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A New Direct Detection System for Antibiotic Resistant Bacteria

Stephen C. Edberg and J. Michael Miller

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1. Introduction

A new system based on the phenotypic expression of antibiotic resistance has been developed. The system, the Defined Substrate Utilization® (DSU®) process, enhances the metabolism of the target microbe while providing inadequate nutrients for the non-target microbes. Accordingly, the target microbe multiples to a much greater extent than the non-target microbes and this differential allows for the specific detection of the target microbe. The non-target microbes multiply insufficiently to be noticed. Incorporated in the DSU® screening tools are substrates, that when metabolized, produces a sensible signal such as color or luminescence. In addition, specific inhibitors to the non-target microbes are included in the formulas. This format allows a more sensitive screen than parallel agar-based methods. In terms of time to positive, the liquid (once the powder is hydrated with sterile water), coupled with the optimization of nutrients generates results in the same time frame as genetic amplification tests. The mixing of the ingredients in powder form permits a much less expensive individual screen than either conventional agar-based or genetic amplification methods. In addition, the format permits utilization of the DSU® tools both in the remote field environment or the clinical microbiology laboratory.

There are two basic protocols utilized for the epidemiological screening of microbes. The first is the conventional agar-based culture technique. Here a specimen is plated on one or more selective agars and incubated 24 to 48 hours. A trained medical technologist examines the colonies and those compatible with the target microbe are subjected to specific biochemical and/or immunochemical testing, and antibiotic susceptibility procedures. These may require from 24 to 72 hours incubation [1]. In an attempt to facilitate the culture protocol, chromogenic substrate media have been introduced for some, but not all, bacteria of epidemiological interest. Chromogenic substrate agars have a short shelf life, must be stored cold and often in
the dark, and the colors differentiating the positive and negative colonies may not always be
distinct, necessitating confirmatory testing [2]. They are generally held 48 hours before calling
a negative, although 48 hours can result in false-positives [3, 4].

The second technique utilizes genetic amplification (GA) to detect the target microbe. A
number of companies make different types of GA. However, in common to them all are: each
requires an expensive instrument, utilizes reagents which are expensive, requires highly
skilled labor in a centralized laboratory, and does not recover living bacteria [5]. In addition,
each is subject to false-positives and false-negatives because of unexpected mutations [6, 7].

A new and novel, one-step, direct epidemiology screening method based on classical pheno-
typic parameters was developed as an alternative to conventional agar-based methods for a
variety of antibiotic resistant bacteria. For example, for *Staphylococcus aureus* (S. aureus), the
key enzyme is coagulase and specificity is achieved equal to that of conventional identification
schemes [8]. The method of Defined Substrate Utilization® (DSU®) optimizes specific, selective,
and differential biochemicals in powder form. Hence, it overcomes the observation by Selepak
and Witebsky that the lot-to-lot variation of commercial plasma was too variable to be used
directly from patient specimens [9]. For each target microbe, an optimum background of
nutrients and inhibitors was generated tailored to the biochemical physiology of that bacteri-
um. Accordingly, the target microbes are preferentially fed while the non-target microbes are
in a nutrient deficit.

The powder format allows optimization of ingredients much more precisely than an agar gel.
Moreover, the overall sensitivity of the DSU® method is enhanced since liquid culture (once
the powder is hydrated with sterile water) detects a lower number of colony forming units
(CFU) than on the surface of agar [10, 11]. The DSU® tools are generally positive from a colony
within 4 to 5 hours and from a human or animal sample in 6-9 hours on average. The bacteria
remain viable in the screens and thus provide the opportunity to further evaluate the target
microbe.

In addition to be able to be done manually by visual observation, an inexpensive instrument
was developed to read and analyze results of all the screens (Pilots Point Monitor 60®, Pilots
Point LLC, Sarasota, FL). One may add any combination of any of the DSU® screening tools
into the instrument and results are generated by an algorithm controlling the analysis. Results
can be sent to cell phones, via Wi-Fi, or in any format to any location. For example, the
instruments may be stationed at various clinics.

This paper shall describe in detail four of the epidemiological screens: methicillin-resistant S.
aureus (MRSA), all S. aureus including MRSA and methicillin-susceptible S. aureus (MSSA),
vancomycin-resistant enterococci (VRE), and carbapenemase-resistant Enterobacteriaceae
(CRE). Screens for vancomycin-intermediate resistant S. aureus (VISA), and ciprofloxacin-
resistant (cipR) bacteria will be described briefly as subsets of the above. In addition, the
principles and function of the instrument will be presented.

While this paper presents six of the DSU® epidemiology screens, the format and phenotypic
expression in the presence of defined nutrients, allows for the rapid development of others.
2. The Defined Substrate Utilization® Method

2.1. Screening Method for *S. aureus*: Both Methicillin-susceptible (MSSA) and Methicillin-resistant (MRSA)

2.1.1. EPI-M® and aureusAlert® — Quantitative analysis with pure and anterior nares cultures

[EPI-M® is the DSU® epidemiology screen for MRSA, aureusAlert® is the DSU® epidemiology screen for all S. aureus – both MSSA and MRSA (Pilots Point LLC, Sarasota, FL)]

2.1.1.1. Pure cultures

S. aureus ATCC 43300 (MRSA), S. aureus ATCC 34591 (MRSA), S. aureus ATCC 25923 (MSSA), S. aureus ATCC 29213 (MSSA), S. epidermidis ATCC 12228, the MRSA USA 600 (6 isolates), MRSA USA 300 (2 isolates), and MRSA USA 100 (2 isolates), plus 5 clinical patient laboratory isolates of MRSA and 5 of MSSA were utilized.

