

## ORIGINAL ARTICLE

# Standardization of an Efficient Technique for the Reporting of CFU/mL in Urine Samples with Bacterial Cell Cytolysis

Estandarización de una técnica eficiente para el reporte de UFC / mL en muestras de orina con citolisis celular bacteriana

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## ABSTRACT

Urinary tract infections (UTIs) are common in women due to the anatomy of the urethra and its proximity to the anus. Bacterial cell cytolysis (BCC) describes the entry of bacteria into the cells of the UTIs. The most frequent BCC is by Gram-negative bacilli, colonizing, adhering and penetrating cells of the urinary tract. BCC is not considered in the CFU / mL count in urine culture using the conventional procedure. The drawback of the technique is that after BCC an innumerable number of bacteria can be located in a single cell and since the release of intracellular bacteria is not promoted, only a single CFU is quantified and not the real set that remains inside the cell. Such under-notification is significant given that the decision to offer an antimicrobial treatment to the patient depends on the count. This study aims to standardize an efficient technique for reporting CFU / mL in urine samples with BCC. Urine sample was collected. Urochemical profile, urinalysis, cytopathology and a plate count were performed to define the initial amount of bacteria, for later comparison against the post-treatment cell lysis bacterial count. To demonstrate the best cell lysis treatment: a randomized 3x2x3x2 full factorial design was used, in triplicate, whose factors were: Lysis method (Vortex®, Low frequency ultrasound (LFU) and Chemical Buffer), speed and time of exposure, and finally material of the sample container (glass or plastic). The technique that generated the greatest recovery and that reduced the quantification error was; LFU of the sample contained in glass, for 30s. However, regardless of the lysis technique, the use of only one substantially improves (63-734%) the bacterial bacillary recovery of urine samples with bacterial cell cytolysis.

**Keywords:** Bacterial cell cytolysis, Chemical and mechanical lysis, Low frequency ultrasound

## RESUMEN

Las infecciones del tracto urinario (ITU) son frecuentes en mujeres debido a la anatomía de la uretra y su proximidad con el ano. La citolisis celular bacteriana (CCB) describe el ingreso de bacterias al interior de las células del TU. La CCB más frecuente es por Bacilos Gram-negativos, colonizando, adhiriéndose y penetrando células de vías urinarias. La CCB no se considera en el recuento de UFC / mL en el urocultivo mediante el procedimiento convencional. El inconveniente de la técnica es que tras la CCB una innumerable cantidad de bacterias pueden situarse en una sola célula y al no promoverse la liberación de las bacterias intracelulares, solo se cuantifica una sola UFC y no el conjunto real que permanecen en el interior de la célula. Tal sub-notificación cobra significancia dado que del recuento depende la decisión de ofrecerle o no un tratamiento

antimicrobiano al paciente. Este estudio pretende estandarizar una técnica eficiente para el reporte de UFC/mL en muestras de orina con CCB. Se colectaron muestra de orina. Se realizó perfil uroquímico, urianálisis, citopatológico y un recuento en placa para definir la cantidad inicial de bacterias, para su posterior comparación contra el recuento bacteriano postratamiento de lisis celular. Para evidenciar al mejor tratamiento de lisis celular: se empleó un diseño factorial completo 3x2x3x2 aleatorizado, por triplicado, cuyos factores fueron: Método de lisis (Vortex®, Ultra sonicación de baja frecuencia (UBF), Buffer-Químico), velocidad y tiempo de exposición, y finalmente material del contenedor de la muestra (vidrio o plástico). La técnica que mayor recuperación generó y que redujo el error de cuantificación fue; UBF de la muestra contenida en vidrio, durante 30s. Sin embargo, sin importar la técnica de lisis, el sólo empleo de una, mejora sustancialmente (63-734%) la recuperación bacilar bacteriana de muestras de orina con citolisis celular bacteriana.

*Palabras clave:* Citolisis celular bacteriana, Lisis química y mecánica, Ultrasonificación de baja frecuencia

## INTRODUCTION

Urinary tract infections (UTIs) cause approximately 20 million doctor visits. The female gender is the most affected and it is presumed that it can be found in a 10:1 ratio with respect to the male gender. By definition, UTIs are considered from the moment that the bacteria appear and multiply in the urinary tract beyond the anterior urethra causing classic symptoms of the pathology, in addition, the diagnosis must consider the response of the host, that is, the Epithelial desquamation and immune response (1,2). The migration of bacteria to the bladder is favored by mechanical, physical and chemical factors. To cause an infection, the responsible microorganisms not only need to colonize the urethra, but also adhere to the uroepithelium; these factors are little considered and applied in the different laboratory analyzes (3).

