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In vitro antimicrobial activity of generic and brand-name Levofloxacin against clinical and ATCC strains *E. coli* and *S. aureus*

Carlos Sánchez-Hoyos[1], Antistio Alviz-Amador[2] and Marlene Duran-Lengua[1,3*]

[1]Farmabac Research Group, Faculty of Medicine, University of Cartagena, Cartagena, Colombia.
[2]Farmacologia y Terapeutica Group, Faculty of Pharmaceutical sciences, University of Cartagena, Cartagena, Colombia.
[3]Faculty of Medicine, University of Cartagena, Cartagena, Colombia.

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***In vitro* antimicrobial activity between generic and brand-name Levofloxacin was evaluated against isolated strains collected in 3 Colombia hospitals: *Staphylococcus aureus* and *Escherichia coli.* Initially, active substance was quantified using the methodologies identified by the United States Pharmacopeia (USP) 38 NF 32, chromatographic conditions were validated and standardized. The minimum inhibitory concentration (MIC) of Levofloxacin was determined in accordance with the Clinical and Laboratory Standards Institute (CLSI). Growth curves were then performed to determine the maximum growth time of the bacteria in order to determine the MIC at the maximum growth time. Different brands evaluated did not present any difference with MIC of 0.125, 0.062, 0.031, 0.062 and 0.125 µg/ml for *E. Coli* ATCC, *E. Coli* Tropical, *S aureus* sensible ATCC, *S aureus* resistant ATCC and *S. luteum*, respectively. The *in vitro* antibacterial activity of levofloxacin against *E coli* Tropically and *S luteum* are reported for the first time.**

Key words: Quinolones, levofloxacin, minimum inhibitory concentration (MIC), *Staphylococcus aureus* and *Escherichia coli* (MeSH).

INTRODUCTION

Fluoroquinolones are the fourth class of antibiotics used in human and veterinary medicine for the treatment of serious bacterial infections. Its broad spectrum of activity and favorable pharmacokinetic properties are the main characteristics that have increased its widespread clinical use throughout the world (Barreto et al., 2017). However, its irrational use has increased the resistance profile, for this reason, it is pertinent to conduct studies that evaluate pharmaceutical alternatives of Levofloxacin, against pathogenic microorganisms such as *S. aureus* and *E. coli* sensitive and resistant, as well as ATCC isolated from hospitals in Colombia (Carvalho et al., 2016; Fariña et al., 2007). Currently, the doubts that arise both in the users and health providers services are very evident

*Corresponding author. E-mail: marlene6817342@yahoo.es.

when it comes to the quality of any generic drug, and their replacement with brand name drugs (Artaza et al., 2016; Medina-Morales et al., 2015). For these reasons, it is necessary to demonstrate that the quality of a drug is not directly related to its value and to dispel the myth "the more expensive the product, the more effective", which may favor communities with less economic capabilities, allowing them access to effective and quality therapy, which until now would be the most affected due to the few numbers of studies.

Bacterial resistance to antibiotics is a global health problem, because the possibility of continuing to successfully treat infections that are now easily treated is in danger (Yılmaz and Aslantaş, 2017). Morbidity, mortality and treatment costs will increase, if these bacterial resistances are not controlled (Pastor-Sánchez, 2006; Jackson et al., 1998; Juste Díez de Pinos et al., 2000; Mandell et al., 2010). The irrational use of antibiotic therapy jeopardizes the possibility of continuing treating with success, infections that are treated with these drugs; to clear up the doubts, we must evaluate the behavior of these substances and certify the suitability of the products used for the therapies indicated, guaranteeing the interchangeability between generic Levofloxacin with its brand name in the treatment of the pathologies caused by *E. coli* and *S. aureus*.

For all drug generic or brand name, especially antibiotics, their efficacy and safety are infallible qualities, since in the opposite case; the patient health is put at risk due to the appearance of bacterial resistances (Sun et al., 2016). According to the surveillance programs in United States, a total of 2008 samples evaluated showed a resistance rate to Levofloxacin of 5% (Cercenado, 2011; Meléndez et al., 2005; Sato et al., 2011).

