Flow cytometry early predicts bacterial susceptibility to antibiotics

Citometria de fluxo prediz precocemente a sensibilidade bacteriana aos antibióticos

La citometría de flujo predice tempranamente la susceptibilidad bacteriana a los antibióticos

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ABSTRACT

Objective: To evaluate a flow cytometry protocol to test bacteria for antimicrobial susceptibility. **Methods:** We used antimicrobial drugs, a strain of *Escherichia coli* (ATCC 25922) susceptible to all antimicrobials, and clinical isolates of *E. coli* resistant to the same drugs. Prior to testing, the susceptibility of all isolates was tested by the disc-diffusion method. For flow cytometry, standard concentrations of antimicrobial drugs were added to *E. coli* bacteria, incubated for 1 to 3 h at 37ºC, stained with propidium iodine and analyzed by flow cytometry. As controls, each bacterial isolate, at each indicated time, was also collected and seeded on Luria Bertani agar plate to enumerate the number of viable cells by the classical method. The growing/survival rate was evaluated in the same samples after incubating for 24h at 37ºC. **Results:** Antimicrobial susceptibility was estimated at 1 and 3 hours but at this time a higher number of viable cells was detected by flow cytometry compared to colonies counting on agar plates. **Conclusion:** Flow cytometry allows early evaluation of bacterial resistance to antibiotics and can be optimized and used as an alternative tool to detect multi-resistant bacterial isolates.

Keywords: Flow cytometry, Antimicrobial resistance, Antimicrobial assay.

RESUMO

Objetivo: Avaliar a citometria de fluxo como um método rápido para testar a suscetibilidade antimicrobiana em bactérias. **Métodos**: Foram usadas drogas antimicrobianas, uma cepa de *Escherichia coli* (ATCC 25922) suscetível a todos os antimicrobianos e amostras clínicas de *E. coli* resistentes aos mesmos antimicrobianos. Antes do teste, a sensibilidade aos antimicrobianos foi avaliada pelo método de difusão em disco. Para a citometria de fluxo, as drogas foram adicionadas à *E. coli* seguida de incubação por 1 a 3 h a 37ºC, e depois coradas com iodeto de propidio. Como controles, os isolados clínicos, em todos os tempos indicados, também foram cultivados em placas contendo ágar Luria Bertani visando quantificar o número de bactérias pelo método tradicional. A taxa de crescimento e sobrevivência foi avaliada nas mesmas amostras após incubação por 24h a 37ºC. **Resultados**: A suscetibilidade bacteriana foi estimada em 1 a 3h, mas nesses momentos um maio número de células viáveis foi detectada pela citometria de fluxo comparada com a contagem de colônias em placas. **Conclusão**: A citometria permite prever precocemente a suscetibilidade da bactéria aos antimicrobianos e pode ser usada como uma ferramenta alternativa para detectar isolados bacterianos multirresistentes.

Palavras-chave: Citometria de fluxo, Resistência aos antimicrobianos, Ensaio antimicrobiano.

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RESUMEN

Objetivo: Evaluar la técnica de citometría de flujo como método rápido para determinar el perfil de sensibilidad de bacterias a los antimicrobianos. **Métodos:** Se utilizaron fármacos antimicrobianos, una cepa de *Escherichia coli* (ATCC 25922) sensible y cepas clínicas del mismo agente resistentes a las moléculas. El perfil de sensibilidad de las cepas fue determinado a través de antibiograma. Para la CF, las bacterias fueron mezcladas con los fármacos e incubadas durante 1 y 3h a 37ºC. A continuación, se añadió a las muestras yoduro de propidio y se procedió el análisis de viabilidad bacteriana. En paralelo, las bacterias tratadas con los antimicrobianos en las mismas condiciones anteriores fueron sembradas en placas de agar para recuento en placa después. **Resultados:** La susceptibilidad de las bacterias a los antimicrobianos fue determinada entre 1 y 3 horas e la técnica de citometría de flujo fue capaz de detectar mayor cuantidad de bacterias viables que el en recuento en placa. **Conclusión:** La citometría permite la predicción precoz del perfil de resistencia de bacterias a los antimicrobianos y se puede utilizar como herramienta alternativa para detectar aislados clínicos multirresistentes.

Palabras clave: Citometría de flujo, Susceptibilidad antimicrobiana, Ensayo antimicrobiano.

INTRODUÇÃO

The use of antimicrobial drugs is central to reduce and control bacterial infectious diseases, to improve transplantation success, as adjuvant therapy on surgeries and immunocompromised individuals, and for improving preterm infants' survival during the neonatal period. As a whole, the use of antibiotics improves human life span expectations (OVERBYE KM e BARRETT JF, 2005; WRIGHT GD, 2007; SPELLBERG B et al., 2013) .

Antibiotics are defined as natural or synthetic molecules that act by interfering on different pathways of bacterial cell metabolism. In general, antibiotic might inhibit the synthesis of protein or the synthesis of the cell wall membrane, inhibit replication of bacterial DNA, and interfere with the biosynthetic pathway of folic acid or by altering bacteria membrane permeability (KAPOOR G et al., 2017). The efficacy of antibiotics is directly related to the concentration used and their availability at the target tissue. Antibiotics might either inhibit bacterial growth (bacteriostatic activity) or lead to bacteria inactivation (bactericidal activity) and are central to anti-microbial therapy (ALOS JI, 2015).

Bacteria resistance to antimicrobial drugs, however, constitutes a major threat to worldwide public health (WORLD HEALTH ORGANISATION, 2012). The continuous and long usage of antibiotics on human and animal population and on the animal food production chain prompted the surge of antimicrobial resistance mediated by different molecular mechanism that prevent antibiotics from interacting with their target molecules on bacteria biosynthetic pathways (KOHANSKI MA et al., 2010; THALLER MC, et al., 2010; ALÓS JI, 2015; HUANG TH, et al., 2015).

Therefore, although aimed to kill bacteria, antibiotics might also trigger the growth of resistant bacteria already existent within the population and, as a consequence, the spread of these new resistant bacterial strains to comingling individual; furthermore, transferring genetic material amongst bacteria helps to speed up antimicrobial resistance genes (LEVY SB e BONNIE M, 2004; BHULLAR K, et al., 2012).

