The Practice of Basic Principles in Routine Diagnostic Bacteriology for Better Diagnostic Value: A Review

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Abstract: The routine medical bacteriology laboratory is saddled with the responsibility of diagnosing the etiological agents of diseases that afflict humans. The ability to control such bacterial infections is largely dependent on the ability to detect the etiological agents and to reliably identify organisms of clinical significance in a cost effective and timely manner. Such services to the hospital patients are usually urgently required by the clinician for the benefit of the end receiver: the patient. Quick as the results may be so desired, basic principles and standard operational procedures still need to be followed without sacrificing efficiency and correct diagnosis at the altar of speed. The bacteriologist needs to adhere strictly to aseptic techniques while carrying out his diagnostic procedures. The benefits of newer diagnostic techniques such as sequence analysis of the 16S rRNA gene, notwithstanding, the bacteriologist still need to view a Gram-stained smear of the specimen microscopically, culture the clinical specimens for purposes of isolating the causative agent of infection, as well as determine the antibacterial susceptibility pattern, without which antibiotic therapy cannot be effectively instituted. These procedures, carefully controlled, have their indispensable value in routine diagnostic bacteriology. Although the newer technologies are reported to be reliable and rapid, users must be mindful of the common cliché, “garbage - in - garbage - out”! In addition, newer technologies are however not readily within the economic reach of most health facilities the world over. This review highlights some important and indispensable principles required by the practicing bacteriologist for effective identification and diagnosis of bacterial infections.

Keywords: Laboratory diagnosis, bacteriology, clinical specimens, valuable techniques.

1. INTRODUCTION

A major role played by health institutions all over the world is that of rendering effective and efficient health care service to those requiring such services. Through a well coordinated team work, health professionals from various disciplines contribute their expertise to effect a holistic treatment for those in need. The health status of any individual is usually determined by three main factors, viz, the individual (host), the disease etiology (pathogen) and the environmental factors. Whenever environmental conditions are so favourable for the pathogen to thrive in, and the host becomes vulnerable, disease or infection sets in. The presence of an infection in a host oftentimes heralds the production of symptoms, although there may be an asymptomatic infection. In addition to the doctor’s clinical assessment of the patient, laboratory investigations are meant to complement, and in most cases, direct the course of effective treatment for the patient.

The purpose of this review therefore, is to highlight those basic diagnostic techniques often overlooked by bacteriologists in their routine bench work. Understanding these principles could go a long way in assisting the clinicians, the bacteriologist and the patient who is at the receiving end.

2. THE CLINICAL SPECIMEN

According to Caroll [1], laboratory procedures used for diagnosing disease conditions cut across microscopic examination of patients’ specimens, the detection of antigen from the infecting agent by immunologic assay, nucleic acid hybridization studies to detect pathogen-specific genes in patients’ specimens, analysis of patients’ specimens and using PCR techniques and serological detection of antibodies. Serology, however, has its pitfalls [2]. In all these, the bacteriologist must realize that irrespective of the technique used, the result of diagnosis can be only as reliable and correct as the original specimen submitted for investigation will allow.

