistant isolates (D2, D3, D9, D10, and D15), this trait may came from a chromosomal copy of erythromycin resistance gene carried by these isolates, where the *mef* (E)/*mel*-containing genetic element Mega is found in at least six distinct chromosomal sites within the pneumococcal genome as it was mentioned by Chancey *et al* (14). Furthermore there are six isolates of *S. pneumoniae* (D4, D8, D11, D12, D13, and D14) were sensitive to erythromycin as they were unable to grow on BHT agar medium containing this antibiotic.

Erythromycin gene carried by Tn*917* in these isolates was sequenced; results illustrated in Figure(2) showed the nucleotide sequence of the amplified product of erm gene. Alignment of these sequences in *S. pneumoniae* isolates with transposon sequences of *S.pneumoniae* standard strains recorded in NCBI are illustrated in Figure(3). Results of alignment showed that erythromycin gene sequence of Tn*917* present in the four bacterial isolates of *S. pneumoniae* was identical (100% identity) with chromosomal erythromycin gene sequences in different standard strains of *S. pneumoniae* and with genomic erythromycin resistance gene in other

strains of this bacterium, which supports the results concluded in this study that the four isolates of *S. pneumoniae* are harboring chromosomal copies of Tn*917* conferring erythromycin resistance. In *S. pneumoniae*, methylation is *erm*(B) mediated in almost all cases (27). Worldwide, the predominant mechanism responsible in *S. pneumonia* is the *Erm*(B) methylase (28). One possible explanation for the presence of genes at different isolates is due to *erm* gene, which was most likely located on Tn*917*.

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  1 TATGATTTTTTAAAGACGGACCCGAAGAGGAACTT G TCT   40
 41 TTTCCCACGGCGACCTGGGAGACAGCAACATCT TTG TGA   80
 81 AAGATGGCA AAGTAAGTGG CTTTATTGATCTTGGGAGAA  120
121 GCGGCAGGGCGGACAAGTGGTATGACATTGCCTTC T GCG  160
161 TCCGGTCGATCAGGGAGGAT ATCGGGGAAGAACAGTATG  200
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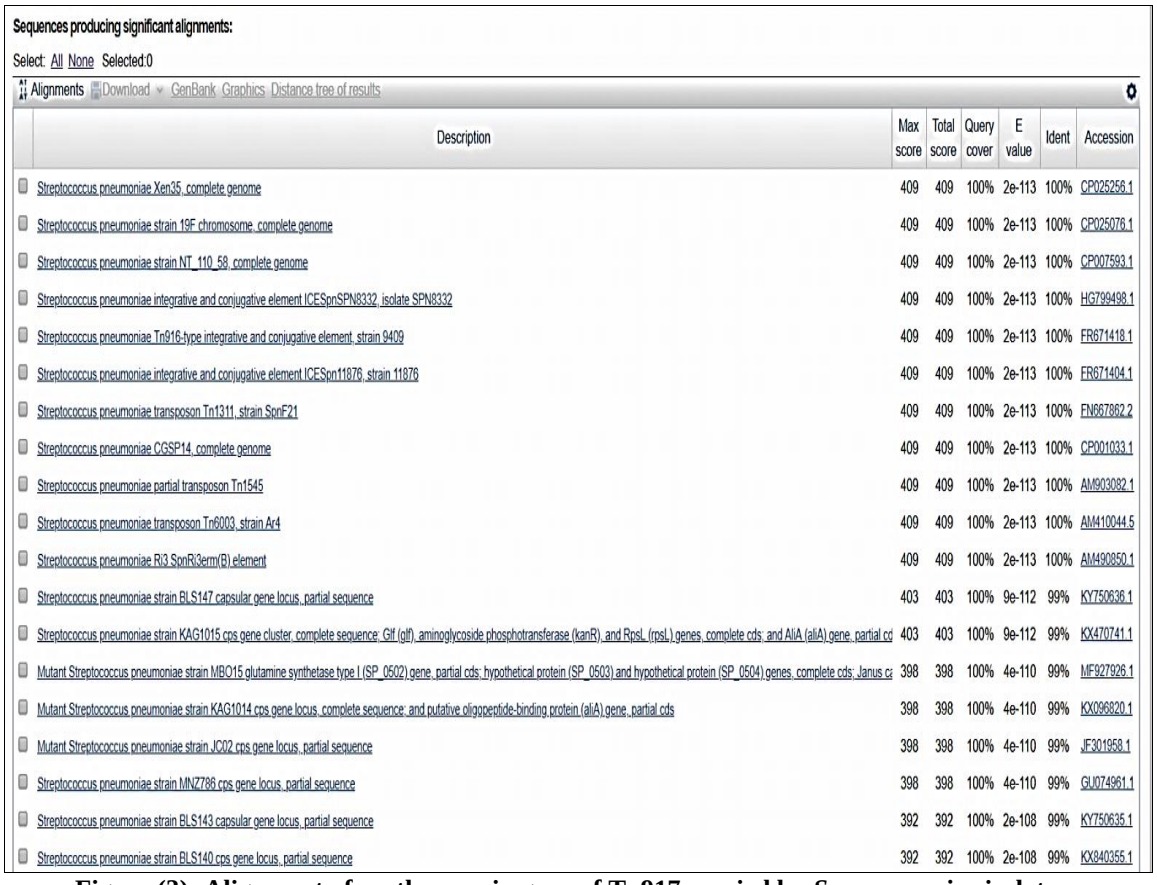
Figure (2): Nucleotide sequence of erythromycin gene of Tn*917* carried by *S.pneumoniae* isolates.