In general, the predicted outcome of antimicrobial resistance on public health might be increased morbidity by infectious diseases, extended hospitalization time, higher risk of complications on surgical procedures, retarded and complicated wound healing, and higher mortality rates (WORLD HEALTH ORGANISATION, 2012).

These outcomes might have a major impact on economics by reducing workers productivity, increasing expenses on diagnosis and disease treatment and hampering the animal food production chain. In this scenario, clinical microbiology laboratories are central to carry out pathogen isolation, identification and antimicrobial drug susceptibility testing prior to advising on proper antibiotic usage during infection (SYAL K, et al., 2017).

Temporally, the time elapsed from sampling up to proper antibiotic indication might be crucial to therapeutic success and it should be as shorter as possible. By using classical methods, it might take up to three days or longer to proper bacteria identification and antimicrobial drug testing; in some cases, it might be too long and could account for by therapy failure. Thus, new tools and method to speed up antimicrobial drugs testing are much needed. As such, the experimental use of flow cytometry to investigate antimicrobial drug susceptibility has been evaluated for bacteria and fungi (EL-MASHAD N, et al., 2012; ESPINAR MJ, et al., 2012; MORALES BP, et al., 2014).

The results already found are encouraging mainly considering the time required to find a proper antimicrobial drug. Flow cytometry analysis allows investigating bacterial viability, replication and membrane integrity and potential (CZECHOWSKA K, et al., 2008; DAVIS C, 2014; HUANG TH, et al., 2015; KHOMTCHOUK KM, et al., 2019). During antimicrobial drug evaluation it allows to analyze the biochemical status of the bacteria cells after antibiotic treatment and to identify viable, but non-cultivable bacteria subpopulations that otherwise would not be detected by conventional methods (DÍAZ M, et al., 2010; LÉONARD L, et al., 2016).

Thus, considering that identifying bacterial susceptibility or resistance to antibiotics is a time-consuming method that could take up to 96h for fastidiously growing bacteria and that for routine and mainly for life-threatening infections, clinicians are required to decide and initiate antibiotic therapy even prior to knowing the susceptibility/resistance status of the infecting bacteria towards the antibiotic of choice, here we aimed to evaluate flow cytometry as a tool to predict the susceptibility or resistance status of bacteria towards several antibiotics.

METHODS

Antimicrobial drugs

The antimicrobial drugs used were those commonly used by clinician to treat different types of infectins: we used enrofloxacin (fluoroquinolones), oxytetracycline (tetracycline), gentamicin (aminoglycoside), cephalexin and ampicillin (β-lactam), and sulfamethoxazole/trimethoprim (diaminopyrimidine). All pure molecules were acquired from Sigma-Aldrich (Brazil) except cephalexin (Aurobindo, Brazil) and gentamicin (Gibco, Brazil).

All stock solutions were prepared as described on the Clinical & Laboratory Standards Institute – CLSI, 2014(“CLSI. Performance Standards for Antimicrobial Susceptibility Testing: Twenty Third Informational Supplement,” 2013) as follows: ampicillin (10µg/ml), cephalexin (30µg/ml), enrofloxacin (5µg/ml), gentamicin (10 µg/ml), sulfamethoxazole/trimethoprim (23.75 and 1.25µg/ml) and tetracycline (30µg/ml). The stock solutions were prepared by diluting antibiotic on ultrapure water, except enrofloxacin and sulfamethoxazole/trimethoprim that were diluted in ethanol. The diluted antimicrobial drugs were then sterilized by filtration (0.22 µM filter, Sterile®) and diluted to working concentration on Luria Bertani (LB) broth (Sigma-Aldrich, Brazil).

Bacterial clinical isolates

We used clinical isolates of *Escherichia coli* that were found resistant to the antibiotics described above when tested by the classical agar-disc diffusion method. A standard strain of *E.coli* (ATCC 25922, Newprov) susceptible to all drugs evaluated here was also used as control throughout the assays.

Flow cytometry evaluation of antimicrobial susceptibility

The clinical isolates of *E. coli* were firstly cultivated in LB agar plates (37ºC for 24h). Isolated colonies were then selected and grown on LB media at 37º C with continuous agitation (200 rpm) until optical density (OD) reached 0.5 at 600 nm. The bacteria was then pelleted by centrifugation (4º C, 10 min., 4.000 rpm) and washed three times with phosphate buffered saline (PBS, pH 7.2). The resulting bacteria pellet was suspended in PBS (1 ml, pH 7.2) and the number of viable cells was counted by flow cytometry.

The susceptibility of bacteria was evaluated in duplicates by adding exactly 10^4 bacteria to the antimicrobial drugs in LB media (1 ml final volume) followed by incubation at 37º C under continuous agitation (200 rpm). Then, after 1, 2 and 3 h, the cells were pelleted and washed (PBS, pH 7.2) three times (4.500 rpm, 5 min). The final bacteria pellet was suspended in 200 µl of PBS (pH 7.2) and stained by adding 5 µl of Propidium Iodate (PI, 1mg/ml, Sigma Aldrich, Brazil) for 10 min at 21ºC in the dark. The bacteria were then evaluated by flow cytometry (FACSVerse Becton Dickinson, USA).

A total of 10.000 bacteria were counted in each sample using a panel of two colors: FITSC and Per-CP for the PI fluorochrome. PI enters damaged bacteria membrane and stains unviable cells that were then differentiated from non-stained cells. A cut-off value was previously set to reduce background and nonspecific fluorescence by counting a population of viable cells only. A control of non-viable, PI-stained cells (positive control) was made by exposing bacteria to 70ºC for 30 min; the control of viable, non-stained cells, were also exposed to PI (negative control).

