

## The Effect of Vancomycin and Some ion Chelators on the Formation of L-forms Bacteria in Gram-Positive Cocci

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### Abstract

**Background:** L-phase variants or cell wall deficient bacteria are strains of bacteria that lack cell wall temporarily. They are pleomorphic, a term that refers to change in size and shape. Recently, unusual antibiotic result was observed in such bacteria.

**Objective:** To investigate the role of vancomycin and some ion chelators like calcium and magnesium on the induction of gram-positive cocci (*Streptococcus faecalis*, *milleri Streptococci*, *Staphylococcus aureus* and *Streptococcus pneumoniae*) to produce L-forms bacteria.

**patients and methods:** In this study, the bacterial isolates were bacteriologically identified, the MIC and sub-MIC of vancomycin were determined of these isolates by using the technique of micro-broth dilution method, hypertonic special culture media designed for induction of L-forms bacteria with different concentrations of CaCl<sub>2</sub> and MgSO<sub>4</sub>.

**Results:** The results indicated that the species *Streptococcus faecalis* was the most affected one especially at the concentration of vancomycin 12.5 mg/l, where this bacterium was affected with this concentration of antibiotic and CaCl<sub>2</sub> at the concentration of 12 g/l and 6 g/l respectively and MgSO<sub>4</sub> at the concentration of 4g/l and provided that at the concentrations of (12+4) g/l and (6+2) g/l of CaCl<sub>2</sub> and MgSO<sub>4</sub>. The *milleri Streptococci*, *Staphylococcus aureus* and *Streptococcus pneumoniae* also were affected at the same concentration of vancomycin (12.5mg/l) and at the concentration of 12 g/l of CaCl<sub>2</sub>. The *milleri Streptococci* and *Streptococcus pneumoniae* also were affected at the concentration of 4 g/l of MgSO<sub>4</sub> and at the concentrations of (12+4) g/l and (6+2) g/l of CaCl<sub>2</sub> and MgSO<sub>4</sub> the *milleri Streptococci* was clearly affected while the *Staphylococcus aureus* and *Streptococcus pneumoniae* were affected only at the concentration of (12+4) g/l of CaCl<sub>2</sub> and MgSO<sub>4</sub> with availability of vancomycin at the same concentration above. The bacterial isolates also were affected at the concentration of vancomycin (6.25) mg/l with CaCl<sub>2</sub> and MgSO<sub>4</sub> but at lower percentage.

**Conclusions:** The vancomycin, CaCl<sub>2</sub> and MgSO<sub>4</sub> showed great effects on the induction of conversion to L-forms where the best growth of L-forms appeared when the vancomycin added at the concentration of (12.5 mg/l) to the media containing (CaCl<sub>2</sub> and MgSO<sub>4</sub>) together especially at concentration (12 g/l) for the CaCl<sub>2</sub> and (4 g/l) for the MgSO<sub>4</sub>.

**Key Words:** Gram positive cocci, Vancomycin, CaCl<sub>2</sub>, MgSO<sub>4</sub>, L- forms.

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### Introduction

The cell wall is an essential structure for virtually all bacteria, forming a tough outer shell that protects the cell from damage by osmotic lysis.

It is the target of some antibiotics like vancomycin. L-form strains are wall-deficient derivatives of common bacteria that have been studied for decades. However, they are difficult to generate and typically require growth for many generations on osmotically protective

media with antibiotics or enzymes that kill walled forms<sup>(1)</sup>.

However, part of the life cycles of many bacteria include phases where they transform into small forms that lose their cell wall. This means that they can no longer be killed by many commonly used antibiotics<sup>(2)</sup>. Conversion to a cell-wall-deficient form called 'L-form', which was first reported in 1935 for *Streptobacillus moniliformis*<sup>(3)</sup>, has been well documented for many species of bacteria. Because of the lack of a rigid cell wall, L-form cells generally show various shapes, for instance, small, large, spherical, irregular and club-shaped<sup>(4)</sup>. L-form bacteria are pleomorphic, a term that refers to their change in size and shape<sup>(2)</sup>.

The cell wall of the Gram-positive bacterium is mainly made from a layer of peptidoglycan 50-100 molecules thick. Glycopeptides and  $\beta$ -lactams act by inhibiting the biosynthesis of peptidoglycan. Thus, they would preferentially induce the development of cell wall-deficient bacteria in Gram-positive bacteria<sup>(5,6)</sup>.

