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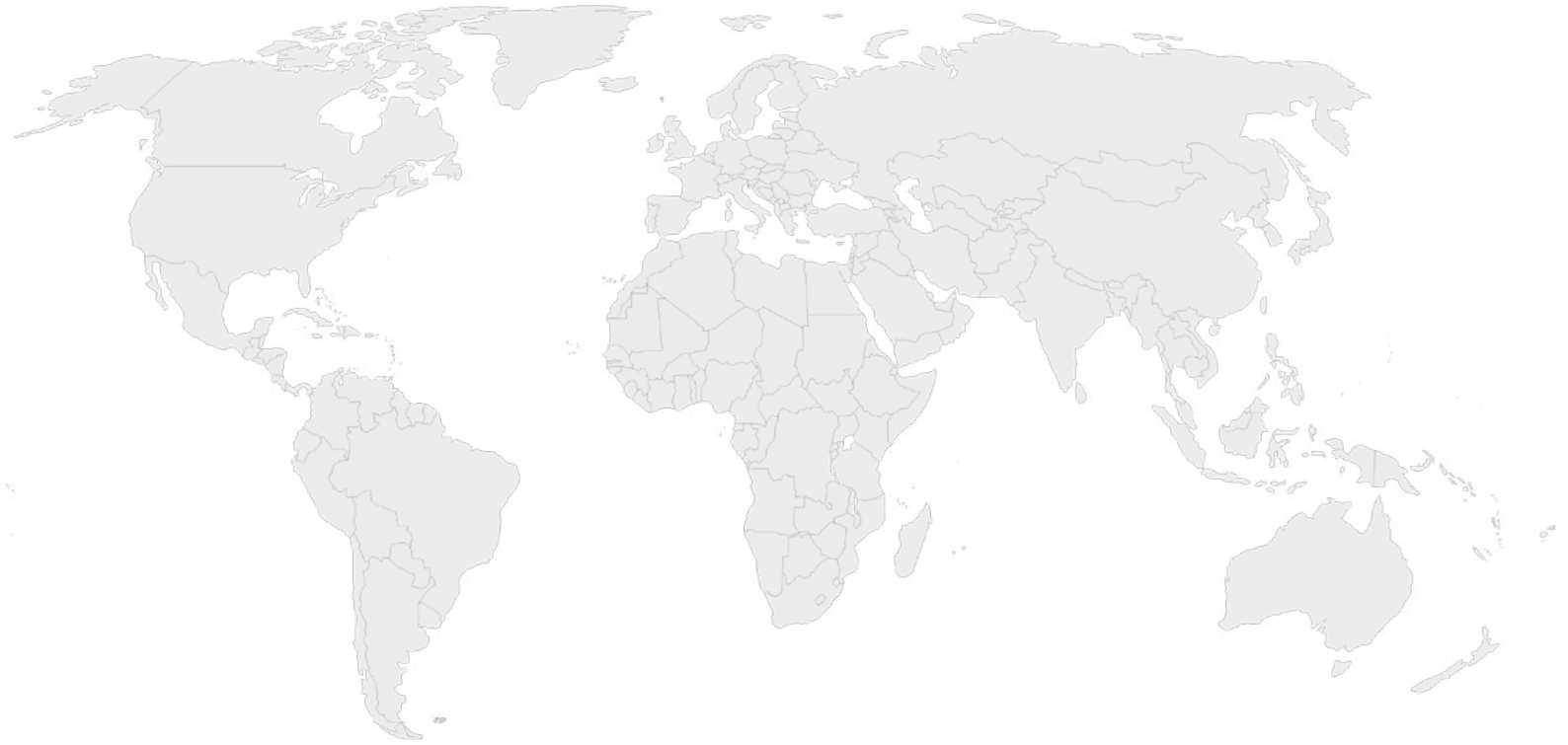

In this Issue

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Letter to the Editor

Biomarkers for harmful alcohol use should be reliable, standardised, and traceable

Only the CDT reference method has been approved by JCTLM and IFCC

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On behalf of the IFCC Working Group CDT (December 2024)

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Dear Editor,

Harmful alcohol consumption is widespread and of major concern. Besides in medical diagnosis and treatment, measurement of overconsumption through biomarkers is often used in forensic and traffic medicine. Traceability and standardisation of biomarkers are required especially in this field, and this is briefly addressed.

None of the modern biomarkers for harmful alcohol use is standardised and traceable yet except carbohydrate-deficient transferrin (CDT). Recently the Joint Committee of Traceability in Laboratory Medicine (JCTLM) has approved the IFCC recognized HPLC reference method for CDT.

Keywords

Carbohydrate deficient transferrin, CDT, Standardisation, Traceability, AUD, Harmful alcohol use, Fitness to drive, DUI

Introduction

Harmful consumption, often called chronic abuse, of alcohol is a major cause of disability and death all over the world. Alcohol consumption resulted in an estimated worldwide 2.6 million deaths (4.7% of all deaths) and 115.9 million DALYs (4.6% of all DALYs) in 2019 [1]. The traditional blood biomarkers for detection and monitoring of excessive alcohol consumption and alcohol use disorder (AUD) include GGT, AST, ALT and MCV. They are still used for this purpose in many countries despite being unsensitive for early detection and also rather unspecific [2]. These biomarkers are called indirect because their increase is based on secondary effects of heavy drinking related to organ or cell damage. Direct biomarkers are products of ethanol metabolism and include PEth, EtG and EtS [3].

Another modern biomarker, carbohydrate-deficient transferrin (CDT), falls somewhat in between the direct and indirect alcohol biomarkers. CDT refers to the disialo glycoform of serum transferrin that is produced in increased levels, in response to prolonged heavy drinking, through action of the ethanol metabolite acetaldehyde [4]. CDT is especially suitable for detecting and monitoring of chronic harmful consumption, not for incidental drinking [5] CDT, which is probably the most studied alcohol biomarker in recent decades, is still relatively unknown and not used in many countries. Even when discussed or reviewed, statements about CDT are repeatedly based on incorrect information and outdated scientific literature [3] using no longer available methods, like a combination of anion exchange chromatography followed by RIA or turbidimetry [6]. The performance of CDT is clearly improved using modern HPLC or CE methods [5].

Alcohol biomarkers are used worldwide as objective measures in medical diagnosis, as well as in forensic tests after driving under influence (DUI) and in fitness-to-drive examinations [7,8]. Especially the latter use implies that the analytical methods used should be reliable, having a high diagnostic accuracy and being standardised and traceable to guarantee a fair judgement about a person's alcohol intake.

A comparison of the diagnostic performance of AUD biomarkers is rather complex and should include the specific time windows, increase and decay kinetics, well defined study populations, analytical and preanalytical interferences, among others [5]. A metrological comparison is preferentially based on ROC curves and a well-chosen study population [5]. In large comparative studies CDT was found favourable to the traditional markers [9,10]. PEth is more sensitive than CDT but depending on the population under study and the consumption level [11,12]. However, this present letter is not about analytical performance, but about the need for standardisation and traceability.

The benefits of standardisation of measurement procedures and having a reference measurement procedure (RMP) are generally acknowledged. To name a few advantages, the improved accuracy and precision of measurement will increase diagnostic accuracy [13]. Method standardisation and uniform cutoffs are also corner stones for developing regulatory guidelines, and important when comparing outcomes of scientific studies. Finally, having an RMP aids in development and improving routine measurement methods.

It is therefore stunning that standardisation of methods is either not discussed at all [14] or incorrectly mentioned even in recent reviews on alcohol biomarkers [3]. The traditional laboratory methods like GGT are standardised by using IFCC procedures [15] but as mentioned before, they lack sufficient specificity.

Although becoming more and more popular, none of the direct alcohol biomarkers like PEth are yet standardised making them vulnerable for dispute in court, especially since the cutoffs are not internationally established [16,17].

It is surprising that the successful IFCC CDT standardisation work [4] is not widely known and implemented. The basis for CDT standardisation was laid by studies performed by the IFCC WG-CDT [4,18,19] starting with a proper selection of the analyte and measurand [18]. Based on an extensive validation study according to ISO15193, an established HPLC method [20] was recognized by the IFCC as the RMP for CDT, to be used for standardisation of all CDT methods on the market [21]. We are now proud to announce that after extensive metrological examinations, the IFCC RMP for CDT was also formally recognized and listed as RMP under database identifier C14RMP1R early 2024 by the Joint Committee for Traceability in Laboratory Medicine (JCTLM) [22]. This is the highest classification available for a reference method.

A distinction has to be made between non-standardised commercial methods and standardised commercial methods since standardisation has not yet been applied to all methods in several countries. Clearly stated, results are only metrologically standardised when measured by the RMP, or by commercial methods that are standardised against the RMP [23]. Standardised results should be expressed as CDT_{IFCC} with an upper level of reference of 1.7% and a cutoff of 2.0% [5,21].

CDT_{IFCC} is currently the only biomarker for alcohol consumption that has an RMP, recognized by both the IFCC and JCTLM, and a set of commercially available commutable reference materials. This means that standardisation is achieved [24]. A patient's CDT_{IFCC} result is traceable to the RMP as required by the JCTLM and the European IVDR 2017/746 regulation.

CDT is also the only FDA approved biomarker in its field and CDT measurement is relatively easy to perform with standard laboratory equipment like HPLC, capillary electrophoresis or immunochemistry / nephelometry.

Summarising, we emphasise the need for traceability and standardisation of biomarkers for harmful alcohol use particularly in forensic and traffic medicine. In addition, we point at the unique status of CDT amongst biomarkers for harmful alcohol use, in fulfilling these demands being JCTLM listed.

Declaration of Conflict of interests

The authors of this article declare that there is no conflict of interest with regard to the content of this manuscript.

Submission declaration

The work described has not been published previously in this form.

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Authorship

All authors have made substantial contributions to all of the following:

The conception of this letter and the publication of underlying studies.

Drafting the article or revising it critically.

Final approval of the version to be submitted.

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Letter to the Editor

Implosion of Grail's Galleri Cancer Screening Test?

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Keywords

Grail, Galleri test, cancer screening, multi-cancer detection

A recent prospective clinical trial using the Galleri Multi-Cancer Detection Test was temporarily put on hold, likely due to poor clinical performance (details were not made public), highlighting the need for its re-evaluation as a new and revolutionary cancer screening tool. In this correspondence we provide several questions that need to be answered by Grail before this technology is disseminated to the general public (although the test is marketed now).

Grail is a multi-billion-dollar biotechnology/diagnostic company, which developed a non-invasive blood test, claiming to detect 50 types of cancer at early and potentially curable stages. Since their first publication [1], we expressed concerns about the sensitivity and specificity of the test (now widely known as the Galleri test) and its suitability for population screening [2-4]. To their credit, Grail conducted large prospective studies [5] to demonstrate the test's clinical capability. The initial promising results, and Grail's anticipated financial success, prompted the next generation sequencing giant Illumina to purchase Grail for $8 billion (2021). Another collaboration of Grail with the UK National Health System (UK-NHS) includes a 3-year prospective trial conducted in parallel with the current standard of care. At the end of the first year, UK-NHS unexpectedly announced that the trial was put on hold, likely due to rather poor clinical performance (no details given) and until they analyze the first-year data which will be available by end of 2026 [6,7]. Legal and financial issues between the interested parties are currently not public [8]. The Grail case has some similarities to the Theranos story, which sent some executives to jail and led to company bankruptcy [9].

One reason that such unfortunate events are happening is the hype that is (intentionally) created around new technologies to make them more attractive to investors. Due to the unknown possible harms of the Galleri test [10], we suggest its withdrawal from the market, until its capabilities and shortcomings become clearer. For example, the ongoing trials will answer the critical question of better survival of those who are screened and the test's associated harms. After in-depth data analysis, Grail has the obligation to make their first-year results transparent, so that interested parties understand the benefits and harms of this screening. For more detailed discussion please see the cited literature [6,7,10].

A partial list of relevant questions related to the Grail-NHS Collaboration includes (these questions were also presented in a more detailed version of our manuscript) [10]: How

many Galleri tests generated equivocal (uninterpretable) results? In such cases, what recommendations are provided to the tested individuals, including money-back refunds? The test currently costs about $1,000. How many results were false positives and how were they confirmed? Were the confirmation tests invasive and produced any harm (including death) due to invasive confirmatory procedures? How many of the detected tumors were indolent? How many patients who developed cancer on follow-up initially had a negative test? Did screened individuals have stage migration from stage III-IV to lower stage? For how many patients was the tumor site correctly identified? Were there complications in trying to locate the tumor in the wrong organ? In patients who test negative at first screening, what explanation would be given to them if the subsequent biannual test discovers a late-stage cancer? Would the testing company be liable for delivering misleading information? We understand that the most fundamental question, "if screening extends overall or disease-specific survival", will require much longer follow-up.

Finally, these and other similar tests should ideally be identified early by regulatory agencies, to protect patients, investors, and other stakeholders from artificially created situations which, at least partially, are motivated by profit [6,7,10].

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Letter to Editor

Highlights from the manifesto on Early Detection and Diagnosis of Cardiovascular Disease: The Role of Laboratory Tests and Emerging Technologies

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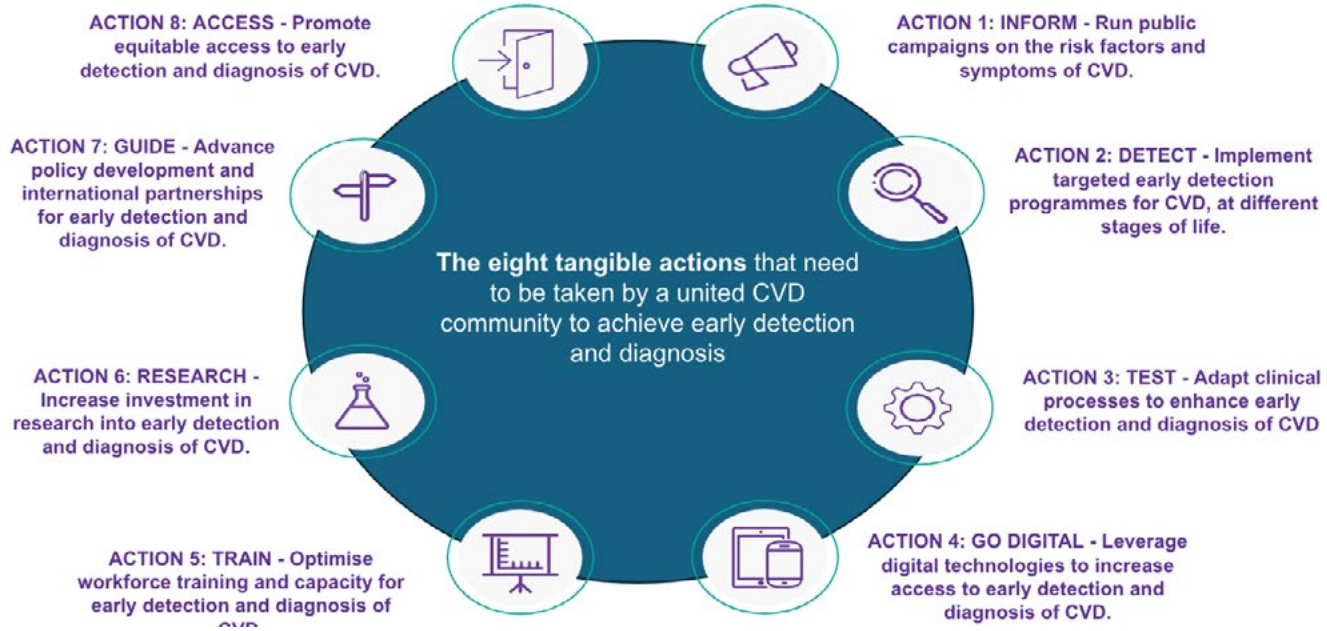
Keywords

Cardiovascular diseases, prevention, emerging technologies, artificial intelligence, biomarkers

Cardiovascular disease (CVD) remains the leading cause of mortality globally, accounting for over 30% of all deaths annually, with projections indicating a rise in CVD-related deaths by more than 60% between 2020 and 2050 [1,2]. Despite significant advancements in treatment, the early detection and diagnosis of CVD are crucial to improving patient outcomes and reducing the socioeconomic burden of the disease. The recently developed manifesto by the Global Heart Hub outlines eight actionable strategies to enhance the early detection and diagnosis of CVD. These eight actionable strategies are summarized in Figure 1.

Figure 1: Eight actionable strategies are summarized.



The eight tangible actions that need to be taken by a united CVD community to achieve early detection and diagnosis. These actions are taken from the GHH manifesto.

