American Journal of Medical Case Reports, 2014, Vol. 2, No. 6, 126-132
Available online at http://pubs.sciepub.com/ajmcr/2/6/5

DOI:10.12691/ajmcr-2-6-5

Virulance Factors Enhancing Microbial Infection in Chronic Osteomylitis and Antibiotic Susceptability Pattern

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Received June 21, 2014; Revised July 10, 2014; Accepted July 15, 2014

Abstract **Aims:** To isolate and identify the causative agents for chronic osteomyelitis in patients and understanding their virulence factors for identification of targets for novel drugs and design of new vaccines. **Study Design:** samples of swab and bone tissues biopsy from chronic osteomylitis patients were collected in order to study the infectious agents by investigating their virulence factors. Place and Duration of study: Twenty five specimens of osteomyelitis were collected from chronic osteomyelitis patients at Surgical Specialist Hospital, Al-Yarmook Teaching Hospital and Baghdad Teaching Hospital during June 2010 to May 2011. **Methodology:** a form was designed to be filled by each patient, which included information about their age, sex, duration of infection, infection site. Specimens were collected using a sterile cotton swab and bunch of biopsy. The pus was taken before the surgery by swabbing an open infected area and from the deeper part of the infected bone, while, biopsies were taken from osteomyelitis patients during surgical management at the operation theater. **Results:** from fifty swab and twenty-five bone tissue biopsy samples, the results showed high prevalence of osteomyelitis in male patients with 84% (21), and 16% (4) in females. It was found that chronic osteomyelitis has a high prevalence among males with 84%, and the highest incidence was recorded at the age group of 30-39 years. Cultural, microscopic examination and bioc-hemical characterization of both swab and biopsy specimens, showed that 50% of the isolates were *Staphylococcus aureus*, 26% *Enterobacter cloacae*, 14% *Pseudomonas aeruginosa*, 6% *Escherichia coli*, and 4% *Klebseilla sp.* From the results of the antimicrobial susceptibility test using different (11) antibiotic discs against *S. aureus*, *Enterobacter cloacae*, *Pseudomonas aeruginosa*, *E.coli*, and *Klebsiella sp*. six isolates of *S. aureus* were selected and designated as (S1, S3, S4, S7) which showed multiple resistances to antibiotics. **Conclusion:** It was found that multiple resistances *S. aureus* isolates were able to produce haemolysin enzyme, capsules, slime layer, biofilm and hyaluronidase enzyme.

Keywords: *Osteomyelitis, S. aureus, hyaluronidase, capsule, slime layer, biofilm*

Cite This Article: RANA A. MOHSIEN, MAYSAA CH. AL –MOHAMMEDAWI, and ABDUL GHANI I. YAHYA, "Virulance Factors Enhancing Microbial Infection in Chronic Osteomylitis and Antibiotic Susceptability Pattern." *American Journal of Medical Case Reports*, vol. 2, no. 6 (2014): 126-132. doi: 10.12691/ajmcr-2-6-5.

1. Introduction

Infection of bone, which is also called osteomyelitis, can be described as acute, sub acute, chronic, haematogenous and exogenous according to the duration and source of infection. [1] Osteomyelitis can become chronic, and can lead to the eventual death of the bone tissue, which is caused by loss of blood supply to the affected bone. This occurs when pus produced within the bone, causes bone abscess which deprives the bone of its blood supply [2].

Chronic osteomyelitis is polymicrobial, which means more than one infectious agent is involved. Many types of microorganisms, including viruses, and fungi, may cause osteomyelitis, but it is usually bacterial in origin. [3] demonstrated that the bacterial osteomyelitis causes substantial morbidity worldwide, despite continued progress toward understanding its pathophysiology and optimal management. *Staphylococcus aureus* and Gram negative bacteria were the most common organisms that causes osteomyelitis.

Another study was done by [4] who found that the dominance of *S. aureus* causes osteomyelitis and proposed that it may be multifactorial, which have an important role to cause infection, such as some enzymes, surface proteins, and toxins which were produced as virulence factors. The purpose of this study was to isolate and identify the bacteria causing agents among chronic osteomyelitis in Iraqi patients, detecting the most efficient antimicrobial agent against isolated osteomyelitis pathogens and the determination of minimum inhibitory concentration (MIC) against the most common bacterial isolates and detection

of some virulence factors of bacterial isolates, *such as* haemolysin enzyme, capsule, biofilm, slime layer and hyaluronidase enzyme production.