Among the factors that affect the initial nonspecific adhesion of bacteria to substrate are ionic or hydrophobic of associations that involve Van der Waals forces,  $Ca^{+2}$ ,  $Mg^{+2}$ , or the double ion layer of K that can neutralize the repellent ionic negative charge between cell walls and bacteria<sup>(7)</sup>; also temperature, pH, and age of the bacteria can affect this interaction<sup>(7,8)</sup>.

In Gram(-) bacteria the outer membrane contains  $Mg^{+2}$  and sometimes  $Ca^{+2}$  as integral compounds and they do not bind as much metal as Gram(+) bacteria do<sup>(8,9)</sup>.

The role of  $Ca^{+}$  in bacteria has been relegated to the cell wall and external environment of the bacterial cell, principally for activating external enzymes<sup>(10)</sup>.

Magnesium is the most abundant intracellular divalent cation at its essential role in all living cells is broadly recognized<sup>(11)</sup>. In prokaryotes, Mg has many different cell functions: bacterial chemotaxis,

enzyme cofactor, maintain integrity of the cell wall /cellular membrane and growth<sup>(11,12)</sup>. The role may differ among bacterial species<sup>(12)</sup>.

This study has been undertaken to investigate the role of vancomycin and some ion chelators like calcium and magnesium on the induction of gram positive cocci (*Streptococcus faecalis*, *Streptococcus milleri*, *Streptococcus pneumoniae*, *Staphylococcus aureus* and *Streptococcus pneumoniae*) to produce L-forms bacteria.

## Patients and Methods

In this study the isolates of *Streptococcus faecalis*, *Streptococcus milleri*, *Streptococcus pneumoniae*, *Staphylococcus aureus* and *Streptococcus pneumoniae* were bacteriologically identified following methods described by Baron, et al<sup>(13)</sup>; Collee et al<sup>(14)</sup> and Willey et al<sup>(15)</sup> according to their culture characteristics (colony morphology and hemolytic reactions on blood agar), Gram staining, biochemical tests, Lancefield classification, capsular polysaccharides and provided that *Streptococcus pneumoniae* was identified according to WHO<sup>(16)</sup> and *Staphylococcus aureus* by using API-Staph..

Determination of the minimal inhibitory concentration (MIC) for vancomycin by using vancomycin vial supplied by Sanofiaventis company while  $CaCl_2$  and  $MgSO_4$  supplied by S.D. finechem. Indian company.

Hypertonic special culture medium (L-forms modified medium) was designed for this purpose which described by<sup>(17)</sup> at which vancomycin was added to it in concentrations of 12.5 mg/l which represent the MIC and 6.25 mg/l which represent the sub-MIC and by adding different concentrations of ions  $CaCl_2$  and  $MgSO_4$ .

The contents of L-forms modified medium are Beef Heart Infusion (4%), Sucrose (20%), Glucose (0.8%), Agar (1%) and concentrations of  $Ca^{+2}$  (1.5,3,6,12) g/l and concentrations of  $Mg^{+2}$

(0.5,1,2,4) g/l . In addition to MIC and sub-MIC of vancomycin (12.5 and 6.25) mg/l .

#### Determination of the Minimal Inhibitory Concentration (MIC):-

The method used for determination of MIC was described by<sup>(18)</sup> and modified by<sup>(19)</sup> by using the technique of micro-broth dilution method which is recommended by the National committee for clinical laboratory standard<sup>(20)</sup> by making double sterile dilution of vancomycin in nutrient broth and adding to all dilution (1ml) of nutrient broth inoculated by loop full of bacteria which used in this study (except the control tube) and incubated in 35°C for 48 h., then determine the MIC and sub-MIC.

McFarland turbidity standard solution was used and provided that *Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC 27853 were used as internal quality control isolates in this study.

#### Induction of L-forms bacteria:-

This method described by Panos etal<sup>(21)</sup> with some modification where the minerals added under the complete sterile

conditions. CaCl<sub>2</sub> was added in the concentrations of (1.5,3,6,12) g/l and MgSO<sub>4</sub> was added in the concentrations of (0.5,1,2,4) g/l.

These minerals were added to L-forms modified medium , the plates were cultivated with colonies of bacterial isolates which used in this study and incubated in (35-37) °c for (48)h. then prepared slides by staining with gram stain and examined under the microscope to observed the conversion of bacterial from the normal shape to L-forms.

#### Results:

The Minimal Inhibitory Concentration (MIC) of vancomycin against the bacterial isolates was 12.5 mg/l and the sub-MIC was 6.25 mg/l.

Different growth of L-form bacteria on the media containing vancomycin in concentration of (12.5) mg/l and CaCl<sub>2</sub> in the concentrations of <sup>(1.5,3,6,12)</sup> g/l as in table (1).