Bacteria plate counting

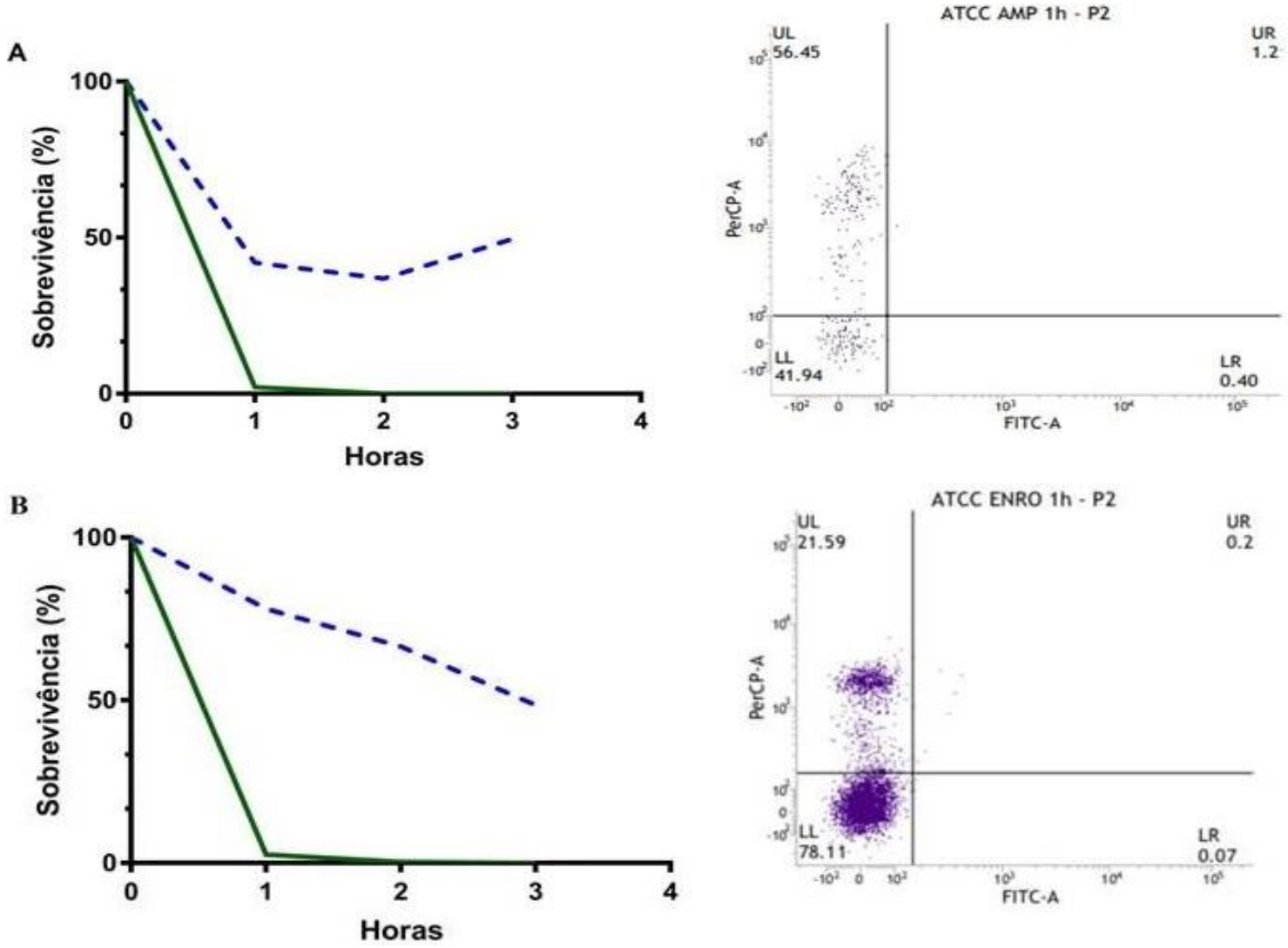
Bacteria survival after exposure to the antimicrobial drugs was also evaluated by the classical method of colonies counting in agar plates. For that, the LB media containing the bacteria (10^4 bacteria/ml) was serially diluted (10^{-1}, 10^{-2} and 10^{-3}) and each dilution was then plated in LB agar in duplicates and incubated at 37ºC up to 24h. For each bacteria isolate and for each antimicrobial drug a positive control (bacteria isolate non-exposed to antimicrobial drug) and negative control (LB media only) were used throughout the experiment.