2. Materials and Methods

2.1. Samples Collection

Twenty five specimens of osteomyelitis were collected from chronic osteomyelitis patients from different hospitals of Baghdad (Surgical Specialist Hospital, Al-Yarmook Teaching Hospital and Baghdad Teaching Hospital) from June 2010 to May 2011. A form was designed to be filled by each patient, which included information about their age, sex, duration of infection, infection site. Fifty specimens were collected using sterile cotton swabs and biopsies were taken from different patches of bone tissues. The pus was taken before the surgery by swabbing an open infected area and from the deeper part of the infected bone, while, biopsies were taken from osteomyelitis patients during surgical management at the operation theater.

2.2. Isolation Characterization and Identification of Bacterial Isolates

Both of swab and biopsy (which was disrupted by mortar) samples were streaked inoculated aerobically on to plates of MacConkey agar, blood agar and Mannitol salt agar and incubated at 37°C for 24 hrs. After incubation, the colonies were sub-cultured onto petri dishes containing brain heart infusion agar and incubated as described above. Routine conventional laboratory techniques including Gram stain, motility, coagulase, oxidase, indole, catalase, dnase, citrate utilization, methylen red, Vogues Proskauer, and carbohydrates fermentation tests such as sucrose, glucose and lactose were all carried out by the methods described by [5,6]. Further identification of bacterial isolates was done at the Central Health Laboratory by using API 20E kits for identification of *Enterobacteriaceae*, while, API *Staph.* kits were used for identification of *Staphylococcus spp*. especially *Staphylococcus aureus*, *Staphylococcus aureus* ATCC 25392 was used as negative control.

2.3. Antimicrobial Sensitivity Test [7]

The disc diffusion method used in this study depends on some modification of Kirby-Bauer diffusion method. After inoculating Muller - Hinton agar medium with an overnight of bacterial culture suspension (1.5 x 10^8) the discs of antibiotic were fixed forceps on the surface of plates and the plates were incubated at 37 ºC for 18-24 hours. After incubation, the diameters of inhibition zones (clear area around discs) were measured by ruler.

2.4. Determination of Minimum Inhibitory Concentration (Mic) [8]

The MIC was determined by agar dilution method. Agar dilutions were prepared using Muller-Hinton agar medium supplemented with two fold serial dilutions of vancomycin, cephalothin, methicillin and tetracycline stock solutions ranging from (20-1280) µg/ml and fresh bacterial isolates were inoculated in this medium, then incubated at 37°C for 24 hours under aerobic conditions. The results of MIC after being incubated for each antibiotic were recorded and compared with the positive reaction to standard in [6].

2.5. Detection of Some Virulence Factors of *Staphylococcus Auerus*

Several virulence factors of highly resistant *S.aureus* isolates were investigated to test their ability to haemolysin, capsule, biofilm, slime layer and hyaluronidase production. [5,9,10,11,12,13].

2.6. Extraction of Plasmid DNA

Plasmid DNA extraction was achieved by Humphreys etal., using simple method for the preparation of large quantities of pure plasmid DNA [14].

2.7. Curing of Plasmid DNA

In order to determine the relationship between plasmid content and virulence factors of *A.hydrophila*, curing experiment was performed on the selected isolate using SDS as a curing agent [15]. After treatment of bacterial isolates with different concentrations of SDS (1-10)%, survivors were analyzes for the presence or absence of antibiotics resistance as result of eliminating the plasmid.

3. Results and Discusion

3.1. Distribution of Osteomyelitis Patients According to Age and Sex

Both swab and bone tissue biopsy specimens of twenty-five osteomyelitis patient samples showed that there were significant differences in prevalence of osteomylitis among males versus females when 84% (21) of them were infected, and only 16% (4) of females showed infection with P=0.024, as shown in Figure 1.

