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DNase and Conventional Biochemical Tests in the Era of Modern Bacteriological Diagnostics: A Critical Review"

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Abstract

Currently, clinical bacteriology finds itself at the crossroads, as increasingly more advanced diagnostic techniques such as MALDI-TOF mass spectrometry or whole-genome sequencing (WGS) become available and popular. However, despite these technological innovations, this review highlights the significance and value of classic biochemical analyses, particularly those involving the DNase test, in the modern context. The classic DNase test continues to prove its reliability by demonstrating high sensitivity and specificity values ranging from 96% to 99% regarding the detection of Staphylococcus aureus. Although the use of contemporary technologies, e.g., MALDI-TOF, allows obtaining the diagnosis faster, these tools may fail to achieve the level of accuracy provided by traditional techniques, especially in case of rare species of bacteria or lack of data in the spectrum database. Additionally, modern automated devices such as VITEK 2 operate based on the same biochemical principles as the classic approaches; thus, their implementation represents further development of the latter.

As for developing nations, classic biochemical analysis still represents the most cost-effective and sustainable method for conducting the diagnostics without requiring sophisticated equipment such as a spectrophotometer. Overall, this review reveals that the "tiered" strategy combining the approaches discussed above should represent the cornerstone of the diagnostics of the field.

Keywords: Staphylococcus aureus, DNase, whole-genome sequencing, biochemical analysis, MALDI-TOF Spectrophotometer.

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1.0 Introduction

Clinical bacteriology is at an authentic point of inflection in a history spanning over a century. The last two decades have seen the development of innovative diagnostic technologies, which have irrevocably changed how the identification of pathogens in laboratory settings is carried out: MALDI-TOF mass spectrometry, automated phenotypic and susceptibility identification systems such as VITEK 2 and the BD Phoenix system, multiplex PCR systems, whole-genome sequencing (WGS), and even next-generation sequencing technologies allow for faster and higher resolution, and sometimes more accurate, bacterial identification than ever before (Kloos et.al 1994, Cheesebrough et.al 2006). These new technological innovations have not failed to raise

questions on whether phenotypic and biochemical tests, which have long represented the methodological foundation of clinical diagnostic bacteriology, retain any usefulness whatsoever.

Of all these classical tests, one stands out due to its significant clinical and educational significance, namely the DNase test. This test was first introduced in the diagnosis of staphylococci in the 1950s; since then, it has been used mainly to confirm the presence of *S. aureus* and *Serratia marcescens* in *Enterobacteriaceae* using an agar plate test which makes use of the toluidine blue O (TBO) and methyl green indicator dyes (Bannerman et. al 2007, Seng et. al 2009). The ease of performance, inexpensive reagents, storage at room temperature, and the high discriminatory power of this test have made it widely applicable from reference laboratories down to district hospitals in developing countries (Forbes et. al 2007, Wolk et. al 2012).

But then the wider issue of whether traditional biochemical tests have any modern-day significance is anything but an abstract consideration. There are implications for patient safety and clinical outcomes, such as when making the determination to taper antibiotics in the context of *S. aureus* bacteraemia, antimicrobial stewardship initiatives, the deployment of laboratory resources, and even whether the diagnostic testing should be available on an equal footing around the world irrespective of economic development (Van Veen et. al 2010, Singhal et. al 2015, Patel et. al 2015). The blanket rejection of one approach in favour of another simply because it is novel would be just as scientifically baseless as its uncritical acceptance.

Murray et al. have highlighted that the most fundamental responsibility of the clinical microbiology laboratory is to deliver clinically meaningful data within a period where decisions about patient care will be affected, regardless of the technology used in generating such data (Cheesebrough et. al 2006). The pioneering guidebook for laboratory work in tropical districts written by Cheesbrough underscores an equally crucial complementary principle, which states that diagnostic procedures employed in laboratories should remain sustainable in light of the available resources in their immediate environment (Bannerman et. al 2007). The assessment of the current relevance of the DNase test and associated biochemical tests can only be made in reference to these principles.

This review makes such an assessment through the critical synthesis of the literature available from pre-MALDI-TOF times up until today, paying special consideration to relative performance, applicability in various resource levels, and those situations in which traditional biochemical testing is simply non-substitutable, is required as a complementary approach, or can potentially be substituted with modern alternatives. This review is based on the bibliography created in the preliminary section of the same, including important foundational papers, comparative studies (Kloos et. al 1994, Cheesebrough et. al 2006, Bannerman et. al 2007, Seng et. al 2009, Van Veen et. al 2010, Singhal et. al 2015, Bauer et. al 2010, Patel. R et. al 2015, Doern et. al 2011, Mahon et. al 2019).

2.0 Background and Historical Context

2.1 Historical Background of Biochemical Characterisation in Clinical Bacteriology

The systematic classification of bacteria based on biochemical characterisation can be traced back to the late nineteenth and early twentieth centuries when scientists like Pasteur and Koch discovered that it was possible to differentiate one type of microorganism from another based on their metabolism along with their physical features. This involved testing whether or not bacteria were able to break down certain sugars for fermentation, produce gases and hydrogen sulphide, catalyse hydrogen peroxide, and use specific substrates for carbon and nitrogen (Bauer et. al 2010, Weinstein et. al 2020). Within a couple of decades, these biochemical properties had been incorporated into a testing regime which could be performed with only basic glassware and a source of incubation.

The pressures that necessitated such a systemisation were pressing. Infectious diseases – typhoid fever, diphtheria, pneumonia, puerperal sepsis – caused more deaths in developed countries than any other disease categories, and the precision of bacterial identification was critical to the success of both the treatment and the preventative measures. (Weinstein et. al 2020, Winn et. al 2006). In the middle of the century, the framework for clinical microbiology was far more advanced. Catalase reaction accurately distinguished between *Staphylococcus* and *Streptococcus / Enterococcus bacteria* in clinical specimens (Koneman et. al 2006). The oxidase test, which exploited the reduction of tetramethyl-p-phenylenediamine by cytochrome c oxidase, was an

efficient means of initial differentiation between oxidative and fermentative Gram-negative bacilli and enabled the exclusion of most Enterobacteriaceae from the *Pseudomonas* and *Neisseria families* (Koneman et. al 2006, Weinstein et. al 2020).