### Bacterial cell cytolysis

Bacterial cytolysis is called the phenomenon of introduction of bacteria to cells of the genitourinary tract, the most frequent bacterial cytolysis observed is that caused by gram negative bacilli, colonizing, adhering and penetrating cells of the upper and intermediate urinary tract. The pathogenicity factors involved with this phenomenon are adhesins, lysozymes and cytolysins (1). The activity generated by the various pathogenicity mechanisms present in bacterial genera, develops great cytoarchitectural modifications in uroepithelial cells. These changes can be reversible or irreversible and can be recognized by observing the cellular characteristics found in the urinary sediment. Once the bacteria have recognized the uroepithelial cell receptors, the adhesion process begins, thereby initiating uroepithelial invasion and multiplication; This process occurs due to one of the bacterial genera (pilis, fimbriae, capsule, etc.), so that each uropathogen adopts a different conformation (adherence pattern) characteristic of the epithelium, for which a total of seven different patterns of adherence

and bacterial cytolysis, when observing the urinary sediment (2,9).

### Problem statement

Leukocyturia encourages bacteria to adhere and subsequently internalize the uroepithelium, establishing and multiplying, a phenomenon recognized as bacterial cytolysis; this event is not considered in the UFC / mL count in the urine culture (3,4). For what we consider that these bacteria that are inside the host cell, must be taken into account, since they significantly influence the final results of the urine culture, since otherwise it is as if a wrong diagnosis was being given (5,8). So far there is no method that is standardized that allows to provide a reliable result, that is, that takes into account the bacteria that are inside the cell in the microbial count.

### Purpose of the study

- To evaluate the different methods to release intracellular bacilli in cytolysis, to generate a more efficient count of CFUs.
- Standardize a technique that allows us to extract the bacteria found inside the uroepithelial cells, in order for them to be considered in the microbial count.

### Justification and use of the results

The purpose of this work is to demonstrate that when modifying the existing bacterial quantification techniques, a marked increase in the bacterial count (expressed in CFU / mL) is observed in direct relation to the number of cells in bacterial cytolysis, improving the results in the urine culture. Regularly in the quantification of samples with cytolysis there is divergence from one analyst to another, so that the unification of the reporting criteria is essential. To unify the criteria, it is essential to standardize the procedures. Through this design, we will obtain a more efficient method for bacterial counting in samples containing bacterial cytolysis, in order to suggest a more accurate procedure for reporting CFUs found. If

the semi-quantitative method of the general bacteriological examination of urine is considered as the beginning of the diagnosis of urinary tract infections, 70% of the patients would be failing to diagnose, since the phenomenon of bacterial cytolysis masks the development of the real bacterial count. If the release of the bacteria present in the uroepithelium develops and is quantified by modified urine culture, the real diagnosis of UTIs can be made in 92% of patients (using the Kass criteria) (6,7).

## MATERIALS AND METHODS

### Sampling

The sample of the first morning urine was collected following the medium jet method and homogenized with gentle circular movements avoiding the formation of bubbles and foam.

### Urochemical study

Pour 5 mL into 3 sterile tubes of 13 x 100. Mark the sterile tubes with the letters A, B and C. To tube A with 5 mL of urine and using reagent strips for urine determination (Axmilab®) the preliminary characteristics that show a possible infection will be evaluated: Nitrites POSITIVE; Glucose NEGATIVE; Leukocytes +2 (> 75 Cell x 40X field), Bacteria (MODERATE/ABUNDANT); Blood: Traces. Samples with such characteristics will continue to be evaluated.

### Cytopathological urinalysis

Tube B should be centrifuged at 2500 revolutions per minute (rpm), for 3 minutes. Once it has been centrifuged, decant 90% of the supernatant, after this step, resuspend the pellet with gentle circular movements in an equivalent volume of Sternheimer-Malbin stain. From tube B: 25 µL of the resuspended is taken and deposited on a clean slide, a coverslip is placed and analyzed under a microscope to verify the presence of cytolysis. Once the presence of cells with bacteria has been identified, a new smear of the sediment is made and later the Gram stain is used. Both preparations already mentioned are observed at 10x to guarantee homogenization and finally the interpretation is carried out at 40x. The presence of urate, oxalate, urea, calcium crystals, as well as squamous cells from the upper, middle or lower pathways should be observed. The search for cells with intracellular bacillary bacteria will be exhaustively described.

