Isolation of Multi Antibiotic Resistance *Serratia marcescens* and the Detection of *AmpC* & *GESβL* Genes by Polymerase Chain Reaction Technique

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ABSTRACT

A total of (420) specimens were collected from Welfar teaching hospital of pediatrics and Al-Kindi hospital, during the period from November, 2007- February, 2008, from patient with ages of (2-120 years and include (300) clinical specimens (which include wound swabs, Sputum, urine and blood specimens), and (120) environmental specimens. Fifty were positive cultures, the identification of microorganism revealed that 21% of the positive cultures were *Serratia marcescens*. The most frequent isolation was from urinary tract infection, followed by wound, respiratory tract infection and blood sepsis. For identification, these isolates cultured on DTC (Deoxyribonucleic acid, Toludin blue, Cefalotin) agar were used and the typical colonies formed red hallo for several millimeters around it. During the identification of *S. marcescens* species Pseudomonas spp. were occasionally present and were easily differentiated by weak or negative DNase reaction, positive oxidase test and forming green colonies on the above culture medium. Multiple antibiotic resistances by the strains are an important subject to be considered of high virulence. Accordingly, isolates in this study were multiple resistances to more than one antibiotic and said to be multidrug resistance. 13 isolates were resistant to (11-12) antibiotics while 12 of the isolates were resistant to (8-10) antibiotics. Molecular method was carried out for detection of *AmpC* and *GES* encoded gene, results showed that among (5) isolates carry the *AmpC* gene which is a type of ESβLs that gives resistance to the bacterium whereas no isolate carrying *GES* gene and this genotyping method for detection ensured the phenotyping method used for ESBL detection as in this method, no isolate gave positive result.
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INTRODUCTION

Serratia marcescens is an aerobic Gram-negative bacillus which can survive well in moist environments (1). S. marcescens has been reported to cause variable infections, including respiratory tract, urinary tract, and wound infections and bacteremia (2). S. marcescens often develops multidrug resistance and tends to spread rapidly in the nosocomial environment, and has been implicated in outbreaks of nosocomial infection both in neonates and adults. S. marcescens is more likely to colonize the respiratory and urinary tracts of hospitalized adults but the gastrointestinal tract in neonates (3).
The high resistance noticed an alternative agents for the prevention and treatment of *S.marcescens* infection is urgently needed (4). *S.marcescens* have been found to be resistant to Gentamicin ,Ofloxacin ,Ampicillin, Chloromphenicol and Amikacin (5), While Chen *et al.* (6) referred that this microorganism shows intrinsic resistance to many antimicrobial agents. *S. marcescens* shows intrinsic resistance to wide variety of antimicrobial agent (multi drug resistance), multi drug efflux pumps are often involved in multi drug resistance in *S. marcescens* (6). Matsuo *et al.* (7) referred that drug efflux pumps is a major cause of multi drug Resistance and has been found to play a major role in intrinsic resistance of *S. marcescens*. Yu *et al.* (8) showed resistance of *S. marcescens* is usually mediated by overproduced AmpC cephalosporinase.

Study of epidemiologic markers is important in an attempt to trace the source of contamination or to prevent patient-to-patient spread (9) . The phenotypic ESBL detection methods described above provide only presumptive identification of an ESBL producer. An alternative approach is to detect the β-lactamase gene. The easiest and most common molecular method used to detect the presence of a β- lactamase is nucleic acid amplification with oligonucleotide primers. PCR with oligonucleotide primers that are specific for a β-lactamase gene is the easiest and most common molecular methods used to detect the prescence of a β-lactamase belonging to a family of enzymes (10).

**MATERIAL AND METHOD**

**Sample collection and isolation of organism:**

A collection of 420 samples were divided into: A:300 clinical specimens were obtained from patients with different infections (included wound swabs, urine and blood samples) referred to (Welfare teaching hospital of pediatrics and Al-Kindi hospital). Beginning of November,2007 until the end of February,2008 ,for children with (2-12) years. B: 120 swabs from different sites of hospital environments included (Welfare teaching hospital of pediatrics,Al-Kindi hospital),which include (operation room, instruments and furnitures, patient beds, wall and floor of hospitals).