The aim of the study is to compare *in vitro* antibacterial activity of a generic and brand name of Levofloxacin previous substance active quantification, following USP38 NF 32 (Pharmacopeia, 2016). The activity of Levofloxacin was measured against 2 strains which are causes of nosocomial infections: *Escherichia coli* and *Staphylococcus aureus*, by determining the minimum inhibitory concentration (MIC), using broth microdilution method. The results obtained allow us to determine which drug offers the highest efficacy *in vitro*. In addition, this study will present results in strains which have not been evaluated microbiologically in Colombia: *Escherichia coli tropically* and *Staphylococcus luteum*.

MATERIALS AND METHODOLOGY

Reagents

Bacterial Culture Media

Solid media: Nutritious Agar, and Trypticase Soy Agar Merck Millipore. Liquid Medium: Thioglycolate Broth, Muller-Hinton Broth and Luria bertani broth Difco.

Antibiotics

Levofloxacin: 3 different batches of Pharmamedic, ADS PHARMA and Sanofi. USP standard of Levofloxacin Sigma Aldrich.

Microorganisms

ATCC: *S. aureus* 43300 (Met-R), *S. aureus* 25923 (Met-S), *E. coli* 25922, purchased from the authorized Techno medical distributor. Clinical isolations were obtained from 3 Colombian Hospitals. *S luteum* and *E coli Tropically* from Microkit SL laboratories

Active principle quantification

The active principle quantification was performed by high-performance liquid chromatography (HPLC) according to USP38 NF-33. Linearity, accuracy, repetitiveness, intermediate precision, selectivity of generic and brand names of levofloxacin drug were determined to validate the analytical methods.

Chromatographic conditions

The high resolution liquid chromatography (Elite Lachrom HITACHI I2350) equipment, equipped with a quaternary pump and a Diode Array Detector (DAD) was used. A reversed phase Merck® C18, 150 × 4.6 mm, particle size 5 µm was used as the analytical column. The mobile phase was a mixture of 0.1% solution of triethanolamine-acetonitrile (80:20), adjusted to a pH of 4.8 with phosphoric acid; filtered and degassed by 0.45 µm membrane. The wavelength was set at 296 nm, with flow rate of 1 ml/min and injection volume of 20 µl. Before using all solutions, the mobile phase was sonicated for 30 min and UV detection was performed at 296 nm for GTX.

Linearity

10 mg of Levofloxacin standard were weighed and taken to a 10 ml graduated volumetric flask, which was completed using mobile phase diluent, thus remaining at a concentration of 1000 ppm. This solution was labeled as standard stock solution. From the stock solution, aliquots of 0.25, 0.5, 0.75, 1.0, and 1.2 ml were taken to 10 ml graduated volumetric flask and adjusted with mobile phase, obtaining concentrations of 25, 50, 75, 100 and 120 ppm, respectively. Each solution was injected into the Chromatograph in triplicate.

Accuracy

Two milliliter of the drug Levofloxacin was taken, which was at a concentration of 5 mg/ml and taken to a 10 ml graduated volumetric flask, it was completed using mobile phase diluent, obtaining a concentration of 1000 ppm. It was labeled as the stock solution. From the stock solution, aliquots of 0.25, 0.5, 0.75 and 1.0 ml were taken to 10 ml graduated volumetric flask and adjusted with mobile phase, obtaining concentrations of 25, 50, 75 and 100 ppm, respectively. Each solution was injected into the chromatograph in triplicate. The obtained data were analyzed and the recovery percentage calculated.

Repetitiveness

From the stock standard and sample solutions, 1 ml aliquots were

Table 1. Percentage of recovery of Levofloxacin estimated by precision test, USP 38.

Levofloxacin accuracy				
Retention time	**Area**	**Theoretical concentration (ppm)**	**Real concentration (ppm)**	**Recovery %**
1.667	6908009	25	23.74732536	94.99
1.66	6886092	25	23.65993038	94.64
1.653	7079861	25	24.43259258	97.73
1.64	13030547	50	48.16120839	96.32
1.64	13264055	50	49.09233156	98.18
1.64	12953915	50	47.855635	95.71
1.633	19556758	75	74.18475483	98.91
1.633	19916697	75	75.62002704	100.83
1.633	19692647	75	74.72661805	99.64
1.633	25765870	100	98.9438554	98.94
1.633	25550126	100	98.08356694	98.08
1.633	25928682	100	99.59307523	99.59
Mean	97.8	Mean Standard Error	0.624928069	
Standard Deviation	1.976196	Variation coefficient	0.02	

taken to 10 ml graduated volumetric flask; mobile phase was adjusted and 100 ppm concentrations were obtained. These solutions were taken to the chromatograph and injected six times each. The obtained data were analyzed and the standard deviation and the coefficient of variation (RSD) were calculated; having an RSD ≤ 2% as acceptance criteria for the runs.