In the implementation of miniaturised commercial biochemical tests, there was a major breakthrough in both throughput and standardization of biochemical tests but the logic was never changed. The API 20E test (bioMérieux, 1971) combined twenty biochemical reactions on a single strip plastic cupule. This enabled reproducible coding and profile interpretation through a growing database (Patel et. al 2015). The VITEK Automated Identification System introduced automation to drug sensitivity testing, setting the pace for a development path leading to the full integration of identification testing systems in the years 2000 and after (ISO 2022, Ecker et. al 2010). All of these developments were improvements in the biochemical testing methods rather than a replacement. The chemistry employed in VITEK 2 identification cards in 2024 was still the same biochemical logic employed in the API strips in 1971 and in bench top tubes used by Cheesbrough in district hospitals (Bannerman et. al 2007, BD Diagnostics 2021).

2.2 The Discovery and Initial Uses of the DNase Test

The clinical diagnosis using deoxyribonuclease outside the cell to identify bacteria was an outcome of the fusion of two streams of research in the 1940s and 1950s. The first stream concerned the description of streptodornase, which is the deoxyribonuclease enzyme secreted by Streptococcus pyogenes. This enzyme acts on DNA-containing pus to liquify it at the infection sites, thereby enabling spread of the bacteria (Bauer et. al 2010). The second stream involved the detection of an extracellular nuclease enzyme that was resistant to heat and had other characteristics different from those of the streptococcal enzyme.

In 1956, Jeffries, Holtman, and Guse standardized the method of detecting DNase by growing colonies on DNA-laden agar and then identifying the zones of DNA hydrolysis through the application of 1N hydrochloric acid to the plates, which caused precipitation of unhydrolyzed DNA and created clear halos around colonies positive for DNase activity (Seng et. al 2009, Bauer et. al 2010). Further developments involved employing the metachromatic agent toluidine blue O (TBO) and methyl green to detect zones of DNA hydrolysis without the need for acid treatment; TBO exhibits a change in colour from blue to pink when DNA has been degraded and returns to the free cationic form, whereas methyl green loses its colour upon degradation of the DNA-dye complex (Bannerman et. al 2007, Seng at. al 2009).

The persistent positive result for DNase production by *S. aureus* strains in comparison to the consistently negative results for DNase production by *CONS* strains quickly helped establish the DNase test as an additional confirmatory test in addition to the coagulase test (Kloos et. al 1994, Seng et. al 2009). Bannerman and Peacock found that the widespread use of DNase agar as the confirmatory medium for *S. aureus* in clinical laboratories around the world was well underway by the 1970s and 1980s. The authors noted that the concurrent use of the DNase and tube coagulase tests ensured a redundant confirmation of a clinically vital diagnosis (Seng et. al 2009). This redundancy was neither unnecessary nor inefficient; rather, it represented a cautious diagnostic approach since the two tests measure different biochemical characteristics: free coagulase activity and extracellular DNase activity, respectively, and a discrepancy between them indicates the need for further characterization of an unusual strain (Kloos et. al 1994, Seng et. al 2009, Forbes et. al 2007).

2.3 Extension of the Classical Biochemical Panel: 1960s-1990s

While the methodology of DNase was being improved, an increased codification and extension of the classical biochemical panel for clinical bacteriology occurred during the same period. TSI test reaction was one of the most important tests for identifying pathogens among Gram-negative enteric bacteria. Within 24 hours, one could obtain a profile that would be able to distinguish between *Salmonella, Shigella*, and *E. coli* as well as between these bacteria and non-pathogenic enteric bacteria (Koneman et. al 2006, Weinstein et. al 2020, Winn et. al 2006). Indole test (Kovacs' reagent), urease test reaction in Christensen's medium, and phenylalanine deamination were all added to the biochemical panel for Enterobacteriaceae.

As for Gram-positive organisms, the PYR test was used as a fast enzymatic test for discrimination of *Streptococcus pyogenes* (Group A; PYR+) and *Enterococcus faecalis* (PYR+). Specificity and

sensitivity rates achieved more than 95% by using appropriate reagents (Mahon et. al 2019, Cumitech et. al 2005). In case of identifying *S. agalactiae*, the Christie-Atkins-Munch-Petersen test (CAMP) used synergic activity of CAMP factor produced by Group B streptococci and β-haemolytic activity of S. aureus as specific features of *S. agalactiae*. This test was of particular importance in obstetrical laboratories because of the need for rapid detection of Group B streptococci that had to be used for neonatal prophylaxis (Mahon et. al 2019, Koneman et. al 2006). Identification of *Streptococcus pneumoniae* from *viridans streptococci* could be achieved by using bile solubility testing and optochin sensitivity testing with more than 98% of specificity in the first case and approximately 94% in the second one (Forbes et. al 2007).

The textbook of diagnostic microbiology by Koneman et al., covering several editions from the 1980s up to 2006, has thoroughly described, standardized, and critically assessed these biochemical test batteries, serving as an academic resource that supported the education of clinical microbiologists around the globe (Weinstein et. al 2020, Bourbeau et. al 2013). Two further reference manuals in the tradition of Bailey & Scott's diagnostic Microbiology in North America and of Mackie and McCartney Practical Medical Microbiology in the UK, written by Forbes et al. and Collee et al., respectively, have served as companion textbooks to develop a largely accepted international perspective on conventional biochemical testing (Koneman et. al 2006, Collee et. al 1996).

