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Portable Diagnostic Platform for Detection of Microorganisms Coliforms and *E. coli*

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Abstract

Portable diagnostic devices are a viable and low-cost alternative for the detection of pathogens, since they reduce the time of analysis of results availability. Ease of sample collection and quick diagnosis allow this new input to be applied in the diagnosis of the main contaminating microorganisms present in the water. Laboratory tests evaluated the technical viability of the diagnostic device, using commercial strains which were inoculated and optimized in the devices and their growth compared to the conventional method in Petri dishes. Samples of 100 µL bacterial suspension were tested and compared with the traditional sample inoculation method. The device viability was determined by detecting characteristic bacterial colonies in a specific culture medium through the colorimetric development of the obtained colonies. The feasibility assessments allow us to affirm that the device enables both qualitative and quantitative detection of the target bacteria present in liquid samples, and is promising to be applied to assess the quality of water, food and environmental surfaces.

Keywords

Portable Device, Bacterial Contamination, Environment

1. Introduction

Diagnostic devices known as Point-Of-Care (POC) are a viable alternative for the diagnosis of microbial pathogens, since they show advantages over methods based on classical microbiological culture, which are laborious and time-consuming, in addition to needing professionals specialized and laboratory

apparatus that are often not easily found in rural areas or distant from urban centers [1] [2] [3] [4]. Currently, there are efforts for the development of POC platforms, for the ease of use and the short diagnostic time allow greater agility in decision making by health professionals in urgent and emergency situations [5].

POC diagnostic platforms that use filter paper and microfluidics allow the implantation and miniaturization of different tests, either diagnostic or analytical. The easy manufacture of microfluidic paper platforms is due to the ease of acquisition and the wide availability of its main input, paper. POC platforms paper is easily adaptable to different types of tests and also has low manufacture cost [6] [7] which makes them an excellent alternative to the development of diagnostics and analytical sensors [8] [9] [10].

Monitoring microorganisms in water and in the environment are a necessity for maintaining populations health, since the ingestion of bacteria and other microorganisms present in water and the environment is responsible for epidemic outbreaks that usually cause death of immunodeficient patients [11]. Traditional methods of detecting microbiological are costly and time-consuming (usually requiring 2 to 3 days) [12] [13].

The presence of environmental contaminants, especially bacterial pathogens and their toxins, viruses and parasites is a major public health problem. Detecting microbial communities in environmental samples is the first step to recognize these organisms and ensure the health of the population by adopting actions of safety against infection.

Currently, there is research developed based on colorimetric tests to assess the presence of bacteria in water samples, such as the lab-on-paper test strip (bioactive paper) that uses intracellular enzyme activity (β-galactosidase or β-glucuronidase) to detect *Escherichia coli* [14], the paper chip immunoassay that uses immobilized antibodies labeled with gold nanoparticles as a signal reporter to detect *E. coli* [15] and also DipTest, a method that consists of paper tape coated with chemoattractant and enzymatic substrate to detect *E. coli* [16]. Some of these tests, however, require prior sample preparation, and none are yet commercially available in several countries, including Brazil.

Thus, the present work we sought to develop a paper-based culture device capable of detecting, identifying and quantifying bacteria of the coliform group using standard bacterial strains, to establish a suitable and functional platform for the on-site diagnosis of these microorganisms.

2. Materials and Method

2.1. Preparation, Sterilization and Assembly of the Culture Device

To build the portable device for the identification of microorganisms coliform and *E. coli*, it was necessary to determine the method and the materials necessary for the development of the diagnostic platform. The platform layout was designed using the software Adobe Illustrator CS6, with a circular pattern of 26

mm in diameter being determined to form the cultivation zone, and around it a quadrilateral pattern to delimit this region. The total size of the paper diagnostic platform was established at 1369 mm^2, including the envelope where the hydrophobic area and the bacterial culture region (hydrophilic area).

The paper used for printing the layout was the qualitative filter paper 50 × 50 of 80 g (Fitec), A4 format. Wax printing allowed the delimitation of the devices hydrophobic and hydrophilic areas, which was performed on a Xerox Phaser printer (model 8000 DP). After printing, the paper was taken to the oven at 120˚C for 2 minutes to allow the wax to melt and to pass through the paper interface.

