

## Inactivation of vancomycin-induced unstable L-forms of *Staphylococcus aureus* by horse serum

Farshad Nojoomi<sup>1,\*</sup>, Qorban Behzadian Nejad<sup>2</sup> and Seyed Davar Siadat<sup>3</sup>

<sup>1</sup> Department of Microbiology, School of Medical Sciences, Military University, Tehran

<sup>2</sup> Department of Microbiology, School of Medical Sciences, Tarbiat Modares University, Tehran

<sup>3</sup> Department of Microbiology, Pasteur Institute of Iran.

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### ABSTRACT

Substitution at codon Ser315 of *katG* gene, a reliable marker for isoniazid (INH) resistance and proliferate in spite of the loss of cell wall is called an L- form. Unstable L- forms can convert to normal cells but stable L-forms cannot. Cell wall - defective state may be induced spontaneously or through the action of an appropriate agent such as antibiotic interfering with the synthesis of cell wall peptidoglycan. Vancomycin is one of these antibiotics which cause L-form formation in some strains of *Staphylococcus aureus* in presence of osmoprotective compounds. Horse serum and some of organic or mineral materials can be used for better isolation of L- forms in vitro. In this study, standard strain of *Staphylococcus aureus* (ATCC 25923) was inoculated to 2 media: i) L phase medium (LPM) agar containing horse serum & ii) LPM agar without horse serum. Sucrose was used in both above mentioned growth media as osmoprotective and vancomycin was used for inducing L-forms. Microscopic examination revealed presence of small and large colonies on LPM agar lacking horse serum and only large colonies on LPM agar containing horse serum after 3 - 4 days. Induced by horse serum were unable to form colony in LPM agar containing horse serum. Results showed that unstable L-forms of *Staphylococcus aureus* in the presence of horse serum neither can survive nor convert to normal cells.

### 1. Introduction

Removal of the bacterial wall may be accomplished by hydrolysis with lysozyme or by blocking peptidoglycan biosynthesis with an antibiotic such as penicillin. In osmotically protective media such as treatments liberate protoplast from gram positive cells and spheroplasts from gram negative cells. If such cells are able to grow and divide they are called L-forms (Jawetz et al., 2004). Sucrose and sodium chloride are the best

osmoprotective compounds added to L-form culture media (Mattman, 2001).

On solid media L- form colonies appear after 3–7 days and their diameter range from 0.1 – 1 mm (Domingue, 1989).

Some L-forms are able to revert to normal bacterial cells with complete cell wall under suitable conditions and after removal of L-forms inducers. Such L-forms are called unstable L-forms while those L-forms which are unable to convert to normal cells even after removal of L-form inducing

\*Corresponding author: Farshad Nojoomi

Farshad Nojoomi - Dept of Microbiology, Medical college,  
Military university of medical sciences, Tehran.  
Tel: 0912 3995517

agent in culture medium are called stable L-forms (Allen, 1991).

*Staphylococcus aureus* is gram positive, facultative anaerobic usually arranged in irregular grape-like clusters. Focal abscess is typical of staphylococcal infection. From any one focus, organisms may spread via the lymphatics and bloodstream to other parts of the body. *Staphylococcus aureus* can cause pneumonia, sinusitis, meningitis, septicemia, enterocolitis, food poisoning and some other infections. Chief sources of infection are shedding human lesions, fomites contaminated from such lesions, and the human respiratory tract and skin. Even now *Staphylococcus aureus* is one of the most important agents of nosocomial infections (Walker, 1999).

In 1944, most strains of *Staphylococcus aureus* were susceptible to penicillins. After massive use of penicillin, 65-68% of staphylococci isolated from hospitals in 1984 were resistant penicillins (eg, methicillin). Even now methicillin-resistant staphylococci produce intermittent hospital outbreaks but majority of its strains are fortunately susceptible to vancomycin. Vancomycin is markedly bactericidal for staphylococci and some other gram positive bacteria. This drug inhibits early stages in cell wall peptidoglycan synthesis (Edmond et al., 1996).