In North Lebanon (29) PCR analysis of the 45 macrolide-resistant *S. pneumoniae* isolates showed that the *erm*(B) was the prevailing gene present in 37.8 % of all strains. Also another study involved the *erm*(B) gene was reported as predominant in several regions, such as Belgium (91.5%), France (90%), Spain (88.3%), Serbia (82.4%), Hungary (82.4%), Poland (80.8%), China (76.5%), Japan (58%) and Italy (55.8%) (30).



Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Streptococcus pneumoniae Xen35, complete genome	409	409	100%	2e-113	100%	CP025256.1
Streptococcus pneumoniae strain 19F chromosome, complete genome	409	409	100%	2e-113	100%	CP025076.1
Streptococcus pneumoniae strain NT_110_58, complete genome	409	409	100%	2e-113	100%	CP007593.1
Streptococcus pneumoniae integrative and conjugative element ICESpnSPN8332, isolate SPN8332	409	409	100%	2e-113	100%	HG799498.1
Streptococcus pneumoniae Tn916-type integrative and conjugative element, strain 9409	409	409	100%	2e-113	100%	FR671418.1
Streptococcus pneumoniae integrative and conjugative element ICESpn11876, strain 11876	409	409	100%	2e-113	100%	FR671404.1
Streptococcus pneumoniae transposon Tn1311, strain SpnF21	409	409	100%	2e-113	100%	FN667862.2
Streptococcus pneumoniae CGSP14, complete genome	409	409	100%	2e-113	100%	CP001033.1
Streptococcus pneumoniae partial transposon Tn1545	409	409	100%	2e-113	100%	AM903082.1
Streptococcus pneumoniae transposon Tn6003, strain Ar4	409	409	100%	2e-113	100%	AM410044.5
Streptococcus pneumoniae R3 SpnR3erm(B) element	409	409	100%	2e-113	100%	AM490850.1
Streptococcus pneumoniae strain BLS147 capsular gene locus, partial sequence	403	403	100%	9e-112	99%	KY750636.1
Streptococcus pneumoniae strain KAG1015 cps gene cluster, complete sequence; Glf (glf), aminoglycoside phosphotransferase (kanR), and RpsL (rpsL) genes, complete cds; and AliA (aliA) gene, partial cd	403	403	100%	9e-112	99%	KX470741.1
Mutant Streptococcus pneumoniae strain MBO15 glutamine synthetase type I (SP_0502) gene, partial cds; hypothetical protein (SP_0503) and hypothetical protein (SP_0504) genes, complete cds; Janus ca	398	398	100%	4e-110	99%	MF927926.1
Mutant Streptococcus pneumoniae strain KAG1014 cps gene locus, complete sequence; and putative oligopeptide-binding protein (aliA) gene, partial cds	398	398	100%	4e-110	99%	KX096820.1
Mutant Streptococcus pneumoniae strain JC02 cps gene locus, partial sequence	398	398	100%	4e-110	99%	JF301958.1
Streptococcus pneumoniae strain MNZ786 cps gene locus, partial sequence	398	398	100%	4e-110	99%	GU074961.1
Streptococcus pneumoniae strain BLS143 capsular gene locus, partial sequence	392	392	100%	2e-108	99%	KY750635.1
Streptococcus pneumoniae strain BLS140 cps gene locus, partial sequence	392	392	100%	2e-108	99%	KX840355.1

Figure (3): Alignment of erythromycin gene of Tn917 carried by *S. pneumoniae* isolates erythromycin gene carried by standard strains of the same bacterial recorded in NCBI.

Conclusions:

Respiratory tracts and cerebrospinal fluid is important source for isolation of *S.pneumoniae* in case of pharyngitis and tonsillitis infections. These isolates were multidrug resistance. Molecular detection of Tn*917* showed that this transposable element carrying erythromycin resistance gene detected in different isolates of *S.pneumoniae* . Not all the antibiotics resistance phenotype of bacterial isolates related to the presence of transposons, but a chromosomal or plasmid copy of the antibiotic resistance gene.

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