All the specimens were streaked on MacConky agar and incubated aerobically overnight at 37C° to detect *S. marcescens*. Non lactose
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fermenting colonies were picked up with sterile loop and incubated on to DTC :(Deoxyribonucleic acid, Toludine blue, Cefalotin), and gelatin agar. The colonies of each representative isolates were then characterized using standard bacteriological methods earlier described (11)

**Antibiotic susceptibility:**

Antibiotic susceptibility test for isolated microorganisms was performed by disc diffusion and broth dilution method.(12) 

**β-lactams:** detected by two standard method (Iodometric method and Capillary tube method.) according to (13).

**Disk antagonism test (DAT):** The disk antagonism test was used to detect the inducibility of β-lactamase. Disks of inducing agent amoxicillin/clavulanic acid (Am/CA) and cephalosporins (ceftazidime and cefotaxime) were placed on the surface of the test bacterial lawn on MHA plates on a lawn of bacterial culture of the suspected inducible AmpC β-lactams producers separated by 15 mm. The plates were examined after overnight incubation at 37°C. If blunting of the cephalosporin disks adjacent to the amoxicillin/clavulanic acid disks occurred, the organisms were considered to produce inducible AmpC β-lactams. (14).

**oligonucleotide primers used in PCR.** The sequences of the primers used in the PCR are listed in below :

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequences</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>AmpC</em></td>
<td>CCTGCAACCTAAGAGCTTCT</td>
<td>15</td>
</tr>
<tr>
<td><em>AmpC</em></td>
<td>GCGCCTGGATGATGTGGTAA</td>
<td>15</td>
</tr>
<tr>
<td>GES1F</td>
<td>AAAGCAGCTCAGATCGGTGT</td>
<td>16</td>
</tr>
<tr>
<td>GES1R</td>
<td>TCATGTGTCCCGATGGTAGA</td>
<td>16</td>
</tr>
</tbody>
</table>

**PCR procedure.** Template DNAs were prepared from each strain as described previously (9). PCR amplification was performed in a 50-ml volume with the TC-5000 PCR system (USA) thermal cycler. Reaction mixtures contained 2μl (each) primer, PCR master mix 25μl , approximately 3 μl of template DNA and completed with 18μl Nuclease Free Water . PCR steps programmed for AmpC & GESβL performed according to Mahlen et al.,2003 and De Vires et al.,2006 respectively. After agarose gel electrophoresis, the ethidium bromide-stained PCR products were visualized under UV light. In the amplification of the intI3 gene, the
annealing step was carried out at 578°C. For more rapid and convenient template DNA extraction, the supernatant of a bacterial culture suspension boiled for 10 min was applied as described previously.

Results and discussion

A total of (420) specimen was included in this study of these. There were (300) clinical specimens, and (120) environmental specimens. The (300) clinical specimens comprises, wound swabs, urine and blood specimens resulted in (50) positive cultures and the identification of microorganism revealed that 21(%) of the positive culture was Serratia spp. (table 1).

Table 1: Number and frequency of *Serratia marcescens* isolated from different clinical specimens

<table>
<thead>
<tr>
<th>Clinical specimens</th>
<th>No. of specimen</th>
<th>No. of isolates</th>
<th>% of isolation from specimen</th>
<th>% of isolation from total isolations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urinary tract infection</td>
<td>150</td>
<td>12</td>
<td>8</td>
<td>57.14</td>
</tr>
<tr>
<td>Wound infection</td>
<td>75</td>
<td>5</td>
<td>6.6</td>
<td>23.8</td>
</tr>
<tr>
<td>Respiratory tract infection</td>
<td>40</td>
<td>2</td>
<td>5</td>
<td>9.52</td>
</tr>
<tr>
<td>Bacteraemia</td>
<td>35</td>
<td>2</td>
<td>5.7</td>
<td>9.52</td>
</tr>
<tr>
<td>Total</td>
<td>300</td>
<td>21</td>
<td></td>
<td>100</td>
</tr>
</tbody>
</table>

