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"Optimization and applications of the Micro Biological Survey method MBS"

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Abstract: Ottimizzazione e applicazioni del metodo *Micro Biological Survey* MBS

Le analisi microbiologiche tradizionali, impiegate nel controllo della qualità e della sicurezza degli alimenti, nel monitoraggio ambientale e in clinica, sono state sviluppate alla fine del XIX secolo. Nonostante i notevoli progressi tecnologici e l'introduzione nell'arco degli ultimi dieci anni di una innumerevole serie di kit commerciali per la rilevazione e la quantificazione dei microrganismi, i metodi culturali classici sono ancora i più utilizzati, principalmente per la relativa semplicità, tecnica e concettuale, e per la loro approvazione da parte di organismi internazionali (ISO, FDA, USEPA, EU). Tali metodi richiedono tuttavia un notevole investimento in termini di tempo, costi e forza lavoro e presentano numerosi svantaggi, per citarne alcuni: un elevata soggettività nella lettura dei risultati, la possibile interferenza delle matrici biologiche analizzate, e soprattutto l'elevato tempo necessario per ottenere risultati definitivi (da 2 a 7 giorni), dipendente dall'abilità dei microrganismi di moltiplicarsi fino a formare colonie visibili ad occhio nudo. Tali problematiche hanno portato allo sviluppo e al raffinamento di metodi microbiologici alternativi. Tali metodi sono molto più rapidi e agevoli dei rispettivi metodi di riferimento, e portano ad un sostanziale miglioramento riducendo le fasi di preparazione e manipolazione dei campioni, accelerando i tempi di risposta e riducendo i costi. Tali metodi spesso includono qualche forma di automazione e i dati possono venire raccolti elettronicamente riducendo la soggettività d'interpretazione dei risultati e l'errore umano. L'obiettivo finale è quello di garantire risultati più sensibili, accurati, precisi e riproducibili rispetto a quelli ottenuti con le metodiche culturali tradizionali. I metodi microbiologici rapidi alternativi includono generalmente metodi immunologici e molecolari. L'affidabilità variabile, i costi elevati e la necessità di laboratori altamente specializzati di alcuni di questi metodi ne hanno tuttavia finora limitato l'adozione.

In tale contesto, il metodo *Micro Biological Survey* (MBS), un metodo alternativo per analisi microbiologiche, è stato sviluppato dall'Università degli Studi "Roma Tre", con l'obiettivo di fornire risultati affidabili diminuendo i tempi di analisi, facilitando le procedure analitiche e l'interpretazione dei dati, aumentando la sensibilità, limitando i costi e permettendo l'esecuzione di analisi anche in assenza di un laboratorio. Il metodo MBS è un sistema colorimetrico per la rilevazione e quantificazione dei microorganismi eventualmente presenti negli alimenti, nelle acque e nei campioni ambientali. A dispetto dei metodi tradizionali, che misurano la capacità delle cellule di moltiplicarsi fino a formare colonie visibili, il metodo MBS misura l'attività catalitica degli enzimi redox nei *pathway* metabolici primari batterici permettendo di stabilire una corrispondenza certa tra attività enzimatica misurata e la concentrazione batterica nel campione analizzato. Il tempo

necessario per il cambiamento di colore è inversamente proporzionale al logaritmo della concentrazione batterica: come una reazione enzimatica, più elevato è il numero di batteri più veloce è il cambiamento di colore. L'analisi MBS si basa sull'utilizzo di fiale di reazione sterili e pronte all'uso che contengono il reagente specifico per l'analisi da condurre. I campioni possono essere inoculati senza alcun trattamento preliminare. Le analisi possono essere eseguite da personale non specializzato ovunque siano necessarie, senza l'uso di strumentazione particolare a parte un semplice termostato. Questo metodo è stato inizialmente concepito per l'analisi degli alimenti e si è dimostrato un sistema affidabile e rapido per valutare la qualità microbiologica di differenti campioni alimentari. Ulteriori studi hanno dimostrato che la semplicità analitica, la ridotta manualità e l'automazione influiscono positivamente anche sui risultati, che mostrano una maggiore riproducibilità e ripetibilità rispetto ai metodi tradizionali. Il metodo MBS rappresenta una promettente alternativa sia ai metodi tradizionali sia ai metodi alternativi finora sviluppati. Le sue caratteristiche lo rendono utile in molti contesti e sono oggetto di questa tesi. In particolare la mia ricerca è stata finalizzata allo studio di applicazioni alternative del metodo MBS. Il primo obiettivo è stato verificare l'efficacia del metodo MBS come metodo alternativo per l'analisi microbiologica dell'acqua potabile, al fine di sviluppare un nuovo *Point of use* test per la valutazione della qualità dell'acqua nei Paesi in via di sviluppo. Il secondo obiettivo è stato di esaminare la possibilità di applicare il metodo MBS in ambito clinico ed in particolare per la rilevazione e quantificazione dei batteri nelle urine e per una valutazione preliminare della loro suscettibilità/resistenza agli antibiotici, al fine di sviluppare un nuovo test al *Point of care* (POCT) per la diagnosi e la gestione delle infezioni delle vie urinarie (IVU). In entrambi i casi la necessità di ottenere risultati accurati e affidabili, in tempi brevi e con una procedura semplice e difficilmente incline ad errori, era una questione centrale per facilitare le analisi e permettere decisioni e azioni correttive rapide.

Nell'ambito dell'analisi dell'acqua potabile ho verificato la possibilità di utilizzare il metodo MBS come metodo alternativo per l'analisi microbiologica dell'acqua dimostrando la sua sensibilità, specificità, linearità e accuratezza. Ulteriori esperimenti sono stati condotti per dimostrare che la determinazione dei coliformi totali in 1 ml di acqua, invece dei 100ml richiesti dalle normative correnti, può essere efficace per dare una valutazione generale della qualità delle acque, specialmente nei Paesi in via di sviluppo, in assenza di strutture e strumentazioni adeguate. Il metodo MBS è stato quindi utilizzato in campo per monitorare la qualità delle acque nella città di Douala in Camerun. Sessantuno siti sono stati selezionati tra 20 quartieri del comune V di Douala. In particolare sono stati presi in considerazione pozzi tradizionali ed incamiciati, utilizzati come fonte di acqua potabile dalla popolazione locale. I dati sono stati collezionati in tre fasi differenti in modo da valutare l'effetto delle variazione meteorologiche inter-annuali sulle caratteristiche delle

falde acquifere. Dai risultati ottenuti è stata osservata una netta correlazione tra la tipologia di pozzo e il livello di contaminazione: il 70% dei pozzi tradizionali è risultato almeno una volta contaminato da coliformi con un elevato livello medio di contaminazione; al contrario il 100% dei pozzi incamiciati è risultato esente da contaminazione dimostrando la sicurezza di questa tipologia di fonte e l'importanza del suo utilizzo in zone rurali e non. Il metodo MBS si è dimostrato un metodo valido ed affidabile per valutare la qualità microbiologica delle acque. La semplicità di esecuzione ed interpretazione dei risultati rendono possibile il suo utilizzo anche in aree rurali da personale locale non specializzato e in assenza di un laboratorio microbiologico.

Per quanta riguarda l'applicazione del metodo MBS come test al *Point of care* per la diagnosi e la gestione delle infezioni delle vie urinarie, la prima fase della ricerca ha riguardato lo sviluppo e validazione preliminare di reattivi MBS specifici per la rilevazione e quantificazione dei batteri nelle urine e una valutazione preliminare della loro suscettibilità/resistenza agli antibiotici. Tali reattivi sono stati testati in uno studio clinico preliminare in collaborazione con l'Azienda ospedaliera Sant'Andrea di Roma che ha evidenziato il grande potenziale del metodo MBS come strumento diagnostico per una rapida e accurata rilevazione dei batteri responsabili di IVU. Uno studio comparativo tra i risultati ottenuti con il metodo MBS e i risultati delle urino colture, eseguite presso il laboratorio microbiologico ospedaliero, è stato condotto utilizzando la *Receiver Operating Characteristics (ROC) analysis*. I risultati hanno dimostrato che il POCT MBS è stato in grado di rilevare la presenza di una concentrazione significativa di batteri nelle urine e quindi diagnosticare una IVU in solo 5 ore (*Area Under the Curve* = 0,93). Inoltre il POCT MBS ha dimostrato una maggiore accuratezza (90,2%), sensibilità (91,2%) e specificità (89,9%) rispetto agli stick urinari comunemente utilizzati per un'indicazione presuntiva di IVU. In un tempo relativamente breve rispetto ai metodi tradizionali il metodo MBS è stato in grado di fornire un accurata indicazione di IVU e una valutazione preliminare della suscettibilità/resistenza agli antibiotici degli agenti infettivi, assicurando una pronta diagnosi e guidando la scelta degli antibiotici prima del classico metodo dell'antibiogramma. Successivamente differenti aspetti legati alla specifica composizione del reattivo, alla procedura analitica e al processo di produzione, sono stati ottimizzati al fine di migliorare ulteriormente le prestazioni del metodo e rispondere ai requisiti essenziali necessari per l'introduzione sul mercato.

Nell'insieme i risultati hanno dimostrato che il metodo MBS è una valida alternativa ai metodi microbiologici tradizionali, sia in campo che in laboratorio. I suoi solidi principi lo rendono estremamente flessibile e adattabile a necessità completamente diverse e permettono di ottenere soluzioni semplificate, rapide e affidabili con un impatto significativo nel campo sociale e nella tutela della salute.

Abstract: Optimization and applications of the Micro Biological Survey method MBS

Traditional microbiological analyses used in food safety, environmental monitoring and clinical diagnosis, were developed at the end of XIX century. Despite advances in technology and the introduction over the past decade of a plethora of commercial assays and kits for the detection and enumeration of a wide range of microorganisms, traditional culture based methods are still the most used, thanks to their relatively technical and scientific simplicity and their acceptance by regulatory agencies (ISO, FDA, USEPA, EU). These methods are however labor intensive, time consuming and present several shortfalls, such as subjectivity in the interpretation of results, the possible interference of matrices and, above all, the prolonged time (from 2 to 7 days) needed to give definitive results, depending on the ability of the organisms to multiply into visible colonies. These reasons have led to the development and refinement of alternative microbiological methods of analysis. These alternative methods are quicker and easier to perform than the corresponding reference methods; they minimize manipulation, provide results in less time, and reduce costs. They also normally involve some form of automation, and often capture data electronically. In particular alternative microbiological method technologies aim to provide more sensitive, accurate, precise, and reproducible test results when compared with conventional, growth-based methods. Rapid methods generally include immuno-based and DNA-based assays. However the variable reliability, cost, novelty and the need of highly equipped laboratories of some of these methods still limit their adoption.

In this context, an alternative method called Micro Biological Survey (MBS) has been developed by "Roma Tre" University in order to meet the need to provide reliable results thus diminishing the time of analysis, facilitating procedures and interpretation of data, increasing the detection sensitivity, limiting costs and allowing analysis also in the absence of a laboratory. The MBS method is a colorimetric system for detection and selective counting of bacteria in food, water and environmental samples. In contrast with traditional methods that measure the capability of cells to grow in discrete colonies, the MBS method measures the catalytic activity of redox enzymes in the main metabolic pathways of bacteria allowing an unequivocal correlation between enzymatic activity and bacterial concentration in the samples. The time required for a color change is inversely related to the logarithm of bacterial concentration in the sample: like an enzymatic reaction, the greater the number of bacteria, the faster the color change. The MBS analysis is performed in disposable ready to use reaction vials that contain the specific reagent for the analysis to perform. Samples can be inoculated without any preliminary treatment. Analyses can be carried out by

untrained personnel and anywhere they are necessary, without the need for any instrumentation other than a thermostatic device. The method was initially conceived for food analysis and it has proven to be a reliable system to evaluate microbiological quality and assess rapidly contamination of different food samples. Further studies have demonstrated that the simple analytical procedure, the reduced labor and automation, positively affect also the analytical performance of the MBS method that displays greater reproducibility and repeatability compared to traditional methods.

The MBS method represents a promising and attractive alternative both to traditional and alternative methods until now developed. Its features make it useful in many contexts and are the subject of this thesis. In particular my research was aimed to study alternative applications of the MBS method. The first aim was to verify the effectiveness of the MBS method as an alternative method for microbiological analysis of drinking water, with the purpose to develop a new point of use test to assess water quality in developing countries. The second aim was to investigate the possibility to use the MBS method in clinical settings and in particular for the detection of bacteria in urine and a preliminary evaluation of their susceptibility/resistance to antibiotics, with the final goal to develop a new Point of care test (POCT) for diagnosis and management of Urinary Tract Infections (UTIs). In both cases the need to obtain rapid, accurate, reliable results in a simple, errorless approach, was a central issue to facilitate investigations, allow fast critical decisions and corrective actions.

In the context of the analysis of potable water I investigated the possibility to apply the MBS method as an alternative method for drinking water analysis demonstrating its sensitivity, specificity, linearity and accuracy. Further experiments were carried to demonstrate that the simple evaluation of total coliforms in 1ml of water samples, instead of 100ml as required by law, could be effective to roughly assess water quality, particularly in developing countries, in the absence of specific facilities and instrumentations. I therefore applied the MBS method on field and in particular in the evaluation of the microbiological quality of water in Douala, Cameroon. Sixty one water points were selected within 20 quarters of the Douala V City council. The water points were divided into dug wells and drilled wells and all of them were used as drinking water sources by the resident population. Data were collected in three different phases in order to study the effects of inter-annual meteorological variation of rainfall and temperature on groundwater characteristics. A strict correlation between the well type and the level of contamination was observed: 70% of the examined dug wells resulted at least once contaminated by total coliforms with a high average contamination; on the other hand, 100% of drilled wells resulted non-contaminated, underlining the safeness of these water sources and the importance of their use in both rural and city areas. The MBS method has therefore proven to be a valid and accurate method to frequently evaluate the

microbiological quality of many water sources. Its simple procedure and interpretation of results can be suitable for use in rural areas, by local personnel, operating without a microbiological laboratory.

Concerning the use of the MBS method as a Point of care test for diagnosis and management of Urinary Tract Infections the first step was the development and preliminary in vitro validation of new MBS reagents for the detection of bacteria in urine and for the evaluation of their susceptibility/resistance to a panel of antibiotics. These were tested in a preliminary clinical study, performed in collaboration with the "Azienda ospedaliera Sant'Andrea" of Rome, that demonstrated the great potential of the MBS POCT as a diagnostic tool for a rapid and accurate detection of bacteria causing UTIs. A comparative analysis between the results obtained with the MBS method and results of urine cultures, conducted by the hospital laboratory, was performed using the Receiver Operating Characteristics (ROC) analysis. Results demonstrated that the MBS POCT was able to reveal the presence of a significant bacterial load in urine, hence diagnose a clinical UTI, in only 5 hours (Area Under the Curve=0,93). More importantly, the MBS POCT showed much higher accuracy (90.2%), sensitivity (91.2%) and specificity (89,8%) compared to urine dipsticks, widely used for a presumptive diagnosis of UTI. In a relatively short time compared to standard methods, the MBS method was able to give an accurate indication of UTI and a preliminary evaluation of the antibiotic susceptibility of the infecting bacteria, ensuring a prompt diagnosis and guiding the antibiotic choice long before the conventional antibiotic susceptibility test is performed. Different issues linked to the specific composition of the reagent, the operating procedures and the manufacturing process were also optimized in order to further improve the overall performance of the MBS POCT and meet the essential premarketing requirements mandatory for all in vitro diagnostic devices.

Together the results obtained demonstrate that the MBS method is an effective, reliable and precise alternative to traditional microbiological testing, proven in laboratory settings and on field trials. It has also been demonstrated that the solid principles of the MBS method make it extremely flexible and adaptable to completely different needs, allowing simplified, rapid, reliable solutions and achieving health-effective and productive results.

1. INTRODUCTION

Detection, quantification and characterization of bacteria are critical issues in many areas, including public health, clinical diagnosis, food safety and environmental monitoring.

Despite advances in technology and the introduction over the past decade of a plethora of commercial assays and kits for the detection and enumeration of a wide range of microorganisms, traditional culture based methods are still the most popular and widely used. Conventional culture methods are the most common category of methods being used by laboratories thanks to their relatively technical and scientific simplicity, the low consumable and equipment costs and their acceptance by regulatory agencies.

Culture test methods used to detect and quantify bacteria are based on the ability of mircoorganisms in a sample to proliferate in or on a specified growth medium, under specified growth conditions. A variety of culture media have been designed to maximize the recovery of different microbial species and are referred by international organizations as the gold standards for detection and quantification of bacteria. Culture dependent methods for enumerating bacterial numbers are known to be biased since bacteria can only be cultivated if their metabolic and physiological requirements can be reproduced in vitro. Microorganisms that will not grow in the selected growth conditions (growth media, temperature) or microorganisms that have lost the ability to reproduce will not be detected. Moreover, although not lacking in sensitivity, these methods can be laborious and depend on the growth of the microorganisms in different culture media (pre-enrichment, selective enrichment, selective plating, identification), which may require several days before results are known [1].

In the past decade, the focus of many researchers has been the study and implementation of improved methods for the isolation, early detection, characterization, and enumeration of microorganisms and their products. In common language, this translates to better methods, automated methods, miniaturized methods, and methods that require less time or expense.

The methods range from simple dip-stick-type tests to very complex automated systems that perform a variety of tests using a variety of techniques. The prices for these methods vary greatly, from a few dollars to hundreds of thousands of dollars. Although these methods are called rapid microbiological methods, many have their roots in other sciences, e.g., chemistry, molecular biology, biochemistry, immunology, immunochemistry, molecular electronics, and computer-aided imaging [2]. These methods can be grouped into three categories: growth-based methods, direct measurement, and cell component analysis. Growth-based methods detect a signal after a short incubation period in liquid or on solid media; examples include detection of CO_2 production by

colorimetric methods or a change in head space pressure and detection of adenosine triphosphate (ATP) by bioluminescence [3].

Direct measurement methods can detect cell viability without requiring growth of the microorganism. One example of a direct measurement method combines fluorescent labeling and laser scanning cytometry to enumerate organisms. Fluorescence-based methods can also be used to enumerate bacteria. In the food and biotechnology industries, for instance, the automated counting of pure cultures by flow cytometry is well established. However, most bacteria are optically too similar to resolve from each other or from debris using flow cytometry, without artificially modifying the target bacteria using fluorescent labeling techniques such as fluorescent antibodies or fluorescent dyes. Differences in bacterial cell size, coaggregation of bacteria and the presence of different contaminating matrices (e.g. mud, food) can also make meaningful counting difficult, if not problematic, by interference with direct, or fluorescence, microscopy [4].

Rapid enumeration of bacteria can also be achieved by using a variety of molecular approaches. Molecular methods have been developed to increase the rapidity of analysis. They are able to achieve a high degree of sensitivity and specificity without the need for a complex cultivation and additional confirmation steps. These methods offer taxonomic information at different levels, such as classes, genera, species or subspecies. Consequently, some of these methods permit the detection of specific culturable and/or non-culturable bacteria within hours, instead of the days required with the cultivation-based methods [5].

Immunological methods are based on the specific recognition between antibodies and antigens and the high affinity that is characteristic of this recognition reaction. Depending on the taxonomic level of the targeted antigens, immunological methods permit detection of antigens at family, genus, species or serotype levels [6,7].

Most of the nucleic acid methods use molecular hybridization properties, which involve the complementary sequence recognition between a nucleic probe and a nucleic target. A hybridization reaction can be realized between a nucleic DNA probe and a chromosomic DNA sequence (DNA–DNA hybridization) or an rRNA or tRNA sequence (DNA–RNA hybridization). Specificity, here, depends on the phylogenetic degree of conservation of the target within the taxonomic target group. The more frequently used nucleic-acid-based methods are the polymerase chain reaction (PCR) and the in situ hybridization (ISH) methods [8]. PCR is designed to facilitate bacterial identification at any level of specificity: strain, species or genus. In recent years, real-time PCR methods have been developed and described for the rapid detection and identification of several bacterial strains. Real-time PCR is a promising tool for distinguishing specific sequences from a complex mixture of DNA and therefore is useful for determining the presence and quantity of pathogen-specific or other

unique sequences within a sample. Real-time PCR facilitates a rapid detection of low amounts of bacterial DNA accelerating therapeutic decisions and enabling an earlier adequate antibiotic treatment [9]. Primers with broad interspecies specificity have been designed to amplify 16S rDNA by PCR and have been used to determine bacterial numbers in complex communities [10].

In situ hybridization techniques use oligonucleotide probes to detect complementary nucleic acids sequences. This method exploits the ability of nucleic acids to anneal to one another in a very specific complementary way to form hybrids. The probes are specific because they are built from, and are complementary to, selected nucleic acids sequences which are unique to a given microorganism, species or group. The probes can target either DNA or RNA molecules. Sequence comparisons make it possible to define targeted regions which are perfectly conserved within different taxonomic levels and consequently specific to these levels, from domains to subspecies. Probes specific to rRNA (mainly 16S and 23S rRNA sequences) have become the standard tools for organism identification. Several oligonucleotide probes are commercially available and the choice of whether to use them or design new, specific ones depends largely on the application [11]. Current work on rRNA in situ hybridization uses fluorescent-labeled nucleotide probes almost exclusively to detect hybridization (FISH). The popularity of the FISH technique is due to its advantages over radioactive labeling, which include sensitivity, speed of visualization of single cells (by means of microscopy or cytometrical devices), stability of the hybridization products, safety, diminished detection time, multiple labels (multiple colors) and ease of use. Depending on the concentration of targeted cells in the sample and to increase resolution, FISH detection can be performed by means of flow or solid-phase cytometry. Flow cytometry enables quantification of the fluorescence intensities for each target-probe hybrid [12].

All analytical methods need to be validated prior to their introduction into routine use [13]. Validation schemes have been established from the major global authorities to assess whether these methods perform at least as well as the corresponding reference methods [14;15,16]. In addition, a European and International Standard, EN ISO 16140, has been developed to provide a common reference protocol for the validation of alternative methods, as well as to determine general principles for their possible subsequent certification [17].

1.1 Conventional and alternative microbiological methods in water analysis

Water intended for human consumption is required to be quality tested for a wide variety of contaminants, including potentially harmful microorganisms. While many water quality parameters such as pH, chlorine and turbidity can be measured in near real-time by on-line measurement instrumentation, microbiological testing presents a unique challenge.