Some strains of *Staphylococcus aureus* in presence of appropriate inducers especially penicillin G, fosfomycin, vancomycin, methicillin or lysozyme are converted to L-forms (Kagan and Weinberger, 1962; Edwards, 1972; Schmid, 1984; Owen et al., 1988; Nojoomi, and Behzadian, 2005).

To isolate Staphylococcal L-forms Vancomycin and other heat labile compounds such as horse serum in suitable concentration are added to culture medium after the end of sterilization.

The aim of this study is to find out why some L-forms do not form colony in these media even under suitable conditions and whether horse serum has any effect on L- form growth which its formation is induced by vancomycin.

## 2. Materials and Methods

*Staphylococcus aureus* standard strain (ATCC 25923) was obtained from diagnostic laboratory of Bu Ali hospital. Biochemical characteristics of this

strain are: coagulase positive, Mannitol positive and DNAase positive.

Bacteria were cultured in LPM medium. Components of LPM are:

Brain heart infusion (BHI) broth	38.00 g / lit
Sucrose	200.00 g / lit
Glucose	10.00 g / lit
L- cysteine	0.20 g / lit
Magnesium sulphate	0.50 g / lit
Sodium chloride	1.50 g / lit
Phenol red	0.02 g / lit
Agar	0.8%

pH was adjusted to 7, after autoclaving the medium, 100 ml/lit of horse serum (obtained from Veterinary college, Tehran university) was added. Vancomycin (Zakaria pharmaceutical Co) 11 µg/ml in distilled water was prepared, filtered through filter paper with pore diameter of 0.22 µm was added to the above sterile medium in a concentration of 10 ml/lit (Banville, 1964; Nimmo, 1969; Takahashi and Tadakoro, 1981a; Takahashi and Ichikawa, 1981b).

Bacterial cells isolated from BHI broth (after 5- 8 hour of growth) was added to petri plates containing LPM agar medium enriched with horse serum and plates containing LPM agar medium without horse serum. Plates were incubated at 37°C for 7 days. Every 24 hour plates were checked for colony formation or any change in colony morphology and appearance. After L- form colony was formed, 1 colony was transferred to BHI broth, incubated for 4 days at 37°C. Every 24 hour, 0.5 ml of of broth was inoculated to plates containing BHI agar. After colony was formed in these plates, smear was prepared, stained with gram stain and biochemical tests were performed.

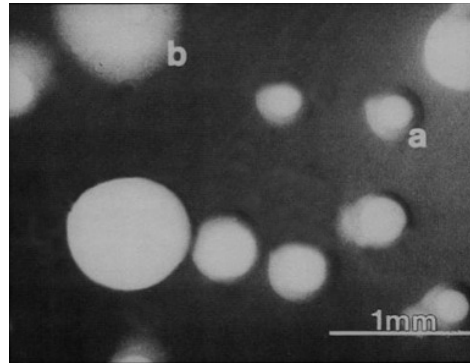
## 3. Results

Three to five days after inoculating cells to both LPM agar media, containing horse serum and lacking horse serum, L-form colonies were formed. Red colour of culture medium was changed to yellow as a result of bacterial growth which made the medium acidic. Microscopic examination by stereomicroscope revealed presence of 2 types of colonies:

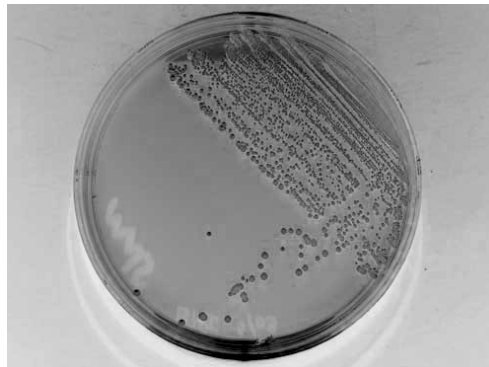
- small colonies with deepened centre (fried egg shaped colonies)
- large and flat colonies (Figure 1)