A suspension of each of the bacterial isolates was made in normal physiological saline to a 0.5 McFarland standard. From this suspension, 0.1 mL was transferred to 9.9 mL of sterile normal saline and vortexed well. Using a quantitative pipette (Rainin, Rainin Instrument LLC, Oakland, CA) 1 and 10 microliters of the suspension was transferred to aureusAlert® and EPI-M® and a dilution series generated. Colony counts were made from the 0.5 McFarland standard and the 9.9 mL normal saline [8].

2.1.1.2. Human surveillance study

Nasal swabs (Culturette™ II, Becton, Dickinson and Company, Cockeysville, MD) were obtained from people entering or leaving a building at the following locations: Yale-New Haven Hospital (New Haven, CT, USA); Walter E. Washington Convention Center (Washington, D.C., DC, USA), George Washington University Hospital (Washington, D.C., DC, USA) and Tampa General Hospital (Tampa, FL, USA). Two swabs were obtained from the same nostril.

2.1.1.3. Conventional procedure

*S. aureus*: Volunteer nasal swab specimens were aseptically transferred to Trypticase Soy Broth (TSB) containing 6.5% NaCl for 24 hours enrichment at 33-35°C. The enrichment samples were subcultured on to Tryptic Soy Sheep Blood Agar (TSBA) plates, and the plates were incubated aerobically for 24 hours at 35°C. Suspicious colonies of *S. aureus* were identified by using standard laboratory methods including catalase, tube or slide coagulase, Gram staining, and mannitol salt agar. Confirmed *S. aureus* colonies were tested for methicillin resistance using the CLSI recommended reference method (e.g., 30 mcg cefoxitin disk) [12]. In addition, an assay for PBP-2 (Remel, Lenexa, KS) was performed.

*MRSA*: Cultures were inoculated into TSB with 6.5% NaCl and incubated for 18-24 hours. A subculture was made to blood agar (BA) plates and a Staphaurex® (Remel, Lenexa, KS) test
performed. In addition, an assay for PBP-2 (Remel, Lenexa, KS) was performed. Antibiotic susceptibility test (AST) was performed by both an agar dilution method (Mueller-Hinton agar with 4 mcg/mL oxacillin, Becton, Dickinson and Company, Cockeysville, MD) and also by the Sensititre™ (Trek Diagnostic Systems, Oakwood Village, OH) microdilution method.

2.1.1.4. Defined Substrate Utilization® screens

All ingredients of the Defined Substrate Utilization® screens are optimized in powder form in a tube and are performed in the same way. Each tube is labelled with the name of the screen, and a specific amount of water is transferred to the tube to hydrate the powder. The sample is added to the tube and incubated at 35°C. A positive result, which can occur any time after the initiation of incubation, is seen as follows:

**Interpretation:**
- **aureusAlert®** – a clot or coalescence forms in the liquid; no observable clot or coalescence is a negative result.
- **EPI-M®** – the liquid changes color from straw-colored to amethyst with an increase in coalescence; no change from straw-colored is a negative result.

Tubes are held a maximum of 24 hours before calling them negative.

Control S. aureus standards ATCC 25923 (MSSA) and ATCC 43300 (MRSA) plus 5 MSSA and 5 MRSA isolates from patients were tested. MSSA and MRSA isolates were diluted from 7 log10 to 1 log10 and incubated at 35°C and 23°C.

Based on quantitative analysis of pure cultures, the Defined Substrate Utilization® screens were able to detect as low as 20 CFU (18 hours of incubation) with both MSSA and MRSA (Table 1; Table 3). Tables 2A and 2B, and Tables 4A and 4B, present the results of the nasal screening of normal subject volunteers. For both the detection of S. aureus and MRSA there was no difference between the Defined Substrate Utilization® screens and conventional methods. aureusAlert® and EPI-M® showed a specificity of 100%.

2.1.2. **aureusAlert® for all S. aureus (MSSA & MRSA)**

![Figure 1. S. aureus Negative – Liquid](image)
Figure 2. S. aureus Positive – Clot

<table>
<thead>
<tr>
<th>MSSA ATCC 25923 (CFU/mL)</th>
<th>Detection Time at 35°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>7 log10</td>
<td>2.0 h</td>
</tr>
<tr>
<td>6 log10</td>
<td>3.0 h</td>
</tr>
<tr>
<td>5 log10</td>
<td>4.0 h</td>
</tr>
<tr>
<td>4 log10</td>
<td>7.0 h</td>
</tr>
<tr>
<td>3 log10</td>
<td>10.0 h</td>
</tr>
<tr>
<td>2 log10</td>
<td>15.0 h</td>
</tr>
</tbody>
</table>