1.1.1 Standard methods for drinking water analysis

Various standard and guideline values have been introduced over the years. Many of these have become legally enforceable, while others have been recommended by appropriate bodies and trade associations [18]. Most waterborne diseases are related to fecal pollution of water sources, therefore water microbiological control is largely based on the need to identify indicators of fecal pollution such as coliforms and *Escherichia coli (E. coli)*, but the use of enterococci and *Clostridium perfringens* is increasing [19]. The two major international bodies, the United States Environmental Protection Agency (USEPA), and the European Union (EU) both include *E. coli* as a mandatory microbial indicator, and the USEPA regulates for total coliforms, via the Total Coliform Rule. Most drinking water guidelines also refer to the use of total estimates of bacterial numbers in water. This measure is generally called 'total heterotrophic plate count' (HPC) or 'standard plate count bacteria', and is considered to represent the general cleanliness of drinking water [20;21].

Currently, three primary techniques for the routine detection and enumeration of indicator and opportunistic bacteria currently are used. They are the multiple tube fermentation (MTF), membrane filtration (MF), and plate count techniques. All methods use media designed to select for the growth and identification of specific organisms. Generally, each method also includes inhibitors, restrictive temperatures, and/or limited nutrients to restrict growth of unwanted species [22].

Heterotrophic plate count standard methods. Heterotrophic plate counts (HPC) or total viable counts (TVC) are enumerations of the general population of heterotrophic bacteria present in water supplies. The enumerations may represent bacteria whose natural habitat is the water environment, or those that have originated from soil or vegetation. HPC measurements are used: to indicate the effectiveness of water treatment processes, thus as an indirect indication of pathogen removal; as a measure of numbers of regrowth organisms that may or may not have sanitary significance; to monitor the performance of filtration or disinfection processes. HPC testing may be used in investigating aesthetic quality, and it is used by some authorities as a marker for some of the underlying causes of some aesthetic problems [23].

Heterotrophic plate count standard methods provide a standardized means of determining the density of aerobic and facultative anaerobic heterotrophic bacteria in water. Standard methods outline three techniques for determining a standard plate count: pour plate, spread plate, and membrane filtration (MF) [24]. The standard plate count (also called the pour plate method) involves pouring 1 ml of the sample into a plate and pouring an agar solution over the top. The sample is allowed to incubate after the agar has set. This is an effective method for quantifying organisms, and also allows more space for the colonies to grow. The spread plate method differs from the pour plate method in that agar plates are poured and set aside to solidify before inoculation with samples. The spread plate method involves spreading 0.1 ml of the sample over a premade agar plate and allowing it to incubate. This method gives more 3-dimensional colonies than the pour plate method that are much easier to identify and count. The pour plate or spread plate methods are typically used for process water and drinking water [25].

The membrane filtration technique is best suited to test large volumes of low-turbidity, low-count (less than 1 to 10 Colonies Forming Units (CFU) /ml) water. Membrane filtration involves filtering a particular volume of a sample to get a higher concentration of bacteria, then rinsing the filter with a sterile buffer before placing it on an agar plate. This is generally the only method used to test sterile water. It can also be used to test drinking water depending on the application. This method provides good colony morphology, making it a good method for identifying the bacteria present in a sample [26].

In all of these standard test methods there is a significant amount of time and labor required, as well as a high chance of manual error; the processes are highly operator-dependent, and as a result the CFU can vary widely when calculated by different operators and different labs. Also the costs associated with these methods can be high, not only the labor costs but also the cost of materials necessary to conduct the testing. It can take between 48-72 hours to get results, and if the sample is improperly diluted so the results are too high or not enough to count, the sample must be retested. Once testing is completed, the process of counting the number of bacterial colonies is complicated and time-consuming, and it is easy to make mistakes during this process.

Total coliform and *Escherichia coli* count standard methods. Coliform bacteria belong to the family Enterobacteriaceae but are further defined by functional characteristics rather than systematic genus and species [27]. The World Health Organization has defined coliforms as any rod-shaped, non-spore-forming, gram-negative bacteria capable of growth in the presence of bile salts or other surface-active agents. Continuing, the definition states that coliforms are cytochrome-oxidase negative and able to ferment lactose at either 35 or 37 °C with the production of acid, gas, and aldehyde within 24 to 48 hours [28]. The total coliform group is the most inclusive indicator

classification, and contamination indicated by the presence of total coliforms is indicative of inadequate disinfection of drinking water also if it does not always correlate properly with the presence of fecal pollution. Monitoring for total coliforms at the treatment plant and in the distribution and storage system is carried out to provide information on the adequacy of drinking water treatment and on the microbial condition of the distribution system. Their presence in groundwater indicates that it is vulnerable to contamination from the surrounding environment so their detection in the water leaving the well should trigger further actions [28;29]. *Escherichia coli* is a thermophilic coliform that produces indole from tryptophan, but also defined now as coliform able to produce β-glucuronidase (although taxonomically up to 10% of environmental *E. coli* may not). The bacterium's only natural habitat is the large intestine of warm-blooded animals and since *E. coli,* with some exceptions, generally does not survive well outside of the intestinal tract, its presence in environmental samples, food, or water usually indicates recent fecal contamination or poor sanitation practices in food-processing facilities. Monitoring for *E. coli* in treated water at the treatment plant and in the distribution system is carried out to provide information on the adequacy of drinking water treatment and on the microbial condition of the distribution system [30].

The US Environmental Protection Agency (EPA) and AFNOR have approved several methods for coliforms and *E. coli* detection: the multiple-tube fermentation (MTF) technique and the membrane filter (MF) technique [31].

The MTF test uses a specified number of tubes (based on the expected population in the sample) containing a specific medium and sample water. After incubation, each tube is examined for growth of the target organism(s). The number of tubes showing growth are matched to a statistically determined table of numbers to yield the most probable number (MPN) of organisms in the sample. The number of presumptive *E. coli* is determined by enzymatic testing on samples that give a positive total coliform test [32]. The incorporation of MUGlu into lauryl tryptose broth used as the medium for the multi-tube fermentation (MTF) technique was first proposed for rapid detection and immediate confirmation of *E. coli* in food and water samples. The presence of methylumbelliferone due to the hydrolysis of MUGlu (positive samples) is detected by exposure to long-wave UV light and visualization of blue-white fluorescence [33] .

Many factors may significantly affect coliform bacteria detection by MTF, especially during the presumptive phase. Interference by high numbers of non-coliform bacteria, as well as the inhibitory nature of the media have been identified as factors contributing to underestimates of coliform abundance. The MTF technique lacks precision in qualitative and quantitative terms. The time required to obtain results is higher than with the membrane filter technique that has replaced the MTF technique in many instances for the systematic examination of drinking water. However, this

technique remains useful, especially when the conditions do not allow the use of the membrane filter technique, such as turbid or colored waters. MTF is easy to implement and can be performed by a technician with basic microbiological training, but the method can become very tedious and labor intensive since many dilutions have to be processed for each water sample. However, it is also relatively inexpensive, as it requires unsophisticated laboratory equipment. Nevertheless, this method is extremely time-consuming, requiring 48 h for presumptive results, and necessitates a subculture stage for confirmation which could take up to a further 48 h [34].

The Membrane Filtration (MF) method consists of filtering a water sample on a sterile filter with a 0.45-mm pore size which retains bacteria, incubating this filter on a selective medium and enumerating typical colonies on the filter. If the organisms sought are present, colonies will grow on the membrane filter. Colonies are examined at 10–15X magnification with a stereoscopic microscope and then identified by size, color, shape and sheen. Typical colonies are counted and the number is reported as the number of colonies per 100 ml of sample. The MF technique is fully accepted and approved as a procedure for monitoring drinking water microbial quality in many countries. *E. coli* is determined from total-coliform-positive samples by the means of specific confirmation media. Different commercial agar media are now available for the detection of *E. coli.* They include classical agar media modified with specific chromogenic and/or fluorogenic substrates for the detection of β-Dglucuronidase activity. Many media and incubation conditions for the MF method have been tested for optimal recovery of coliforms from water samples [35].

The predominant concern about MF is its inability to recover stressed or injured coliforms. A number of chemical and physical factors involved in drinking water treatment, including disinfection, can cause sub lethal injury to coliform bacteria, resulting in a damaged cell unable to form a colony on a selective medium. Exposure of bacteria to products like chlorine may result in injury and increased sensitivity to bile salts or to the replacement surface-active agents (sodium desoxycholate or Tergitol 7) contained in some selective media. Other authors have suggested that chlorination affects various functions of coliform bacteria activity, such as catalase enzymatic activity. Metabolically active bacteria produce hydrogen peroxide (H_2O_2), which is usually rapidly degraded by the catalase. Injured coliforms with reduced catalase activity accumulate toxic hydrogen peroxide, to which they are extremely sensitive [36]. The high number of modified media in use is a reflection of the fact that no universal medium currently exists which allows optimal enumeration of various coliform species originating from different environments and present in a wide variety of physiological states. A significant advantage of the MF technique over the MTF method is that with MF, the examination of larger volumes of water is feasible, which leads to greater sensitivity and reliability. MF also offers a quantitative enumeration comparatively to the

semi-quantitative information given by the MTF method. MF is a useful technique for the majority of water quality laboratories as it is a relatively simple method to use. Many samples can be processed in a day with limited laboratory equipment by a technician with basic microbiological training. Nevertheless, since this method is not sufficiently specific, a confirmation stage is needed, which could take a further 24 h after the first incubation period on selective media.

Comprehensively these methods have limitations, such as duration of incubation, antagonistic organisms interference, lack of specificity to the coliform group and a weak level of detection of slow-growing or stressed coliforms. Indeed, depending on the environmental system, only a small portion (0.1–15%) of the total bacterial population can be enumerated by cultivation-based methods. The proportion of non-culturable bacteria may be affected by unfavorable conditions for bacterial growth during culturing or by their entry into viable or active but non-culturable states (VBNC or ABNC) [37].

1.1.2 Alternative methods for drinking water analysis

Conventional microbiological methods display some important limitations linked mainly to the time taken to produce a result and the inability of many methods to recover all of the microorganisms that might be present in a sample. The time taken to produce a result relates to the incubation period required for conventional methods, which rely on agar as a growth medium, or for microorganisms to grow in broth culture. Such methods are relatively slow, and results are only available after an incubation period (somewhere between two to 10 days, depending on the application). A further limitation is culturability and the issue of "viable but non-culturable (VBNC) microorganisms." Many bacteria, although maintaining metabolic activity, are non-culturable due to their physiology, fastidiousness, or mechanisms for adaptation to the environment.

While some adaptations to cultivation media and cultivation conditions have improved these methods, it has become increasingly evident that conventional cultivation-based detection methods do not suffice to describe all the major bacteriological events that occur during drinking water treatment and distribution.

The new European Drinking Water Directive (EU DWD) defines reference methods for the enumeration of microbiological parameters in drinking water. Member states may use alternative methods in cases where they have demonstrated that these methods produce results that are at least as reliable as those produced by the reference methods [38].

Cultivation independent methods for total heterotrophic counts. In the last decade, cultivation independent detection methods, such as adenosine triphosphate (ATP) and flow cytometry total cell count [40;41] have gained foremost interest because these methods are accurate, rapid, quantitative, detect both cultivable and uncultivable microorganisms, and are easy to perform. Using cultivation independent methods, many studies irrefutably demonstrated that the total bacterial cell concentration in water is several orders of magnitudes higher in comparison to heterotrophic plate count (HPC) results [39].

ATP is a parameter that assesses active biomass by quantifying the activated, energy-rich metabolic compounds present in all viable cells. ATP is used as primary energy currency for all bacteria, and therefore it is a parameter suited for the quantification of the active biomass [42]. It has been promoted for its applicability in drinking water and also found a wide application including the analysis of groundwater, assessment of biofilters in water treatment plants, drinking water and biofilms in drinking water distribution systems [43]. An ATP measurement requires 5 minutes per sample, and has additionally the advantages that the equipment is simple to use and relatively sensitive and affordable. However, ATP is not yet widely used in the drinking water industry, due to a lack of knowledge on average ATP concentrations in natural bacteria. Also significant differences can exist between the cellular ATP content of different bacteria cultivated under different conditions or in different physiological states [44].

Flow cytometry (FCM) is a routine method for characterizing cellular biology that has been used since the 1970s. FCM is fast, highly reproducible and it detects all bacteria irrespective of their culturability. It can be used to enumerate the bacteria in a water sample within 20 minutes (inclusive of the required staining time) and it provides a high degree of reproducibility. This method can be used for direct enumeration of the total cell concentrations in water, staining and detection of specific cellular features such as viability, or staining and enumeration of specifically targeted cells with antibodies. A lucrative feature of FCM is that it is fast, accurate and quantitative [45;46;47].

Enzymatic methods for detection and quantification of total coliforms and *E. coli*. The use of microbial enzyme profiles to detect indicator bacteria is an attractive alternative to classical methods. Enzymatic reactions can be group-, genus- or species-specific, depending on the enzyme targeted. Moreover, reactions are rapid and sensitive. Thus, the possibility of detecting and enumerating coliforms through specific enzymatic activities has been under investigation for many years now. Chromogenic and fluorogenic substrates produce color and fluorescence, respectively, upon cleavage by a specific enzyme. In most tests, the detection of total coliforms is based on the β-galactosidase activity. The enzyme substrates used are chromogenic substrates such as ONPG (o-

nitrophenyl-β-D-galactopyranoside), CPRG (chlorophenol red-β-D galactopyranoside), X-GAL (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) or cyclohexenoesculetin-β-D-galactoside. Rapid assays for detection of *E. coli* are based on the hydrolysis of a fluorogenic substrates, 4-methylumbelliferone glucuronide (MUG) by β-glucuronidase, an enzyme found in *E. coli*. The end product is fluorescent and can be easily detected with a long-wave ultraviolet lamp. Several commercial tests were then developed based on the defined substrate technology: Colilert (IDEXX Laboratories, Portland, ME, USA), Colisure (Millipore corporation, Bedford, MA, USA), Coli-Quick (Hach, Loveland, CO, USA). Most of these are available for a presence/absence response and for enumeration by the multi-tube technique. Numerous and very extensive comparisons between these commercial tests and the classical MTF and MF approaches have been performed to enumerate total coliforms and *E. coli* in various types of waters [48;49;50]. The main conclusion of these studies was that these tests were effective for the detection of the coliforms from varied source waters, usually as sensitive as the MTF approach for the detection of *E. coli* and sometimes more sensitive for the detection of total coliforms. A major concern with any assay based on enzyme activity, is the interference that can be caused by the presence of other bacteria. In addition, the use enzyme can be found in numerous organisms. This could lead to the detection of a number of false positive organisms [51;52].

Molecular methods for detection and quantification of total coliforms and *E. coli*. Over the last 10 years, attempts have been made to use immunological methods for the detection of water quality indicators in drinking water. An ELISA using a monoclonal antibody against the enterobacterial common antigen (ECA), a lipopolysaccharide which is linked within the outer membrane of Enterobacteriaceae, was developed and refined. However, since an epitope can be present in more than one antigenic agent, a rigorous specificity testing of the monoclonal antibodies synthesized with closely and distantly related bacterial strains must precede the routine testing of environmental samples. Most likely due to this problem of cross-reactivity with other bacterial strains, the use of immunological methods for the detection of coliforms and *E. coli* has not yet been successful [53;54].

The PCR method has often been described for the detection and identification of microorganisms in foods, soils, sediments and waters. Application of this method to the detection of microorganisms in drinking water is, however, recent [55;56]. Since the total coliform group of organisms is a diverse group containing many genera and excluding some that are closely related, the application of PCR technology to the detection of this group of organisms is difficult. Primers to detect total coliforms in water are mainly based on the *lacZ* gene, encoding the β-galactosidase protein [57]. For the specific detection of *E. coli*, regulatory region (uidR) and the uidA gene encoding the β-D-

glucuronidase (GUD) enzyme are used with success. Many MUG-negative *E. coli* strains, which include the pathogenic E. coli of serotype O157:H7, were detected after PCR amplification of the uidA gene. Despite its sensitivity, it is difficult to use PCR for the quantification of microorganisms. Two techniques have mainly been developed for DNA quantification by PCR: the most-probable-number PCR and the competitive PCR (addition of a DNA competitor for the PCR stage to calibrate the PCR efficiency). However, these techniques have not been applied up to now for the quantification of coliforms in drinking water. Thus, at present, it does not seem possible to quantify coliform density in drinking water using PCR [58].

1.1.3 Point-of-use tests for drinking water analysis

Microbiological analysis carried out with traditional methods based on bacterial growth, and innovative methods based on genetic and/or enzymatic testing, are expensive or time-consuming and, more importantly, require skilled technicians and sophisticated laboratory equipment and/or expensive reagents. Alternative point-of-use methods using portable, disposable and ready to use devices have been developed in order to meet the need to provide rapid, reliable results, facilitating procedures and interpretation of data, ensuring high analytical performance, limiting costs and allowing analysis in the absence of a laboratory. Their use is crucial in many situations and helps to overcome a great number of economic and logistic restrictions.

A new microbiologic indirect indicator test measuring hydrogen sulfide production using paper strips has been developed for screening bacteriological contamination of drinking water in developing countries. Because hydrogen sulfide is easy and inexpensive to measure, this has been suggested as a new indicator in developing countries. However, hydrogen sulfide can be produced via other mechanisms than bacterial metabolism, and so hydrogen sulfide production is, in effect, measuring an indicator (hydrogen sulfide presence of bacteria) of an indicator (bacteria of human health risk). In addition, there is currently no quantitative test for hydrogen sulfide production, although some are currently being investigated [59].

Presence/Absence (P/A) testing is the simplest direct testing method for microbiological indicators that can be used as a point-of-use test in developing countries. Pre-prepared, disposable, sterile plastic bottles pre-filled with media, such as Hach P/A with MUG broth or IDEXX Colilert, both of which test for total coliforms and *E. coli,* are commercially available to perform presence/absence analysis. The procedure involves adding a liquid or powdered media to 100 ml of water and incubating for 24-72 hours at 25-35°C. A color change (for total coliform or fecal coliform bacteria) or UV-fluorescence (for *E. coli)* indicates the presence of bacteria. These tests are certainly simple and applicable also in the presence of minimal equipment. However, at low contamination levels

(<10 CFU/100 ml), they can report false negative results, they are not quantitative, and do not allow analytical flexibility.

The only commercially available quantitative on field test is commercialized by IDEXX and can be performed using a sterile, commercial tray with 97 wells of different sizes for simplified MPN testing. To use the tray the sample is mixed with a media and poured into the top of the tray. The tray is sealed using a sealer, and incubated for 24 hours. Yellow and fluorescent wells of each size are counted and compared to a statistical chart which converts the counts to numerical total coliform and *E. coli* results. This method cannot be used by totally untrained personnel and though the analytical procedure is easy the sensitivity range is small (from 1 max of 2490 CFU/ml). Moreover, Vessels and *QuantyTray* are quite bulky compared to other microbiological kits and also the incubator and *QuantyTray Sealer* are bench space consuming.

In all cases (P/A analyses and quantitative analyses) results can be read by an operator only at the end of the analysis (exactly after 24 hours). Due to the need to detect fluorescence for *E. coli* testing, it is possible to analyze only clear water samples and analyses are not possible for mud containing water, which makes them unsuitable in many settings.

1.2 Conventional and alternative methods for Urinary Tract Infections (UTI) diagnosis and management

Although molecular diagnostic approaches utilizing antigen detection or DNA hybridization and amplification techniques are being applied to the diagnosis of many infections, Urinary Tract Infections (UTI) are still generally diagnosed as they have been for decades. The gold standard for the diagnosis of a urinary tract infection is the detection of the pathogen in the presence of clinical symptoms [60].

The common signs and symptoms of acute uncomplicated lower UTI include: dysuria, frequent and urgent urination, suprapubic pain or tenderness, and possibly hematuria. There is also agreement that the presence of vaginal symptoms (e.g., vaginitis, urethritis) should prompt alternative diagnoses. Clinical manifestations suggestive of pyelonephritis include fever (temperature >38°C), chills, flank pain, costovertebral-angle tenderness, and nausea or vomiting, with or without symptoms of cystitis. Dysuria is also common with urethritis or vaginitis, but cystitis is more likely when symptoms include frequency, urgency, or hematuria; when the onset of symptoms is sudden or severe; and when vaginal irritation and discharge are not present [61;62;63].

1.2.1 Standard methods for UTI diagnosis and management

Diagnosis and management of a urinary tract infection is a multistep process which includes the determination of the concentration of pathogens, and the identification of the responsible bacteria, as well as their susceptibility to various antibiotics, the so called antibiogram. The pathogen is detected and identified by urine culture. This also allows an estimate of the level of the bacteriuria. Many laboratories define 10^5 CFU/ml urine as the threshold. However, this threshold misses many relevant infections. There are therefore other recommendations that recommend the diagnosis of UTI from a count of 10^3 cfu/ml, depending on the types of bacteria detected [64].

Routine bacterial urine cultures. The laboratory diagnosis of urinary tract infection requires quantitative urine culture on standard agar media not only because it helps to document infection, but also because it is necessary for determination of the identity of the infecting microorganism(s) and for antimicrobial susceptibility testing. This is particularly true because of the increased incidence of antimicrobial resistance. The most commonly used criterion for defining significant bacteriuria is the presence of 10^5 CFU per milliliter of urine.

Urine cultures have traditionally been performed using sheep blood agar, a nonselective medium, and a selective medium such as MacConkey agar, cysteine lactose electrolyte-deficient (CLED) agar, or eosin methylene blue (EMB) agar. Also chromogenic media have been reported to be an acceptable alternative to traditional media for the isolation of urinary pathogens and offer another option for diagnostic laboratories [65].

Such assays require repeated culturing of a sample and take over 48 hours in order for bacterial colonies to be grown, counted, and exposed to antibiotics using conventional clinical methods. Since the patient cannot remain untreated during this rather prolonged period before definitive diagnosis is obtained, physicians prescribe broad spectrum antibiotics prior to antibiogram results [66].

Non-culture Methods. Key findings pointing toward UTI include pyuria (which can, however, have other causes), hematuria (a finding helpful mainly in excluding alternative diagnoses such as urethritis and vaginitis), and significant bacteriuria.