*S. marcescens* isolated from urinary tract infections in frequency of (57.14%) was the most frequent, followed by wounds and respiratory tract infections, blood sepsis (23.8%) respectively. Wound cultures showed mixed infection with Pseudomonas spp., while one of the positive blood cultures was *S. liquificans* and has been excluded from this study. Contamination of hospital environment was detected in (120) specimens collected from different sites including walls, floors, and hospital beds (table 2). (18) revealed that *S. marcescens* was rarely isolated from clinical specimens. Study results matched with (19), who could isolate 23 clinical isolates referred to *S. marcescens* from different sites of the body and this
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also matched with (20), who obtained 6 isolates of *S.marsceecn* from total number of 32 different Gram negative isolates mostly from urine, pus, throat swabs and blood specimens. The highest frequency of isolation of *S.marsceecn* was from UTI (57.14%) from total *S.marsceecn* isolates, this result matched with the findings of (21), who stated that *S.marsceecn* tend to colonize urinary tract of adults in patients.

Most of the isolates responsible for hospital environment contamination were Gram negative bacilli, the most frequent isolates were *Pseudomonas* spp., Gram positive bacteria were also observed as a source of hospital environment contamination. This result supported by (22) study which showed that the environmental screening yielded 4 strains of *S.marsceecn*. That matched with (23), who reported that environmental cultures were positive for *S.marsceecn* in 1.4% and confirmed that *S.marsceecn* was endemic in neonatal intensive care unit and belonged to one genotype.

**Table2: Number and frequency of *Serratia marcescens* isolated from different environmental specimens**

<table>
<thead>
<tr>
<th>Environmental specimens</th>
<th>No. of specimen</th>
<th>No. of isolates</th>
<th>% of isolation from specimen</th>
<th>% of isolation from total isolations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Walls and floors</td>
<td>40</td>
<td>2</td>
<td>5</td>
<td>50</td>
</tr>
<tr>
<td>Patient beds</td>
<td>40</td>
<td>1</td>
<td>2.5</td>
<td>25</td>
</tr>
<tr>
<td>Hospital equipments</td>
<td>40</td>
<td>1</td>
<td>2.5</td>
<td>25</td>
</tr>
<tr>
<td>Total</td>
<td>120</td>
<td></td>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>

**Identification**

Strains of *S. marsceecn* formed typical colonies on DTC agar forming red hallo for several millimeters around the colony. Colonies of *Pseudomonas* spp. were occasionally present, but these were easily differentiated because of their weak or negative DNase reaction, positive oxidase test and usually forming green colonies on the above culture medium. After 24 hrs. Of incubation *S. marsceecn* colonies appeared as circular, opaque, convex, with 2-15 mm diameter, it's color ranging from dark red to white. The use of MacConky agar supplemented with sorbitol
for primary isolation improved considerably the isolation rate of S. marscencens and detection from clinical specimens easier and time and cost efficient. (24) referred that Deoxyribonuclease (DNase) produced by S.marscencens is characteristic feature which is useful in distinguishing this organism from closely related members of Enterobacteriaceae.

**Antibacterial susceptibility testing:**

Revealed that all strains of S.marscencens showed 100% sensitivity to 4 antibiotics that were Imipineme, Aztereonam, Meropenem and Ciprofloxacine. On the other hand the S.marscencens strains were 100% resistant to 10 antibiotics which were Cephtaxin, Cephradin, Cephtazidime, Cefixime, Cephalothin, Penicilline, and were less resistance to 3 antibiotics that were. Amoxacilline, Erythromycine, Tetracycline 96%, 92%-68% respectively (fig.1) In addition, S.marscencens strains are variably sensitive to Vancomycin, Gentamicin and Amikacine. As shown in (fig.1) the most sensitive isolates were those which isolated from one of the two blood cultures (B24) as it showed sensitivity to (9) different antibiotics followed by wound isolates (W21) UTI isolate (U12) and one environmental isolate (E1) they were sensitive to 8 different antibiotics. The third isolate in sensitivity was environmental isolate (E2) which was susceptible to (7) different antibiotics the next isolate (E3, E4) environmental isolates (U5, U6, U8, U10, U11, U16, U17, U19, U20) are UTI isolates and one blood isolates B25 showed sensitivity to 6 different antibiotics U7 and R23 were sensitive to 5 antibiotics U9 and U14 were sensitive only 4 antibiotics from above results we can notice that the most sensitive isolate was blood culture isolate by being sensitive to 9 antibiotics and the most resistant isolates were urinary tract isolates by being sensitive to only 4 antibiotics and resisting 14 antibiotics. This result was supported by the study of (25), who was indicated that all S.marscencens isolates are sensitive to ciprofloxacine, also the result matched with (26) who was noted that S.marscencens is resistant to most of the antibiotics except carbapenemens. As well as (27) indicated that S. marcescens was generally susceptible to imipenem, followed by ciprofloxacine and ofloxacin by Agar diffusion susceptibility testing.