Intermediate precision

From the standard stock solution aliquots of 0.5, 0.75 and 1 ml were taken to 10 ml graduated volumetric flask, to which volume was completed with mobile phase and a solution was obtained with concentrations 50, 75 and 100 ppm, respectively. These solutions were injected in duplicate. This procedure was carried out by three different analysts on different days. The data obtained were analyzed and the relative standard deviation (RSD) obtained.

Antimicrobial activity

The minimum inhibitory concentration (MIC) of 2 generic, 1 USP standard and 1 brand name of levofloxacin drug were evaluated using the broth micro dilution method, described by the Clinical and Laboratory Standards Institute (CLSI, 2015). Ten dilutions were prepared for each drug, performing serial double dilutions from 64 to 0.0075 µg/ml on bacterial suspensions at a concentration of 5×10^5 CFU/ml, in 96-well microtiter plates CLSI (2011). Initially beginning with a concentration of levofloxacin drugs (5 mg/100 ml) which was diluted with Muller-Hinton broth at pH 7.3 to obtain a stock solution of 64 µg/ml, the solution was diluted to obtain an intermediate solution at a concentration of 8 µg/ml after which, doubling-dilution series of the antibiotic solutions of 8 µg/ml to 0.015 µg/ml were performed. 50 µl of each dilution was dispensed into the wells of the Microplates and 50 µl of the inoculum was added to each one, to obtain final bacterial concentrations of 5×10^5 CFU/ml. A well that contains inoculum without antibiotic was used as a positive control and one containing antibiotic dissolved in broth without inoculum as a negative control. The turbidity of the actively growing broth culture was adjusted to an optical density equivalent to a 0.5 McFarland standard using a Thermo Scientific Multiskan EX® spectrophotometer at 620 nm. All assays were conducted in triplicate.

Statistical analysis

The analytical datas, such as linearity, accuracy, repetitiveness and intermediate precision, were tested for each alternative through descriptive statistics. MIC values between doubling-dilution series of the antibiotic solutions, positive control and negative control for each alternatives, were tested using one-way analysis of variance (ANOVA), followed by a tukey test for multiple comparisons with significant statistical difference at $p < 0.05$.

RESULTS AND DISCUSSION

Linearity

The results obtained indicate that the system for determining levofloxacin is able to explain the response (Area) from the use of the concentration variable. Therefore, in the concentration range between 25 and 120 ppm the linearity conditions of the analytical system are satisfied, this is demonstrated by obtaining a correlation coefficient r = 0.9982 and a determination coefficient $R^2 = 0.9965$.

Accuracy

Table 1 shows the levofloxacin recovery percentage values, which reached between 94.64 and 100.83%, which are within the acceptance criteria of 92% as a minimum. The values of RSD remained below 2%, this

Table 2. Percentage of recovery of Levofloxacin estimated by system repeatability test, USP 38.

Standard Levofloxacin				
Retention time	**Área**	**Theoretical concentration (ppm)**	**Real Concentration (ppm)**	**Recovery %**
1.827	24370246	100	93.3787448	93.3787448
1.82	24311355	100	93.14391441	93.14391441
1.82	24385487	100	93.43951894	93.43951894
1.813	24337761	100	93.24920947	93.24920947
1.813	24415376	100	93.55870261	93.55870261
1.813	24302249	100	93.10760385	93.10760385
Mean	93.31294	Standard deviation	0.176377963	
Variation Coefficient	0.001890177	Mean Standard Error	0.055775609	

Table 3. Percentage of recovery of Levofloxacin estimated by method's repeatability test, USP 38.