This article aims to summarize the key messages of the manifesto, emphasizing the expectations from laboratory tests and emerging technologies [2]. One of the unique features of this manifesto is that it was patient-led, global, and developed in collaboration with key stakeholders, with many contributors. These aspects highlight the importance of the messages shared.

Public Awareness and Targeted Detection

Inform

Public campaigns are essential to educate individuals about the risk factors and symptoms of CVD. These campaigns should be culturally tailored and leverage diverse media to reach a broad audience, empowering individuals to monitor their cardiovascular health and seek timely medical advice [2].

Detect

Implementing targeted early detection programs at different life stages is critical. These programs should identify high-risk individuals through biomarker testing, clinical support tools, and consideration of genetic, metabolic, and lifestyle risk factors. Collaboration with patient organizations and health authorities is necessary to adapt these programs locally [2].

Enhancing Clinical Processes and Digital Integration

Test

Enhancing clinical processes to facilitate early detection and diagnosis of CVD includes increasing access to rapid, point-of-care testing (POCT) in primary care and community settings. Redesigning patient care pathways to ensure thorough investigation of potential cardiac symptoms and underlying causes is paramount [2].

Opportunities and Challenges with POCT

Optimize

While POCT may face skepticism from the community due to concerns about accuracy and reliability, it presents significant opportunities. POCT allows for immediate results, enabling quicker decision-making and treatment initiation. This is particularly beneficial in rural or underserved areas where access to centralized laboratories is limited. The convenience and rapid turnaround time of POCT can lead to better patient adherence and engagement in their healthcare [3,4]. Furthermore, the improvement of the performances of POCT tests over last years is significant.

Implications of Seeking Reimbursement for Biomarker Testing and New Technologies

Incentivize

Securing reimbursement for biomarker testing and emerging technologies is crucial for their widespread adoption. Reimbursement policies that support the cost of NP testing can improve access to these critical diagnostic tools, particularly in primary care settings. In regions where NP testing is reimbursed, there is higher utilization, leading to earlier detection and improved patient outcomes. Policymakers must design reimbursement strategies that incentivize high-quality, patient-centered care, promoting the adoption of innovative diagnostic technologies [3,4].

Task Shifting
Expanding Roles of Healthcare Professionals

Reallocate

Expanded Remit of Healthcare Professionals
The concept of task shifting involves reallocating certain tasks from healthcare providers to other trained professionals, such as general practitioners, nurses, pharmacists and other groups of health care professionals. By performing POCT and initial screenings, these healthcare professionals can identify patients who need further evaluation by a physician, thus reducing the diagnostic burden on doctors and specialists. This approach not only optimizes the use of available resources but also ensures that patients receive timely care. To ensure competency, clinical laboratory professionals must certify healthcare professionals involved in diagnostic testing as competent. This certification process should include rigorous training and continuous education to keep pace with advancements in diagnostic methods. By doing so, we can maintain high standards of care and ensure the reliability and accuracy of test results [3].

Multidisciplinary Team Approach

Collaborate
The laboratory community is integral to a multidisciplinary team approach. Specialists in laboratory medicine, clinicians, and other healthcare professionals must collaborate to define clinical needs and interpret test results and develop comprehensive care plans. This collaboration ensures that patients receive accurate diagnoses and appropriate treatments in a timely manner [4].

Promoting Research and Rapid Patient Profiling
Enhance

Role in Research
The laboratory community plays a pivotal role in promoting research and developing rapid patient profiling tools. By participating in research initiatives and clinical trials, laboratories can contribute to the discovery of new biomarkers and diagnostic methods. Rapid patient profiling tools developed through such research can provide real-time insights into a patient's health status, facilitating personalized treatment plans [3,5].

Implications for the Laboratory Community

Invest
The adoption of new diagnostic technologies and the expansion of specialist in laboratory medicine roles have significant implications for the laboratory community. Continuous education and training are necessary to keep pace with advancements in diagnostic methods. Laboratories must also invest in state-of-the-art equipment and maintain rigorous quality control standards to ensure the accuracy and reliability of test results [4,5].

Conclusion
The manifesto for early detection and diagnosis of CVD outlines a comprehensive approach involving public awareness, targeted detection programs, enhanced clinical processes, digital integration, workforce training, research investment, policy development, and equitable access. Specialists in laboratory medicine and laboratory tests, particularly NP testing, and emerging technologies play a critical role in achieving these goals. By implementing these strategies, we can significantly reduce the burden of CVD, improve patient outcomes, and foster economic resilience and public health globally.

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Review Article

Increasing the impact and value of laboratory medicine through effective and AI-assisted communication

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Keywords

artificial intelligence, communication, impact, laboratory medicine, value proposition

Abstract

Effective communication is pivotal in maximizing the impact and value of laboratory medicine (LM) within healthcare. This review explores diverse strategies to enhance communication among healthcare providers, patients, laboratory personnel, and the general public. Key strategies include improving interdisciplinary collaboration through clear reporting, regular multidisciplinary meetings, and consultative services. Enhancing patient communication involves providing accessible test results via patient portals, developing educational materials, and fostering direct patient-provider communication. Implementing efficient information systems by integrating laboratory information systems with electronic health records and using automated alerts ensures timely data sharing and critical value notifications. Continuous education and training for healthcare providers and laboratory staff will keep them updated on advancements and improve communication skills. Fostering a culture of open communication encourages feedback and transparency, while leveraging technology such as telemedicine, mobile health applications, and artificial intelligence--(AI)-driven tools enhances real-time consultations and personalized insights. AI can be used to assist communication through providing advanced data analysis, personalized patient insights, enhanced communication, streamlined workflows, and demonstrable impact through research and analytics. These strategies collectively ensure accurate conveyance of critical information, improving patient and public insight and leading to better patient outcomes and more informed clinical decisions.

1. Introduction

Effective communication is a cornerstone in maximizing the impact and value of laboratory medicine in healthcare. It ensures that critical information is accurately conveyed, understood, and acted upon by the four main groups of stakeholders: healthcare providers, patients, and laboratory personnel and the general public.

In this article, we ask the following questions: *How can we improve communication between the laboratory and external stakeholders such that there is greater value and impact and how can we use new developments such as artificial intelligence (AI) to assist this process?*

One of the most important roles of a laboratory is to communicate results to stakeholders in the most efficient and cogent manner. The manner in which results are presented is largely geared towards clinicians and not to patients. Simply providing results to patients is not sufficient to facilitate participation in care because many may not be able to make sense of the results in the way the results are presented. *How can information be presented such that the recipient obtains the maximum value from the results?*

There are several different ways in which communication can enhance the value and impact of laboratory medicine. By employing these various communication strategies, the critical role of laboratory medicine in diagnosing, treating, and managing diseases can be more widely recognized and appreciated [1-4].

When considering the groups of stakeholders, the communication strategy has to be tailored to the particular group. The patients and general public should be considered uppermost in efforts to demonstrate the impact of laboratory medicine because these groups drive the demand for laboratory testing and it has the greatest impact on their lives.

2. Communicating with patients and the public

2.1 Enhancing communication with patients and education

Enhancing patient communication and education includes providing accessible test results through patient portals [1], developing educational materials, and ensuring effective direct communication between patients and providers.

Provide clear, simple and accessible test results [5]

Patients should have access to their laboratory results through patient portals integrated with electronic health record (EHR) systems [1]. Clear explanations of what the results mean and how these results impact their health can be provided alongside the raw data. Providing links to reputable sources for further reading can enhance understanding. However, often the design of patient portals may be geared towards presenting results in a table [1-4] and this presentation form is primarily geared towards clinicians. Providing patients with clear, easy-to-understand explanations of their test results helps them appreciate the importance of laboratory medicine. Accompanying results with visual aids, such as charts or infographics, can make complex information more accessible. Information portals for patients will require designs that feature information tailored to the medical context [4] and this is where AI-driven portals will find future value.

Public awareness and advocacy

Educational Materials and campaigns [6]

Laboratories and healthcare providers should develop educational materials that explain the use and purpose and implications of common laboratory tests. Brochures, videos, and online resources (eg. LabTestsOnline) can empower patients to understand their health better and engage more actively in their care.

Public health campaigns that educate the general public about common laboratory tests, their purposes, and their impact on health can increase awareness. These campaigns can use various media, including social media, brochures, and community workshops. National and community-level campaigns can highlight the role of laboratory medicine in public health. Topics can include the importance of screening programs, the impact of early diagnosis, and the contribution of laboratory medicine to disease prevention.

Media Engagement

Engaging with media outlets to share stories of how laboratory medicine has made a difference in patient outcomes can raise public awareness. Interviews with laboratory professionals and success stories from patients can humanize the science and highlight its value.

Social Media Presence

Utilizing social media platforms to disseminate information about laboratory medicine can reach a broader audience [7, 8]. Infographics, videos, and Q&A sessions can engage the public and increase understanding of the field.

Effective use of patient-provider communication, feedback and transparency [9]

Direct communication between patients and healthcare providers regarding test results ensures that patients can ask questions and receive personalized explanations. This can alleviate anxiety, improve understanding, and encourage adherence to follow-up plans. Fostering a culture of open communication encourages feedback from healthcare providers and patients, and emphasizes transparency in communication about laboratory processes.

Encouraging Feedback [10, 11]

Creating a culture where feedback is encouraged and valued can lead to improvements in laboratory services. Healthcare providers and patients should be able to provide feedback on the clarity of reports, the accessibility of information, and the overall communication process [10]. Such feedback can produce beneficial short-term emotional changes for laboratory

professionals and this can assist with low morale or staff burnout [10]. It therefore becomes important for laboratories to encourage such feedback in the same way as complaints may be entertained and recorded in the patient-facing context [11].

Transparent Communication [12, 13]

Transparency in communication about laboratory processes, potential delays, or issues with test results builds trust among healthcare providers and patients. Clear and honest communication helps manage expectations and reduces misunderstandings. A number of strategies have been proposed to assist entities involved in health care to increase trust amongst patients [12]. Of relevance to laboratory medicine is the need to develop standards, training and accountability systems [12].

Using AI for communication with patients

Developments in AI have led to the development of large language models (LLMs) that are probabilistic natural language processing systems trained on vast amounts of data. Generative AI applications such as chatbots can generate responses in a conversational style. It has been shown that chatbots can effectively communicate health information. It was hypothesized that this technology could be utilized in clinical decision support. However, multiple studies have indicated that, in its current form, this technology is too prone to errors and limitations to be effective in clinical settings. On the other hand, it has been found that chatbots can provide higher-quality responses and display more empathy than some physicians when answering patient questions. Chatbots have the potential to be used in a diagnostic setting to convey interpretative information on laboratory results. One cross-sectional study of 1134 reports examined the use of chatbots to simplify anatomical pathology reports [14]. The study found that chatbots could simplify anatomical pathology reports but there were inaccuracies and hallucinations indicating that such simplified reports should be reviewed by laboratory/healthcare professionals before transmission to the patients. There is support amongst laboratory professionals [15] for the use of AI as this could boost productivity and reduce errors.

Critical result reporting

AI can play a crucial role in the communication of critical results to clinicians or patients. Secure messaging apps offer significant advantages over traditional phone calls, particularly in enhancing the timeliness of reporting results [16, 17]. When healthcare organizations shift from reporting critical values by telephone to utilizing a secure messaging app, they can not only meet but often exceed accrediting agency standards. This transition enhances efficiency by delivering results directly to the appropriate person, reduces the time required to report outcomes, and provides a reliable communication record with automatically time-stamped messages upon sending, receiving, and reading. Additionally, built-in reporting features offer precise data on the communication process. The use of secure messaging apps that are coupled with AI-driven autoverification will facilitate rapid reporting of critical value results in clinical laboratories.

3. Communicating with healthcare providers including requesting clinicians

3.1 Clear and Concise Reporting [18]

Laboratory results must be reported clearly and concisely to clinicians. Standardized reporting formats, including the use of structured data and electronic health records (EHR), facilitate the easy interpretation of results. Avoiding jargon and using standardized terminology ensures that clinicians from different specialties can understand and utilize the information effectively. Reference has been made to the use of critical result reporting above.

3.2 Regular Multidisciplinary Meetings [19]

Improving interdisciplinary collaboration involves clear and concise reporting, regular multidisciplinary meetings, and consultative services to facilitate accurate interpretation and utilization of laboratory data. Holding regular meetings that involve laboratory personnel, clinicians, nurses, and other healthcare providers promotes interdisciplinary collaboration. These meetings can discuss complex cases, unusual findings, and ensure that laboratory data is integrated into patient care plans and laboratory operational challenges are understood. Regular interdisciplinary meetings and case discussions that include laboratory specialists can highlight the diagnostic value of laboratory tests. These discussions can showcase real-world examples of how laboratory results have led to accurate diagnoses and successful treatments. Anatomical pathologists are particularly adept at this because they often have to present tissue and biopsy results at clinicopathological meetings with different clinical specialties. Clinical chemistry generally finds a great partnership with endocrinology but this does not preclude interaction with intensive care/critical care specialists and nephrologists as examples.

3.3 Consultative Services [20]

Laboratories should offer consultative services where laboratory professionals, such as pathologists or clinical chemists, can discuss results with clinicians. This is generally an established part of laboratory medicine but will be greatly facilitated by the regular multidisciplinary meetings referred to above. This helps in the proper interpretation of complex test results and in deciding subsequent steps in patient management.

3.4 Educating health care providers

Integrating laboratory medicine into clinical pathways and guidelines ensures that healthcare professionals understand when and why specific tests are needed. Clear guidelines help providers appreciate the role of these tests in improving patient outcomes. Offering Continuing medical education (CME) courses focused on laboratory medicine keeps healthcare providers updated on

the latest advancements, testing protocols, and their clinical implications. Interactive sessions, case studies, webinars and online courses can make this education more engaging.

3.5 Implementing efficient information systems to assist communication

Integration of laboratory information systems (LIS) with electronic health records

Implementing efficient information systems focuses on integrating laboratory information systems (LIS) with electronic health records (EHR) for seamless data sharing, and employing automated alerts and reminders for critical laboratory values. Integrating LIS with EHR ensures seamless and real-time sharing of laboratory results with healthcare providers. This integration reduces delays, minimizes errors, and ensures that clinicians have immediate access to vital information.

Timely communication and automated alerts and reminders [21, 22]

Ensuring that laboratory results are communicated promptly to the relevant healthcare providers prevents delays in diagnosis and treatment. Real-time notifications and alerts can help keep providers informed and responsive. Automated systems can be set up to alert clinicians about critical values or significant changes in laboratory results. Reminders for follow-up tests or monitoring can also be automated, ensuring that important actions are not overlooked [16, 17].

Standardized reporting formats

Using standardized formats for reporting laboratory results ensures consistency and clarity, making it easier for healthcare providers to interpret and act on these results. Structured reports with clear conclusions and recommendations facilitate better clinical decision-making.

Feedback Mechanisms

Implementing feedback mechanisms where healthcare providers can discuss the relevance and clarity of laboratory reports with laboratory staff can lead to continuous improvement in communication practices.

4. Communicating with laboratory staff

4.1 Laboratory staff training [23]

Laboratory staff should also receive ongoing training to remain conversant with the latest technologies, methodologies, and communication strategies. This ensures that they can provide accurate information and effective consultations. Communication training [24] is particularly important because it is an aspect that is neglected in many training curricula. The inability of scientists and in this instance, laboratory professionals to communicate with the public, has led to mistrust [24].

4.1.1 Training in communication skills for laboratory staff

Workshops and Seminars

Conducting workshops and seminars for healthcare providers and laboratory staff on effective communication strategies ensures that all team members are skilled in conveying the value of laboratory tests. Role-playing scenarios and interactive sessions can enhance learning.

Certification Programs

Certification programs that include modules on communication skills specific to laboratory medicine can ensure that laboratory professionals are equipped to explain their findings clearly and effectively.

5. Leveraging Technology for Enhanced Communication

Telemedicine, mobile health applications and AI-driven tools can be used to enhance communication in laboratory medicine and provide real-time consultations and provide personalized feedback to patients.

5.1 Telemedicine and Remote Consultations and telehealth integration [25, 26]

Telemedicine platforms can facilitate consultations between laboratory specialists and clinicians, especially in remote or underserved areas. This ensures that even distant healthcare providers can access expert advice and interpretations. Integrating laboratory results into telehealth consultations allows patients and providers to discuss results in real-time, enhancing understanding and immediate decision-making. Visual aids can be shared on-screen to explain complex results.

5.2 Mobile Health Applications [27-30]

Mobile health applications can be used to deliver laboratory results, provide explanations, and offer follow-up advice directly to patient smartphones. This enhances accessibility and ensures timely communication. Developing mobile applications that notify patients of their test results and provide explanations and next steps can enhance patient engagement and understanding. Apps can also offer reminders for follow-up tests or appointments.A number of mobile apps have been developed for patients and health care professionals (reviewed in [27, 29]. In these studies [27, 29] it was found that mobile apps for laboratory medicine that deal exclusively with interpretation of results represented half of all apps and apps designed for patients were of the poorest quality, indicated that there is considerable scope for improvement.