Whenever possible and before antibiotic therapy is instituted, it is most desirable to aseptically collect enough representative specimens for prompt examination. Here, emphasis must be laid on the requirement that in handling any clinical specimen, the bacteriologist must engage his laboratory work under aseptic conditions. Aseptic techniques involve developing both manual dexterity in safely handling microorganisms and mental dexterity in thinking ahead about what one is doing with the microorganisms. The concept of aseptic technique began with the pioneering work of Dr. Ignaz Phillip Semmelweis, a Hungarian
physician of German extraction. Fondly referred to as the “Saviour of Mothers”, he discovered that the incidence of puerperal fever could be drastically reduced by the use of hand disinfection in obstetrical clinics [3]. He proved the capability of reducing mortality from about 10% - 35% to below 1% through hand washing with chlorinated lime solution. Other basic safety procedures, often disregarded by routine bench workers include, (a) the need to wear laboratory coats and gloves, (b) the provision of discard jars containing appropriate concentration of disinfectants for collecting used slides, contaminated pipettes etc, (c) the need for proper decontamination of the laboratory bench with suitable disinfectant before and after laboratory work. In carrying out laboratory procedures such as biochemical testing of isolates (e.g. testing *Staphylococcus aureus* for presence of catalase enzyme), special precautions must be taken to prevent the contamination of the skin or workbench by aerosols generated by the reaction. For example, at the point of transferring a test colony of *S. aureus* onto a few drops of hydrogen peroxide placed on a glass slide, rather than leave the entire preparation exposed, the slide could be placed in a Petri dish, while the cover is used to shield the vulnerable parts of the body from the aerosol generated in a positive catalase reaction.

### 3. COOPERATION BETWEEN THE DOCTOR’S CLINIC AND THE BACTERIOLOGIST’S LABORATORY

This important necessity is sometimes taken for granted by practitioners, resulting to these personnel working at cross-purposes to the detriment of the patient’s health. The channel of communication is often strengthened when collective attention is focused on the patient. The prompt collection of sufficient and representative amount of the patients’ clinical specimen(s) is a sine qua non to a successful laboratory diagnosis. It must also be stressed that it is essential to view any specimen microbiologically. In order words, the possibility of encountering any type of microbe from the patient’s specimen may not be ruled out. Hence, the provision of enough clinical history alongside the laboratory request-form could help prevent what would otherwise result in a futile exercise; indeed, a wild-goose chase!

### 4. WHEN DO WE CONSIDER AN INFECTION SIGNIFICANT?

The infectious agents encountered in the practice of medical microbiology are legion and no single test will permit isolation or characterization of all the potential pathogens. It is therefore imperative for one to be conversant with the human microbiota in order to be able to adjudge which is a likely pathogen, or a member of the resident flora. There is need for caution here! An infection is said to occur whenever a microbe successfully invades the primary defense armour of the human body, attaches to and multiply within susceptible cells, sometimes spreading to other organs and tissues of the host’s body. Many infections are caused by organisms that are permanent or transient members of the normal flora. For example, coagulase-negative *S. aureus*, hitherto regarded as a commensal when living on the human skin, has been isolated from patients suffering from bacterial meningitis [4]. The incidence of significant bacteriuria involving coagulase-negative *S. aureus* has also been reported by Souvenir et al. [5]. *Escherichia coli*, a normal resident of the gastrointestinal tract of healthy individuals, are the most frequently documented uropathogens [6], just as it usually can be isolated from infected wounds [7, 8]. The relative numbers of specific organisms found in a culture are important when members of the normal flora are the cause of infection. *The lesson here is that the presence of an organism in an anatomical site other than its “normal” residence must be further investigated by the bacteriologist.* “In the early forties, it was important to know the source of a culture, for its identification might depend on whether it was isolated from above (Klebsiella) or below (Escherichia) the umbilicus. Today we are not surprised to isolate an infectious organism (bacteria) from a respiratory site or vice versa”. In urine bacteriology, the number of leucocytes, erythrocytes, casts, and bacteria/ml are counted and the bacteria identified. Normally, there will be none or less than 20/mm³ of leucocytes or other cells and a colony count of less than 10⁴ organisms per milliliter. The presence of 10⁵/ml of a single type of enteric Gram-negative rod is strongly suggestive of urinary tract infection, especially in men, while young women with acute dysuria and urinary tract infection will have 10² – 10⁵/ml. Sometimes urine culture may be negative in the presence of clinical signs of urinary tract infection. In this case the possibility of an undisclosed disease condition, an obstruction in the urinary tract or tuberculosis of the bladder should be further investigated. On the other hand, a bacterial count of the same type of organism from two consecutive specimens exceeding 10⁵/ml indicates an ongoing urinary tract infection and a significant bacteriuria [1, 9, 10].