Assessment for pyuria and bacteriuria is often performed with the use of commercially available dipsticks that test for leukocyte esterase, an enzyme released by leukocytes, and for nitrites, since some bacteria reduce urinary nitrates to nitrites. The dipstick test is most accurate for predicting UTI when the presence of either leukocyte esterase or nitrite is considered a positive result, with a sensitivity of 75% and a specificity of 82%. These tests have low sensitivity, high specificity, low positive-predictive values, and high negative-predictive values. Taken together, the performance characteristics of these tests make them useful as a way to rule out bacteriuria on the basis of a

negative test result. Indirect dipstick tests are informative, but less sensitive, than microscopic examination of the urine; however none of these tests establish the diagnosis of UTI [67].

Bacteriuria can be detected microscopically using Gram staining of uncentrifuged urine specimens, Gram staining of centrifuged specimens, or direct observation of bacteria in urine specimens. The performance characteristics of the test are not well-defined. The urine Gram stain test has the important advantage of providing immediate information as to the nature of the infecting bacterium or yeast (rarely infectious agents such as microsporidia) and thereby guiding the physician in selecting empiric antimicrobial therapy. However the Gram stain test has the disadvantage of being too labor intensive for it to be practical for most clinical microbiology laboratories. Moreover it is an insensitive test because it does not distinguish between living and dead bacteria [68].

Standard antibiotic susceptibility testing. Determination of bacterial resistance to antimicrobials is an important part of the management of infections in patients. A limited number of methods for antimicrobial susceptibility testing (AST) of medically important microorganisms have survived the maturation of modern diagnostic clinical microbiology.

In many countries, the disk diffusion method is the most commonly used method in clinical laboratories. This test provides the greatest flexibility and cost-effectiveness; however, the test takes at least 24 h and there are limitations in its accuracy. Another is broth dilution testing, which has attained reference standard status to which all other AST methods are currently compared during development, verification, validation, and clinical trials. Several automated systems are now available that provide rapid antimicrobial susceptibility data. The major limitations of these methods include the requirement for relatively large numbers of viable organisms, complicated pre-analytical processing, limited organism spectrum, analytical variability, time to results, and cost [69].

The disk diffusion method. The disk diffusion method of Kirby and Bauer is simple and practical and has been a well standardized method routinely used. The test is performed by applying a standard bacterial inoculum to the surface of a large (150 mm diameter) Mueller-Hinton agar plate. Up to 12 commercially-prepared, fixed concentration, paper antibiotic disks are placed on the inoculated agar surface. Plates are incubated for 16–24 h at 35°C prior to determination of results. Simultaneous growth of the bacteria and diffusion of the antimicrobial compounds occurs. Growth occurs in the presence of an antimicrobial compound when the bacteria reach a critical mass and can overpower the inhibitory effects of the antimicrobial compound. The zones of growth inhibition around each of the antibiotic disks are measured to the nearest millimeter. The diameter of the zone is related to the susceptibility of the isolate and to the diffusion rate of the drug through the agar medium [70]. The zone diameters of each drug are interpreted using the criteria published by the

Clinical and Laboratory Standards Institute (CLSI, formerly the National Committee for Clinical Laboratory Standards or NCCLS). The results of the disk diffusion test are "qualitative," in that a category of susceptibility (i.e. susceptible, intermediate, or resistant) is derived from the test rather than an minimum inhibitory concentration (MIC). The disk diffusion approach for AST has been standardized primarily for commonly encountered, rapidly growing bacterial pathogens and is applicable to neither anaerobes nor fastidious species that demonstrate marked variability in growth rate from strain to strain. The advantages of the disk method are the test simplicity that does not require any special equipment, the provision of categorical results easily interpreted by all clinicians, and flexibility in selection of disks for testing. It is the least costly of all susceptibility methods. The disadvantages of the disk test are the lack of mechanization or automation of the test [71].

Broth dilution methods. Broth dilution methods may be used to determine the minimum concentrations of antibiotics that are required to inhibit or kill microorganisms. Drugs under study are typically tested at 2-fold doubling ($\log_2$) serial dilutions (e.g. 4, 8, 16 μg/ml, and so on), with the lowest concentration of each antibiotic that inhibits visible growth of organisms designated as the minimum inhibitory concentration (MIC). The concentration ranges tested vary with the antimicrobial agent, the pathogen under study, and the infection site. The broth macrodilution approach is both reliable and well standardized and is of particular utility in research studies and in testing of a single antimicrobial agent for 1 bacterial isolate. The principal disadvantages of the macrodilution method are the tedious, manual task of preparing the antibiotic solutions for each test, the possibility of errors in preparation of the antibiotic solutions, and the relatively large amount of reagents and space required for each test [72].

The miniaturization and mechanization of the test by use of small, disposable, plastic "microdilution" trays has led to its widespread use in both clinical and reference laboratories. Standard trays contain 96 wells, each containing a volume of 0.1 ml that allows approximately 12 antibiotics to be tested in a range of 8 two-fold dilutions in a single tray. Microdilution panels are typically prepared using dispensing instruments that aliquot precise volumes of preweighed and diluted antibiotics in broth into the individual wells of trays from large volume vessels. Following incubation, MICs are determined using a manual or automated viewing device for inspection of each of the panel wells for growth [73].

An example of a commercially available manual broth microdilution (BMD) systems is Sensititre (TREK Diagnostic Systems, Cleveland, OH). Examples of automated BMD platforms include the BD Phoenix (Becton Dickinson, Franklin Lakes, NJ), Microscan (Siemens Healthcare Diagnostics, Deerfield, IL), and Vitek (bioMérieux, Marcy l'Etoile, France)[69].

The advantages of the microdilution procedure include the generation of MICs, the reproducibility and convenience of having pre-prepared panels, and the economy of reagents and space that occurs due to the miniaturization of the test. There is also assistance in generating computerized reports if an automated panel reader is used. The main disadvantage of the microdilution method is some inflexibility of drug selections available in standard commercial panels [70].

1.2.2 Alternative methods for UTI diagnosis and management

The standard routine clinical-practice tests currently used to diagnose UTIs regularly misdiagnose infection and antibiotics are often not prescribed when appropriate. Urine cultures followed by classical antibiotic susceptibility testing are not always performed since they delay achievement of results and are laborious and time consuming. This practice has many undesirable consequences, both short term and long term: unsuccessful treatments leading to chronic infections, increased health care costs and increased antibiotic resistance by a growing number of bacterial strains. Given these concerns, it is obvious that a rapid and accurate method of UTI diagnosis and bacterial susceptibility to antibiotics would offer significant health benefits [74].

Alternative methods for UTI diagnosis. Rapid diagnosis methods based on PCR are used to complement conventional culture methods. They have been developed in order to bypass the need for culturing especially with regard to shortening the time to result. Although such PCR assays are fast and very sensitive, they typically require species and strain-specific probes that may or may not be available for a particular organism. Additionally, amplification methods like PCR suffer from contamination problems, complex interpretation of results, as well as high costs [75]. In the diagnosis of UTI, applied real-time PCR methods are presently limited to the detection of single pathogens or the Gram status. A new multiplex real-time PCR test for the detection of 25 common blood stream pathogens (SeptiFastH, Roche Diagnostics GmbH, Penzberg, Germany), introduced recently assures time saving with, however, not very high sensitivity and specificity [76].

Some studies have also hypothesized that bacterial colonization of shed urothelial cells and high levels of urinary ATP (i.e. >50 nmol/l; presumably as a result of a proinflammatory response involving the purinergic system) is a powerful marker of UTI [77].

Another approach is based on the investigation of urine interleukin levels. Active bacterial infection in the lower urinary tract initiates a local inflammatory response including epithelial release of cytokines, acting as neutrophil chemoattractants, acute phase response activators and endogenous pyrogens. In symptomatic UTI, the uroepithelium is triggered to secrete IL-6 and its level to reflect the severity of the infection. Also the investigation of urine interleukin-8 levels was found to be useful for early diagnosis of urinary tract infections [78].

Alternative methods for antibiotic susceptibility testing. Currently, AST is typically accomplished using either classical manual methods or growth-dependent automated systems, such as the Becton, Dickinson Phoenix, the Siemens Micoscan WalkAway, or the bioMérieux Vitek 2, all of which are based on BMD testing. The major limitations of these methods include the requirement for relatively large numbers of viable organisms, complicated preanalytical processing, limited organism spectrum, analytical variability, time to results, and cost. At present, nonphenotypic, mostly nucleic acid-based AST methods, cannot detect all resistance markers, are expensive, and have not been widely adopted [79]. Recent advances have led to the development of genotypic assays suitable for antibiotic susceptibility testing. A universal method for measuring the inhibitory effects of antimicrobial agents on common bacterial pathogens, using universal primers and quantification of DNA copies, has been recently proposed. Presently, the major drawback of this method, as compared with conventional assays, is the lack of automation and costs [80].

Matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS), a powerful tool for the rapid identification of organisms with medical importance, may also prove to be of value as an AST method. Several approaches have been explored, including: documenting the activity of antibiotic-inactivating enzymes; confirming the presence of a PCR product indicative of antimicrobial resistance; observing changes in the protein spectrum of an organism in the presence or absence of an antimicrobial agent that correlate with susceptibility changes [81].

Also Flow cytometry (FC) has been proposed as an alternative AST. It permits observation of changes in the morphology, physiological and metabolic activity, and viability of microorganisms after exposure to antibiotics. Through a process of staining with nucleic acid dyes that do not permeate the cell walls of healthy organisms, the proportion of cells in a dying or dead state (and everything in between) can be rapidly assessed by examining emission spectra after the cells pass individually through a flow channel and when the dye is excited by a laser. Although significant advances in the design and performance of FC instrumentation have occurred, the technology has not yet emerged as a major player in the AST market, although commercial assays have been launched [82].

1.2.3 Point of Care Tests for UTI diagnosis and management

In suspected UTIs, empirical antibiotic treatment is usually started before the laboratory results of urine culture and antibiogram are available because of the long time required for these analyses. This can result in therapeutic failure in case of resistant strains, and ultimately lead to the spread of antibiotic resistance among pathogens responsible for both community and nosocomial acquired infections [83]. The availability of a rapid, user-friendly, accurate and inexpensive test that could

quickly detect a clinically significant bacterial load in urine, would allow physicians to properly decide which patients really need antibiotic treatment, thereby improving therapeutic success and reducing the emergence of antibiotic resistance.

Point-of-care testing (POCT), or bedside testing, is defined as medical testing at or near the site of patient care. These tests offer rapid results, allowing for timely initiation of appropriate therapy, and/or facilitation of linkages to care and referral. Most importantly, POC tests can be simple enough to be used at the primary care level and in remote settings with no laboratory infrastructure [84].

POCT are used in a wide variety of health care settings and encompass a wide variety of procedures and technologies [85]. Rapid biochemical dipstick tests are available and currently used as predictors of bacterial UTI, but must often be correlated with other testing and clinical information. In fact, the dipstick urinalysis can provide false positive and negative results when compared with the gold standard culture method, demonstrating a low sensitivity and positive predictive value [86;87]. Moreover they do not provide information on susceptibility/resistance of bacteria to antibiotics.

Also, classic and alternative antibiotic susceptibility testing methods cannot be performed at the bedside and always require an urine culture, that lengthens the time occurred to obtain results. For these reasons currently no POCT are available for diagnosis and management of urinary infections.

1.3 The Micro Biological Survey Method

The Micro Biological Survey method (MBS) is an alternative, fast and simple colorimetric system for quantitative and qualitative microbiological analyses validated according to ISO 16140 (2003) [17]. The MBS analysis is performed in disposable ready to use reaction vials that contain the specific reagent for the analysis to be performed. The vials' content changes color in times that are inversely proportional to the bacterial concentration in the sample.

In order to allow selective detection and quantification of bacteria and meet nutritional requirements of different microorganisms, MBS reagents contain different classes of compounds:

Nutrient. These include sources of organic carbon, nitrogen, minerals (phosphorus and sulfur) as well as growth factors (amino acids, purines, pyrimidines) vitamins and trace elements (Mg, Fe, Mn) that stimulate bacterial growth.

Selective agents. The selectivity is accomplished in several ways. For example, organisms that can utilize a given sugar are easily screened by making that sugar the only carbon source in the medium. On the other hand, selective inhibition of some types of microorganisms can be achieved by adding

organic substances, dyes, antibiotics, salts or other specific inhibitors which affect the metabolism or enzyme systems of the organisms.

Indicators. Organic substances capable of changing color as a result of variations of the chemical and physical state of the medium.

Reducing agents. Organic substances capable of releasing electrons to the respiratory chain, and that are not easily oxidized by the oxygen dissolved in solution.

Vaseline. Organic substance used to avoid an external input of oxygen into the reaction vial; as highly hydrophobic compared to reagent, it sets at the interface with the air excluding the oxygen input.

1.3.1 Micro Biological Survey method biochemical principles

In contrast with traditional methods that measure the capability of cells to grow in discrete colonies, the MBS method measures the catalytic activity of redox enzymes in the main metabolic pathways of bacteria allowing an unequivocal correlation between enzymatic activity and bacterial concentration in the samples. Detection of bacterial metabolism (enzymatic activity) is possible thanks to the presence of a redox indicator called TMPD (N, N, N ', N'tetrametil-p-phenylene diamine hydrochloride). In its oxidized form, TMPD displays a blue coloration, while it becomes yellow in its reduced form; its potential, of about 250 mV, is very close to the one of cytochromes belonging to the mitochondrial electron transport chain of aerobic and anaerobic bacteria, which goes from100 to 300 mV. For this reason, this indicator can be easily reduced by cytochromes and, at the same time, can be directly oxidized by oxygen, which has a potential of about 820 mV. TMPD is an anphipathic molecule, capable of crossing the cytoplasmatic and mitochondrial membranes and rapidly reduce cytochrome c [88] (Figure 1).

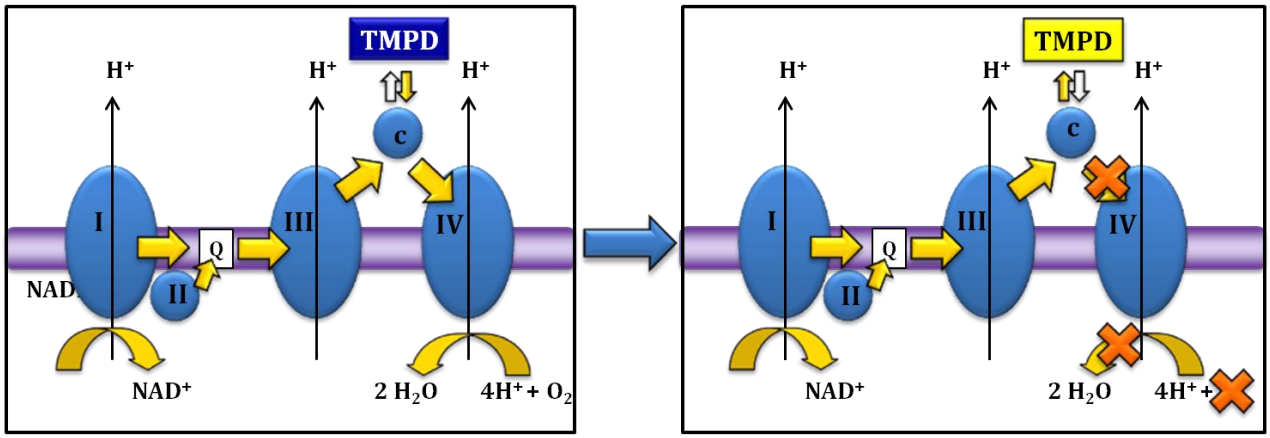

Figure 1. TMPD oxidation of TMPD in presence of oxygen (left); reduction of TMPD in absence of oxygen (right).

Starting from the inoculation of the sample, bacteria are in a lag phase and, consequently, the oxygen present in the vial oxidizes the indicator, which becomes blue. When bacterial growth reaches the log phase, being in the presence of oxygen, all electrons are transported toward the IV complex of the respiratory chain favoring reduction of oxygen, leaving TMPD in its oxidized blue form. Once all the oxygen present in the vial has been consumed by bacteria and is completely reduced to a point of saturation, electrons are transferred to the TMPD, which will be reduced, turning yellow. To prevent the passage of atmospheric oxygen in the solution, making this reaction possible, an appropriate amount of vaseline (1.5 ml) is included in the reaction vials acting as a "cap".

This kind of reaction is well visible for the MBS reagent, i.e. total viable count reagent (TVC vials), for which the starting color blue becomes yellow in the presence of bacteria thanks to the reduction of TMPD (Figure 2).

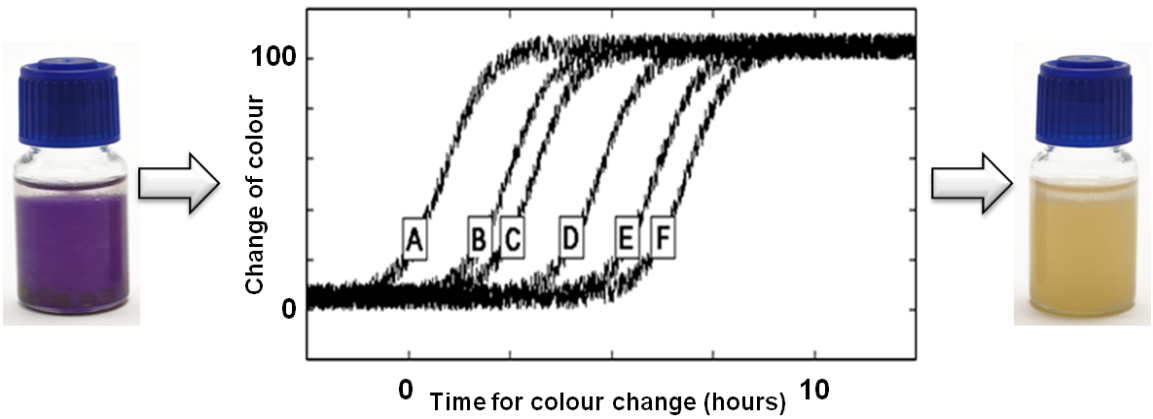

Figure 2. Color change of the MBS vials. The starting color blue (left), changes to yellow (right) in the presence of bacteria. In the absence of bacteria the color remains blue. The time required for colour change is inversely related to bacterial concentration ([A]>[B]>[C]>[D]>[E]>[F])

In some MBS reagents, instead, the color change is determined by the presence of Phenol Red, an indicator of soil acidification that occurs through fermentation processes. This indicator changes color from red to yellow between the pH range that goes from 8.2 to 6.4. For these MBS reagents it is, therefore, possible to observe the color change, from red to yellow, once TMPD is completely reduced, only in the presence of bacteria able to grow in the selective medium and to ferment the source of carbohydrates of the medium, for example in the MBS reagent for detection and quantification of total coliforms and *E. coli* (COLI vials) [89].

The time required for a color change is inversely related to the logarithm of bacterial concentration in the sample: like an enzymatic reaction, the greater the number of bacteria, the faster the color change [90]. Table 1 shows an example of the correlation between the time taken by an MBS reagent to change color (expressed in hours) and the bacterial concentration of the sample analyzed (expressed as log CFU/ml or g). Each MBS reagent shows different times of reaction (meaning the time taken to change color in relation to bacterial concentration of the sample), therefore each reagent has its own specific calibration curve.

Table 1. Example of correlation between bacterial concentration (expressed as CFU/g or ml) and time taken for the vials to change color (expressed as hours).

Time for color change (hours)	Contamination (CFU/g or ml)
< 3.30	$> 10^6$
3.30	10^6
6.30	10^5
9.30	10^4
12.30	10^3
16.00	10^2
19.00	10
22.00	1
> 24.00	0

1.3.2 Micro Biological Survey method analytical procedure

The analytical procedure of the MBS method is extremely simple, allowing analysis also in the absence of an equipped laboratory and specialized personnel. The simple execution and interpretation of results, together with the analytical principle of the method, results in a significant reduction of time and costs while assuring the same performance, in terms of sensitivity, repeatability and reproducibility, of traditional methods of analysis [91].

For the MBS analysis, samples can be directly inoculated in the reaction vials without any preliminary treatment. Before starting the analysis vials must be opened, filled with 10 ml of sterile distilled water and shaken until the reagent is completely dissolved. The final color required to start the analysis (specific for each type of reagent) is achieved in 5-10 minutes. To start the analysis, 1 ml for liquid samples (water or a homogenate of a solid or semi-solid matrix), and approximately 1g for solid samples (or directly the swab for surfaces), can be directly introduced in the vials. The vials must be then incubated in a thermostatic device that maintains the given temperature of incubation depending on the type of microorganisms being sought (30°, 37° or 44°C). Results are obtained monitoring color variation of the vials at different times, referring to the color scale provided with the test kit. After the analysis, the safety cap is pushed down; this opens the tank that releases the sterilizing compound into the vials; in a few minutes all microorganisms are eliminated and vials can be disposed as non-pathogenic waste.

To complete the analysis it is necessary to relate the time taken by the vial to change color to the bacterial concentration of the sample. This can be done using the reference table provided with the kit.

1.3.3 Micro Biological Survey Multireader

The MBS Multireader (MR) is a modular optical device that is made to incubate multiple samples, automatically detect the color change of the reaction vials and calculate the bacterial concentration in the sample. This device automates the analysis process, performing simultaneously 8 independent analyses and makes it unnecessary to check the color change of the vials. The device stations are independent from each other, therefore it is possible to perform at the same time analyses at different temperatures.

The MR is controlled by a computer with a specific software that is able to automatically detect the color change of the vials and calculate, according to the time taken for the vials to change color, the number of microorganisms in the sample. The analysis progression can be followed and monitored

in real time. The final result is automatically recorded in a database from which it is possible to recover the results of interest and build a printable analysis report (Figure 3).

Figure 3. MBS Multireader

The MR was conceived in successive steps dividing the apparatus into different sections. In particular, these sections can be identified with the following subsystems:

Heating system and temperature control of the reaction cell. The reaction cells that lodge the vials are constituted by an aluminum pipe of opportune diameter that can be maintained at a constant temperature. The incubation temperature is variable since this can vary according to the typology of analysis to perform. The temperature range goes from 30 ° C to 44 ° C. The circuit is constituted by a sheet in kapton on which are placed thin resistive copper tracks (the resistivity of about 20 ohms / m is provided by the small size of the deposit of copper). The structure was studied considering the holes for the LEDs, the phototransistor and the temperature sensor.

