Disk Diffusion Bioassays for the Detection of Antibiotic Activity in Body Fluids: Applications for the Pneumonia Etiology Research for Child Health Project

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To draw inferences about the putative etiologic agents of severe pneumonia, the Pneumonia Etiology Research for Child Health (PERCH) project must be able to objectively assess antibiotic pretreatment in enrolled participants. This review is focused on the disk diffusion bioassay, a simple laboratory method to assess recent antibiotic treatment. In this method, a sensitive indicator organism is used to detect antimicrobial activity in body fluid specimens that have been inoculated on a filter paper disk and placed on agar growth medium. We reviewed and present several variations on the disk diffusion method as applied to serum or urine, including specimen handling, choice of indicator organism and medium, and incubation steps. Although there are limitations to the disk diffusion method, its low cost, ease of use, and ability to broadly detect antibiotic pretreatment make it an appealing method for epidemiologic studies such as PERCH.

Detecting antibiotic activity in body fluids is useful for verifying therapeutic concentrations of antibiotics, monitoring for drug toxicity, and assessing adherence to treatment regimens. In the Pneumonia Etiology Research for Child Health (PERCH) project, the presence of antimicrobial agents in specimens of body fluids is of interest due to the potential interference of these agents with some microbiological tests. Most importantly, if antibiotic treatment is received before specimen collection, the sensitivities of bacteriologic cultures are reduced [1, 2]. As a result, the inferences about pathogen-specific etiology will be biased toward viruses and antibiotic-resistant bacteria [2]. Although parents may be asked about their child’s history of antibiotic use, this information is often unreliable. Taken together, etiology studies of children with respiratory infections in Argentina [3], Pakistan [4] the Philippines [5, 6], and Uruguay [7] found antibiotic activity in urine specimens of approximately one-half of the children whose parents reported no pretreatment with antibiotics. A study in Thailand similarly found serum microbial activity to be a more accurate measure of antibiotic pretreatment than parental reports [1]. For these reasons, a simple and reliable laboratory method to screen for antibiotic activity in body fluids at the time specimens are collected for microbiological testing would enhance the interpretation of microbiological data in PERCH.

Tests of antimicrobial activity in body fluid specimens can be broadly categorized into 2 groups: nonmicrobiological assays and microbiological assays (bioassays). Nonmicrobiological assays use chemical and physical methods (such as high-performance liquid chromatography), whereas bioassays rely on a susceptible microorganism to indicate the presence of an antimicrobial agent. Bioassays tend to be simpler and less
costly to perform than nonmicrobiological assays and can simultaneously detect multiple types of antibiotics [8]. One of the simplest and most widely used bioassay methods, and the focus of this review, is a diffusion bioassay method known as disk diffusion. Disk diffusion methods date back to the 1940s [9], are inexpensive, and can be easily performed in laboratories with bacterial culture capabilities. Because disk diffusion assays detect antibiotic activity of many classes, they are suitable for use when there is no requirement to determine the presence of specific antibiotic types or concentrations. The method involves inoculating small filter paper disks with the body fluid specimen of interest and placing the disks on an agar plate that has been inoculated with an indicator organism. The plate is then incubated, typically overnight at 35°C–37°C. A specimen with sufficient concentration of antimicrobials will create a zone of bacterial inhibition as it diffuses through the agar. This indicates the presence of antimicrobial activity in the body fluid specimen. Positive and negative controls are usually run in parallel with the test specimen to assure that the assay is performing as expected. Because inhibition zones correspond to a relative concentration of the antimicrobial compound, standard curves can be created using known standards to obtain a quantitative estimate of concentration if the specific compound is known [9, 10].

Because of the attributes mentioned above, disk diffusion is the chosen methodology for PERCH. Disk diffusion methods can vary on many factors including specimen type, indicator organism, medium, and incubation conditions. Because variations could affect results and interpretation, we reviewed the available methods to select the most appropriate method for standardization across the PERCH study sites. This review summarizes the data on disk diffusion methods that are contained in a variety of publications over a long period.

HISTORY OF THE DISK DIFFUSION METHOD

Prior to the introduction of the disk diffusion assay, the most common method for detecting penicillin in body fluid specimens was the cylinder plate method [11]. This method used small (approximate diameter, 8 mm) glass or stainless steel cylinder tubes to hold the antibiotic-containing substance on the surface of an agar plate. Zones of inhibition in the agar were measured from the borders of the tubes. A modification of this method using filter paper disks was first reported in the literature by Vincent and Vincent in 1944 [9]. In addition to being simpler than the cylinder plate method, disk diffusion was found to be more sensitive for detecting penicillin in serum specimens [9, 12]. The authors hypothesized that the improved sensitivity might be due to more consistent contact of penicillin with the surface of the agar and more even diffusion from the disk. Using Staphylococcus aureus as the indicator organism, they reported a lower limit of detection of 1/16 (0.0625) U of penicillin per milliliter of test fluid and noted that the method worked equally well on serum, spinal fluid, and urine specimens. A 1955 monograph by Grove and Randall [13] described assay methods for all antibiotics commonly in use at the time, including a more sensitive cylinder plate method capable of detecting penicillin concentrations as low as 0.005 U/mL with use of Micrococcus luteus (formerly Sarcina lutea) as the indicator organism. Several investigators later modified the methods of Grove and Randall for use with filter paper disks, particularly after the introduction of gentamicin in the 1960s, when the need to determine blood levels for assessment of toxicity became more pressing [14–18].