5.3 Using AI and machine learning to improve communication and understanding of laboratory medicine [31-33]

AI-driven tools can help interpret laboratory results and provide clear explanations to both healthcare providers and

patients. These tools can identify patterns, predict outcomes, and suggest next steps based on the latest medical evidence.AI can be used to assist with communication from the laboratory to external stakeholders. It has the potential to help improve the understanding of the value and impact of laboratory medicine among healthcare providers, patients, and the general public (summarised in Table 1). With the use of advanced data analysis, personalized insights, and streamlined communication, AI can highlight the critical role laboratory medicine plays in modern healthcare.

5.3.1 Advanced Data Analysis and Interpretation

Automated Data Interpretation

AI algorithms can analyze large volumes of laboratory data quickly and accurately, providing automated interpretations of complex results. This can help healthcare providers or patients understand the implications of test results more clearly and make informed decisions. A study of 3200 patients showed that patients who received interpretations on abnormal results showed a much higher rate of followup (71% vs 49%) [34]. Patients stated that a significant benefit of this was the time factor compared to receiving interpretations from a doctor. It has been found in a prior study [35] that 26% of results are not followed up by patients and auto-generated interpretations will definitely increase the potential of results being followed up [34].

Predictive Analytics

AI can predict disease progression and treatment outcomes by analyzing historical data and identifying patterns [36, 37]. Predictive analytics can demonstrate the long-term value of laboratory tests in managing chronic diseases and improving patient outcomes [38]. In a study of close to 230 000 patients and results from nine laboratory tests, the data were subjected to visual analytics and used to predict the likelihood of Acute kidney injury (AKI) [37]. Clinical laboratories produce vast amounts of data and there is growing interest in using data to make decisions and improve patient care [38]. Challenges such as data accessibility, lack of resources and data literacy are still impediments to exploiting data literacy to the fullest extent [38].

Anomaly Detection

AI systems can detect anomalies and trends that might be missed by human analysts. Identifying these patterns can lead to early diagnosis and intervention, showcasing the importance of routine laboratory testing [39, 40]. In one example, a clustering approach was used to detect anomalies/outliers in HER data [40].

5.3.2 Personalized Patient Insights

Personalized Health Reports

AI can generate personalized health reports for patients, explaining their laboratory results in an understandable and context-specific manner. These reports can include visual aids, such as graphs and charts, to help patients comprehend their health status [41-44].

Risk Assessment and Management

AI tools can assess individual patient risks based on their laboratory results and medical history. Providing personalized risk assessments and management plans highlights the value of laboratory medicine in preventive healthcare.

Tailored Health Recommendations

By analyzing patient data, AI can offer tailored health recommendations and follow-up actions. This personalized approach helps patients see the direct benefits of laboratory tests in managing their health.

5.3.3 Enhancing Communication and Education

Natural Language Processing (NLP)

AI-powered NLP can translate complex laboratory data into plain language, making it easier for both healthcare providers and patients to understand [45-47]. This ensures that critical information is communicated effectively and comprehensively. An example of a large language model is GatorTron [47] which was developed from millions of patient records and contains > 90 billion words of text. Another example is Med-Bert [48].

Virtual Health Assistants

AI-driven virtual health assistants [49] can interact with patients and healthcare providers, answering questions about laboratory tests and their implications. These assistants can provide 24/7 support, improving accessibility to information.

Educational Platforms

AI can power educational platforms that offer interactive learning modules about laboratory medicine. These platforms can adapt to the user's knowledge level, providing customized content that enhances understanding.

5.3.4 Streamlining Workflow and Decision-Making

Integrated Decision Support Systems

AI can integrate with Laboratory Information Systems (LIS) and Electronic Health Records (EHR) to provide real-time decision support. AI-driven alerts and recommendations can guide clinicians in interpreting laboratory results and determining the next steps.

Workflow Optimization

AI can optimize laboratory workflows by automating routine tasks and managing resources efficiently. This ensures that laboratory staff can focus on more complex tasks, improving overall productivity and accuracy.

Real-Time Data Sharing

AI systems can facilitate real-time data sharing and communication between laboratories and healthcare providers. Instant access to up-to-date information ensures that clinical decisions are based on the latest available data.

5.3.5 Demonstrating Impact Through Research and Analytics

Clinical Research and Outcomes Studies

AI can analyze data from clinical research and outcomes studies to demonstrate the impact of laboratory medicine on patient care. Publishing these findings can help healthcare providers and policymakers understand the value of laboratory tests.

Cost-Benefit Analysis

AI can perform cost-benefit analyses by comparing the costs of laboratory testing with the benefits in terms of improved patient outcomes and reduced healthcare expenses. This information can be crucial for decision-makers in healthcare organizations.

Population Health Management

AI can analyze population health data to identify trends and the impact of laboratory medicine on public health. This can help in planning and implementing effective public health strategies and policies.

5.3.6 Public Awareness and Advocacy

Interactive Health Dashboards

AI-powered health dashboards can present laboratory data and health insights to the public in an interactive and engaging way. These dashboards can highlight the role of laboratory medicine in maintaining public health.

Social Media and Public Engagement

AI can analyze social media trends and public sentiments to tailor communication strategies that raise awareness about the importance of laboratory medicine. Targeted campaigns can educate the public and dispel misconceptions.

AI-Driven Storytelling

AI can generate compelling stories based on real-world data, illustrating the life-saving impact of laboratory medicine. Sharing these stories through various media channels can resonate with a broader audience [50].

Table 1: Summary of the applications of AI in enhancing the value and impact of laboratory medicine.

Application in improving the impact of Laboratory Medicine	Key Components	Role
Advanced Data Analysis and Interpretation	Automated Data Interpretation	analyze large volumes of laboratory data and provide interpretations
	Predictive Analytics	predict disease progression and treatment outcome
	Anomaly Detection	detect anomalies and trends
Personalized Patient Insights	Personalized Health Reports	explaining laboratory results in an understandable and context-specific manner
	Risk Assessment and Management	assess individual patient risks
	Tailored Health Recommendations	offer individualized health recommendations
Enhancing Communication and Education	Natural Language Processing (NLP)	translate complex laboratory data into plain language
	Virtual Health Assistants	can provide 24/7 support
	Educational Platforms	interactive learning modules
Streamlining Workflow and Decision-Making	Integrated Decision Support Systems	real-time decision support
	Workflow Optimization	automate routine tasks and manage resources efficiently
	Real-Time Data Sharing	real-time data sharing and communication between laboratories and healthcare providers

Demonstrating Impact Through Research and Analytics	Clinical Research and Outcomes Studies	data from clinical research and outcomes studies demonstrate the impact of laboratory medicine on patient care
	Cost-Benefit Analysis	by comparing the costs of laboratory testing with the benefits in terms of improved patient outcomes and reduced healthcare expenses
	Population Health Management	analyze population health data to identify trends
Public Awareness and Advocacy	Interactive Health Dashboards	present laboratory data and health insights to the public
	Social Media and Public Engagement	tailor communication strategies that raise awareness about the importance of laboratory medicine
	AI-Driven Storytelling	compelling stories based on real-world data

6. Specific AI tools that can assist with improving the understanding and impact of laboratory medicine (Table 2)

6.1. Machine Learning (ML) Platforms

TensorFlow and PyTorch

These open-source ML platforms provide powerful tools for building, training, and deploying machine learning models [51-53]. They can be used to analyze complex laboratory data, identify patterns, and predict outcomes. TensorFlow and PyTorch can be used to develop models that predict the likelihood of a patient developing a particular disease based on their clinical history, laboratory results, and other data or applied to create models that predict patient outcomes, such as the likelihood of recovery or relapse, based on their medical data [54].

Scikit-learn

This Python library is user-friendly and ideal for implementing basic to advanced machine learning algorithms [55, 56]. It can be applied to various data analysis tasks in laboratory medicine, such as clustering and classification [57]. In patients with non-alcoholic fatty liver disease, machine learning models were developed to predict the risk of hepatocellular carcinoma [57]. The model was able to predict the development of carcinoma with more than 90% accuracy[57].

6. 2. Natural Language Processing (NLP) Tools

spaCy and NLTK

These NLP libraries can process and interpret large volumes of textual data from laboratory reports, converting complex medical terminologies into plain language [58, 59]. They enhance the clarity and accessibility of information for both healthcare providers and patients. In one example, EHRs were interrogated in patients with lung cancer to identify clinical phenotypes such as cancer staging, treatment recurrence and organs affected to provide additional insights into patient health. Comparisons were made between scispaCy, medspaCy, Flan-T5-xl, Flan-T5-xxl, Llama-3-8B, GPT-3.5-turbo, and GPT-4 [58]. The study highlighted the potential of GPT-4 for accurate phenotype extraction and improved care [58]. In another example, EHRs were screened for a number of different parameters, including laboratory data [59] using a natural language processing tool, EXTEND (EXTraction of EMR Numerical Data).

IBM Watson Natural Language Understanding

This tool provides advanced text analysis capabilities, including sentiment analysis, entity recognition, and keyword extraction [60-62]. It can help in summarizing laboratory reports and providing actionable insights.IBM Watson has been used to infer gene-gene relationships and identify novel biomarkers [60].

6.3. AI-Powered Virtual Assistants

Chatbots (e.g., Microsoft Bot Framework, Google Dialogflow)

AI chatbots can interact with patients and healthcare providers, answering queries about laboratory tests, results, and implications [63, 64]. They provide real-time, accessible support, enhancing understanding and engagement. A chatbot was used in a general practice to collection information for 3 months [63]. The information was used to monitor health status and provide health recommendations to patients and significant reductions in patient ailments was observed and the primary care physician was able to response rapidly to patients [63]. ChatGPT was used at a conference to answer questions and the answers were compared to those provided by expert faculty [64]. The AI chatbot provided answers comparable to experts suggesting that future development should be anticipated in the use of AI Chatbots to answer questions in laboratory medicine.

Virtual Health Assistants (e.g., HealthTap, ADA Health)

These AI-driven assistants can offer personalized health advice based on laboratory results, medical history, and current

symptoms [65, 66]. They help patients understand their test results and make informed health decisions.ADA health was used to screen patients with mental disorders [65]. Good agreement was obtained for mental disorders in adulthood when comparing psychotherapists with the performance of the app. In another example, a virtual assistant called "Paola" was used to carry out a 12 week evaluation of physical activity and diet [66]. The virtual health assistant performed well during the structured weekly check-ins, but showed performance errors mainly when queries were outside the capabilities of the virtual assistant. However, dietary compliance was high [66].

6.4. Predictive Analytics Tools

IBM Watson Health

In addition to its NLP capabilities, Watson Health leverages AI to analyze health data and provide predictive insights [62]. It can help healthcare providers anticipate disease progression and tailor treatment plans based on laboratory results.

SAS Advanced Analytics

SAS provides comprehensive analytics solutions that can process and analyze vast amounts of laboratory data, offering predictive insights and supporting clinical decision-making [67].

6.5. Data Visualization Tools

Tableau and Power BI

These tools offer advanced data visualization capabilities, transforming complex laboratory data into intuitive and interactive dashboards [68, 69]. Visualizations help in understanding trends, patterns, and the impact of laboratory tests. Tableau and Power BI are being used across the healthcare spectrum to analyze data pertaining to health care provider analytics, medical device analytics and pharmacy analytics to improve efficiency and reduced costs.

D3.js

This JavaScript library allows for the creation of dynamic and interactive data visualizations on web platforms [70]. It can be used to present laboratory data in an engaging and comprehensible manner [71]. Data visualization plays a pivotal role in healthcare by enabling providers and researchers to make well-informed decisions from extensive datasets. Through effective visualization, healthcare professionals can detect trends, patterns, and anomalies that may not be immediately apparent in raw data. Additionally, it helps researchers present their findings in a more digestible and comprehensible manner, facilitating better interpretation and actionable insights by stakeholders.

D3.js offers valuable applications in various health tech scenarios, such as:

Electronic Health Records (EHRs)

D3.js can be employed to craft interactive visualizations of EHR data, allowing healthcare providers to discern trends and patterns that may suggest specific health conditions, ultimately leading to more informed patient care decisions.

Clinical Trials

Clinical trials generate complex datasets essential for medical research, but interpreting this data can be challenging. D3.js can be utilized to develop interactive visualizations of clinical trial data, enabling researchers to analyze the information in real-time, thereby identifying trends and patterns critical to their studies.

Health Analytics

D3.js can be instrumental in creating interactive visualizations of health analytics data, providing healthcare providers and researchers with the ability to explore the data dynamically and pinpoint relevant trends and patterns for their research.

6.6. Clinical Decision Support Systems (CDSS)

MedAware

These AI-driven CDSS tools integrate with electronic health records (EHR) to provide real-time decision support, based on laboratory results and patient data [72]. They assist healthcare providers in making accurate and timely clinical decisions. Medaware is a medication safety monitoring platform that can predict and avoid adverse drug events [73]. MedAware was compared to an existing CDSS and it was found that more than 60% of MedAware generated alerts were not detected by the existing CDSS in a 4 year data collection period [73, 74].

DXplain

This is an AI-based diagnostic decision support system that provides differential diagnosis suggestions based on clinical findings and laboratory data [75]. It helps in interpreting complex cases and improving diagnostic accuracy. A clinical decision support system was found to improve diagnostic accuracy in a group of 87 Family medicine residents and this had the potential to decrease diagnostic errors and improve patient safety [75].

6.7. Personalized Medicine Platforms

Foundation Medicine

This platform uses AI to analyze genetic data from laboratory tests, providing insights into personalized cancer treatments [76]. It highlights the role of laboratory medicine in tailoring treatments to individual patients.In a study of patients with lung cancer, considerable racial inequities were identified in next generation sequencing results [77]. Racial disparities were also identified in genomic profiling of prostate cancer patients [78] in a large retrospective analysis of more than 11 000 patients.

Flatiron Health
Flatiron Health leverages AI to integrate clinical and laboratory data, offering personalized treatment recommendations and improving cancer care [79]. It can be used to assist physicians adhere to evidence-based guidelines during cancer diagnosis and treatment helping to standardize treatments in haematology-oncology [79].

6.8. Automated Quality Control Systems

Bio-Rad Unity Real-Time
This AI-powered quality control system monitors laboratory testing processes in real-time, identifying errors and ensuring accuracy [80]. It enhances the reliability and value of laboratory results.

Abbott AlinIQ
AlinIQ uses AI to optimize laboratory operations, including quality control, workflow management, and data analysis, ensuring high standards and efficient laboratory practices [81].

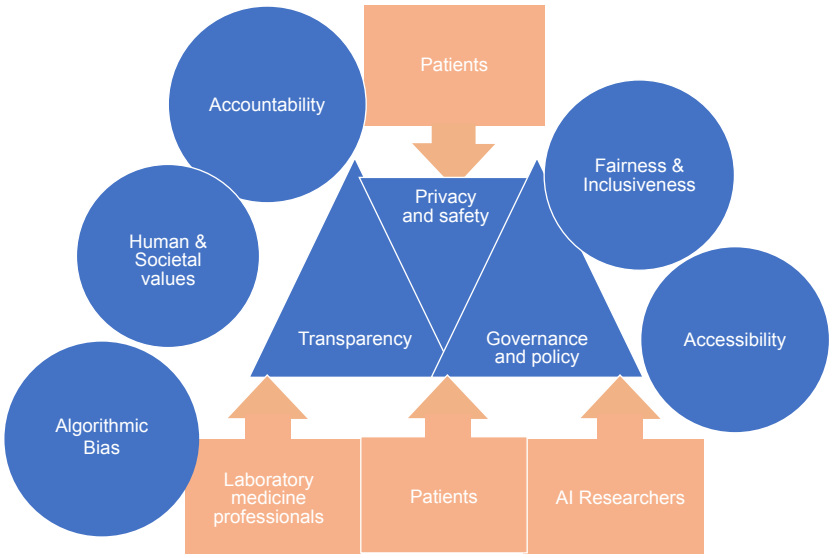
Table 2: Summary of the AI tools available to improve understanding of laboratory medicine.