The temperature control system is provided by a chip (U3) that measures the surface temperature of the aluminum pipe. The chip controls directly the heating circuit, furnishing or removing power when needed. Another safety chip that controls the programmed temperature is placed in contact with the cylinder base (also in aluminum). If the temperature exceeds 55 ° C, due to a failure of the first chip, the second chip intervenes blocking the heating circuit. The different chips were set using a thermocouple in order to ensure a very precise and accurate measurement of the temperature inside of the reaction vial. With these devices, the planned temperature is assured with a discard of ± 0.5 ° C.

Optical system of illumination and detection of the reaction cell. The MR is able to give a picture of the colorimetric state of the reaction vials thanks to a particular optical system composed

by a light emission device, made of 3 LEDs centered on the Red, Green and Blue bands symmetrically arranged around the receiving photodiode. Such configuration allows only the detection of scattered light (reflected and diffused) excluding transmitted and absorbed light in order to avoid the receiver saturation due to the clearness of the initial solution. The LEDs display a very high efficiency and brightness (from 15000 → 22000 mcd). The signal detection device is a light sensor (U1), consisting of a wide-band phototransistor. At the color sensor exit, the RGB signals (calculated in µA) are sent to an amplifier (U2) that amplifies them to fit the entry of the ADC (Analog to Digital Converter) that converts them into digital signals (proportional to exit current values). The particular constitution of the sensor allows it to have a very good response in the visible spectrum (400 - 700 nm). Measurements are performed a minimum every 8 seconds and data are processed in order to allow calculations.

Motherboard (PCB). All of the different MR systems are integrated in the motherboard of processing and complete control of the different analysis cells (8). The heart of the PCB is the PIC18F97J60 10-bit microcontroller (Microchip Semiconductor), which ADC can receive up to 16 analogue channels. The microcontroller is assigned to control all the functions of the MR, to receive all the details set by the user through the computer and to send the measurements at the control program (installed on the computer). Through a series of planned and specific controls, it determines if the analysis result is negative (no significant color changes) or positive (color changes due to bacterial growth), calculating directly the final result. The PCB is interfaced with the computer through 2 alternative serial ports, an USB and an Ethernet. This type of connection, apart from using the common standards of connection, grants the simultaneous use of different MR and the simultaneous analysis of several independent samples. The PCB is powered with 12 V by an external power feeder in order to operate only at low voltage, avoiding problems related to the isolation and therefore ensuring a simple procedure for the CE mark of the device.

Firmware. The code, or software, resident in the PIC microcontroller is written by C++ language. It enables an interface between the hardware drive and the main user software interface. It also enables the control of the functions of the whole system directly from a computer. A PCW compiler included in the MPLAB IDE version 6 package is used to allow all these operations. The functions used are all available in the library of the compiler. The microcontroller is placed on the PICDEM 2 PLUS demo board connected to the system. Thanks to this system it is possible to change the set point values (LEDs intensity, Temperature values, etc.) having a "total" control of the functions of the PIC controller and the whole system.

MBS Multireader Manager software. The software must perform a great amount of controls and calculations with particular attention to the measures detected and to the combinations of these.

This software is written in java language that is very common and easily integrative in the actual HTML browsers. To detect the color change and end the analysis, the system needs to verify the occurrence of different conditions which are established and recorded in the operation system, according to the type of analysis to be carried out.

A specific algorithm, which specifies the stop conditions required for each analysis, must be implemented in the software for each reagent. When the MR is able to detect the stop conditions, due to the color change, the time required for color change is recorded and the concentration of bacteria in the sample is calculated thanks to specific correlation equations different for each reagent and matrix.

User interface. The User interface is very simple and user-friendly. Analysis progression can be followed and monitored in real time.

For each station it is possible to visualize:

- the availability or unavailability of the location indicated by a green or red light respectively;
- the box “CFU <”, that indicates the current “real time” result

When the analysis is ended the CFU box shows the final result that will be automatically recorded in the database in order to be retrieved when needed. The database contains all the information referred to Operators, Customers, Samples and Analysis that can be specified before starting the analysis. From the database it is possible to recover the results of interest and build a printable Analysis report that includes all the information referred to the sample and the analyses performed.

2. AIMS

Conventional microbiological analyses are labor intensive, require extensive manual operations and display well-known inherent limitations, especially concerning the long time needed to obtain results. In the last decades, technological development in microbiological instrumentation has been progressing at an accelerated rate and with a level of sophistication difficult to predict just a few decades ago. A myriad of new analytical methods have emerged through the application of technological advances in molecular biology, chemistry and biochemistry, immunology and immunochemistry, nanotechnology and electronics. New technical platforms appropriate for the detection, enumeration and identification of microorganisms have been recently developed and numerous options exist already in the market. The range of technologies is broad and many currently available rapid methods undoubtedly provide significant benefits to the user, as they have advantages over traditional methods in a wide range of applications. However, by virtue of cost, complexity, and throughput, many of the newly developed technologies are not being implemented into routine laboratory analysis and certainly cannot be used in particularly challenging settings.

There are many areas in which it is critical to provide timely data in order to allow faster decisions and definitive actions, and there still is an urgent need to develop new technologies that could provide rapid, accurate and sensitive results, allowing, however, to simplify analytical procedures and data handling, reduce costs and perform analysis at the point in which they are necessary.

In this context the Micro Biological Survey method (MBS) represents a promising and attractive alternative both to traditional and alternative methods until now developed. The method was initially conceived for food analysis and it has proven to be a reliable system to evaluate microbiological quality and rapidly assess contamination of different food samples [92]. Further studies have demonstrated that the simple analytical procedure, the reduced labor and automation also positively affect the analytical performance of the MBS method, which displays greater reproducibility and repeatability compared to traditional methods [93]. My research was aimed at studying alternative applications of the MBS method, taking advantage of its particular characteristics. The first aim was to verify the effectiveness of the MBS method as an alternative method for microbiological analysis of drinking water, with the purpose to develop a new point of use test to assess water quality in developing countries. The second aim was to investigate the possibility to use the MBS method in clinical settings and, in particular, for the detection of bacteria in urine and a preliminary evaluation of their susceptibility/resistance to antibiotics, with the final goal to develop a new Point of care test for diagnosis and management of Urinary Tract Infections.

3. MATERIALS AND METHODS

3.1 Application of the MBS method for the evaluation of drinking water quality

Culture media. All dehydrated media were dissolved in distilled water, sterilized in an autoclave at 121°C for 15 minutes, under the pressure of 1 atmosphere.

Brain heart infusion broth (Sigma Aldrich, St Luis, MO, USA). Highly nutritious general-purpose growth medium used for the cultivation of a wide variety of fastidious and nonfastidious microorganisms. It is specified in many references for the culture of microorganisms and is cited by NCCLS, the National Committee for Clinical Laboratory Standards, for preparing the inoculum used in antimicrobial susceptibility tests.

Plate count agar (Liofilchem, Roseto degli Abbruzzi, Italy). General-purpose growth medium commonly used to assess or to monitor "total" or viable bacterial growth of a sample. It is recommended for the determination of plate counts of microorganisms in food, water or waste water.

MacConkey agar (Liofilchem, Roseto degli Abbruzzi, Italy). Selective and differential culture medium for bacteria used for the isolation and differentiation of Gram-negative enteric bacilli, and it conforms to Harmonized USP/EP/JP Requirements.

MBS reagents. The MBS vials are made in PET (Polyethylene Terephthalate), provided with a screw cap for a secure closure and a safety cap that includes a tank containing a sterilizing chemical compound that must be manually injected in the vial, after testing, for proper disposal. All the components of the MBS reagents are micronized together (granulometry < 10 micron) in a mortar and then distributed in defined amounts, in each vial. Before closure, 1.5 ml of vaseline is added to each vial. After preparation, the vials are sterilized, using Gamma (γ) rays (5kGy), by a specialized company. Vials so sterilized are ready for use. MBS vials' shelf-life is generally 6 months at room temperature and 1 year at 4°C.

MBS TVC vials. The MBS TVC reagent allows the growth, detection and quantification of nonfastidious as well as moderately fastidious aerobic or microaerophilic microorganisms. The TVC reagent was validated according to ISO 16140 [89]. The presence of bacteria is detected by a color change from blue to yellow.

MBS COLI vials. The MBS COLI reagent allows the growth, detection and quantification of coliform bacteria, defined by WHO as rod-shaped, Gram negative, non spore-forming, cytochrome oxidase negative microorganisms fermenting lactose in the presence of bile salts and other surfactants [28]. The TVC reagent was validated according to ISO 16140 [89]. The presence of bacteria is detected by a color change from red to yellow.

3.1.1 Preliminary study

Samples collection. 50 naturally contaminated water samples were collected from domestic (10 samples) and industrial (10 samples) distribution systems and from water wells (30 samples) of the agricultural region located south of Rome (province of Latina).

MBS method procedure for the analysis of 100 ml of water. Analysis of 100 ml of water with the MBS method was carried out using TVC and COLI vials for the detection and quantification of mesophilic aerobic bacteria or total viable cells (TVC) and total coliforms, respectively. The MBS analysis of 100 ml of water samples was performed using a filtering device made of a polycarbonate body in which is inserted a polycarbonate filter of 0.45µm. The filtering device was assembled and sterilized in autoclave at 121°C for 15 minutes and then used as a normal syringe filter. 100 ml of water samples were filtered through the filtering device and the filter was then inserted into the MBS vials previously filled with 10 ml of sterile distilled water. After the insertion of the filter, TVC and COLI vials were incubated at 37°C for 30 hours in an the MBS Multireader that automatically detects the change of color of the vials. All experiments were carried out in triplicate.

Reference method of membrane filtration. The analysis of 100 ml of water samples with the reference method of membrane filtration was performed using filters with pores of 0.45µm. The filters were incubated on Plate Count agar at 37°C for 48 hours for total viable count and on MacConkey agar at 37°C for 24 hours for total coliforms detection. The results were obtained counting the plates with a number of colonies between 30 and 300. All experiments were carried out in triplicate.

Linearity and accuracy of the MBS method. Different naturally contaminated water samples coming from domestic and industrial distribution systems and from private wells were analyzed both with the MBS method and with the reference methods. The results were then compared in order to demonstrate linearity and accuracy.

Correlation between the analysis of 1ml and 100ml of water samples. 100 ml and 1 ml of naturally contaminated water samples were analyzed with both the MBS method and the reference

methods. For the MBS method, 100 ml was analyzed as previously described using COLI vials; 1 ml was analyzed simply inoculating COLI vials with 1 ml of water samples. COLI vials were incubated at 37°C for 30 hours in MBS Multireader that automatically detects the change of color of the vials. For the analysis of 100 ml the membrane filtration technique was used as the reference method: water samples were filtered and the filters were incubated on MacConkey agar at 37°C for 24 hours. For the analysis of 1ml the Plate count technique was used as the reference method: 1ml of the samples was plated on MacConkey agar at 37°C for 24 hours. The results were obtained counting the plates with a number of colonies between 30 and 300 and were compared in order to verify a possible correlation.

3.1.2 Application of the MBS method on field in the evaluation of the microbiological quality of water in Douala, Cameroon

Study area. Douala, the economic capital of Cameroon, is located between 4°04' latitude north and 9° 45' longitude east, and it is situated near the Atlantic coast 1 m above sea level within the Congo-Guinean phytogeographic zone [94]. It is characterized by a typical warm and humid equatorial climate, with an average annual temperature of 27.0°C (80.6°F), an average humidity of 85% and two rainy seasons extending from March to June and from September to November. The soil is made up of coastal sands, black mud of mangrove swamps and fluvial deposits.

Data collection. Data were collected in three different phases in order to study the effects of inter-annual meteorological variation of rainfall and temperature on groundwater characteristics. The first phase took place in November 2012, within the rainy season, characterized by heavy and frequent precipitations and by a moderate temperature of 30-38°C. The second phase took place in January 2013, when the rainfalls were absent and the temperature was very high, with an average of 40-45°C. The third phase took place in March 2013, at the beginning of the rainy season and at high temperature of 40-45 °C. Sixty one water points were selected within 20 quarters of the Douala V City council. The water points were divided into dug wells and drilled wells and all of them were used as drinking water sources by the resident population. All the dug wells were between 1.5 and 6 m deep while drilled wells were all deeper than 20 meters.

After taking the GPS (LG Marquee™) coordinates of the water points, water samples were collected in 50 ml sterile plastic containers (BD Falcon tubes), treated with thiosulfate to inhibit the antimicrobial activity of the chlorine eventually used to treat water, immediately stored in a refrigerator box and transported to the field lab, set up in the Douala V City council, for analysis.

The period between the sample collection and the analysis was in all cases less than 6 hours. All wells were analyzed in each phase and for each sample analyses were performed in triplicate.

MBS method procedure for quantitative evaluation of total coliforms. Analysis with the MBS method was carried out using COLI vials for the detection and quantification of total coliforms. To carry out analyses, the vials were filled with 10 ml of sterile distilled water and 1 ml of the samples was added to each vial. After inoculation the vials were incubated at 37°C for 24 hours in a thermostat. The change of color was monitored at two different time intervals corresponding to three levels of contamination. The color change was checked after 14 and 24 hours: a color change to yellow after 14 hours indicates high contamination (total coliforms concentration higher than 100 CFU/ml), a color change to yellow within 24 hours indicates contamination (total coliforms concentration lower than 100 CFU/ml) and no color change after 24 hours indicates no contamination (absence of total coliforms). All experiments were carried out in triplicate. Independent analyses using traditional methods were carried out in some of the drilled wells by the "Louis Pasteur Labo" (Laboratoire Multidisciplinaire d'Analyses de Biologie Médicale).

Georeference. Georeference was carried out using the Oziexplorer software (ver 3.95.5r) by D&L Software Pty Ltd., Brisbane Australia, licensed to G.A.. Douala map was downloaded from Google Maps. Geographical coordinates refers to WGS 84 Datum.

3.2 Application of the MBS method as a Point of Care Test for Urinary Tract Infections diagnosis and management

Bacterial strains. Different bacterial strains coming from the ATCC (American Type Culture Collection) culture bank were tested: *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 12600, *Enterococcus faecalis* ATCC 29212, *Pseudomonas aeruginosa* ATCC 27853. These microorganisms were chosen because frequently responsible for Urinary Tract Infections (UTI). The pure cultures were stocked in glycerol (70%).
Artificially contaminated urine samples were prepared diluting in filter-sterilized urine from healthy donors, bacteria coming from pure cultures of the above indicated strains.

Antibiotics. The antibiotics tested in this phase were trimethoprim-sulfamethoxazole (TMP-SMZ), ciprofloxacin (CIP) and amoxicillin-clavulanic acid (BAN) chosen as the gold standard for the treatment of UTI and prescribed by the Infectious Disease Society of America ISDA [95].

Culture media. All dehydrated media were dissolved in distilled water, sterilized in an autoclave at 121°C for 15 minutes, under the pressure of 1 atmosphere.

Brain heart infusion broth (Sigma Aldrich, St Luis, MO, USA). Highly nutritious general-purpose growth medium used for the cultivation of a wide variety of fastidious and nonfastidious microorganisms. It is specified in many references for the culture of microorganisms and is cited by NCCLS, National Committee for Clinical Laboratory Standards, for preparing the inoculum used in antimicrobial susceptibility tests.

Tripticase soy agar (Sigma Aldrich, St Luis, MO, USA). General-purpose growth media that supports the growth of nonfastidious as well as moderately fastidious microorganisms. It is used for a wide range of applications, including culture storage, enumeration (counting), isolation of pure cultures, or simply general culture.

Muller Hinton agar (Sigma Aldrich, St Luis, MO, USA). General-purpose growth media recommended for use in the cultivation of a wide variety of microorganisms. Mueller Hinton Agar is recommended for disk diffusion sensitivity testing of non-fastidious organisms, which may be used in internationally recognized standard procedures.

MBS reagents. The MBS UBC vials containing the reagents described below were prepared as previously described for MBS TVC and COLI vials.

MBS UBC reagent. For the quantitative evaluation of bacteria in urine a new MBS reagent called Urine Bacterial Count (UBC) was studied in order to allow the growth and detection of all aerobic living bacteria possibly present in urine. The composition of the UBC reagent is reported in the following table. The presence of bacteria is detected by a color change from blue to yellow.

Ingredients	*g/l*
Nitrogen source B	5
Nitrogen source C	15
Nitrogen source Ye	1
Carbon source S	1
Buffer system He	10
Buffer system Tr	2,5
Redox indicator T	0,5
Oxidizing agent FeCN	0,1

In the preliminary phase the reagent was prepared dissolving the ingredients in distilled water according to the formula; the liquid preparations were filtered and distributed in sterile vials (10 ml each). Sterile vaseline was added in order to allow the MBS analysis.
In the rest of the experimentation the reagent was prepared according to the standard MBS procedure.

MBS UBC reagent added with antibiotics. For the evaluation of susceptibility/resistance of bacteria the UBC MBS reagent was supplemented with three antibiotics chosen among those more frequently used in the treatment of UTIs and prescribed by the Infectious Disease Society of America: amoxicillin-clavulanic acid (BAN) ciprofloxacin (CIP) and trimethoprim-sulfamethoxazole (TMP-SMZ). Antibiotics were added at breakpoint concentrations according to EUCAST (European Committee of Antimicrobial Susceptibility Testing) tables (www.eucast.org) in order to reproduce the conditions tested in a classic antibiogram.
The developed MBS reagents were:

- UBC+ BAN (UBC reagent + amoxicillin-clavulanic acid at a concentration of 8 mg/l)
- UBC + CIP (UBC reagent + ciprofloxacin at a concentration of 1 mg/l)

- UBC + TMP-SMZ (UBC reagent + trimethoprim-sulfamethoxazole at a concentration of 4 mg/l;

In the preliminary phase the UBC reagent with antibiotics was prepared dissolving the ingredients in distilled water, according to the formula; the liquid preparation was filtered and distributed in sterile vials (10 ml each). Sterile vaseline was added in order to allow the MBS analysis. In the rest of the experimentation the reagent was prepared according to the standard MBS procedure.

3.2.1 Preliminary in vitro validation of new MBS reagents for the detection of bacteria in urine and for the evaluation of their susceptibility/resistance to a panel of antibiotics

Preliminary tests. The effect of urine on color and pH of the new MBS UBC reagent was studied: 1ml of sterile urine samples with pH values ranging from 5 to 6 were inoculated in the UBC vials. pH was measured using pH test Strips (Sigma Aldrich, St Luis, MO, USA) and color was observed before and after 30 minutes from inoculation.

Sensitivity testing. The sensitivity of the UBC reagent was tested for different bacterial strains belonging to the species most frequently responsible for Urinary Tract Infections. Serial dilutions of different ATCC strains (*Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 12600, *Enterococcus faecalis* ATCC 29212, *Pseudomonas aeruginosa* ATCC 27853) were analyzed both with the MBS method and the reference method of plate count.

MBS method. Samples were inoculated in the UBC vials prepared as described previously and incubated in a thermostat at 37°C for 24 hours. A color change from blue to yellow indicates the presence of bacteria and the time for color change should be inversely related to the bacterial concentration in the sample according to the basic principles of the MBS method. The persistence of a blue color indicates the absence of bacteria detected with the MBS method. Analyses were also carried out in order to verify the sterility of the reagent by inoculating control UBC vials with 1ml of sterile water. Five different dilutions of each bacterial strain were tested in duplicate.

Reference method. The standard plate count method for the enumeration of colony forming units (CFU/ml) on Tripticase Soy Agar media (Sigma Aldrich, St Luis, MO, USA) was used as the reference method for the assessment of bacterial concentration in the samples. 1 ml of the samples was plated using the pour plate technique and incubated in a thermostat at 37°C for 24 hours. The results were obtained counting the plates with a number of colonies between 30 and 300. All experiments were carried out in triplicate.

Linearity and accuracy of the MBS UBC reagent. Urine samples artificially contaminated with different concentrations of ATCC strains (*Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 12600, *Enterococcus faecalis* ATCC 29212, *Pseudomonas aeruginosa* ATCC 27853) were inoculated in the UBC vials prepared according to the standard MBS procedure and incubated in the MBS Multireader at 37°C for 24 hours. Nine different dilutions of each bacterial strain were tested in triplicate with the MBS UBC reagent. The standard plate count method for the enumeration of colony forming units (CFU/ml) on Tripticase Soy Agar media (Sigma Aldrich, St Luis, MO, USA) was used as the reference method for the assessment of bacterial concentration in the samples as previously described. The results were then compared in order to demonstrate linearity and accuracy.

Evaluation of susceptibility/resistance of bacteria to a panel of antibiotics. Standard inocula obtained from overnight cultures at 37°C of different ATCC strains (*Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 12600, *Enterococcus faecalis* ATCC 29212, *Pseudomonas aeruginosa* ATCC 27853) were analyzed both with the MBS method and the reference method of Kirby Bauer antibiotic susceptibility test. All experiments were carried out in triplicate.

MBS method. 1 ml of standard inocula (~10^5 CFU/ml) were inoculated in the UBC vials with and without antibiotics and incubated in the MBS Multireader at 37°C for 24 hours. Bacteria were considered susceptible to an antibiotic if the Multireader did not detect bacterial growth in the specific vial supplemented with the antibiotic of interest; bacteria were considered resistant if the Multireader detected bacterial growth in times comparable to those observed analyzing the same samples with the UBC vial without antibiotic, meaning that bacteria were able to grow regardless of the presence of antibiotics. Bacteria displayed an intermediate susceptibility to the antibiotic under study if bacteria grew slower in the vial supplemented with antibiotics compared to the UBC vial without antibiotics, meaning that the antibiotic affected bacterial metabolism and growth.

Kirby-Bauer disk diffusion method. The standard antibiotic susceptibility testing was performed with the Kirby-Bauer disk diffusion method. For each microorganism tested, using an aseptic technique, a sterile swab was placed in the culture broth (adjusted at a turbidity of 0,5 Mc Farland corresponding to approximately 10^8 CFU/ml) and, after gently removing the excess liquid, was streaked on Muller Hinton agar plates to obtain a bacterial loan. Disks containing a standard concentration of the tested antibiotics (30 µg amoxicillin clavulanic acid, 5 µg ciprofloxacin and 1,25 µg trimethoprim-sulfamethoxazole) were dispensed on the plate. Plates were incubated for 24 hours at 37°C. If present, zone sizes of complete inhibition were measured with a ruler and compared with the accepted cut off values for each antibiotic/microorganism pair.