Masking tape 48 mm wide (Adelbras® Sleeve) was also used to cover and seal the device, and holes were made in the tape with a 25.4 mm paper punch (Ek tools TM/MC). To cover the hole formed in the adhesive tape, Polydimethylsiloxane (PDMS) polymer Sylgard® 184 (Dow Corning) was added to allow gas exchange, in addition to allowing the visualization of bacterial colonies that grow on the paper.

After assembling the portable culture device, in order to promote sterilization in the device's autoclave, 100 µL of distilled water was added over the hydrophilic part of the paper, to prevent the wax from entering the hydrophilic region during the sterilization process. The process followed that recommended for the sterilization of microbiological culture media.

2.2. Culture Medium and Preparation of Test Strains

The Chromocult® coliform agar (Merck) culture medium was used for microbial growth and multiplication, which was prepared according to the manufacturer's instructions.

Control strains of *Klebsiella pneumoniae* (ATCC 13883) and *Escherichia coli* (ATCC 25922) belonging to the collection of the Centro de Pesquisa em Medicina Tropical (CEPEM) of Fundação Osvaldo Cruz (FIOCRUZ) were used to carry out the in vitro evaluation tests of device efficiency. The strains used were kept at −80˚C and were resuspended in Luria-Bertani (LB) Broth. In order to rule out the possibility of culture contamination, biochemical tests were performed using the modified Rugai and Araujo identification means (Newprov), which consists of nine biochemical tests in a single tube to identify bacteria belonging to the Enterobacteriaceae family [17]. After identity confirmation, the strains were kept frozen at −20˚C in 70% glycerol and resuspended in LB broth for use in the tests.

For the optimization tests, the bacteria were cultivated on nutrient agar for 24 hours, and the obtained colonies were suspended in 0.9% saline solution. McFarland 0.5 scale, which measures the absorbance in a spectrophotometer at 625 nm, indicated turbidity values between 0.08 and 0.132, which points to an estimated 1.5×10^8 cells/mL [18]. Then, the serial dilution of *K. pneumoniae* and *E. coli* bacteria was performed, standardized at 0.5 on the McFarland scale, in

concentrations 10^7, 10^6, 10^5, 10^4, 10^3, 10^2 and 10^1 CFU/mL.

2.3. Culture Device Optimization Tests

Tests to determine the ideal volume of medium to be deposited in the device were performed in triplicate, with evaluation of 300, 350, 400, 450, 500, 550 and 600 µL volumes. For this test, the bacterial concentrations (*K. pneumoniae*) of 10^3, 10^2 and 10^1 CFU/mL were used, with 100 µL of the bacterial dilution inoculated in each device, and then triplicates were incubated in an oven at 37˚C for 24 hours.

A comparative analysis of the bacterial growth in the device with the conventional culture was also carried out using a Petri dish of 5 cm in diameter, the same volume of sample used in the device, performed the pour plating. Seven concentrations were prepared, in triplicate, of the bacterial suspension (10^7, 10^6, 10^5, 10^4, 10^3, 10^2, 10^1 CFU/mL) standardized on the McFarland ladder 0.5, inoculated into the devices and Petri dishes and incubated in an oven at 37˚C for 24 hours.

For results analysis, the photographic record of the devices and Petri dishes was performed and analyzed by color intensity, through the pixel count, using the color measurement of the color histogram in the range between red, green and blue (RGB) using the Image J software, since the target bacteria show colony staining in this color range when grown in the Chromocult coliform agar culture medium, which made it possible to prepare the analysis curves and compare the results.

The comparisons were performed considering the culture staining intensity, using the pixel value of each triplicate and each microbial concentration, from which the value of the white sample (plates and devices without bacterial growth) was subtracted. Thus, the greater the intensity of the staining in the analyzed area, the greater the amount of bacteria.

2.4. Statistical Analysis

The mean, standard deviation and standard error of the results obtained in the optimization of the devices were calculated, using the model simple linear regression and the model Boltzmann sigmoidal for analysis and statistical modeling, in the OriginPro 2018 software (OriginLab®). Analytical curves were constructed to determine the sensitivity of the method through the equation obtained by the curve, being assessed the coefficient of determination (R^2), correlation (r), angular and slope, in addition to the analysis of variance ANOVA (Test F) to determine equality between the means.

The limits of detection (LOD) and quantification (LOQ) (CFU/mL) were also estimated considering the LOD three times the value of the standard deviation of the white divided by the angular coefficient of the line (slope) and the LOQ was ten times the standard deviation of the white, divided by the slope of the line [19].