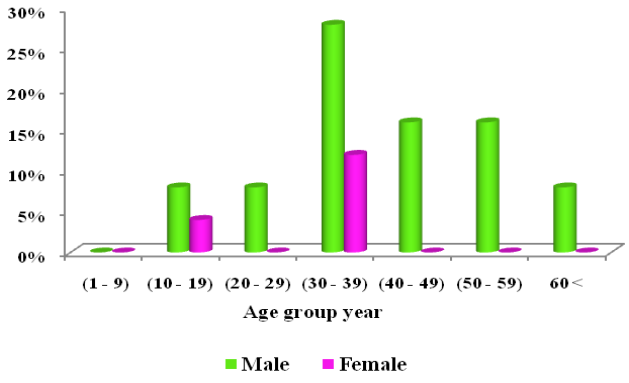

Figure 1. Distribution of osteomyelitis according to the patient's gender (21 were males and 4 were females) and from different age groups (1-9, 10-19, 20-29, 30-39, 40-49, 50-59 and 60<)

Our results were similar to those of [16] who found that the percentage of males was 80.36% and 19.64% for females among osteomyelitis patients. Also, [17] found that a high percentage of osteomyelitis patients were among males with 65% and females 35%.

This was due to the deterioration of the security situation in the country, due to that almost all of these

patients residence area were from hot spot risk area of Al Sader city, Shula and Shaeb, and especially males who were more exposed to trauma and accidents than females in Baghdad as in Figure 2.

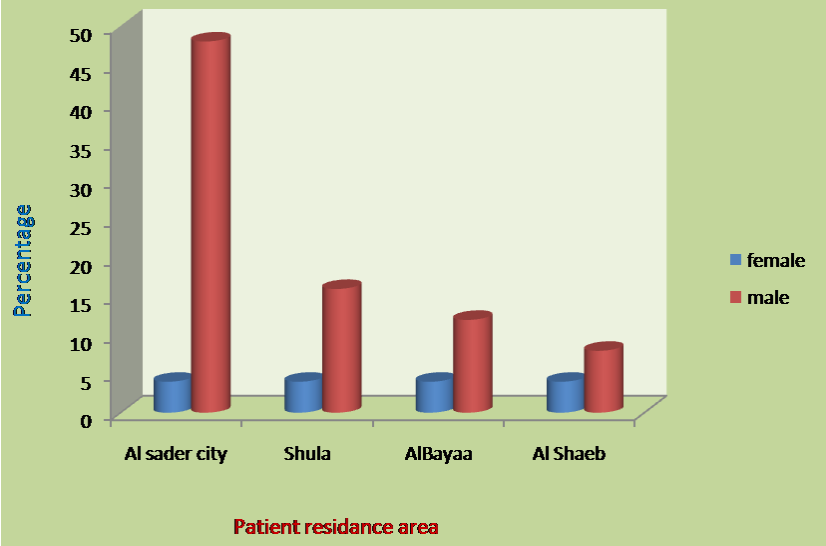

Figure 2. shows patients residence area in Baghdad

During 2011 some 2,771 civilians were killed and some 7,961 civilians were wounded, most of the violence was concentrated in and around Baghdad city capital.

On the other hand, result of various age groups ranging from teenager to over sixty years old showed that 12% prevalence of within the age group (10-19) was distributed between males and females as 8% and 4% respectively. Also, 8% (2) were the prevalence of males among age groups (20-29) and > 60. As well as, 28% (7) of patients were males and 12% (3) were females among aged group (30-39) and 16% (4) males were found among aged group (40-49) and (50-59) among osteomyelitis patients. Such findings were close to those of [18] who found a high prevalence of osteomyelitis in the age group (30-39) years (28.75%).

3.2. Identification of Bacterial Isolates

Out of the 25 cases of osteomyelitis, the bacteria were isolated in pure culture from 50 swab and biopsy osseous tissue samples obtained during a surgical procedure. Also, depending on cultural, microscopical examination and biochemical characterization, the results showed that 50 isolates of bacteria causing osteomyelitis were obtained as follows; 25 of them (50%) were identified as gram positive *Staphylococcus aureus*, 13 (26%) *Enterobacter cloacae*, 7 (14%) *Pseudomonas aeruginosa*, 3 (6%) *Escherichia coli* and 2 isolates (4%) were *Klebsiella* sp. as shown in Figure 3. Results of this study came according to that of [19] who found that *S.aureus* was the most isolated bacteria from the osteomyelitis patients with percentage (40%). While, [16,17] found that the highest percentage of infections (87%) was caused by *S. epidermidis*, while, *S. aureus* was caused by only 2% in osteomyelitis patients.