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Multiple antibiotic resistances:

Multiple antibiotic resistances are an important subject to be considered, since it has a role in controlling and directing the remedy policy in bacterial infections. Moreover, multiple resistances are a sign of higher virulence. In this study most of the isolates of *S. marcescens* showed multiple resistances to more than one antibiotic table (5).

*S.marcseccens* isolated from the clinical and environmental sources were divided into two groups (A and B) depending on the antibiotics they resist (table 6). Accordingly, the above table shows that group (B) was the dominant group in regard to antibiotic resistance, where 13 (52%) isolates were resistant to (11-12) antibiotics while group (A) 12 of the isolates were resistant to (8-10) antibiotics. A collective result obtained out of table (6) indicates that the dominant pattern of resistance among *S.marcseccens* isolates of all groups was the resistance to all 11-12 antibiotics. (28) described an outbreak of multi-drug resistant *S.marcseccens* Infection and colonization involving adults admitted to surgical intensive care unit. (6) *S.marcseccens* shows intrinsic resistance to a wide variety of antimicrobial agent (multi drug resistance) multi drug efflux pumps are often involved in multi drug resistance in *S. marcescens*.

**Table3: Multi-drug resistance of Serratia marcescens**

<table>
<thead>
<tr>
<th>Isolates symbol</th>
<th>No. of isolates</th>
<th>% of isolates</th>
<th>No. of antibiotics resistat by isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>S₁</td>
<td>1</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td>E₂/E₃/U₄/B₁</td>
<td>4</td>
<td>16</td>
<td>9</td>
</tr>
<tr>
<td>E₄/E₅/W₆/U₇/U₈/U₉</td>
<td>7</td>
<td>28</td>
<td>10</td>
</tr>
<tr>
<td>W₁/W₄/W₅/U₂/U₃/U₄/U₁₁/B₁/S₂</td>
<td>10</td>
<td>40</td>
<td>11</td>
</tr>
<tr>
<td>U₁/U₁₀/U₁₂</td>
<td>3</td>
<td>12</td>
<td>12</td>
</tr>
</tbody>
</table>
Table 4: Classification of *S. marcescens* according to number of antibiotic resistant

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of antibiotics resistat by isolates</th>
<th>No. of isolates</th>
<th>% of isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>8-10</td>
<td>12</td>
<td>48</td>
</tr>
<tr>
<td>B</td>
<td>11-12</td>
<td>13</td>
<td>52</td>
</tr>
</tbody>
</table>

**β-lactamase detection: A-Capillary tube method**

To detect the ability of *S. marcescens* isolates to produce β-lactamase enzyme depending on pencilloic acid production as a result of β-lactamase enzyme action. This method is considered one of the rapid in testing the of β-lactamase. Out of (25) isolates 5(20%) were β-lactamase producers, one (4%) isolate gave result after (10) minutes. While 4(16%) isolates gave result after (5) minutes. Whereas 20(80%) isolates were β-lactamase negative (table 7).