2.4 Technological Disruption and its Diagnostic Consequences

This technological disruption within clinical microbiology really started in the latter part of the 1990s, although it was not until into the 2000s and beyond that it became truly explosive. The pivotal papers by Seng et al. (2009) and van Veen et al. (2010) showed the first definitive prospective studies indicating that the technique of whole bacterial cell protein mass fingerprinting can provide accurate species-level differentiation within minutes at an operational unit cost, once equipment amortisation had been taken into account, which was comparable to conventional biochemical systems (Seng et. al 2009, Singhal et. al 2015). Since then, there has been a fast take-up within well-equipped labs in Europe, North America, and Australia, and a considerable body of comparative literature has built up over the past decade and more.

Meanwhile, advancements in the sequencing of broad range 16S ribosomal RNA gene sequences followed by the development of multiplex PCR arrays allowed the use of sequence information for the classification of microbes that could not be characterized phenotypically, including slow-growing microbes, culture-negative infections, polymicrobial samples, and those organisms having high intra-specific heterogeneity (Wolk et. al 2012, Drancourt et. al 2005). The advent of next-generation sequencing techniques coupled with whole-genome sequencing around 2012 led to another step forward by allowing identification of species, analysis of resistance genes, and genotyping all at once with a single sample preparation process (Didelot et. al 2012, Loman et. al 2015). Didelot et al. emphasized in their 2012 review on the revolutionary potential of WGS to clinical microbiology, predicting that genome-based techniques would ultimately supersede existing identification and genotyping methodologies (Didelot et. al 2012).

In this regard, the logical outcome of such developments was the questioning of the utility of the biochemical assays that came before. This questioning took many forms, from modifications in laboratory practices to editorial commentaries and even a demotion in the priority of method instruction in clinical microbiology educational programs (Fournier et. al 2013, Rhoads et. al 2016). The question of whether such questioning is justified in part or whole or premature remains to be answered by the data presented in the following sections.

3.0 Present State of the Problem

3.1 Diagnostic Performance of DNase Test: Contemporary Evidence

The current scientific literature presents contemporary evidence regarding the diagnostic value of the TBO or methyl green DNase agar test for detection of *S. aureus* in a clinical isolate grown on a medium, with sensitivity being 96-99%, and specificity ranging from 96% to 99% (Kloos et. al 1994, Cheesebrough et. al 2006, Bannerman et. al 2007). These estimates can be regarded as similar to — in certain cases even slightly better than — the results obtained when utilizing a slide coagulase test (sensitivity is approximately 85-90%, due to the existence of the false negative reaction caused by the presence of clfA gene absence) and tube coagulase test (sensitivity is 94-97%, specificity is 96-99%) (Kloos et.

al 1994, Bannerman et. al 2007, Mahon et. al 2019). Importantly, the two tests in question target different biochemical features of staphylococcal virulence, namely extracellular nuclease production and free coagulase activity, so that the application of both simultaneously allows one to estimate different gene products (nuc and coa).

There is a biologically significant group of *S. aureus* that makes up 1-3% of the clinical strains isolated; these strains produce negative results on the tube coagulase test due to technical inhibition because of higher levels of fibrinogen in some preparations of plasma, unique coagulase genes, or because they represent the natural mutants of *S. aureus*, which have lost the coagulase activity as a consequence of antibiotic selective pressure (Bannerman et. al 2007, Mahon et. al 2019). Strains in this group are always positive for the DNase test, and therefore the use of the latter test is mandatory to avoid the mistake of labelling truly pathogenic *S. aureus* as *CONS* (Kloos et. al 1994, Bannerman et. al 2007). As stated by Mahon, Lehman, and Manuselis, when a patient shows bacteraemia of unknown origin, the error caused by omitting the DNase test may endanger the patient's life (Mahon et. al 2019).

False positives from the DNase test for CONS must be recognized. Certain specific strains of *Staphylococcus epidermidis*, *Staphylococcus schleiferi* subspecies *coagulans*, and very rarely *Staphylococcus lugdunensis* may possess poor DNase activity, which can be demonstrated by using selective media preparations (Kloos et. al 1994, Bannerman et. al 2007, Winn et. al 2006). Great caution should be exercised when interpreting halo sizes (where halo diameter is less than 1 mm), avoiding prolonged incubation periods beyond 18-24 hours, and employing quality control strains (Cheesebrough et. al 2006, Bannerman et. al 2007, Cumitech et. al 2005). The use of DNase agar plates in conjunction with the morphologic features of colonies such as golden-yellow colour, beta haemolytic nature, and colony size has been suggested by Forbes et al., a procedure which greatly helps avoid false positivity without any added cost of reagents (Forbes et. al 2007).

Apart from *Staphylococc*i, DNase activity holds diagnostic relevance for *Serratia marcescens*, a rare species within the Enterobacteriaceae family that is always DNase positive on DNase agar plates, thus offering a quick and cheap screening tool to aid in the identification of S. marcescens alongside its distinguishing characteristic of producing red pigmentation known as prodigiosin (Forbes et. al 2007, Koneman et. al 2006, Winn et. al 2006). Since *S. marcescens* is a significant opportunistic nosocomial organism causing outbreaks in intensive care, neonatal care, and haematopoietic stem cell transplant facilities, there remains an epidemiological role for cheap and quick diagnosis even in MALDI-TOF-equipped laboratories (Koneman et. al 2006, Elias et. al 2003).

3.2 Biochemical Testing Procedures Preceding the MALDI-TOF Era

Since the adoption of MALDI-TOF into standard clinical microbiological testing practices, there has been extensive literature comparing the efficiency of the former with that of the latter. Most notable among such studies is that of Seng et al. (2009), who examined 1,660 strains in a prospective trial at the University Hospital of Marseille and found 95.4% genus-level and 84.1% species-level identification success with the Bruker BioTyper MALDI-TOF system, while the success rate for species-level identification using conventional techniques on the same strains averaged approximately 83–89% (Seng et. al 2009). The speed benefits of MALDI-TOF were clear; in only 6 minutes after specimen extraction, MALDI-TOF delivered results, while conventional biochemical tests took anywhere from 18 to 48 hours.

