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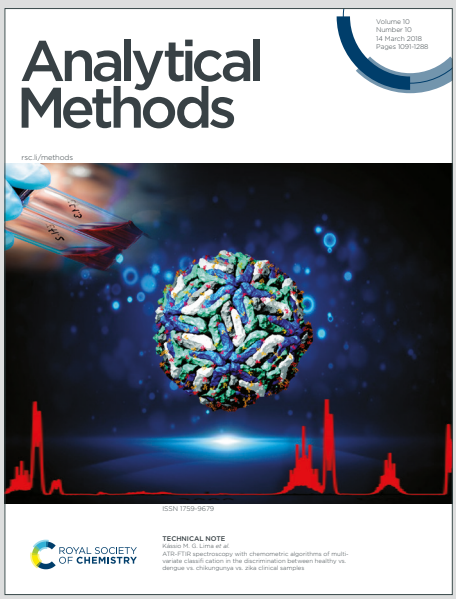

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Fast Antibiotic Susceptibility Test Integrated Microfluidic Chips for Detection of Carbapenem/Colistin-Resistant Bacteria by a Smartphone- based Analysis

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Abstract: Although many phenotypic methods have been developed for the detection of antibiotic-resistant bacteria, this emerging field still requires more rapid, practical, and economic approaches. Herein, we report a colorimetric phenotypic antimicrobial susceptibility test integrated into a microfluidic chip (mc-AST) for the detection of carbapenem (Car) and colistin (Col) resistant bacteria. A key component of the mc-AST is anthocyanin, a natural pH indicator that changes color based on the pH of the reaction environment. The mc-AST contains corresponding antibiotics to suppress the growth of susceptible bacteria. The color changes in the mc-AST can be detected by the naked eye and through image processing using a smartphone. The novelty of this study lies in the first-ever integration of an anthocyanin-based antibiotic susceptibility test into a microfluidic chip. This integration provides a resistance profile for multiple doses of antibiotics on a single chip. Ultimately, the mc-AST reduces the workload and delivers results within 2 hours.

Keywords: Antibiotic Susceptibility Test; Colorimetric Assay, Natural pH Indicator; Microfluidic Chips; Carbapenem/Colistin-Resistant Bacteria; Smartphone-based Analysis

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Introduction

Antimicrobial resistance (AMR) can be considered as a silent epidemic that has been growing stronger for years. The current methods have not been fully effective in prevention and management of AMR. World Health Organization (WHO) and the United Nations (UN) have taken serious responsibilities in preparation global action plan to combat resistant microorganisms. [1] The UN General Assembly mentioned the critical importance of AMR with High-Level Meeting in 2016. The countries have been encouraged to develop national action plans to reduce AMR and to secure people against threats of bacterial AMR in the future.[2] Despite preventive measures and innovative decisions, the number of deaths associated with bacterial AMR is estimated to be 4.95 million in 2019. Unfortunately, the worldwide death count due to AMR is estimated to reach 10 million by 2050. The health economic impact of this disease is estimated to be $100 trillion at US.[1] The top six pathogens responsible for resistance-related deaths are *Acinetobacter baumannii*, *Klebsiella pneumoniae, Escherichia coli, Staphylococcus aureus, Streptococcus pneumoniae* and *Pseudomonas aeruginosa*.[3] Carbapenem resistance has been reported at the critical level in the list of "Bacterial Priority Pathogens" announced by the WHO Core Package of Interventions to Support National Action Plans. The WHO updated the Bacterial Priority Pathogens List (BPPL) in 2024, which includes 15 families of antibiotic-resistant bacteria grouped into critical, high and medium categories for prioritization. Carbapenem-resistant *Acinetobacter baumannii* is ranked first in the BPPL. [4, 5] The announced plan reported that AMR could be prevented with four basic components, one of which is early, rapid and accurate diagnosis. The 33 research priorities for bacterial and fungal infections are grouped into four themes. Investigation and evaluation of rapid point-of- care (POC) diagnostic tests ranks 6th behind prevention parameters. [5] This was highlighted as a priority that because more effective methods are highly needed for pathogen detection, rapid identification of antibiotic resistance and implementation of urgent and accurate treatment. Rapid diagnostic tests and/or methods are still required to help healthcare professionals make the right choice of antibiotics. These tests will shorten the treatment process and limit the use of antibiotics. Except malaria and HIV, only 19% of people in low- and middle-income countries have access to simple diagnostic tests to guide antibiotic prescribing in primary care.[5,6]

The current gold standard for antimicrobial susceptibility testing (AST) is culture-based methods such as agar diffusion, disc diffusion and broth dilution. These methods rely on the bacterial growth. However, they present several drawbacks including 18-24 hours (hrs) incubation time, intensive labor work and multiple steps [7]. Apart from phenotypic methods, genotypic methods

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are also available like polymerase chain reaction (PCR), which aims to detect resistance genes, gives rapid results, spectroscopic methods for monitoring bacterial growth and mass spectrometric methods for biochemical analysis. However, they have several disadvantages, such as the need of expensive equipment, specialized staff and high cost.[8,10] In view of these problems, there is a high demand for rapid, cost-effective, user-friendly and accurate POC tests. Microfluidics has become popular as a promising diagnostic technique for AST development. The importance of POC diagnostics was highlighted during the pandemic. In the fight against antibiotics, it will be the most important tool with high specificity analysis. Features such as rapid results, high efficiency, minimal sample requirements and cost effectiveness are the main reasons for choosing microfluidics based POC diagnostics.[7,11] The results of color-based microfluidic sensors are based on the detection and evaluation of individual colors. Mathematical models can be used to accurately determine colors. The CIELab formula, one of the models developed for this purpose, divides each color into three components. Lightness (0-100) is the space between black and white, a is the space between green and red (-128, +127) and b is the space between blue and yellow (-128, +127). All colors that the human eye can see and distinguish can be represented in this space. Based on this formula, the formula ΔE Lab is used to calculate the difference between two colors. This formula is also used in the analysis of test results.[12]

$$\Delta E^*_{ab} = \sqrt{(L^*_2 - L^*_1)^2 + (a^*_2 - a^*_1)^2 + (b^*_2 - b^*_1)^2}$$

Color space values categorized as RGB (red, green, blue), HSV, HSL, CMYK and CIE give results proportional to the concentration of analyte. However, color image processing techniques can be performed in computer-based software. In this case, the end user must be a professional. In recent years, smartphones have become an indispensable tool for POC testing. This is due to their popularity, portability and improvements in software.[13,14] Smartphone-based POC assays are preferred because of their widespread availability, accessibility, usability, low cost and rapid analysis.[15] In many tests that make direct measurements from different samples such as urine and plasma, the analysis is based on color image processing using smartphones. To protect the analysis results from external factors, standardization is ensured by the development of a photo box and phone apparatus.[11,16-18]

Here, we have developed a colorimetric microfluidic chip-integrated AST (mc-AST) that gives results in 2 hrs. The mc-AST was designed for detection of carbapenem (Car) and colistin (Col) (last resort but have high resistance rates) antibiotic resistant bacteria. The colorimetric mc-AST solution contains anthocyanin groups, which are pH indicators extracted from plants,

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culture media and appropriate antibiotics. The current test solution is deposited into the microfluidic chip, revealing the resistance profile to multiple doses of antibiotics on a single chip. As the bacteria continue their vital activities in the chip-well, acidic volatile gases (AVG) are released. The susceptible bacteria do not release AVG owing to inhibition of their growth and then the pH remains constant and therefore, no color change is observed. In contrast to that, when bacterial strains are resistant towards antibiotics, color and pH values of mc-AST are changed due to production of AVG. We developed a mobile application that performs color image processing to increase the sensitivity of mc-AST results based on color change. We also developed a mobile phone apparatus to standardize the results. Microfluidic chips reduce the workload of AST, which requires a time-consuming process, and reveals the resistance profile to several different doses at the same time. Thus, a fast, sensitive and innovative mc-AST was developed.