AI Tool/Method	Function	Impact on Understanding
TensorFlow and PyTorch	Machine learning platforms for data analysis	Enhanced data analysis and pattern recognition in laboratory results
Scikit-learn	Machine learning library for clustering and classification	Improved analysis of laboratory data
spaCy and NLTK	NLP libraries for processing and interpreting textual data	Clearer translation of complex medical terminologies
IBM Watson Natural Language Understanding	Advanced text analysis capabilities	Summarized and actionable laboratory reports
Chatbots (Microsoft Bot Framework, Google Dialogflow)	AI chatbots for real-time support	Accessible and responsive patient-provider communication
Virtual Health Assistants (HealthTap, ADA Health)	AI-driven assistants for personalized health advice	Improved patient engagement and understanding of test results
IBM Watson Health	Predictive analytics for disease progression	Demonstrated long-term value of laboratory tests
SAS Advanced Analytics	Comprehensive analytics solutions	Supported clinical decision-making
Tableau and Power BI	Data visualization tools	Intuitive dashboards for trend and pattern recognition
D3.js	JavaScript library for dynamic data visualizations	Engaging presentation of laboratory data
MedAware	Clinical decision support systems	Real-time decision support based on laboratory results
DXplain	Diagnostic decision support system	Improved diagnostic accuracy through differential diagnosis suggestions
Foundation Medicine	Personalized medicine platform for genetic data analysis	Highlighted role of laboratory medicine in personalized cancer treatments
Flatiron Health	Integration of clinical and laboratory data	Enhanced personalized treatment recommendations
Bio-Rad Unity Real-Time	Automated quality control system	Ensured accuracy and reliability of laboratory results
Abbott AlinIQ	Optimization of laboratory operations	High standards and efficient laboratory practices

7. Ethical considerations

Notwithstanding the desirability of improving communications in laboratory medicine, a number of ethical issues need to be considered [82, 83] in the implementation of any of the recommendations discussed in this article. Whilst a detailed consideration is beyond the scope of this article, readers should always be cognisant of the ethical guidelines surrounding the use of patients' laboratory data. These include (Figure 1), amongst others, stewardship, and confidentiality of patient data; transparency in the development of software applications and scientific study and publication of AI applications and how commercial interests may impact on these in a rapidly growing field.

Figure 1: Key considerations in the use of AI to implement enhanced communication methods in laboratory medicine. Adapted from [83].



Conclusion

Effective communication is pivotal in maximizing the impact and value of laboratory medicine in healthcare. By improving interdisciplinary collaboration, enhancing patient education, engaging patients, implementing efficient information systems, promoting continuous education, fostering a culture of open communication, and leveraging technology, the full potential of laboratory medicine can be realized. By educating healthcare providers, engaging patients, fostering clear communication within healthcare teams, raising public awareness, leveraging technology, and promoting professional development, the critical contributions of laboratory medicine to healthcare can be better recognized and valued. These strategies ensure that critical information is accurately conveyed, leading to better patient outcomes, more informed clinical decisions, and a more cohesive healthcare system. These strategies not only improve the appreciation of laboratory medicine but also lead to better patient outcomes and more efficient healthcare delivery.

AI tools have the potential to significantly enhance the understanding of the value and impact of laboratory medicine. By providing advanced data analysis, personalized insights, streamlined communication, workflow optimization, and compelling demonstrations of impact, AI can ensure that the critical contributions of laboratory medicine are widely recognized and appreciated. Healthcare providers can offer better diagnostic accuracy, personalized care, and more informed clinical decisions, ultimately highlighting the critical role of laboratory medicine in modern healthcare. As AI continues to evolve, its integration into laboratory medicine will further highlight the essential role this field plays in improving healthcare outcomes and public health.

Abbreviations

EHR, Electronic Health Records, LIS, Laboratory information system, LLM, Large language models, LM, Laboratory medicine

Conflict of interest

None.

Ethical clearance

Approval from local institutional ethical clearance was not required.

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Authors' contribution

Tahir Pillay conceived the idea, retrieved literature and wrote the article.

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Research Article

Spreadsheet for patient-based quality control analysis and evaluation (SPAE)

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Abstract

Introduction: Patient-based quality control (PBQC) is an alternate quality control technique to conventional (internal) quality control. It uses patient results generated for clinical care to monitor the analytical performance through statistical analysis. The use of PBQC in routine laboratory is impeded by lack of familiarity and appropriate informatics tool.

Method: A Spreadsheet for PBQC Analysis and Evaluation (SPAE, based on Microsoft Excel) is developed. It incorporates IFCC recommended features for PBQC informatics tool that has been automated, including data visualization, data (Box-Cox) transformation, extreme value treatment (winsorization) and user parameter selection (block size, acceptable false positive rate, desirable bias for detection).

Results: Following parameter selection and data input, the spreadsheet automatically calculates the winsorization limits, transformed values, performance verification metrics such as false positive rates and number of results affected before error detection (NPed) – a performance metric for how sensitive the PBQC model detects the predefined error (bias). The verified PBQC model can be used for routine monitoring. The performance of the spreadsheet tool was verified against an independent model based on Python. Laboratory users can download the tool at https://github.com/HuiQi96/PBQC/blob/main/ PBQC_model_v2.2.zip.

Discussion: The SPAE is a simple-to-use desktop tool that lowers the barrier for laboratory users to adopt PBQC in their quality control system. In addition, the spreadsheet can be used as an educational tool, such as when conducting a workshop, to help laboratory users better familiarize themselves with the PBQC concepts and used for independent verification of the output of another informatics tool.

Introduction

Patient-based quality control (PBQC) uses patient results generated for routine care to monitor for potential changes in analytical performance [1-3]. It has gained recognition as an alternative quality control practice to conventional quality control in the latest ISO 15189:2023 document [2]. However, the routine implementation of PBQC is beset by the lack of suitable informatics capability in the instrument middleware or laboratory information system [3,4]. This report describes and provides a fully functional, end-to-end informatics tool encoded in a spreadsheet (Microsoft Excel, Spreadsheet for patient-based quality control analysis and evaluation, SPAE) to lower the barrier of adopting PBQC in routine laboratory practice (downloadable from https://github.com/HuiQi96/PBQC/blob/main/PBQC_model_v2.2.zip). In addition, the spreadsheet can be used as an educational tool, such as when conducting a workshop, to help laboratory users better familiarize themselves with the PBQC concepts and used for independent verification of the output of another informatics tool. Laboratory users are encouraged to get acquainted with basic PBQC concepts before using the spreadsheet tool [1-3].

Material and Methods

The following features were considered during the development of the spreadsheet tool, considering the recommendations from the IFCC Working Group on Patient-Based Real-Time Quality Control [6,7]. These include:

1. Ability to visualize the distribution of the laboratory data to assess for skewness and the need for data transformation to approximate normal distribution
2. Ability to select appropriate PBQC model and parameters, including block size, acceptable false positive rate (which determines the control limits), the acceptable bias for detection, winsorization limit (which converts extreme values to the predefined limit), perform auto-optimized Box-Cox transformation of data (if necessary)
3. Ability to assess the effect of the selected parameters above on the performance of the PBQC model (see below)
4. Ability to verify the performance of the PBQC model using established performance parameters such as false positive rates, detection rates and numbers of patient results affected before error detection (NPed)
5. Ability to monitor the ongoing performance (i.e. error detection) using the selected and verified PBQC model and an alert of any error detected

These features were coded into five separate spreadsheets ('Input', 'Training', 'Verification', 'Output', 'Routine'. For this tool, the moving average and moving median are adopted as the PBQC algorithms. The laboratory user can directly input their local data to customize the PBQC parameter setting for optimal performance in routine practice. Visualization tools were also coded to show the overall data distribution (in the histogram) and in control charts to allow users to better appreciate the effects of different parameters on the PBQC model.

Results

Figure 1 shows the 'Input' spreadsheet where laboratory users can define the acceptable false positive rate (%), the desirable magnitude of bias (expressed as %) to be detected, Box-Cox transformation ('Yes' / 'No'), winsorization (%) and three choices of block size. The acceptable false positive rate affects the control limit of the PBQC model. It can be determined based upon the operational consideration and risk tolerance of the laboratory. A higher acceptable false positive rate will tighten the control limit and improve error detection but can produce higher false alarm (as defined by the user). For example, a 5% false alarm rate for a laboratory analyzing 1000 samples will produce 50 PBQC false alarms daily. Generally, keeping the false positive rate as low as possible is desirable, starting with 0%.

The user defines the desirable magnitude of bias (%) to be detected, which may be determined according to the Milan consensus (e.g., biological variation, or state-of-the-art). The predefined bias will be used to assess the performance of the PBQC model subsequently during the verification step. Box-Cox transformation may be selected if the data distribution appears skewed. When selected, the spreadsheet will determine the optimal lambda based on the distribution of the laboratory data to approximate a normal distribution.

Winsorization is the statistical technique to convert an extreme laboratory value to a predefined limit. For example, if a winsorization limit of 150 mmol/L is selected, a laboratory value of 165 mmol/L will be converted to 150 mmol/L. This conversion helps reduce the effects of outlier (or extreme) results while keeping the data instead of removing it and is preferred [5]. The selection of 'Winsorization %' in the tool will convert the most extreme data outside the predefined percentages bilaterally to the below-mentioned limits. For example, a winsorization limit of 95% will convert the highest 2.5% and the lowest 2.5% values to the winsorization limit. Winsorization is recommended if extreme values are common in the laboratory. However, it should generally be kept at >90% (i.e., not more than the most extreme 10% laboratory values are converted to the winsorization limits). An overly strong winsorization setting (e.g., 60%, thereby converting the 'most' extreme 40% laboratory data) can overly constrain the distribution of the laboratory data, leading to a poorer bias detection rate.

The three choices of block size for the moving average and moving median algorithms can be defined and adjusted based on the performance seen during the verification step. In general, a larger block size has the effect of reducing the variability of the moving statistics, which produces smaller control limits. While a smaller control limit may be associated with better error detection capability, the improvement may be offset by the need

for more samples within the (larger) block size to be affected by the error before the moving statistics exceed the control limits. Moreover, the smaller control limits (due to larger block size) may also be associated with increased false positive rate. The interplay between block size, NPed (a metric for sensitivity for error detection) and false positive rate requires simulation and depends on the distribution of the data.

Figure 1: 'Input' spreadsheet where laboratory users can define the acceptable false positive rate (%), desirable bias detection (%), winsorization (%) and block size.

Predefined setting	
Acceptable False Positive Rate(%)	0
Desirable bias for detection (%)	5
Box-cox transformation	No
Box-cox lambda	#N/A
Winsorisation %	98
Winsorisation lower limit	#NUM!
Winsorisation upper Limit	#NUM!

Method parameters	
Method1	Med
Method2	Avg
Block size1, n1	5
Block size2, n2	10
Block size3, n3	20

Column	Method	block size, n	lower control limit	upper control limit
MMed1	Med	5	#NUM!	#NUM!
MMed2	Med	10	#NUM!	#NUM!
MMed3	Med	20	#NUM!	#NUM!
MAvg1	Avg	5	#NUM!	#NUM!
MAvg2	Avg	10	#NUM!	#NUM!
MAvg3	Avg	20	#NUM!	#NUM!

Once the preliminary settings are made, the laboratory user can input historical data of their laboratory into the 'Training' spreadsheet (Figure 2). The format for data input should include 'ID' (e.g. sample ID), 'date' (in "YYYY-MM-DD" format) and measurement (the numerical laboratory results) into the blue cells. It is recommended that at least six months, and ideally one year, of data, be used to ensure adequate variation in the data is incorporated into the model. Of note, the laboratory should first remove data with any symbols (e.g. "<", ">" or "#" etc.) or non-numerical (e.g. "NA", "INV", "Error", "INSUFF" etc.) from the dataset prior to input into the spreadsheet. Examples of such data may include results falling outside analytical measurement interval or those associated with errors such as insufficient sample.

Once the 'Training' is input, the optimal lambda (if Box-Cox transformation was selected) and winsorization limits (if a predefined percentage is input) will automatically be calculated and displayed in the 'Input' page (Figure 3). A frequency histogram is automatically produced to allow the user to visualize the distribution of the laboratory data for skewness (to determine whether Box-Cox transformation is necessary) and the presence of extreme values (to determine how much winsorization is required). In the example in Figure 3, the Box-Cox transformation was not selected since the data appeared normally distributed. Still, a mild winsorization (99.5%) was chosen to transform the most extreme 0.5% of laboratory results (bilaterally). The control limits for each block size of the moving average and median algorithms are also auto-calculated and displayed.

Figure 2: 'Training' spreadsheet where the laboratory user inputs at least 6-12 months of historical laboratory results.

ID	date	measurement
1	2020-01-01	138.00
2	2020-01-01	141.00
3	2020-01-01	145.00
4	2020-01-01	139.00
5	2020-01-01	137.00
6	2020-01-01	141.00
7	2020-01-01	140.00
8	2020-01-01	143.00
9	2020-01-01	140.00
10	2020-01-01	140.00
11	2020-01-01	144.00
12	2020-01-01	141.00
13	2020-01-01	141.00
14	2020-01-01	140.00
15	2020-01-01	139.00
16	2020-01-01	141.00
17	2020-01-01	137.00
18	2020-01-01	135.00
19	2020-01-01	137.00
20	2020-01-01	136.00
21	2020-01-01	141.00
22	2020-01-01	138.00
23	2020-01-01	137.00

Result	
min date=	2020-01-01
max date=	2020-10-26
min date (number)=	43831
max date (number)=	44130
number of days=	300

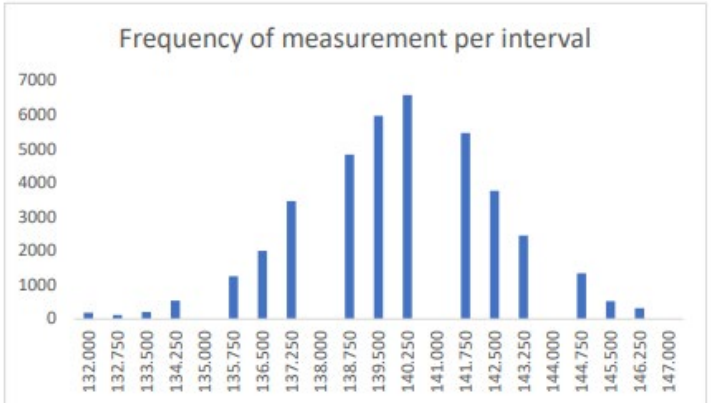


Figure 3: 'Input' spreadsheet shows the auto-populated parameters, including winsorization limits (if defined) and control limits of each block size of the moving average and moving average algorithms once the data has been entered into the 'Training' spreadsheet.