3.2.2 Preliminary clinical study of the developed MBS reagents as a POCT, in collaboration with the hospital "Azienda Ospedaliera Sant'Andrea" of Rome

MBS POCT. The MBS Point of Care Test is an in vitro diagnostic medical device that was developed to meet the need of rapid, accurate, user-friendly and inexpensive tests for Urinary Tract Infections (UTI) diagnosis, that could be confidently used by healthcare professionals at the patient's bedside. The device is specifically designed for the early detection of UTI and for a first evaluation of the susceptibility/resistance of infecting bacteria to a panel of antibiotics.
The device is made of ready to use, disposable, reaction vials containing the Urine Bacterial Count (UBC) reagent alone or with the addition of different antibiotics. Analyses are performed thanks to a thermostatic optical reader, the MBS Multireader, that automatically detects the color change of the vials.

Study design. A prospective diagnostic accuracy evaluation study was performed in collaboration with the Department of Emergency Medicine of the Azienda Ospedaliera Sant'Andrea of Rome. Between November 2013 and July 2014, a total of 122 patients with clinically suspected UTIs was enrolled upon hospital admission. Criteria for the clinical suspicion of UTI were: high levels of leukocytes in urine, their cloudy appearance and/or abnormal color and the presence of a catheter left in place for more than 72 hours. Urine samples from indwelling urinary catheters were obtained from the sampling port using the aseptic technique. The port is usually situated in the drainage tubing, proximal to the collection bag, ensuring the freshest sample possible. Urine samples of non-catheterized patients were obtained via the clean-catch midstream technique. Urine samples were collected in the morning in disposable sterile urine collection containers, split into two aliquots and immediately analyzed with the MBS method and using the routine urine culture / antibiogram protocols in use by the hospital laboratory. Among 122 urine samples, 72 were analyzed also using urine dipsticks.

Ethical Committee. Approval of the preliminary clinical trial was obtained on 14.01.2013 from the Ethical Committee of the Azienda Ospedaliera Sant'Andrea, constituted according to DM 12.05.2006 following Good Clinical Practice The authorization was given on the basis of the declaration that the patients were duly informed and consenting. In addition, the clinical trial did not require any change in the normal diagnostic and therapeutic strategy, only to collect a small (1-2 ml) sample of the patient's urine in addition to that already required for the planned routine

laboratory tests. The results from the laboratory analysis with the results obtained from the new device were compared in an absolutely anonymous way.

Hospital laboratory tests. Urine specimens were inoculated onto blood agar and selective CHROM agar plates using a calibrated 0.010 ml loop and streaked manually. All culture plates were incubated at 37°C for at least 24 h before reading. Bacterial counts were determined by the dilution plating method. A positive result was defined by the presence of a concentration of bacteria $\geq 10^5$ colony-forming units (CFU)/ml. The number and identification of different organisms were primarily assessed by colony color and morphology. The definitive identification was performed using the VITEK® MS (BioMérieux Italia S.p.a., Florence, Italy). The VITEK® 2 (BioMérieux Italia S.p.a., Florence, Italy) automated system with 64-well cartridges was used for antibiotic susceptibility testing according to the CLSI recommendations [96].

Urine dipstick test. A small volume of urine from the sterile container was drawn with a sterile syringe, and then applied to the urine dipstick (DIRUI, DIRUI Industrial Co., Ltd, Changchun, China). A positive result was defined in the presence of both leukocytes and nitrite. The test was carried out immediately after urine collection at the patients' bedside by the hospital personnel.

Urine Bacterial Count using the MBS POCT. According to the MBS method, for the MBS POCT analysis, 1 ml of urine samples was manually injected in the UBC vials immediately after urine collection. Vials were then incubated in the MBS Multireader at 37°C up to 27 hours in order to check the time taken for the vials to change color also when analyzing urine samples that did not display a significant bacteriuria but only a low bacterial concentration. Afterwards, through the ROC analysis, it was possible to identify the cut off time limit able to distinguish between the presence or absence of a significant bacteriuria maximizing sensitivity and specificity.
After analysis, small aliquots of the vials' content (that can be assimilated to a bacterial preculture) can be taken out to perform bacterial identification using standard techniques; otherwise, the vials' content can be sterilized simply by pressing the cap on top of the vials and then disposed as "Non-Hazardous Waste".

Diagnostic accuracy evaluation of the MBS POCT. The outcomes of the MBS POCT were compared with the outcomes of the reference test for urine culture. The performance characteristics were evaluated by the Receiver Operating Characteristic analysis using MedCalc statistical software (MedCalc software bvba, Belgium) [22]. This analysis is widely used in medicine to determine the validity of a diagnostic test and to find its optimal cut off limit. In particular, the Area under the ROC Curve (AUC) is a measure of how well a parameter can distinguish between two diagnostic

groups, which is subjects with and without a UTI. An area under the curve equal to 1 is that of a test that displays 100% sensitivity and 100% specificity. Accuracy, sensitivity and specificity of urine dipsticks for the diagnosis of UTIs were also evaluated according to Friedman *et al.* [98].

Antibiotic susceptibility testing using the MBS POCT. Presumptive antibiotic susceptibility was assessed by using UBC vials supplemented with three different antibiotics (UBC+BAN, UBC+CIP, UBC+TMP-SMZ). For the MBS POCT analysis, 1 ml of each urine sample was manually injected in the UBC vials supplemented with antibiotics immediately after urine collection. Vials were then incubated in the MBS Multireader at 37°C for 27 hours. After analysis vials were sterilized simply by pressing the cap on top of the vials and then they were disposed as "Non-Hazardous Waste". Bacteria were predicted to be susceptible if, following urine inoculation in the vial supplemented with the specific antibiotic, they did not induce a color change of the medium, suggestive of absence of growth in the presence of antibiotic. Bacteria were instead predicted to be resistant to the antibiotic if causing a color change in times proportional to those determined analyzing the same urine samples in the UBC vial without antibiotic.

Statistical evaluation. The outcomes of the MBS POCT for antibiotic susceptibility test were compared with the outcomes of the reference standard test of antibiograms. Accuracy, Sensitivity, Specificity as well as Positive Predictive Value (PPV) and Negative Predictive Value (NPV) were evaluated according to Friedman *et al.* [98].

3.2.3 Optimization of the MBS UBC reagent composition and the operating procedure for the evaluation of susceptibility/resistance to antibiotics, using the optimized UBC reagent added with antibiotics

Optimization of the MBS UBC reagent composition. Different ingredients, especially growth promoting agents that could favor growth of slow-responding bacteria, were tested alone or in combination at different concentrations. In the following table are reported all the ingredients and the different concentrations tested.

Ingredients	*Functions*	*Tested concentrations (g/l)*
Gly	Growth promoting agent	0; 1; 1,5; 3; 12;
Cys	Growth promoting agent	0; 0,01; 0,03; 0,05; 0,06; 0,1; 1
SP	Growth promoting agent	0,5; 1,5; 2; 10
SS	Growth promoting agent	0,1; 1
AC	Growth promoting agent	1
CI	Growth promoting agent	1; 2,5
FeCN	Oxidizing agent	0,1; 0,2; 0,3; 0,4; 0,6
BM	Redox indicator	0,004
NaCl	Selective agent	3

In the preliminary phase the reagents were prepared dissolving the ingredients of the previous UBC reagent in distilled water according to the formula and adding the different ingredients alone or in combination at different concentrations; the liquid preparations were filtered and distributed in sterile vials (10 ml each). Sterile vaseline was added in order to allow the MBS analysis. In order to find the optimal formulation, different preparations were tested with different bacterial strains belonging to the species most frequently responsible for Urinary Tract Infections. Serial dilutions of different ATCC strains (*Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 12600, *Enterococcus faecalis* ATCC 29212, *Pseudomonas aeruginosa* ATCC 27853) were analyzed both with the MBS method and the reference method of plate count. All experiments were carried out in triplicate.

MBS method. Samples were inoculated in the previous UBC vials and the optimized UBC reagent prepared as described before and incubated in a thermostat at 37°C for 24 hours. A color change from blue to yellow indicates the presence of bacteria and the time for color change should be inversely related to the bacterial concentration in the sample according to the basic principles of the MBS method. The persistence of a blue color indicates the absence of bacteria detected with the MBS method. Analyses were also carried out in order to verify the sterility of the reagent inoculating control UBC vials with 1ml of sterile water.

Reference method. The standard plate count method for the enumeration of colony forming units (CFU/ml) on Tripticase Soy Agar media (Sigma Aldrich, St Luis, MO, USA) was used as the

reference method for the assessment of bacterial concentration in the samples. 1 ml of the samples was plated using the pour plate technique and incubated in a thermostat at 37°C for 24 hours. The results were obtained counting the plates with a number of colonies between 30 and 300.

Linearity and accuracy of the optimized UBC MBS reagent. Linearity and accuracy were determined analyzing urine samples artificially contaminated with different concentrations of ATCC strains (*Escherichia coli* ATCC 25922, *Enterococcus faecalis* ATCC 29212, *Pseudomonas aeruginosa* ATCC 27853) and a clinical isolate *S. aureus* strain, as previously described.

Optimization of the analytical procedure for the evaluation of susceptibility resistance to antibiotics using the optimized UBC reagent added with antibiotics. Different sizes of bacterial inocula were tested with the optimized UBC vials with and without antibiotics. Artificially contaminated urine samples were obtained using overnight cultures at 37°C of different ATCC strains (*Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 12600, *Enterococcus faecalis* ATCC 29212, *Pseudomonas aeruginosa* ATCC 27853). The contamination mimicked the one of naturally contaminated urine samples. The samples were analyzed both with the MBS method and the reference method of Kirby Bauer antibiotic susceptibility test as previously described.

3.2.4 Study of a new standardized manufacturing process

UBC MBS lyophilized vials production. UBC lyophilized vials were produced by Sclavo Diagnostics International S.r.l. (Siena, Italy).

Linearity and accuracy of the MBS UBC lyophilized vials. Linearity and accuracy were determined analyzing urine samples artificially contaminated with different concentrations of ATCC strains (*Escherichia coli*, *Staphylococcus aureus, Enterococcus faecalis, Pseudomonas aeruginosa*) as previously described. Experiments were conducted in parallel using also plastic UBC vials produced according to the standard MBS procedure.

Stability tests. Lyophilized vials were stored at room temperature and 4°C for 240 days and the performance was evaluated after 1, 2, 4, 6 and 8 months. Control analyses were carried out with vials that were lyophilized a few days before.

Physico-chemical tests. The color of the reagent was evaluated immediately after dissolution and after 5, 10 and 20 minutes. Also, the pH of the vials was measured and compared to the control value in order to verify the stability of the reagents stored at different temperatures.

Sensitivity tests. The vials stored at room temperature and 4°C were tested analyzing serial dilutions of *Escherichia coli* ATCC 25922 and the results obtained with the MBS method were compared with the results obtained with the reference method of plate count on Tripticase soy as previously described.

3.2.5 Set up of the optimal detection parameters of the MBS Multireader

MBS Multireader (MR) Software. The MR software is written in Java language that is very common and easily integrative in the actual HTML browsers. The output generated by the software is a curve that correlates intensities of the signals with time. Curves' behavior can be analyzed taking into consideration mainly two parameters: the intensity coefficients C of the three signals (CR, CV, CB) that increase significantly during the color change, and the first derivative or slope of the three signals (slope R, slope G, slope B) that are used to find the exact point of color change. In order to detect the color change and end the analysis, the system needs to analyze in "real time" the curves' behavior and verify the occurrence of different conditions according to the type of analysis to be carried out. The selected parameters are:

- the lowest signal amplification coefficient for the different signals;
- threshold limit, time of decrease and percentage of decrease of the first derivative or slope usually of the most significant signal/signals.

Curve analysis. An analysis of the intensity coefficients and the first derivatives was performed for each signal for 300 performed analyses in order to find the range between which these parameters were included and test all the possible stop conditions that could potentially fit the detection needs. The results were used to generate a table of 3600 combinations.

Fast Loader Research (FLR). FLR is a calculation software developed by MBS srl. This software can be implemented with a table of combinations that is run together to each output generated by the MBS MR software. FLR is able to calculate the exact result (expressed in hours) that will be obtained for each analysis and for each stop condition separately.

Data analysis. A dedicated program (Excel Macro) was set up in order to compare the results obtained with the FLR software with those expected with a tolerance of 5%. This program was able to take into consideration results obtained for 300 analysis and find the percentage of success of each stop condition separately.

4. RESULTS AND DISCUSSION

4.1 Application of the MBS method for the evaluation of drinking water quality

The first part of my study was focused on the application of the MBS method for drinking water analysis. The final aim was to successfully use the MBS method for a simplified and effective microbiological analysis of drinking water.

4.1.1 Preliminary study

Preliminary studies were carried out in order to verify the possibility to apply the MBS method to the analysis of drinking water. The parameters total viable count (TVC) and total coliforms were considered as two of the parameters mostly used for monitoring the overall quality of water. Standard regulations require the evaluation of these parameters in 100 ml.

In order to evaluate specificity and sensitivity of the MBS method compared to the traditional method of membrane filtration, according to national and international standards, 50 naturally contaminated water samples were analyzed both with the MBS method and with the reference methods.

A comparative presence/absence analysis was carried out: of the 100 analyses performed, 61 were positive with both methods without the presence of false negatives (sensitivity 100%) and 39 were negative with the total absence of false positives (specificity 100%) (Table 2).

Table 2. Comparison analysis of the results obtained both for total viable count and for total coliforms on 100ml of different water samples between the MBS method and the reference method.

	Reference method		
MBS method	Present	Absent	Total
Positive	61 (PA)	0 (PD)	61
Negative	0 (ND)	39(NA)	39
Total	61 (N_+)	39 (N_-)	100(N)

PA: positive agreement, **NA**: negative agreement, **ND:** negative deviation (false negatives), **PD:** positive deviation (false positives), **N:** total number of samples (NA+PA+PD+ND), $\mathbf{N_+}$**:** total number of positive results obtained with reference method, $\mathbf{N_-}$**:** total number of positive results obtained with reference method.

Linearity and accuracy of the MBS method. Linearity is the ability of the method when used with a given matrix to give results that are in proportion to the amount of analyte present in the sample, that is, an increase in analyte corresponds to a linear or proportional increase in results as indicated by ISO 16140 (2003). The quantitative results obtained with the MBS method analyzing the previously described water samples in terms of time taken for the vials to change color were compared with the results obtained with the reference method of membrane filtration.

This was achieved graphically as illustrated in Figure 4 (a and b) by plotting bacterial concentrations (expressed as the log of CFU/ml) obtained with the reference method with the time occurred for color change of the identical water samples analyzed with the MBS method both for TVC and for total coliforms. A linear inverse relationship between the MBS method and the bacterial concentration can be observed in both cases. The equations of the curves and their correlation factors (R^2) were calculated (Table 3).

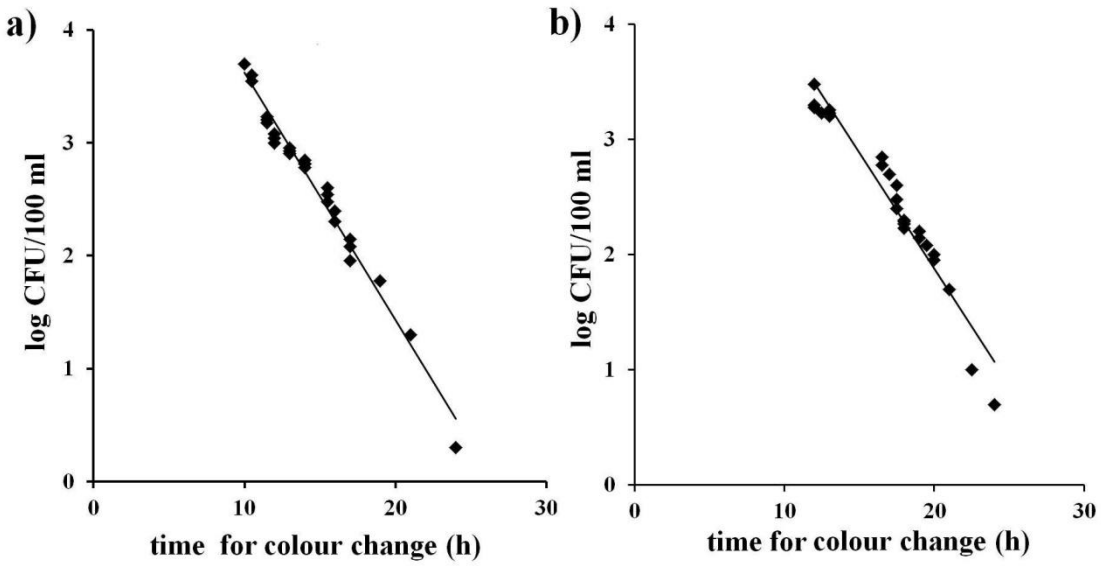

Figure 4. **Linearity: correlation line between the analytes concentration and the time occurred for color change in the MBS vials using naturally contaminated water samples**. Bacterial concentrations (expressed as the log of CFU/100 ml) obtained with the reference method of membrane filtration are plotted against the time occurred for color change of the identical samples analyzed with the MBS methods. Panel a): total viable count; Panel b): total coliforms. The straight line represents the linear regression analysis including all the points. Each point is the mean of three different analyses.

Table 3. Equations of the linearity lines and their correlation factors (R^2) for TVC and total coliforms.

	Linear equation	R^2
TVC	y = -0,22 x + 5,80	0,98
total coliforms	y = -0,20 x + 5,90	0,94

The equations of the curves were used to determine, from the time occurred for color change, the bacterial concentrations (expressed as the log of CFU/100 ml) so as to demonstrate the accuracy of the MBS method.

Accuracy is the degree of correspondence between the response obtained by the reference method and the response obtained by the alternative method on identical samples (ISO 16140, 2003). A perfect correlation between the bacteria number (expressed as log CFU/100 ml) obtained with the traditional method of membrane filtration and the alternative MBS method for the different water samples was observed both for TVC and for total coliforms (Figure 5 a and b). The straight lines obtained were close to the ideal y = x (slope = 1.00), with values of correlation factor (R^2) which further confirm the high equivalence between the reference method and the alternative MBS method.

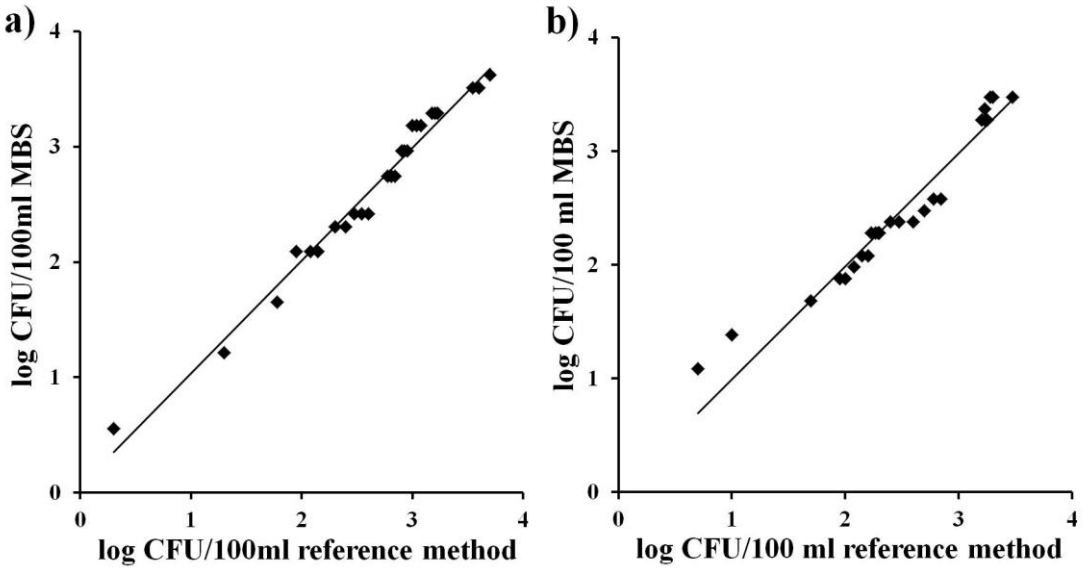

Figure 5. Accuracy: correlation line between the alternative MBS method and the reference method using naturally contaminated water samples. Bacterial concentrations (expressed as the log of CFU/100 ml) obtained with the MBS method are plotted against bacterial concentrations (expressed as the log of CFU/100 ml) obtained with the reference method on the identical samples: a) total viable count; b) total coliforms. Continuous line linear regression analysis: slope=0.98 (R^2= 0.98) for total viable count; slope=0,99 (R^2=0.94) for total coliforms. Each point is the mean of three different analyses.

Correlation between total viable count at 37°C and total coliforms. To further simplify the analysis, a comparative study between the two parameters total viable count and total coliforms in different water samples was carried out.

The correlation between the two parameters was achieved graphically by plotting the concentration of total viable cells (expressed as the log of CFU/100 ml) against the concentration of total coliforms obtained with the MBS method. Figure 6 shows that in the presence of total coliforms a strict correlation between the two parameters can be observed. This result confirms the possibility to use only the parameter total coliforms to have a good evaluation of the microbiological quality of water. Total coliform count is in fact the main bacteriological parameter that has been used to determine the general quality of drinking water worldwide. This test could therefore be used as the starting point to determine the biological quality of drinking water.

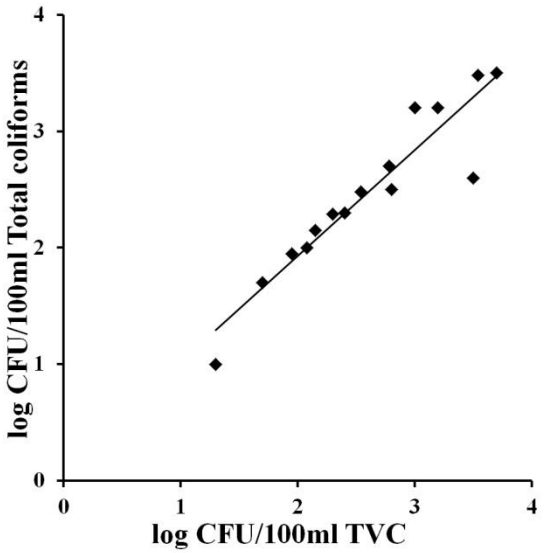

Figure 6. Correlation between the parameters total viable count and total coliforms in water samples coming from domestic and industrial distribution systems. Bacterial concentrations (expressed as the log of CFU/100 ml) obtained with the MBS method for total viable count are plotted against the bacterial concentrations (expressed as the log of CFU/100 ml) obtained with the MBS method for total coliforms on the identical samples. Continuous line linear regression analysis: slope=0.91 (R^2= 0.87). Each point is the mean of three different analyses.

