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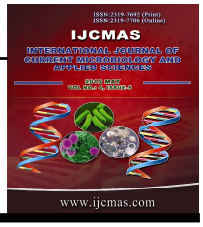

Original Research Article

Prevalence of *erm* Genes among Methicillin Resistant *Staphylococcus aureus* MRSA Iraqi Isolates

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A B S T R A C T

Eighty four *staphylococcal* bacterial isolates were obtained from Medical city in Baghdad during the period August to November 2013The results revealed 74 isolated were *S. aureus* isolates identified by routine chemical tests, in genotypic identification has been appeared (61 out of 74) isolates contained *mec* A (MRSA), phenotypic screening about MLSB was showed(18 out of 74) *S. aureus* isolates is constitutive resistance to erythromycin cMLSB phenotype (24.32%), (6 out of 74) *S. aureus* isolates sensitive to erythromycin (9.83%), (4 out of 74) *S. aureus* isolates inducible resistance to erythromycin D-shape (6.55%), (9 out of 74) *S. aureus* isolates showed MS phenotype which is resistance to erythromycin and sensitive to clindamycin without D-shape (14.75%) and finally (29 out of 74) *S. aureus* isolates showed intermediate resistance to erythromycin, while genotypic screening about MLSB was showed prevalence of *erm* A gene (7.35%), of *erm*C gene (5.88%) and no *erm* B gene recovered from *S. aureus* isolates.

Introduction

S. aureus bacteria consider the most frequent agent for causing hospital a queried infection additional to the great ability to adapt itself to numerous conditions and successful clones can be epidemic and even pandemic by its ability to spread from one *S. aureus* is a common pathogen, although continuous progress in the medical and diagnostic field, it is a causative agent to a vast numbers of infections incorporating soft tissue infections, impetigo, septicemia toxic shock and scalded skin syndrome, Conventionally methicillin resistance *Staphylococcus aureus* (MRSA) was consider a Hospital- Acquired infection (HA) (Patrick *et al.*, 2009). The large threading is currently represented by methicillin resistance *S. aureus* MRSA that usually carries additional antibiotic resistance determinants, therefore warranting the usage of the last-barrier drug like glycopeptides. Today the emergence of MRSA with reduce sensitivity to vancomycin has increased particularly (Mohammadi *et al.*, 2014). The control of such hospital-acquired infection demands spendy surveillance programs containing

patient isolation and contact precaution, with great impact on costs (Costella *et al.*, 2010).

S. aureus isolates qualify to have a resistance to Erythromycin, this resistance commonly associated with resistance to other Macrolides. It is found three genes in *S. aureus* responsible for this resistance (*erm*A, *erm*B and *erm*C) these genes encoding to methylase enzymes which play role in modifying ribosomal target site leading to MLSB phenotype (Zmantar *et al.*, 2011). MLSB phenotype resistance maybe occur constitutive or inducible form after exposure to inducer which is macrolide molecule, induction test have been done by double diffusion disc test erythromycin and clindamycin disks are utilized in test (Fiebelkorn *et al.*, 2003).

The present study aims to identification of *S. aureus* isolates and investigate the occurrence of MRSA among *S. aureus* isolates in Iraqi hospitals by detecting mobile genetic elements *mec*A gene, the isolates were also studied for phenotypic screening about constitutive and inducible resistance toward Macrolide- Lincosamide,- Streptogramin B additional to genotypic screening about MLSB genes *erm* A, *erm* B and *erm* C prevalence among MRSA – MSSA isolates.

Material and Methods

Collection and diagnosis of bacterial isolates:

Eighty four staphylococcal bacterial isolates were obtained from Medical city in Baghdad during the period August 2013 to November 2013. It was divided between seventy five isolates from clinical samples from patient and nine isolates from hospital environment. The source of clinical isolates distributed as (n=5) isolates from Blood, (n=15) isolate from urinary tract infection, (n=43) skin infection, (n=2) eye swab, (n=5) ear swab, (n=3) nasal swab, one isolate from seminal fluid and lastly one isolated from sputum. Each isolate was identified according the morphology, routine biochemical tests according to Atlas *et al.* (1995) and confirmed by EPI Staph test.