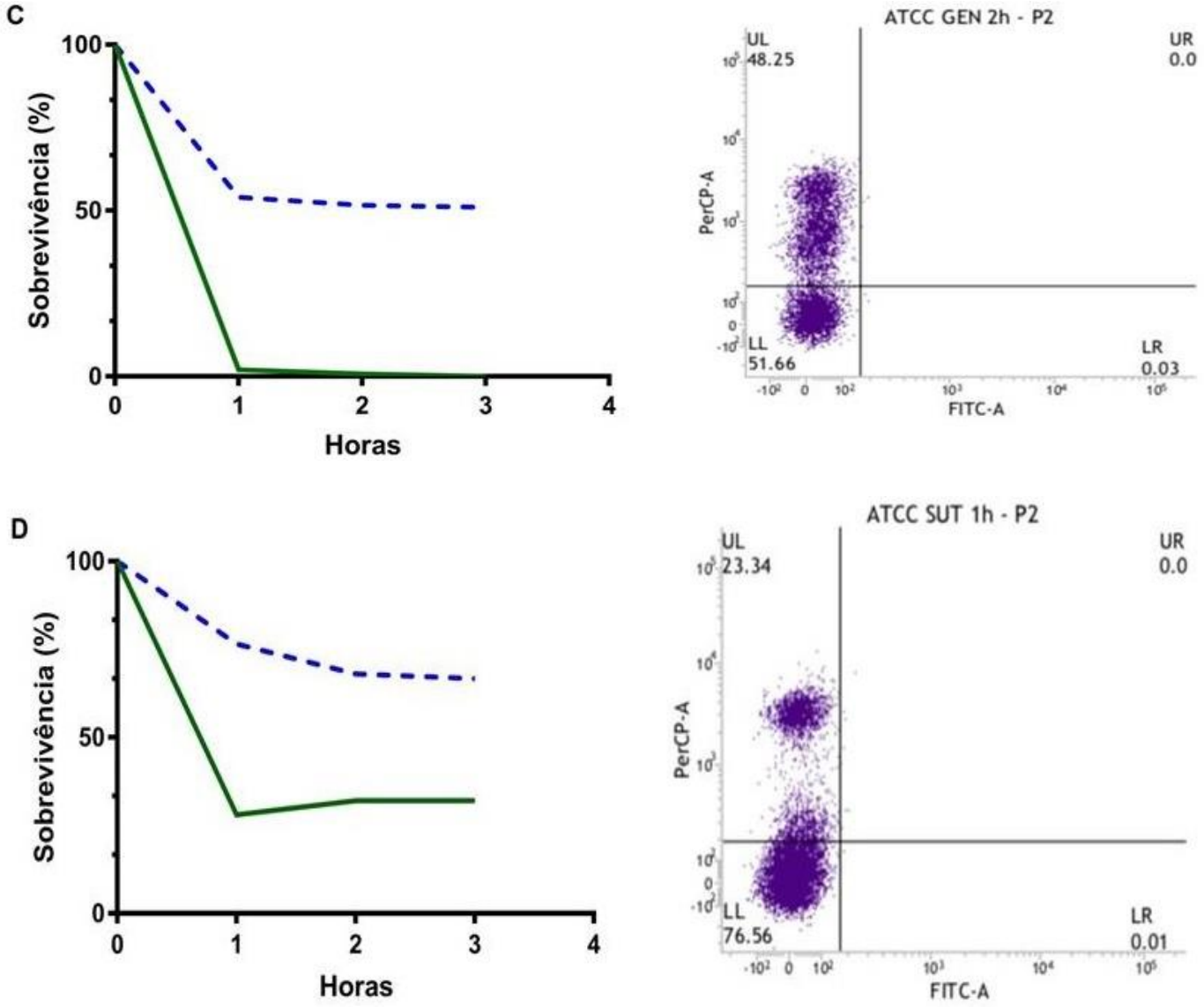
RESULTS

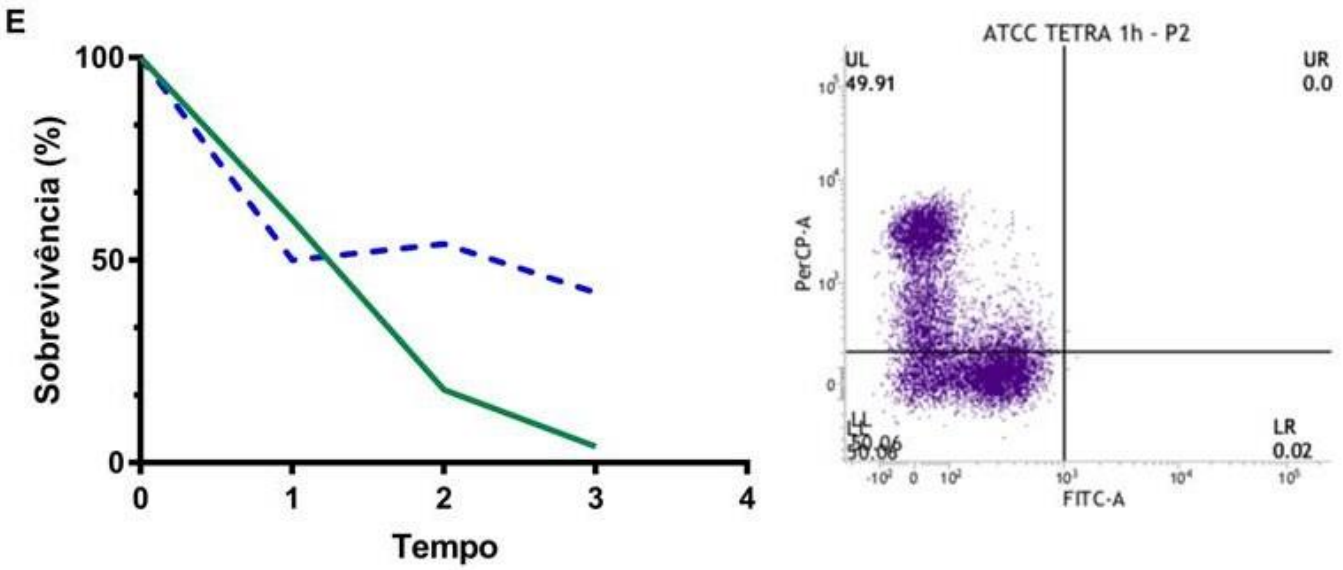
The procedure was standardized using a reference strain of *E. coli* (ATCC 25922) susceptible to all antimicrobial drugs indicated herein. The bacteria cell viability analysis was carried out by flow cytometry and by counting the number of colonies growth on LB agar plates. Bacterial survival was lower than 10% after 1 h exposure to ampicillin, enrofloxacin, and gentamicin when evaluated by the agar plate assay (colony counting); at the same time, for these antibiotics, a higher number of bacteria considered viable were detected by flow cytometry (**Figure 1**).

A constant rate of bacterial survival regarding to time of exposure to antibiotics was detected by flow cytometry. Compared to CFU counting, flow cytometry efficiently detected bacterial survival after exposure to 5 out of the 6 antimicrobials used here. When analyzed by agar plate colony counting, the number of CFU found on agar plates increased exponentially (**Figure 2**).

Figure 1 - Flow cytometry (doted line) and plate counting (continuous line) analysis of *Escherichia coli* (ATCC 25922) survival rate after exposure to different antimicrobial drugs (A-F).