Table 1. Detection time of Quality Control Clone ATCC 25923 in aureusAlert®

<table>
<thead>
<tr>
<th></th>
<th>Conventional Positive</th>
<th>Conventional Negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>aureusAlert® Positive</td>
<td>450</td>
<td>20</td>
<td>470</td>
</tr>
<tr>
<td>aureusAlert® Negative</td>
<td>10</td>
<td>1787</td>
<td>1797</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>460</td>
<td>1807</td>
<td>2267</td>
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</table>

(A)

<table>
<thead>
<tr>
<th>Site</th>
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<th>Conventional (Positive / Negative)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tampa General Hospital</td>
<td>69 / 322</td>
<td>72 / 322</td>
</tr>
<tr>
<td>Yale-New Haven Hospital</td>
<td>63 / 289</td>
<td>58 / 289</td>
</tr>
</tbody>
</table>

(B)

Table 2. A&B. Detection comparison of S. aureus nasal swabs in aureusAlert® and conventional methods
2.1.3. EPI-M® (MRSA)

![Image](image1.png)

**Figure 3.** MRSA Negative – Straw-colored

![Image](image2.png)

**Figure 4.** MRSA Positive – Amethyst

<table>
<thead>
<tr>
<th>MRSA ATCC 43300 (CFU/mL)</th>
<th>Detection Time at 35°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>7 log10</td>
<td>2.0 h</td>
</tr>
<tr>
<td>6 log10</td>
<td>3.0 h</td>
</tr>
<tr>
<td>5 log10</td>
<td>4.0 h</td>
</tr>
<tr>
<td>4 log10</td>
<td>6.5 h</td>
</tr>
<tr>
<td>3 log10</td>
<td>14.0 h</td>
</tr>
<tr>
<td>2 log10</td>
<td>18.0 h</td>
</tr>
</tbody>
</table>

**Table 3.** Detection time of Quality Control Clone ATCC 43300 in EPI-M®
A New Direct Detection System for Antibiotic Resistant Bacteria

http://dx.doi.org/10.5772/59345

<table>
<thead>
<tr>
<th></th>
<th>Conventional Positive</th>
<th>Conventional Negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>EPI-M® Positive</td>
<td>49</td>
<td>2</td>
<td>51</td>
</tr>
<tr>
<td>EPI-M® Negative</td>
<td>1</td>
<td>480</td>
<td>481</td>
</tr>
<tr>
<td>Total</td>
<td>50</td>
<td>482</td>
<td>532</td>
</tr>
</tbody>
</table>

(A)

<table>
<thead>
<tr>
<th>Site</th>
<th>EPI-M® (Positive / Negative)</th>
<th>Conventional (Positive / Negative)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tampa General Hospital</td>
<td>33 / 317</td>
<td>32 / 317</td>
</tr>
<tr>
<td>Yale-New Haven Hospital</td>
<td>19 / 217</td>
<td>18 / 217</td>
</tr>
</tbody>
</table>

(B)

Table 4. A & B. Detection comparison of MRSA nasal swabs in EPI-M® and conventional methods

Described here is a method to detect S. aureus, both MSSA and MRSA, directly from a human, animal, or environmental sample. The key innovation was to optimize all ingredients and generate a stable, ready-to-use powder. The system’s central ingredient is an enhanced coagulase substrate. The enhanced coagulase reaction overcame the observation by Selepak and Witebsky that rabbit plasma variability precluded the ability to directly detect S. aureus directly from samples. In one form, aureusAlert®, all S. aureus are detected. Using the aureusAlert® as a base, the addition of a mecA inducer specifically distinguishes MRSA (EPI-M®). By employing the definitive tests in the right milieu, as low as 20 CFU of MSSA and MRSA could be detected.

This study demonstrated that the sensitivity was equivalent to conventional methods by direct comparison and molecular methods from literature references. The time to detection was in the clinically useful range for the epidemiological screening of anterior nares (Table 1; Table 3). Because coagulase was chosen as the core detection system, a specificity of 49/49 for EPI-M® was seen from sampled subjects. Likewise, aureusAlert® being an enhanced coagulase test also showed complete agreement with conventional identifications.

The bacteria remain viable in the aureusAlert® and EPI-M® epidemiology screening tools. The tubes can be transported, as is, from the field for further analysis (e.g., antibiotic susceptibility testing, molecular fingerprinting). The powder format offers great flexibility of use. It has the ability to gather information from a broad spectrum of sampling: in the field, in satellite facilities, in clinics, and in large volume central laboratories. It requires no skilled labor. Because it is a stable powder, it can be inexpensively transported. Its cost is 20% of genetic amplification and 75% of conventional processing surveillance samples.

2.1.4. Additional S. aureus screen: EPI-VISA®

The screen for vancomycin-intermediate resistant S. aureus (VISA) utilizes the EPI-M® base with the substitution of 3 mcg/mL (3 mg/L) of vancomycin for cefoxitin. The colors produced are the same as the EPI-M®.
2.2. Screening Method for Vancomycin-resistant Enterococci (VRE)

Both asymptotically colonized and infected patients serve as reservoirs for transmission of
VRE, as well as contaminated surfaces and patient care equipment. European Union and
United States public health agencies have developed recommendations for preventing the
transmission of vancomycin resistance within and among hospitals and nursing homes.
Current surveillance methods require a number of sequential steps and needs a special series
of culture media and identification tests. A highly skilled and trained medical technologist is
needed to perform the analysis. Molecular tests are expensive and require special equipment.