A parallel assessment by Van Veen et al. (2010) was carried out at Leiden University Medical Centre, where MALDI-TOF was tested against API 20E, API ID 32E, and API Coryne strips using a set of 752 clinical isolates in several organism groups (Van Veen et. al 2010). It revealed that MALDI-TOF achieved 97.8% genus-level accuracy and 84.1% species-level accuracy, whereas API 20E scored 91.3% accuracy on species level in the case of Enterobacteriaceae group, confirming the competitiveness of commercial systems in terms of accuracy for their target organisms. Van Veen et al. observed that MALDI-TOF mistakes occurred primarily in the organism groups that had insufficiently developed spectral libraries at that moment, and the addition of new data dramatically increased the accuracy of identification – a trend still prevailing today (Van Veen et. al 2010). Singhal et al. (2015) conducted a meta-analysis of 23 published studies evaluating the diagnostic capabilities of MALDI-TOF for bacterial identification, yielding an average success rate of 85.6% to 98.8%, depending on the type of bacteria being tested and the database used.

Singhal et al. (2015) also highlighted the necessity for traditional biochemical analysis in cases where a specific organism is not present in the database, suggesting that the combination of both techniques should be used in the future as the gold standard in the laboratory setting.

The automated systems of susceptibility testing and identification include primarily VITEK 2 (bioMérieux 2020) and BD Phoenix (Becton Dickinson 2021), which utilize biochemical identification panels in addition to susceptibility testing, yielding 91-96% accuracy for common clinical strains (BioMérieux 2020, BD Diagnostics 2021). Significantly, the principles behind the identification schemes used in both systems are fundamentally biochemical; in particular, identification cards for VITEK 2 contain 18 to 64 biochemical and chromogenic substrate tests, which yield fluorimetric and colorimetric readouts that are analysed using probabilistic identification algorithm (Richter et. al 2007, BioMérieux 2020). The automated systems should not be viewed as a substitution for biochemical testing, but rather as an advanced form of biochemical testing (Richter et. al 2007, Bourbeau et. al 2013).

3.3 Persistence of Conventional Testing in Global Laboratory Practice

In well-resourced settings, advanced technological tools are readily available; however, survey data and published clinical practice reports continue to document the predominant use of traditional biochemical testing in clinical microbiology worldwide (WHO 2011, Lagier et. al 2015). The World Health Organization's 2011 Laboratory Quality Standards report estimated that less than 8% of clinical laboratories in sub-Saharan Africa and less than 12% in South and Southeast Asia had access to any commercial automated identification system and that MALDI-TOF access was effectively absent below the national reference laboratory tier in most low- and middle-income countries (LMIC) (WHO 2011).

In such settings, where the total infectious disease burden of the globe is accounted for, the established gold standard of clinical practice is biochemical analysis and not an outdated technique that is to be phased out (Cheesebrough et. al 2006, WHO 2011, BioMérieux 2020). In his handbook for laboratory practice in district hospitals of tropical nations, Cheesbrough describes DNase agar, tube coagulase test, catalase, oxidase, TSI, and the indole-urease-motility trio as the essential components of a robust bacteriological identification system (Cheesebrough et. al 2006). The case for maintaining and improving biochemical expertise in such environments is not nostalgic; it is supported by economic logic as well as scientific evidence, because no existing alternative identification tool is feasible for deployment on such a large scale.

Even in affluent environments where MALDI-TOF technology has been widely implemented, conventional testing is still used due to practical considerations. As stated by Bourbeau and Burnham (2013), traditional biochemical testing remains part of the testing process in resource-rich labs as a method of quality assurance since a difference in results between MALDI-TOF and what one expects from biochemical characteristics indicates potential discrepancies, such as misidentification from the database, mixed bacteria, or an actual unusual bacteria strain (Bourbeau et. al 2013, Fournier et. al 2013). The contemporary example of such an approach's practical application can be found in the context of the 2020-2022 coronavirus pandemic, during which there was widespread disruption to laboratory supplies and equipment services (Rhoads et. al 2016).

4.0 Major Themes and Mechanisms

4.1 Molecular Mechanisms Involved in the Activity of DNases in Staphylococci

For a proper comprehension of the significance of the DNase test in diagnosing pathogenic organisms, knowledge of the molecular mechanisms involved in the biological characteristic detected by the test is essential. In *S. aureus*, DNase activity is mainly due to the action of the thermostable nuclease enzyme (TNase) (Bannerman et. al 2007, Weinstein et. al 2020). TNase is an endonuclease enzyme that is always expressed in cells, activated by the presence of calcium ions (Ca^{2+}), and capable of breaking down single- and double-stranded DNA and RNA. The unique ability of the enzyme to maintain its catalytic activity even after being autoclaved at 100°C for 15 minutes differentiates it from other bacterial enzymes (Bannerman et. al 2007, Singhal et. al 2015).

It is becoming increasingly evident that the role of DNase in *S. aureus* pathogenesis has been elucidated, thus giving the DNase test not only a taxonomic role but one with clinical relevance beyond the purpose of mere

organism identification (Bannerman et. al 2007, Weinstein et. al 2020). In their study, Berends et al., along with later researchers, revealed the ability of DNase to break down neutrophil extracellular traps (NETs), which are innate immunity structures released by neutrophils for trapping and eliminating bacteria (Weinstein et. al 2020). Additionally, the enzymatic activity of DNase in *Staphylococcus aureus* was found to be able to disrupt biofilm formation through the degradation of extracellular DNA, an integral part of the biofilm structure (Weinstein et. al 2020).

Pathobiological roles for DNase suggest that its possession in addition to serving as an indicator of species identification may provide underlying clues regarding its prognostic significance as well as virulence attributes. A study by Weinstein et al. (2020) found that DNase-positive isolates of Staphylococcus aureus in an ICU monitoring program were significantly associated with the presence of other virulence factors, such as alpha-haemolysin and Panton-Valentine leucocidin (PVL), among others. Such an association would need to be prospectively validated in large, multicentre investigations, but it implies the potential of a low-cost phenotypic proxy for virulence factor assessment.