Levofloxacin Sample				
Retention time	**Área**	**Theoretical concentration (ppm)**	**Real Concentration (ppm)**	**Recovery %**
1.83	24695054	100	94.67393064	94.67393064
1.82	24718643	100	94.76799279	94.76799279
1.813	24204589	100	92.7181804	92.7181804
1.802	24405801	100	93.52052189	93.52052189
1.813	24382798	100	93.42879644	93.42879644
1.82	24438024	100	93.64901248	93.64901248
Mean 94.0	Standard Deviation	0.788607358		
Variation Coefficient	0.008407949	Mean Standard Error	0.249379543	

indicates that the methodology yields acceptable results according to USP 38 (Van et al., 2017). These results are similar to those shown by Aragon-Martinez in a study conducted on plasma samples (Aragon-Martinez et al., 2017).

Repetitiveness

The recovery percentage for each injection were calculated, and average values of 93.31% for the system and 93.79% for the method were obtained, indicating that the method and system met the requirements to perform the test (Tables 2 and 3).

Intermediate precision

The RSD values obtained were between 0.01% and 0.05%. Likewise, the calculation of the recovery percentage was made for each run; the average value obtained was 102.58% (Tables 4, 5 and 6), this is due to analyst linked errors during the preparation of the solutions, evidenced when finding the real concentration of these solutions. These results show that the analytical method is accurate, since the USP38 accepts as a minimum value or acceptance criterion for this parameter, an RSD less than or equal to 4% and a recovery percentage greater than or equal to 95%.

Generic and brand name comparison

The comparison test between generic and brands name levofloxacin drug yielded very similar results in terms of areas under the curve and the recovery percentage, with an average of 97.76% for the generic drug and 97.11% for the commercial one. Both cases had a variation coefficient (RSD) of 0.01. The generic and brand name drug vials concentration were determined using the formula described in the methodology, obtaining a concentration of 4.861 mg/ml for the generic drug and 4.826 mg/ml for the brand name one (Tables 7 and 8), corresponding to 97.22% for the generic drug and 96.52% for the brand name one of the reported concentration (5 mg/ml). In this study, the analytical quantification of active principle of generic and brand names levofloxacin shows that there were no differences from the point of view of the concentration reported in the tag of the different drugs evaluated. This is directly

Table 4. Percentage of recovery of Levofloxacin estimated by intermediate precision test, day 1, USP 38.

Levofloxacin intermediate precision day 1				
Retention time	**Área**	**Theoretical concentration (ppm)**	**Real Concentration (ppm)**	**Recovery %**
1.667	14893895	50	55.5913885	111.18
1.66	14543607	50	54.19460007	108.39
1.653	21842411	75	83.29889425	111.07
1.64	21754631	75	82.94886774	110.60
1.64	28145689	100	108.4334858	108.43
1.64	28042377	100	108.0215248	108.02
Mean	109.62	Standard Deviation	1.480942954	
Variation Coefficient	0.01	Mean Standard Error	0.468315282	

Table 5. Percentage of recovery of Levofloxacin estimated by intermediate precision test, day 2, USP 38.

Levofloxacin intermediate precision day 2				
Retention time	**Área**	**Theoretical concentration (ppm)**	**Real concentration (ppm)**	**Recovery %**
1.612	13323093	50	49.32774811	98.66
1.62	14140852	50	52.58859722	105.18
1.613	20695054	75	78.72375898	104.97
1.613	20718643	75	78.81782113	105.09
1.64	24782798	100	95.02381361	95.02
1.653	25038024	100	96.04153823	96.04
Mean	100.83	Standard deviation	4.806603534	
Variation Coefficient	0.05	Mean Standard Error	1.519981498	

Table 6. Percentage of recovery of Levofloxacin estimated by intermediate precision test, day 3, USP 38.

Levofloxacin intermediate precision day 3				
Retention time	**Área**	**Theoretical concentration (ppm)**	**Real concentration (ppm)**	**Recovery %**
1.613	13003039	50	48.05151905	96.10
1.6	12607513	50	46.47434216	92.95
1.593	19428611	75	73.67376316	98.23
1.587	20121383	75	76.43622124	101.91
1.573	25563412	100	98.13654543	98.14
1.583	25145610	100	96.47054203	96.47
Mean	97.30	Standard deviation	2.96379407	
Variation Coefficient	0.03	Mean Standard Error	0.937233978	

related to the quality of levofloxacin used for *in vitro* antibacterial activity assay. In the Figure 1 you can see the recovery percentage means between generic and brandname levofloxacin drugs without significant differences

Hospital strains growth curves

E. coli and *S. aureus* ATCC strains, both sensitive and resistant, showed a maximum growth from 5 and up to 15 h, which is consistent with what was reported by the CLSI (2014) and computer simulated methods (Jorgensen and Turnidge, 2015; Cattaneo et al 2009). On the other hand, *E. coli tropically* and *S. luteum* canariensis strains reached up to 23 h, which is also compatible with that reported by Microkit SL laboratories in the technical annexes provided. Microkit laboratories strains grow faster in time than ATCC strains. Hospital strains presented the following timing of maximum growth; 16 h

Table 7. Percentage of recovery of Levofloxacin generic drug, USP 38.