Results and Discussion

In this study, we prepared natural pH indicator based colorimetric test solutions integrated to microfluidic chips fabricated with various designs for detection of carbapenem and colistin resistant bacteria. The colorimetric responses were witnessed by a naked eye and smartphone readouts containing Red-Green-Blue (RGB) and Delta-E (ΔE) analysis. The anthocyanin molecules used as a key component in colorimetric test change reaction color in the presence of antibiotic resistance bacteria. As bacteria grow, their metabolism produces AVG. The organic acids produced in this process cause the pH of the medium to decrease. In the test medium, anthocyanins act as pH indicators, so they protonate as the medium becomes acidic, changing color from blue (basic) to pink. Bacterial metabolism is inhibited by carbapenem/colistin, preventing acid production. The stable structure of the anthocyanin molecules is retained and their initial blue color is maintained due to the absence of AVG in the medium. The test response time relies on bacteria concentration and microfluidic chips designs. The **Scheme 1** clearly show preparation of colorimetric microfluidic chip based test and detection of corresponding bacteria by a naked eye and digital image analysis based upon color change.

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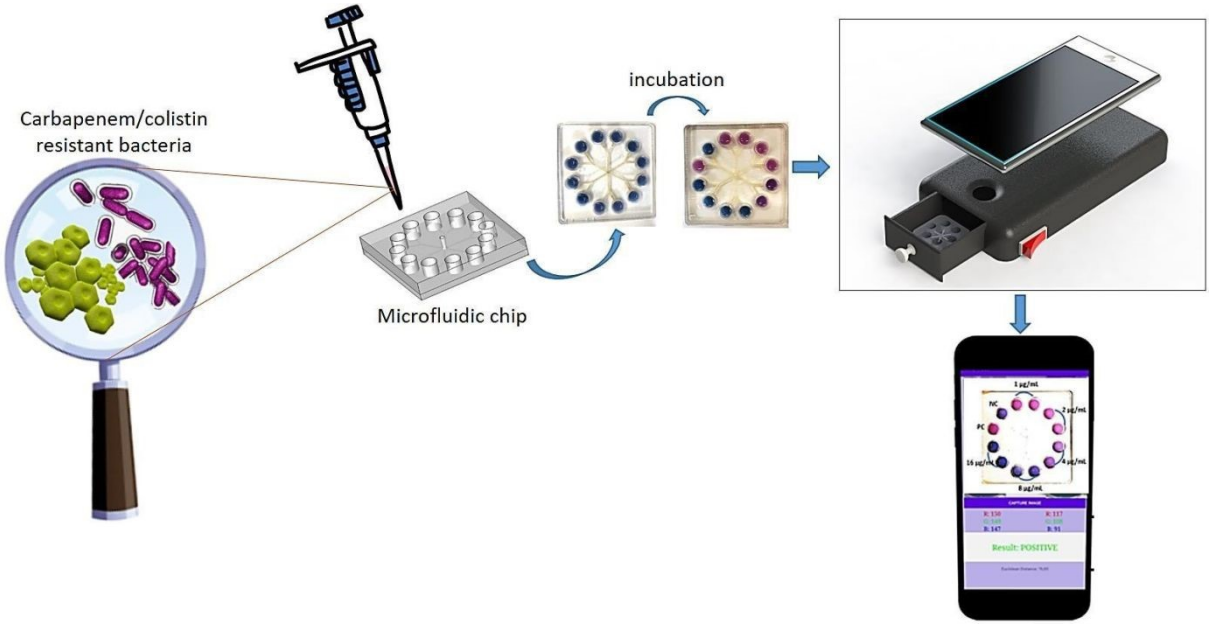
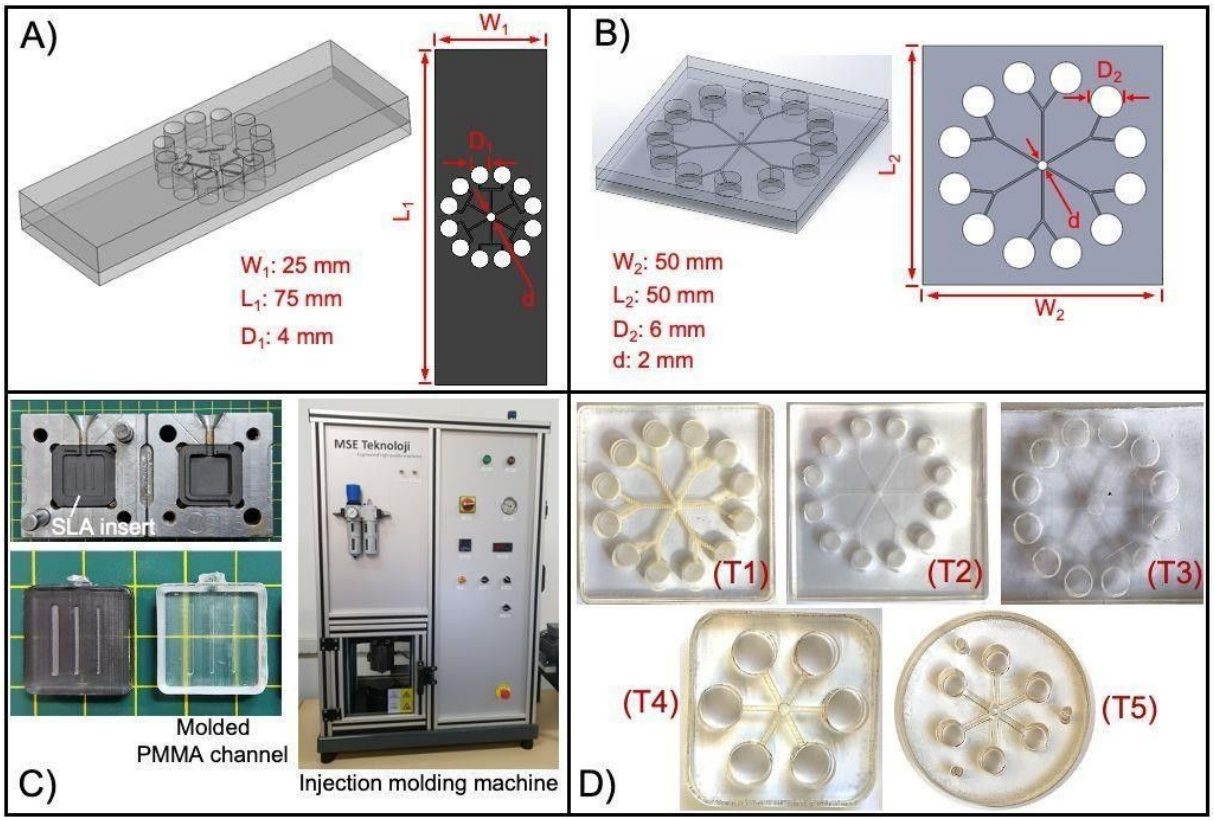
Scheme 1.



Schematic illustration of colorimetric test integrated microfluidic chips for detection of carbapenem and colistin resistant bacteria by a naked eye and smartphone analysis.

It is worthy to mention that integration of colorimetric test solutions to various microfluidic chips. These microfluidic chips were fabricated with different designs and stages.

Fig. 1



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Microfluidic chip designs and stages A) First generation design B) Second generation design C) Production stages to be used in mass production. Appearance of microfluidic chips prepared with different designs D) 12-well microfluidic chips (T1, T2, T3) and 6-well microfluidic chips (T4, T5).