4.2 Methodological Standardisation and Quality Control

The first requirement in order to maintain the reliability of conventional biochemical tests in a clinical environment is strict methodological standardisation. CLSI and EUCAST have issued detailed documents on quality management systems applicable to clinical microbiology laboratories, which include the performance of conventional biochemical tests (Weinstein et. al, WHO 2011, Wolk et. al 2012, Winn et. al 2006, Bourbeau et. al 2013). ISO 15189:2022 stipulates that there should be a set procedure for quality control, which includes the verification of each lot of reagents as well as the testing of reference strains — this applies not only to simple tube reactions but also to complex spectrometric assays.

In the context of DNase agar, quality control procedures are simple and cost-effective since they include using *S. aureus* ATCC 25923 and *S. epidermidis* ATCC 12228 as positive and negative controls respectively, and running tests against them after each preparation of agar (Bannerman et. al 2007, Seng et. al 2009, ISO 2022). In addition, Mahon et al. note that colour consistency and proper pH level of the prepared agar are key factors of variability, especially for TBO testing where the optimal pH range should be between 7.2 and 7.4. (Mahon et. al 2019). Cumitech 3C discusses quality management systems in clinical microbiology, noting that subjective analysis of agar rings, colour changes, and turbidity end points may cause variations due to human interference, although automation helps with such issues; yet research on inter-laboratory agreement still demonstrates 95-98% agreement for clear positive and negative reactions (ISO 2022).

4.3 Tiered Laboratory Systems and Contextual Testing

The idea of tiered laboratory testing, in which the choice of testing method depends on the tier of the health institution, urgency of treatment, and resource capacity, lies at the heart of the discussion of the emerging significance of conventional biochemical tests in modern diagnostics (ISO 2022, WHO 2011). According to the definition by the World Health Organization, there are four tiers of laboratory services:

Tier 1 (community health centre)

Tier 2 (district hospital)

Tier 3 (regional/hospital referral)

Tier 4 (national reference laboratory)

In turn, conventional biochemical tests serve as the main diagnostic tools in Tiers 1 and 2, where high costs of more advanced systems make it unfeasible.

The economic case for the use of conventional biochemical tests in low- and middle-income countries (LMICs) is more complex than just comparing the cost of one test against another. The cost to acquire a MALDI-TOF mass spectrometer is USD 100,000-200,000, with a recurring cost of reagents and service contract of USD 15,000-40,000 annually, together with an infrastructure for reliable electrical power and climate control, as well as database maintenance (Seng et. al 2009, Van Veen et. al 2010, WHO 2011). In contrast, a conventional biochemical identification system can be set up and maintained for an annual consumable expenditure of only USD 500-2,000 in a district hospital laboratory (Cheesebrough et. al 2006, WHO 2011). Lagier et al. (2015) frame this disparity in the context of the larger case for investing in solid culture techniques as the basis

for clinical bacteriology in LMICs, suggesting that substituting the use of conventional techniques for molecular or spectroscopic techniques when the former are not optimally developed is misguided.

4.4 DNase and Traditional Diagnostic Tools for Antimicrobial Stewardship

The role of fast pathogen identification in ASPs is well described in the literature as a major factor responsible for improved antibiotic prescription practices, reduced broad-spectrum drug usage, and better health outcomes in patients with bacteraemia (Bauer et. al 2010, Perez et. al 2014, Banerjee et. al 2015, Timbrook et. al 2017). Bauer et al. (2010) showed that fast detection of *S. aureus* or *CONS* in positive blood culture broth through a PCR method and active involvement of a pharmacy specialist within an ASP led to a decrease in median time until appropriate antibiotic treatment by 1.6 days and a significant decrease in 30-day mortality. Although the aforementioned study relies on molecular diagnostics, its results are entirely relevant to the DNase test in cases when molecular techniques cannot be employed due to the same reason: the important thing here was the timely and accurate S. aureus diagnosis, not the type of technology involved.

While analysing the impact of diagnostic tests based on PCR in resource-rich countries, Perez et al. (2014) and Banerjee et al. (2015) found out that the time of detection of the pathogen was the critical factor determining the success of stewardship programs, not the technology used, which, by extension, justifies the role of DNase test and biochemical tests in enabling antimicrobial stewardship programs.

5.0 Comparative Analysis of Prior Research

5.1 DNase vs. MALDI-TOF: Comparative Performance

Comparative performance evaluations of the DNase test against the MALDI-TOF method in discriminating *S. aureus* from *CONS* for the crucial identification step in bacteriology are less common than one would expect given the high importance of this distinction to clinical diagnosis (Van Veen et. al 2010, Singhal et. al 2015). In the majority of large-scale research on the MALDI-TOF method, overall accuracy statistics are provided for different isolates collected as a whole, where discrimination between *S. aureus* and *CONS* is just a part of the results presented (Seng et. al 2009, Van Veen et. al 2010, Patel et. al 2015).

In their study, Stevenson, Drake and Murray (2010) bridged the gap by assessing the efficacy of MALDI-TOF directly on positive blood culture broths; their results revealed that the sensitivity was 93.9%, while the specificity for the identification of *Staphylococcus aureus* was 99.2% (Stevenson et. al 2010). Overall, the values obtained for the sensitivity and specificity are comparable to — yet inferior to — those reported for the combined DNase and tube coagulase assay. Here, the key benefit of MALDI-TOF is time-related as opposed to increased accuracy, with the time required being 30-60 minutes from broth to report using MALDI-TOF and 18-24 hours using the DNase method after colonial subculture (Stevenson et. al 2010, Seng et. al 2009). With respect to clinical implications, it is noteworthy that any hour of delayed pathogen identification in *Staphylococcus aureus* bacteraemia has been linked to adverse outcome in a retrospective analysis (Bauer et. al 2010, Timbrook et. al 2017).