3. Results

Constructing the diagnostic platform for detection, identification and quantification of bacteria of the coliform group present in the water and in the environment used specific chromogenic culture media that were injected into a platform constructed with the aid of masking tape, filter paper impregnated with wax and PDMS sheets (Figures 1(A)-(C)).

The device manufacturing follows the steps below:

1) Unroll masking tape from package (2 joined tapes), measure 13 cm with a ruler and cut it.

2) Open the orifices in both sides of the masking tape using the 25 mm punch, carefully marking the orifice opposite sides so that, when de device is closed, the holes are correctly overlapped (Figure 1 (1)).

3) Attach a 28 mm diameter PDMS film over each hole (Figure 1 (2)).

4) Place a 37 mm × 37 mm paper sheet printed with wax on the top of every hole PDMS, align the culture area and the hole on the tape and glue the hydrophobic part of the paper to the masking tape (Figure 1 (3)).

5) Fold both short edges to create a 5 mm wide border to ease handling and avoid sticking (Figure 1 (4)). Close the device carefully to keep the hydrophilic paper parts parallel and apply pressure through the device to seal the parts. This finishes the device manufacturing process.

The average time of construction of the device is 2 minutes and 10 seconds ±0.57, measured by the observation of 12 volunteers.

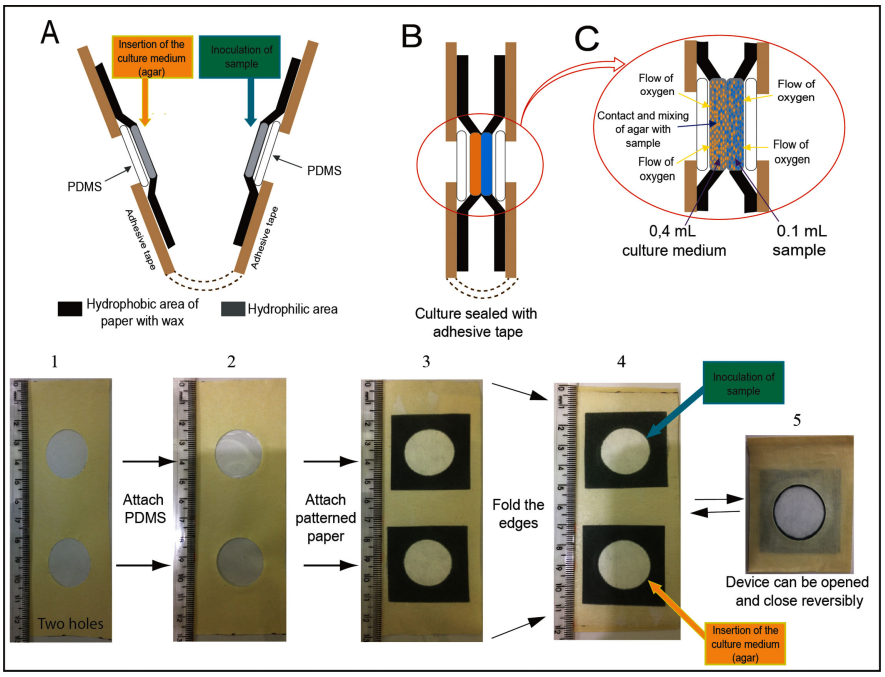

Figure 1. Scheme of manufacture of portable culture device. (A and B) Representation of areas of culture growth on a paper platform printed on wax and glued on adhesive tape; (C) Demonstration of the contact between culture medium and sample and oxygen flow; (1 - 5) device manufacturing steps. Source: Edited figure from Funes-Huacca *et al.* [20].

In order to ascertain the ideal volume of culture medium for microbial growth in the portable culture device, tests were performed with different volumes of culture medium (300, 350, 375, 390, 400, 425, 450, 475, 500, 550, and 600 µL). It was observed that volumes of medium above 500 µL overflowed from the hydrophobic boundaries, while volumes below 390 µL determined a slight dryness of the culture medium during the incubation period, which was harmful to bacterial development. Thus, 400 µL of culture medium was set as the standard volume, since it allows the proper development of the colonies without the medium overflowing from the device hydrophobic areas. The use of specific chromogenic media made possible to identify the target bacteria. The incubation time able to provide colonies visualization was between four and six hours, depending on the bacterial concentration, which varied from 10^7 to 10^1. The exponential growth phase can be observed after five hours of incubation for the highest concentrations (10^7 and 10^6) and after seven hours for the other concentrations; entry into the stationary phase occurred after 16 hours of incubation in the highest concentrations and 14 hours in the lowest concentrations (10^2 and 10^1) (**Figure 2**).