A new, simple, and one-step screening method for the detection of VRE (as defined as a
minimum inhibitory concentration (MIC) to vancomycin of 6 mcg/mL) directly from rectal/
perirectal specimens was compared to the Food and Drug Administration (FDA) reference or
predicate method. The screening tool (EPI-V®, Pilots Point LLC, Sarasota, FL) contains all the
required ingredients in a stable powder form, in a standard test tube. The presence of VRE in
a rectal swab is denoted by the production of a two sequential biochemical reactions inside the
test tube: substrate hydrolysis representing bile-esculin followed by pyrrolidonyl arylamidase
(PYR) substrate hydrolysis. Bile-esculin plus PYR is the generally accepted identification of
the genus Enterococcus [13].

2.2.1. Standard culture method

The FDA predicate VRE culture method is Bile Esculin Azide agar with 6 mcg/mL vancomycin
(BEAV) (Remel, Lenexa, KS) [14]. Specimens consisted of 400 sequential human rectal/
perirectal surveillance swabs obtained as part of the ongoing surveillance program. The
collection device was a CulturetteTM II (Becton Dickinson and Company, Cockeysville, MD).
There was no prior notification of individuals collecting the specimens. The Culturette™ II
swab was twirled vigorously in 1 mL of pH 7.0, 50 mM HEPES (Sigma-Aldrich, St. Louis, MO)
buffer so that each of the two methods would be challenged with the same inoculum. From
the HEPES buffer, 0.1 mL of the extracted patient specimen was plated on the surface of the
BEAV agar plate. After incubation for 24 hours at 35°C in ambient air, colonies that were brown-
black were gram-stained. Colonies showing gram-positive cocci in chains were then tested by
the PYR reaction (Becton-Dickinson, Cockeysville, MD). The MIC to vancomycin from PYR
positive colonies was performed by the Etest® method (bioMérieux, Durham, NC).

2.2.2. EPI-V®

The EPI-V® screening tool is in a powder format, in a flange-capped test tube, and stored at
room temperature. To use, 3 mL of sterile water was added to dissolve the powder. To this
solution, 0.1 mL of the extracted patient's specimen was added. EPI-V® test tubes were
incubated for a maximum of 24 hours at 35°C in ambient air to call a specimen negative.
However, color development at any time, denoted a positive result. Test tubes demonstrating
a brown-black color (bile-esculin positive) were tilted to coat the disk present in the EPI-V®
cap. This cap contains an enhanced reagent for the detection of PYR hydrolysis [15]. The
development of a bright fuchsia color within 15 seconds is a positive reaction for PYR. Positive bile-esculin and PYR results demonstrated the presence of VRE.

In order to determine if a false-positive or false-negative reaction had occurred from the EPI-V®, approximately 0.1 mL was subcultured from all tests tubes to the surface of a Bile Esculin Azide agar plate containing 6 mcg/mL vancomycin. Plates were incubated at 35°C for 24 hours in ambient air. All colonies compatible with Enterococcus were then identified to species. If an Enterococcus species was isolated, an Etest® MIC to vancomycin was performed. All isolates were identified to species using the Vitek 2 system (bioMérieux, Durham, NC).

The EPI-V® tool, from a diluted specimen, demonstrated a sensitivity of 107% and a specificity of 98.4%. Table 5 presents the comparison of the EPI-V® results versus the Bile Esculin Azide (BEAV) culture method. Table 6 presents the time course of the 120 positive analyses by the EPI-V® tool.
The EPI-V® screening tool consists of both a substrate in the liquid (a bile-esculin substrate) and a detection disk for a second biochemical reaction (PYR).

The EPI-V® tool showed excellent sensitivity and specificity relative to the Bile Esculin Azide FDA referenced method and required only a maximum of 20 hours. In addition, the EPI-V® tool offered several significant advantages over the FDA referenced method. These included: no skilled technologist time required, simple quality control, highly conserved incubator and refrigerator space, and approximately 1/4-1/3 the cost of agar-based methods. The simplicity and ease of use of the EPI-V® epidemiology screen allows for more efficient and timely processing of large numbers of patient surveillance specimens for VRE. This new screen offers the clinical microbiologist, hospital epidemiologist, or infection control practitioner, flexibility as to the timing of collection and number of rectal or perirectal patient surveillance cultures.

### 2.3. Screening Methods for Antibiotic Resistant Enterobacteriaceae

Carbapenems are a class of antibiotics made by modifying penicillin. This chemical modification keeps part of the penicillin molecule, called the beta-lactam structure, and makes the rest artificial. Therefore, carbapenems are referred to, with other classes of penicillin modified antibiotics, as semi-synthetic penicillins. The modifications were made because bacteria rapidly became resistant to penicillin. Chemists added bits and pieces of chemical structures to penicillin to avoid this resistance. The bacteria figured out how to make enzymes to chemically destroy these new modifications. After 70+ years of this war between chemists and bacteria, the bacteria have topped the chemists by making a class of enzymes called carbapenemases [16].
The original carbapenem was first used in the U.S. in 1976. Since then better forms of this class have been made chemically. Currently, the most common carbapenem antibiotic used is meropenem. In the fight between chemists and bacteria, meropenem is the last antibiotic standing. If a bacterium is resistant to meropenem, there are no good alternatives [16].