In the first step, all wells of the chips were spiked with 8 ppm carbapenem to detect antibiotic resistant bacteria. This was a proof-of-concept for simultaneous color change, as this high concentration would be expected to inhibit susceptible strains while resistant strains continue to live. We prepared test solution composed of 25% RCE extract as a source of anthocyanin acting pH indicator and carbapenem resistant bacterial strain suspension at pH 8 with blue color. The 1200 µL and 600 µL of test solution was deposited into T1 type 12-well and T4 type 6-well microfluidic chips respectively. After 2 hours (hrs) incubation, blue color of the test solution in all 12-well and 6-well microfluidic chips was converted into pinkish color as shown in Figure 2A and 2B, respectively. The content and volume of colorimetric test solution in 12-well and 6-well microfluidic chips remained the same, except replacing antibiotic type from carbapenem to colistin, for analysis of colistin resistant bacteria. The goal of this experiment is to prove that the color change is simultaneous in wells loaded with the same dose of antibiotic. This was done by controlling the flow of liquid through the channels in the designed microfluidic chips. The microfluidic chips were left for 2 hrs incubation, then distinct occurrence of pink color was observed in both 12-well and 6-well microfluidic chips as presented in Figure 2C and 2D. We believe that the color changes in the each well rely on protonation of anthocyanin molecules. Although colorimetric tests include antibiotics, resistant bacteria continuously grow and make reaction environment acidic owing to their AVOCs production. The hydroxyl groups of anthocyanin molecules were protonated at acidic pH and their electron density was changed, both of which induce color change from blue to pink. While colorimetric responses were seen by a naked eye, the color changes in the each well was analyzed by smartphone based ImageJ software including Red/Blue (R/B) and Delta-E (ΔE) calculation. As an example, the clear differences in R/B and ΔE values before and after addition of antibiotics resistant bacteria were produced in Figure 2E and 2F, respectively, which can be considered as a semi-quantitative and supportive results for colorimetric detection.

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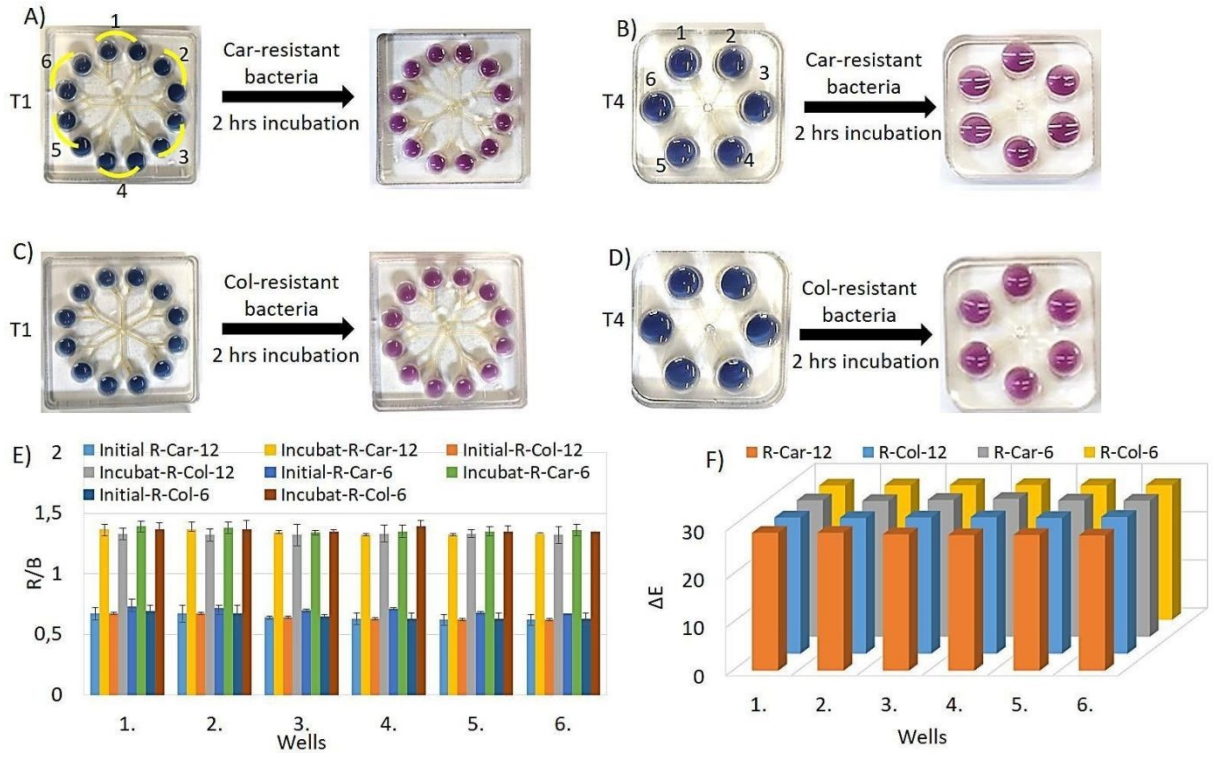
Fig. 2



Color change observed in the presence of resistant bacteria in T1 type 12-well microfluidic chips and T4 type 6-well microfluidic chips. A) Carbapenem resistance analysis in T1 type microfluidic chips B) Carbapenem resistance analysis in T4 type microfluidic chips C) Colistin resistance analysis in T1 type microfluidic chips D) Colistin resistance analysis in T4 type microfluidic chips. Antibiotic: 2 ppm, Bacteria: 3 McFarland. E) RGB analysis. F) Delta E analysis.

In order to show how type of microfluidic chips affect response time of the test solution, we combined the same test solution contained carbapenem resistant bacterial strain with T2 type and T3 type 12-well microfluidic chips. We demonstrated that dark and light blue color of initial test solutions were turned to dark and light pink colors in 2 hrs incubation in T2 type (Figure 3A) and T3 type (Figure 3B) microfluidic chips. These visual colorimetric readouts were analyzed with ΔE and R/B calculations presented in Figure 3C and 3D, respectively. The remarkable and acceptable differences in ΔE and R/B values were calculated in 2 hrs incubation.

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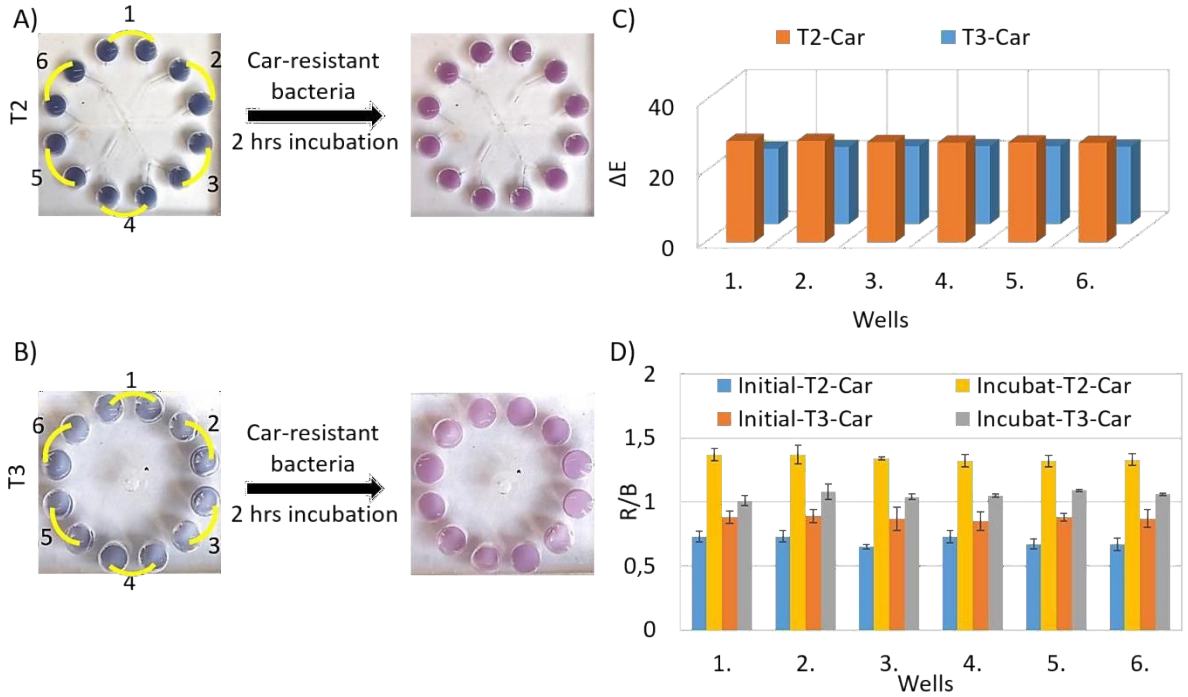
Fig. 3



Color change observed in the presence of Carbapenem-resistant bacteria in 12-well microfluidic chips A) Carbapenem-resistant bacteria in Type 2 microfluidic chips B) Carbapenem-resistant bacteria in Type 3 microfluidic chips C) RGB analysis D) Delta E analysis.