VARIATIONS ON THE DISK DIFFUSION METHOD

A summary of disk diffusion methods for urine and blood specimens reported in the literature since 1955 are shown in Supplementary Tables 1 and 2, respectively. Some report on the use of a disk diffusion method to assess treatment compliance or to detect unreported antibiotic pretreatment in an epidemiologic cohort. Others describe the systematic validation of a specific disk diffusion method, typically conducted in one of two ways. The first validation method involves the creation of serial dilutions of a sterile test substance spiked with a known concentration of antibiotic [15, 19–25]. This can be used to develop a standard curve, determine lower limits of antibiotic detection, and estimate the antibiotic concentration of an unknown specimen. The second validation method involves assaying the body fluid specimens of human subjects following their treatment with a known dose of antibiotic [16–18, 22, 24, 26, 27]. This method allows for an estimation of the time interval for which the bioassay can reliably detect antibiotic activity after the last treatment dose. It is also used to evaluate the incidence of false-negative and false-positive bioassay results in clinical specimens.

CHOICE OF URINE, SERUM, OR PLASMA SPECIMENS

Although urine and blood specimens are both commonly tested for antibiotic activity, urine is generally accepted as being more sensitive than serum or plasma because chemical compounds are often concentrated in urine. However, only a few published studies directly compare antimicrobial activity in the blood and
INOCULATION AND STORAGE OF FILTER PAPER

The most commonly described method of applying the test body fluid specimen to the filter paper disk (typically 6 mm in diameter) is to use forceps to dip the disk in the fluid and then press the disk to the side of the bottle to drain any excess fluid. However, this dipping method was found unsatisfactory by Simon and Yin [20], who reported that a filter paper disk could absorb 0.03–0.05 μL of fluid, leading to inconsistent volumes of test specimen. An alternative method is to use a pipette to drop the desired amount of fluid (3–20 μL, depending on the method) onto the disk after it has been placed on the agar plate [14]. It has been suggested that the use of disposable micropipettes is less prone to operator error than are the use of larger pipettes, reliance on capillary action, or dipping of filter paper in the specimen [35].

A wide variety of practices for the storage of the inoculated filter paper have been reported. Some validation studies report no loss in penicillin sensitivity after filter paper strips are left at room temperature for 5 days [18], whereas others found a 50% decrease in rifampicin concentration after inoculated filter paper is left at room temperature for only 24 hours [27].

INDICATOR ORGANISMS

The indicator organism used in the disk diffusion bioassay depends on the intended use of the bioassay. Assays that are designed to detect a small range of antimicrobial agents may use an organism with limited sensitivity, whereas assays designed to detect a wide range of antimicrobial agents typical utilize a broadly sensitive indicator organism. M. luteus, S. aureus, and Bacillus subtilis are 3 of the most commonly used indicator organisms [10]. M. luteus has most often been used to detect penicillin [13], whereas S. aureus and B. subtilis are commonly used to detect a broad range of antibiotics [36, 37]. Other common indicator organisms include Escherichia coli and Streptococcus pyogenes, often used in combination with other indicator organisms to detect various classes of antibiotics.

INOCULATION OF THE ASSAY PLATE

There are 3 ways to inoculate the assay plate with the indicator organism: a broth culture growth may be mixed with a single molten agar layer [8, 14], added to a thin agar layer which is spread over a solid base agar layer [5, 9, 15, 18, 19], or streaked on the surface of the agar with a sterile swab [4, 16, 17, 25]. The earliest disk diffusion method recommended the use of a peptone broth as a seeding medium in order to grow a more diffuse (less granular) growth [9]. After the culture is grown, the bacterial suspension may be standardized
colormetrically [20] or, as reported by more recent studies, adjusted to a McFarland suspension of 1 or (more commonly) 0.5 [6, 25, 38, 39].