Correlation between the analysis of 100 ml and 1 ml of water samples. According to international standards some parameters must be evaluated in 100 ml of water samples. The possibility to apply the MBS method for the analysis of 100 ml of water has been successfully demonstrated. However, the execution of this type of analysis requires minimal equipment and is more laborious and time consuming. Because the next step of the study considered the use of the

MBS method on field and in particularly difficult conditions, a preliminary study to verify the margin of error between the analysis of 1ml and the analysis of 100 ml was carried out. This can be also very important for turbid water and water with suspended particles that is difficult to filter.

100 ml and 1 ml of naturally contaminated water samples were analyzed with both the MBS method and the reference methods. After the demonstration of the good correlation between the results obtained analyzing the same samples with both the reference method and the MBS method (data not showed), the MBS results obtained from the analysis of 1 ml of the sample were normalized for 100 ml and then compared to the results obtained analyzing 100 ml.

Figure 7 shows that in the presence of a concentration of coliforms greater than 10^2 the analysis of 1ml underestimates the bacterial concentration of 0.5 log units. This result can be acceptable considering that the Most Probable Number technique, a standard method used for water analysis, has a given uncertainty of about 1 log unit.

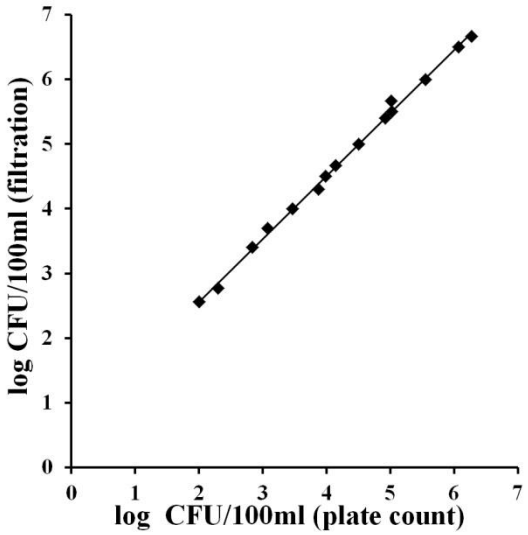

Figure 7. Correlation between the analysis of 1 ml and 100 ml of naturally contaminated water samples. Total coliforms concentrations (expressed as the log of CFU/100 ml) obtained using the MBS method for the analysis of 100 ml of the samples are plotted against the bacterial concentrations (expressed as the log of CFU/100 ml) obtained analyzing 1 ml of the identical samples and normalizing the results for 100 ml. Continuous line linear regression analysis: slope=0.97 (R^2= 0.99). Each point is the mean of three different analyses.

4.1.2 Application of the MBS method on field for the evaluation of the microbiological quality of water in Douala, Cameroon

The MBS method was applied to evaluate the microbiological quality of water in the city of Douala. According to the results obtained in the preliminary studies the concentration of total coliforms was enumerated in 1ml of water samples. Sixty one water points were selected within 20 quarters of the Douala V City council. The water points were divided into dug wells and drilled wells and all of them were used as drinking water sources by the resident population.

Data were collected in three different phases in order to study the effects of inter-annual meteorological variation of rainfall and temperature on groundwater characteristics.

Analysis with the MBS method was carried out using COLI vials for the detection and quantification of total coliforms. In order to distinguish three levels of contamination the change of color was monitored visually at two different time intervals. The operator simply looked at the vial color after 14 and 24 hours: a color change to yellow after 14 hours indicates high contamination (total coliforms concentration higher than 100 CFU/ml), a color change to yellow within 24 hours indicates contamination (total coliforms concentration lower than 100 CFU/ml) and no color change after 24 hours indicates no contamination (absence of total coliforms). Absence of coliforms can be simply assessed monitoring the color change of the vials after 24 hours (Table 5).

Table 5. Levels of contamination determined by the time of color change of the MBS vials. Contamination levels correlated with the bacterial concentrations expressed in CFU/ml are determined by the change of color of the MBS vial controlled by visual inspection after 19 and 24 hours. 1ml of water samples to be tested was inoculated into the MBS vials. The starting color of the MBS vials for total coliforms was red. Depending on coliform concentration into the water sample, the color of the MBS vials turns to yellow (presence of coliform bacteria) or remains red (absence of coliform bacteria).

Color after 14 hours	Color after 24 hours	Bacterial concentration (CFU/ml)
Yellow	Yellow	$> 10^3$ = high contamination
Yellow	Red	$1< x< 10^3$= contamination
Red	Red	No contamination

From the obtained data we have assessed that 70% of the dug wells analyzed in the first phase were contaminated, while 100% of the analyzed drilled wells were not contaminated. This first phase took place in November 2012, within the rainy season, characterized by heavy and frequent precipitations and by a moderate temperature of 30-38°C. Very similar results were obtained during

the second phase that took place in January 2013, when the rainfalls were absent and the temperature was very high, with an average of 40-45°C: 62.5% of the dug wells analyzed in this second phase resulted contaminated while 100% of the analyzed drilled wells resulted not contaminated. During the third phase that took place in March 2013, when the rainfalls begin and the temperature was still very high, with an average of 40-45°C, it resulted that 50% of the dug wells analyzed were contaminated while 100% of the analyzed drilled wells were not contaminated. Figure 8 shows the level of coliform contamination found in the water samples analyzed with the MBS vials for total coliforms in the three different phases.

Independent analysis performed by the "Louis Pasteur Labo" (Laboratoire Multidisciplinaire d'Analyses de Biologie Médicale) confirmed the absence of coliforms contamination in some of the drilled wells analyzed with the MBS method (data not shown).

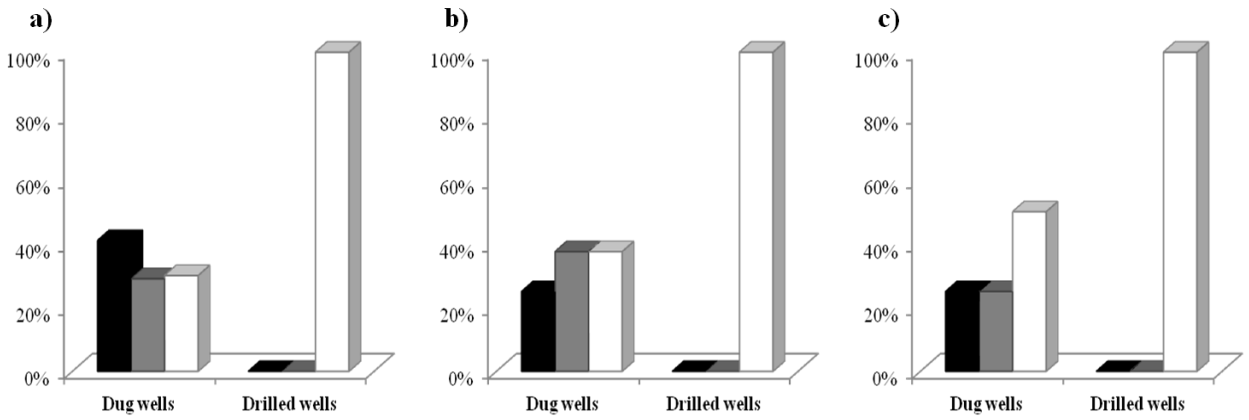

Figure 8. Coliform contamination of water samples from drilled wells and from dug wells. The type of water point and the coliform contamination level of the water samples tested with the MBS method are shown together with the examination phase. Black bars mean high contamination by coliform (> 100 CFU/ml). Grey bars mean low coliform contamination (between 1 and 100 CFU/ml). White bars mean no coliform presence. Data were collected three times during the year: a) November 2012, rainy season, heavy and frequent precipitations and moderate temperature, 30-38°C; b) January 2013, beginning of dry season, no rainfall and very high temperature, 40-45°C; c) March 2013, end of the dry season, high temperatures, 40-45 °C.

A strict correlation between the well type and coliform concentration was observed: 70% of the examined dug wells resulted at least once contaminated by total coliforms with a high average contamination; on the other hand, 100% of drilled wells resulted non-contaminated, underlining the safeness of these water sources and the importance of their use in both rural and city areas.

Also during the raining period the number of highly contaminated water samples increased from 25% (observed in dry periods) up to 41% (observed in the rainy season) (Figure 9). This can be explained by the fact that this water resource is vulnerable not only to anthropogenic pollution but also influenced by the effects of rainfall which varies not only throughout the year but also fluctuates from year to year. The heavy and frequent rainfall and the higher level of the phreatic layer of groundwater observed in November could be responsible for the higher levels of contamination due to the greater possibility of infiltration, draining or streaming of water from septic pits.

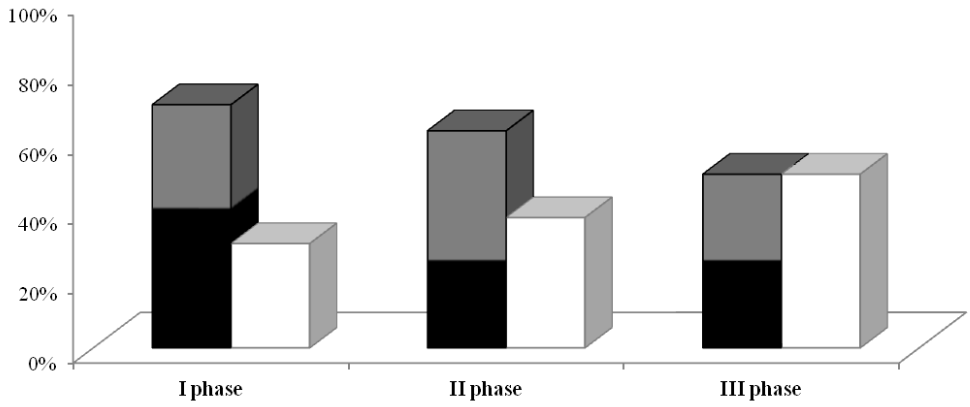

Figure 9. Levels of coliform contamination of dug wells in the three different phases. The level of coliform contamination of dug wells tested with the MBS method are shown together with the examination phase. Black means high contamination level (coliform concentration > 100 CFU/ml). Grey means low contamination level (between 1 and 100 CFU/ml). White bars mean no coliform presence. Phase I: November 2012, rainy season, heavy and frequent precipitation and moderate temperature, 30-38°C. Phase II: January 2013, beginning of the dry season, no rainfall and very high temperature, 40-45°C. Phase III: March 2013, end of the dry season, high temperatures, 40-45 °C.

Figure 10 shows the map of Douala with the geo-references of the sites where water samples were collected. The different colors of the spots indicate the highest level of coliform contamination found in the water samples analyzed with the MBS vials for total coliforms. Black spots mean high contamination by coliform (> 100 CFU/ml). Grey spots mean low coliform contamination (between 1 and 100 CFU/ml). White spots mean no coliform presence. No correlation between the level of coliform bacteria contamination and the position of the water points was observed.

Figure 10. Georeference of the collected water points in Douala (Cameroon). Georeference was carried out using Oziexplorer software. The color of the water points refers to the level of coliform contamination of the water samples tested with the MBS method. Black spots mean high contamination by coliform (> 100 CFU/ml). Grey spots mean low coliform contamination (between 1 and 100 CFU/ml). White spots mean no coliform presence.

4.2 Application of the MBS method as a Point of Care Test for Urinary Tract Infections diagnosis and management

4.2.1 Preliminary in vitro validation of new MBS reagents for the detection of bacteria in urine and for the evaluation of their susceptibility/resistance to a panel of antibiotics

The main purpose of the study was to develop and test specific MBS reagents for the quantitative evaluation of bacteria in urine and for the qualitative evaluation of their susceptibility/resistance to a panel of antibiotics used in the treatment of Urinary Tract Infections.
For the quantitative evaluation of bacteria in urine a new MBS reagent called Urine Bacterial Count (UBC) was studied in order to allow the growth and detection of all aerobic living bacteria possibly present in urine. The specific composition of this reagent was developed adding to the ingredients mostly used in non-selective microbiological growth media, in order to favor the growth of viable bacteria, the particular MBS detection system made of a redox indicator that changes color from blue to yellow as a measure of bacterial consumption of oxygen, in order to allow detection and quantification of bacteria. An oxidizing reagent was also added so as to avoid interference with oxidizing substances possibly present in biological samples.

Preliminary experiments were carried out in order to evaluate the effect of urine on color and pH of the new MBS reagent and to evaluate the effectiveness of the buffer system. pH was measured and color was also observed before and after 30 minutes from inoculation. No variation was observed, demonstrating that urine does not interfere with the MBS analysis (data not shown).

Sensitivity testing. The sensitivity of the UBC reagent was tested for different bacterial strains (*Escherichia coli, Staphylococcus aureus, Enterococcus faecalis, Pseudomonas aeruginosa*) belonging to the species most frequently responsible for Urinary Tract Infections. The same samples were analyzed also with the reference method in order to verify the presence of bacteria. The results obtained are reported in Table 6. All tested bacteria caused color change in the MBS UBC reagent in less than 24 hours also at very low concentrations. Moreover, comparable bacterial loads determined comparable responses with the MBS method. Together these results demonstrate the sensitivity of the UBC reagent to all tested ATCC strains and the repeatability of results regardless of the bacterial strain tested.

Table 6. Sensitivity testing of the MBS UBC reagent. Results obtained analyzing with the UBC reagent different concentrations of *E. coli* (ATCC 25992), *S. enterica* ser. Thyphimurium (ATCC 14028), *E. faecalis* (ATCC 29212), *P. aeruginosa* (ATCC 27853*) and S. aureus* (ATCC 12600)[1].

Microorganisms	*Dilution*	*TC (h)*	*SD*	*Colony count TSA (CFU/ml)*
E. coli ATCC 25922	CTRL	NCC	-	-
	10^{-1}	2	±0,5	~10^{8}
	10^{-3}	6	±0,7	~10^{6}
	10^{-5}	9	±0,8	~10^{4}
	10^{-7}	11	±0,6	1,4x10^{2}
	10^{-9}	14	±0,6	3
E. faecalis ATCC 29212	10^{-1}	1,5	±0,8	~10^{8}
	10^{-3}	5	±0,5	~10^{6}
	10^{-5}	8,5	±0,7	~10^{4}
	10^{-7}	12	±0,8	1,6x10^{2}
	10^{-9}	15	±0,7	2
P. aeruginosa ATCC 27853	10^{-1}	3	±0,6	~10^{8}
	10^{-3}	6	±0,5	~10^{6}
	10^{-5}	9,5	±0,5	~10^{4}
	10^{-7}	12	±0,6	1,7x10^{2}
	10^{-9}	14,5	±0,8	3
S. aureus ATCC 12600	10^{-1}	3,5	±0,6	~10^{8}
	10^{-3}	7	±0,5	~10^{6}
	10^{-5}	10	±0,7	~10^{4}
	10^{-7}	13	±0,6	1,2x10^{2}
	10^{-9}	16,5	±0,8	3

TC=Time for color change; SD= Standard deviation

[1]*Five different dilutions of each bacterial strain were tested in duplicate with the MBS UBC reagent. The color of the MBS vials changed from blue to yellow. The values are mean ± SD of 5 experiments carried out in triplicate.*

Linearity and accuracy of the UBC MBS reagent. Linearity and accuracy of the MBS method were evaluated according to ISO 16140, 2003. Linearity is the ability of the method when used with a given matrix to give results that are in proportion to the amount of analyte present in the sample, that is, an increase in analyte corresponds to a linear or proportional increase in results. The

quantitative results obtained with the MBS method in terms of time taken for the vials to change color were compared with the results obtained with the reference method of plate count.

This was achieved graphically as illustrated in Figure 11 (a) by plotting bacterial concentrations (expressed as the log of CFU/ml) obtained with the reference method with the time occurred for color change of the UBC vials. A linear inverse relationship between the MBS method and the bacterial concentration can be observed. The equation of the curve and correlation factor (R^2) were calculated (Table 7).

Table 7. Equation of the linearity line and correlation factor (R^2) for the UBC reagent.

	Linear equation	R^2
UBC	y = -0,68 x + 10	0,96

The equation of the curve was used to determine, from the time occurred for color change, the bacterial concentrations (expressed as the log of CFU/ml) so as to demonstrate the accuracy of the MBS method.

Accuracy is the degree of correspondence between the response obtained by the reference method and the response obtained by the alternative method on identical samples (ISO 16140, 2003). A very good correlation between the bacteria number (expressed as log CFU/ml) obtained with the traditional method of plate count and the alternative MBS method was observed (Figure 11 b). The straight line obtained was close to the ideal y = x (slope = 1.00), with a correlation factor (R^2) which further confirm the high equivalence between the reference method and the alternative MBS method.

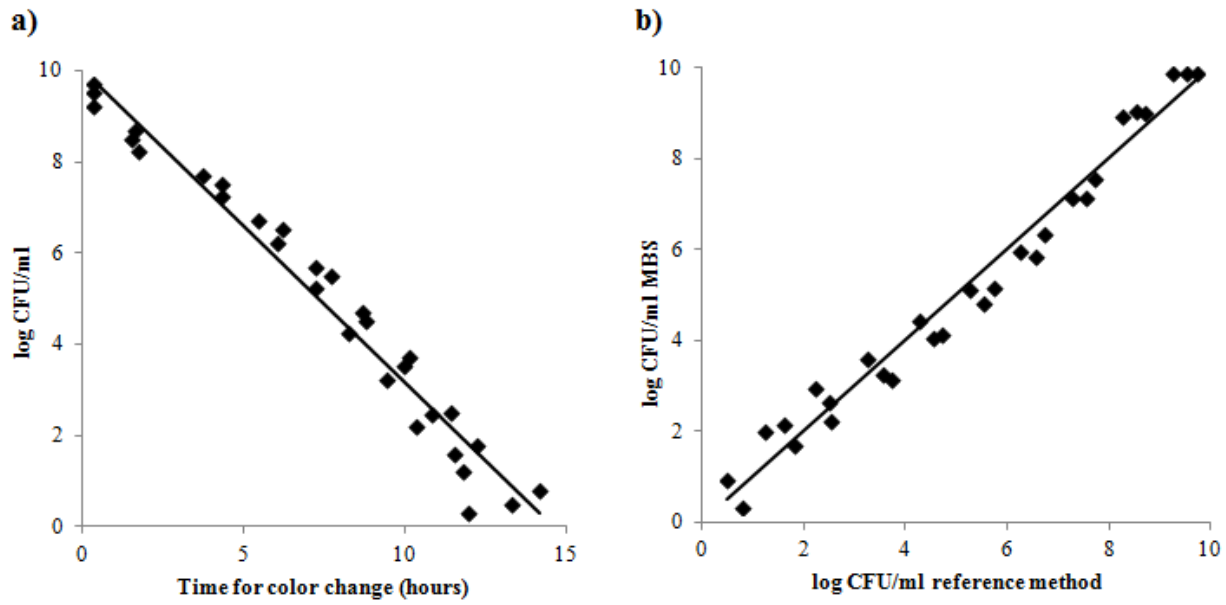

Figure 11. **Linearity and accuracy of the UBC reagent: a) correlation line between the analytes concentration and the time occurred for color change in the MBS UBC vials**. The straight line represents the linear regression analysis including all the points (R^2= 0,96). Nine different dilutions of each bacterial strain were tested in triplicate with the MBS UBC reagent. Each point is the mean of three analyses; **b) Correlation line between the results obtained with the MBS method and the reference method**. Continuous line represents the linear regression analysis: slope=0.91 (R^2= 0.87).

Evaluation of susceptibility/resistance of bacteria to a panel of antibiotics. UBC MBS reagents supplemented with three different antibiotics, chosen among those more frequently used in the treatment of UTIs and prescribed from the Infectious Disease Society of America, were used to evaluate the susceptibility/resistance of bacteria to a panel of antibiotics. Standard inocula (~10^5 CFU/ml) of different ATCC strains (*Escherichia coli, Staphylococcus aureus, Enterococcus faecalis, Pseudomonas aeruginosa*) were tested with UBC vials with and without antibiotics. Results obtained with the MBS method were compared to the ones obtained with the reference Kirby Bauer antibiotic susceptibility test performed by Muller-Hinton agar diffusion commercial disks (Table 8). Bacteria were considered susceptible to an antibiotic if the Multieader did not detect bacterial growth in the specific vial supplemented with the antibiotic of interest; bacteria were considered resistant if the Multieader detected bacterial growth in times comparable to those observed analyzing the same samples with the UBC vial without antibiotic, meaning that bacteria were able to grow regardless of the presence of antibiotics. Bacteria displayed an intermediate

susceptibility to the antibiotic under study if bacteria grew slower in the vial supplemented with antibiotics compared to the UBC vial without antibiotic, meaning that the antibiotic affected bacterial metabolism and growth.

Table 8. Evaluation of susceptibility/resistance of bacteria to a panel of antibiotics. Comparison between results obtained with the MBS method and results obtained with the reference method of Kirby-Bauer. Results of the MBS method were obtained using the UBC vials added with antibiotics (UBC + BAN, UBC + CIP, and UBC+ TMPZ). Experiments were carried out in triplicate .

	Co-amoxiclav		*Ciprofloxacin*		*Co-trimoxazole*	
	MBS	Kirby-Bauer	MBS	Kirby-Bauer	MBS	Kirby-Bauer
E. coli ATCC 25922	S	S	S	S	S	S
E. faecalis ATCC 29212	R	R	S	S	I	I
P. aeruginosa ATCC 27853	R	R	S	S	R	R
S. aureus ATCC 12600	S	S	S	S	S	S

"S" means that bacteria resulted sensitive to the antibiotics under study and the Multireader did not detect bacterial growth. "R" means that bacteria resulted resistant to the antibiotics under study and the Multireader detected bacterial growth. "I" means that bacteria resulted intermediate to the antibiotics under study and the Multireader showed a variability in the results.

The results were in agreement with those obtained with the standard antibiotic susceptibility test and indicated that *E. coli*, *S. enterica* ser. Thyphimurium and *S. aureus* were sensitive to BAN CIP and TMP-SMZ; *E. faecalis* was resistant to BAN, sensitive to CIP and has an intermediate sensitivity to TMP-SMZ; *P. aeruginosa* was resistant to TMP-SMZ and BAN and sensitive to CIP.