### 5. THE MEDIA ROOM AND CULTURE MEDIA

This aspect of microbiological work, by my estimation, is the hub around which the wheel of
diagnostic bacteriology revolves. Regrettably, the “kitchen”, as the media room is sometimes colloquially referred to, is oftentimes left in the hands of amateur “cooks”, with resultant poor outcomes. Just as the media room and facilities therein are expected to be in top sanitary condition, so also are the processes involved in media preparation. Problems may arise when control strains do not give expected results on certain prepared media, and if such media are inoculated with specimens that are not easily obtainable e.g. cerebrospinal fluid from a neonate. Since all culture media mimic the natural and essential nutrients which are present in the living tissue and which we try to provide for within a Petri dish, adequate and correct measurements of the dehydrated powder, distilled water, and additives and other supplements must be strictly carried out according to the manufacturers’ recommendations. The need for specimen preservation must not be overlooked. For instance, if urine specimen cannot be examined immediately after collection, it can either be refrigerated at 4°C or preserved in 1.8% boric acid. The acid is dissolved in the specimen by shaking. Numerous techniques have been developed for the examination of urine samples [11-13]. Their usefulness however, varies according to the facilities available to the bacteriologist. For more precise interpretation, however, a formal plating technique (using a standard inoculating loop) must be used, as this gives adequate qualitative and quantitative information [14-16]. Cysteine Lactose Electrolyte Deficient (CLED) agar, blood and MacConkey’s agars have been used successfully by various laboratories as culture media for urine specimens. The precise composition of a satisfactory medium depends on the species being cultivated due to the great variety of nutritional requirement. However, depending on the nature of the experiment some desired adjustments in the composition of culture media ingredients could be made [17].

6. SELECTIVE USE OF CULTURE MEDIA

The bacteriologist appears to me like a detective who sets out to disclose the identity of microorganisms hidden within a clinical specimen. Without the luxury of the more complex molecular and immunological techniques, he could provide a presumptive identification of the causative agent of an infection merely on the basis of microscopic examination of specimens and studying the growth and biochemical characteristics of isolated microorganisms. Thus, knowledge of diverse numbers of bacteria makes for easy identification of isolates from clinical specimens. Frequently a medium is used to select and grow specific microorganisms or to help identify particular species present in a specimen from nature. For example, the use of blood agar, eosin methylene blue agar, MacConkey agar and mannitol salt agar as selective and differential media for isolating *Streptococcus pyogenes* (a β – haemolytic streptococcus), faecal *E. coli* which produce a metallic sheen, lactose fermenting *E. coli* which appear as red colonies, and *S. aureus* respectively, has practical utility when investigating clinical specimens in diagnostic bacteriology [16].

7. THE PATHOGEN E. COLI O157:H7

*E. coli* O157:H7 is an emerging pathogen of much public health concern and therefore regular screening for the presence of this pathogen in man and his immediate environment is an important proactive measure for early detection of infection and the establishment of necessary control measures. Mostly due to the need to cut cost in the management of laboratory services, most bacteriologists do not routinely search for *E. coli* O157:H7 together with other enteric pathogens in their isolation procedures [18]. For instance, for the differential isolation of this organism from suspected specimens, the less costly MacConkey agar is used. According to Raji et al. [19], the major problem with *E. coli* O157:H7 is that it is not detected by the usual methods used to isolate and identify “traditional” enteric bacterial pathogens. MacConkey agar, which contains lactose as fermentable sugar is a suitable indicator medium for isolating almost all strains of *E. coli* which can ferment lactose.