CULTURE MEDIUM AND INCUBATION

Culture plates of 100 mm in diameter are typically used for the disk diffusion bioassay and can test 2–8 filter paper disks at a time, depending on the size of the zones of inhibition used to define antibiotic activity. Larger plate methods that can test more disks have also been described [35]. It is important to have an agar layer of even thickness [9], and a thinner agar layer may improve the sensitivity of the assay [40]. The choice of medium depends largely on the antibiotic to be assayed, as variation in the pH or salt concentration may have an effect on the ability of the antibiotic to diffuse through the agar. Mueller-Hinton agar is currently the most frequently used medium [1, 5, 6, 25, 28, 38, 39, 41, 42]. If selective antibiotic detection is desired, certain compounds can be added to the medium to deactivate specific antibiotics. For example, semicarbazide hydrochloride can be added to eliminate streptomycin activity and β-lactamase can be added to inactivate penicillins and cephalosporins [14, 21]. Incubation times are frequently “overnight” (16–18 hours) and sometimes up to 24 hours, although incubation times as short as 5, 4, and 1.5 hours have been described [14, 19, 22]. The standard incubation temperature is consistently 35°C–37°C unless Bacillus stearothermophilus is the indicator organism, in which case incubation is at 56°C [25, 39, 42].

DETECTION OF ANTIBIOTIC ACTIVITY

Timing of Specimen Collection

In their 1999 study of 3 indicator organisms, Liu et al [25] describe the time from last antibiotic dose to specimen collection as the most important factor affecting results of the disk diffusion method. The amount of time that an antibiotic can be detected in the urine or serum specimen after the last dose varies greatly depending on the antibiotic and dose. In urine specimens, β-lactam antibiotics and erythromycin could be detected for up to 12 hours after the last dose [25]; penicillin could be detected for up to 18 hours after the last dose [17]; and rifampin, clindamycin, tetracycline, cotrimoxazole, and ciprofloxacin could be detected for up to 48 hours after the last dose [25, 27]. False-negative results are therefore possible if specimen collection is delayed. For serum specimens, no publications could be found that reported the maximum time since last dose that the antibiotic could be detected (although one validation study of plasma reported that antibiotics could be detected up to 8 hours after the last dose [43]). This is probably because most validation assays for serum specimens have focused on monitoring drug toxicity levels and thus report the shortest interval after treatment that the antibiotic can be detected (as short as 60 minutes [44]).

Lower Limits of Detection

Almost all of the published methods consider any zone of inhibition around the filter paper disk to be indicative of antibiotic activity. One study was able to correlate urine bioassay zone sizes with the approximate time since the last treatment with penicillin [17]. Lower limits of detection depend on the antibiotic and the indicator organism, varying widely from <0.01 to 20 μg of antibiotic per milliliter of specimen. In both serum and urine specimens, chloramphenicol has consistently lower sensitivity and requires greater concentrations to be detected compared with other antibiotics [6, 20, 25, 41].

ANTIBIOTIC DETECTION BIOASSAY METHODS FOR PERCH

PERCH will use the disk diffusion assay for the detection of antibiotic activity owing to its ease of use, low cost, and ability to broadly detect antibiotic pretreatment. Because the disk diffusion method will also be used for antibiotic susceptibility testing of isolates, the consistency of particular methods within the study contributes to its appeal. The antibiotic bioassay methods will be standardized across all PERCH site laboratories. We will use 6-mm filter paper disks and a 0.5 McFarland suspension of the S. aureus ATCC strain 25923 in normal saline as the indicator organism, which was selected because it is commonly used and easy to acquire. The suspension will be streaked onto a Mueller-Hinton agar plate with a sterile swab. Next, 20 μL of the body fluid will be applied with a pipette onto the filter paper disk, which will then be placed onto the streaked agar plate. Incubation will be overnight (18–24 hours) at 35°C–37°C, and any zone of inhibition will be interpreted as a positive result. Commercial antibiotic susceptibility disks will serve as positive controls, and disks inoculated with sterile saline will serve as negative controls. These will be included on every plate. Specimens from control participants will be tested using the same methods and in the same facility as are the case specimens. We plan to test serum specimens but will also test urine specimens if urine collection rates are high.

CONCLUSIONS

More than 60 years after first being described in the literature, disk diffusion bioassays remain an inexpensive and straightforward method to detect the presence of antibiotics in blood and urine specimens. Because these methods are
widely used, it will be possible to compare PERCH results for antibiotic activity with results of other studies. As noted in this review, the disk diffusion bioassay is not without limitations. The specimen-organism, organism-antibiotic, and specimen-antibiotic relationships all have the potential to influence the assay in unexpected ways [8]. As a result, false-negative and false-positive results have been observed in the literature and often cannot be explained. The most significant limitation of the assay may be the short window of time following the last antibiotic dose within which it can reliably detect antibiotic activity in the body fluid specimen. Despite these limitations, analytic validation studies have demonstrated the method to be reliable under controlled conditions, and in field studies it has been shown to provide valuable information in conjunction with parental reports of antibiotic pretreatment [1].

Supplementary Data

Supplementary materials are available at Clinical Infectious Diseases online (http://cid.oxfordjournals.org). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyrighted. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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