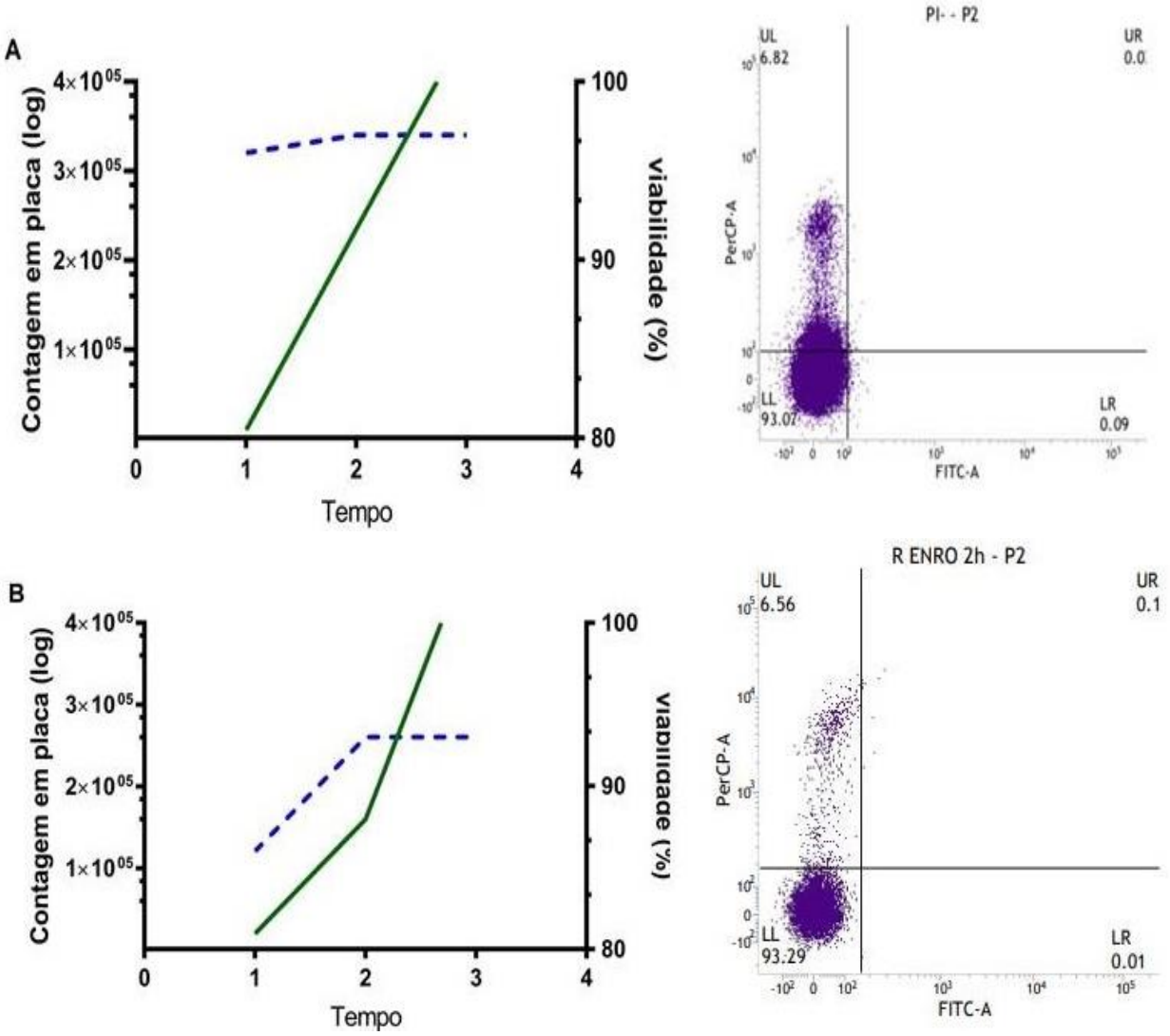

C
Sobrevivência (%)
100
50
0
0
1
2
3
4
Horas
ATCC GEN 2h - P2
UL
48.25
UR
0.0
LL
51.66
LR
0.03
PerCP-A
FITC-A
D
Sobrevivência (%)
100
50
0
0
1
2
3
4
Horas
ATCC SUT 1h - P2
UL
23.34
UR
0.0
LL
76.56
LR
0.01
PerCP-A
FITC-A