Genotypic detection to screening about methicillin resistant *S. aureus* isolates MRSA

The prevalence of MRSA isolates was done by using specific primers and amplicon sizeas list in table 1 to detecting *mec*A gene (responsible for methicillin resistance) (Cabrera *et al.*, 2010). Template DNA was prepared by simple boiling methods. Briefly, few isolated colonies of overnight growth bacteria were suspended thoroughly in 5 ml of TE buffer and boiled in water bath for 5 min. after centrifugation the supernatant was separated and applied as template of DNA. PCR mixture was prepared by adding 12.5µl of GoTaq®Green master Mix (2X) promega, 5µl template DNA, 1.5µl from each forward and reverse primers with final concentration 1 poml /µl, finally volume was completed to 25µl by adding nuclease free water. PCR condition was usually started the process with initial denaturation step at (95^{o}C/30 min) followed by repeated cycles (35 times) which consists from denaturation step at (94^{o}C/30 sec.) annealing step at (53^{o}C/30 sec.) then extension step at (72^{o}C/30 sec.) followed by final extension step at (72^{o}C/10 min). PCR products were detected in 1 % agarose gel for 1 hr. at 50 V, stained with ethidium bromide and visualized by transilluminator.

Phenotypic screening of (MLSB) in *S. aureus* isolates

Double diffusion disc method (D-shape) was used to screening about MLSB phenotype

by using Clindamycin (2 µg/disc) and Erythromycin (15 µg/disc) discs were placed at the distance (15-20 mm) edge-to-edge on the surface of medium). The process was carried as recommended in CLSI (2013) isolates. Three phenotypes will be noticed as following

1- Constitutive (cMLSB) phenotype: isolates appeared resistant to each erythromycin and clindamycin discs with circular inhibition zone around both discs.
2- Inducible (iMLSB) phenotype: isolates appeared resistant to erythromycin and sensitive to clindamycin with flat inhibition zone D-shape toward erythromycin disc
3- Macrolides resistance and clindamycin sensitive (MS) phenotype: isolates appeared resistance to erythromycin and sensitive to clindamycin without D-shape phenomenon.

The results summarized in (figure 1)

Genotyping detection of MLSB

The prevalence of *erm* A, *erm* B and *erm* C genes were determined to genotypic screening about macrolides resistant by PCR. There were used Multiplex PCR to screening about *erm* A and *erm* C genes and uniplex PCR to screening about *erm* B gene, Process was done by using specific primers with the amplicon size (Martineau *et al.*, 2000) were listed in table 1. PCR mixture was prepared as described previously. PCR condition for each primer was started the process with initial denaturation step at (96^{o}C/30 min) followed by repeated cycles (40 times) which consists from denaturation step at (94^{o}C/30 sec.) annealing step at (56^{o}C/30 sec.) then extension step at (72^{o}C/30 sec.) followed by final extension step at (72^{o}C/10 min). PCR products were detected in 1 % agarose gel for 1 hr. at 50 V, stained with ethidium bromide and visualized by transilluminator.

Results and Discussion

Bacterial isolation and identification

Staphylococcus aureus is a commensal bacteria which almost colonizes the nose of healthy persons. *S. aureus* bacteria can cause wide spectrum of infection, beginning from skin and soft tissue infections to invasive diseases. Because *S. aureus* have numerous virulence factors make it have ability to colonize and distribute of different environments. It have been observed

Rapid emergence of MRSA In the last two dedicate associated with complicated the control of infection (Gopal *et al.*, 2008). The collected isolates were initially diagnosed as *Staphylococcus.* to confirm biochemical tests were done to confirm the identification, *Staphylococci* which showed negative reaction in oxidase test, positive reaction in catalase test, also most of them (n=74) showed positive reaction in coagulase test (COPS) and few isolated (n=10) showed negative reaction (CONS) coagulase positive Finally, to confirm accurate identification at generic and species level was used API Staph system which applied on all isolates including those with coagulase negative results. The results appeared similarities to the previous identifications tests as shown (table 2)

According to the results have been shown in (Table 2), the highest percentage of collected *S. aureus* isolates from skin infection (51.35%), Al-Talib and his colleagues (Al-Talib *et al.*, 2009) were revealed in their research *S. aureus* isolates

was the predominant pathogen recovered from burn wound (33.6%) which is colonized and infected skin tissues more than other bacterial species.