Generic levofloxacin				
Retention time	**Área**	**Theoretical concentration (ppm)**	**Real concentration (ppm)**	**Recovery %**
1.787	13197225	50	48.82584406	9765
1.78	13246582	50	49.02265722	98.05
1.773	19307821	75	73.19210786	97.59
1.773	19536842	75	74.10533892	98.81
1.767	25526874	100	97.99084859	9799
1.76	25142543	100	96.45831223	96.46
Mean	97.76	Standard deviation	0.770234	
Variation Coefficient	0.01	Mean Standard Error	0.243569	

Table 8. Percentage of recovery of Levofloxacin brand name drug. USP 38.

Brand name levofloxacin				
Retention time	**Área**	**Theoretical concentration (ppm)**	**Real Concentration (ppm)**	**Recovery %**
1.74	13051547	50	48.24494679	96.49
1.74	12936915	50	47.78784677	95.58
1.74	19534091	75	74.09436919	98.79
1.733	19529731	75	74.0769835	98.77
1.733	25119726	100	96.36732847	96.37
1.733	25197243	100	96.67643083	96.68
Mean	97.11	Standard deviation	1.34627227	
Variation Coefficient	0.01	Mean Standard Error	0.425728672	

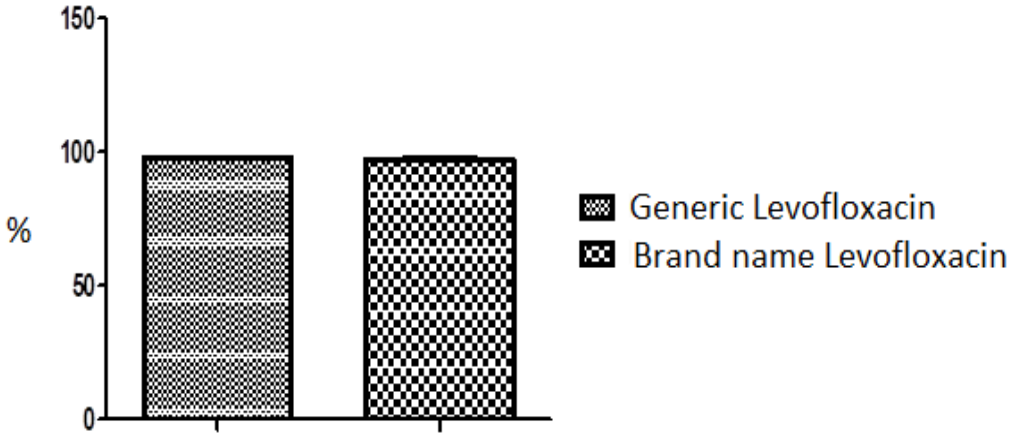

Figure 1. Recovery percentage between generic and Brand name levofloxacin drugs.

S. aureus, 18 h *E. coli*, showing a time of maximum growth greater than ATCC strains, previously evaluated and presenting no abnormalities with respect to growth. It is possible to observe and differentiate the stages from medium assimilation that is less than 5 h to the stage of death, which in the case of *S. aureus* is between 18 and 20 h, and for *E. coli* it is observed from 19 h. Hence, the timing of maximum growth of the isolates is greater than ATCC. Comparing Microkit SL laboratories strains to those isolated from hospitals and to ATCC, it was observed through absorbance performed by turbidimetry tests, that hospital strains had the highest cellular