As a further systematic study, we investigated stability and specificity of colorimetric test solution integrated into four types of microfluidic chips including T1 type 12-well (Figure 4A), T2 type 12-well (Figure 4B), T4 type 6-well (Figure 4C), and T5 type 6-well (Figure 4D) microfluidic chips towards carbapenem susceptible strain. After 2 hrs incubations, no color change was observed by a naked eye in all microfluidic chips. We interpret that growth of susceptible strain was inhibited due the presence of 8 ppm carbapenem in test solution, then pH values of reaction solution remained the same. As long as pH of reaction solution is not changed, the pH indicator does not loss or gain proton, then color of initial test solutions are not changed. In addition to that, the test solution was quite stable because its initial color was constant before and after addition susceptible bacterial strain.

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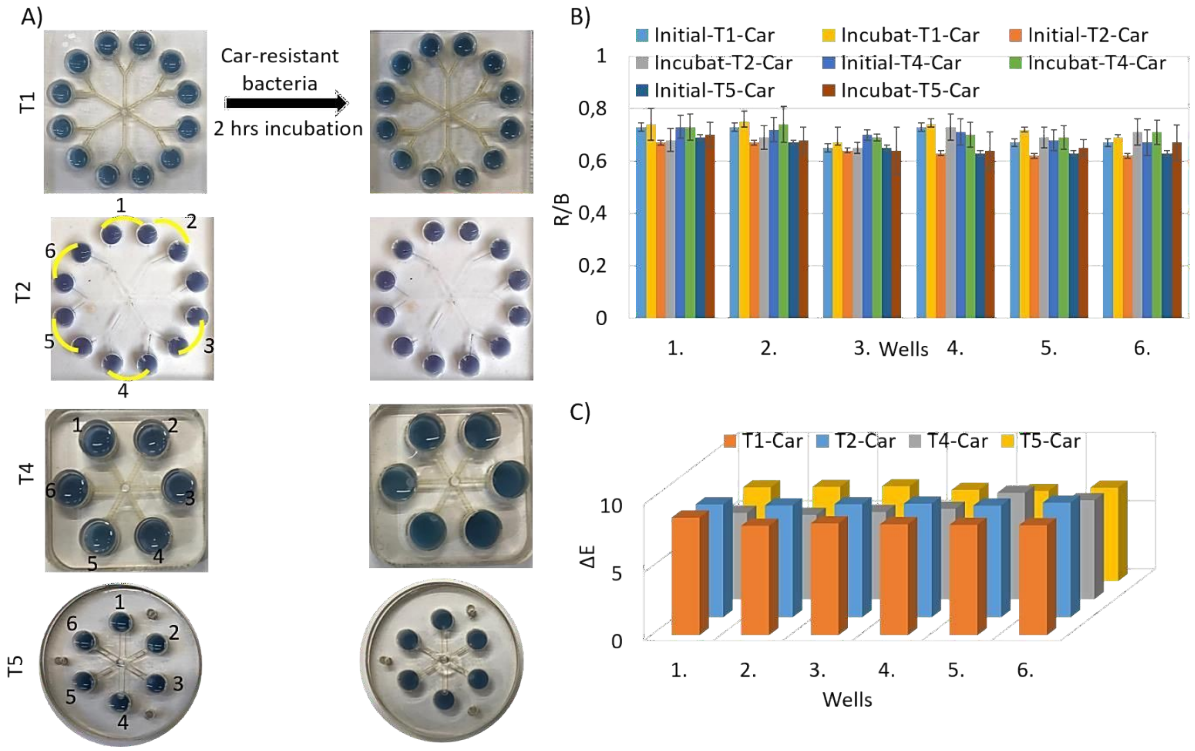
Fig. 4



Colorimetric results observed in 12-well microfluidic chips (T1 and T2) and 6-well microfluidic chips (T4 and T5) in the presence of carbapenem susceptible strain. Antibiotic: 32 ppm, Bacteria: 3 McFarland. A) Carbapenem- susceptible bacteria in Type 1, Type 2, Type 4, and Type 5 microfluidic chips B) RGB analysis C) Delta E analysis.

We manipulated concentration of antibiotics in test solution in order to determine how antibiotic affect detection of target bacteria. We prepared blue color test solutions with a series concentration of carbapenem (1 ppm, 2 ppm, 4 ppm, 8 ppm and 16 ppm) in each well of T1 type of microfluidic chips for detection of carbapenem susceptible strain bacteria (Figure 5A). For instance, while initial blue color test solutions with 1 ppm, 2 ppm, 4 ppm and 8 ppm were turned clear pink color in the presence of Carbapenem-resistant bacteria within 2 hrs incubation, however, 16 ppm carbapenem included test solution was turned into purple color (Figure 5B). We propose that Carbapenem resistant bacteria actively grown in 8 ppm carbapenem included test solution, but they partially or slowly grown in test solution with 16 ppm carbapenem. The test solution called “Negative Control (NC) containing 32 ppm carbapenem completely inhibited growth of the resistant bacteria, then blue color of the test solution was not changed. The Positive Control (PC) test solution does not contain antibiotic, then its blue color was converted into pink color due to bacterial growth. In the presence of carbapenem susceptible strain, blue color of test solutions with 1 ppm and 2 ppm was turned to pink color (Figure 5B).