4.2.2 Preliminary clinical study of the developed MBS reagents as a POCT, in collaboration with the hospital "Azienda Ospedaliera Sant'Andrea" of Rome

Patients' characteristics. The mean patient age was 77 years old (40-49 years of age, 1%; 50-59 years of age, 11%; 60-69 years of age, 11%; 70-79 years of age, 25%; 80-89 years of age, 37%; 90-100 years of age, 14%). Most of the patients were catheterized (89%) and already under antibiotic therapy (80%). The antibiotics administered to the patients were: 28% Fluoroquinolones, 19% Cephalosporins, 16% Penicillins, 10% Carbapenems, 9% Glycopeptides, 9% Nitroimidazoles, 7% Macrolides, 1% Oxazolidinones and 1% Polymyxins. In few cases, more than one antibiotic was administered to the same patient.

Laboratory results. Urine cultures were performed by the hospital laboratory and usually results were obtained after 48-96 hours. A positive result was found for 34 patients. Fungal infections were found in 10 patients but considered negative for bacterial UTI. Polymicrobic associations between fungi and bacteria, found in only 2 patients, were considered positive. Regarding bacterial UTIs, *Enterococcus faecalis* and *Escherichia coli* were the most frequent causative agents (26% and 20%, respectively), followed by *Klebsiella pneumonia* (11%), *Proteus vulgaris* (9%), *Pseudomonas aeruginosa* (9%), *Staphylococcus aureus* (4%). Polymicrobic associations were observed in 19 patients (56%). Antibiograms performed by the hospital laboratory yielded results 48-96 hours after urine sampling. Resistance to amoxicillin-clavulanic acid, ciprofloxacin and trimethoprim-sulfamethoxazole was observed in 38%, 84% and 69% of the isolates respectively, following the Clinical and Laboratory Standards Institute (CLSI) procedure.

Diagnostic accuracy evaluation of the MBS POCT. To verify the diagnostic performance of the MBS POCT, quantitative results obtained using the UBC vials without antibiotics, in terms of time taken for the vials to change color, were compared to the ones of conventional urine cultures (positive or negative). The comparative analysis was performed using the Receiver Operating Characteristics curve analysis, using MedCalc statistical software (MedCalc software bvba, Belgium) [99]. This analysis is widely used in medicine to determine the validity of a diagnostic test and to find its optimal cut off limit. In particular, the Area under the ROC Curve (AUC) is a measure of how well a parameter can distinguish between two diagnostic groups, which is subjects with and without a UTI. An area under the curve equal to 1 is that of a test that displays 100% sensitivity and 100% specificity.

The AUC of the MBS POCT was 0.93, very close to the theoretical limit of 1, demonstrating the overall accuracy of the method. The combination of optimal sensitivity and specificity was obtained at 5 hours. At this time the maximum percentage of true positive and true negative results was observed with 90.2% accuracy, 91.2% sensitivity, and 89.8% specificity (Figure 12).

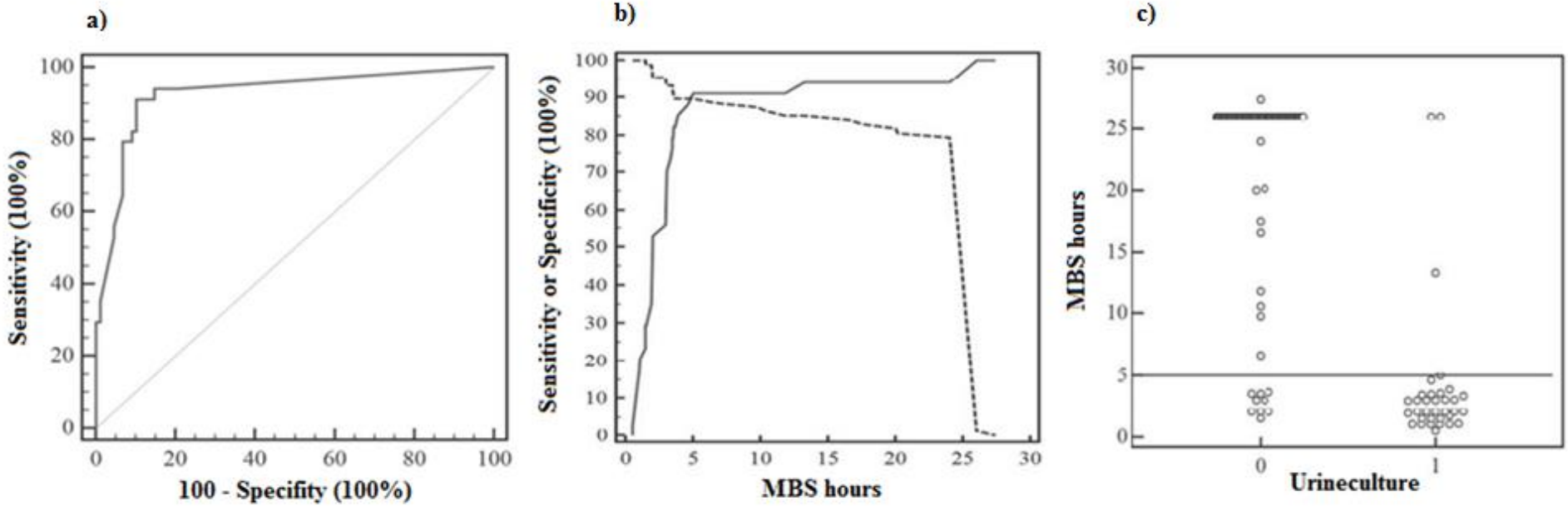

Figure 12. ROC analysis of the MBS POCT. a) Area Under the Curve. The ROC curve shows an AUC = 0.93 with 95% confidence interval 0.873 to 0.981. **b) Sensitivity and specificity of the MBS POCT.** Maximum for combined sensitivity and specificity is obtained at 5 hours. MBS hours stand for the time taken for the vials to change color. The solid line represents sensibility, the dotted line specificity. **c) Dot plot of the MBS POCT.** Distribution of true positive (1) and true negative (0) results. MBS hours stand for the time taken for the vials to change color. The cut-off limit of 5 hours is shown as a straight line.

Comparison between MBS POCT and urine dipstick for UTI diagnosis. The effectiveness of the MBS POCT in diagnosing UTI was also compared to that of urine dipsticks, using the standard urine culture as the reference method. Urine dipsticks are widely used for fast and cheap presumptive diagnosis of UTI. Positivity criteria for UTI were color change within 5 hours in the MBS POCT and positivity for both leukocytes and nitrite in the urine dipsticks. Results are shown in Table 9. It can be seen that there is a poor agreement between urine cultures results and urine dipstick, which yielded 16 false negative and 6 false positive results out of 72 samples analyzed. Notably, a general agreement between results obtained with the MBS POCT and urine cultures was observed. The MBS POCT displayed a much higher accuracy, sensitivity and specificity compared to urine dipsticks. In fact, it gave only 3 false negative and 9 false positive results out of 122 analyses.

Table 9. Comparison between MBS POCT and urine dipstick to diagnose UTIs. MBS POCT and urine dipstick positive and negative results are compared to urine cultures results.

		Urine culture		
		Positive	Negative	Total
MBS POCT (color change in ≤ 5 h)	Positive	31	9	40
	Negative	3	79	82
	Total	34	88	122
		Urine culture		
		Positive	Negative	Total
Urine Dipstick (Leukocytes positive Nitrite positive)	Positive	10	6	16
	Negative	16	40	56
	Total	26	46	72

Accuracy, sensitivity and specificity of urine dipsticks for the diagnosis of UTIs were also evaluated according to Friedman *et al.* [98] and compared to those of the MBS method (Table 10).

Table 10. Statistical evaluation between MBS POCT and urine dipstick to diagnose UTIs. Accuracy, sensitivity and specificity of the MBS POCT and urine dipstick compared to urine cultures results calculated according to Friedman *et al.* [98].

Method	Accuracy (%)	Sensitivity (%)	Specificity (%)
MBS POCT	90.2	91.2	89,8
Urine dipstick	69.4	38.5	87.0

Results demonstrate that the MBS POCT is able to reveal the presence of a significant bacterial load in urine, hence diagnose a clinical UTI, in only 5 hours. More importantly, the MBS POCT showed much higher accuracy, sensitivity and specificity compared to urine dipsticks. It should also be emphasized that positive vials can also be considered as a bacterial pre-culture ready to use for further microbiological analysis, e.g. bacterial identification. This method has therefore the advantage of providing quantitative information on the bacterial load, which is essential for UTI diagnosis, as well as providing samples for identification and susceptibility testing with traditional methods of analysis.

Antibiotic susceptibility testing using the MBS POCT. To verify the possibility to use the MBS POCT for a direct susceptibility testing of infecting bacteria, urine samples of 31 patients, resulting positive for UTI with both UBC vials and urine culture, were tested in parallel with antibiotic-free and antibiotic-supplemented UBC vials. MBS POCT considers positive to bacterial growth the color change of the vials and negative to bacterial growth the lack of color change, within 5 hours. In the susceptibility test, an antibiotic resistance induces a color change (positive to bacterial growth), while susceptibility does not induce a color change (negative to bacterial growth). Results shown in Table 11 were used to determine accuracy, sensitivity, specificity, as well as Positive Predictive Value (PPV) and Negative Predictive Value (NPV) for the MBS vials supplemented with the three different antibiotics (Table 12), relative to the standard CLSI method. Although values varied depending on the antibiotic, a similar trend was observed, resulting in sensitivity>accuracy>specificity for all three antibiotics. Results showed a good concordance of true resistant (true positive) results for the UBC vials supplemented with ciprofloxacin and UBC vials supplemented with trimethoprim-sulfamethoxazole, underlined by a PPV of 89% and 68% respectively; a high presence of false resistant (false positive) results for the UBC supplemented with amoxicillin-clavulanic acid, underlined by a PPV of 44%. The highest probability of a true susceptibility (true negative) result was obtained with the UBC vials supplemented with amoxicillin-clavulanic acid, demonstrated by an NPV of 83%.

Table 11. Comparison between MBS POCT for the evaluation of susceptibility/resistance of bacteria in urine to amoxicillin-clavulanic acid, ciprofloxacin and trimethoprim-sulfamethoxazole and antibiograms results. Resistant for the MBS method = color change; susceptible for the MBS method = No color change

Amoxicillin clavulanic acid		**Antibiogram**		
		Resistant	Susceptible	Total
MBS method	Resistant	11	14	25
	Susceptible	1	5	6
	Total	12	19	31
Ciprofloxacin		**Antibiogram**		
		Resistant	Susceptible	Total
MBS method	Resistant	25	3	28
	Susceptible	2	1	3
	Total	27	4	31
Trimethoprim-sulfamethoxazole		**Antibiogram**		
		Resistant	Susceptible	Total
MBS method	Resistant	19	9	28
	Susceptible	2	1	3
	Total	21	10	31

Table 12. Performance of MBS POCT in antibiotic susceptibility. Accuracy, sensitivity, specificity, Positive Predictive Value (PPV) and Negative Predictive Value (NPV) for the MBS POCT compared to urine culture antibiograms for amoxicillin-clavulanic acid, ciprofloxacin and trimethoprim-sulfamethoxazole calculated according to Friedman *et al*. [21]

	Accuracy	*Sensitivity*	*Specificity*	*PPV*	*NPV*
UBC+ BAN	52 %	92 %	26 %	44 %	83 %
UBC+ CIP	84 %	93 %	25 %	89 %	33 %
UBC+ TMP-SMZ	65 %	90 %	10 %	68 %	33 %

The results obtained with the MBS POCT for the evaluation of susceptibility/resistance to antibiotics were not so satisfying. Conventional antibiotic susceptibility tests must be performed using a standard bacterial inoculum, and the activity of antimicrobial agents is referred to that inoculum. This condition could not be reproduced in the MBS POCT analysis. The greater concentration of bacteria occurring in the MBS vials was affected by the antibiotic of interest to a minor extent, leading to a partial misinterpretation of results. However, it should be emphasized that the detection of false resistant strains is much less significant than the detection of false susceptible strains.

4.2.3 Optimization of the MBS UBC reagent composition and the operating procedure for the evaluation of susceptibility/resistance to antibiotics, using the optimized UBC reagent added with antibiotics

The preliminary clinical trial performed in collaboration with the "Azienda Ospedaliera Sant'Andrea" demonstrated the great potential of the MBS POCT as a diagnostic tool for a rapid and accurate detection of bacteria causing UTIs but also pointed out its limits.

The UBC reagent for the diagnosis of UTIs showed very high accuracy, sensitivity and specificity but displayed some false negative results. Furthermore, the results obtained with the UBC reagents for a presumptive evaluation of bacterial susceptibility/resistance to a selected panel of antibiotics

were not fully satisfying. The reasons behind these analytical problems however were linked only in part to the specific composition of the reagent and more deeply to the operating procedures and the manufacturing processes.

Different issues linked to the specific composition of the reagent, the operating procedures, the manufacturing process and the development of the new optical reader with particular attention to the investigation and verification of the best parameters for detection were examined. All these factors, in fact, contribute to the overall performance of the MBS POCT and needed to be optimized and standardized in order to meet the essential premarketing requirements mandatory for all IVDs.

Optimization of the MBS UBC reagent composition. The composition of the UBC reagent needed to be optimized in order to decrease the presence of false positive results. Different ingredients, especially growth promoting agents that could favor growth of slow-responding bacteria, were tested alone or in combination at different concentrations. In order to find the optimal formulation, sensitivity tests were performed on different bacterial ATCC strains (data not shown). The obtained results led to the development of an optimized UBC formulation that promoted the growth of bacteria, improved the color change behavior without penalizing the starting and end color and the pH (Table 13).

Table 13. Composition of the optimized UBC reagent.

Reagents	*g/l*
Nitrogen source B	5
Nitrogen source C	15
Nitrogen source Ye	1
Carbon source S	1
Buffer system He	10
Buffer system Tr	2,5
Redox indicator T	0,5
Oxidizing agent FeCN	0,1
Growth promoting agent SP	0,5
Growth promoting agent AC	1

The sensitivity of the optimized UBC reagent was compared to the one of the previous UBC reagent for different ATCC strains (*Escherichia coli, Enterococcus faecalis, Pseudomonas aeruginosa*), tested in the first phase, and for a clinical *Staphylococcus aureus* isolate that gave a false negative result during the clinical trial. The same samples were analyzed also with the reference method in order to verify the presence of bacteria. The results obtained are reported in Table 14.

Table 14. Sensitivity testing of the optimized MBS UBC reagent. Results obtained analyzing with the UBC reagent different concentrations of *E. coli* (ATCC 25992), *S. enterica* ser. Thyphimurium (ATCC 14028), *E. faecalis* (ATCC 29212), *P. aeruginosa* (ATCC 27853*)* and a clinical isolate that gave a false negative result during the clinical trial [1].

	Colony count TSA (CFU/ml)	UBC		Optimized UBC	
		TC (h)	*SD*	*TC (h)*	*SD*
S. aureus (Clinical specimen)	~10^8	1,67	±0,4	1,47	±0,4
	~10^6	5,66	±0,6	3,34	±0,4
	~10^4	8,81	±0,5	5,61	±0,5
E. coli ATCC 25922	~10^8	1,10	±0,4	1,19	±0,4
	~10^6	2,55	±0,4	2,41	±0,3
	~10^4	5,48	±0,6	5,13	±0,4
E. faecalis ATCC 29212	~10^8	1,87	±0,4	1,61	±0,4
	~10^6	3,22	±0,6	3,28	±0,4
	~10^4	6,03	±0,5	6,27	±0,5
P. aeruginosa ATCC 27853	~10^8	1,45	±0,4	1,59	±0,3
	~10^6	2,69	±0,5	2,70	±0,5
	~10^4	5,51	±0,4	5,50	±0,4

TC=Time for color change; SD= Standard deviation

[1]*Three different dilutions of each bacterial strain were tested in triplicate. The color of the MBS vials changed from blue to yellow. The values are mean ± SD of 5 experiments carried out in triplicate.*

The optimized UBC reagent gave very good results tested with collection strains and was able to facilitate the growth and decrease the time of detection of the clinical isolate that was slow responding in the clinical trial.

Linearity and accuracy of the optimized UBC MBS reagent. Linearity and accuracy of the optimized UBC reagent were determined for all bacterial strains and the clinical isolate. The quantitative results obtained with the MBS method in terms of time taken for the vials to change color were compared with the results obtained with the reference method of plate count on Tripticase Soy agar.

This was achieved graphically as illustrated in Figure 13 (a) by plotting bacterial concentrations (expressed as the log of CFU/ml) obtained with the reference method with the time occurred for color change of the UBC vials. A linear inverse relationship between the MBS method and the bacterial concentration can be observed. The equation of the curve and correlation factor (R^2) were calculated (Table 15).

Table 15. Equation of the linearity line and correlation factor (R^2) for the optimized UBC reagent.

	Linear equation	R^2
UBC	y = -0,71x + 10,2	0,97

The equation of the curve was used to determine, from the time occurred for color change, the bacterial concentrations (expressed as the log of CFU/ml) so as to demonstrate the accuracy of the MBS method. A very good correlation between the bacteria number (expressed as log CFU/ml) obtained with the traditional method of plate count and the alternative MBS method was observed (Figure 13 b). The straight line obtained was close to the ideal y = x (slope = 1.00), with a correlation factor (R^2) which further confirm the high equivalence between the reference method and the alternative MBS method.

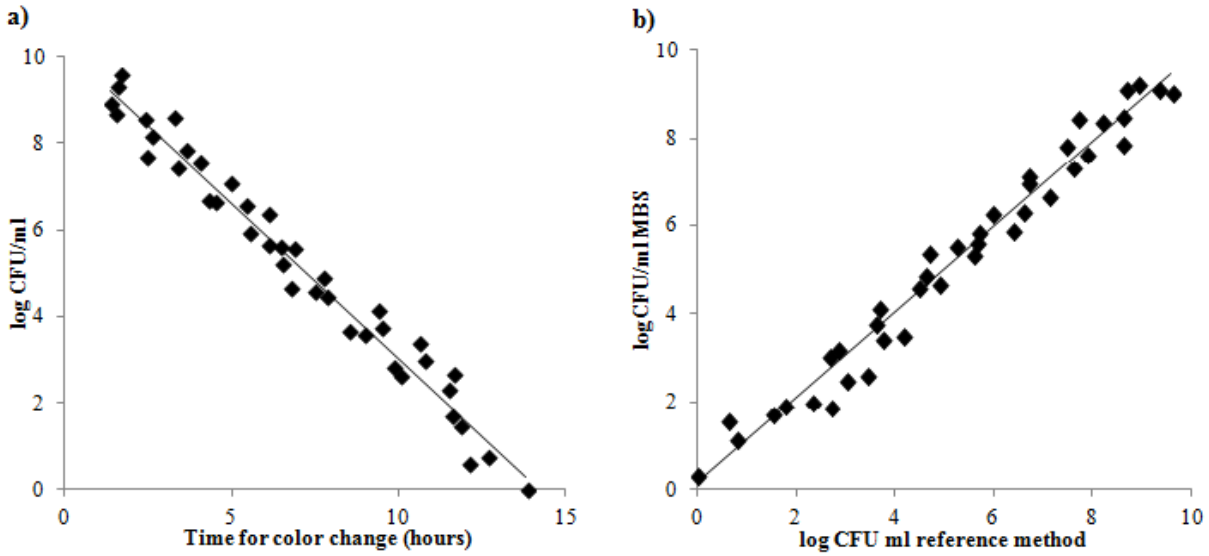

Figure 13. **Linearity and accuracy of the optimized UBC reagent: a) correlation line between the analytes concentration and the time occurred for color change in the UBC vials**. The straight line represents the linear regression analysis including all the points (R^2= 0,97). Nine different dilutions of each bacterial strain were tested in triplicate with the MBS UBC reagent. Each point is the mean of three analyses. **b) Correlation line between the results obtained with the MBS method and the reference method**. Continuous line represents the linear regression analysis: slope=0.97 (R^2= 0.97).

Optimization of the analytical procedure for the evaluation of susceptibility/resistance to antibiotics using the UBC reagent added with antibiotics. For the UBC vials added with antibiotics the aim was to find a new operating procedure that could improve results of the evaluation of bacterial susceptibility/resistance to antibiotics. The main reason behind the unsatisfactory MBS method results in the clinical trial is due to the MBS POCT detection of many false resistant results, probably as a consequence of a very high concentration of bacteria inoculated in the vials. From the results obtained analyzing urine samples collected from patients with confirmed urinary tract infections, the concentration of infecting bacteria was always much higher than the standard bacterial concentration that was tested in the in vitro phase. Probably the bacterial metabolism was detected before antibiotics had an effect, leading to a color change of the vial even if inoculated with bacteria susceptible to the tested antibiotic. The inoculum size has a profound impact on antibiotic susceptibility results [100]: increasing inoculum size can diminish the activity of certain antimicrobial agents. The inoculum size has, in fact, been recognized as the single most important variable in susceptibility testing for both bacteriostatic and bactericidal antibiotics [101]. Experiments were carried out in order to find the best size of bacterial inoculum. Different sizes of

bacterial inocula of artificially contaminated urine samples were tested with the UBC vials with and without antibiotics. The contamination mimicked the one of naturally contaminated urine samples. In Table 15 are reported the results obtained testing a resistant (*Enterococcus faecalis*) and a susceptible (*Pseudomonas aeruginosa*) strain.