The successful differential isolation of *E. coli* O157:H7, however, requires the incorporation of sorbitol (rather than lactose) as fermentable sugar into the MacConkey agar medium. The diagnostic value of this is that while sorbitol is fermented by all other *E. coli* strains after 24hr incubation, *E. coli* O157:H7 serotype doesn’t. In view of the health conditions associated with infections with this pathogen it might be worthwhile if physicians in developing countries, query *E. coli* O157:H7 infection whenever the patient experiences bloody diarrhea, and microbiology laboratories should consider routinely searching for *E. coli* O157:H7 using sorbitol MacConkey agar supplemented with cefixime-tellurite.
8. THE PURE CULTURE CONCEPT

The early pioneers of bacteriology realized too well the importance of working with a pure culture. This aspect of work requires much ingenuity and patience but its practicability is most times compromised in most busy hospital laboratories. In their natural microbiota, bacterial organisms live as mixed populations of different species. Many difficulties in identification arise as a result of the use of an impure culture as starting material. As a “detective”, the onus rests upon the bacteriologist to skillfully identify the specie(s) of interest. Therefore, before an organism can be identified, it must be obtained as a ‘pure culture’ such that when replated, the descendants of a single colony will be like the parent. It is a futile exercise to attempt to carry out any characterization tests on an impure culture. Oftentimes, many isolations are made on selective or differential media which themselves contain substances that suppress or inhibit the growth of some organisms. It is good practice, therefore, to replate the colonies picked from a selective medium onto a non-inhibitory and preferably an indicating medium, before a culture can be assured to be pure.

9. PERFORMANCE AND CONTROL OF ANTIBIOTIC SUSCEPTIBILITY TESTS

Antibiotic susceptibility testing, like antibiotic usage, has fallen into misapplication by many laboratory scientists. It is doubtful if tests on the same organism, done in different laboratories, would result in consistent recommendations for treatment. Antibiotic susceptibility testing is supposed to act as a guide to antibiotic therapy, leading to continuance or alteration of therapy. It is usually designed to determine which antibiotics are likely to be effective in curing the infections in question. The classical disk-diffusion technique is a convenient and most commonly used method which requires that a source of antimicrobial agent is applied to the surface of a well-dried agar medium (usually dried at 37°C for 20 to 30 minutes in an incubator.) During incubation, antibiotic diffuses radially from the disk into the medium, inhibiting the growth of a sensitive organism to a degree partially dictated by the susceptibility of the organism. Other factors that could affect the zone of inhibition around the antibiotic disk have been well documented by Brown and Blowers [20]. It is important that the bacteriologist clearly defines and apply the criteria for reading and interpreting results of the antibiotic susceptibility tests. It is a far cry from the normal practice if a ‘that – zone – looks – about – the right – size’ attitude is adopted by the bacteriologist. It is a basic scientific principle that tests should be controlled, and no matter how urgent test results are required for use by the clinician, efficiency and accuracy in the test procedures must not be compromised. For example, in interpreting the correct zone size of a disk-diffusion test, Cremer [21] regards an organism as sensitive (implying that the infection should respond to treatment with normal doses of antibiotic under normal circumstances) when a zone radius is the same size as or is larger than the control or is not smaller by more than 3mm. A zone that is more than 3mm in radius but is smaller than the control by more than 3mm in radius is reported as moderately sensitive, while a zone of radius of 2mm or less is reported as resistant. The approved performance standard for antimicrobial susceptibility testing has been documented [22] and remains a useful manual for the practising bacteriologist.

Despite the usefulness of antimicrobial susceptibility testing, antibiotic resistance has over the years remained an intractable and worrisome problem for medical personnel and patients. Many reasons have been attributed to this phenomenon [23-26]. Correct identification of the causative organism of bacterial infection as well as adopting proper procedures for carrying out susceptibility testing will no doubt assist in curbing antibiotic resistance. Although some physicians may simply be interested in the Gram result and corresponding antibiogram in order to initiate antibiotic management of an infected patient, it is important that organisms are correctly identified so as to have correct epidemiological data of causal agents in a given disease state, as well as for infection control purposes.