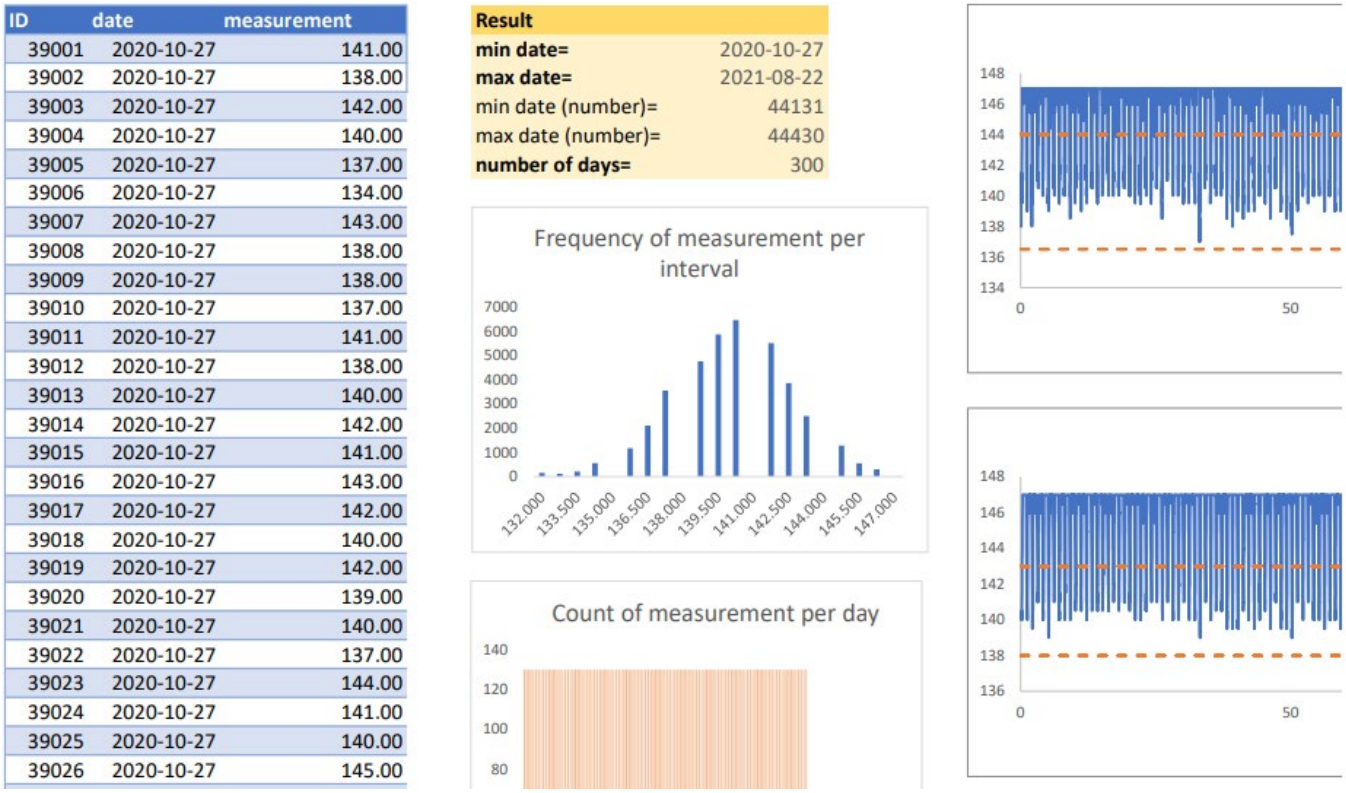
Predefined setting	
Acceptable False Positive Rate(%)	0
Desirable bias for detection (%)	5
Box-cox transformation	No
Box-cox lambda	#N/A
Winsorisation %	99.5
Winsorisation lower limit	132.000
Winsorisation upper Limit	147.000

Method parameters	
Method1	Med
Method2	Avg
Block size1, n1	10
Block size2, n2	20
Block size3, n3	30

Column	Method	block size, n	lower control limit	upper control limit
MMed1	**Med**	**10**	136.500	144.000
MMed2	**Med**	**20**	138.000	143.000
MMed3	**Med**	**30**	138.000	142.500
MAvg1	**Avg**	**10**	137.200	143.400
MAvg2	**Avg**	**20**	138.300	142.550
MAvg3	**Avg**	**30**	138.800	142.233

Next, the laboratory user can input another set of (verification) data in the blue cells of the 'Verification' spreadsheet. Here, the predefined 'desirable bias to be detected (%)' will be introduced into the verification dataset (i.e., bias is simulated in the 'verification' dataset). The bias will be introduced once for each day of data in the verification dataset. Several charts are shown, including the data distribution, the number of results for each day of the dataset, and the control charts of the data with the simulated bias introduced (Figure 4).

Figure 4: 'Verification' spreadsheet shows the distribution of the data, the number of measurements for each day, and the control charts showing the data with the simulated bias.



ID	date	measurement
39001	2020-10-27	141.00
39002	2020-10-27	138.00
39003	2020-10-27	142.00
39004	2020-10-27	140.00
39005	2020-10-27	137.00
39006	2020-10-27	134.00
39007	2020-10-27	143.00
39008	2020-10-27	138.00
39009	2020-10-27	138.00
39010	2020-10-27	137.00
39011	2020-10-27	141.00
39012	2020-10-27	138.00
39013	2020-10-27	140.00
39014	2020-10-27	142.00
39015	2020-10-27	141.00
39016	2020-10-27	143.00
39017	2020-10-27	142.00
39018	2020-10-27	140.00
39019	2020-10-27	142.00
39020	2020-10-27	139.00
39021	2020-10-27	140.00
39022	2020-10-27	137.00
39023	2020-10-27	144.00
39024	2020-10-27	141.00
39025	2020-10-27	140.00
39026	2020-10-27	145.00

Following this, the performance parameter of the PBQC model, based on the parameters selected, will be displayed in the 'Output' spreadsheet for each block size (Figure 5). The parameters include percentage detected (i.e., number of days of bias detected/ number of days bias was introduced), ANPed (average number of patient results affected before error detected, which is the average number of patient results between the bias is introduced and the bias is detected). MNPed (median NPed), 95Nped (95th percentile Nped), false positive (false alarm before bias introduction). Each performance criteria will be automatically ranked with the highest-performing parameter highlighted in the green cell. An overall best combination of the best parameters (i.e., the combination with the greatest number of highest performing parameter/ green cells) will be indicated as 'Preferred' and applied in the 'Routine' spreadsheet. Details of the parameter selected is also displayed at the bottom of the spreadsheet. The laboratory user may further tune/ modify the PBQC parameters in the 'Input' spreadsheet to improve the performance of the PBQC model as necessary.

Figure 5: The 'Output' spreadsheet shows the performance parameters of the user-defined PBQC models. The best-performing parameters are highlighted in green cells, and the PBQC model producing the preferred (overall best) combination of performance is indicated.

Method	block size	Perc detected(%)	ANPed	MNPed	95NPed	False positive (%)	Preferred
Med	10	100	5	5	6	0	Preferred
Med	20	100	8	8	11	0	
Med	30	100	11	12	15	0	
Avg	10	100	5	5	7	0.015	
Avg	20	100	7	7	9	0.0125	
Avg	30	100	8	8	12	0.01	

Best metrics	
max per detected (%)	100
min ANPed	5
min MNPed	5
min 95NPed	6
min fasle positive (%)	0

Method parameters for real-time data	Value
Method	Med
Block size	10
lower control limit	136.500
upper control limit	144.000
Acceptable False Positive Rate(%)	0
Desirable bias for detection (%)	5
Box-cox transformation	No
Boc-cox lambda	#N/A
Winsorisation %	99.5
Winsorisation lower limit	132.000
Winsorisation upper Limit	147.000

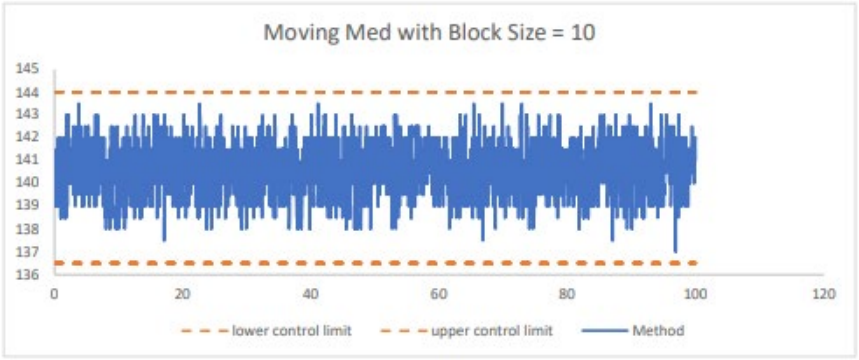
Recommended preferred set of method Parameters is Moving Med with Block Size = 10 to detect 5% bias for 0% of Acceptable False Positve Rate

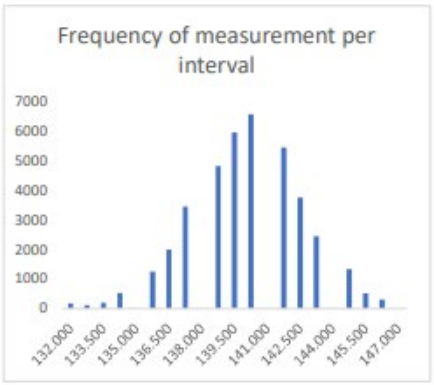
Once the laboratory user is satisfied with the performance of the PBQC model, routine laboratory data can be input into the 'Routine' spreadsheet. The optimized parameters based on the 'Verification' spreadsheet will be automatically adopted. A control chart shows the running PBQC model, and if bias is detected, it will be indicated in the box (Figure 6). The performance of the spreadsheet tool was independently verified using an independent PBQC model built using the same parameters in Python (see Supplemental Material).

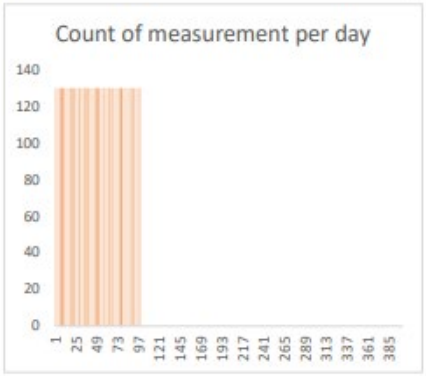
Figure 6: 'Routine' spreadsheet shows the user-optimized PBQC model running with routine laboratory data. Any bias detected will be flagged and displayed in the box.

ID	date	measurement
78001	2021-08-23	139.00
78002	2021-08-23	144.00
78003	2021-08-23	144.00
78004	2021-08-23	137.00
78005	2021-08-23	141.00
78006	2021-08-23	139.00
78007	2021-08-23	140.00
78008	2021-08-23	141.00
78009	2021-08-23	139.00
78010	2021-08-23	140.00
78011	2021-08-23	138.00
78012	2021-08-23	139.00
78013	2021-08-23	141.00
78014	2021-08-23	139.00
78015	2021-08-23	145.00
78016	2021-08-23	141.00
78017	2021-08-23	139.00
78018	2021-08-23	138.00
78019	2021-08-23	140.00
78020	2021-08-23	142.00
78021	2021-08-23	139.00
78022	2021-08-23	143.00
78023	2021-08-23	136.00
78024	2021-08-23	141.00
78025	2021-08-23	138.00
78026	2021-08-23	141.00
78027	2021-08-23	136.00
78028	2021-08-23	143.00
78029	2021-08-23	140.00
78030	2021-08-23	141.00
78031	2021-08-23	143.00
78032	2021-08-23	142.00
78033	2021-08-23	141.00
78034	2021-08-23	139.00
78035	2021-08-23	144.00
78036	2021-08-23	144.00
78037	2021-08-23	134.00
78038	2021-08-23	140.00
78039	2021-08-23	139.00

Result	Value
min date=	2021-08-23
max date=	2021-11-30
min date (number)=	44431
max date (number)=	44530
number of days=	100
average count per day =	130
number of positive detected =	0
percentage of positive detected (%)=	0
first sequence number of positive detected =	0
first day of positive detected =	0
first date of positive detected =	NONE







Discussion

Patient-based quality control has several advantages over conventional internal quality control. They include better error detection capability, fewer concerns over non-commutability and potentially lower costs to perform. However, the main barriers to adoption include a lack of informatics capability and familiarity with parameter selection/ optimization [3]. This report introduced a spreadsheet tool containing many of the recommended features for a PBQC informatics tool [6,7]. The SPAE tool was deliberately coded as a spreadsheet (Microsoft Excel) owing to its generally widespread use, as well as avoiding concerns related to privacy and cybersecurity when using web-based tools. It is envisioned that laboratory users can download this tool from https://github.com/HuiQi96/PBQC/blob/main/PBQC_model_v2.2.zip and perform the desired analysis from a desktop computer.

The SPAE tool also allows the user to input the key PBQC parameters and visualization the changes in data distribution or control chart to better appreciate the interaction between the PBQC parameters and the data distribution and PBQC performance. This should allow the laboratory users to gain familiarity and confidence with the PBQC concepts and techniques. Additionally, more complex optimization functions have been deliberately automated to simplify user experience. The SPAE tool is suitable for running PBQC retrospectively, either periodically to assess for potential errors missed by conventional internal quality control approaches or when an analytical error is suspected (e.g. due to failed internal quality control). This spreadsheet is also well suited as an educational tool for laboratory users.

A limitation of the SPAE is the lack of direct integration with the laboratory information system, which necessitates separate data extraction to perform PBQC. Nonetheless, this spreadsheet tool may serve as a baseline template for interested middleware or laboratory information system vendors to consider emulating in their software to implement some of the recommended features [6]. Another limitation of this tool is the availability of only two standard, simple PBQC models (moving average and moving median), which may limit its detection of more specialized errors such as increased imprecision (more optimally detected by the moving standard deviation approach [8]) or small biases (potentially more optimally detected by the moving positive rate [8,9]). The use of spreadsheet, while convenient and more commonly accessible, is computationally less efficient. When large amount of data is input into the tool or a computer with lower processor specification, it may take some time (up to a few minutes) to complete the analysis.

The SPAE described in this study adds to a growing list of freely available tool for implementing PBQC to meet varying laboratory requirements. They include an online parameter optimization tool [5] and the QC Constellation [10], which provides more complex PBQC algorithms (e.g., exponentially weighted moving average and cumulative sum algorithms). Collectively, this improves the accessibility of the PBQC informatics tool and reduces the barrier for adoption.

Research Funding
None received.

Author contributions
Hui Qi Low, Tze Ping Loh, Chun Yee Lim: Conceptualisation, Development, Investigation, Software, Analysis, Write-up. Hyun-Ki Kim, Sollip Kim, Tony Badrick: Writing – review and editing.

Conflict of Interests
None to declare.

Ethics approval
Not applicable as this study did not involve human subjects.

Consent for Publication
Consent to submit has been received explicitly from all co-authors, as well as from the responsible authorities. Authors whose names appear on the submission have contributed sufficiently to the scientific work and therefore share collective responsibility and accountability for the results.

Data availability
The data and spreadsheet tool described in this study is available as Supplemental Material accompanying this study.

Acknowledgement
This spreadsheet tool has been developed as part of a one-day workshop to educate and promote the use of patient-based quality control under the APFCB traveling lectureship program. Interested reader may contact the corresponding author for further details on the workshop.

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Supplemental Material

A: Screenshot from PBQC python code.

A.1 Input:
A.1.1: Predefined setting table.

predefined setting	value
Accpetable False Positive Rate (%)	0
Desirable bias for detection (%)	5
Box-cox Transformation	No
Box-cox lambda	None
Winsorisation (%)	99.5
Winsorisation lower limit	132.0
Winsorisation upper limit	147.0

A.1.2: Method parameter table.

Method parameters	value
Method1	Med
Method2	Avg
blocksize1, n1	10
blocksize2, n2	20
blocksize3, n3	30
number start added bias per day	30

A.1.3 Control limit table.

Method	block size	lower control limit	upper control limit	column
Med	10	136.5	144.0	**MMed1**
	20	138.0	143.0	**MMed2**
	30	138.0	142.5	**MMed3**
Avg	10	137.2	143.4	**MAvg1**
	20	138.3	142.55	**MAvg2**
	30	138.8	142.233333	**MAvg3**

A.2 Output:

A.2.1 Performance result table.

Method	block size	Percentage of detection (%)	ANPed	MNPed	95NPed	False positive (%)	Sum Best	Preferred
Med	10	100.0	4.0	5.0	6.0	0.0	5	Preferred
	20	100.0	8.0	8.0	11.0	0.0	2	NaN
	30	100.0	11.0	11.0	15.0	0.0	2	NaN
Avg	10	100.0	4.0	5.0	7.0	0.015385	3	NaN
	20	100.0	6.0	7.0	9.0	0.012821	1	NaN
	30	100.0	8.0	8.0	12.0	0.010256	1	NaN

A.2.2 Method parameter for routine table.

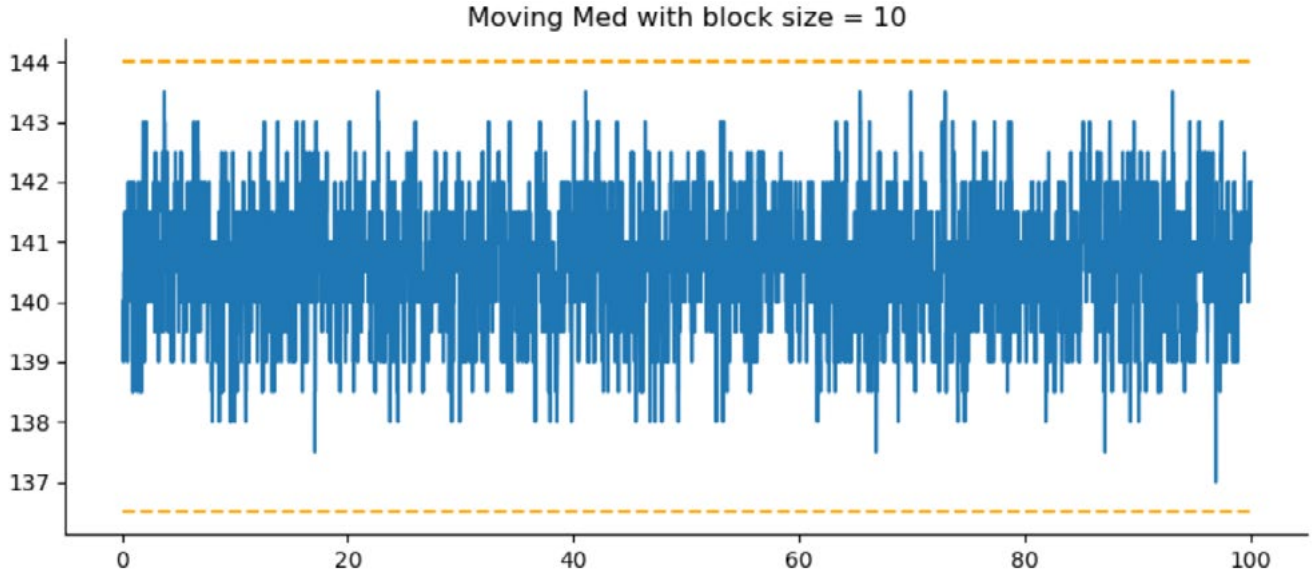
Method parameters for real-time data	value
Method	Med
Block size	10
lower control limit	136.5
upper control limit	144.0
Acceptable False Positive Rate(%)	0
Desirable bias for detection (%)	5
Box-cox Transformation	No
Box-cox lambda	None
Winsorisation (%)	99.5
Winsorisation lower limit	132.0
Winsorisation upper limit	147.0

A.3 Routine

A.3.1: Routine summary table.