Table 5: β-lactamases production by Capillary tube method from *S.marcescens*

<table>
<thead>
<tr>
<th>Result</th>
<th>No. of isolates</th>
<th>% of isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>+++</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>++</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>+</td>
<td>4</td>
<td>16</td>
</tr>
<tr>
<td>-</td>
<td>20</td>
<td>80</td>
</tr>
</tbody>
</table>

+++ Through 5-10 min  ++ Through 10-15 min  + After 15 min  - No production

**B-Iodometric method:**

Iodometric method is important in primary investigation for β-lactamase enzymes production. The method depends on the reaction between Iodine and starch to form blue of dark violet complex, while during β-lactamase enzyme production it will hydrolyse penicilline G and produce inactive pencilloic acid that reduce Iodine to Iodide, the last have no ability to react with starch, so color will be changed to white, (table 8) shows that 6(24%) isolates out of 12 isolates were able to produce β-lactamase enzyme,
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One isolate gave rapid positive result that indicate its ability to produce large amounts of β-lactamase enzyme.

**Table 6: β-lactamases production by Iodometric method from Serratia marcescens**

<table>
<thead>
<tr>
<th>Result</th>
<th>No. of isolates</th>
<th>% of isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>6</td>
<td>24</td>
</tr>
<tr>
<td>Negative</td>
<td>19</td>
<td>76</td>
</tr>
</tbody>
</table>

From the above, noticed slightly difference between Iodometric & Capillary tube method. Six isolates of *S.marscecens* produced β-lactamase enzyme by Iodometric method, but only 5 isolates of *S.marsceccens* produced β-lactamase enzyme by Capillary tube method. As shown in (figure 2).

![Figure 2: Difference between two methods (Iodometric & Capillary tube) for β-lactamases production](image)

(29) pointed that the production of β-Lactamase enzyme may be insufficient which may be found in the periplasmic space, as the presence of enzyme in this site one of the causes that leads to difficulties in detecting it. Results also showed difference in the time of production the enzyme, and this result is similar to (30) result which showed that the difference in the time of production the enzyme may belong to the difference in the enzyme concentration in periplasmic space , in addition to the presence of stimulatory factors for producing factor which the antibiotic have a role in decreasing or increasing the enzymatic activity.
**Extended Spectrum β-Lactamase production:**

With the spread of AmpC and ESBLs producing strains all over the world, it is necessary to know the prevalence of these strains in hospitals. Detection of resistant isolates would allow doctors to formulate a policy of empirical therapy in high-risk units where infections due to resistant organisms. The method that used in this study to detect ESBL producing bacteria was the disk approximation test. All the (25) bacterial isolates used in the study have no synergy zone of inhibition between β-lactam antibiotic and Amoxicillin /Clavulanate which mean that they are no ESBL producers. The test done by using different antibiotics which where Augmentin (Clavulanic acid /Amoxicillin), Cefotaxime, Ceftazidime and Aztreonam. The result of this study was in agree with the findings of (31) which showed that from 24 isolates of S.marsecens non susceptible to Cefotaxime ,only one ESβLs producer was found by using ESBLs screen test. The result of this study was in agreement with the finding of Cheng et al. (2006) finding that showed 5(12%) ESβLs producers were identified out of 123 non repetitive isolates of S.marcescens and they recommended that the detection and report of ESβLs production by S.marcescens in clinical laboratories must be made mandatory. in the same way (15) showed that S.marcescens isolates are AmpC type β-lactamase Producers that indicate by imipinem and cefoxitin.