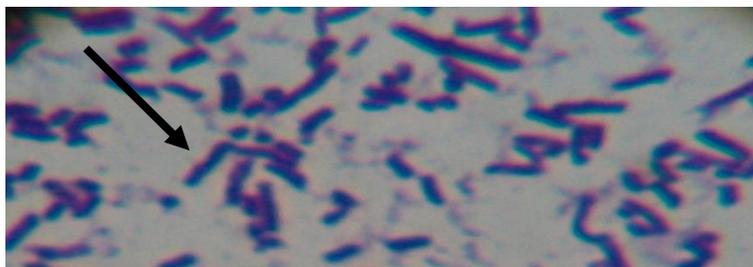
**Table (1) Growth and L-form bacteria on the media contain different concentrations of CaCl<sub>2</sub> and vancomycin (12.5) mg/l**

Organisms	CaCl <sub>2</sub> and Vancomycin							
	Growth in 1.5 g/l	L-form	Growth in 3 g/l	L-form	Growth in 6 g/l	L-form	Growth in 12 g/l	L-form
<i>Streptococcus faecalis</i>	-	-	-	-	++	+++	+++	+++
<i>Streptococcus milleri</i>	-	-	-	-	-	-	++	+++
<i>Staphylococcus aureus</i>	-	-	-	-	-	-	++	+++
<i>Streptococcus pneumoniae</i>	-	-	-	-	-	-	++	++

Where : +++ is heavy growth , ++ is moderate growth, + is scanty growth, - is no growth.

where in the concentration of 12 g/l *Streptococcus faecalis* showed heavy growth of swelled moderate circular entire smooth and few number of the same cells but in large size with large number of

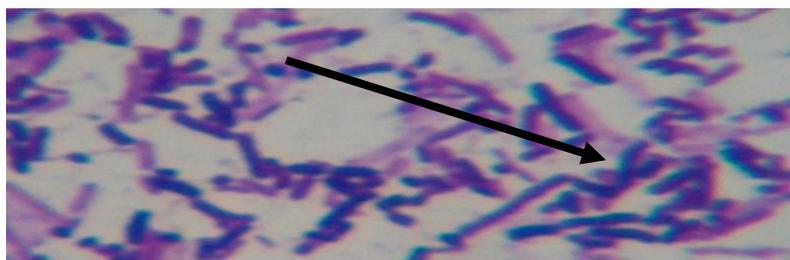
swelled, spherical, elongated cells arranged in chains or as a single cell with pleomorphism, which represent the L-form under the microscope, figure 1-a.



**Figure 1-a:** *Streptococcus faecalis* in the concentration (12.5) mg/l of vancomycin and 12 g/l of  $\text{CaCl}_2$  under the microscope (100X).

In the concentration of 6 g/l *Streptococcus faecalis* showed moderate growth of swelled, circular shaped entire umbonate smooth with large number of

L-form under the microscope where it is similar to L-form which appeared in the concentration of 12g/l. figure 1-b.



**Figure 1-b:** *Streptococcus faecalis* in the concentration (12.5) mg/l of vancomycin and 6 g/l of  $\text{CaCl}_2$  under the microscope (100 X).

While in the concentrations of 3 g/l and 1 g/l *Streptococcus faecalis* didn't give any response.

*milleri Streptococci* in the concentration of 12 g/l showed moderate growth of swelled small circular entire smooth colonies (larger than the normal size) with large swelled ovoid bodies arranged in chains or as a single cell with pleomorphism which represent the L-form under the microscope.

*Staphylococcus aureus* in the same concentration (12 g/l) showed moderate growth of large size (larger than the normal size) swelled circular entire smooth colonies and under the microscope which appeared as swelled large size grape-like clusters and as swelled large size single cocci and some

cells are red color a gram-negative bacteria where these swelled large size clusters and cells which represent the L-form.

*Streptococcus pneumoniae* in the same concentration (12 g/l) showed moderate growth of swelled small round colonies with swelled large lancet-shaped (diplococci) and some arranged in short chains in the line of long axis under the microscope which represent the L-form.

However in other concentrations *milleri Streptococci*, *Staphylococcus aureus* and *Streptococcus pneumoniae* didn't give any response.

Different growth of L-form bacteria on the media containing vancomycin in concentration of (12.5) g/l and  $\text{MgSO}_4$  in the

concentrations of (0.5, 1, 2, 4) g/l as in table (2).