2.3.1. What is CRE?

The clinical microbiology laboratory, where infections are diagnosed, divides bacteria into various shapes (spheres are cocci, cylinders are rods) and armor (gram-positive or gram-negative). A particular class of antibiotic generally has activity against either gram-negative or gram-positive bacteria, but not both. Carbapenems were developed to treat the gram-negative bacteria found in hospitals. Gram-negative bacteria in hospitals are associated with a very large morbidity and mortality, plus tremendous cost.

The gram-negative bacteria that have become resistant to carbapenems are known as CRE, or carbapenem-resistant enterics. Until a short time ago carbapenem resistance was only in one type of enteric, called *Klebsiella pneumoniae* (*K. pneumoniae*); therefore resistant bacteria were called *K. pneumoniae carbapenemase* (KPC). Unfortunately resistance has spread to bacteria related to Klebsiella and we now use the term CRE. These enteric bacteria normally live in the intestines of humans [16].

2.3.2. What is the CRE problem?

CRE is primarily a nosocomial associated problem. The likely reasons include the following: the use of large amounts of various antibiotics that select for the CRE bacteria; the transport from patient to patient of CRE on the hands of health care workers, including doctors and nurses; an environment such as a hospital room in which the bacteria can be deposited to infect new residents. For example, the CRE bacteria can remain alive in hospital rooms for a very long time period. In one study, after 30 days significant numbers of CRE could be found in a hospital room, and even after 100 days CRE were recovered. Death rates of up to 50% can be seen in patients with CRE isolated from the blood. The CDC has identified CRE as one of the prime problems for hospitalized patients [16].

2.3.3. Impediments to the control of CRE

*Lack of funding*: There is no billing code for CRE under Medicare and Medicaid. Therefore, reimbursement for the actual laboratory testing is not available. While there would be a huge financial benefit to the prevention of infections (in addition to the obvious benefit to the patient) hospitals do not balance costs and benefits between departments.

*Lack of a systems approach*: Many hospitals are now actually networks of hospitals. It has been difficult to achieve uniformity of action across the networks.

*Lack of a diagnostic test*: The IDSA says “New, rapid accurate diagnostic tests are sorely needed. Unfortunately, there is little impetus for companies…”
Need for highly skilled labor: Current methods require several days and utilize highly skilled and costly labor [16].

2.3.4. Methods for the detection of Carbapenemase-producing Enterobacteriaceae

2.3.4.1. Centers for Disease Control Method

The CDC method utilizes a series of sequential steps and can require up to 4 days to determine either positive or negative [17].

Procedure:

Step 1. Day One
1. Aseptically, place one 10-μg ertapenem or meropenem disc in 5 mL trypticase soy broth (TSB).
2. Immediately inoculate the broth with the rectal culture swab.
3. Incubate overnight at 35 ± 2°C, ambient air.

Step 2. Day Two
1. Vortex and subculture 100 μl of the incubated broth culture onto a MacConkey agar plate.
2. Streak for isolation.
3. Incubate overnight at 35 ± 2°C, ambient air.

Step 3. Day Three
1. Examine the MacConkey agar for lactose-fermenting (pink-red) colonies.
2. More than one colony morphology may represent different species of Enterobacteriaceae.

NOTE: Carbapenemases are known to exist in several different species of gram-negative bacilli including species of Enterobacteriaceae and Pseudomonas aeruginosa (P. aeruginosa). However, carbapenemases are more common in lactose-fermenting species of Enterobacteriaceae (e.g. K. pneumoniae and E. coli) than in non-lactose fermenting Enterobacteriaceae (e.g. Serratia marcescens and some Enterobacter spp.) and P. aeruginosa. In this procedure, it is suggested that laboratories focus their efforts on detection of resistant lactose-fermenting bacteria to reduce workload. Healthcare facilities that have identified clinical infections with carbapenemase-producing non-lactose fermenting gram-negative species should consider altering this procedure to include characterization of colonies with a morphology that is consistent with those species.

It may be necessary to subculture representative colonies of each morphology type to a non-selective media for isolation and/or for susceptibility testing. Screen representative isolated colonies using a phenotypic test for carbapenemase production, such as the Modified Hodge Test (MHT) or test for carbapenem susceptibility using a standardized method and follow the CLSI guidelines for identification of carbapenemase-producing Enterobacteriaceae [12].
Step 4. Day Four

1. For CRE and/or MHT-positive isolates, perform species-level identification.

2.3.4.2. Hydrolysis of Carbapenem Methods

Rapid Detection of Carbapenemase-producing Enterobacteriaceae

This method takes advantages of the observation that the hydrolysis of imipenem results in an acid molecule. Therefore, the authors incorporate a pH indicator that changes from red to yellow when acid is produced in sufficient amounts. One advantage of the method is that by using a pH change, a broad spectrum of carbapenemases can be detected. A significant disadvantage is that imipenem is not stable and the whole formula must be made fresh each time it is used. This aspect seriously inhibits the method’s use [18].