On LPM medium supplemented with horse serum only large colonies were grown. After inoculating bacteria from each colony to BHI broth, L- forms of "b" type grew after 30 hours which made the broth opaque. On subculturing, normal colonies were formed (Figure 2). While, inoculation of bacteria from L-forms of "a" type did not cause opacity in the broth even after 4 days and no colony grew on BHI agar.

Smears prepared from yellow, opaque, convex and round colonies formed on BHI agar and staining them according to gram staining method showed single, diplo, tetrads and staphyl form (grape – like irregular clusters) of gram positive cocci under light microscope (Figure 3). Biochemical tests specially catalase, coagulase, DNAase and acid production from mannitol confirmed similarity between these and standard strain (ATCC 25223).



**Figure 1.** A variety of staphylococcal L-form colonies observed at 3-5 days after inoculation on LPM Agar lacking horse serum : a) small colony, b) large colony.magnification,×25



**Figure 2.** Normal colonies of *Staphylococcus aureus* observed at 24h after inoculation on BHI Agar



**Figure 3.** Gram positive cocci arranged in single, diplo, pairs, tetrads and grape-like irregular clusters. Magnification,×1000

#### 4. Discussion

Small colonies with deepened centres are formed by bacteria having lost their cell wall completely. These cells are unable to revert to primary form having cell wall on subsequent cultures. These are called as stable L-forms. Horse serum does not inhibit growth of L-forms. L-forms can grow and reproduce on LPM agar only in the presence of osmoprotective agents while on inoculation to non – hypertonic media like BHI agar or broth these can not survive.

Bacteria which save a part of their cell wall, form large colonies. In case of removal of L-form inducing agents from the medium, these bacteria revert to normal cells with complete cell wall. These cells are called unstable L-forms. Horse serum prevents growth of such cells. These do not grow on LPM agar or similar culture media containing horse serum.

Cytotoxic effect of high density lipoprotein (HDL) of human serum on bacterial L-forms is well known. But mechanism for the growth inhibition remains to be clarified. In 1968, Kalmanson and his colleagues reported that human serum has bactericidal activity against protoplasts (Kalmanson et al., 1968). In 1972, Matsuoka and his coworkers proved that horse serum has negative effect on growth of Staphylococcal L-forms (Matsuoka et al., 1972). Studies of Shimokawa on two standard strains of *Staphylococcus aureus* (KD101 & 209 P) showed that heat inactivated horse serum has inhibitory effect on penicillin induced L-form colony formation in *Staphylococcus aureus* when include in an osmotically stabilized culture medium (Shimokawa et al., 1994). At last, inhibitory effect of human high density lipoproteins on penicillins ampicillin and fofomycin – induced *Staphylococcus aureus* L-forms were showed by Ikeda and Shimikawa independently (Ikeda, 1993; Shimokawa and Nakayama, 1997).

As mentioned above, we can conclude that HDL present in horse serum is the most effective factor in inhibiting growth and reproduction of unstable L-forms of *Staphylococcus aureus* in vitro.

By virtue of lacking peptidoglycan, L-forms are resistant to antibiotics that affect bacterial growth at the site of cell wall like penicillins & cephalosporins. Thereby, by elimination of drug from the vicinity of L-forms, these can synthesize

complete cell wall, causing disease condition and symptoms. So L-forms are responsible for recurrence and chronizing infection after treatment is over. Results obtained in this research confirm that some lipoproteins of human serum specially HDL can act as a part of innate immune system against L-forms. On the other hand, unstable L-forms in the presence of HDL of serum can not survive. So, the concept that L-forms are responsible for recurrence and chronic infections needs further research & work.

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