**Table (2) Growth and L-form bacteria on the media contain different concentrations of MgSO<sub>4</sub> and vancomycin (12.5) mg/l**

Organisms	MgSO <sub>4</sub> and Vancomycin							
	Growth in 0.5 g/l	L-form	Growth in 1 g/l	L-form	Growth in 2 g/l	L-form	Growth in 4 g/l	L-form
<i>Streptococcus faecalis</i>	-	-	-	-	++	++	+++	+++
<i>milleri Streptococci</i>	-	-	-	-	-	-	++	+++
<i>Staphylococcus aureus</i>	-	-	-	-	-	-	-	-
<i>Streptococcus pneumoniae</i>	-	-	-	-	-	-	++	+

Where *Streptococcus faecalis* in the concentration of 4 g/l showed heavy growth of L-form where the colonies which appeared as large size like the fried eggs and some of swelled moderate

circular entire smooth colonies with large number of L-form bacteria which appeared as long axis of the chains contain large spherical shaped elongated cells and some singly under the microscope, figure 2-a.

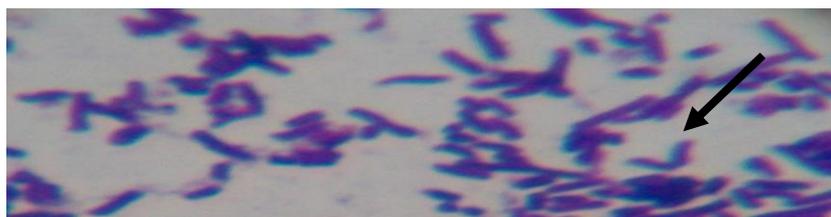


Figure 2-a : *Streptococcus faecalis* in the concentration (12.5) mg/l of vancomycin and 4g/l of MgSO<sub>4</sub> under the microscope (100 X).

In the concentration of 2 g/l *Streptococcus faecalis* showed moderate growth of swelled, moderate size, circular entire smooth colonies on the culture media and

under the microscope L-form bacteria which appeared as single and chains of large spherical elongated cells and some of swelled rod-like forms cells, Figure 2-b.

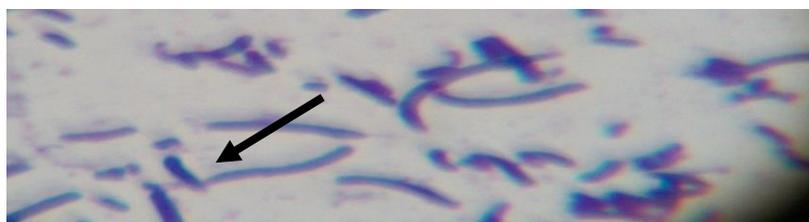


Figure 2-b: *Streptococcus faecalis* in the concentration (12.5) mg/l of vancomycin and 2g/l of MgSO<sub>4</sub> under the microscope (100 X).

*milleri Streptococci* in the concentration of 4 g/l showed moderate growth of large size colonies like the fried eggs and others appeared as moderate size, swelled, circular shaped colonies with large number of large swelled cocci elongated bodies in

chains and singly which represent the L-form.

*Streptococcus pneumoniae* in the same concentration (4 g/l) showed moderate growth of swelled large mucoid round colonies and some of swelled, small round colonies on the media and under the

microscope the L-form bacteria appeared as swelled, large size diplococcic (pairs) Different growth of L-form bacteria on the media containing vancomycin in concentration of (12.5) mg/l and

and in short chains.

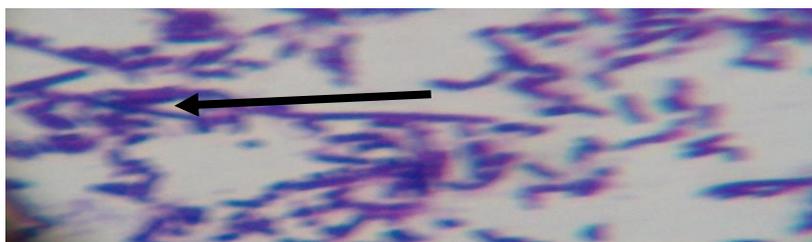
(CaCl<sub>2</sub>+MgSO<sub>4</sub>) in the concentrations of (1.5+0.5) g/l, (3+1) g/l, (6+2) g/l and (12+4) g/l as in table (3).

