Bulletin of Environment, Pharmacology and Life Sciences
Bull. Env. Pharmacol. Life Sci., Vol 3 [Special Issue V] 2014: 50- 58

Online ISSN 2277-1808
Journal's URL:http://www.bepls.com
CODEN: BEPLAD
Global Impact Factor 0.533
Universal Impact Factor 0.9804

ORIGINAL ARTICLE

A new method for detection of *Mycobacterium tuberculosis* by biosensor based on Conductivity and zeta potential

Pourmir Ahmadreza[1], Farsizaban Masomeh[2*],Nakhzari Moghadam Hadi[3] ,Poorjangi Mahsa[4]
[1]Faculty of Medicine, Department of Medical Physics, Medical School, Zahedan University of Medical Sciences, Zahedan, I.R.Iran.
[2]Faculty of Medicine, Department of Medical Physics, Medical School, Zahedan University of Medical Sciences, Zahedan, I.R.Iran.
[3]Faculty of Medicine, Department of Medical Physics, Medical School, Zahedan University of Medical Sciences, Zahedan, I.R.Iran.
[4]Medical Students Research Center, Zahedan University of Medical Sciences, Zahedan, I.R.Iran
E-mail: ma_farsizaban@yahoo.com

ABSTRACT
Based on danger of Mycobacterium tuberculosis, detection in time and that rapid is much important. Although the classic methods of detection for example methods of culture-based in low concentrations of bacilli to have ability of diagnosis, but they answer in the long time and method of smear microscopy (AFB) answers in comparatively short time but it is not answer in low concentrations In the other hand the methods PCR and ELISA two problems mentioned about, but are very expensive and those aren't operable everywhere. With progress of biosensors technology, the use from Conductivity meter for detection of the few number of Mycobacterium tuberculosis bacillus is important. In this study we measurement conductance and zeta potential in different concentrations bacillus based on CFU/ml and observed that in every two case increased with the increase of concentration bacterium so linear.
With comparison between size of colony, conductance and zeta potential distinguished relative change of size of colony's is less from relative change of zeta potential and relative change of conductance. Therefore probably with increase concentration of bacteria, the hydrophobic effect is important.
Therefore with regard to results of this study and the pattern of increase of conductance and zeta potential can to use for to take measure number of bacterium. The complete this study probably can to make available possibility to build of tool that with use from that be able to distinguish number of bacterium by employ a portable diagnosis system, so fast and careful acceptable and clinical available necessary preliminary for control to be increased spreading of Mycobacterium tuberculosis.
Key words: Mycobacterium tuberculosis, zeta potential, hydrophobic effect, size particle Conductivity

Received 29.05.2014 Revised 30.06.2014 Accepted 22.09. 2014

INTRODUCTION

Mycobacterium tuberculosis is a pathogenic bacterium that causes tuberculosis [1].Tuberculosis is caused by Mycobacterium tuberculosis and can infect the lungs (pulmonary) as well as the kidneys, lymph nodes, bones and joins (extra-pulmonary) [2]. Tuberculosis is a major public health problem that it may emerge as a complication of acquired immune deficiency syndrome (AIDS) infection [1]. To appear of drug-resistant species and AIDS epidemic are from important original reasons renewed spreading of tuberculosis .Therefore the rapid diagnosis and treatment of infectors is considered crucial for the effective control of tuberculosis [1]. Consequently, it is necessary to use new methods for fast and sensitive diagnosis especially on the basis of spreading and distribution Mycobacterium tuberculosis [1]. In numerousness regions use traditional methods especially culture-based methods is time consuming and laborious. Although in spite of to take time, be able detection few concentrations of bacteria. The methods of PCR, Latex agglutination, ELISA and Radiometric detection [3 , 4] cannot provide the detection results in real-time. Therefore for direction development of sensitive, accurate, rapid and capable methods in unexpanded regions and effective prevention from tuberculosis infection, it is necessary of use from another methods [1].

With the improvement of biosensor technology for disease diagnostic guardianship from biological [4, 5,8], to be found abundant applications. Biosensors act specificity and they are with high sensitive which allows the detection of a broad spectrum of materials similar sputum, salvia, serum and urine with minimum samples preparation [4]. Charged groups existent in bacterium cell wall may associate or dissociate upon charges in pH or ionic strength of the suspending fluid, but also upon approach of a charged surface, either of another bacterium or a substratum that may induce changes in the conformation of charged molecules based on kind of adhesion to surface. Moreover the bacterial cell surface maybe penetrable to solvent, and solutes in particular ions, either due to the presence of a peptidoglycan layer. Inside the bacterial cell wall charged groups are present and the distribution of these double layer interactions in bacterial adhesion and interaction with other surfaces [6].