On the other hand, [20] stated that *Enterobacter* bone and joint infection are usually implicated in osteomyelitis in adults and children and the occurrence of *Enterobacter cloacae* was also found to be the second agent responsible for predisposing infection with a percentage of 26%. A study done by [21] found that hospitalized patients suffering from chronic osteomyelitis especially open fracture, are more predisposed to infections with Gram negative bacilli bacteria such as *Klebsiella spp.*, *Pseudomonas spp.* and *E. coli*.

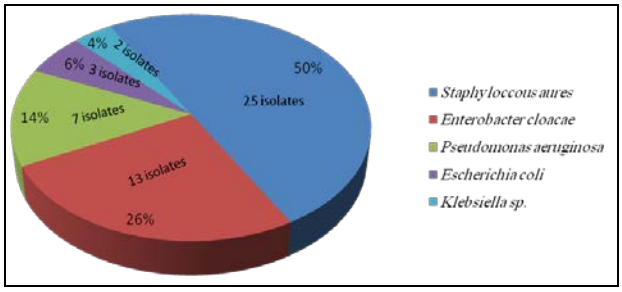

Figure 3. Percentages of bacterial isolates detected in the osteomyelitis patients

3.3. Antimicrobial Susceptability of Bacterial Isolates

From a total of 50 Gram negative and positive bacterial isolates, 12 of them were randomly selected to study their antimicrobial susceptibility toward different antibiotic groups. They were 6 isolates of *S. aureus*, 3 of *Enterobacter cloacae*, and one for each of *E. coli*, *Klebsiella sp.* and *Pseudomonas aeruginosa*. Results showed that all *S. aureus* isolates were completely (100%) susceptible to ciprofloxacin, chloramphenicol, novobiocin and rifampicin. In contrast, all of these isolates were totally resistant to methicillin, vancomycin, cephalothin and tetracycline followed by clindamycin, gentamicin and penicillin with the percentage of 83% as in Table 1. These results were in agreement with the study of [22] who found that *Staphylococcus* isolates were resistant to multiple antibiotics, including methicillin, tetracycline and vancomycin. *S. aureus* easily develops antibiotic resistance either by mutation which causes permanent alteration in the DNA base sequence of the organism or by transfer of genetic information from one organism to another, either through chromosomal recombination (transformation, transduction, conjugation or by extra-chromosomal–plasmid- transfer). In contrast, [2] found that 50% of *S. aureus* isolates were sensitive to

ciprofloxacin, but all (100%) to vancomycin, while, [23] reported complete resistance to ciprofloxacin, and low percentage (40%) of resistance to vancomycin, methicillin, cephalothin, as well, he revealed the total resistance percentage of *Enterobacter cloacae* to amoxicillin, cephalothin and penicillin among osteomyelitis patients. In contrast, the results were not in agreement with the study of [16] who found that the resistance to tetracycline was 25% for *Enterobacter* isolates which were sensitive to chloramphenicol. Also, resistance of *Enterobacter spp.* and *S.aureus* to the β-lactam antibiotic is due to their production of β-lactamase enzyme which break down the β-lactam ring in the structure of β-lactam antibiotics, such as penicillins group and cephalosporins group. [24].

Table 1. Antimicrobial susceptibility of *S.aureus* bacterial isolates

Bacterial isolate and symbol	ME	P	VA	CIP	C	KF	TE	CN	NV	CM	RA
S.aureus (1)	R	R	R	S	S	R	R	R	S	R	S
S.aureus (3)	R	R	R	S	S	R	R	R	S	R	S
S.aureus (4)	R	R	R	S	S	R	R	R	S	R	S
S.aureus (5)	R	S	R	S	S	R	R	R	S	R	S
S.aureus (7)	R	R	R	S	S	R	R	R	S	R	S
S.aureus (10)	R	R	R	S	S	R	R	S	S	S	S

R: resistant S: sensitive ME: Methicillin (5 µg/disc) P: Penicillin (10 µg/disc) VA: Vancomycin (30 µg/disc) CIP: Ciprofloxacin (5 µg/disc) C: Chlorophenicol (30 µg/disc) KF: Cephalothin (10 µg/disc) TE: Tetracycline (30 µg/disc) CN: Gentamycin (10 µg/disc) NV: Novobiocin (30 µg/disc) CM: Clindamycin (2 µg/disc) RA: Rifampacin (5 µg/disc).