**Table (3) Growth and L-form bacteria on the media contain different concentrations of (CaCl<sub>2</sub> + MgSO<sub>4</sub>) and vancomycin (12.5) mg/l**

Organisms	(CaCl <sub>2</sub> + MgSO <sub>4</sub> ) and vancomycin							
	Growth in (1.5+0.5)g/l	L-form	Growth in (3+1) g/l	L-form	Growth in (6+2) g/l	L-form	Growth in (12+4) g/l	L-form
<i>Streptococcus faecalis</i>	-	-	-	-	++	+++	+++	+++
<i>Streptococci milleri</i>	-	-	-	-	++	+++	+++	+++
<i>Staphylococcus aureus</i>	-	-	-	-	-	-	++	+++
<i>Streptococcus pneumoniae</i>	-	-	-	-	-	-	++	+++

In the concentration of (12+4) g/l *Streptococcus faecalis* showed heavy growth of large size colonies like the fried eggs with large number of L-form bacteria

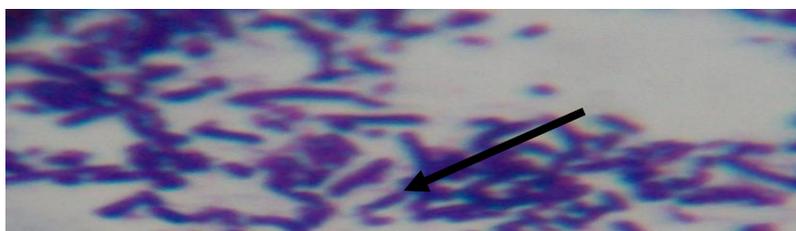
which appeared as chains of large swelled spherical elongated cells and singly with pleomorphism under the microscope , figure 3-a.



**Figure 3-a : *Streptococcus faecalis* in the concentration (6.25) mg/l of vancomycin and (12+4)g/l of (CaCl<sub>2</sub>+MgSO<sub>4</sub>)g/l under the microscope (100X).**

*Streptococcus faecalis* in the concentration of (6+2) g/l showed moderate growth of swelled, moderate circular entire umbonate

smooth and some of large size colonies like the fried eggs with large number of L-form under the microscope, figure 3-b.



**Figure 3-b : *Streptococcus faecalis* in the concentration (6.25) mg/l of vancomycin and (6+2) g/l of (CaCl<sub>2</sub>+MgSO<sub>4</sub>)g/l under the microscope (100X).**

*milleri Streptococci* in the concentration of (12+4) g/l showed heavy growth of L-form on the culture media which appeared as swelled moderate circular shaped colonies and some of like the fried eggs colonies with large number of swelled,

large size , ovoid cells in chains and singly with pleomorphism under the microscope.

*milleri Streptococci* give response to conversion to L-form in the concentration of (6+2) g/l where in this concentration this bacteria showed moderate growth of

swelled, moderate size, circular entire smooth colonies with large number of L-form under the microscope which is similar to L-form that appeared in the concentration of (12+4) g/l.

*Staphylococcus aureus* in the concentration of (12+4) g/l showed moderate growth of L-form which appeared as swelled large size colonies circular shaped with large number count of L-form under the microscope which appeared as large single cocci and swelled grape-like clusters and some cells are red color a gram-negative bacteria.

In the same concentration (12+4) g/l *Streptococcus pneumoniae* showed moderate growth of swelled large mucoid round colonies which appeared under the microscope as large elongated diplococci (arranged in pairs) and arranged in very short chains which represent the L-form.

The addition of vancomycin in the concentration of (6.25) mg/l on the media contain different concentrations of CaCl<sub>2</sub> (1.5, 3, 6, 12) g/l showed different growth of L-form in the concentration of 12 g/l as in table (4).

**Table (4) Growth and L-form bacteria on the media contain different concentrations of CaCl<sub>2</sub> and vancomycin (6.25) mg/l.**

Organisms	CaCl <sub>2</sub> and vancomycin							
	Growth in 1.5 g/l	L-form	Growth in 3 g/l	L-form	Growth in 6 g/l	L-form	Growth in 12 g/l	L-form
<i>Streptococcus faecalis</i>	-	-	-	-	-	-	++	+++
<i>milleri Streptococci</i>	-	-	-	-	-	-	++	+++
<i>Staphylococcus aureus</i>	-	-	-	-	-	-	+	++
<i>Streptococcus pneumoniae</i>	-	-	-	-	-	-	-	-

Where in this concentration *Streptococcus faecalis* showed moderate growth of L-form which appeared as swelled, moderate circular colonies and under the microscope which appeared as chains of large number of swelled, large size spherical elongated cells or as a single cells.

*Milleri Streptococci* in the same concentration (12) g/l showed moderate growth of swelled small size (larger than the normal size) round entire smooth colonies with large number of L-form under the microscope.