Bacterium cell surface is a highly dynamic surface responding strongly to environmental changer through adsorption of ions and macromolecular components and in contact with an electrolyte solution, an electric double layer (EDL) forms. In this situation a space distribution from charges coming from the solution will become equilibrated with the surface charges [6,7].

The effective dissociation constants as valid for bacterial cell surface groups also depend on environmental properties, such as the local hydrophobic effect and electric potential. At physiological pH values between 5 and 7 most bacterial strains are negatively charged, as the number of carboxyl and phosphate groups exceeds the number of amino groups (1 &7)

The important methods of determine bacterial cell surface charge include particular micro-electrophoresis (1) and dielectric spectroscopy (5). In dielectric spectroscopy, The frequency dependence of the complex conductivity of a bacterial suspension to AC wave frequency is measured, from which in formation on the special electric double layer of bacteria can be derived (1).Detection of Mycobacterium tuberculosis based on micro-electrophoresis reveals the electro kinetic potential (zeta potential) (9). Zeta potential (ζ) is defined as the electric potential at the hydrodynamic plane of shear or, otherwise called, the slip plane. The slip plane is the hypothetical interface between a liquid layer adjacent to the bacterium surface and the liquid moving relative to the surface during electrophoresis. The slip plane is generally assumed to be located within several nanometers of the bacterium surface. (1,10,11).Assuming thin double layers low surface conductance in the case of fixed charge surfaces, movement toward high or low electrolyte concentration can be obtained by appropriately tuning the parameters, and even opposite motion for the same type of change (positive or negative) can be achieved. The direction of motion depends on its magnitude and the strong dependence found for the velocities on both size and electrolyte concentration (1,611,12,13). Ions in fluid strongly absorption substrate in a closed layer and consequently become immobile. By use a external electric field be able to send out the number of them from this layer and send to direction of electrode of possessing opposite charge. (6,11,13,14).The particles of effective zeta potential move towards the electrode of opposite sign.

The electrophoresis mobility increases with the particle zeta potential and decrease with the viscosity of the media. The exact Coefficient of correlation between them depends on the size of the particles (6 &18). The dielectric walls of cells in contact with liquid nearly always develop a surface charge, with the corresponding counter ionic double layer in the liquid phase. The ions in this layer move towards the oppositely charged electrode. The polarized particles not only interact with each other but also modify the field intensity in the region of the cell. (6,15,16,17).

Conductance is the ability of a solution of possessing metal ions to pass an electric current consequently of AC fields and special frequencies. In solutions the current is carried by ions whereas in metals, it is carried by electrons. Conductance measured by applying an electrical current (I) between two electrodes and measuring the resulting voltage. During this process, the cautions migrate to the negative electrode, the anions to the positive electrode. Conductance is typically measured in aqueous solutions of electrolytes. The charge of the ions in solution facilitates the conductance of electrical current and the conductance of a solution increase with concentration (19 &20).

The impermeability of the cell envelope is not a static entity, but rather a dynamic structure which is altered in response to growth phase and environmental conditions of the mycobacterium (21, 22, 23). The mycobacterium cell envelope consists of three main layers: The cytoplasm membrane, thecell wall and the capsule. The mycobacterium cytoplasm membrane appears to resemble a typicalbacterial membrane and containing lipids, glicolipids and proteins while the capsule which appears to be limited to the pathogenic mycobacterium, contains polysacharides and excreted proteins.

The cell wall as being asymmetric and to consist of an inner segment of Mycolic acid attached to a peptidoglycan- arabinogalactan scaffold(figure1) and an outer segment of non-covalently associated lipids and proteins (23,24). The outer layer of the cell wall is actually a lipid layer, referred to as themycobacterium outer membrane (mom). The lipids of cell wall contain glycolipids and phospholipids (such as outer membrane).