Detection of Carbapenemase Producers in Enterobacteriaceae by Use of a Novel Screening Medium

Different concentrations of several carbapenem molecules were tested, and finally, ertapenem was added to Drigalski agar medium at a concentration of 0.25 mg/mL. ZnSO₄ (70 mg/mL) was added to improve expression of metallo-beta-lactamases (MBLs) by MBL producers. Cloxacillin (250 mg/mL), which is a cephalosporinase (AmpC-type beta-lactamase) inhibitor, was used to prevent growth of isolates expressing high levels of cephalosporinases, such as Enterobacter cloacae, Enterobacter aerogenes, Morganella morgannii, and Serratia marcescens. These isolates are clinically significant sources of carbapenem resistance associated with an outer membrane permeability defect [19].

2.4. Screening Method for Carbapenem-resistant Enterobacteriaceae (CRE)

EPI-CRE® is a selective and differential medium containing 2 mcg/mL of meropenem, intended for use in the qualitative detection of gastrointestinal colonization with carbapenem-resistant Enterobacteriaceae (CRE) to provide epidemiological data. It is based on classical biochemicals in an optimized form. EPI-CRE® is performed with rectal swabs and fecal specimens from patients to screen for CRE colonization. The presence of CRE is established when the color of the medium changes from red to yellow. In addition, a violet precipitate may also be observed. Specimens should be collected, handled and disposed of in compliance with accepted guidelines and in accordance with all regulations. Cotton swabs should be avoided with preference to synthetic fibers such as Dacron or rayon. A generally available reference is the Manual of Clinical Microbiology [8]. EPI-CRE® is performed with rectal swabs and fecal specimens which should be collected and handled following recommended guidelines [13].

2.4.1. EPI-CRE®

2.4.1.1. Procedure

1. Inoculate the sample into the medium. If a swab is used, twirl the swab in the medium for 10 seconds and express excess fluid from the swab before removing and discarding into disinfectant. Do not use a cotton swab.
2. Incubate the tube(s) at 34-36°C.

3. The tube(s) may be examined any time up to 24 hours for a color change from red to yellow. If the medium has not become yellow in 24 hours, it is negative.

4. At any time after the start of incubation, if a yellow color is observed, EPI-CRE® is positive. In addition, a violet precipitate may be observed.

*Figure 7. CRE Negative – Red*

*Figure 8. CRE Positive – Yellow with violet precipitate*
2.4.1.2. Note on the biology of EPI-CRE®

EPI-CRE® detects ONLY living bacteria. It is 100% specific (based on Bergy’s Manual and the Manual of Clinical Microbiology).

**PCR positive and EPI-CRE® negative:** Genetic amplification is not to be used as a “test of cure”. The reason is that PCR and other genetic amplification methods will detect dead bacteria. Therefore, if a patient is treated intra-nasally or systemically with an effective antibiotic, the bacteria may be killed but their genetic material still is present. EPI-CRE® only detects living bacteria so this situation is not a limitation with this method.

2.5. Screening Method for Ciprofloxacin-resistant (cipR) Bacteria

EPI-FLOX® is a selective medium that allows the differentiation of ciprofloxacin-resistant from ciprofloxacin-susceptible bacteria. EPI-FLOX® contains 2 mcg/mL of ciprofloxacin, intended for use in the qualitative detection of ciprofloxacin-resistant (cipR) bacteria to provide epidemiological data. It is based on classical biochemicals in an optimized form. EPI-FLOX® is performed with urethral swabs and specimens, or rectal swabs and fecal specimens from patients to screen for cipR bacterial colonization. The presence of cipR bacteria is established when the color of the medium changes from red to yellow. In addition, a violet precipitate may also be observed.

The base formula of the EPI-FLOX® is the same as that of EPI-CRE® with the substitution of ciprofloxacin for meropenem. Therefore the procedure for use and the color changes are the same.

2.5.1. Note on the utility of the antibiotic resistant Enterobacteriaceae format

Because the base formula is designed to select for lactose positive Enterobacteriaceae, changes in the antibiotic can easily produce tailored screens. For example, by decreasing the concentration of meropenem to 0.25 mcg/mL, an epidemiological screen for OXA-48 can be produced.

3. Instrument: Pilots Point Monitor 60® (PPM 60®)

As described above, each of the DSU® epidemiology screens can be done manually. However, there are advantages to be able to place the screens in an instrument. Among these are:

*Standardization:* the instrument will read and interpret the screens in a completely uniform manner.

*Positives reported when detected:* the instrument monitors the color change of each screen every 15 minutes. Once the algorithm determines that a significant change has occurred compared to a control the results are reported at that time. Therefore there is continuous monitoring and reporting. Another benefit is that response time will be shortened.

*Satellite screening:* Because results are available from the instrument via Wi-Fi, internet, and intranet, screening at various locations can be controlled from a central location.
Epidemiological pattern analysis: Either from a central location or from satellite laboratories, data can be analyzed to determine if any increase of any of the epidemiological threats from any location is occurring.