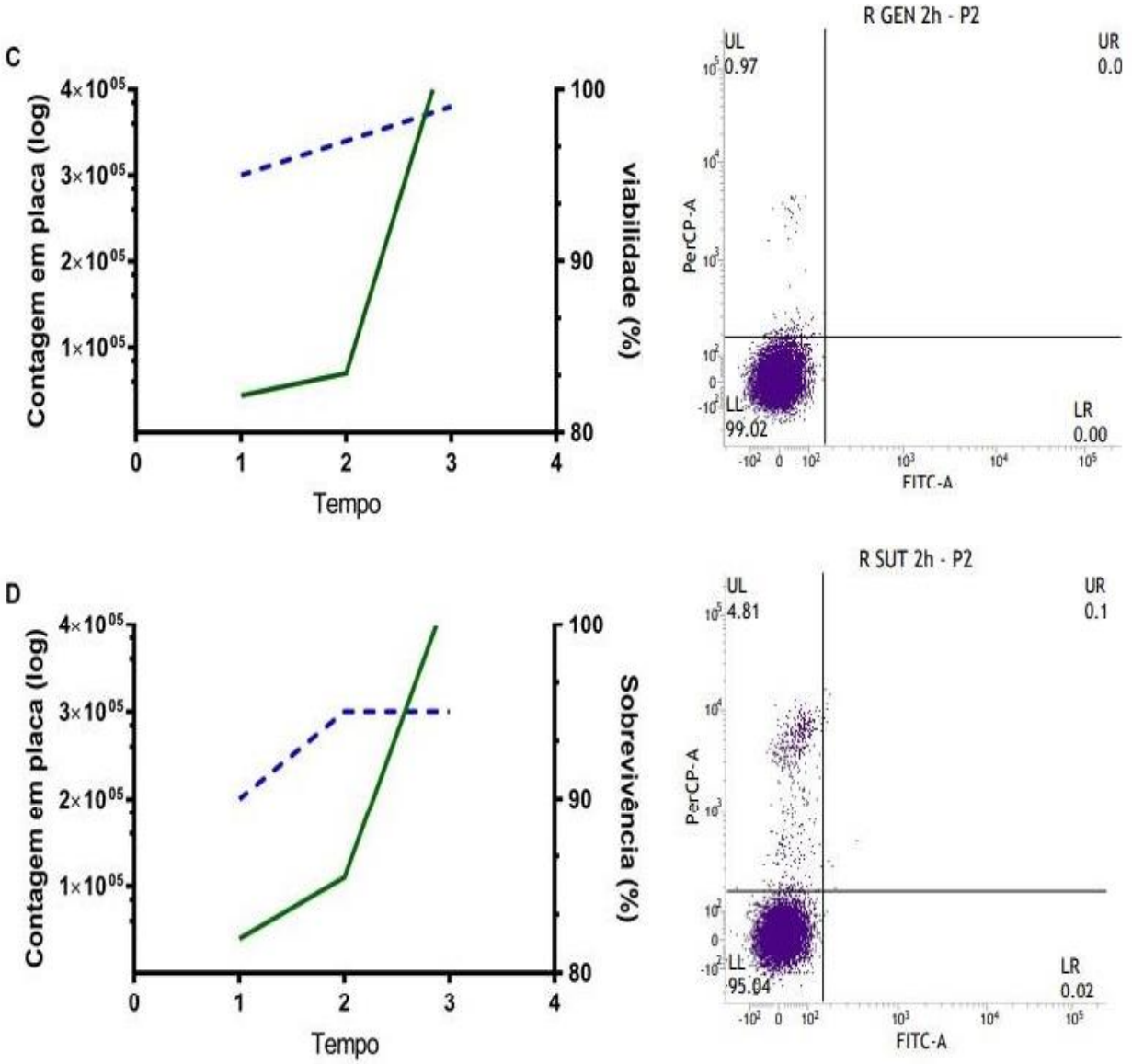


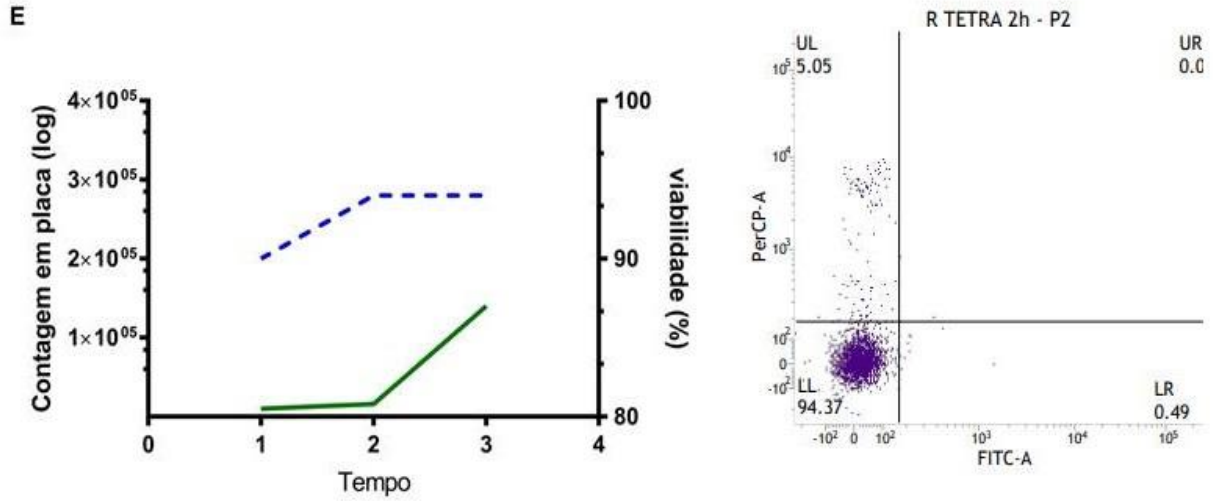
Notes: The percentile rate of survival was evaluated prior to (0h) and after exposure (1, 2 and 3h) to the antimicrobial (left panel). The right panel figures represent the bacteria counting by flow cytometry. The low left corner indicates the viable bacteria subpopulation (not stained) whereas the left upper corner contains the non-viable (PI stained) bacteria subpopulation. The data represents the matching results of one out of three different experiments.

Source: Anziliero EB, et al., 2020.

Figure 2 - Multi resistant *E. coli* growth rate evaluation by flow cytometry and plate counting.




C
Contagem em placa (log)
4×10^05
3×10^05
2×10^05
1×10^05
100
90
80
viabilidade (%)
0
1
2
3
4
Tempo
R GEN 2h - P2
UL
0.97
UR
0.0
PerCP-A
LL
99.02
LR
0.00
FITC-A
D
Contagem em placa (log)
4×10^05
3×10^05
2×10^05
1×10^05
100
90
80
Sobrevivência (%)
0
1
2
3
4
Tempo
R SUT 2h - P2
UL
4.81
UR
0.1
PerCP-A
LL
95.04
LR
0.02
FITC-A



Notes: The absolute number of bacteria is represented by the left panel figures and was measured prior to (0h) and after (1, 2 and 3h) exposure to the antibiotics, by flow cytometry (doted line) and plate counting (continuous line). The right panel indicates the number of viable and non-viable bacteria measured by flow cytometry. The lower left corned indicates viable cell (non-stained) whereas the upper left corner indicates the nonviable cell population (PI stained). The data represents the matching results of one out of three different experiments.
Source: Anziliero EB, et al., 2020.

DISCUSSION

Here, we used flow cytometry as a tool to evaluate antimicrobial susceptibility in bacterial cells and we opted to use PI only mostly because when compared to other stains it is easily found in most laboratories and has a low cost. Our essays measured, in real time, the fluorescent intensity obtained by exposing clinical isolates of *E.coli* (resistant or susceptible) to different antimicrobial drug classes followed by PI staining. Thus, bacteria cells exposed to antimicrobial drugs were considered viable or nonviable according to their ability to allow the entrance of PI through the cell membrane; the increase on the emitted fluorescence, in this case, was a clear indication of a damaged membrane and the cells was considered death.

We succeeded in determining the antimicrobial susceptibility for 5 out of 6 antimicrobial drug classes (83.33%) routinely used in medical centers and, with the exception of cephalexin, the cell viability was indicated as soon as 1 hour after exposure to the drugs. Compared with the classical antimicrobial susceptibility test, flow cytometry analyses of cell susceptibility was much faster and thus represents a viable alternative tool that might support the earlier choice of antimicrobial drugs usage by medical staff, contribute to reduce morbidity and mortality rates on critical patients, and reduce the surge of antimicrobial drug resistance within the target bacteria population.