### Direct count (basal)

A basal count of colony forming units (CFU) was performed on the urine samples that presented cytolysis using Trypticasein Soy Agar (BD-Dixon™). Four plates were inoculated with the aforementioned medium, the first directly; the second with the 1:100 dilution; the third with the 1: 10,000 dilution and the fourth of 1:1,000,000, these were inoculated by means of the surface extension technique. And then the number of CFU / mL of urine was reported.

### Post-treatment count

After having the report of each urine sample, those with the highest cytolysis (tube C) are selected and each treatment described below is applied to them. From the initial number of CFU / mL, it is taken as a reference to make the post-treatment dilutions and continue to quantify them with surface extension. A post-treatment gram stain was added to compare the amount of cell lysis and observe the release of intracellular bacilli.

### Results analysis plan

Design of experiments: A randomized 3x2x3x2 factorial design will be used in triplicate experimentation. The following table (Table 1) shows the variables and their levels:

**Table 1.** Variables and levels of the 3x2x3 design of experiments

**Tabla 1.** Variables y niveles del diseño de experimentos 3x2x3x2

Variable (factors)	Levels
Cell lysis technique	1. Vortex® stirring
	2. Low Frequency Ultrasonication (1 W/cm <sup>2</sup> )
	3. Tris-HCL® Chemical Buffer Lysis
Speed (only for mechanical methods)	1. High 2800 rpm
	2. Low 500 rpm
Time (treatment exposure time)	1. 30 s
	2. 2 min
	3. 5 min
Tube type	1. Glass
	2. Plastic.

As response variable: It is the increase in the concentration of CFU / mL in the samples with cytolysis after having submitted them to each of the experiments. Increase in CFU / mL = (Count after lysis experiment / Baseline Direct Count) \* 100.

The program to be used for the analysis of the CFU increment values of each procedure performed will be JMP 5.1.0 and thus obtain the best technique to perform in samples that present bacterial cytolysis.

## RESULTS AND DISCUSSION

The fact of applying a lysis treatment, either by Vortex, Lysis Buffer and / or Low Frequency Ultrasound (LFU) generated an increase in the count compared to the initial count without lysis treatment. The lysis technique that showed the greatest efficiency in the recovery of the bacterial cells trapped inside the epithelial cells was LFU, followed by Vortex and finally, the lysis buffer. However, statistically there is no significant difference ( $p > 0.05$ ) between the levels of the type of technique factor, so any technique could be used without discrimination to improve the microbial bacillary recovery of urine samples with cytolysis by 63-734 %. Similarly, no significant difference ( $p > 0.05$ ) is found between techniques when evaluating them based on the quantification error. Regarding the factor of agitation speed in Vortex® and type of material for LFU, the high speed was the one that showed the greatest increase in the recovery of intracellular bacterial cells with respect to the lower speed. On the other hand, for the type of material used in LFU, the one that showed the greatest recovery of intracellular bacterial cells was glass, showing increases of up to 1273 % with respect to plastic, which showed maximum increases of 832 % over the initial count without LFU. However, no statistically significant difference ( $P > 0.05$ ) was shown between the levels of the speed factor and type of material used.

## CONCLUSION

Regardless of the lysis technique, vortex speed, type of container material used for the LFU and the exposure time in each of the techniques, the simple use of a cell lysis technique substantially improves the recovery of bacteria cells trapped inside invaded epithelial cells (cells with cytolysis). The technique that generates the greatest recovery and that limits the quantification error to a greater degree was the use of LFU from 5 mL of sample contained in a glass tube, for 30 s at room temperature 22-25 ° C. However, since no statistically significant difference is found between the factors and levels tested, it is suggested to use any cell lysis technique interchangeably. It is suggested to continue the research, increasing the treatments and including a more level of technique that combines the lytic

chemical power of the Tris buffer solution, vortex agitation and the micro-vibration in LFU generated by glass microbeads, in order to test if better results are obtained in bacterial cell recovery in samples with bacterial bacillary cell cytolysis.

### *Conflict of interest:*

The authors declare that they have no conflict of interest.

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