When comparing with the conventional method, the mean of the inflection points obtained in the curves was 8.22 ± 1.05 (**Figure 3**). Thus, the bacteria entered the exponential growth phase one hour and 34 minutes earlier in conventional culture when compared to growth in the culture device, just as the entry into the stationary phase occurred approximately one hour earlier, however these differences did not influence the final incubation time.

To evaluate the device effectiveness in detecting and quantifying the target bacteria, 100 µL of the bacterial suspensions (direct inoculation) were inoculated in conventional culture medium in Petri dishes and in the device. Analytical curves (**Figure 4(A)** and **Figure 4(B)**) were designed showing the intensity of the devices staining, due to the logarithmic quantity of Colony Forming Unit (CFU).

The data of evaluation by linear regression from the analytical curves resulted in a coefficient of determination (R^2) of 0.9891 for the device and 0.9894 for the conventional culture method, showing a good correction of the estimated value and the observed value. The behavior of the portable culture device in relation to conventional bacterial growth enabled to observe an excellent correlation between the two methods (r = 0.9775), however the slope (0.84) and the intercept (0.36) (**Figure 5**) being different 0 and 1, demonstrate a statistically significant difference between the two methods, where p = 0.28, indicate that there is a difference in equivalence between the methods, a fact explained because the conventional method has greater availability of nutrients (greater volume of culture medium) and space. Since the device in the culture device, the cultivation area is twice as smaller and the volume twelve times smaller than in the Petri dish.

The Limits of Detection (LOD) attained by staining intensity for the direct inoculation method in the portable culture device and in the conventional method were 1.053 CFU/mL and 0.977 CFU/mL, respectively, and the Limits of

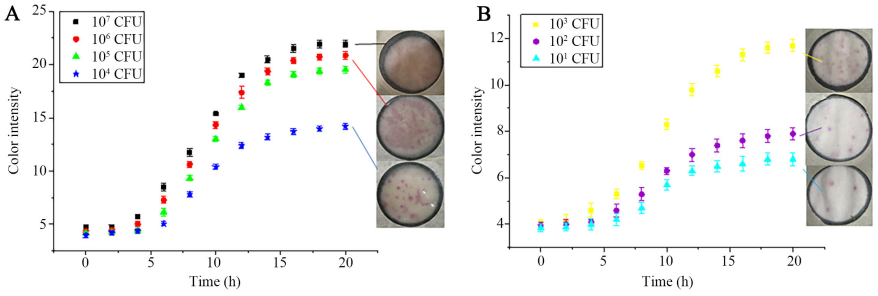

Figure 2. Bacterial growth time *Klebsiella pneumoniae* grown in the device, photodocumented every two hours for 20 hours. (A) Bacterial growth curve in concentrations of 10^4 to 10^7; (B) Bacterial growth curve in concentrations of 10^1 to 10^3.

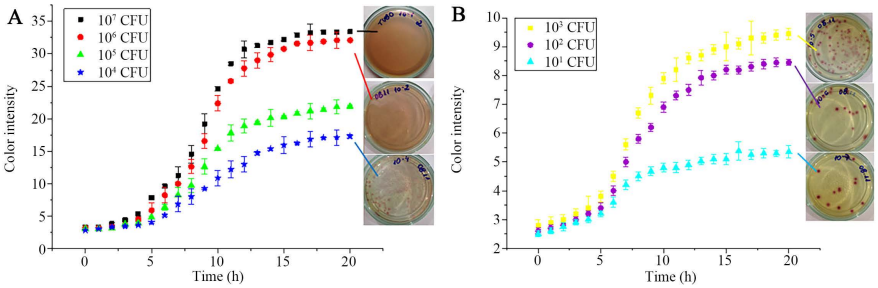

Figure 3. Bacterial growth time *Klebsiella pneumoniae* grown in the Conventional method, photodocumented every two hours for 20 hours. (A) Bacterial growth curve in concentrations of 10^4 to 10^7; (B) Bacterial growth curve in concentrations of 10^1 to 10^3.