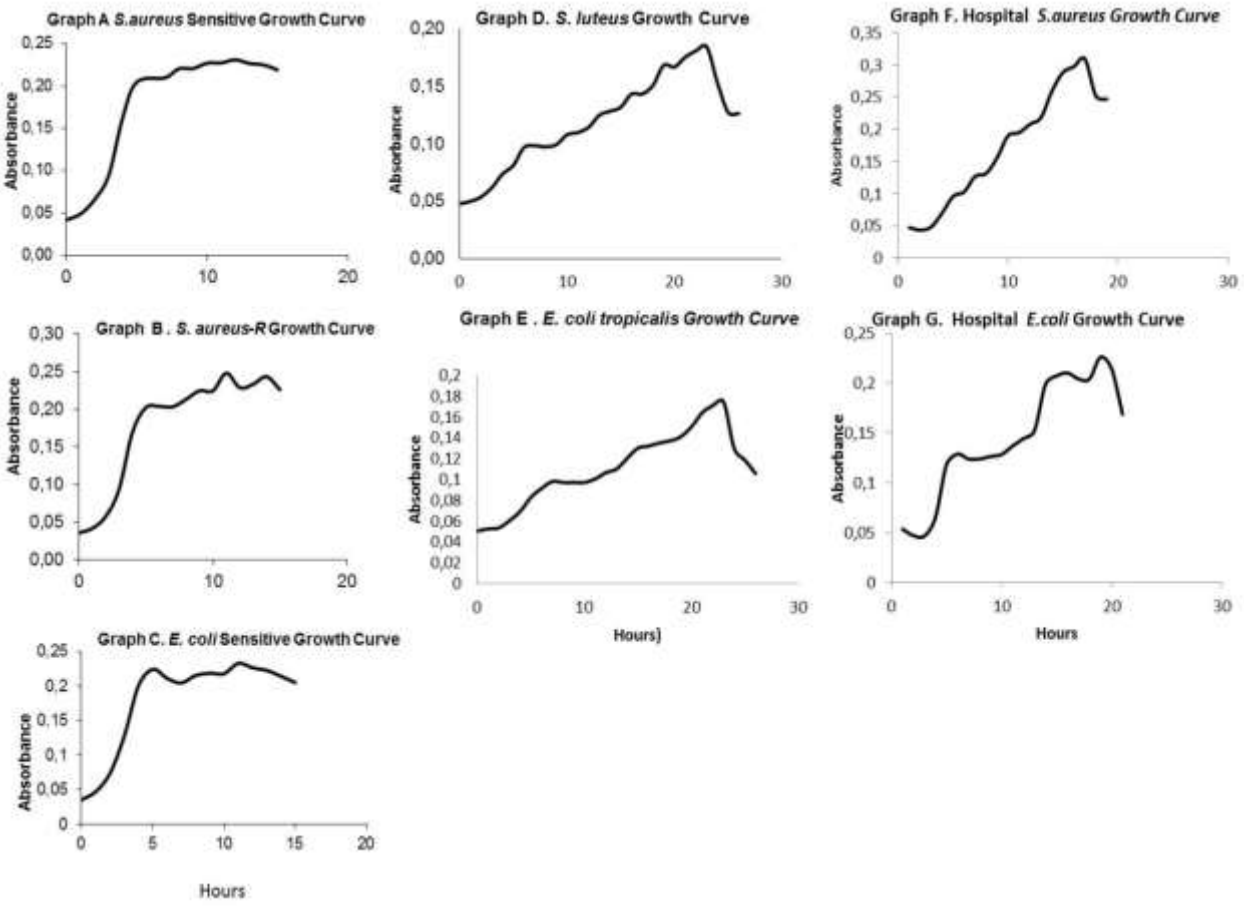

Figure 2. Growth curves A. *S aureus* sensitive ATCC B. *S aureus* resistant, ATCC C. *E coli* ATCC. D. *S luteum*, E. *E coli Tropically*, F and G. *S aureus* and *E coli clinical* isolated.

concentration over time of maximum growth (Figure 2). Although Microkit SL laboratories strains obtained the longest time of maximum growth, they presented less bacterial concentration. Thus, comparing hospital isolated strains; it can be concluded that those obtained from ICU had a higher bacterial concentration, in a smaller time unit. The MIC values of standard, generic and brand name of levofloxacin are presented in Tables 9 and 10. Overall, the MIC of different levofloxacin drugs against *S. aureus* and *E coli* evaluated were found much lower than that reported by Martínez et al. (2004) and Van Bambeke (2005). On the other hand, MIC of levofloxacin was determined against to *E. coli tropically* and *S. luteums*, for the first time in Colombia.