Result	value
min date	2021-08-23
max date	2021-11-30
number of days	100
average count per day	130.0
number of positive detected	0
percentage of positive detected(%)	0.0
first sequence number of positive detected	NaN
first day of positive detected	NaN
first date of positive detected	NaT

A.3.2 Control chart with selected moving statistic result.



Research Article

International Consensus on ANA Patterns (ICAP) classification tree revisited: A single centre report on four nuclear patterns from a tertiary care centre in India

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Keywords
Antinuclear antibody, ANA patterns, ICAP, Connective tissue diseases, IIF, LIA

Abstract

Background: ICAP describes ANA patterns from AC- 0 to AC-29. They are further marked for competent or expertise reporting depending on ease of identification. There are some debatable patterns in ICAP which share similar features with a few others yet have a distinct identity and few others which are not addressed by ICAP but are described by BCA like Quasi-homogenous. This study analysed four nuclear patterns with overlapping features, namely Homogenous, speckled, Dense Fine Speckled70(DFS70) and quasi-homogenous to identify challenges posed in their identification due to overlapping features.

Methods: All samples which were reported as positive for the above four nuclear patterns (n=388) by IIF using HEp-2 cell were included in the study. LIA was performed on 103 such samples to look for association between the ANA patterns and specific antibody detected by LIA.

Results: DFS70 pattern is a rare pattern and existed in combination with other autoantibodies thus making its identification difficult on IIF. Homogenous pattern corresponded to AC- 29 (anti-topoisomerase, anti-Scl 70) which was probably due to wrong identification. Mixed pattern i.e speckled and homogenous was associated with Sm and U1sn RNP antibodies.

Conclusions: DFS 70 is a pattern with overlapping features of both homogenous and speckled and calls for expertise reporting. More awareness is required about AC 29 pattern as it is an overlap of five different components. Its identification poses significant challenges and is rightly placed in the expert reporting by ICAP. Mixed pattern (speckled and homogenous) referred to as Quasihomogenous by BCA needs to be addressed by ICAP.

Introduction

Antinuclear antibodies (ANA) are directed against nuclear self-antigens and are the hall mark of systemic autoimmune rheumatic diseases (SARD). The prevalence of autoimmune diseases is 3%–5% in developing countries with significant female preponderance [1]. A significant increase has been observed in overall incidence and range of autoimmune diseases after COVID -19 infection [2]. Many methods are available for detection of ANA of which indirect immunofluorescence (IIF) using Human epithelial cancer (HEp-2) cell line is considered to be the gold standard. IIF is the most commonly used screening tool on suspicion of SARDs or connective tissue diseases (CTDs) [3]. Different patterns are visualised under the fluorescent microscope which correspond to the different nuclear, cytoplasmic or mitotic antigens targeted by autoantibodies. Each pattern hence obtained gives vital clue to the probable antigen targeted and the clinical disease associated. A recent classification of the International consensus on ANA patterns (ICAP) based on HEp-2 patterns in the diagnosis of ANA-associated autoimmune diseases intends to achieve harmonisation in ANA reporting across the world [4]. This classification generates 29 different types of fluorescence patterns numbered from AC 1 to AC 29 and a negative pattern numbered AC 0. The patterns are distributed into 3 groups: nuclear, cytoplasmic, and mitotic. Each of these patterns is expected to reflect some clinical relevance. The disease associations may be confirmed further by specific tests like Enzyme linked immunoassay (ELISA), Line-immunoassay (LIA), chemiluminescence immunoassay (CLIA), FEIA (Fluorescent enzyme immunoassay) etc. to identify the antigen targeted. However not all anti-nuclear antibodies are associated with disease [5]. One pattern believed to be associated with apparently healthy individuals is the dense fine speckled pattern (AC-2), but this association only holds if the targeted antigen is confirmed as monospecific for DFS70.As per literature, DFS 70 is associated with non- autoimmune conditions [6]. At a titre of 1:40 serum dilution, 25–30% of healthy individuals may show ANA positivity, which increases with age [7]. In contrast to ICAP, The Brazilian Consensus on Autoantibodies (BCA) recognizes 34 different positive staining patterns observed by IIFT on HEp-2 cells. BCA proposed the distribution of these patterns into 5 groups: nuclear, cytoplasmic, nucleolar, mitotic, and complex. Nucleolar staining is a separate entity in the BCA which is included in the nuclear group in the ICAP classification and the complex patterns (CPs) which are not addressed by the ICAP classification are included in BCA [8]. The terms “mixed pattern” or “composite pattern” were introduced to describe those cases where more than one cellular component is targeted by a single antibody in the same sample or an overlap of patterns is observed due to the presence of more than one antibody [8]. The nuclear pattern as a whole is invariably the most common pattern reported in all studies worldwide [9]. Amongst this, the homogenous and speckled patterns are the most frequently reported. The dense fine speckled (AC-2) and anti-topoisomerase I (AC-29) patterns were previously often considered homogeneous, speckled or even mixed patterns due to overlapping features and the distinction till date is difficult at competent level of reporting [4]. This study was undertaken to understand the demographic profile, clinical manifestations and laboratory findings associated with these four similar yet different nuclear patterns, namely homogenous, speckled, DFS-70 and mixed (homogenous and speckled) in a tertiary health care set up in northern India.

Material and Methods

This is an analytical cross sectional study that was carried out in Biochemistry Central lab, AIIMS Patna in collaboration with departments of General Medicine, Dermatology, Paediatrics and Orthopaedics. The study was carried out after obtaining Institute ethical clearance from October 2023 to February 2024 with reference to letter number Ref.No.AIIMS/Pat/IEC/2023/1150. All samples for which an ANA screening by IIF (HEp-2) was advised by the treating clinician and subsequently processed were included in the study. All the samples which yielded a positive ANA screening report reporting four nuclear patterns, namely, homogenous (AC-1), speckled (AC-4 and AC-5), dense fine speckled ((AC-2) as per ICAP classification and mixed [homogenous and speckled] or quasi homogenous as per BCA classification(BCA3), were included as the study group. A total of 2294 samples were received for ANA screening by IIF HEp-2 during the study period. A total of 388 (16%) samples were positive for the four nuclear patterns committed in the study. The patients were tracked using Electronic medical records using Hospital information system for further details. Relevant history was taken by administering drafted questionnaire after obtaining informed consent. Data was collected and entered in MS excel.

Samples were processed using kits of Euroimmun (Lubeck, Germany), IIFT Mosaic HEp 20-10/Liver(Monkey) based on indirect immunofluorescence technique. The indirect immunofluorescence (IIF) test is considered gold standard for the determination of antibodies against nuclear antigens including cytoplasmic and mitotic components. Antibodies against cell nuclei can be determined on numerous substrates. The BIOCHIP technology facilitates different substrates to be combined in one test field (multiplex test) and incubated with individual patient serum. The substrate combination HEp-2 or HEp-2010 cells with primate liver allows dual confirmation of few patterns in a single approach. Combinations of HEp-2 cells and primate liver as substrate are incubated with diluted patient sample(1:100). Specific antibodies of classes IgA, IgG and IgM attach to the antigens if a positive reaction is obtained. The attached antibodies are stained with fluorescein labelled anti-human antibodies in a second step and visualised with the fluorescence microscope by Euroimmun (EUROSTAR) by two readers. The four ANA patterns were identified as follows keeping in mind ICAP guidelines and our past experience:

A. Homogenous pattern: The AC-1 pattern is characterized by a homogeneous nucleoplasm during interphase with an intensely

stained chromatin mass in a homogeneous hyaline fashion in mitotic cells. Primate Liver is positive for homogenous pattern and is associated with autoantibodies to double stranded DNA (dsDNA), nucleosomes, and histones, which are mostly related to Systemic Lupus Erythematosus (SLE).

B. Dense Fine speckled [DFS70]: The AC-2 pattern is characterized by a heterogeneity in the size and brightness of speckles in the nucleoplasm during interphase with a heterogeneously speckled chromatin in the metaphase plate. It is associated with autoantibodies against the dense fine speckled protein of 70 kD (DFS70) also known as lens epithelium-derived growth factor protein of 75 kD i.e LEDGF/p75.Primate liver is largely negative.

C. Speckled pattern: The AC-4(Fine speckled) and AC-5 (coarse speckled) are identified by fine, tiny and coarse speckles across the nucleoplasm respectively. Nucleoli is not stained. Mitotic cells do not reveal any staining of chromatin mass. This pattern corresponds to antibody against antigens U1sn RNP (uridine 1 small nuclear ribonuclear protein),SSA-Ro/SSB/La(Sjogren syndrome), Sm (smith antigen)etc.. Primate liver shows positive reaction.

D. Mixed pattern (homogenous and speckled): It has characteristics of both the speckled and homogenous pattern with nucleus of primate liver also showing positive reactivity.

E. Anti-Topoisomerase I(AC29): This pattern is characterized by five key elements: (1) prominent nuclear compact fine speckled pattern in interphase cells, (2) consistent strong fine speckled staining of condensed chromatin in mitotic cells, (3) strong staining of nucleolar organizing region (NOR) associated with condensed chromosomes in mitotic cells, (4)weak and delicate cytoplasmic weblike staining radiating from the perinuclear area to the plasma membrane, and (5) inconsistent staining of the nucleoli. Additionally, the Topoisomerase I-like pattern exhibits a subtle hazy interface between the nuclear fine speckled staining and the cytoplasmic staining. Unlike typical nuclear patterns with sharp borders, the Toposiomerase I nuclear staining displays a blurry border where the fine speckling in the nucleus extends into the adjacent cytosolic region to nucleus of hepatocytes. This pattern corresponds to antibody against antigen Scleroderma -70(Scl-70).

As a confirmatory testing for specific antigens, further line immunoassay (LIA) testing was performed using kits developed by Human diagnostics (Germany). The instrument used was fully automated HUMABLOT 44FA(Germany). LIA is a qualitative test which reveals reactivity of antibody to antigens coated as distinct lines on a membrane. A total of 18 antigens were capable of being detected in a single approach by ANA profile kit based on LIA(IMTEC-ANA-LIA-XL). Specific nuclear antigens are applied to nitrocellulose strips at equal distances which are then placed on respective rows of the incubation tray. A buffer containing blocking protein is added to rehydrate and to block free binding sites on the strips against unspecific binding. The membrane strips are incubated with prediluted serum samples (1:100) after discarding the blocking buffer. Autoantibodies present in the patient's sample bind to the antigens according to their specificity and are traced by alkaline phosphatase conjugated anti-human-IgG antibodies that appear as blue stained bands on the strips. The nuclear and associated cytosolic antigens applied as thin bands on a nitrocellulose membrane in our study included 18 antigens namely, dsDNA, Nucleosome, histone, SmD1, proliferating cell nuclear antigen(PCNA), Po(RPP-ribosomal P Protein), SS-A/Ro60, SS-B/Ro52, SS-B/La(Sjogren syndrome), CENP-B(centromere protein B), Scl-70, U1-snRNP, AMA-M2(anti-mitochondrial antibody), Jo-1, PM-Scl(polymyositis-Scleroderma), Mi-2, Ku and DFS 70. Qualitative measurement of IgG class of antibodies found in human serum against these antigens helps in diagnosis of a wide number of diseases which constitute SARDs. Patient's result were categorised age and sex wise. The results were graded as negative (-), equivocal (0), (+), (++), (+++) depending on intensity of band with reference to cut off control. The test result is negative if no band is recognised or if the band exhibits a smaller intensity in comparison to the cut -off control, equivocal if the intensity of the band and the intensity of the cut off control do not significantly differ and positive if a band exhibits a stronger staining in comparison to the cut-off control. Functional cut off serves the purpose of quality control.

Statistical Analysis

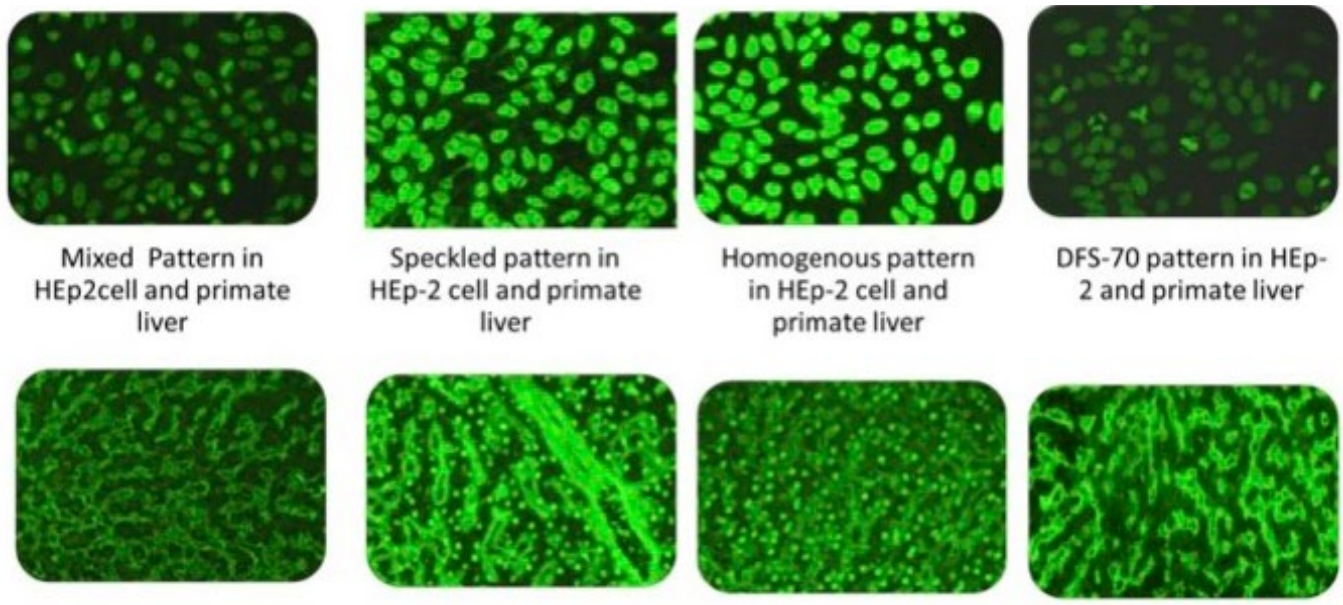
Descriptive statistics, such as means, standard deviations and ranges for continuous variables and frequencies and percentages for categorical variables was conducted to outline the baseline characteristics of the sample. The primary analysis involved chi square test to explore the association between categorical variables especially different ANA patterns (homogenous, speckled, DFS 70 and mixed: quasi-homogenous) and the antigens identified. All analyses were conducted using JAMOVI 2.3.28 [10,11].