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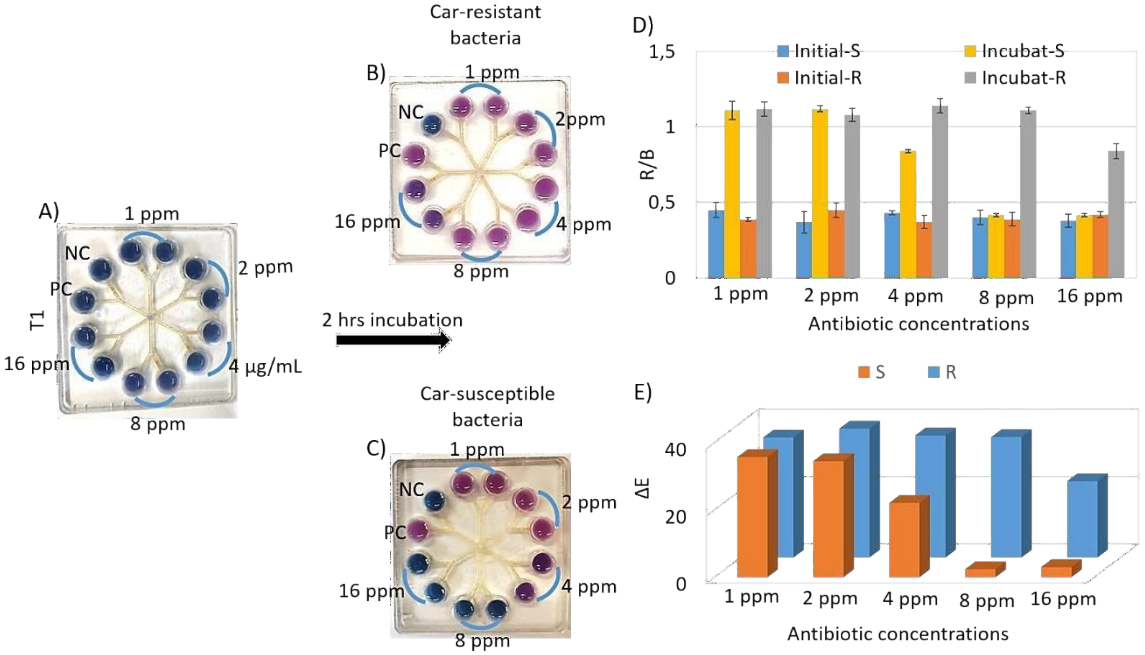
We claim that antibiotic used with 1 ppm and 2 ppm was not able to suppress the growth of these susceptible bacteria. Additionally, 4 ppm carbapenem used in test solution partially inhibited bacterial growth since not clear color change was observed. The test solutions including 8 ppm, 16 ppm and 32 ppm (Negative Control) carbapenem completely stopped growth of susceptible bacteria. The blue color of test solution without antibiotic rapidly turned to pink color in the growth of susceptible bacteria. All colorimetric responses in the presence of resistant and susceptible bacteria in microfluidic chips observed by a naked eye were well-consistent with R/B and ΔE analysis, respectively presented in Figure 5D and 5E.

Fig. 5



Analysis of carbapenem-resistant and susceptible strains in the anthocyanin-based phenotypic carbapenem susceptibility test integrated into T1 type 12 well microfluidic chips. A) Appearance of the test with carbapenem-resistant strain added before incubation B) Color change of the test with carbapenem-resistant strain added at the end of incubation period C) Color change of the test with carbapenem-susceptible strain added at the end of incubation period D) RGB analysis E) Delta E analysis. NC: 32 ppm. Bacteria: 3 McFarland.

In order to elucidate effect of microfluidic chips type, we adapted all experiments made in Figure 5 to Figure 6 by just only T1 type 12-well microfluidic chips with T2 type for detection of carbapenem resistant bacteria. We revealed that initial blue color test solutions with 1 ppm, 2 ppm, 4 ppm, 8 ppm and 16 ppm carbapenem concentrations in T2 type 12-well microfluidic chips (Figure 6A) were clearly turned to pink color in the presence of Carbapenem-resistant bacteria within 2 hrs incubation (Figure 6B). The behavior of negative Control (including 32 ppm and positive Control (no antibiotic included) gave the same response with

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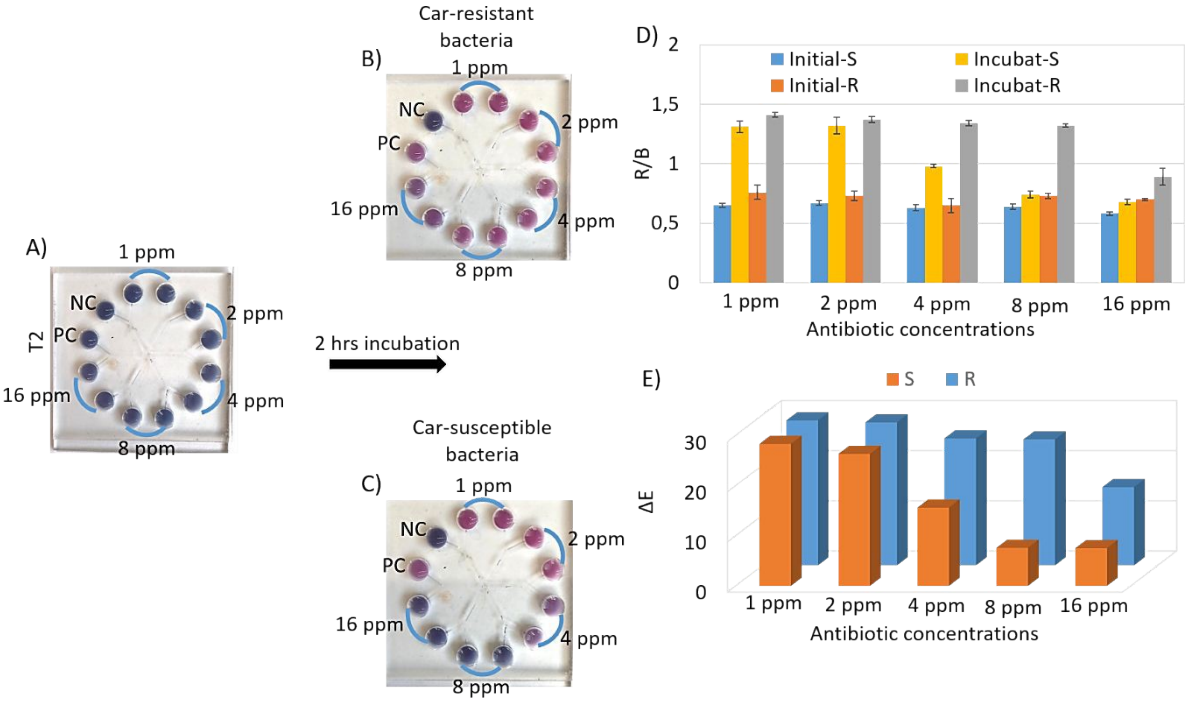
Figure 5B. The carbapenem susceptible strains were added into each well of T2 type microfluidic chips, while no bacterial growth was observed in 8 ppm and above carbapenem concentrations, but these bacteria continued to grow in test solution including under 8 ppm carbapenem concentrations (Figure 6C). The smartphone based digital imaging processing system produced the same results for R/B and ΔE analysis in Figure 6D and 6E, respectively as given in Figure 5D and 5E.

Fig. 6



Analysis of carbapenem-resistant and susceptible strains in the anthocyanin-based phenotypic carbapenem susceptibility test integrated into T2 type 12 well microfluidic chips. A) Appearance of the test with carbapenem-resistant strain added before incubation B) Color change of the test with carbapenem-resistant strain added at the end of incubation period C) Color change of the test with carbapenem-susceptible strain added at the end of incubation period D) RGB analysis E) Delta E analysis. NC: 32 ppm. Bacteria: 3 McFarland.

We further examine how 6-well microfluidic chips called “T4 type” influence detection of carbapenem resistant compared to other microfluidic chips. We demonstrated that initial blue color test solutions with 1 ppm, 2 ppm, 4 ppm and 8 ppm carbapenem was changed to pink color in the presence of carbapenem-resistant bacteria (Figure 7A and 7B). When carbapenem concentration increased to 16 ppm, blue color of the test solution was turned to purple color in the 2 hrs incubation due partial growth of carbapenem-resistant bacteria. The Negative Control test solution (including 32 ppm carbapenem) completely inhibited growth of the bacteria, then initial blue color remained consistent. While carbapenem susceptible strains changed the color

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of the test solutions including 1 ppm and 2 ppm carbapenem from blue to pink (Figure 7C). While the test solution with 4 ppm carbapenem was converted from blue to purple, but no susceptible bacterial growth was observed in 8 ppm and above carbapenem concentrations. The R/B and ΔE calculations in Figure 7D and 7E, respectively were well consistent with colorimetric responses visualized by a naked eye.