None of the strains exceeded the ranges reported by literature. However, we must take into account the significant difference between MIC values presented by Clinical Isolated strain of Colombian hospital, which were higher with respect to ATCC and MICROKIT SL laboratory strains. Although, they do not exceed the limits established by the above referenced studies, this outcome could be in relation with the increase of levofloxacin resistance (Kao et al., 2016), reason for which its use in clinic has decreased in Colombian Hospital.

Table 11 shows the results of confirmative tests confirming that the data obtained by turbidimetric method used in the present study were correct. Furthermore the MIC values remained the same as in the previous trials, confirming the low efficacy against clinical isolates compared with standard strains of *E coli* and *S aureus* for both generic and brand name of levofloxacin

Conclusions

No significant differences existed in active principle substance concentration between the different brands of levofloxacin evaluated by a precise, repetitive and accurate method. This is directly related to the quality of levofloxacin used for *in vitro* antibacterial activity assay. When comparing the differences between generic and brand name levofloxacin, the differences in MIC values were very minimal. It was possible to demonstrate that

Table 9. Standard, generic and commercial Levofloxacin MIC in the maximum growth time for each strain.

Strains	Levofloxacin MIC (µg/ml) (standard)	Levofloxacin MIC (µg/ml) (Generic) Ads pharma	Levofloxacin MIC (µg/ml) (Generic) Pharmedic	Levofloxacin MIC (µg/ml) (commercial) Sanofi
E. coli ATCC	0.125	0.125	0.125	0.125
S. aureus-R ATCC	0.0625	0.0625	0.0625	0.0625
S. aureus- S ATCC	0.03125	0.03125	0.03125	0.03125
*E. coli tropically**	0.0625	0.0625	0.0625	0.0625
*S. luteums**	0.125	0.125	0.125	0.125
*S. aureus***	4	4	4	4
*E. coli***	8	8	8	8

*MICROKIT SL laboratory strains, **Clinical Isolated strain.

Table 10. Standard, generic and commercial levofloxacin MIC, 24 hours after the maximum growth time for each strain.

Strains	Levofloxacin MIC (µg/ml) (Standard)	Levofloxacin MIC (µg/ml) (Generic) Ads Pharma	Levofloxacin MIC (µg/ml) (Generic) Pharmedic	Levofloxacin MIC (µg/ml) (Commercial) Sanofi
E. coli ATCC	0.25	0.25	0.25	0.25
S. aureus-R ATCC	0.125	0.125	0.125	0.125
S. aureus-S ATCC	0.0625	0.0625	0.0625	0.0625
E.coli tropically*	0.125	0.125	0.125	0.125
*S. luteums**	0.25	0.25	0.25	0.25
*S.aureus***	8	8	8	8
E.coli **	>8	>8	>8	>8

*MICROKIT SL laboratory strains, **Clinical Isolated strain.

Table 11. Results of all confirmative tests.

Strains	Levofloxacin MIC (µg/mL) (Standard)	Levofloxacin MIC (µg/mL) (Generic) Ads Pharma	Levofloxacin MIC (µg/mL) (Generic) Pharmedic	Levofloxacin MIC (µg/mL) (Commercial) Sanofi
E. coli ATCC	0.125	0.125	0.125	0.125
S. aureus-R ATCC	0.0625	0.0625	0.0625	0.0625
S. aureus-S ATCC	0.03125	0.03125	0.03125	0.03125
*E. coli tropically**	0.0625	0.0625	0.0625	0.0625
*S. luteums**	0,125	0.125	0.125	0.125
*S.aureus***	4	4	4	4
E. coli **	8	8	8	8

*MICROKIT SL laboratory strains, **Clinical Isolated strain.

generic and brand names levofloxacin showed similar *in vitro* antimicrobial activity against Clinical and ATC strain of *S. aureus* and *E. coli*.

However the clinical isolation strain presented MIC to be more elevated that ATCC strain for both generic and brand name levofloxacin, this outcome could be the relationship with the increase of levofloxacin resistance, and this would explain the low efficacy of quinolones in clinic. This study reported for the first time the levofloxacin MIC against two specific strains from Colombia that had never been evaluated against *E. coli tropically* and *S. luteum*.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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