However *Staphylococcus aureus* in the concentration of 12 g/l showed scanty

growth of L-form bacteria which appeared as large colonies (larger than the normal size) swelled, circular entire with large grape-like clusters and large size of swelled single gram-positive cocci and some of gram-negative cocci under the microscope.

The addition of vancomycin in the concentration of (6.25) mg/l on the media contain different concentration of MgSO<sub>4</sub> (0.5, 1, 2, 4) g/l showed moderate growth of *Streptococcus faecalis* and *milleri Streptococci* in the concentration of 4g/l of MgSO<sub>4</sub> with large number count of L-form bacteria under the microscope as in table (5).

**Table (5) Growth and L-form bacteria on the media contain different concentrations of  $MgSO_4$  and vancomycin (6.25) mg/l**

Organisms	MgSO <sub>4</sub> and Vancomycin							
	Growth in 0.5 g/l	L-form	Growth in 1 g/l	L-form	Growth in 2 g/l	L-form	Growth in 4 g/l	L-form
<i>Streptococcus faecalis</i>	-	-	-	-	-	-	++	+++
<i>milleri Streptococci</i>	-	-	-	-	-	-	++	+++
<i>Staphylococcus aureus</i>	-	-	-	-	-	-	-	-
<i>Streptococcus pneumoniae</i>	-	-	-	-	-	-	-	-

Where in *Streptococcus faecalis* L-form appeared as long chains of swelled, spherical shaped elongated cells and swelled rod-like forms and some of large size single cocci under the microscope and on the culture media which appeared as large size colonies like the fried eggs and few of moderate size, circular shaped, entire swelled colonies.

L-form of *milleri Streptococci* which appeared as chains of circular shaped elongated cells swelled and some of swelled single cocci under the microscope and on the culture media which appeared

as large colonies like the fried eggs and some of swelled, small size (large than the normal size) circular shaped entire colonies.

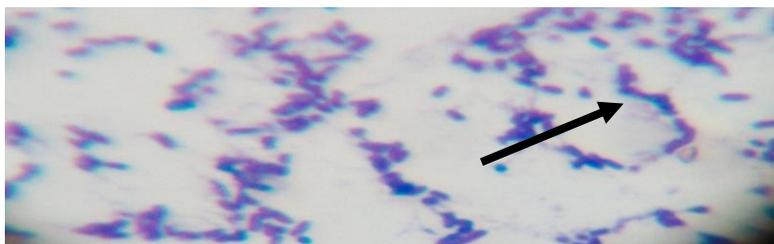
The addition of vancomycin in the concentration of (6.25) mg/l on the media contain different concentrations of ( $CaCl_2$  and  $MgSO_4$ ) in the concentrations of (1.5+0.5) g/l, (3+1) g/l, (6+2) g/l and (12+4) g/l showed moderate growth of *Streptococcus faecalis* in the concentrations of (12+4) g/l and (6+2) g/l as in table (6).

**Table (6) Growth and L-form bacteria on the media contain different concentrations of ( $CaCl_2 + MgSO_4$ ) and vancomycin (6.25) mg/l.**

Organisms	( $CaCl_2 + MgSO_4$ ) and Vancomycin							
	Growth in (1.5+0.5) g/l	L-form	Growth in (3+1) g/l	L-form	Growth in (6+2) g/l	L-form	Growth in (12+4) g/l	L-form
<i>Streptococcus faecalis</i>	-	-	-	-	++	++	++	+++
<i>Milleri streptococci</i>	-	-	-	-	-	-	++	+++
<i>Staphylococcus aureus</i>	-	-	-	-	-	-	++	++
<i>Streptococcus pneumoniae</i>	-	-	-	-	-	-	-	-

Where in the concentration of (12+4) g/l the colonies appeared as large size like the fried eggs with large number of large size circular shaped elongated cell arranged in chains and single cells which represent the L-form under the microscope.

While in the concentration of (6+2) g/l *Streptococcus faecalis* appeared as swelled moderate size, circular shaped and under the microscope the number of L-form is less than the number in the concentration of (12+4) g/l, figure 4.



**Figure 4 :** *Streptococcus faecalis* in the concentration (6.25)mg/l of vancomycin and (6+2)g/l of (CaCl<sub>2</sub> +MgSO<sub>4</sub>) g/l under the microscope (100X).

*milleri Streptococci* in the concentration of (12+4) g/l showed moderate growth of swelled circular shaped colonies larger than the normal size and some of large size colonies like the fried eggs with large number of swelled, large size, spherical elongated cells adhere in chains and in single cells which represent the L-form.