In addition, 98.4% of heat-treated bacteria were identified as nonviable after staining with PI indicating that the method efficiently marked only cells with damaged membranes. We found that flow cytometry, as used here, could not replace the classical method of bacterial susceptibility testing; accordingly, after flow cytometry analysis of bacteria exposed to the drugs for 1h we found 30 to 50% of viable cells whereas, at the same time, the percentile of survival, measured as colony forming units (CFU) in a plate assay was much lower. Furthermore, a similar result was observed when bacteria cells were analyzed at 3h post-exposure: we found unstained cells by flow cytometry but no bacteria colonies on the plate assay.

However, we believe the contrasting results should not rule out the use of flow cytometry to evaluate antimicrobial susceptibility in bacteria. The results indicate that using only a single fluorochrome (PI) to evaluate bacteria viability might not suffice to predict whether the bacteria is indeed nonviable and that the use of other fluorochromes should be evaluated altogether, and longer exposure periods should be considered (EL-MASHAD N, et al., 2012). Here, we used PI only in that our original hypothesis was that a single fluorochrome would suffice to differentiate viable from nonviable cells. However, considering that PI entry depends on injured cell membranes, that bacteria are permeable to several antibiotics and bacteria killing mechanisms might not target or alter cell membrane permeability, the use of additional fluorochromes such as SYTO9, DiBAC4, SYBRgreen and Acridine orange would contribute with the possibility of using flow cytometry for early prediction of cell viability and efficient indication of antibiotic to clinicians (FREIRE JM, et al., 2015; LÉONARD L, et al., 2016).

Because our main goal was to evaluate the use of flow cytometry to early detection of antimicrobial susceptibility, the rate of agreement (κ index) between the results using different methodologies (flow cytometry and plate counting) were not estimated here; however, the results indicate that there is no correlation between flow cytometry evaluation (detects high percentile of viable cells) and plate counting (low number of resulting colonies on agar plates). We believe that this might be explained by the fact that within the target bacteria there could be viable but non-cultivable bacteria subpopulations that are unable to grow on LB agar plates. And growth inhibition confirmation depend on each isolate or strain growing rates and the drug evaluated in that there might be differences within strains regarding their ability to overcome drug exposure.

However, the ability of drug-exposed injured bacteria growing after returning to a favorable environment is still in debate and would need deeper investigation. Furthermore, we observed that cell viability measured by plate assay is steadily reduced temporally but remains constant when analyzed by flow cytometry, except for enrofloxacin in which it decreased slowly (**Figure 1**) (LÉONARD L, et al., 2016). By using flow cytometry, we were unable to evaluate bacteria cells exposed to cephalexin. Cephalexin inhibits the synthesis of the peptidoglycan layer on cell membrane and lyses bacteria; this might expose and damage bacteria DNA that becomes refractory to PI binding turning this “ghost bacteria cells” undetected by flow cytometry; that is, ghost bacteria cells become entwined reducing the possibility of PI binding. Thus, another fluorochrome should be

evaluated on cephalexin-treated bacteria and other cephalosporin antibiotics should be used when using flow cytometry as a tool to measure bacteria viability. Another possibility is that we used a Gram-negative bacteria and cephalexin is more effective toward Gram-positive microorganisms (KOHANSKI MA, et al., 2010; COATES AR, et al., 2011).

Interestingly, flow cytometry analysis of antimicrobials-exposed clinical isolates of *E. coli* predicted with 90% the likelihood of resistance within the population; and, as expected, the number of CFU found on agar plates increased exponentially from $1x10^4$ up to $5x10^5$ cells. In addition, flow cytometry analysis indicated a constant rate of bacterial survival regarding to time of exposure. Compared to CFU counting, flow cytometry efficiently detected bacterial survival after exposure to 5 out of the 6 antimicrobials used here. The only exception was cephalexin, as discussed earlier.

Therefore, considering the easiness and time spend on analysis (3h) we found flow cytometry testing as a viable and efficient procedure to identify antimicrobial drug resistance on clinical samples. In addition, because knowledge on bacteria drug resistance is central to medical staff to initiate antimicrobial therapy, an assay that allows earlier decision on antibiotic usage is fundamental mainly to patients infected with multi resistant microorganisms and on life-threatening situations.

Bacterial infection are a major threat to human and animal health and the continuous usage of antimicrobial drugs on veterinary and human medicine, and on animal food production chain might trigger the surge of antimicrobial drug resistance (SPELLBERG B, et al., 2013; HUANG TH, et al., 2015). Thus, treatment of bacterial infections should be carried out only after ascertain bacteria susceptibility by laboratory testing; otherwise, improper antimicrobial usage might select resistant bacterial within the target population increasing morbidity and mortality rates (CAMPION EW e MORRISSEY SA, 2013; FREIRE JM, et al., 2015; HUANG TH, et al., 2015). Thus, proper use of tools aimed to evaluate antimicrobial susceptibility is deemed necessary and the improvement of antimicrobial susceptibility testing are in great demand.

Analysis of bacterial viability is based on indirect methods aimed to evaluate the bacterial physiological status but not the bacteria capability to multiply (STIEFEL P, et al., 2015; BALOUIRI M, et al., 2016). Thus, analytical methods for analyzing antimicrobial drug resistance targeting bacterial DNA, the membrane potential or indicators of oxidation-reduction reactions should be explored aiming to reduce the likelihood of triggering antimicrobial resistance on target bacteria (STIEFEL P, et al., 2015).

The integrity of cell membrane is a criterion to distinguish viable from death cells. Intact or impermeable membranes are a hallmark of viable cells; in contrast, nonviable cells have permeable or disrupted membranes allowing the entry of foreign molecules (CZECHOWSKA K, et al., 2008). However, within a population there might be cells with intact membrane but are non-cultivable (KHOMTCHOUK KM, et al., 2019).

In contrast, alterations on the cell membrane integrity might occur during exponential cell growth or in extreme nutritional conditions. Thus, in these cases, the external environmet and the physiological status of the cells might impact on the evaluation of cellular viability and growth (SHI L, et al., 2007; DÍAZ M, et al., 2010; LÉONARD L, et al., 2016; KHOMTCHOUK KM, et al., 2019).