We systematically investigate stability of the test colorimetric test solutions in detection of carbapenem-resistant strains (Figure 8A). We prepared test solutions with blue color at pH 8 and stored them at +4 °C and -20 °C for 1 month, 3 months and 6 months. We added carbapenem-susceptible bacterial suspensions into well no 1 and no 4 including 8 ppm carbapenem, of 1 month, 3 months and 6 months stored test solution stored at +4 °C and -20 °C, initial blue color of the test solutions was not changed. We conclude that both the test solutions were quite stable and no susceptible bacteria were grown. However, the same test solutions under the same experimental parameters were used for detection of carbapenem-resistant bacteria. The regardless of any storage time of the test solutions, the carbapenem-resistant bacteria deposited into well no 2 and no 5 changed blue color to pink at +4 °C and -20 °C with 2 hrs incubation. We claim that the test solutions stored at any temperatures and till 6 months can be used as freshly prepared for detection of antibiotic-resistant bacteria. In terms of the smartphone based digital image processing, the colorimetric responses were supported by R/B and by ΔE analysis, respectively presented in Figure 8B and 8C. The distinct differences in R/B ratio and ΔE value allow us to distinguish antibiotic resistant bacteria compared to susceptible ones.

Fig. 7

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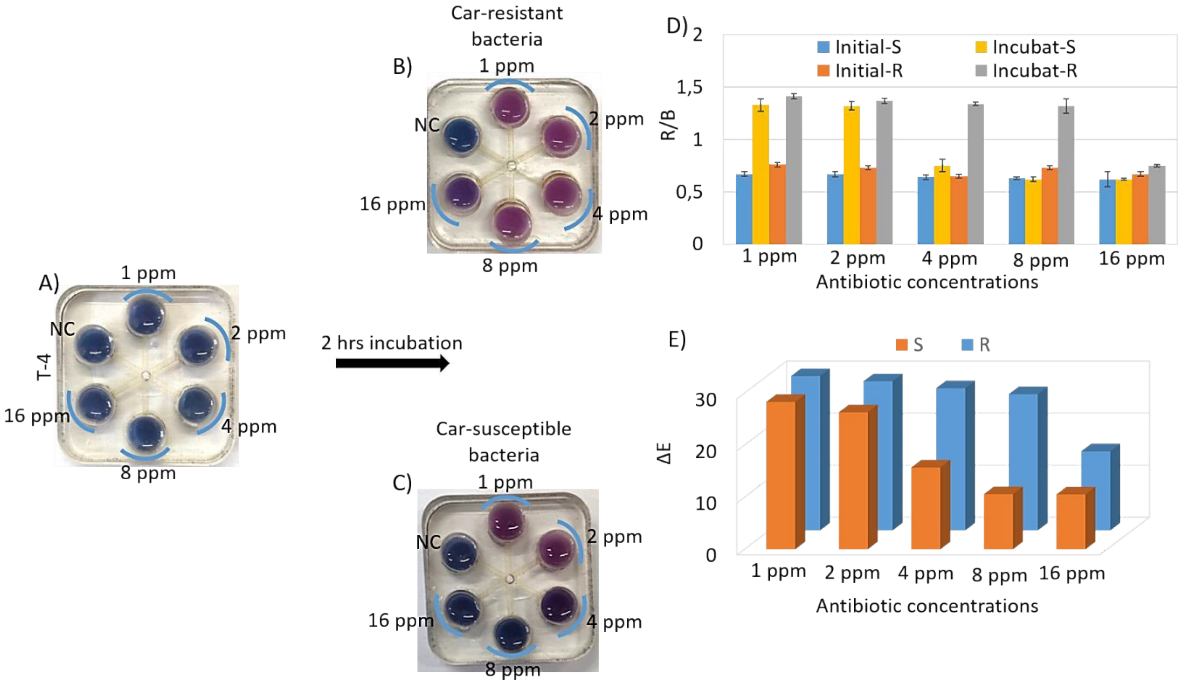
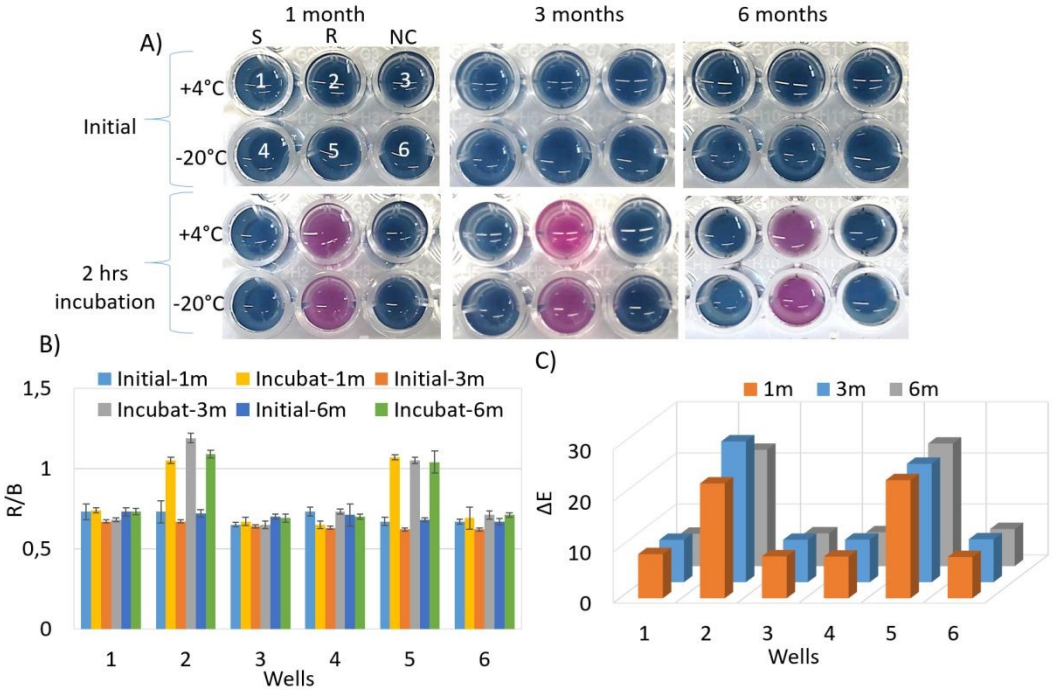


Analysis of carbapenem-resistant and susceptible strains in the anthocyanin-based phenotypic carbapenem susceptibility test integrated into T4 type 6 well microfluidic chips. A) Appearance of the test with carbapenem-resistant strain added before incubation B) Color change of the test with carbapenem-resistant strain added at the end of incubation period C) Color change of the test with carbapenem-susceptible strain added at the end of incubation period D) RGB analysis E) Delta E analysis. NC: 32 ppm. Bacteria: 3 McFarland.