As well, the results of the antimicrobial susceptibility of the three isolates of *Enterobacter cloacae* showed that they were resistant to ampicilllin, amoxicillin, cephalothin, tetracycline, vancomycin, rifampicin and penicillin, two of which are resistant to chloramphenicol and trimethoprim, while only one isolate is a resistant to gentamicin. Nevertheless, all of them are susceptible to nalidixic acid and ciprofloxacin. Furthermore, results of antimicrobial susceptibility of *Pseudomonas aeruginosa* and *E. coli* isolates showed that they were susceptible to ciprofloxacin but resistance to all other groups of antibiotics. *Klebsiella sp.* isolate was susceptible to chloramphenicol and resistance to all other groups of antibiotic. As shown in Table 2.

Table 2. Antimicrobial susceptibility of some Gram negative bacterial isolates

Bacterial isolate and symbol	AM	P	C	CN	SXT	KF	NA	TE	VA	RA	AMX	CIP
E. cloacae (6)	R	R	S	S	S	R	S	R	R	R	R	S
E. cloacae (12)	R	R	R	R	R	R	S	R	R	R	R	S
E. cloacae (13)	R	R	R	S	R	R	S	R	R	R	R	S
P. aeruginosa	R	R	R	R	R	R	R	R	R	R	R	S
E. coli	R	R	R	R	R	R	R	R	R	R	R	S
Klebsiella sp.	R	R	S	R	R	R	R	R	R	R	R	R

R: resistant S: sensitive AM: Ampicillin (10 g/disc) P: Penicillin (10 µg/disc) SXT: Trimethoprim (25 µg/disc) CIP: Ciprofloxacin (5 µg/disc) C: Chlorophenicol (30 µg/disc) KF: Cephalothin (10 µg/disc) TE: Tetracycline (30 µg/disc) CN: Gentamycin (10 µg/disc) AMX: Amoxicillin (10 µg/disc) NA: Nalidixic acid (30 µg/disc) RA: Rifampacin (5 µg/disc) VA: Vancomycin (30 µg/disc).

3.4. Minimum Inhibitory Concentration (MIC)

The results of the MIC of methicillin, vancomycin, cephalothin and tetracycline which were determined using four *S.aureus* isolates, showed that MICs of methicillin were 320 for S1, 80 for each of S3 and S7, and 40 µg/ml for S4. For vancomycin, MICs were 160 for S1, 640 for S3, 80 for S4 and 40 µg/ml for S7. In cephalothin, MICs were 320 for S1, 80 for S3, 640 for S4 and 160 µg/ml for S7. For tetracycline, MICs were 320, 80, 640 and 40 µg/ml for S1, S3, S4 and S7, respectively, as shown in Table 3. The results were close to the study of [25] who found that MIC of all *S. aureus* isolates obtained from osteomyelitis patients, was 625 µg/ml for each of tetracycline, cephalothin and vancomycin. [23] Found that MICs of *S. aureus* isolates obtained from bone infection were 40 µg/ml for each of methicillin, vancomycin, and tetracycline. From antimicrobial susceptibility and MIC results of *S. aureus* isolates from osteomyelitis, four highly resistance isolates (S1, S3, S4 and S7) were further studied and their ability to produce some of the virulence factors.

Table 3. The MICs of *S.aureus* isolates from osteomyelitis patients

Bacterial isolates	Methicillin	Vancomycin	Cephalothin	Tetracyclin
S1	320 (µg/ml)	160 (µg/ml)	320 (µg/ml)	320 (µg/ml)
S3	80 (µg/ml)	640 (µg/ml)	80 (µg/ml)	80 (µg/ml)
S4	40 (µg/ml)	80 (µg/ml)	640 (µg/ml)	640 (µg/ml)
S7	80 (µg/ml)	40 (µg/ml)	160 (µg/ml)	40 (µg/ml)

3.5. Detection of Some Virulence Factors by *Staphylococcus Aureus*

Studying the virulence factors of *S.aureus* is an important factor used in the identification of targets for novel drugs and design of new vaccines. So, the four *Staphylococcus aureus* isolates from osteomyelitis patients showed complete haemolysis on blood agar due to β- haemolysin production. In this regard, [26] found that 58.9% of *Staphylococcus aureus* strains in osteomyelitis patients, possessed β-haemolysis characteristics. In contrast, [16] found in their study on haemolysin as virulence factors of *Streptococcus spp.* in osteomyelitis patients, a percentage of 75% secreted haemolysins. Another important virulence factor of many bacteria, which can play a protective role for the pathogen is capsule formation as in *S. aureus*. The capsule may enable *S. aureus* to invade the bone tissue and cause osteomyelitis. All four isolates of *S. aureus* obtained from