Flow cytometry-based techniques, as used here, could allow for several possibilities of bacterial quantification mainly when used in association with fluorochromes that selectively enter viable or nonviable cells (LÉONARD L et al., 2016; KHOMTCHOUK KM et al., 2019). Propidium Iodide, for instance, has been used to evaluate cell death because it only enters cells with injured membranes and intercalates with DNA turning the cell fluorescent when analyzed by flow cytometry; however, PI might accumulate in a few bacteria species or in intact bacteria cells during the exponential growth phase (SHI L, et al., 2007; STIEFEL P, et al., 2015).

In contrast, the SYTO9 fluorochrome (green fluorescent nuclei acid stain), frequently used to mark total proteins, when used alone, stains all bacteria regardless of their membrane integrity and viability (STIEFEL P, et al., 2015). Thus, the association of SYTO9 and PI to stain bacterial cells could be remarkable useful to

evaluate bacteria viability by flow cytometry. Accordingly, PI would enter cells with damaged membranes preventing the entry of SYTO9 that, in turn, would enter the remaining viable cells allowing for a better differentiation between those two populations (FREIRE JM, et al., 2015; STIEFEL P, et al., 2015; LÉONARD L, et al., 2016).

Thus, we found that by using flow cytometry we could anticipate the identification of antimicrobial drug resistance. Overall, in a shorter period of time we improved the likelihood of choosing the right drug to be used and this is highly relevant mainly to treat infections caused by multidrug resistance bacterial strains.

CONCLUSION

We determined the antimicrobial susceptibility for 5 out of 6 antimicrobial drug classes (83.33%) routinely used in medical centers and, with the exception of cephalexin, cell viability was indicated as soon as 1 hour after exposure to the drugs. We found that 98.4% of heat-treated bacteria were identified as nonviable by PI staining indicating that the method efficiently marked only cells with damaged membranes. A limitation of this study is that flow cytometry might not replace the classical method of bacterial susceptibility testing because the equipment in not readily available in most laboratories. A second limitation is that flow cytometry might not be useful to evaluate antibiotics that damage bacteria DNA, which became refractory to PI binding.

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REFERENCES

1. ALÓS JI. Resistencia bacteriana a los antibióticos: una crisis global. Enferm. Infecc. Microbiol. Clin, 2015; 33: 692–699.
2. BALOUIRI M, et al. Methods for in vitro evaluating antimicrobial activity: A review. J. Pharm. Anal, 2016; 6:71–79.
3. BHULLAR K, et al. Antibiotic resistance is prevalent in an isolated cave microbiome. PLoS One, 2012; 7: 1–11.
4. CAMPION EW, MORRISSEY S. A Different Model — Medical Care in Cuba. N. Engl. J. Med, 2013; 368: 297–299.
5. CLSI. Performance Standards for Antimicrobial Susceptibility Testing: Twenty Third Informational Supplement, 2013.
6. COATES AR, et al. Novel classes of antibiotics or more of the same? Br. J. Pharmacol, 2011; 163: 184–194.
7. CZECHOWSKA K, et al. Use of flow cytometric methods for single-cell analysis in environmental microbiology. Curr. Opin. Microbiol. 2008; 11: 205–212.
8. DAVIS C. Enumeration of probiotic strains : Review of culture-dependent and alternative techniques to quantify viable bacteria. J. Microbiol. Methods 2014;103: 9–17.
9. Díaz M. et al. Application of flow cytometry to industrial microbial bioprocesses. Biochem. Eng. J. 2010; 48: 385–407.
10. EL-MASHAD N, et al. Susceptibility tests of oropharyngeal. Quest, 2012; 266–273.
11. ESPINAR MJ, et al. A novel flow cytometric assay for rapid detection of extended-spectrum. Clin. Microbiol. Infect, 2012; 1–8.
12. FREIRE JM, et al. Monitoring antibacterial permeabilization in real time using time-resolved flow cytometry. Biochim. Biophys. Acta, 2015; 1848: 554–560.
13. HUANG TH, et al. Rapid cytometric antibiotic susceptibility testing utilizing adaptive multidimensional statistical metrics. Anal. Chem, 2015; 87: 1941–1949.
14. KAPOOR G, et al. Action and resistance mechanisms of antibiotics: A guide for clinicians. J. Anaesthesiol. Clin. Pharmacol, 2017; 33(3): 300–305.
15. KHOMTCHOUK KM, et al. Quantitative assessment of bacterial growth phase utilizing flow cytometry. J. Microbiol. Methods, 2019; 167: 105760.
16. KOHANSKI MA, et al. How antibiotics kill bacteria: From targets to networks. Nat. Rev. Microbiol, 2010; 8: 423–435.
17. LÉONARD L, et al. Recent advances on multi-parameter flow cytometry to characterize antimicrobial treatments. Front. Microbiol, 2016; 7:01225
18. LEVY SB, BONNIE M. Antibacterial resistance worldwide: Causes, challenges and responses. Nat. Med, 2004; 10: S122–S129.
19. MORALES BP, et al. Determination of the minimum inhibitory concentration of Cryptococcus neoformans and Cryptococcus gattii against fluconazole by flow cytometry. Medical Mycology, 2014; 52: 90-98.

20. OVERBYE KM, BARRETT JF. Antibiotics: Where did we go wrong? Drug Discov. Today, 2005; 10: 45–52.
21. SHI L, et al. Limits of Propidium Iodide as a Cell Viability Indicator for Environmental Bacteria. Cytometry, 2007; 71: 592–598.
22. SPELLBERG B, et al. The Future of Antibiotics and Resistance. New Engl. J. Med, 2013; 368: 299–302.
23. STIEFEL P, et al. Critical aspects of using bacterial cell viability assays with the fluorophores SYTO9 and propidium iodide, BMC Microbiology, 2015; 15: 1–9.
24. SYAL K, et al. Current and emerging techniques for antibiotic susceptibility tests. Theranostics, 2017; 7: 1795–1805.
25. THALLER MC, et al. Tracking acquired antibiotic resistance in commensal bacteria of galápagos land iguanas: No man, no resistance. PLoS One, 2010; 5: 3–6.
26. WORLD HEALTH ORGANISATION. The evolving threat of antimicrobial resistance: Options for action. WHO Publ. 2012; 1–119. 1
27. WRIGHT GD. The antibiotic resistome: The nexus of chemical and genetic diversity. Nat. Rev. Microbiol, 2007; 5: 175–186.