Genotypic method to screening about MRSA

Detecting of MRSA isolates have been done by mobile genetic element *mec*A and using polymerase chain reaction PCR, this technique characterized with (93.8 to 100 %) sensitivity and (98.6 to 100) specificity, In recent years, clinicians and researchers have been excerpted huge genomic information from clinical samples especially in clinical bacteriology leading to major transformation in diagnostic way, belong to the diagnostic in PCR (Ratnayake and Olver, 2011). The results revealed (61 out of 74) isolates contained *mec*A (82.43%) is MRSA and (13 out of 74) isolates did not contained *mec* A (17.57%) is MSSA

In fact the significant of rapid diagnostic pathogen in clinical samples play critical role in improve patient care because the accurate identification of pathogen at species level and antibiogram sensitivity consider the first line in treatment and control on infectious diseases (Croxatto *et al.*, 2012).

Phenotypic detection of (MLSB)

This method is concluded by detecting the resistances to erythromycin disc, inducible resistance to clindamycin which occur when erythromycin diffuse to ward clindamycin disc will produce flattening inhibition zone of clindamycin disc in the margin adjust to the erythromycin disc to forming D-shape (Sedighi *et al.*, 2009).

Resistance in *S. aureus* bacteria have done if it have *erm* genes, erythromycin can attach to mRNA leading to get the cell start codon of methylase gene as a result methylation is done (Zmantar *et al.*, 2011), so changing in erythromycin binding site has been occurred also overlapping in binding site of the three mentioned classes account for cross-resistance, resistance to erythromycin produced not only by expression of *erm* genes constitutive way but also by inducible way if present of erythromycin molecules which induce production of methylase (Stefanie and Gallert, 2014).

The results of this study have been revealed four distinct resistance phenotypes, as shown in (table 3).

As shown in (table 3) the results of double diffusion disc (18 out of 74) *S. aureus* isolates is constitutive resistance to erythromycin cMLSB phenotype (24.32%), which is distributed to(13 out of 61) MRSA (21.31%) and MSSA (3 out of 13) (38.46%), (6 out of 74) *S. aureus* isolates sensitive to erythromycin (9.83%) all of them is MRSA, (4 out of 74) *S. aureus* isolates inducible resistance to erythromycin D-shape (6.55%) also all of them is MRSA, (9 out of 74) *S. aureus* isolates showed MS phenotype which is resistance to erythromycin and sensitive to clindamycin without D-shape (14.75%) and finally (29 out of 74) *S. aureus* isolates showed intermediate resistance to erythromycin distributed between MRSA and MSSA as shown in (table 3). MLSB are antibiotics used commonly to treat *S. aureus* infection, additional to clindamycin which is used frequently to treat skin and superficial infection especially with patients have allergy to penicillin as alternative drug (Sedighi *et al.*, 2009). In the current study, it was observed a higher prevalence of the cMLSB phenotype in the MSSA isolates compared with MRSA isolates The results of this study disagree with others studies