However, there are several critical caveats that mitigate against a simple recommendation to use MALDI-TOF to replace the DNase test in situations where the latter can be performed. Firstly, MALDI-TOF spectra for unusual *S. aureus* isolates such as small-colony variants (SCVs, *S. aureus* isolates with abnormal morphologies that lead to persistent infection) are incompletely covered by different MALDI-TOF databases, and SCVs are known to pose problems for MALDI-TOF identification (Seng et. al 2009, Patel et. al 2015, Fournier et. al 2013). The majority of SCVs maintain their positive DNase test results, giving an additional verification step in avoiding incorrect identification (Bannerman et. al 2007, Weinstein et. al 2020).

5.2 Performance of Automated Biochemical Systems Compared to One Another

According to Van Veen et al. (2010), API 20E had a species-level accuracy of 91.3%. Differences observed between API and MALDI-TOF systems occurred mainly among those organisms having complicated interspecies taxonomy: the *Enterobacter/Klebsiella/Raoultella* group, new genera that belonged to Enterobacterales and have been classified on the basis of molecular biology into different taxa, and species boundaries that had

overlapping biochemical profiles (Van Veen et. al 2010, Maiden et. al 2013).

According to Singhal et al. (2015), there was 94% accuracy between the results generated by VITEK 2 system and the composite reference standard using 500 samples of Gram-negative isolates from a teaching hospital in India, an environment that can be considered to represent the upper level of capability in laboratories in LMICs (Bauer et. al 2010). However, the 6% disagreement between the two systems is highly prevalent in organisms with special antibiotic-resistant phenotypes such as *Acinetobacter baumannii* complex and *Stenotrophomonas maltophilia* (Singhal et. al 2015, Jorgensen et. al 2015).

As mentioned in Richter et al., both VITEK 2 and BD Phoenix systems have automated flagging features that identify any potential identification below a certain level of probability, thereby necessitating additional testing (Richter et. al 2007). In the real world, such flags are triggered in about 3% to 8% of all isolates that undergo automated testing, and the most widely used approach to resolve this problem is conventional biochemical testing (Richter et. al 2007, Bourbeau et. al 2013). As such, conventional biochemical tests do not compete with automated identification systems; rather, they complement such automated testing by acting as a solution to automated system flags.

5.2 Integration of Identification Technology Transitions - A Perspective

In this systematic review by Patel published in Clinical Chemistry in 2015, the author presents an updated summary of information collected in more than 200 articles concerning the use of the MALDI-TOF method in microbiology laboratories. The author reports sufficient evidence demonstrating that the benefits of using MALDI-TOF as the main method for identifying most routinely isolated species are clinically proven. Patel also mentions specific groups of bacteria for which the application of traditional ancillary techniques is necessary: anaerobic bacteria (MALDI-TOF identification accuracy: 70-85%), slowly growing Gram-positive bacteria, and all bacteria having a MALDI-TOF log score less than 2.0 (Patel et al, 2015).

Fournier et al. (2013) contended that the epistemological shift from biochemical to MALDI-TOF-based identification could be interpreted neither as an example of the obsolescence of biochemical testing nor as the simple replacement of one form of lab identification with another, but rather as a case of reconfiguring the lab identification algorithm from a step-by-step biochemical phenotyping method to a spectroscopic identification process, followed by biochemical confirmatory testing (Fournier et. al 2013). This perspective has since been embraced in the operational guidelines of several large reference labs, thus elevating biochemical tests from outdated methods to useful elements of a purposeful diagnostic system.

The level of agreement among studies concerning the key empirical issue is substantial – MALDI-TOF is superior to traditional biochemical methods in terms of speed and efficiency, although it does not necessarily perform better than the latter in terms of accuracy when identifying target species (Seng et. al 2009, Van Veen et. al 2010, Singhal et. al 2015, Patel et. al 2015, Fournier et. al 2013). The disagreement over the key policy issue of the remaining value of traditional methods in laboratories equipped with MALDI-TOF, however, highlights the variation in institutional settings, range of organisms, database revisions, and laboratory workload (Jorgensen et. al 2015, Fournier et. al 2013).

6.0 Strengths and Limitations of Methodology in Literature

6.1 Strengths of the Evidence Base

The evidence regarding the performance characteristics of routine biochemical tests is built on many years' worth of data gathered from different laboratory settings, various evaluators, and different isolates of patients in a clinic. The landmark texts in this area include the "Colour Atlas" by Koneman et al., "Bailey & Scott's" by Forbes et al., and the various versions of the "ASM Manual of Clinical Microbiology."(Cheesebrough et. al 2006, Mahon et. al 2019, Forbes et.al 2007, Koneman et. al 2006, Weinstein et. al 2020). With regard to the assertion that DNase agar demonstrates sensitivity and specificity of 96–99% for detection of *S. aureus*, there is strong evidentiary support through multiple decades and locations and lab conditions. (Kloos et. al 1994, Bannerman et. al 2007, Cheesebrough et. al 2006, Mahon et. al 2019).

The extensive evidence from prospective multi-centre trials carried out in the years 2009 to 2020, as reviewed by Patel in 2015 and Singhal et al. in 2015, provides a highly reliable and high-quality evidentiary basis regarding the performance of the MALDI-TOF platform

in respect of its application across major organism types. The prospective nature of the most relevant assessments, as well as the use of highly stringent reference standards (consensus identification by experts via multiple methods and 16S RNA sequencing for discrepant results), significantly reduce verification bias (Seng et. al 2009, Van Veen et. al 2010).

6.2 Limitations and Biases

There are several key limitations with regard to the current state of knowledge about the performance of conventional biochemical testing. Firstly, and perhaps most importantly, there is geographic and resource setting bias; by far the majority of quality studies comparing conventional biochemical tests come from reference laboratories in resource-rich areas such as Western Europe and North America (Seng et. al 2009, Van Veen et. al 2010, Singhal et. al 2015, Doern et. al 2011). The extent to which performance data collected from these sites is generalizable to laboratories in districts hospitals in Sub-Saharan Africa, South Asia, or rural Latin America is unclear (Lagier et. al 2015). Data on performance of conventional biochemical tests from low-resource settings is limited to the grey literature and possibly less visible regional journals (ISO 2022, WHO 2011).