Observation and Results

A total of 2294 samples were received for ANA screening.528 samples (23%) were from the IPD and 1766 (76.98%) samples were from the OPD. A total of 388 samples (16%) were reported as positive for the four nuclear patters included in our study.444 samples (19.3%) tested positive for other ANA patterns excluding the four described here. The total positivity rate was 35.3% including all the other ANA patterns. 105 (27.1%) and 283(72.9%) patients from IPD and OPD respectively tested positive for the four nuclear patterns. A total of 294(75.8%) females and 94(24.2%) males were positive for the nuclear patterns included in our study. The maximum number of patients (142) with a positive nuclear pattern belonged to the 21-40 age group amounting to 36.6% of the total patients. The medical specialities accounted for 301(77.6%) positive four nuclear

ANA patterns included in our study whereas 87(22.4%) positive nuclear ANA patterns were from surgical specialities. Amongst the specialities, General Medicine Department accounted for 42% of the positive nuclear samples included in our study, orthopaedic department accounted for 18.3% followed by pulmonary department at 10.6%, gastroenterology at 8.8%, paediatrics at 6.2% and dermatology at 5.2% followed by the other departments amounting for the remaining 9.0%.The most common pattern reported as per our study was the speckled pattern(69.8%) followed by homogenous(14.2%),mixed (13.9%) and the least common pattern reported was DFS 70 at 2.1%. The most common symptom reported was joint involvement (63.7%) followed by fever (51.8%) and fatigue (46.9%). Amongst the laboratory parameters, the most common finding reported was anaemia (60.3%). A total of 103 samples were also subjected to a confirmatory testing for ANA for specific antigen detection by LIA. As this was a non-funded study, samples which were advised for further testing by LIA by the treating clinicians were included(n=103) excluding the rest (n=285). The most common antibody observed on LIA in 103 patients was against U1snRNP (24.27%) followed by Ku (18.44%) and SSA-Ro60 (16.5%). Only 2 persons amongst 103 had antibody against Mi-2. No antibody was recorded against CENP-B and Jo1. We also looked for any association between ANA screening patterns and specific autoantibodies detected by LIA. Significant association was noted between mixed pattern and U1 snRNP(p=0.048) and Sm D1(p=0.042). Significant association was noted between homogenous pattern and dsDNA(p=0.024) and Scl 70(p=0.008). No significant association was noted between speckled pattern and any of the antigens detected on ANA profile by LIA. DFS70 pattern was not taken into account because of a small sample size(n=2) for which both ANA screening and corresponding reports by LIA were available.100% negative association was noted between the four nuclear patterns included in our study and CENP-B and Jo-1. Figure 1 shows the four different positive nuclear patterns obtained in our study (a) homogenous(b) speckled (c) mixed (homogenous and speckled) and (d) dense fine speckled (DFS-70).

Figure 1: Depicts Mixed,speckled,homogenous and DFS -70 patterns on HEp-2 cell line and Primate liver by IIF.



Discussion

Autoimmune testing is an important diagnostic aspect in a clinical setup. ANA screening is one of the most frequently performed tests as a first line investigation in a suspected case of SARD. HEp-2 cell line serves as an efficient substrate for the ANA test. However, it significantly increases the sensitivity, which often leads to a high-false positive rate. A biochip that incorporated primate liver along with the HEp-2 cells was introduced to further improve the performance of HEp-2 cell line especially in cases of ambiguity. Further reflex testing can be performed by commonly available techniques like ELISA, LIA etc to confirm the targeted antigen. Our study was performed to identify and evaluate the four similar yet distinct nuclear patterns, namely homogenous (AC-1), speckled (AC-4 and AC-5), dense fine speckled -70(AC-2) and mixed pattern (homogenous and speckled together also known as Quasi homogenous by BCA for ANA patterns) from a tertiary health care in a northern state of India, Bihar. Such a study to address the two debatable patterns on ANA screening, namely DFS-70 and Quasi Homogenous is probably the first of its kind in India.

The findings revealed many similarities with previously published studies. Sixteen percent (16.3%) positivity was reported for the four nuclear patterns on ANA screening in a time frame spanning 5 months. The total positivity rate was 35.3% including all the other ANA patterns. As the study was not population based, prevalence could not be estimated but a rough

calculation of disease burden could be proposed. The patterns included all grades of intensity reported. Few other studies from India in past have reported a prevalence of 38.2% (Sebastian et al, n=5066) and 18.9% (Minz et al, n=650) ANA positivity based on HEp-2 screening which included all ANA patterns reported in the study [12,13].A study conducted by Guo et al in China was a population based study on a sample size of 20970 people and the total positivity rate was 5.9% [14]. Female population was most commonly affected accounting for 75% of the total positivity in our study. Female predominance in the field of autoimmunity is already established. Beeson et al attributed estrogen as a potential modulator of autoimmunity [15]. Hayter et al proposed microchimerism, implication of X-chromosome encoded genes and random inactivation phenomena as the potential causes for female predominance amongst autoimmune diseases [16]. The most common age group afflicted in our population was 21-40 years accounting for 36.6% of the total positives for nuclear pattern. Table 1 depicts age and sex distribution of four different nuclear patterns in our study. Our study is in accordance with the findings of Beeson et al who has reported that autoimmune disorders are high in the 20 to 50 age group, while Hayter et al observed that the 20 to 29 age group presented with the highest prevalence of autoimmune disorders [15,16]. Cataudella et al have reported the highest prevalence of autoimmune diseases in the reproductive age group [17]. ANA positivity was maximum in the 30 to 39 age group patient population with a mean age of 37 ± 18 years in a study by Gupta et al from central India[9]. In a study from central India authors have emphasised that childbearing may be accountable for initial antigen stimulation or breach in tolerance to self-antigens contributing to the event of autoimmunity [9].

Table 1: depicting age and sex distribution of pour nuclear patterns in our study.

Sex wise distribution			
Age (years)	**Total number of females positive**	**Total number of males positive**	**Total**
0-20	52	17	69 (17.7%)
21-40	107	35	142 (36.6%)
41-60	108	22	130 (33.5%)
61-80	27	20	47 (12.1%)
Total	294	94	388

Screening tests that have high false positive rate have many undesirable consequences. When requested minus appropriate clinical rationale, tests that screen for rare diseases but have a high rate of false positivity can lead to multiple problems including misdiagnosis and potentially harmful follow-up testing and even inappropriate treatment. ANA is such a test with a very low positive predictive value. Out of a total of 2294 samples received for ANA testing by HEp-2 cell line ,388 were positive for ANA for the four nuclear patterns amounting to a positivity of 16.3%. Out of 819(35.7%) requisitions raised by the department of General Medicine at our hospital,163 were positive for the four nuclear patterns amounting to 19.9% positivity. In a study by Banhuk et al, 14 (8.1%) ANA test was advised by rheumatologists and 158 (91.9%) by physicians from other specialties. Amongst the positive results for ANA, 16.7% were advised by rheumatologists and the rest by other departments, whereas in the negative group, rheumatologists requested only 6.2% of the tests [18] implying that rheumatologists were more trained to evaluate and identify rheumatological diseases. The chances of ANA result being positive in a case suspected of a rheumatological disease by a rheumatologist was high as compared to other specialities. In a study in Korea, ANA positivity rate was 14.4% and varied according to the requesting department, with the highest rate for rheumatology (19.9%). ANA associated rheumatic disease (AARD) was diagnosed in 645 (0.69%) among all ANA tested patients. The diagnosis rate varied according to the requesting department with the highest for rheumatology and hemato-oncology (1.73% and 1.23% respectively). However, diagnosis of SARD was made in less than 1% of ANA tested subjects for all other departments. AARD was diagnosed in 4.74% among all ANA positive patients. SARD diagnosis rate among ANA positive patients was the highest for rheumatology, followed by nephrology and hemato-oncology (8.7%, 6.95%, and 6.86%, respectively). SARD diagnosis rate was the lowest among both ANA tested and ANA positive patients when requested by orthopaedics (0.14% and 1.23%, respectively). However, AARD diagnosis was made in only 1.73% of ANA tested patients even when it was requested from rheumatology thereby questioning the predictive value [19]. Patients with the highest pretest probabilities for AARD, as associated with the initial presenting symptoms are most likely referred to rheumatology. However even in those patients with a positive ANA test, the diagnosis of a rheumatic disease being made was very less. As we did not have rheumatology department, the maximum number of patients with fever and musculoskeletal complaints were attended to by General Medicine department. Hence, the maximum number of ANA positivity for nuclear samples (42%) was reported from the department of General Medicine followed by the department of Orthopaedics. Table 2 depicts the percentage positivity of samples from different departments for ANA screening test using IIF.

Table 2: depicting positivity rate from different Departments.

Department	Total number of requisitions raised on HIS[OPD]	Total number of requisitions raised on HIS[IPD]	Total number of requisitions raised inclusive of IPD and OPD	Total number of samples positive for four nuclear patterns included in our study	Positivity [%]
Dermatology	60	24	84(3.6%)	20	5.15
Gastroenterology	201	26	227(9.8%)	34	8.76
General Medicine	540	279	819(35.7%)	163	42.01
Orthopaedics	526	4	530(23.1%)	71	18.3
Paediatrics	66	84	150(6.5%)	24	6.19
Pulmonary Medicine	152	42	194(8.45%)	41	10.57
Others	221	69	290(12.6%)	35	9.02
Total	1766	528	2294	388	100

Amongst the four common nuclear pattern the most common pattern noted was speckled (69.8%), a finding which is very much in agreement with previous studies. In a study by Ramachandran et al in India, the most common pattern reported was speckled (52.9%) followed by homogenous (27.5%) in a study involving 204 SLE patients [20]. In alignment with our findings, a research from Saudi Arabia found speckled pattern (79.5%) as the most frequently reported followed by the homogeneous pattern (11.4%) [21]. DFS -70 pattern is a very rarely reported pattern and its frequency across world- wide studies have varied from 0.3% to 27% [22]. DFS 70 accounted for 2.1% of the total of four nuclear patterns in our study. The DFS 70 pattern is frequently observed in people without autoimmune diseases and with a positive ANA test [22]. DFS 70 is also known to have a positive association with diseases with an autoimmune basis like Raynaud's disease and idiopathic fibrotic alveolitis [23]. However, some studies have observed that patients with DFS 70 pattern were diagnosed with rheumatic autoimmune diseases [18]. Mixed pattern (homogenous and speckled) has been named as Quasi homogenous as per BCA for ANA screening using HEp-2 cells and describes a pattern not included in ICAP. We have frequently observed this pattern on HEp-2 screening for ANA (13.9% in this study) lately especially post COVID-19 pandemic. The nomenclature used in our study to describe this pattern is mixed (homogenous and speckled) and is characterised by staining characteristics resembling both homogenous and speckled patterns on HEp-2 cells. The primate liver substrate offered by Euroimmun which was used in our study also showed a positive reaction which could be used to differentiate this pattern from DFS 70 which showed a similar reaction on HEp-2 cell line but does not elicit any reaction on primate liver. Table 3 depicts the distribution pattern of the four different nuclear patterns in our study.

Table 3: Pattern of distribution of the four nuclear patterns in our study.

ANA screen pattern	% distribution
DFS 70	2.1%
Homogenous	14.2%
Mixed [homogenous and speckled]	13.9%
Speckled	69.8%

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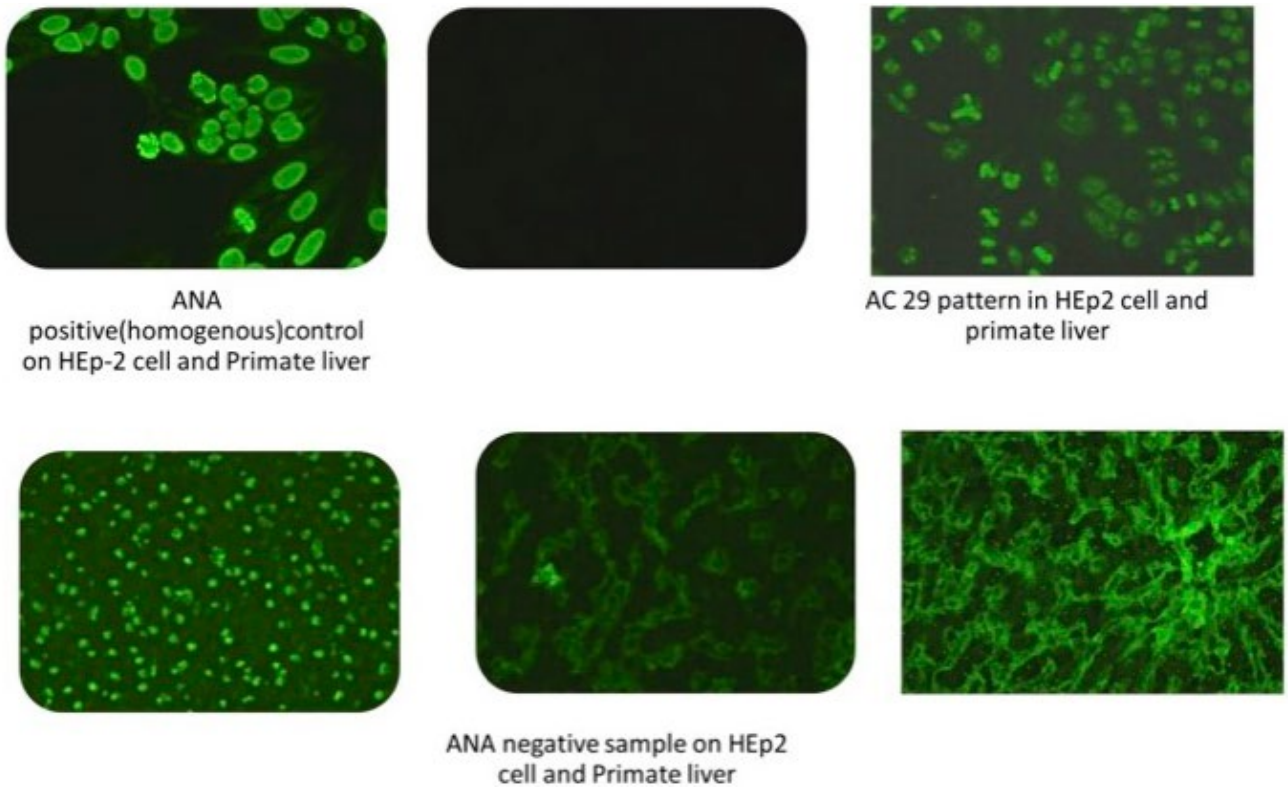
Table 4: Depicting correlation between the three nuclear patterns and antibodies detected against 16 antigens by LIA in 103 samples(DFS 70 was excluded amongst nuclear patterns as only two were positive for which LIA results were available ,CENP B and Jo-1 were excluded from ANA profile anti body list as none of the two were positive in 103 samples).

Antigens/patterns	**dsDNA**			**Nucleosome**			**Histone**		
	ABSENT	PRESENT	p value	ABSENT	PRESENT	p value	ABSENT	PRESENT	p value
homogenous	70.80%	29.20%	0.024	75%	25%	0.075	75%	25%	0.1.26
mixed	91.70%	8.30%		91.70%	8.30%		91.70%	8.30%	
speckled	92.30%	7.70%		92.30%	7.70%		90.80%	9.20%	
Antigens/patterns	**SmD1**			**PCNA**			**P0[RPP]**		
	ABSENT	PRESENT	p value	ABSENT	PRESENT	p value	ABSENT	PRESENT	p value
Homogenous	83.30%	16.70%	0.042	100%	0	0.218	91.70%	8.3%	0.599
mixed	75%	25%		91.70%	8.3%		100%	0%	
speckled	95.40%	4.60%		98.5%	1.50%		92.30%	7.7%	
Antigens/patterns	**SSA/Ro60**			**SS-A/Ro52**			**SSB/La**		
	ABSENT	PRESENT	p value	ABSENT	PRESENT	p value	ABSENT	PRESENT	p value
homogenous	87.50%	12.50%	**0.64**	91.70%	8.30%	0.362	79.20%	20.80%	0.063
mixed	75%	25%		75%	25%		66.70%	33.30%	
speckled	83.10%	16.9%		87.70%	12.30%		90.80%	9.20%	
Antigens/patterns	**Scl-70**			**U1-snRNP**			**AMA-M2**		
	ABSENT	PRESENT	p value	ABSENT	PRESENT	p value	ABSENT	PRESENT	p value
Homogenous	79.20%	20.80%	0.008	62.50%	37.5%	0.048	95.8%	4.20%	0.411
mixed	100%	0%		58.30%	41.70%		91.70%	8.3%	
speckled	96.9%	3.10%		83.10%	16.90%		98.50%	1.50%	
Antigens/patterns	**PM-Scl**			**Mi-2**			**Ku**		
	ABSENT	PRESENT	p value	ABSENT	PRESENT	p value	ABSENT	PRESENT	p value
Homogenous	95.80%	4.20%	0.458	100%	0%	0.568	75%	25%	0.48
mixed	83.30%	16.70%		100%	0%		91.70%	8.3%	
speckled	90.80%	9.2%		96.90%	3.10%		81.50%	18.50%	
Antigens/patterns	**DFS-70**								
	ABSENT	PRESENT	p value						
Homogenous	87.50%	12.50%	0.232						
mixed	91.70%	8.30%							
speckled	96.90%	3.10%							

Percentages are calculated row wise.