Mycolic acid (MA) represent 50% of the dry weight of the mycobacterium cell wall and have a basic structure of R^2CH (oH) CH(R') COOH in which R' represents an alkane branch (C_{22}-C_{24}) and R^2 an alkyl chain (C_{30}-C_{60}) containing different functional groups. The major different between the MAs is the different in overall chain lengths. But MAs also differ with regards to the intra-chain lengths between the functional groups (23, 25,26). The mycobacterium, deficient in synthesis long chain MA, had on altered cell wall in which the electron transport region, characteristic of the mycolate layers was absent and the cell wall was thermally unstable and permeable to hydrophobic drugs. The fluid state and integrity of the cell wall of each mycobacterium is determined by the MA composition (23,24,25). For the reason that the mycobacterium cell wall contains large quantites of lipids, and special its much hydrophobic in comparison with other bacteria cause much resistance of this bacterium in front of immunological system and its much non-permeability (23,27,28,29).

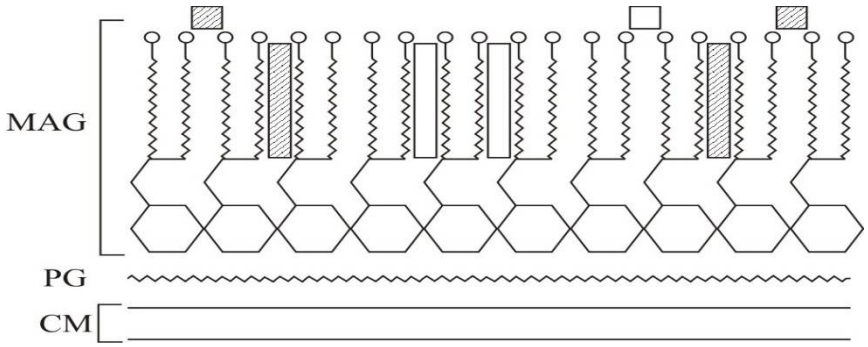
Intensive research in to the virulence factors that determine the pathogenic of M. tuberculosis havebeen carried out since the bacillus was discovered. When M. tuberculosis cells grow in a liquid medium without detergent, they from tight bundles or cords, consisting of bacilli in which the orientation of the long axis of each cell is parallel to the long axis of the cord. M. tuberculosis cords were first observed by Robert koch in 1882, but knowledge of their significance increased in 1947 with studies by Middlebrook et al. In 1953 Bloch isolated a toxic glycolipid from M. tuberculosis and related it to the virulence of the bacillus and to cording. Bloch named the glycolipid cord factor and later it was identified as trehalose dimycolate (TDM) (28, 29, 30, 31, 32).

TDM, the most abundant lipid produced log virulent M-TB, has long been a puzzling substance because it changes from non-toxic to highly toxic when injected in an oily vehicle. Bloch, Noll and their collaborators published evidence that cord factor contributes to the virulence of M-TB. With the development of better analytic methods, TDM was found in all mycobacterium, not just virulent M-TB. Therefore the idea that cord factor was a virulence factor of tuberculosis was widely dismissed. Few of the data linking TDM to the virulence of tuberculosis had been challenged. The real problem was that the activation to toxicity of a glycolipid by oil could not be explained or investigated by the prevailing paradigms of science.

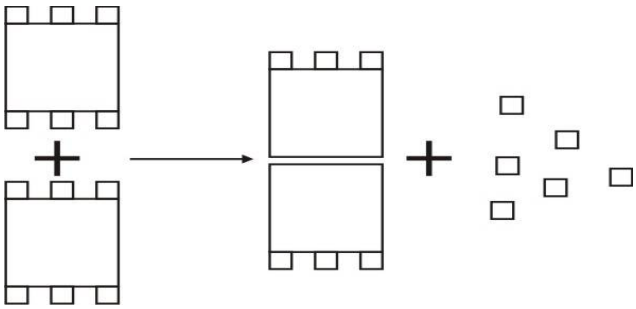
Retzinger demonstrated that TDM spontaneously formed a crystalline monolayer on hydrophobic surfaces(figure2) that is rigid and stable. The monolayer is a two dimensioned crystal of regular linear arrays of hydrophilic (trehalose) and hydrophobic (mycolate) domains (23, 31, 32). The mycolic acid groups are entirely covered. TDM is complex other lipids in mycobacterium cell walls. It forms an asymmetrical intercalated layer with lipoarabinomannan (LAM).

The size and interlocking short and long hydrocarbon chains of mycolic acid contribute to the extraordinary stability and impermeability of mycobacterium cell walls (23, 29, 30).

In this study is tried with the comparison of results of zeta potential, particle size and conductancemeasurement and then theoretical subjects to be connected to structure of M.TB cell wall and its hydrophobic effect, a step takes in the road to find of relatively careful standard for detection of number of bacterium in any sample.