10. NEWER TECHNOLOGIES VERSUS TRADITIONAL METHODS OF BACTERIAL IDENTIFICATION

Many scientists would no doubt sometimes pause for a while to contemplate the depth of inspiration, the uncommon wisdom and scientific knowledge displayed by pioneers such as Louis Pasteur, Robert Koch, Antony Van Leeuwenhoek, Richard Petri, Fanny and Walter Hesse and a host of others who laid the foundation for the discipline of microbiology. Before the advent of advanced automated technologies for bacterial identification, bacteriological diagnosis has been traditionally based on bacterial culture, the observation and analysis of phenotypic expression such as shape and colour, and biochemical testing of metabolic end products characteristic of the studied microbes. For example, specific media were devised
for recognizing production of acid and/or gas under appropriate and selective conditions. However, these phenotypic methods may not be so reliable since they depend on the expression of the chosen characters which may vary as a result of environmental factors or strain variations. The Gram staining technique had been in use and still remains a useful practical tool for bacterial identification and classification and can offer valuable information for clinical diagnostics including aiding in deciding what antibiotic disk to apply for testing bacterial susceptibility to antibiotics. There is need to understand the principle of the Gram staining reaction in order to control it and achieve better results.

In the staining procedure, a crystal-violet iodine complex is formed within the protoplast of the stained cells. Gram-positive bacteria are able to retain basic dyes (such as crystal violet and methyl violet) at a higher pH than the Gram-negative species, showing an isoelectric point of pH 2-3 as compared with pH 4-5. The more acidic character of their protoplasm, which is enhanced by treatment with iodine, may partly explain the stronger retention of basic dyes by Gram-positive cells. It is also known that decolourization with either acetone or ethanol dissociates the lipid layer from the Gram-negative cells, leading to the primary stain diffusing freely out of the Gram-negative cells, but not from the Gram-positive cells, whose cell walls are less permeable. Also, the decolourizer causes dehydration in the thicker Gram-positive cell walls, closing the pores as the cell wall shrinks during dehydration. This effectively blocks the diffusion of the crystal violet-iodine complex, and the bacterial cells remain stained.

Gram positivity is a feature of relatively young bacterial cells; as they age, the cells lose this characteristic and apparently become Gram-negative. Consequently, younger cells about 24 hours old are preferably stained. Genuinely Gram-negative bacteria do not retain the primary stain which is easily removed by the decolourizer. Also, the decolourization is critical in differentiating Gram-positive bacteria from Gram-negative ones, as excessive decolourization will remove all the stain from Gram-positive cells rendering them false Gram-negative. Preston and Morrell’s modification of Gram’s Method is reported as giving reliable results without the need for taking great care in adjusting the duration of decolourization [27]. It would be advisable as a matter of policy for workers in each laboratory to adopt a standard Gram staining procedure, but not without controlling the staining technique. Recommended quality control cultures for assessing the correctness of the Gram staining technique include Escherichia coli NCTC 10418 (pink to red Gram-negative bacilli), Oxford Staphylococcus aureus NCTC 6571 (blue to purple Gram-positive cocci), and haemolytic Streptococcus group A NCTC 8198 (blue to purple Gram-positive cocci).

In practice traditional microbiological techniques can take days to provide results and there are limitations in identifying certain species of microorganisms. The nucleic acid amplification technology has however opened a new horizon for microbial detection and identification. Unlike the phenotypic methods earlier mentioned, the nucleic acids involved in the expression of a character will remain present in the organism and even though environmental factors have suppressed expression they remain a more reliable material for microbiological analysis. In addition, molecular methods become very relevant where culture procedures fail to identify the causal organism due to one or more of the following reasons: (i) prior antibiotic therapy, e.g. treatment of acute meningitis with intravenous benzyl penicillin, (ii) where specialized cell culture techniques are required, e.g. Chlamydia spp. and Coxiella burnetti, (iii) where the organism is slow growing, e.g. Mycobacterium spp, (iv) where the organism is fastidious in nature, such as the HACEK group of organisms in the case of endocarditis [28]. The HACEK group of bacteria includes Haemophilus parainfluenzae, Aggregatibacter species (Aggregatibacter aphrophilus, Aggregatibacter actinomyctemcomitans, and Aggregatibacter segnis), Cardiobacterium species (Cardio-bacterium hominis and Cardiobacterium valvarum), Eikenella corroden, and Kingella species.