*Staphylococcus aureus* in the concentration of (12+4) g/l showed moderate growth of swelled, large size colonies, round smooth with large size, spherical cells, swelled arranged in large irregular clusters, Gram-positive cocci and some cells are gram-negative cocci.

## Discussion

Vancomycin is effective primarily against gram-positive organisms. It has been lifesaving in the treatment of methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-resistant *Staphylococcus epidermidis* (MRSE) infections, as well as enterococcal infections with the emergence of resistant strains, it is important to curtail the increase in vancomycin-resistant bacteria (for example, *Enterococcus faecium* and *Enterococcus faecalis*) by restricting the use of vancomycin to the treatment of serious infections caused by  $\beta$ -lactam resistant, gram-positive microorganisms, or for patients with gram-positive infections who have a serious allergy to the  $\beta$ -lactamase.<sup>(22)</sup>

The mechanism of vancomycin is inhibition of bacterial cell wall synthesis and this effect referenced by<sup>(23)</sup> that the bacitracin and vancomycin inhibiting the

bacterial cell wall synthesis in a manner different than the penicillin and cephalothin and stimulate the production of L-forms bacteria when growth on a medium of known osmolarity, the L-forms bacteria appear to have the ability to stay above the normal average of osmolarity but the mechanical arrangement of osmolarity for L-forms bacteria till now is not well explained. Study of these shapes which is losing cell wall of bacteria give us some of view essential processes in bacteria.<sup>(24)</sup>

The exposure of bacteria to antibiotics, antiserum, different chemical factors and tap water this is leading the bacteria to be converted to L-forms, staying and growing L-forms after exposure to one of these induction factors leading this bacteria to be more resistant than the original one and when the bacteria convert to L-forms their infectivity become unclear in all conditions and possibility of this process to occur inside the living body through the process of phagocytosis also through bacteriophage and treatment with antibiotics.<sup>(23,25,26)</sup>

In this study hypertonic special culture medium were used which described by<sup>(17)</sup> and provided that<sup>(4)</sup> explained the conversion technique to L-forms bacteria is consisting of a single overnight incubation in a rich hypertonic medium in the presence of the  $\beta$ -lactam cefsulodin. This antibiotic inhibits the transpeptidase activity of the major peptidoglycan-synthesizing enzymes, penicillin-binding proteins (PBPs)<sup>(27,28)</sup>.

In hypertonic media, the cells become spherical, osmosensitive, and

heterogeneous in size, traits associated with L-forms<sup>(3)</sup>.

The difficult problem that face who is working in medical field is determining the significant percentage of isolated L-forms bacteria form patients and after reinfection it is keeping resistance to body defense system<sup>(29)</sup>.

Results showed that the colonies of L-forms bacteria which isolated in this study on the hypertonic culture media appeared as circular shaped this is in agreement with<sup>(30)</sup> stated that L-forms bacteria appears either circular or irregular in shaped and this occur either spontaneously as in *Streptobacillus moniliformis* or by induction with electrical shock or by osmolarity or through antibiotics which inhibits the cell wall.

In the state of conversion bacteria to L-forms it is losing completely or partially to the cell wall and sometimes return to the normal shape after removal of the induction factor and this named the unstable L-form or it stays and don't return to its original shape even when removing the induction factor and continue in growth and development this named the stable L-form.

Results indicated that the species *Streptococcus faecalis* was the most effective one especially at concentration of vancomycin 12.5 mg/l and at concentrations (6,12) g/l of CaCl<sub>2</sub> and (2,4) g/l of MgSO<sub>4</sub> and at (6+2)g/l of (CaCl<sub>2</sub> and MgSO<sub>4</sub>) and (12+4) g/l of (CaCl<sub>2</sub> and MgSO<sub>4</sub>).

To explain these results<sup>(21)</sup> showed that the L-forms bacteria needs high concentrations of salts and sucrose during studying the L-forms bacteria of *Streptococcus faecalis* where the salts and sucrose give as organizer or osmotic stabilizer and osmotic requirement different according to strain and bacterial type, and also observed that the internal osmosis is higher than this found in media and it is increased by doubling L-forms bacteria and explained that the cytoplasmic membrane play great role in helping bacteria to overcome changes that occur in

osmotic pressures or this membrane became more elasticity at L-forms.

The sucrose has active role in induction of bacteria to conversion to L-forms which is used in 20% to make this effect<sup>(24)</sup>.