have showed higher frequency of constitutive resistance to erythromycin in MRSA isolates Bottega *et al.* (2014) have been mentioned (20 out of 29; 68.9%) MRSA have cMLSB also (Seif *et al.,* 2012) have been observed found this phenotype in (52.3%) MRSA isolates. On the other hands, it have been observed iMLSB phenotype among clinical samples of *S. aureus* specially MRSA isolates more frequently in urine, blood and general secretions, this founding relatively similar with Bottega *et al.* (2014) he mentioned (3 isolates of MRSA showed D- test positive (2.1%). Furthermore the results disagree with anther author mentioned toprevalence of MS phenotype among MRSA is high (44.6%) (Lyall *et al.,* 2013). In fact macrolides, lincosamides and streptogramin B all of them have same target site which is protein biosynthesis, erythromycin belong to macrolides and can induce cross-resistance against two others groups iMLSB, So *S. aureus* isolates which appear resistance to erythromycin will resist to linocosamides and strptogramin B (Stefanie and Gallert, 2014). The prevalence of iMLSB phenomenon is found in all *Staphylococcus* species pathogenic and non-pathogenic to human so *Staphylococcus* isolates can play critical role as reservoir for resistance genes and possibly consider the source of spread them to environment, because the little concentration of erythromycin even pico-molar can induce resistance against lincosamides so it consider potential risk for human health (Stefanie and Gallert, 2014).

Genotypic detection about MLSB

This study have been revealed the prevalence of *erm* A gene (7.35%) in five out of sixty-eight isolates of *S. aureus* have different phenotypically resistance forms to erythromycin all five isolates is MRSA, (4 out of 5) isolates harboring with *erm* A gene have constitutive resistance to MLSB and only one isolates appeared MS that is meaning resistance to erythromycin and sensitive to clindamycin as shown in table (3-3). This finding is agree with Zmantar *et al.* (2011) observed incidence of *erm* A (7.7%) in MRSA isolates while disagree with Paniagua-Contreras *et al* (19) have been observed incidence of *erm* A gene (100%), *erm* B gene (100%)and *erm* C (9.5%) in MRSA isolates.

others founding in this study present of *erm* C gene (5.88%) which is recovered from four out of sixty-eight isolates, two of them showed cMLSB phenotype and harboring with *erm* A gene. on the other hands two isolates recovered from blood and sputum showed inducible MLSB with D-shape phenomenon, unfortunately none of our MRSA isolates harboring with *erm* B gene. This finding is correspondence with many studies have been done to investigate harboring the clinical isolates of *S. aureus* showed *erm*A and *erm*C predominant *erm* genes found in *S. aureus* and CON (Gherardi *et al.,* 2009). *S. aureus* isolates can resistant to macrolides by two mechanism, ATP-dependent efflux pump which encoded by *mrs* A gene also macrolides efflux effected by the role of membrane protein which coded by *mef* gene (Zmantar *et al.,* 2011). Another mechanism which is done by *erm* genes family which have nearly forty types of erm genes, expression only one type can lead to resistance against MLSB antibiotics (Stefanie and Gallert, 2014). The resistance presented by single alteration in ribosomal target site, *erm* genes encode to N6-demethylase which play the role in N6 demethylation of an adenine residue in the 23S rRNA causing conformational alteration in ribosome and increase resistance of *S. aureus* strains to MLSB group (Martineau *et al.,* 2000). As it mentioned previously in

phenotypic detection of MLSB, four isolates of MRSA showed D-shape, two of these isolates (MRSA NI and MRSA S21) had none of three resistance genes, it maybe belong to the high diversity of this gene family specially in clinical isolates additional to many different spices of *Staphylococcus* and high rate of horizontal gene transfer with them.

Table.1 Primers used for PCR amplification

primer	Primer sequences 5……..3	Origin	Product size (bp)	Reference from
*mec*A F	GTAGAAATGACTGAACGTCCGATAA	Oligo Data, South, Africa	314	Cabrera *et at*., 2010
*mec*A R	CCAATTCCACATTGTTTCGGTCTAA			
*erm*A F	TAT CTT ATC GTT GAG AAG GGA TT	Oligo Data, South, Africa	139	Martineau *et al.*, 2000
*erm*A R	CTA CAC TTG GCT TAG GAT GAA A			
*erm*B F	CTA TCT GAT TGT TGA AGA AGG ATT	Oligo Data, South, Africa	142	Martineau *et al.*, 2000
*erm*B R	GTT TAC TCT TGG TTT AGG ATG AAA			
*erm*C F	CTT GTT GAT CAC GAT AAT TTC C	Oligo Data, South, Africa	190	Martineau *et al.*, 2000
*erm*C R	ATC TTT TAG CAA ACC CGT ATT C			