Figure(1)-The mycobacterium cell consists of three main layers: Cell membrane(CM),Peptidoglicane(PG),Micolylarabinogalactan(hydrophobe)(MAG)



Figure(2)- crystalline monolayer on hydrophobic surfaces

MATERALS AND MTHODS

The bacterium used in this study obtained from culture of sputum on Lowenstein-Jensen medium patients referred to Institute pasture of Iran with concentration of 0.5 Mac Farland (3×10^8 CFU/ml) and all materials and solutions is bought from SIGMA company. Electrolyte utilized was 0.9% $(\frac{w}{v})$ NaCl the bacterial suspension diluted to final concentration of bacterium 1 CFU/ml concentration reached to 10^8 CFU/ml and in the next steps concentration reached to 1 CFU/ml).

The changes of conductance of bacterial suspension in different concentrations of bacterium (CFU/ml) measurement by CONDUCTIVITY METER made in RADIOMETER Copenhagen Company of Denmark, CD MZE-207452 model and it reported based on μs(figure3). Electrodes were fabricated from style stainless with 3cm long and 0.7cm interval space. The obtained results statistical analysed with zet pw32 software. Measurements were taken for 4 min, at 1 min interval.

For microscopic observations of cord factor and distribution of bacteria in Culture of sputum on Lowenstien-Jensen medium used from a optical microscope ZEISS (Standard 20) model made in Germany with magnify X100 and Invert Microscopy ZEISS model with magnify X40 .For the measurement of zeta potential and particle size, used from apparatus of DLS model ZETA plusmade in Brookhaven company of U.S.A.

Statistical analysis of results obtained with SPSS Ver. 16 software's and every test repeated 3 times and results based on student-t test compared. For this study, p-value was less than 0.05 (95% Confidence interval).

Figure(3): CONDUCTIVITY METER

RESULTS

The images of bacteria and colony of bacteria in the different concentrations of bacterium showed in figure4 and image of cord factor showed in figure 5.

A) B)

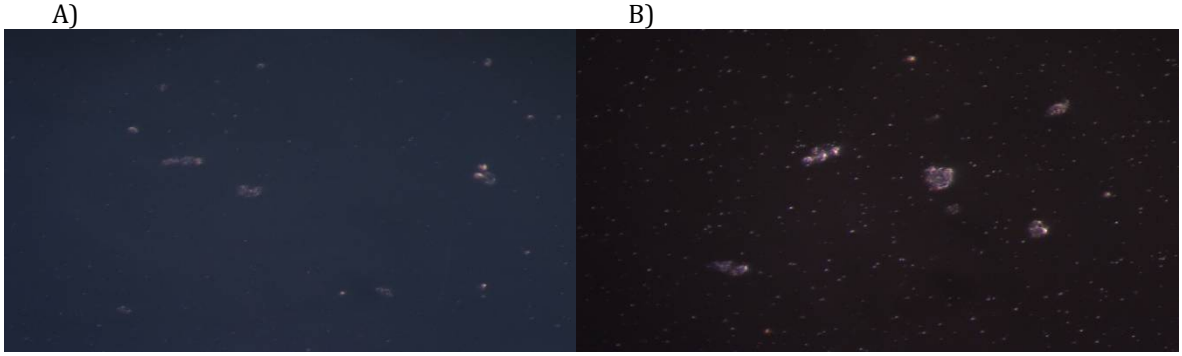


Figure (4)- The image of Invert microscope (magnify X40) bacteria colonies contain of A) few number of bacterium and B) colonies contain of more number of bacterium.

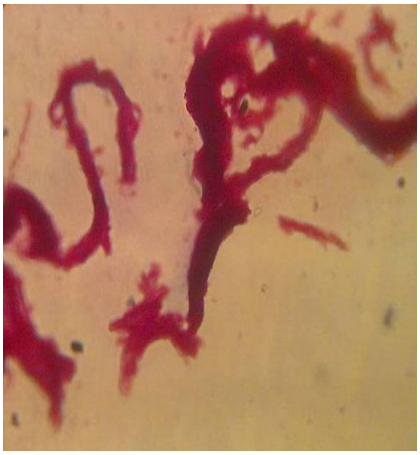


Figure (5)-. Image of optical microscope (magnify X100) from cord factor in method of Zichl- Neelsen.

The results of measurements showed particle size of bacteria increased linearity with increase of concentration of bacteria (figure6).

Consideration of figure 6 show in Concentrations 0-10^6, 10^6-10^7 and 10^7-10^8 (CFU/ml), $\frac{\delta S}{\delta \rho}$ (changes of particle concentration) equal to 10^{-3}, 10^{-5} and 10^{-8} that is change of size particle of one bacterium in every change phase.