Other sophisticated techniques including scanning electron microscopy [29], fatty acid analysis [30], sequence analysis of the bacterial 16S rRNA gene [31, 32] have been used successfully for bacterial identification. Whereas a pure culture is required for proper identification and characterization using the traditional cultural methods, these newer technologies are reported to be rapid, reliable and may not require a pure culture to be able to identify bacterial agents [33]. Scanning electron microscopy aids in detecting and identifying morphological features of bacterial cells [34].
Fatty acids abound in bacterial cell membranes and their profiles which can be determined by gas chromatography, distinguish the various bacteria on the basis of their physical properties [35]. According to Eren et al. [31], the regularity and constancy of the 16S rRNA gene in bacterial cells make it useful for detecting bacterial cells in natural specimens and confirming phylogenetic relationship between species.

Strategies based on polymerase chain reaction (PCR) have been reported to be faster and highly sensitive for microbiological identification [36], but they are not optimized for resource-limited environments. Pure PCR products of the gene can be sequenced and aligned against bacterial DNA database for purposes of identification [32]. Although the PCR operates on the basis of three simple reactions i.e., denaturation of the DNA double strands, annealing of the oligonucleotide primers, and extension of the primers, different modifications or protocols have been developed to meet specific purposes. Different criteria dictate the appropriate protocol to employ for bacterial diagnosis. If speed is an important factor of the assay, e.g. the detection of meningococcal DNA in cerebrospinal fluid from children with suspected meningitis, employment of the real-time assays should be adopted [37]. Where multiple targets are involved as in the determination of multiple respiratory pathogens from sputum, the multiplex PCR format should be employed [28]. It must be noted that PCR protocols may be affected by contamination from various sources, leading to false results; hence there is utmost need to control the reactions.

Ralph Weissleder, and colleagues at Massachusetts General Hospital in Boston, USA, has pioneered the magneto-DNA nanoparticle system for rapidly identifying bacteria. This hybridization assay involving probes which target bacterial 16S rRNAs was designed to detect amplified target DNAs using a miniaturized nuclear magnetic resonance (NMR) device the size of a microscope slide. The system is reported to be robust, rapid and is able to simultaneously diagnose a panel of 13 bacterial species in clinical specimens within 2 hrs compared with conventional culture (procedural time, 3-5 days). In view of the high sensitivity exhibited by the system (down to single bacteria), it could potentially be used for the early diagnosis or detection of rare pathogens in dilute samples [38].

CONCLUSION

These advanced techniques though attractive are, unfortunately, not within the common reach of many laboratories, especially those in the developing countries of the world. The Gram staining techniques, microscopy and the culture of clinical specimens for purposes of isolating the causative agent of infection, still have their indispensable value in routine diagnostic bacteriology. Robert Koch (1843 – 1910) did not have to wait for Dr. Kary Banks Mullis to invent the PCR technology before elucidating his postulates for proving the etiology of microbial infections. What is required, therefore, is for the bacteriologist to exercise much caution and wisdom as an “investigating detective”, and work on his or her specimens painstakingly, adhering to all safety precautions while applying aseptic techniques to arrive at useful and reproducible results. It is a very wise decision, for instance, never to hastily discard clinical specimens or inoculated media until all tests have been satisfactorily completed and results issued out to the clinician. Had Alexander Fleming hurriedly disposed his ‘contaminated’ agar plate, without giving it a little further thought, humanity would probably have had to wait for a longer period of time before reaping the benefits of Penicillin!

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