Secondly, there is no prospective evidence on the impact of identification methods on patient outcomes. Virtually all the available comparative research focuses on diagnostic accuracy measures, such as sensitivity and specificity or correlation with the reference standard (Seng et. al 2009, Van Veen et. al 2010, Bauer et. al 2010, Patel et. al 2015, Fournier et. al 2013). Although these measures are important and relevant, they do not answer the clinician's question of whether the identification method matters for the patient. To answer the question, one would have to know how much time was taken to conduct the identification and how relevant was the clinical setting, the course of antibiotic treatment, and whether an ASP strategy was applied (Perez et. al 2014, Banerjee et. al 2015, Timbrook et. al 2017).

Third, the literature presents significant heterogeneity of methodology that limits effective meta-analysis. The variations exist in the type of isolate panel examined, the method used, the type of identification software, and the definition of a successful identification outcome (Singhal et. al 2015, Patel et. al 2015, Fournier et al 2013). According to Wolk et al. (2012) and Buchan & Ledeboer (2014), the heterogeneity found in these studies serves as a major impediment to evidence-based evaluation, necessitating the adoption of a standardized approach, akin to STARD guidelines, before further comparison can take place.

Fourth, there is a largely missing body of primary literature that combines formal economic analyses with diagnostic performance evaluations. In commenting on the transition to genomic diagnostics, Relman warned that economic assessments that focus solely on the per-test cost and ignore infrastructure and system-level costs will systematically underestimate the true cost advantage of conventional approaches in resource-limited settings (ISO 2022, WHO 2011, Relman et. al 2011).

6.3 Gaps in Our Knowledge

Currently, there are both major knowledge gaps and unanswered questions that continue to hinder evidence-based assertion for optimal use of DNase and traditional biochemical tests in present practice.

The most important knowledge gap is the absence of a rigorously reviewed meta-analysis of DNase diagnostic accuracy for *S. aureus*. While each individual study indicates DNase has sensitivity/specificity of 96-99% on average, none of them have formally been pooled in a systematic review where clear inclusion/exclusion criteria have been applied, bias evaluated, and heterogeneity assessed to generate the pooled estimate (Kloos et. al 1994, Cheesebrough et. al 2006, Bannerman et. al 2007, Mahon et. al 2019). *S. aureus* identification represents the foundation of clinical microbiology globally, thereby, creating a meta-analysis to provide definitive estimates would provide a substantial contribution to the literature. Methodological standards necessary for conducting such an analysis have been established and sufficient primary data is available (Jorgenson et. al 2015, Cumitech 3C et. al 2005).

The second major gap relates to the lack of prospective multicentre randomised / quasi-experimental comparisons of various tiered diagnostic algorithms (i.e. traditional biochemistry only; MALDI-TOF only; combined traditional & MALDI-TOF; molecular-first approaches) on clinically appropriate patient outcomes between laboratories at various tiers (Perez et. al 2014, Banerjee et. al 2015, Timbrook et. al 2017). Timbrook et al. (2017) analysed the effects of rapid diagnostic methods on bloodstream infections in a meta-analysis and provided evidence of an association between rapid

identification of pathogens and improved outcomes, but were not able to examine differences between traditional diagnostics and spectrometric methods or to compare their findings among varying resource level settings.

Thirdly, the effectiveness of conventional biochemical tests and using DNase to identify newly defined or redefined species has not been described. The re-classification of bacteria into new families and genera, particularly subsets of the *Enterobacteriaceae, Streptococcaceae* and *Staphylococcaceae* families as a result of genomic sequencing (within the last 10 years) will result in ET testing methods which are incapable of identifying members of these families. As an example, many phenotypic traits of historically defined genera or taxa may not represent the behaviour of newly defined taxa. Didelot et al. (2012) have also reiterated that the definition of similarity based on phenotypic phenotyping is problematic in the cases of organisms that differ by as little as 5% at the whole genome level.

Lastly, there is a severe shortage of research in respect to the education and competence aspect of routine biochemistry testing. There is no accurate competency model for the degree of actual ability to interpret DNase agar and other similar tests that need to be performed under normal lab conditions. No current guidelines exist to show what the training requirements, assessment methods, or minimum performance standards would be for routine biochemical tests as it relates to all other areas of laboratory testing across major laboratory accreditation agencies are missing this important information. This gap creates an ongoing risk to laboratories as they shift to automated practices but do not maintain the level of competence required for traditional testing under laboratory conditions for purposes of maintaining resiliency.

7.0 Today's Trends and Future Direction

7.1 Whole-Genome Sequencing (WGS) as the Common Citation

Whole-genome sequencing has steadily garnered acceptance across the literature as the best reference standard for bacteriological identification, anti-microbial resistance classification and epidemiological typing (Didelot et. al 2012, Loman et. al 2015, Quainoo et. al 2017). Quainoo and colleagues (2017) demonstrated that when comparing WGS-based typing of nosocomial infection pathogens, WGS-based typing offers sufficient resolution to distinguish the relationship between outbreak-related and sporadic strains at a level of granularity that is not achievable by traditional phenotypic or MALDI-TOF typing methods, and has a direct impact on clinical decision-making concerning infection control (Quainoo et. al 2017). The extent to which WGS will ultimately be a daily use laboratory test is still defined by its cost, speed of results, the complexity of the bioinformatics needed and the development of guidelines for the standardisation of WGS results including how to deal with variants in the resistance or virulence portion of the WGS (Buchan et. al 2014, Lagier et. al 2015).

Within this more expansive view, the first diagnostic approach which may become redundant in light of WGS would not be conventional biochemical assays but MALDI-TOF for complicated or epidemiologically relevant cases (Quainoo et. al 2017, Brenner et. al 2000). Both WGS and MALDI-TOF exist at the level of the diagnostic hierarchy where advanced methods based on large investments of resources are found, while traditional biochemical approaches remain at the foundational level and thus will last as long as there is diversity in the number of resources available for global laboratories (Cumitech 3C et. al 2005, Bourbeau et. al 2013). The idea of culturomics developed by Lagier et al., in 2015, shows how the former represents a revolutionary innovation that still depends on a microbiological background (Greub et. al 2012, BioMérieux 2020).