Fig. 8



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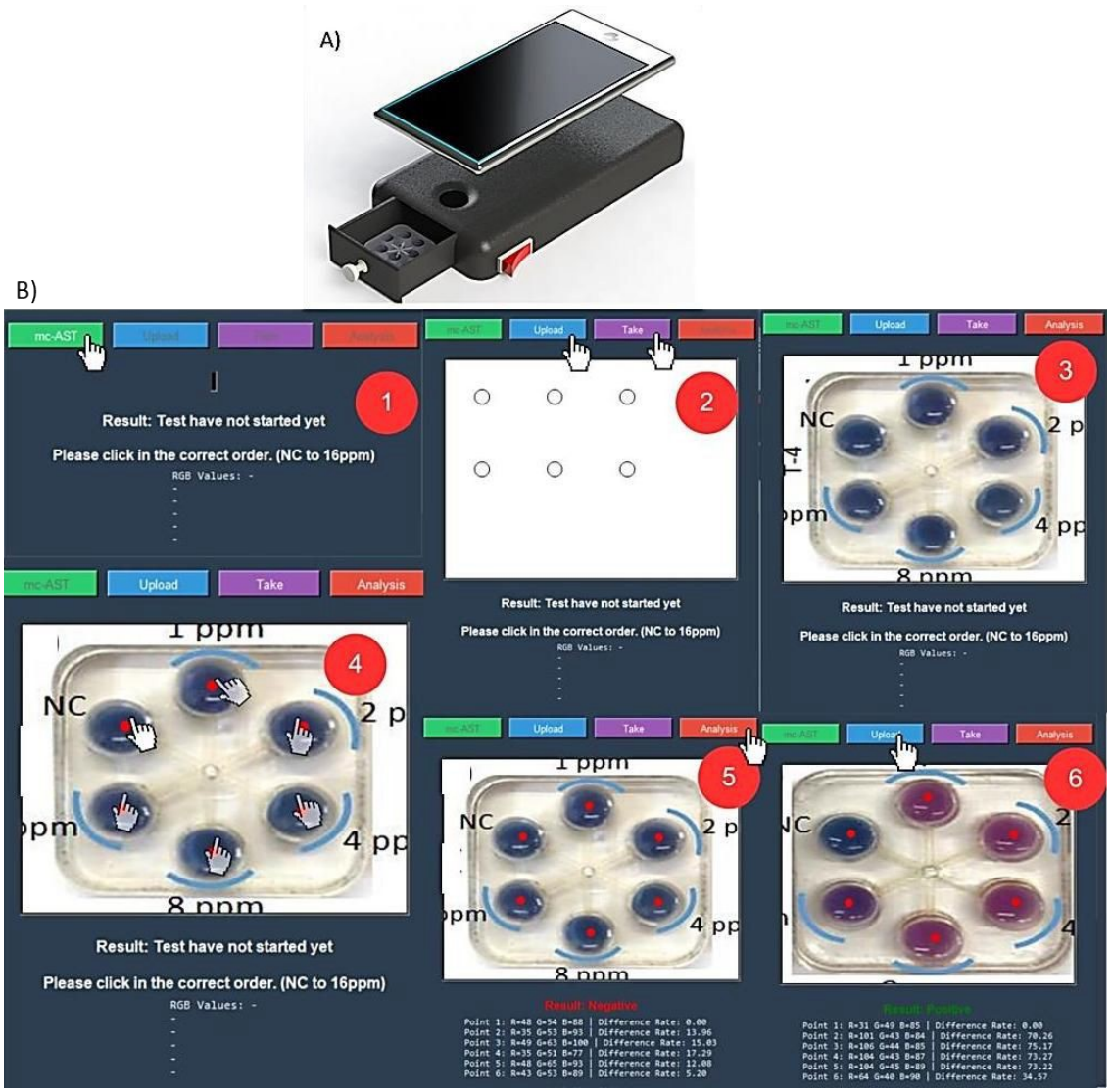
Color changes observed in the presence of resistant and sensitive bacteria in test solutions incubated at +4 °C and -20 °C for 1, 3 and 6 months. A) Colorimetric results B) RGB analysis C) Delta E analysis.

Fig. 9



Smartphone platform and mobile application interfaces A) Components of the smartphone platform B) Interfaces of smartphone applications.

3D-printed smartphone platform was developed and used to analyze digital images with R/B and ΔE calculations as illustrated in Figure 9. Figure 9A shows that the components of the smartphone platform composed of the camera, LED, and sample holder coupled with computer assisted design (CAD) software. In terms of working mechanism, the initial blue of test solutions (pH 8) were converted to pink color in the presence of colistin or carbapenem resistant clinical bacterial strains. The interfaces of the smartphone application are shown in Figure 9B. The mobile application provides the test results through a user friendly interface.

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Compared to our earlier manual assays, the microfluidic platform has simplified the analytical workflow by replacing repetitive dilutions with a single-step procedure for loading the sample using ready-to-use reagents. Relying on the device's internal fluidic layout rather than manual volume adjustments significantly minimises user-induced errors during sample distribution. Furthermore, reducing the necessary equipment to just one chip and pipette tip removes labour-intensive steps, demonstrating the platform's potential for high-throughput applications.

Conclusion

In summary, we have developed microfluidic chip-integrated a colorimetric phenotypic antimicrobial susceptibility tests (mc-AST) for detection of carbapenem (Car) and colistin (Col) antibiotic resistant bacteria. Our study demonstrated that mc-AST was efficiently used for reducing detection time and revealing the resistance profile to several different doses at the same time. The antibiotic resistant bacteria released AVG during their growth and made reaction environment acidic, in which anthocyanin molecule called “pH indicator” was protonated and change the color change in the chip-well, respectively. The color changes were observed by a naked eye and digital imaging processing (ΔE and R/B analysis). The mc-AST showed great stability in time interval of 1, 3 and 6 months.

Materials and Methods

Materials and Instruments: Tryptic soy agar (Merck, Germany), agar (Merck, Germany), skimmed milk medium (Difco, USA), meat extract (Acumedia, UK), NaCl (Isolab, Türkiye), peptone (Mast Diagnostic, UK), carbapenem (Merck, Germany) and colistin (Merck, Germany) were all purchased from the companies indicated.

Microorganisms: Resistant and susceptible bacterial pathogens: *Acinetobacter baumannii* ATCC BAA-1710, *Acinetobacter baumannii* ATCC BAA-1792, *Klebsiella pneumoniae* ATCC 13883, *Klebsiella pneumoniae* ATCC BAA-3067 and *Escherichia coli* ATCC 25922 were obtained from Erciyes University, Faculty of Pharmacy, Pharmaceutical Microbiology research laboratory ATCC culture collection. All pathogens were stored in skim milk medium at -20 °C and regenerated prior to the experiments. The optical density was determined by spectrophotometer (Azure Ao, Azure Biosystems, Inc.).

Red Cabbage (Brassica oleracea) Extraction: Red cabbage (*Brassica oleracea L.; family Brassicaceae*) is used to extract anthocyanin, one of the main components in antibiotic susceptibility testing. In the first stage of extraction, the leaves of this plant, which is rich in

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cyanidin-3-diglucoside-5-glucoside, are separated, cleaned, and cut into small pieces. One hundred grams of the plant material was boiled in 100 grams of distilled water for 30 minutes in a 1:1 wt/wt ratio. Finally, the extract is filtered with Whatman No. 1 filter paper. The obtained extract is purple and has a pH value of 7.0. The extract was stored in amber-colored glass bottles at 4 °C for use in the test content.[19]

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Design of Microfluidic Chips: Microfluidic chips were designed within the Microfluidics and Lab-on-a-Chip Research Group at Bilkent University. The first-generation design comprised a single inlet (d: 2 mm) and twelve outlet wells (D1: 4 mm), interconnected via microchannels with a width of 0.4 mm and a height of 0.2 mm (Figure 2A). The first-generation prototype was manufactured using micromilling at the Micro Manufacturing Laboratory at Middle East Technical University. The chip was fabricated on a 3 mm thick polymethyl methacrylate (PMMA) substrate with standard microscope slide dimensions of 25 × 75 mm, using a CNC milling machine (PROXXON FF500/BL CNC Milling Machine, PROXXON GmbH, Hetzerath, Germany). The microchannels were machined using a 0.4 mm diameter end mill, with a spindle speed of 4000 rpm, a feed rate of 30 mm/min, and a depth of cut of 0.1 mm. The terminal cavities of the channels were created using a 2 mm diameter end mill at the same spindle speed, with an increased feed rate of 50 mm/min. During the experiments, it was observed that sharp corners at the bifurcation points of the microchannel create cleaning difficulties. In the second-generation design, these corners were rounded to facilitate more effective maintenance and reduce the risk of contamination. The second-generation design retained the overall architectural layout (Figure 2B); however, the outlet wells were redesigned with a reduced diameter of 3.3 mm. Consequently, the volume of each outlet well increased from 38 µL to 200 µL. The manufacturing of the second-generation design was also performed via milling (PROXXON FF500/BL-CNC) on two PMMA plates, each with a thickness of 3 mm, forming a structure with overall dimensions of 50 mm × 50 mm. The microchannels were machined using a 0.4 mm diameter tool at a spindle speed of 4000 rpm and a feed rate of 30 mm/min. A 6 mm diameter tool was used to drill the holes and cavities. On one of the plates, microchannels with a width of 0.4 mm and a depth of 0.2 mm were patterned, along with 6 mm diameter through-holes located at the ends of the channels. The second PMMA plate featured 6 mm diameter, 2 mm deep cavities positioned to align with the holes on the first plate. The patterned PMMA plates were then exposed to chloroform vapor for 3 minutes to locally soften the surfaces, enabling temporary adhesion. Subsequently, the plates were thermally bonded under a hot press by applying a force of 1000 N at 72°C for 30 minutes.