Inexpensive cost: the instrument is designed to minimize cost. As shall be discussed below the instrument’s function is to produce light and measure a change. Data are transferred to a computer – either a personal computer (PC) or an information technology (IT) system – for analysis. This format lowers the cost of the instrument.

Single instrument for all screens: Because of the light source chosen (see below) all of the screens, regardless of color, can be performed in one instrument. Moreover, wavelengths from 360nm through the entire visible light can be accurately measured, which allowed both fluorescent (366nm) and visible light measurements. The ability to use both ultraviolet and visible wavelengths permits the development of two separate biochemical identification systems. The separation of two biochemicals can allow for the detection of two separate target microbes (if each is specific for each microbe) of an enhanced specificity for a single target microbe (if the two together provide enhance specificity over one alone).

3.1. Principle of the instrument

The choice of the light source is critical to the generation of a functioning inexpensive instrument. Instead of using laser, fluorescence polarization, light scattering, reflectance spectrophotometry that are expensive and functionally limited, an inexpensive instrument that uses a white light generator that passes through the sample was generated. Absorption of one or more of the colors allows other to pass through. In addition an overall measure of turbidity, called luminosity, is also measured. For EPI-M® the coagulase coalescence change enhances the process.

The principal of the instrument is based on the way the human eye sees color.

There are three primary colors that the cones see, which translate into the full range of colors.
In the analysis program the light coming out of the sample is broken down as shown below. The first measure, which is called the “L” value, is essentially luminosity. It determines a scale from black to white. The second measure is the “a” value, which measures a change from green to red. The third measure is the “b” value, which measures a change from blue (and effectively includes as shown below yellow). Any particular reading can be determined exactly on the graph below by knowing the “L”, “a”, and “b” values. The sensitivity of the analysis program is enhanced because it can relate the changes of any combination of “L”, “a”, and “b” values.

An example of a typical analysis is shown below for an EPI-M® MRSA analysis with standard ATCC MRSA 43300. The tube is scanned every 15 minutes and “L”, “a”, and “b” values
generated (see the spreadsheet below for the individual values). The analysis program then translates the values into graphical form, which are shown above the spreadsheet. The analysis program then determines by a combination of “L”, “a”, or “b”, or any combination thereof, if there is a significant change compared to a control. In this analysis 3 log10 of ATCC 43300 was detected in 14.5 hours.

3.2. The instrument

The inside of the Pilots Point Monitor 60° is shown below. It is designed to accept the largest number of screens with the smallest footprint. It is approximately 0.8 meters in diameter. In the current configuration, 6 instruments can be connected to one PC. In practice the instrument cannot be opened. The tube (as shown in the opened instrument below) in practice is added to the instrument by sliding the blue plastic bar below the PPM 60° logo so as to expose only one slot. The instrument via a barcode on the test tube recognizes the name of the screen and all the demographics of the patient. The carrousel moves past the light generator with each shift of position every 15 minutes. There is an incubator in the instrument that can be adjusted +/-0.2°C.

Below is a diagram of the working aspects of the instrument: 46 is the carrousel block, 28 is the wall of the carrousel, 50 are the holes in the carrousel into which the tubes are place, 34 is the white light source, and 32 is the detector which reads the amount and quality of light (“L”, “a”, and “b”) that is represented by 54.
Below is the instrument as it would be used.

As the scanning of the tubes progresses, the information is sent to a PC or to a central IT station. This flexibility allows both large and small laboratories, epidemiologists in the field, researchers studying the prevalence of antibiotic resistant bacteria in animals, people, and even surfaces to use one system.
3.3. Enhanced sensitivity of the Defined Substrate Utilization® tools

3.3.1. Agar compared to liquid

Compared to agar-based microbial detection systems, liquid with the same formulas are always more sensitive, meaning liquid has the ability to detect a smaller number of bacteria than agar and in shorter period of time [10]. The reasons for this difference are based first on the fact that on the surface of agar, bacteria must make colonies to be observed. A visible colony requires a minimum number of bacteria. The surface of agar has a surface tension that inhibits the spreading of the bacterial colony. By comparison in liquid, the bacteria are free to divide in three dimensions.

In addition, the nutrients from the agar must enter the colony from only one direction – from the agar vertically into the colony. Likewise the waste products, which are inhibitory to the growth of the microbial colony, diffuse in close proximity to the developing colony thus
inhibiting the colony’s lateral development. The production of a colony can be thought to reach development when the nutrients below the colony (all that is available to it) combined with the production of waste products cause the bacteria to stop multiplying and expanding. By contrast, a bacterium in liquid suffers no such inhibitions. Nutrients, including the metabolizable substrate, are available from all directions. Therefore, there is no inhibition for the development of the bacteria. Likewise any waste products diffuse away from the developing bacteria nidus into the liquid, away from the nidus.