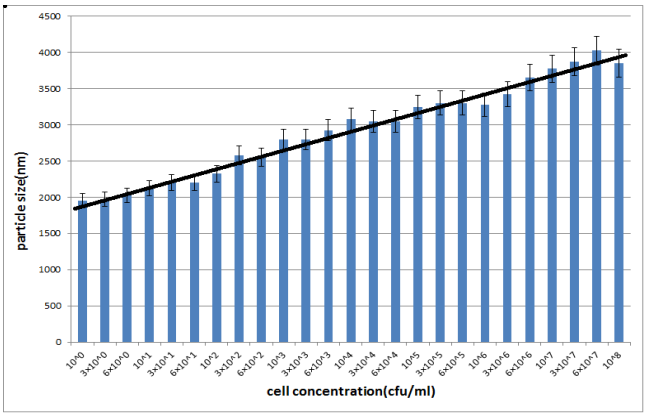


Figure (6) - The graph of changes particle size of bacteria in scale of nanometer in the different concentrations (CFU/ml).

Zeta potential increased Linearity with increase concentration of bacteria with slope 10^{-5} (0-10^5 CFU/ml), nearly o (10^5-10^6 CFU/ml) and 10^{-8} (10^6-10^8 CFU/ml) (Figure 7)

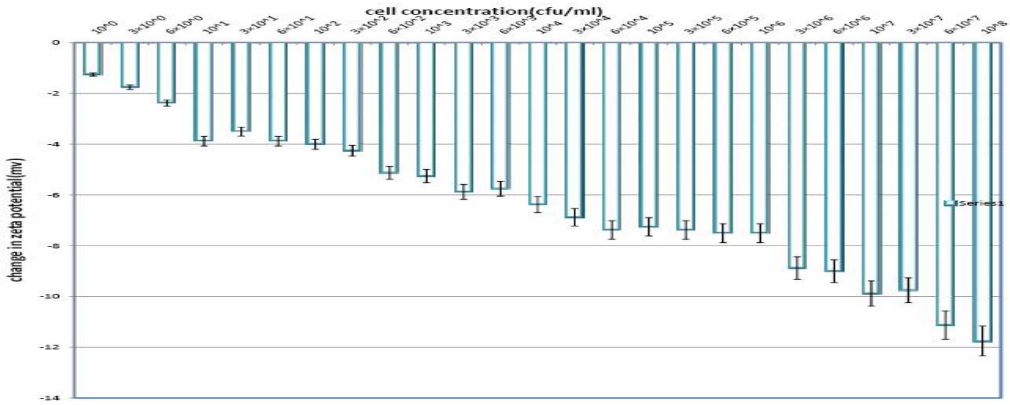


Figure (7) - graph changes of zeta potential (mV) in different concentrations of bacterium (CFU/ml).

In this graph showed three different region related to slope of changes of zeta potential in different concentrations of bacterium potential region of phase 1 (0-10^5 CFU/ml) phase 2 (10^5-10^6 CFU/ml) and phase 3 (10^6-10^8 CFU/ml). The slope of changes in phase 1 is 10^{-5}, in the phase 2 is nearly 0 and in the phase 3 is 10^{-8}.

Increase of zeta potential is obtained from decrease of conductance. Increase concentration of bacteria with coefficient of 10, but quantity of zeta potential increase with linear slope.Consideration of figure 7 showed in concentrations 0-10^5, 10^5-10^6, 10^6-10^8 (CFU/ml), $\frac{\delta\zeta}{\delta\rho}$ (changes of zeta potential to compare with changes of concentration) equal to 10^{-5}, 0 and 10^{-8} that is change of zeta potential of one bacterium in every change phase.

The conductance of bacterial suspensions increased with increase of concentration of electrolyte. The increase of conductance is linear and equal to 0.6 (0-10 CFU/ml), 10^{-6} (10-10^6 CFU/ml) and 10^{-7} (10^6-10^8 CFU/ml) (figure 8). Consideration of figure 8 show in concentrations 0-10, 10-10^6 and10^6-10^8 (CFU/ml), $\frac{\delta G}{\delta\rho}$ (changes of conductance to compared with changes of concentration) equal to 0.6, 10^{-6} and 10^{-7} that is change of conductance of one bacterium in every change phase .