osteomyelitis patients, were able to produce capsule in the two different used (capsule stain and negative stain methods). The results were close to the study of [25] who found the ability of capsule formation in most *Staphylococcus* isolates from osteomyelitis patients by using capsule stain method. Also, he used the negative stain in addition to capsule stain method and showed that the capsule stain was better than the negative stain method for detection of bacterial capsules. In our result, we found that the capsule stain method was more clear and accurate than the second one (negative stain) due to staining in both cell and its background which made the capsule more clear and accurate. Also, the probability of the loss of some cells that were not heat fixed to the slide during washing procedures as in negative stain method, thus the capsule stain method was better than the negative stain method.

Slime layer production may reflect the microorganism's capacity to adhere to specific host tissue and thereby produce invasive microcolonies. Many researchers consider it as a significant virulence factor for some strains of Staphylococci [9,28]. All four isolates of S.*aureus* from osteomyelitis patients were able to produce a slime layer by using the Congo red agar method as shown in Figure 4. Colonies appeared to be tightly bound to each other and had a black color and a rough surface, whereas non producing isolates showed smooth colony morphology and were loosely bound to each other and also had a pink color. The advantage of using a Congo red agar method which is described by [10], is its rapid, sensitive, and reproducible as well as the advantage that colonies will remain viable in the medium. Furthermore, it is not subject to the inter batch variation of media, which affects the reproducibility of the other methods such as Christensen method. This result was close to the study of [25] who showed that the rate of slime layer production positiveness was 77.8% for *S.aureus* isolates among osteomyelitis patients.

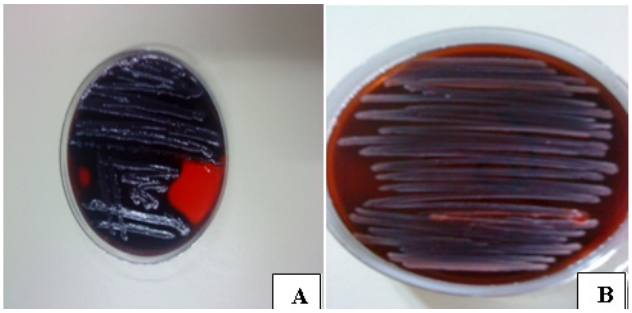

Figure 4. A- Slime layer production of *S. aureus* isolates shown the black colonies on Congo red agar. B- Non- producing isolates of slime layer shown the pink colonies on Congo red agar

Table 3-3. ELIZA reader values revealed the biofilm producer *S. aureus* isolates by TCP method

Staphylococcus isolates	Mean OD values	Biofilm formation
Control isolate	0.187	Non
S. aureus (7)	0.87	Moderate
S. aureus (1), (3), and (4)	1.768, 2.09 and 2.5	High

Our results showed that all four isolates of *S.aureus* from osteomyelitis patients, were able to produce biofilm. The results of three highly producer *S.aureus* isolates showed that the OD_{490} nm value of ELIZA reader was more than 1.500 (1.500< OD_{490} nm) while, only one moderate producer *S.aureus* isolate was in value ranged between 0.5-1.5 (0.500< OD_{490} nm < 1.500). Non producer isolates (control) showed the OD_{490} nm value less than 0.5 (0.500 > OD_{490} nm) as shown in Table 3.

[29] Found that the use of TCP method was more sensitive (96.2%) and specific (94.5%) with high accuracy (97.3%) in terms of discriminating between biofilm producers and non-producers. Consequently, high variability was observed and classification in biofilm positive and negative was difficult by tube method. The results were close to the study of [8] who showed that the rate of biofilm production positiveness was 74.4% using the TCP method for *S.aureus* isolates from osteomyelitis patients. As well, [4] found that 66.6% of *S.aureus* isolates using TCP method were able to form biofilm among patients with chronic disease.