3.3.2. Optical parameters

Microbiological optical systems have over the years moved to a microtiter format. The microtiter structures are either the classical 96 well microtiter tray, or a variation, or a microtiter format in a vertical series of cupules, as exemplified by the Vitek 2 system (bioMérieux, Durham, NC). The decrease in the light path has significant negative effects on both sensitivity and the time to detection. The reason for this is that the optics of light absorbance is governed by the Beer-Lambert law. As shall be discussed below, the intensity of light is logarithmically related to the length of the light path. This means that a decrease in the light path results in a log decrease in the amount of measurable light that reaches the detector. This relationship was presented as the linear relationship between absorbance and concentration of an absorbing species.

The general Beer-Lambert law is usually written as:

\[ A = a \, \text{\(\lambda\)} \, b \, c \]

where \(A\) is the measured absorbance, \(a \, \text{\(\lambda\)}\) is a wavelength-dependent absorptivity coefficient, \(b\) is the path length, and \(c\) is the analyte concentration.

When working in concentration units of molarity, the Beer-Lambert law is written as:

\[ A = \epsilon \, b \, c \]

where \(\epsilon\) is the wavelength-dependent molar absorptivity coefficient with units of \(\text{M}^{-1} \text{cm}^{-1}\) 

Regarding instruments, measurements are usually made in terms of transmittance (\(T\)), which is defined as:

\[ T = \frac{I}{I_0} \]

where \(I\) is the light intensity after it passes through the sample and \(I_0\) is the initial light intensity.

The relation between \(A\) and \(T\) is:

\[ A = -\log T = -\log \left( \frac{I}{I_0} \right) \]

and this formula is the one that is generally associated with the Beer-Lambert law.

The PPM 60\(^{\circ}\) instrument uses traditional 1 centimeter (cm) light path test tubes. By comparison, the light path of the well formatted microtiter trays is 0.1 cm and the light path of the Vitek 2 system even less. Therefore, the differences in the light paths inherently make the PPM 60\(^{\circ}\) instrument on the order of 100 times more sensitive. In terms of number of bacteria per mL, 6
log\(10/\text{mL}\) of bacteria is the lowest limit of detection in a 0.1 cm light path (based on turbidity, without a chemical signal, such as the release of a chromophore from a hydrolysable substrate or change in a pH indicator) and in a 1 cm pathway, the limit increases to 8 log\(10/\text{mL}\).

Practically, because bacteria in log phase have a generation time of 15-20 minutes, the time to detection in the PPM 60\(^\circ\) instrument is much less than other methods, and significantly less than the observation of colonies on agar.

3.3.3. Example of data produced by the PPM 60\(^\circ\) and analysis of a positive

Below are the results from the PPM 60\(^\circ\) from a patient isolate of MRSA from the Mt. Sinai Hospital, New York City, NY, USA (courtesy of Dr. Camille Hamula). The initial inoculum was less than that found in the nose at 6.0 x 10\(^4\) CFU.
There was a significant change beginning at 9 hours and the positive was called at 11.25 hours.

3.3.4. Quantification of CFU/mL by the PPM 60® instrument

The time to a positive is proportional to the number of bacteria in the sample. Bacteria go through four stages of growth in liquid (but not agar): lag phase, log phase, stationery phase, and decline phase.

It is well known that the greater the number of bacteria in a sample, the shorter the lag phase. Lag phase is the time that the bacteria acclimate to a new environment. When bacteria enter a new environment, there is a heterogeneous population in terms of physiological health. Each of the cells acclimates to the new environment and leave lag phase and enter log phase at
different times. By analogy it is similar to a kettle of popcorn kernels; one adds the kernels and all are in the same oil at the same temperature. The “healthier” ones pop first, the number of pops increase, and then there are a very large number of pops. Then there is a declining number of pops. Likewise with bacteria, some leave lag phase and enter log phase before others. The greater the number of bacteria, the shorter is the overall time the population goes from lag phase to log phase. In the PPM 60® instrument as discussed above, once the bacteria enter log phase they are dividing every 15-20 minutes. Therefore, the more bacteria in a population that leaves lag phase and enters log phase, the shorter the time to a positive since each bacteria multiplying produces a signal.

Below are examples of the ability of the PPM 60® to provide quantitative information with a variety of target bacteria.

3.3.4.1. MRSA

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Positive Result</th>
<th>Total CFU</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clinical Isolate 50153</td>
<td>6.25 hrs</td>
<td>24,000</td>
</tr>
<tr>
<td>Clinical Isolate 43681</td>
<td>6.0 hrs</td>
<td>14,800</td>
</tr>
<tr>
<td>Clinical Isolate 42869</td>
<td>8.5 hrs</td>
<td>10,400</td>
</tr>
<tr>
<td>Clinical Isolate 51667</td>
<td>9.0 hrs</td>
<td>4,800</td>
</tr>
<tr>
<td>ATCC 43300</td>
<td>9.75 hrs</td>
<td>4,000</td>
</tr>
</tbody>
</table>
3.3.4.2. VRE

![Graphs showing data for VRE strains](image1)

3.3.4.3. CRE

![Graphs showing data for CRE strains](image2)
Acknowledgements

Lize-Mari Russo is thanked for excellent technical assistance with this project. SCE is the developer and patent holder of the DSU® epidemiology screens including EPI-M®, aureusAlert®, EPI-VISA®, EPI-V®, EPI-CRE® and EPI-FLOX®.

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References