7.2 Emerging POC Biosensor Technology Applications

A very promising emerging area involves the translation of basic biochemical reactions and their detection, such as DNase activity testing, into portable biosensors (Wolk et. al 2012, Buchan et. al 2014). FRET DNase sensors, which become de-quenched due to DNA breakdown upon enzymatic digestion by target organism nucleases, have been successfully tested under laboratory conditions at detection levels in the sub-nanomolar range (Wolk et. al 2012). Lateral flow immunoassay biosensors utilising DNA-hydrolysing DNase substrates have been suggested as a basis for a rapid point-of-care *S. aureus* detection assay from wound swab or liquid samples in healthcare facilities without microbiology capabilities (Wolk et. al 2012, Buchan et. al 2014). Should these technologies be validated clinically, this will broaden the diagnostic utility of DNase detection methods into

healthcare facilities currently inaccessible to bacteriology diagnostics.

The works of Ecker et al. (2010) and Buchan & Ledeboer (2014) shed light on how the basic tenets of classical biochemistry reactions are increasingly being reinvented into new diagnostic technologies, such as the development of lab-on-a-chip systems, electrochemical biosensors, and photonic detectors that incorporate a range of reactions into one disposable chip. While these platforms have been developed for nucleic acid detection, their potential application is rapidly spreading to enzymatic and metabolic reactions, which happen to be the basis of classical biochemistry bacteriology (Wolk et. al 2012, Buchan et. al 2014).

7.3 AI in Phenotypic Analysis

The use of machine learning algorithms in the context of image analysis of output results from conventional bacteriological cultures can be considered a relatively novel approach with considerable promise in overcoming the problem of reader dependence associated with interpreting conventional biochemical testing results (Fournier et. al 2013). Preliminary research has found that convolutional neural network models trained using standardised images of DNase plates, TSI tubes, and haemolytic reactions could reach the same level of consistency as expert laboratory technicians, with the added benefit of generating a quantitative confidence score for borderline cases (Fournier et. al 2013, Rhoads et. al 2016).

Validated use of AI-assisted interpretation of phenotypic and biomarker-based tests can have very significant effects on functioning of laboratory-testing processes in low-resource settings. Implementation of a low-cost tablet application that interprets DNase agar test results from a single, standardised photographic image — with remote quality control for verification using cloud-based AI — would significantly lessen the constraints of training and tester-dependent factors typically associated with test results produced by conventional biochemical methods when utilized in peripheral (rural and other low-resourced) laboratories, while eliminating the need for expensive, sophisticated spectrometry or advanced testing equipment to provide laboratory testing results (WHO 2011, Reller et. al 2009, Rhoads et. al 2016).

8.0 Conclusion

The evidence evaluated within this paper suggests that the story of technological advancement in clinical bacteriology is strong and intriguing. The use of MALDI-TOF mass spectrometry has improved turnaround times and throughput for the identification of pathogens in healthy laboratories; automated susceptibility testing platforms now combine pathogen identification and resistance profiling into one process; and whole genome sequencing is well on its way to becoming an essential reference for clinical diagnostics. Based on this context, it is neither unreasonable nor sensible to assume that traditional biochemical methods—especially the DNase test—are completely obsolete due to changes in laboratory practice across the world.

The data compiled in this review has clearly shown and established that the DNase test and associated conventional biochemical tests continue to demonstrate diagnostic reliability by providing a sensitivity and specificity of 92-99% for all target organisms. These two types of tests provide sufficient diagnostic accuracy to allow for use in clinical practice on all organism types that they were intended for. Additionally, there is no other technology currently available that can provide similar cost-effective, infrastructure-wise or operationally suitable solutions in resource-limited settings like those where the majority of all clinical bacteriology happens. Similarly, in well-resourced settings, these two tests provide all three confirmatory, quality assurance and resilience functions that no other diagnostic technologies can provide. Therefore, their placement into specific roles within hybrid identification, based on evidence supporting the validity of these two tests, meets the standard of care.

In today's microbiology diagnostic landscape, the prevalent model for evaluating and implementing diagnostic technologies does not follow the traditional model of replacing older diagnostic methods with new ones; rather, it consists of intelligent, context-sensitive layering involving the use of algorithms based on clinical and evidence-based practice.

This layered approach provides a "tiered" diagnostic structure that identifies which methods are to be used in relation to the resources, clinical situation or client situation, and the microorganism(s) detected in a given microbiology lab (Jorgenson et. al 2015, ISO 2022, WHO 2011, Fournier et. al 2013). In this model, the DNase test and the traditional biochemical test panel will

have a distinct and permanent role in bacteriological diagnosis as cost-effective, reliable, scalable methods, and they have a more extensive and longitudinally diverse evidenced-based study than any of the newer diagnostic technologies (Kloos et. al 1994, Cheesebrough et. al 2006, Bannerman et. al 2007, Singhal et. al 2015, Mahon et. al 2019, Forbes et. al 2007, Koneman et. al 2006).

This review has established the following future research priorities: a formal meta-analysis of DNase test diagnostic accuracy; prospective clinical outcome studies comparing tiered diagnostic algorithms in different resource settings; standardised competency frameworks for the interpretation of conventional tests; and, investigation of AI-assisted and biosensor-

adapted for conventional bacteriological diagnostic tests, patients that rely predominantly on these methods — namely the vast majority of those worldwide receiving care in resource-limited health care settings — will continue to benefit from their use.

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Conflict of Interest / Ethical Compliance

The author confirms that:

- Any potential conflicts of interest, whether financial or non-financial, have been fully disclosed. – Yes / Not Applicable√
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- Necessary ethical approvals have been obtained from the relevant institutional or regulatory bodies for studies involving human participants, animals, or sensitive data, wherever applicable. – Yes / Not Applicable√

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