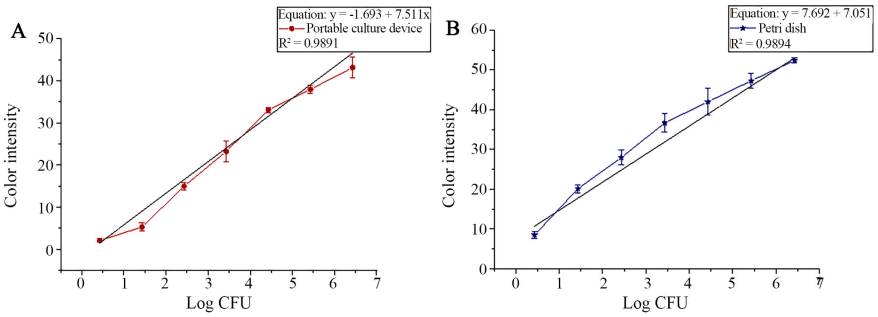

Figure 4. Analytical curves of staining intensity of the device area (A) (25 mm in diameter) and Petri dish (B) (50 mm in diameter) referring to the growth of *K. pneumoniae*.

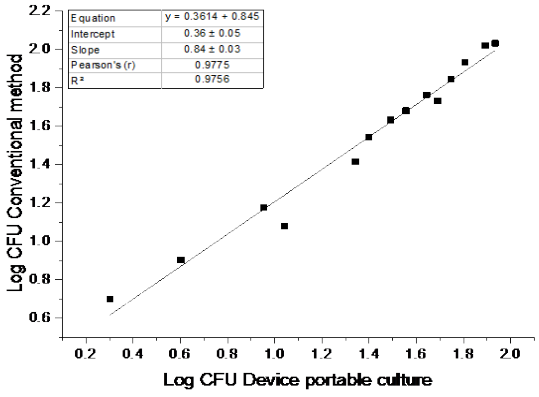

Figure 5. Graph showing linear regression between the portable culture device and the conventional method ($\alpha = 0.05$).

Quantification (LOQ) were 3.299 CFU/mL and 3.259 CFU/mL. When comparing LOD and LOQ detected in the portable culture device and in the conventional method, there was no statistically significant difference through the ANOVA variance test with linear adjustment, which demonstrated $p > 0.05$.

4. Discussion

The POCs platforms were developed as an accessible methodology to provide quick diagnostics, in loco and close to the patient, to hasten the treatment of illnesses and provide adequate therapeutic resources [5]. Countless infectious diseases are treated without a correct diagnosis as to the pathology, leading to mistaken or unnecessary treatments applied to the patient, and causing undesired side effects, such as the predominance of microorganisms resistant to antibiotics [2]. On the other hand, the early detection and monitoring of a possible pathogen reduces the search for health systems and the patient mortality, reducing public health expenditures, which are major problems faced mainly in poor and developing countries [21].

There are several types of POC tests available for diagnostics. However, there is a predominance in use of microfluidic systems [22], because they are built on a porous matrix that allows the flow of liquids, in a miniaturized way, for the use of small sample volumes, besides eliminating sample processing steps and presenting low aggregate cost [23] [24].

Microfluidic paper-based analytic devices (μPADs) are used for analyzes involving colorimetric detection, where channels are produced using barriers capable of guiding the flow of fluids by capillarity until they find substrates in the detection zones. These barriers can be made by wax printing on the paper, creating a hydrophilic and hydrophobic contrast, responsible for confining the samples without interacting or compromising the analysis [7] [25] [26] [27]. They are largely used in clinical analyzes, in urine, blood and saliva samples, especially for verifying glucose [25] [28], uric acid [29], total proteins [30], enzymes [31] and DNA [32], as well as infectious diseases, most of them focusing on tests for antigens or antibodies, such as immunochromatography [23].

Development of devices for environmental analysis have been on the rise in recent years, especially paper-based platforms with colorimetric detection designed to evaluate contaminants in water, in particular those using enzymatic substrates [14] [16] and the paper chips using immobilized antibodies for the detection of microbial agents such as *E. coli* [15].

In this sense, we developed a portable culture device based on the POC premises, seeing as it is produced using simple components and is easy to build and to apply, besides presenting a quick response time and reliable results [33] [34]. However, our methodology was based on culture and the product designed to be versatile, used with different samples, such as water, food or environment surfaces.

The layout designed for printing in wax ink on filter paper created a vertical

and horizontal barrier, confining the culture medium and the sample in a hydrophilic zone. Several studies point out that wax is suitable for the development of diagnostic platforms, reporting it to be adequate, effective, cheap and useful [7] [35] [36].