Another virulence factor also studied is hyaluronidase using two different methods; turbidity reduction assay and plate method [11,13] and it was found that all four isolates of *S.aureus* from osteomyelitis patients were able to produce the hyaluronidase enzyme. The principle of using turbidity reduction assay for detection of hyaluronidase was based on reduction in turbidity of media when inoculated with hyaluronidase producing *S.aureus* isolates, after 30 min of incubation. The medium appeared as a clear broth (positive result) while, non-hyaluronidase producing isolate medium remained as a turbid broth (negative result). The second method (plate method) was done by determining the diameter zone of producing *S. aureus* isolates which were 27 mm, 18 mm, 7 mm and 6 mm for *S. aureus* isolates (4), (7), (1) and (3), respectively, as shown in Figure 5. This means that *S. aureus* isolates (4) and (7), respectively, were highly producers of hyaluronidase than the *S. aureus* isolates (1) and (3) that produced the enzyme in moderate level according to the diameter sizes of hydrolyzed zones in plates, at the ratio of 50%: 50%.

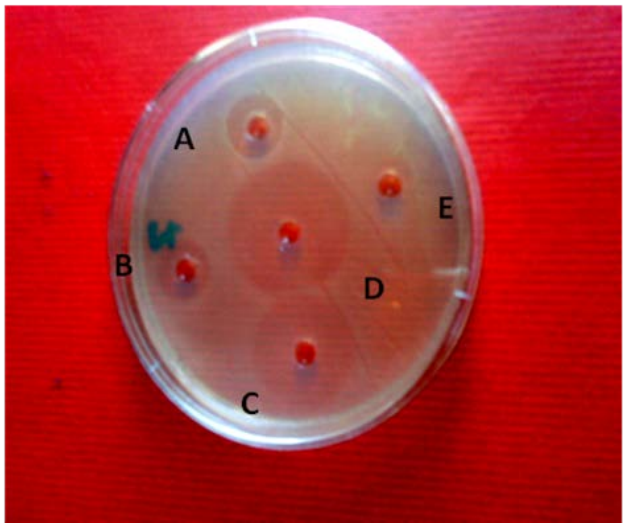

Figure 5. Production of hyaluronidase enzyme by *S. aureus* isolates using plate method showed the symbol [A] represented *S. aureus* (1) (7mm), the symbol [B] represented *S. aureus* (3) (6mm), the symbol [C] represented *S. aureus* (7) (18mm), while the symbol [D] represented *S. aureus* (4) (27mm) and the symbol [E] represented the control isolate

The non-degraded substrate by the enzyme precipitates as a conjugate with the albumin, leaving a hydrolyzed zone around those colonies which produce soluble enzymes that attack the hyaluronic acid. Plate method was a rapid screening to distinguish between hyaluronidase

producing and non producing isolates so, it was a qualitative test. Turbidity reduction assay was a quantitative test, as it was used to distinguish between enzyme producing and non producing isolates but it could not differentiate among producing isolates which could produce it in high or moderate level. These results were related to the study of [11] who found that only *S.aureus* isolates which cause bone infections possess hyaluronidase enzyme. This would suggest that hyaluronidase represented yet another potential virulence factor employed by *S.aureus* to cause disease and may represent a diagnostically important characteristic for distinguishing *S. aureus*.

3.6. Plasmid Profile and Eliminating Some of Antimicrobial and Virulance Factors of *S. Aureus* Isolates

Plasmid profile of the clinically isolated *S.aureus* S3, S4 and S7 were studied by extraction genomic DNA according to Humphreys etal., using simple method for the preparation of large quantities of pure plasmid DNA [14]. Results mentioned in Figure 6 showed that these isolates have two mega plasmids after electrophoresis on agarose gel. The molecular sizes of *S.aureus* mega plasmids ranged from 12.5 - 15 Kb approximately.