Table 15. Evaluation of the best inocolum size to test with the optimized MBS UBC reagent added with antibiotics. Results obtained analyzing with the optimized UBC reagent added with antibiotics (UBC+BAN) with different inocula of *E. faecalis* (resistant strain) and *P. aeruginosa* (susceptible strain) [1]

		E. faecalis (resistant)				*P. aeruginosa (susceptible)*			
		UBC		UBC+ BAN		UBC		UBC+ BAN	
Inocula	CFU/ml	*TC (h)*	*SD*	*TC (h)*	*SD*	*TC (h)*	*SD*	*TC (h)*	*SD*
1ml	~ 10^8	1,52	±0,3	4,86	±0,4	1,28	±0,3	1,71	±0,4
0,5 ml	~ $5x10^7$	1,95	±0,3	5,51	±0,4	1,80	±0,4	1,62	±0,5
4 drops	~ 10^7	3,00	±0,4	11,44	±0,5	2,35	±0,3	3,46	±0,5
1 drop	~ $5x10^6$	3,82	±0,4	NCC	±0,5	3,37	±0,4	3,78	±0,5

TC=Time for color change; SD= Standard deviation; NCC= No color change

[1]*Four different inocula of each bacterial strain were tested in triplicate with the MBS UBC reagent. The values are mean ± SD of 5 experiments carried out in triplicate.*

The inoculum of 1 drop appeared to be the best to discriminate between susceptible and resistant bacteria. When tested with increasing sizes of inocula, in fact, even though the antibiotic was able to affect bacterial growth increasing the time for color change of the susceptible strain, it was not able to inhibit completely bacterial growth. In some cases (inoculum size equal to 1ml or 0,5 ml) also for susceptible bacteria color change occurred within 5 hours, leading to a false resistant result. This inoculum was used to test the susceptibility resistance of different bacterial strains to antibiotics. Results obtained with the MBS method were compared to the ones obtained with the reference Kirby Bauer antibiotic susceptibility test performed by Muller-Hinton agar diffusion commercial disks (Table 16).

Table 16. Evaluation of susceptibility/resistance of bacteria to a panel of antibiotics using inocula of 1 drop. Comparison between results obtained with the MBS method and results obtained with the reference method of Kirby-Bauer. Results of the MBS method were obtained using the optimized UBC vials added with antibiotics (UBC + BAN, UBC + CIP, and UBC+ TMPZ). Experiments were conducted in triplicate .

	Co-amoxiclav		*Ciprofloxacin*	
	MBS	Kirby-Bauer	MBS	Kirby-Bauer
E. coli	R	R/S	S	S
E. faecalis	S	S	S	S
P. aeruginosa	R	R	S	S
S. aureus	S	S	S	S

"S" means that bacteria resulted sensitive to the antibiotics under study and the Multireader did not detect bacterial growth. "R" means that bacteria resulted resistant to the antibiotics under study and the Multireader detected bacterial growth.

The results obtained with the new operating procedure were in good agreement with the ones obtained with the reference method. Results were always obtained in less than 8 hours and no susceptible bacteria caused color change in the reaction vials.

4.2.4 Study of a new standardized manufacturing process

As prescribed by law, all in vitro diagnostic medical devices (IVDs) must be designed and manufactured in order to achieve standardized performances in terms of analytical sensitivity, diagnostic sensitivity, analytical specificity, diagnostic specificity, accuracy, repeatability and reproducibility. The original manufacturing procedure did not assure these features and currently could not obtain authorization to enter the market. Lyophilization appeared to be a new manufacturing process that could assure maximum reproducibility, greater stability and certified sterility, as required by law for all IVDs. UBC MBS vials were produced according to the new production process by Sclavo Diagnostics S.r.l. Glass instead of plastic vials were used according to the standard procedure.

Linearity and accuracy of the MBS UBC lyophilized vials. The new vials containing the lyophilized reagent were tested in order to verify if the different production process could modify

the analytical performance of the test or influence the detection parameters. Linearity and accuracy of the lyophilized UBC vials were evaluated. Experiments were conducted in parallel using also plastic UBC vials produced according to the standard MBS procedure. Approximately the same results were obtained (data not shown) demonstrating that the lyophilization process did not interfere with the analytical performance.

Stability tests of the UBC MBS lyophilized reagent. Stability studies evaluate how the quality of a determined product is influenced by time and environmental factors such as temperature, humidity and light. Stability tests were performed in order to determine the stability of the lyophilized reagent. Lyophilized vials were stored at room temperature and 4°C for 240 days and the performance was evaluated after 1, 2, 4, 6 and 8 months. Physico-chemical tests were performed to evaluate the intrinsic properties of reagents. Results of the physico-chemical tests confirmed the stability of the reagent: starting and end color of the vials remained the same up to 8 months in both storage conditions and also pH values did not vary significantly (data not shown).

Sensitivity tests were performed in order to evaluate the analytical performance of the lyophilized reagent (Figure 14). The quantitative results obtained with the different vials in terms of time taken for the vials to change color were compared with the results obtained with the reference method of plate count on Tripticase Soy agar. Control analyses were carried out with vials that were lyophilized a few days before. The equations of the curve and correlation factor (R^2) were calculated in order to verify if the different storage conditions could modify the analytical performance (Table 17).

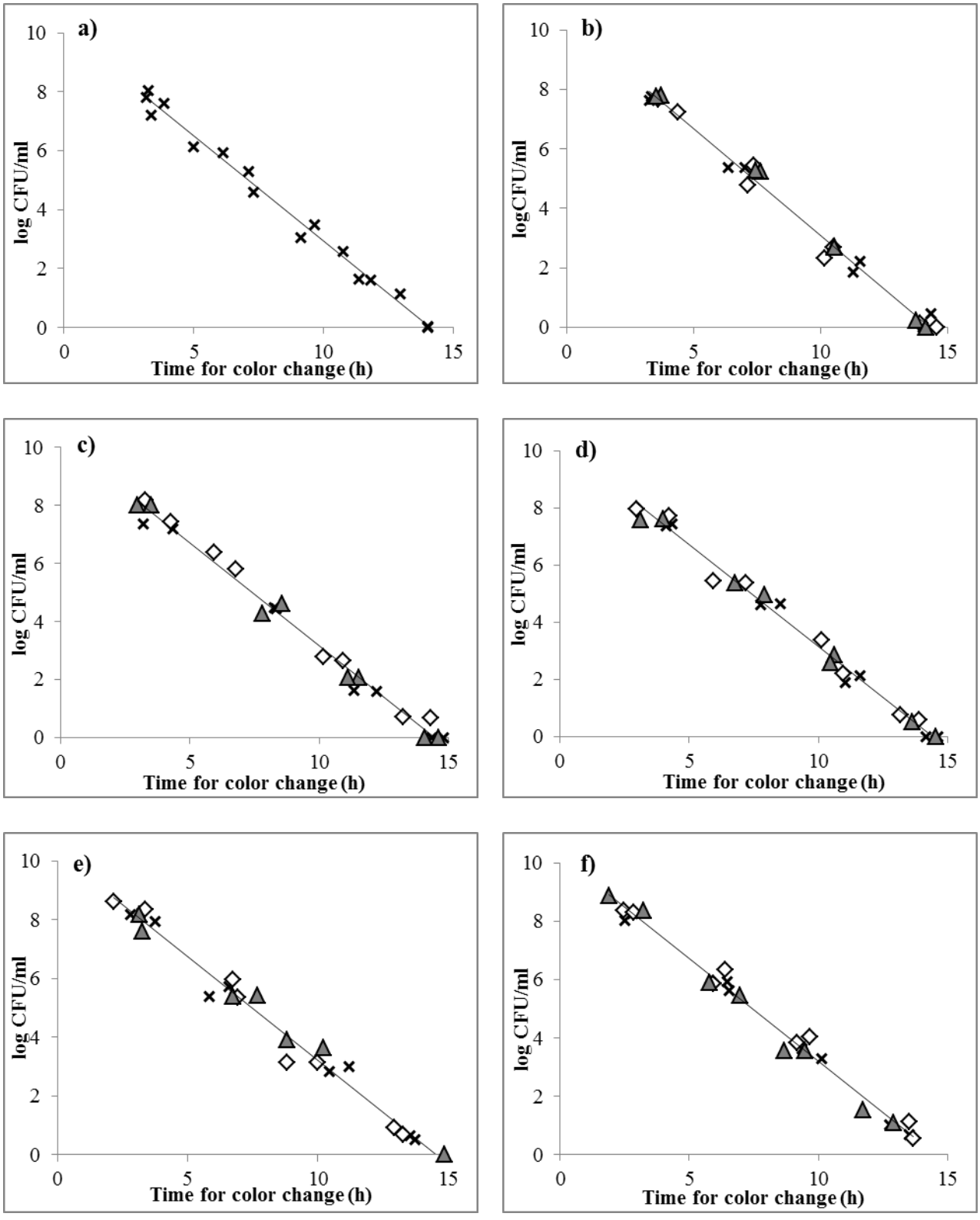

Figure 14. Sensitivity stability tests. The quantitative results obtained with the different vials in terms of time taken for the vials to change color were compared with the results obtained with the reference method. Each point is the mean of three analyses. X= CTRL lyophilized vials; Δ = lyophilized vials kept at 4°C;◇ = lyophilized vials kept at room temperature. a) T=0; b) T= 1 month; c) T= 2 months; d) T= 4 months; e) T= 6 months; f) T= 8 months.

Table 17. Equations of the linearity lines and correlation factors (R^2) of the lyophilized UBC vials stored at different conditions after 1, 2, 4, 6 and 8 months.

	Linear equation	R^2
T=0	y = -0,71x + 10,04	0,99
T= 1 month	y = -0,72x + 10,20	0,99
T= 2 months	y = -0,71x + 10,27	0,99
T= 4 months	y = -0,72x + 10,27	0,99
T= 6 months	y = -0,70x + 10,27	0,98
T= 8 months	y = -0,70x + 10,24	0,99

The analytical performance of lyophilized vials remained the same up to 8 months for both conditions so that storage can be confidently safe up to 1 year at room temperature and up to 2 years at 4°C, doubling the storage time of the powdered reagent.

4.2.5 Set up of the optimal detection parameters of the MBS Multireader

The MBS Multireader is a modular optical device that is made to incubate multiple samples, automatically detect the color change of the reaction vials and calculate the bacterial concentration in the sample. The output generated by the software is a curve that correlates intensities of the signals with time. Curves behavior can be analyzed taking into consideration mainly two parameters: the intensity coefficients C of the three signals (Red, Green and Blue) that increase significantly during the color change and the first derivative or slope of the three curves (Red, Green and Blue) that are used to find the exact point of color change (Figure 15).

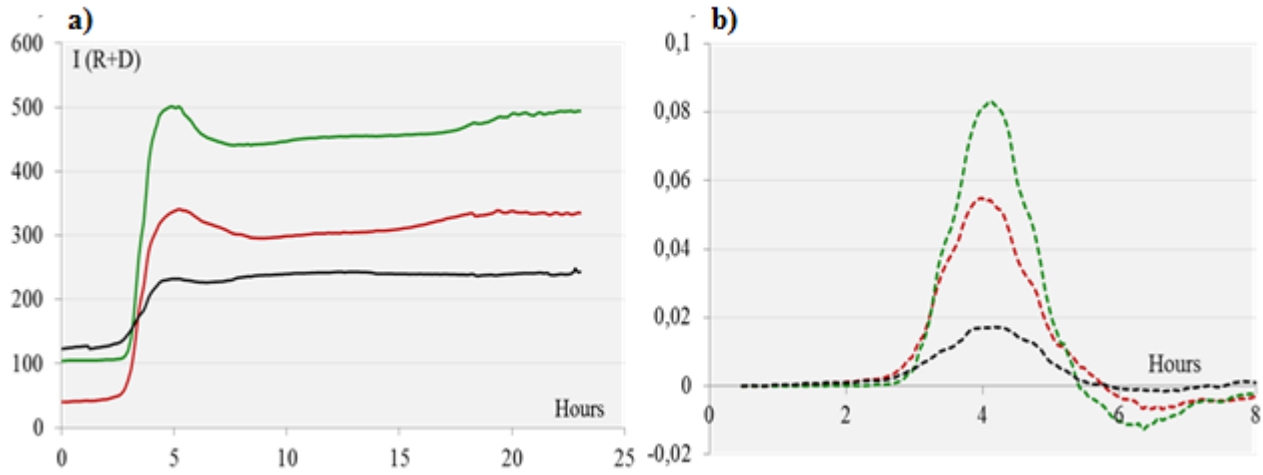

Figure 15. MBS MR output. a) Intensity coefficients (Red, Green and Blue); b) slopes (Red, Green and Blue curves)

A specific algorithm that specifies the stop conditions required for each analysis must be implemented in the software for each reagent. This algorithm was not already available for UBC analysis because each reagent acts in a different way. Till now for all experiments the time taken for the vials to change color was defined empirically at the end of the analysis. This was possible because the software output detected the entire progression of the curve and the absolute maximum value was easily visualized. However, in order to provide results immediately it was necessary to identify the best stop conditions. The most significant signals, the lowest signal amplification coefficients and their slope conditions (threshold limit, time of decrease and percentage of decrease) needed to be specified to allow the software to discriminate relative from absolute maximum values, exclude noise and identify precisely the time taken for the vials to change color and stop at that time.

A statistical evaluation of the best detection parameters was performed in order to define the best calculation algorithm. 300 curves were analyzed using the Fast Loader Research software that was set up in order to test 3600 combinations. The percentage of success for each combination was calculated (Figure 16).

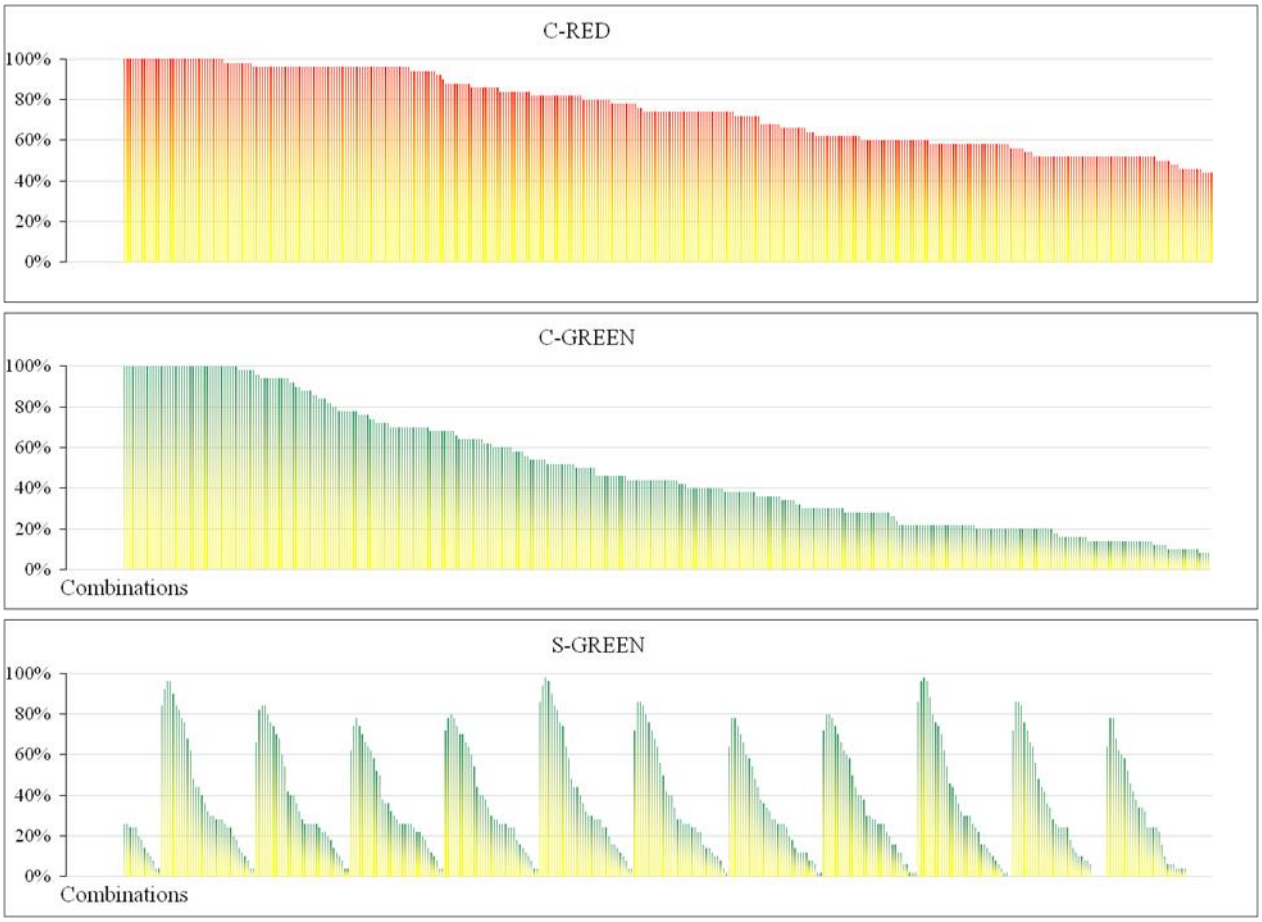

Figure 16. Percentage of success of the stop conditions referred to the intensity coefficients of the Red and Green signals and the Green slope.

The obtained results showed that the most significant parameters were the intensity coefficients of the Red and Green signals and the Green slope. In particular, the lowest signal amplification coefficient was of x2.5 for the red signal and x3 for the green signal.

The optimal Green slope conditions were:

- threshold limit = 0,013
- time of decrease = 2700 or 3600 seconds
- percentage of decrease = 0.4%.

The detection parameters found for the MBS MR ensure the maximum sensitivity of the optical reader. They were used to set up a new optical reader, called MR4. This new optical reader was developed in collaboration with the Department of Physics of "Tor Vergata" University and was designed according to the requirements of all relevant medical device directives in the European Union. It maintains the same optical detection and incubation systems and shares with the previous reader the principle of acquisition of data, the calculation algorithm and the software. What changes

drastically is the way data are processed and elaborated. In fact, whereas in the old reader all the elaboration was performed by the computer, the new MR4 reader includes an advanced calculator that enables the device itself to perform all calculations without the need of a computer. Moreover, the device is furnished with a TDK lambda power supply that can be used in medical settings (Figure 17). These features meet the ultimate goal of the study, which was to develop a Point of Care Test that could be completely standalone – not computer managed – and used in a medical setting not compromising, directly or indirectly, the clinical condition or the safety of the patients or the safety or health of users.

Figure 17. MR4 new optical reader. a) external view; b) motherboard

5. CONCLUDING REMARKS

Alternative rapid microbiological methods are based on technologies which can be growth-based, viability-based, or surrogate-based cellular markers for a microorganism (e.g. nucleic acid-based, fatty acid-based) and include any microbiological technique or process that increases the speed or efficiency of isolating, culturing, or identifying microorganisms when compared with conventional methods. They have been developed in order to provide more rapid, sensitive, accurate, precise, and reproducible test results when compared with conventional, growth-based methods. Moreover alternative methods should aim to simplify analytical procedures and interpretation of results and help laboratories reduce worker subjectivity, increase labor efficiency and reduce errors. They should require minimal training and equipment, be stand-alone and ensure the most error prone processes. Unfortunately not all alternative methods share these features. Most rapid methods are technically more complex than culture methods and require skilled personnel and equipped laboratories. They require high upfront capital investments, and the cost per test is high compared with that of culture tests.

The MBS method is an alternative system for selective counting of bacteria. It is based on a colorimetric survey performed in single-use disposable reaction vials in which samples can be inoculated without any preliminary treatment. The MBS method measures the catalytic activity of redox enzymes in the main metabolic pathways of bacteria, allowing an unequivocal correlation between the observed enzymatic activity and the number of viable cells present in the samples. The time required for a color change is inversely related to the logarithm of the bacterial concentration. The MBS method has been developed in order to meet the need to provide reliable results, thus diminishing the time of analysis, facilitating procedures and interpretation of data, increasing the detection sensitivity, limiting costs and allowing analysis also in the absence of a laboratory. The cost of consumables and equipment is very competitive compared with other microbiological kits and traditional methods of analysis and the decreased cost of materials is also accompanied with a decrease in labor costs.

The aim of my PhD work was to study two different applications of the method that clearly demonstrate its potential. The MBS method was used as an alternative method for microbiological analysis of drinking water and was applied on field as a point of use test to assess water quality in developing countries. Moreover it was employed for the detection of bacteria in urine and a preliminary evaluation of their susceptibility/resistance to antibiotics and tested in a preliminary clinical trial as a point of care test for diagnosis and management of Urinary Tract Infections.

In both cases the need to obtain rapid, accurate, reliable results in a simple, pass–fail approach, was a central issue to facilitate investigations, allow fast critical decisions and corrective actions.

In the context of the analysis of potable water the MBS method has proven to be a valid method to frequently evaluate the microbiological quality of many water sources since it is accurate, sensitive, easy to use and permits rapid assessment limiting costs of instrumentation and personnel. Compared to other rapid microbiological test kits currently used for water analysis the MBS method represents a unique technology, which stands above others for ease of use, analytical flexibility, simultaneous detection and quantification of bacteria along with a broad quantification range. Moreover the stand alone equipment does not need a dedicated space and can be easily used by untrained technicians. Its simple procedure and interpretation of results can be suitable for use in rural areas by local personnel, operating without a microbiological laboratory.

Concerning the use of the MBS method as a Point of Care Test (POCT) for diagnosis and management of Urinary Tract Infections, the MBS POCT resulted as a valuable diagnostic tool for a rapid and accurate detection of bacteria causing UTIs. Thanks to this method it could be possible to have, in a relatively short time compared to standard methods, an accurate indication of UTI and a preliminary evaluation of the antibiotic susceptibility of the infecting bacteria, ensuring a prompt diagnosis and guiding the antibiotic choice long before the conventional antibiotic susceptibility test is performed. The optimization process resulted in an improvement of different issues linked to the specific composition of the reagent, the operating procedures and the manufacturing process contributing positively to the overall performance of the MBS POCT and leading to a standardization that meets the essential premarketing requirements mandatory for all In Vitro Diagnostic Devices. Moreover the set up of the new optical reader was the final step in the development of a POCT completely standalone, not computer managed, which can be used in a medical setting not compromising, directly or indirectly, the clinical condition or the safety of the patients nor the safety or health of users. Its introduction into routine use in the Emergency Department could thus improve turn-around time for the medical decision making to give or not antibiotic treatment, drastically decreasing the time required to achieve the laboratory-based diagnosis, improving patients' outcome, reducing antibiotic resistance, and ultimately saving money.

In conclusion the MBS method has proven to be an effective, reliable and precise alternative to traditional microbiological testing, confirmed in laboratory settings and on field trials. It has also been demonstrated that the solid principles of the MBS method make it extremely flexible and adaptable to completely different needs, allowing simplified, rapid, automated, reliable solutions and achieving health-effective and productive results.

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7. APPENDIX

PUBLICATIONS:

Bottini G., Losito F., **Arienzo A**., Priolisi F. R., Mari A., Visca P. and Antonini G. A New Method For Microbiological Analysis That Could Be Used For Point-Of-Care Testing (POCT). *The Open Emergency Medicine Journal*. **2013**;5:13-15.

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