Table.2 distribution of Staphylococci belong to source of isolation

Source of isolation	No. of isolated	*S. aureus*	*S. epidermis*	Percentage of *S. aureus* from total isolates 74
Skin infection	43	38	5	51.35 %
UTI	15	12	3	16.21 %
Blood	5	3	2	4.05 %
Ear infection	5	5	0	6.75 %
Nasal infection	3	3	0	4.05 %
Eye infection	2	2	0	2.70 %
Sputum	1	1	0	1.35 %
Seminal fluid	1	1	0	1.35 %
Environmental of hospitals	9	9	0	12.16 %
Total isolates	84	74	10	100%

Table.3 Phenotypic results of S. aureus isolates toward MLSB

S. aureus	MRSA 61 out of 74	100%	MSSA13 out of 74	100%	total
Both Erythromycin -clindamycin resistance cMLSB phenotype	13	21.31	5	38.46	18
Erythromycin resistance-clindamycin sensitive D-shape iMLSB phenotype	4	6.55	0	0	4
Erythromycin resistance-clindamycin sensitive no D-shape MS phenotype	9	14.75	0	0	9
Both erythromycin- clindamycin sensitive	6	9.83	0	0	6
Erythromycin intermediate-clindamycin sensitive	3	4.91	1	7.69	4
Erythromycin intermediate- clindamycin intermediate	9	14.75	3	23.07	12
Erythromycin intermediate- clindamycin resistance	11	18.03	1	7.69	12
Erythromycin resistance- clindamycin intermediate	6	9.83	2	15.38	8
Erythromycin sensitive- clindamycin intermediate	0	0	1	7.69	1
Total	61	100	13	100	74

Table.4 Prevalence of erm A, B and C among MRSA isolates

Strain	Isolation site	cMLSB	iMLSB	MS	ermA	ermB	ermC
MRSA U2	Urine	-	-	+	+	-	-
MRSA U4	Urine	+	-	-	+	-	-
MRSA S12	Skin	+	-	-	+	-	-
MRSA S24	Skin	+	-	-	+	-	+
MRSA SP1	Sputum	-	+	-	-	-	+
MRSA B1	Blood	-	+	-	-	-	+
MRSA Y1	Eye	+	-	-	+	-	+

Figure.1 Diagram explain D-Test negative and positive results on Müller-Hinton agar (designed according to this study)