Although the method used is suitable for prototyping, the chip must be compatible with injection molding for high-volume manufacturing.[20] Considering the channel dimensions in the second-generation design, it was assessed that the design could also be fabricated using injection molding to support scalability. To evaluate this, injection molding trials were

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conducted at the Micro Manufacturing Laboratory. During the trials, an insert mold featuring channels with widths of 0.2 mm, 0.5 mm, and 1.0 mm (each with a height of 0.2 mm, resulting in aspect ratios of 1, 0.4, and 0.2, respectively) was produced using a stereolithography (SLA) 3D printer (Form 3, FormLabs, Somerville, MA, USA) (Figure 2C). PMMA pellets were injected into the mold using a plunger-type injection molder (IM_2500_30_300, MSE Technology, Istanbul, Türkiye) at a barrel temperature of 230 °C. The results confirmed that the smallest channels (0.2 mm wide with an aspect ratio of 1.0) could be successfully replicated using this method, demonstrating the moldability of the features on the microfluidic chips.

Preparation of Fast Antibiotic Susceptibility Test-Integrated Microfluidic Chips: Colorimetric assays containing anthocyanins were prepared in solution form with minor modifications of the reported studies.[21,24] In brief, the first step in preparing the test was sterilizing the basic components — 10 g/L peptone, 1 g/L meat extract, and 75 g/L salt — in an autoclave at 121 °C for 15 minutes. The second step was to adjust the red cabbage extract solution to pH 8.0 with 1 M NaOH solution. After filter sterilization, the solution was added to the test medium at a ratio of 1:1.

Firstly, carbapenem/colistin solution prepared by serial dilution is added to the wells of the microfluidic chips as positive control, negative control, 1-2-4-8-16 ppm doses. Tests for carbapenem/colistin-resistant bacteria have been developed in accordance with the European Committee on Antimicrobial Susceptibility Testing (EUCAST) 2026 guidelines. For susceptible strains of *Acinetobacter baumannii*, *Klebsiella pneumoniae*, and *Escherichia coli*, these concentrations were determined based on the concentration ranges corresponding to the antibiotic MIC cutoff values specified in the EUCAST 2026 guidelines. Bacteria surviving in wells containing antibiotics at these concentrations are considered resistant strains. This design provides a rapid testing platform for detecting carbapenem/colistin-resistant bacteria. [25] The antibiotic volumes added in this step were standardized to 5 µL. They were dried at 37°C in a static, closed enviroment in an incubator without shaking.

The test solution containing anthocyanin and the bacterial suspension to be tested for resistance profile were mixed in a 1:1 ratio. Antibiotic-loaded microfluidic chips were also dried in an incubator and then dispensed through the central inlet hole so that each well contained 100 µL of anthocyanin-containing test medium. 1200 µL (for 12-wells chip) and 600 µL (for 6-wells chip) of test solution were added to the wells. 200 µL and 300 µL of test media were tested and did not provide the desired yield. The microfluidic chips were incubated for four hours at 37 °C in a stationary medium (without shaking) after the inoculation. The channels were filled

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with mineral oil and put inside a humidified Petri dish to avoid the test solution from entering them or returning to the central intake hole, as well as minimize evaporation and possible cross-contamination. Throughout the experiment, this setup eliminated desiccation and maintained a consistent reaction volume. The color change in the wells is recorded over time.

Digital Image Processing: For digital image processing, microfluidics are placed on a white background and photographed. The captured images are saved in JPEG format. ImageJ software (National Institutes of Health) was used to analyze color changes in the microfluidic wells. ImageJ software was used to calculate the RGB (red, green, blue) analysis averages and the Euclidean distance (Delta E) in the wells. To quantify color change, all pixels in the images were divided into red, green, and blue components, and the mean values of the R, G, and B channels were calculated. In color image processing, the Delta E formula, derived from the CIE 1976 Lab color difference formula, was applied to compare initial and incubation color changes. The ΔE formula is based on measuring color differences between two images.[26,27]

$$\Delta E = [(\Delta L)^2 + (\Delta a)^2 + (\Delta b)^2]^{½} \qquad (1)$$

L*, a*, and b* in the formula represent the dimensions of the CIE Lab color space. The a-axis ranges from red (+a) to green (-a); the b-axis ranges from yellow (+b) to blue (-b); and the L-axis ranges from black (0) to white (100). According to these parameters, the color difference is low in similar images and increases in different images.[28,29] The ΔE value is a numerical indicator of the limits at which the human eye can distinguish between two colors. Thus, the color difference in the developed colorimetric test provides a more accurate indication of the presence of bacteria.

Development of a Smartphone Application: A custom smartphone application with image processing functionality was developed using Python. The application has the ability to run from Android 4 to Android 10. The mobile application does not require an Ethernet connection to access the camera and files on the device. The main menu consists of a "Please click in the correct order" button to launch the menu. This interface provides instructions for capture the image and analysis steps. After photographing the microfluidic chips, the Red Green Blue (RGB) values of each wells of microfluidic chip image are calculated. These values are used in the Euclidean distance formula. After analysis, all RGB values, the Euclidean distance result, and the final test result are displayed at the bottom of the screen. The Euclidean distance (ED) formula (Equation 2) is shown below.[30,31]

$$ED^2=(R_2–R_1)^2+(G_2–G_1)^2+(B_2–B_1)^2 \qquad (2)$$

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(2) For values of 25 and above, the screen displays positive, confirming the presence of antibiotic resistant bacteria. Based on repeated experiments with susceptible and resistant standard strains, we defined a threshold value for the Euclidean distance. The Euclidean distance (ED) values for the datasets illustrated in Figures 5, 6, and 7 are provided in the supplementary material for reference. To obtain reliable and highly accurate results the camera should be held in the same position as much as possible to avoid focusing on the background or other objects. This is not always possible. Therefore, a smartphone platform has been developed to standardize the distance of the camera from the chips and to achieve an even lighting environment. Thanks to the platform, reliable results can be obtained.

Versatile 3D-Printed Smartphone Platform Design: A smartphone platform was designed for imaging the chips. This design is a more compact version of our previous platform. [32] The platform consists of two components: a drawer for holding the chips and a stand for the smartphone. The drawer includes a slot to position the chips and prevent movement. The stand features an opening aligned with the smartphone camera. Inside the stand, a battery and a white LED light are integrated. The system is controlled via an on/off switch. The platform was fabricated using an Ultimaker S3 3D printer with black tough poly(lactic acid) (PLA) at 20% infill. Figure 9A presents an overview of the design, which has been modified in this study to specifically fit the Xiaomi Redmi 10S model by adjusting the slot in the drawer and the camera opening.

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Author Contributions

C.C.Y. ran all experiments as the first author. The project was conveyed and designed by I.O. as a correspond author. N.I., P.S., M.A.A., N.Y.D., M.D., B.C., E.Y., G.C.S., Y.K.G., E.Y. contributed to the experiments. N.I., P.S., M.A.A., N.Y.D., M.D., B.C., E.Y., G.C.S., Y.K.G., E.Y. and I.O. supervised all experiments. All authors wrote the manuscript.

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Data Availability Statement

The data supporting this article have been included as part of the Supplementary Information.