Also the results indicated that the *milleri Streptococci*, *Staphylococcus aureus* and *Streptococcus pneumoniae* were affected at the concentration of vancomycin 12.5 mg/l and at the concentration 12g/l of CaCl<sub>2</sub> the species *Streptococcus pneumoniae* and *milleri Streptococci* were affected at the concentration 4 g/l of MgSO<sub>4</sub> and at the same concentration of vancomycin (12.5 gm/l).

Where the L-forms of these types of bacteria under the microscope appeared as swell to large size bodies and some undergo elongation this is in agreement with<sup>(25)</sup> stated that the isolated L-forms bacteria form positive types of gram-stain rods and spherical bacteria swell to large bodies and some undergo elongation this occur in all types and in the same way as this process occur at other types of bacteria. L-forms bacteria that are growing to large bodies become straight at each other and may destroyed at others and give shape like granules and at some cases these shapes appear small with large number. There is many stimulators that stimulate the cell to convert to L-forms like the high concentrations of organic components such as dl, L-phenylalanine, Glycin, Raffinose, caffeine, methionine, also the light minerals and doses that less lethal of heavy minerals that induce all the kinds of spherical and rod shape of gram-positive and gram-negative stain also cooling, and thought that these shapes make genetic role in rearrangement of genetic characteristics to converted strains.

Also the results of this study showed that the best growth of L-forms appeared when the vancomycin added at the concentration of 12.5 mg/l to the media contain CaCl<sub>2</sub> and MgSO<sub>4</sub> together especially at concentration (12 g/l) for the CaCl<sub>2</sub> and 4 g/l for MgSO<sub>4</sub>. The species *Streptococcus faecalis* was clearly affected

at the concentration of vancomycin (6.25 mg/l) and at the concentration (6+2) g/l and (12+4) g/l of  $\text{CaCl}_2$  and  $\text{MgSO}_4$ , *milleri Streptococci* and *Staphylococcus aureus* were affected at the same concentration of vancomycin (6.25 gm/l) and at the concentration (12+4) g/l of  $\text{CaCl}_2$  and  $\text{MgSO}_4$ . Therefore the calcium and magnesium ions with vancomycin play important and essential role for conversion of bacteria to L-forms.

To explain the role of calcium for conversion of bacteria to L-forms<sup>(31)</sup> in studying the effect of different concentration of calcium on *E. coli* observed that the increase in calcium level inside the cell through the cellular division and when changes happened in environment and showed that calcium is playing active role in regulation of life cycle of cell by organization the protein that is controlling the skeletal enzymes. L-forms for this bacteria that lost natural peptidoglycan can grow and divide but when the media lost  $\text{Ca}^{++}$  it will stop cell division and become spherical, swell and may form vacuoles then lysis occur. When  $\text{Ca}^{++}$  removed from media these cells become long and form multinucleated filamentous forms, while high concentration of  $\text{Ca}^{++}$  affect the direction of flagellar rotation in *Bacillus subtilis*; and cause the bacteria to tumble while at lowers concentration they swim and when reading  $\text{Ca}^{++}$  in media cell will retain the growth<sup>(3,32,33,34,35)</sup>.

When  $\text{Ca}^{++}$  decreases with presence of good amount of  $\text{Mg}^{++}$  leading to decreases the rate of bacterial growth.

When  $\text{Mg}^{++}$  decreases less than 0.1 mmol will notice elongation of the bacterial cell, when  $\text{Ca}^{++}$  concentration decrease less than 0.25 mmol lead to swelling of the bacterial cell and forming vacuoles<sup>(36)</sup>.

The intracellular concentration of magnesium appears to be similar between Gram-negative and Gram-positive bacteria; they have an absolute growth requirement for magnesium that cannot be replaced by other ions<sup>(8,12)</sup>.

On the other hand the  $\text{CaCl}_2$  is important need for growth and can't be replaced by  $\text{MgSO}_4$  and typical concentration to be added is 0.15% and for  $\text{MgSO}_4$  is 0.05%<sup>(30)</sup>.

In this study some L-forms of *Streptococcus faecalis* and *milleri Streptococci* showed transition to pleomorphic and this is in agreement with<sup>(4,37)</sup> stated that transition of bacteria to pleomorphic L-forms in response to stress factors has been considered as a potential of microbes for survival under unfavorable conditions.

In conclusion vancomycin,  $\text{CaCl}_2$  and  $\text{MgSO}_4$  showed great effect in the induction of conversion to L-forms, which is very important for knowing the virulence of bacteria and their resistance for body defense and chronic cases or staying diseases.

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