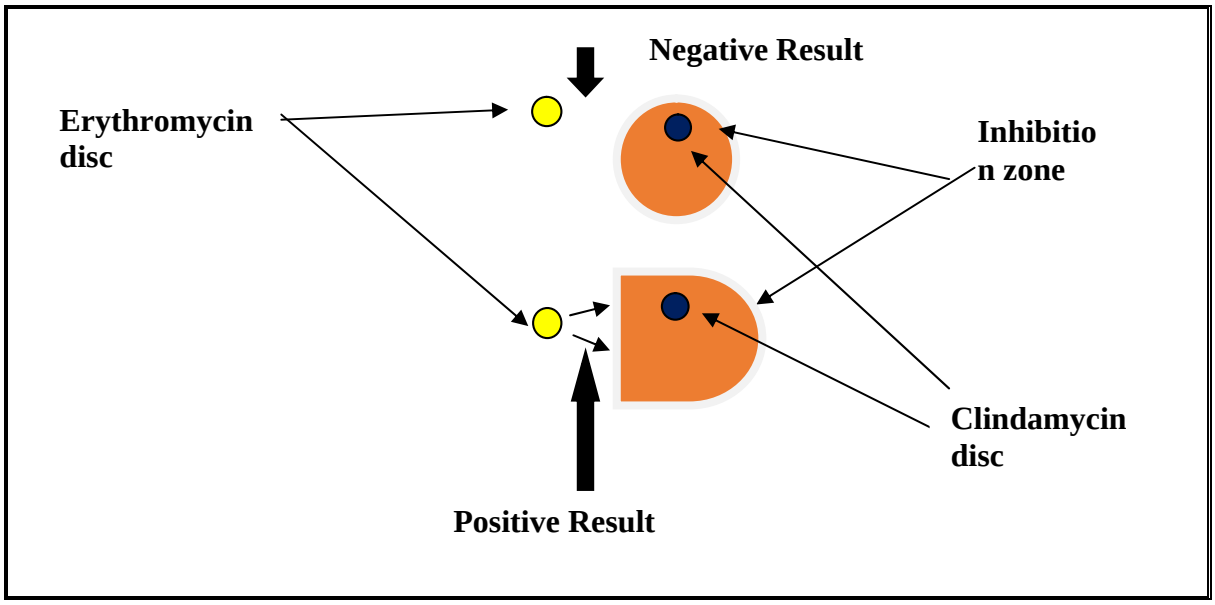


Figure.2 D- Shape positive iMLSB for S. aureus isolates

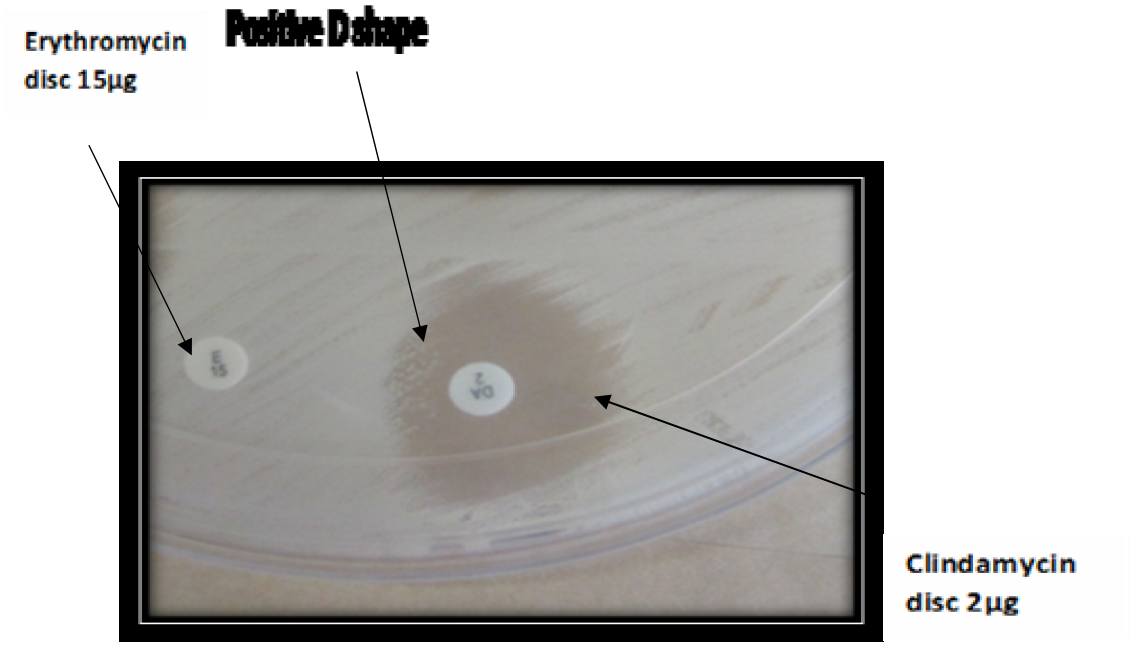


Figure.3 Agarose gel electrophoresis (1% agarose, 7V/cm, for 90 min) for mobile genetic element mec A (amplified size 314 bp) compared with (100 bp) DNA ladder line M DNA Ladder; lines 2,3,6 and 7 positive results of bands; lines 1and 5 negative results