The materials used in the construction of our portable culture platform combine durability, resistance to different temperatures, and can be sterilized in an autoclave, without compromising its integrity [35]. The use of adhesive tape provides the device's architecture, provides structural support for the paper, simplifies the handling and ensures the containment of the analytical region [6] [20] [37]. The presence of two windows in the adhesive tape covered by PDMS films provides the necessary gas flow for bacterial development, ensuring the diagnostic platform functionality [20] [38].

Moreover, the analytical components in the POC favor microbial detection by colorimetry, because we have added a chromogenic culture medium that contains enzymatic components able to react and release a stable chromogen in the presence of specific enzymes from the target bacterial groups. Also, the Chromocult® agar medium has substances that inhibit Gram-positive and some Gram-negative bacteria, such as Tergitol 7 [39] [40], thus assuring the results reliability to detect and quantify total coliforms and *E. coli*. The enzymatic components present in Chromocult® agar, such as 6-chloro-3-indolyl-β-D-galactoside, were tested on paper strips for the production of a test for water diagnostics, where it, with other chemical components, was effective in the colorimetric detection of *E. coli* [16], for the substrate reacts with the presence of the enzyme β-galactosidase present in the coliform group bacteria.

The volume of medium optimized and applied on the paper platform can be absorbed through the paper fibers so that, when interacting with the sample, it provides an efficient bacterial development, with values comparable to the conventional methodology, as demonstrated in the statistical analyzes. It is noteworthy also that the culture miniaturization had no interference on microbial multiplication, and the oxygen entry window provided by the PDMS contributed to the good performance of microbial growth. Since, in closed circuits, coliform bacteria tend to consume oxygen constantly during their multiplication, with a greater need for this during the first hours of incubation [41], the PDMS windows were sufficient to supply this microbial need.

Bacteria can be detected by colorimetry few hours after the incubation, an advantage over conventional culture techniques, since it is possible to visualize the color change with the naked eye or use color intensity analysis software in order to determine the presence of target microorganisms qualitatively and semiquantitatively. Therefore, it was possible to infer that, using semiquantitative analysis, LOD was close to 1 CFU/mL and the LOQ was approximately 3 CFU/mL, which demonstrate the device ability to quantify CFU by colorimetry in low amounts of bacterial cells, which can be an efficient resource in the detection and quantification of coliform bacteria, for there was no statistically significant difference in the results between this method and the conventional one.

Our method is limited to the use of liquid samples, with low volumes, and high volumes of samples can overflow the hydrophilic limits of the culture device, in addition to being developed to qualitatively and quantitatively evaluate coliform bacteria and *E. coli*, limiting their use to these microorganisms.

Thus, the presented diagnostic platform can be used as an alternative method to conventional plating for the detection of coliforms and *E. coli*, although it has shown a significant difference in colony count when comparing the methods, they showed a high correlation, evidenced by the value of r, thus not excluding the possibility of its use, since the purpose of is to perform direct analysis in the field, by colorimetric analysis, avoiding the need for a complete laboratory infrastructure, requiring only adding a portable microbiological over for incubating the sample. Thus, it is possible to obtain results more quickly, so that intervention measures are taken more quickly, in places where access to laboratories is restricted.

Additionally, the platform has a low cost and is developed with materials that are easy and quick to manufacture and to analyze, thus making it a great alternative for the fast and reliable pathogen identification. This is important to improve the quality of life and maintain the health of populations that they live in regions that are difficult to access or that need to travel great distances to have access to laboratory infrastructure, which is very common in developing countries.

5. Conclusion

In summary, we have demonstrated the development and the applicability of a portable culture device capable of quantifying bacteria in 100 µL of liquid samples evaluated by colorimetry. Our portable culture device presents an excellent alternative to be incorporated in the evaluation of microbiological quality in liquid samples applied directly in the field, and can also be used as an alternative culture method for microbiology laboratories. However, additional optimizations should be performed to determine different ways of obtaining and applying samples in the device aimed at detecting pathogens in the environment, such as water and water percolating surfaces.

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Authors' Contributions

Natália Faria Romão: Conceptualization, Investigation, Writing—Original Draft,

Visualization. Nathalia Aparecida Costa Pereira: Formal analysis, Data curation. Maribel Elizabeth Funes Huacca: Methodology, Validation, Supervision. Luciana Gatto Brito: Writing—Reviewing and Editing, Project administration, Funding acquisition.

Conflicts of Interest

The authors declare there are no conflicts of interest.

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