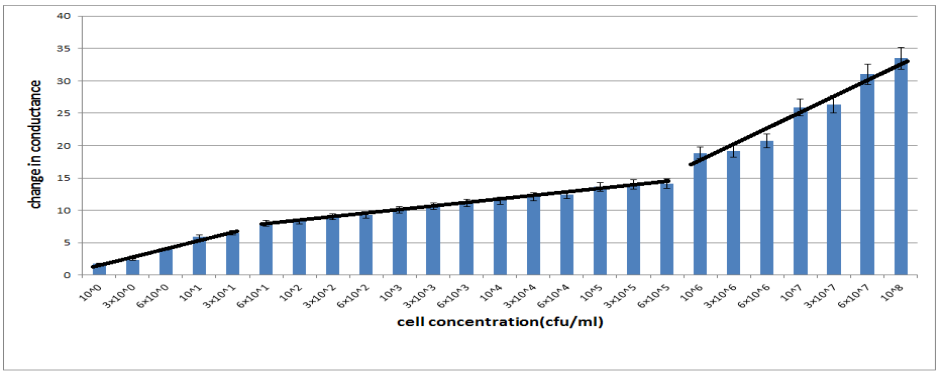


Figure (8) - graph changes of conductance (μs) bacterial suspension in different concentration (CFU/ml)

The slope of changes of zeta of <10^5 CFU/ml is more than slope of changes of particle size but inthe concentration of $\geq 10^5$ CFU/ml the slope changes of zeta potential decreased and come near to the slope of particle size.

On the other hand in the different regions of concentration the slope of changes of zeta potential and the slope of changes of conductance are similar.

In concentration on <10^5 CFU/ml the slope of change of conductance is nearly equal to the slope of change of particle size but in concentrations on $\geq 10^5$ CFU/ml the slope of change of conductance is more than the slope of change of particle size.

DISCUSSION AND CONCLUSION

In this work, reported the results of the studies of zeta potential measurements and conductance measurements but theoretical studies show that the change of zeta potential and the change of conductance are similar. The diffuse layer ions between the shear plane and the outer plane do conduct a current inside the shear plane that reduces the mobility for a given value of zeta potential [6, 7, 9, 10]. Two different mechanisms of ion conduction may be distinguished in the electric double layer. The first one corresponds to the charge transference above the slipping plane. The second mechanism is related to ionic movements that occur between the bacterium surface and the slipping plane. Thus the electric double layer is characterized by two parameter ζ and conductance [6, 7, 9, 12, 13]. The surface conductance modified the electric potential around the bacterium and affects the conversion of mobility into zeta potential [12, 13]. Electrophoresis mobility increases with decreasing salt concentration. However, bacteria conduct part of the current, which leads to a reduction of mobility, particularly when the conductance of the solution is low (6, 9). The hydrophobic effect and electrophoresis mobility of bacteria are complementary. Hydrophobic effect increased with increasing dilution rate, whereas electrophoresis mobility did not change markedly [9].

Mycobacterium tuberculosis is a hydrophobic bacterium and has low electrophoresis mobility. but isolation of a hydrophobic bacterium with a low zeta potential (ζ) from a natural sample would be very difficult. This kinds of bacteria adhesion very strongly to surfaces and to each other (cord factor). Therefore this hydrophobic bacteria with low zeta potential (ζ) could have escaped classical microbiological isolation techniques.

Another explanation for the difficulty in finding such bacteria could be that hydrophobic effect combined with low electrical change [6, 9, 10, 34, 35].

In spite of this, Variation in surface characteristic such as change of conductance, change of zeta potential and hydrophobic effect, play a less important role, at least in relation to bacterial surface charge and in high-ionic strength environments. This may be due to elevated concentrations of ions. But in low-ionic-strength environments, the microbial surface hydrophobic effect and charges are of greater importance [36, 37, 38, 39, 40].

The present study showed that a large variability in parameters can be existent and that can be indication of the M.TB. In this study, the lack of statistical correlation between surface charge, hydrophobic effect and other parameters is due to the complexity of our system. Linearity of changes of zeta potential and conductance of bacteria can be with completion of studies and accomplishment of other tests, a important result for measurement of unknown concentration of bacterium in a sample.

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CITATION OF THIS ARTICLE
Pourmir A, Farsizaban M, Nakhzari Moghadam H ,Poorjangi M. A new method for detection of Mycobacterium tuberculosis by biosensor based on Conductivity and zeta potential. Bull. Env. Pharmacol. Life Sci., Vol 3 [Spl Issue V] 2014: 50-58