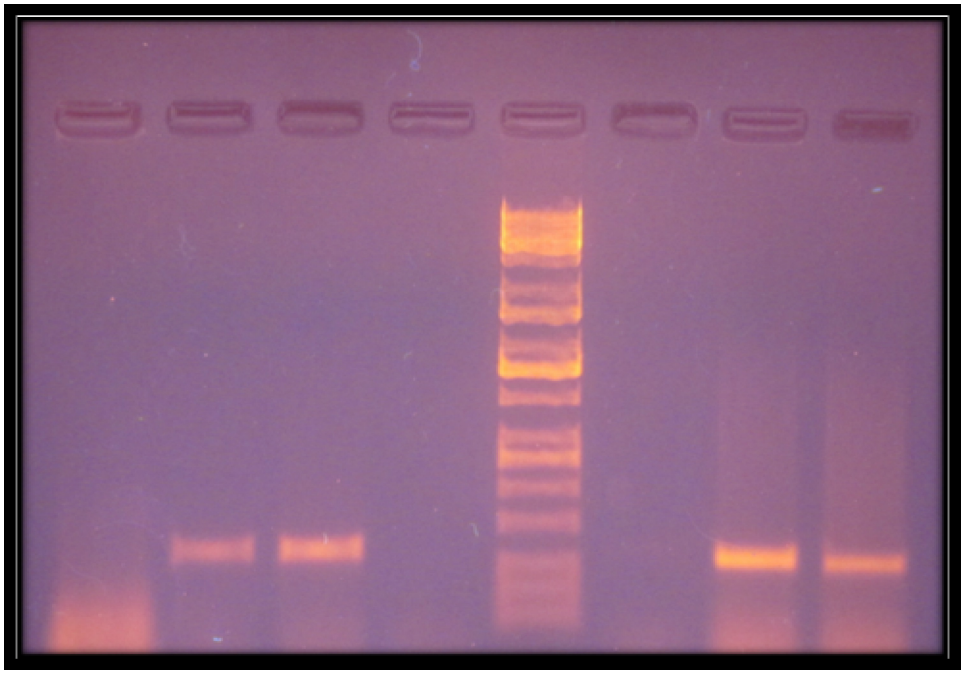
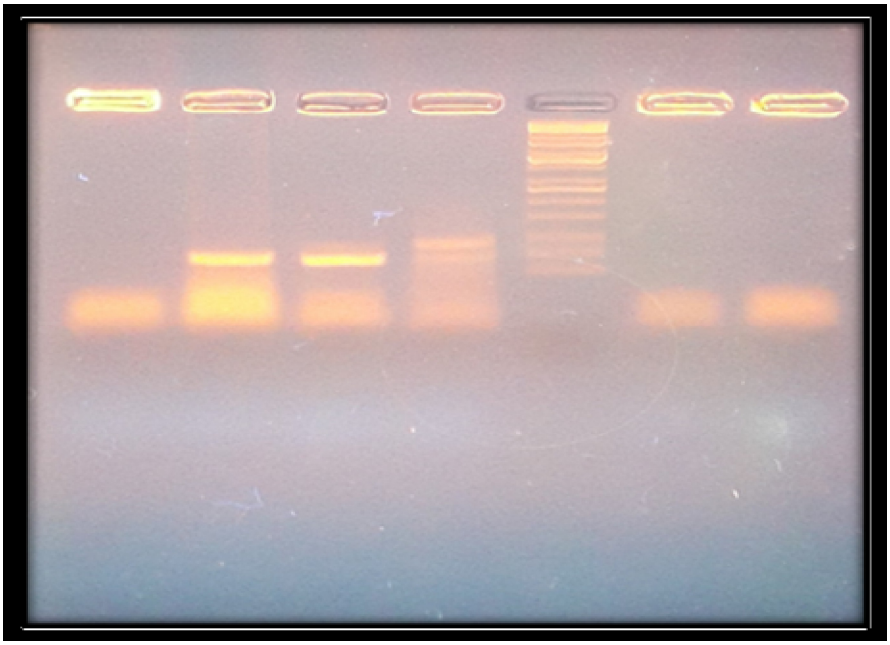


Figure.4 Agarose gel electrophoresis (1% agarose, 7V/cm, for 60 min) for *ermA* gene (amplified size 139 bp) and *ermC* (amplified size 190 bp) compared with (100 bp) DNA ladder line M DNA Ladder; lines 2,3 and 4 positive results of bands; lines 1,6 and 7 negative results



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