**Polymerase chain reaction:**

After all the S.marcescens isolates were tested for its ability to produce β-Lactamase enzyme two phenotypic methods (Iodometric and Capillary) were carried out and there were different results of production the enzyme. After that two tests of detection the extended Spectrum β-Lactamase was done by double disks diffusion method. All (25) isolates didn't show any ability to produce the GESBLs, while (5) isolates showed ability to produce Extended Spectrum β-Lactamase type AmpC by PCR as shown in fig4-3. Because all of that genetic method was adapted using specific primers to detect the presence of the genes in the bacteria, (25) isolates were tested and the results showed that there is no presence of GESβL n all of them, while 5 isolates showed the genes encoded for AmpC enzyme and this result agreed with sensitivity test results as they was the most resistant isolates to β-Lactamase enzymes antibiotics (multiple drug resistant). The environmental isolates of S.marsecens didn't seem to have
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genes encoded for GESβL and ampC, whereas among (10) clinical isolate (5) was carrying ampC gene and this indicates its pathogenicity as it hold the chromosomal gene that encoding for resisting the antibiotics. The 5 isolates that was holding the AmpC gene encoding for β-lactamase enzyme production was those which were collected from urinary tract U5, U9, U14 and U6 and one isolate from respiratory tract R 23. These Five isolates exhibited either resistance or reduced susceptibility to Augmentin, Cefixime and Cefepime with MICs 1024 µg/ml for each of them except U6 which have MIC of 512 µg/ml to Cefixime and U5 which have MIC of (256) µg/ml to Cefepime. On the other hand the isolates were susceptible to Ciprofloxacin and Imipenem with MIC of 4 µg/ml for each one except U6 that exhibited MIC of for Imipenem and MIC of 2µg/ml to Ciprofloxacin by all (5) isolates. This result agree with (32) found five *S.marscecens* isolated from UTI were positive for chromosomal AmpC. And agree with (15) which showed the amplified DNA segment (AmpC gene) have molecular weight near to 1100 base pair. While the other (20) *S. marcescens* is a lots were negative for chromosomal AmpC, Indication that another mechanism, in addition to the AmpC is also involved in the determination of the resistance phenotype of *S. marcescens* strains. (33) referred that *S. marcescens* strains poses different mechanism of resistance to the expanded spectrum Cephalosporin, beside AmpC expression, Acquisition of Ambler class A extended spectrum β-lactamase (ESβLs) or class B metalo β-lactamase also confers resistance cephalosporin's (34).The different phenotype resistance pattern of *S. marcescens* may be due to, regulation of the AmpC expression occurs at multiple levels, which might be involved in the mechanism(s) that determines the differing AmpC activity of *S. marcescens* strains (15). The process that results in the emergence of these β-lactamase resistant *S. marcescens* strains in the hospital is unclear, but frequent use of extended spectrum cephalosporin's could result in the emergence of resistance strains producing a chromosomal AmpC in fact based on in vitro antibiotic selection experiments, (35) proposal that increased use of extended spectrum cephalosporin's may eventually, leading to the emergence of extended-spectrum cephalosporin's-resistant strains. Our isolates were tested for primary detection of β-lactamase enzymes prescens .Among (25) isolates of *S.marcescens* only 4 gave positive result that shows its ability to produce β-lactamase enzyme. Number of studies pointed that *S. marcescens* have the ability to produce chromosomal β-
lactamase enzymes that stimulated by the antibiotic which have β-lactam ring. (36) found that all *S.marcescens* isolates (77) were AmpC producers that chromosomally encoded. (37) showed of 72 isolates, 16 isolates were de repressed AmpC and 22 isolates produced extended spectrum β-lactamase, the most prevalent ESβLs were CTX-M enzyme followed by TEM-S2 SHV-12. (2) referred that ESβLs production occurred in about 19% of *S.marcescens* isolates. Not all ESβL producers universally resistant to any of ESBLs. They vary in their substrates specificity and may not phenotypically express resistance to its own substrate. (38). The coexistence of both ESβLs and AmpC-type β-lactamase may result in false negative tests. AmpC type β-lactamase resists inhibition by clavulnate and hence obscures the synergistic effect of clavulnate and cephalosporin against ESβLs (10).

**Figure 3:** Agarose gel electrophoresis and Ethidium bromide staining. to detect GESβL gene Lane M, molecular size DNA ladder (123 bp DNA Ladder); lanes 1-5, DNAs isolated from *S. marcescens* samples and all of these showed Negative PCR bands; lanes 6-7, negative control; The PCR was carried out in duplicate. The photocomposition of the figure was obtained with Gel documentary Camera.
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**Figure 4:** Agarose gel electrophoresis and ethidium bromide staining. To detect AmpC gene, Lane M, molecular size DNA ladder (123 bp DNA Ladder); lanes 1-14, DNAs isolated from S.marcescens samples and only lanes 4,7,8,11,12 showed Positive PCR bands; lanes 15, negative control; The PCR was carried out in duplicate. The photocomposition of the figure was obtained with Gel documentary Camera.

This survey showed that PCR is comparable in respect to the discrimination and reproducibility for epidemiological studies of *S. marcescens* strains in nosocomial outbreaks.

**REFERENCE**


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