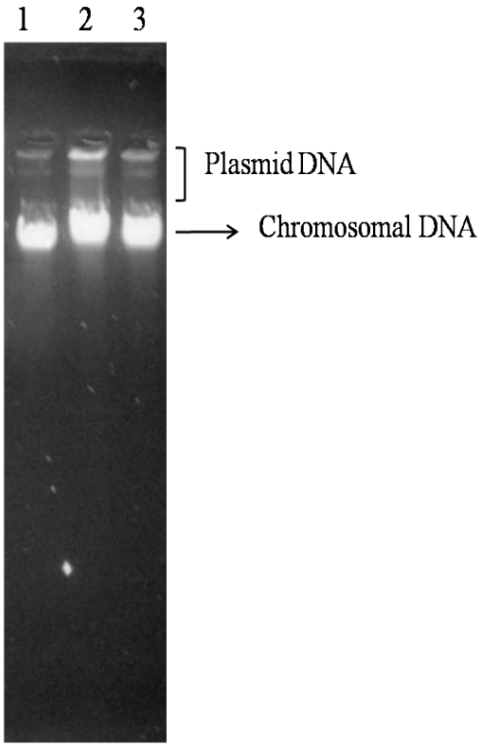

Figure 6. Electrophoresis pattern of genomic DNA of clinically isolated S. aureus (line1 presented S3, line 2 presented S4, and line 3 presented S7) after extraction and running on agarose gel (0.8%) in TBE buffer (0.5X, 5V/cm, 2hr). Stained with ethidium bromide and visualization done under U.V. light

Plasmid curing for S.aureus S3, S4 and S7 isolates was investigated using SDS, the highest concentration of SDS that allows the growth of S.aureus isolates was 8% for S3 and S7 and was 7% for S4, After treatment with SDS, a total of 100 derivatives colonies were randomly selected and tested on a selective medium containing the appropriate antibiotic to which the wild type was resist (methicillin, vancomycin and cephalothin) in order to detect the cured colonies which lost their ability to conferring the resistance phenotypes to those antibiotics and to determine whether the genes responsible for virulence factors and antibiotic resistance are chromosomally located or encoded by plasmid. Results showed that 18 from the total selected colonies of S3 cured derivatives were unable to grow in the presence of methicillin and cephalothin and became sensitive to these antibiotics and was unable to produce slime layer and hemolysin enzyme. One of these colonies was selected randomly and examined for the presence of its own plasmid by extraction of genomic DNA and electrophoresis on agarose gel. In this regards the same was for S7 derivatives of cured cells when 9 of them were unable to produce hemolysin, hyluronidase, slime layer and showed susceptibility to methicillin, vancomycin, this due to that cured of mega plasmids companied with changing in phenotypic expression of some of virulence factors of cured derivatives as in Figure 7.

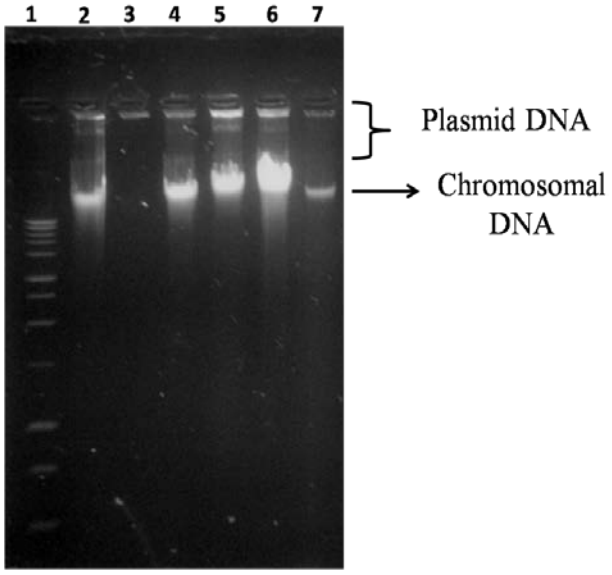

Figure 7. Electrophoresis pattern of genomic DNA of clinically isolated S. aureus (line1 presented ladder =1kb ƛ DNA(10000 bp- 240 bp), line 2 and 3 presented S3 and cured derivative respectively, line 4and 5 presented S4 its derivatives, and line 6 and 7 presented S7 and cured derivative) after extraction and running on agarose gel (0.8%) in TBE buffer (0.5X, 5V/cm, 2hr). Stained with ethidium bromide and visualization done under U.V. light.)

While, the picture was in opposite with S4 cell derivatives when no evidence for phenotypic changes were observed for their virulence factors and resistance to antibiotics. This referred that these plasmids were responsible for resistance methicillin and cephalothin in *S.aureus* S3 and S7 and expressed some of virulence factors and when these plasmids were eliminated from bacteria the resistance and production of hemolysin, hyluronidase and slime layer were not possible.

On these notes, we concluded that high prevalence rate of osteomyelitis infection was found to be caused by *Staphylococcus aureus* organism and were completely resistance to each of methicillin, vancomycin, cephalothin and tetracycline. Beside that, they were more virulence once due to their ability to produce hemolysin, capsule, slime layer, biofilm and hyaluronidase enzyme and some of these virulence factors were encoding by plasmid.

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