These findings are in agreement with other studies in past except for the homogenous pattern which was associated with Scl70 additionally in our study. Dellavance et al. in 2009 documented a composite HEp-2 pattern linked to anti-Topoisomerase I antibodies, commonly known as anti-Scl-70. The incorporation of the Topoisomerase I-like pattern into the ICAP algorithm was initially proposed at the 2nd ICAP edition at the 12th Dresden Symposium on Autoantibodies (DSA) in 2015, Dresden, Germany and at the fourth ICAP meeting in 2017, there was a consensus that the Topoisomerase I-like pattern be designated as AC-29[28]. Although autoantibodies to Topoisomerase I may be reported as homogeneous, they typically reveal a composite AC-29 HEp-2 IIF pattern and clinical suspicion of Systemic Sclerosis may call for follow-up testing for antibodies to Scl-70. In the past there have been instances of the dense fine speckled (AC-2) and topoisomerase I-like (AC-29) patterns often being considered homogeneous, speckled or even mixed patterns [4]. It is very much possible that the AC -29 pattern could have been wrongly been reported as homogenous pattern in our study due to overlapping features. AC-29, anti- topoisomerase I (previously known as Scl 70) is hence rightly placed in Expert level reporting as per ICAP ANA classification tree and calls for more awareness and expertise to be rightly reported. Figure 2 depicts positive control pattern, negative ANA sample pattern and anti-topoisomerase antibodies i.e Scl 70 (AC 29 pattern) on HEp-2 cell and primate liver obtained in our study.

Figure 2: Depicts positive ANA control, negative ANA control and anti-topoisomerase pattern (AC-29) on HEp-2 cell line and primate liver by IIF.



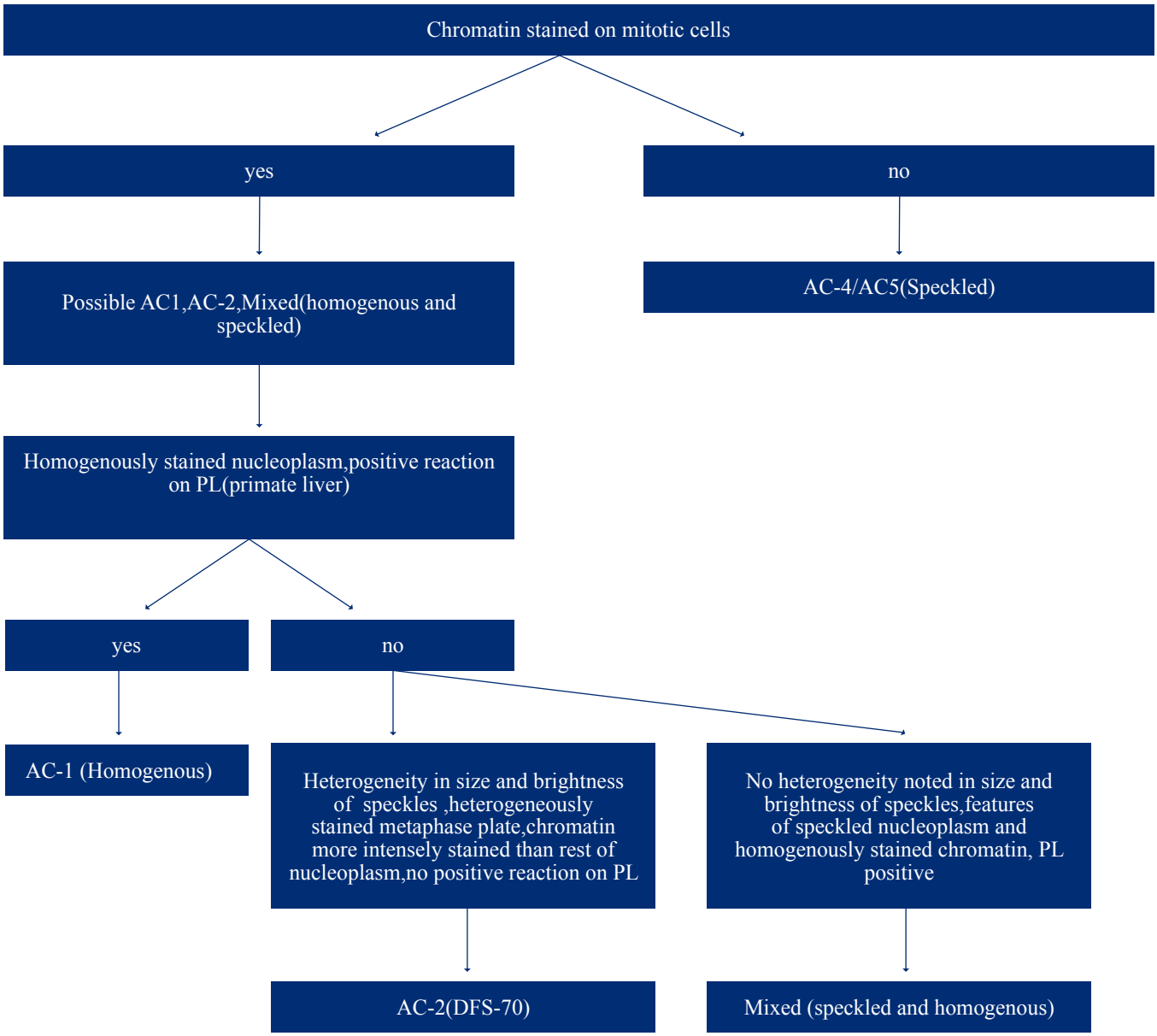
Interestingly the speckled pattern in our study, despite being the most frequently reported, failed to show association with any particular antigen detected by LIA. Mixed pattern in our study corresponded to antibodies to U1snRNP and SmD1 antigens. Antibody to U1-snRNP targets the U1snRNP.Autoreactive B cells and T cells target the U1-sn RNP in several rheumatic diseases including SLE and Mixed connective tissue disease (MCTD). In our study a total of twelve cases of mixed patterns were reported on ANA screening by HEp-2. When followed up by LIA, two of them were associated with a single antibody on LIA, six of them had more than two antibodies associated and four had no antibody associated. The mixed pattern has been associated with antibodies to U1snRNP and SmD1in our patient population and previous studies have been reported where SLE was found to be associated with two different clusters of antibodies: Sm/anti-RNP or Ro/La autoantigens. Both are proteins often involved in RNA binding activities. The Sm/RNP cluster was associated with a higher prevalence of serositis in comparison to the Ro/La cluster [29].

Elevated antibody titers against U1snRNP are also linked to MCTD. Originally defined in 1972, MCTD is characterized by the presence of anti-U1snRNP antibodies alongside overlapping clinical features reminiscent of SLE, Systemic Sclerosis, idiopathic inflammatory myositis, and rheumatoid arthritis.

U1-snRNP is one of the five snRNPs constituting the mammalian spliceosome, a crucial macromolecular complex responsible for post-transcriptional processing of pre-messenger RNA (pre-mRNA). This process involves intron removal and exon ligation to produce mature RNA for translation into proteins [30,31]. The five snRNPs—U1, U2, U4, U5, and U6—each comprise a unique small nuclear RNA molecule, specific associated proteins, and seven common core proteins known as Smith (Sm) proteins. The term "Smith protein" originates from the patient whose blood sample contained antibodies specific to the Sm complex. Autoantibodies against Sm and 'RNP', which refers to U1-specific proteins and U1-snRNA, target distinct molecular entities. While Sm proteins are present in all five snRNPs, autoantibodies against Sm precipitate all snRNP RNA molecules, whereas anti-RNP autoantibodies precipitate only U1-specific RNA, sparing other unique RNA molecules. U1-snRNP comprises U1-snRNA, the seven common core Sm proteins, and three U1-specific proteins: U1-70K, U1-A, and U1-C [30,31]. The prevalence of anti-RNP autoantibodies varies across diseases, with detection rates of 30–40% in SLE patients and nearly universal presence in MCTD patients, where high titers of anti-RNP antibodies are diagnostic criteria for MCTD [30,31]. Anti-Sm antibodies are included in the Systemic Lupus Erythematosus International Collaborating Clinics (SLICC) classification criteria for Lupus. The prevalence of anti-U1snRNP as the most common antibody on LIA, followed by Ku and the occurrence of multiple antibodies in combination suggests the likely existence of polyautoimmunity phenomena in the patient population. LIA is a platform which enables the detection of multiple autoantibodies in a single approach and may prove a valid tool in times to come.

The authors propose an algorithm based on ICAP and BCA guidelines along with their experience in approaching the identification and distinction of four nuclear patterns described above (Figure 3).

Figure 3: Algorithm suggested for evaluation of four nuclear patterns on IIF by HEP-2.



Limitations of our study

The study was carried out as a short term research for a limited duration as per guidelines of ICMR. It could have probably benefitted otherwise from a larger sample size spanning a longer duration. Moreover the number of samples included in LIA (n=103) were restricted as the work was non funded and only those samples were included for which the investigation was routinely ordered as part of patient care services. More studies in future are required to substantiate the results of this study.

Conclusions

1. Laboratory investigations play a very supportive role in the diagnosis of SARDs. Approach to laboratory investigation of a suspected case of SARD starts with IIF and is usually followed by reflex testing in positive cases using ELISA or LIA for identification of specific antigens.
2. Female predominance is noted with respect to a positive ANA screening test and the young and middle age group (20-40 years) are mostly affected.
3. Speckled pattern is the most common nuclear pattern noted in our study although no significant association with any specific antibody could be elicited in our study. Homogenous pattern was found to be most commonly associated with anti-dsDNA and anti Scl-70 antibodies while mixed pattern was associated with anti-SmD1 and antiU1snRNP antibodies.
4. Correlation of homogenous pattern with Scl-70 has not been reported very frequently in literature. Scl-70 corresponds to anti -Topoisomerase-I antibodies (AC 29). Scl-70 is a difficult pattern to identify because of its complex characteristics and is rightly placed under competent level of reporting by ICAP. This pattern deserves more attention and it may have been wrongly interpreted as homogenous (AC-1) in our study because of similarities in microscopic morphology.
5. DFS-70 which is currently placed under competent level reporting by ICAP needs to be reviewed as its identification is not devoid of challenges due to overlapping features with other three nuclear patterns namely, speckled, homogenous and mixed (homogenous and speckled). Anti-DFS 70 antibody was not reported singly in our study by LIA and was found in combination with other antibodies thus making correlation and interpretation even more difficult.
6. Mixed pattern as reported in our study mostly corresponded to antibodies against SmD1 and U1snRNP antigens. This pattern finds a mention in BCA as Quasihomogenous (features between speckled and homogenous) but is not depicted in the ANA classification tree by ICAP. This pattern has been on the rise post COVID-19 pandemic in population being catered by our hospital for SARDs and maybe addressed by ICAP.
7. Combination of multiple autoantibodies on LIA points towards the existence of polyautoimmunity in the present times highlighting the need for LIA as a detection tool.

Ethical clearance

The study has been approved by Institute Ethical committee at AIIMS Patna. The study has been completed in compliance with the ethical principles for medical research involving human subjects, in accordance with the Declaration of Helsinki.

Conflict of intertest

None declared.

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Data availability

All relevant data has been uploaded with the manuscript. Any additional data may be obtained from corresponding author on reasonable request.

Author contributions

All authors have contributed to the design and implementation of the research, to the analysis of the results and to the writing of the manuscript.

Consent for publication

All authors have consented for publication of the manuscript.

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Research Article

Practices and insights for diabetes mellitus testing in Sri Lanka, Singapore and the Philippines

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Diabetes mellitus, Harmonization, HbA1c, Laboratory testing, Plasma glucose, Urine albumin

Abstract

Objectives: Considering The pivotal role of biochemical testing for the management of diabetes mellitus, we studied the current status of diabetes testing and reporting in three countries of the Asia-Pacific region.

Methods: A survey of 254 practicing pathology laboratories comprising of 40, 11 and 203 laboratories from Sri Lanka, Singapore and the Philippines was conducted under the auspices of the Asia-Pacific Federation for Clinical Biochemistry and Laboratory Medicine (APFCB) Working Group for Diabetes Testing Harmonization using Survey Monkey and Google Forms.

Results: The country response rate varied from 40% to 88%. A diagnostic threshold of 6.5% (48 mmol/mol) for HbA1c is reported by 51%, 22% and 90% of the participant laboratories in Sri Lanka, Singapore and the Philippines, respectively. All participants in Singapore and 86% of the laboratories in Philippines use NGSP-certified methods for HbA1c. Traceability to Certified Reference Materials for both glucose and HbA1c results was confirmed by 74% of Sri Lankan laboratories. For albuminuria testing, early morning spot urine albumin to creatinine ratio is recommended by 56%, 75% and 69% of the laboratories in Sri Lanka, Singapore and the Philippines, respectively, while 16%, 50% and 26% of the laboratories recommended 24-hour urine collection.

Conclusion: There is a lack of harmonization in diabetes testing and reporting practices both across and even within the three countries surveyed. Scientific bodies or professional associations have an important role in harmonization of laboratory testing and reporting of results for the diagnosis and management of diabetes mellitus.

Introduction

Biochemical testing for the diagnosis and management of diabetes mellitus (DM) is not standardized globally. The lack of standardization impacts the prevalence, as well as management strategies. The age-adjusted prevalence of DM from 2019 to 2030, is expected to increase from 11.4% (87 million) to 12.2% (115 million) for the South-East Asia region [1]. The proportion of undiagnosed diabetes in South-East Asia is 51.2% as against the worldwide figure of 44.7% [2].

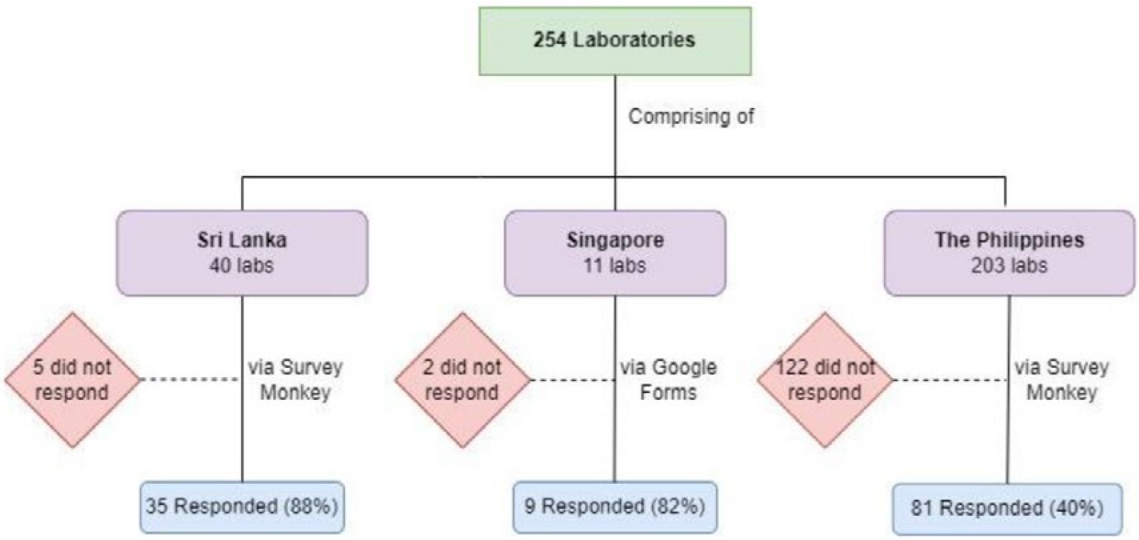
High proportion of undiagnosed diabetes represents a serious gap in healthcare. Type 2 diabetes mellitus (T2DM) has an asymptomatic stage of up to seven years [3], during which complications may develop. Hence, it is important to diagnose the disease early to enable early therapeutic and lifestyle interventions.

Biochemical investigations are essential in the diagnosis of diabetes, monitoring treatment response, and assessment of diabetic complications such as nephropathy and cardiovascular diseases. Fasting plasma glucose and haemoglobin A1c are the important diagnostic tests for T2DM in adults whilst oral glucose tolerance test (OGTT) is essential for the diagnostic confirmation of gestational diabetes mellitus (GDM). Considering the significant economic burden of T2DM across low, middle and high- income countries [4], and the perspective that harmonization of practices worldwide may lead to better patient outcomes [5], we aimed to investigate the current status of laboratory testing practices related to T2DM in laboratories across Sri Lanka (South Asia), Singapore and Philippines (South-East Asia) by administering a survey. The survey has been conducted similar to a previous survey conducted in India [6], under the auspices of the Asia Pacific Federation of Clinical Biochemistry and Laboratory Medicine (APFCB) Working Group for Diabetes Testing Harmonization.

Materials and Methods

A web-based survey was circulated to 254 laboratories in year 2020-2021 comprising 40, 11 and 203 laboratories in Sri Lanka, Singapore and the Philippines, respectively (Figure 1). Survey Monkey was used by Sri Lanka, and Philippines while Singapore used Google Form. The College of Chemical Pathologists of Sri Lanka, and Singapore Association of Clinical Biochemists facilitated the distribution of the surveys to the laboratories in the respective countries while the country representative to APFCB Working Group for Diabetes Testing and Harmonization facilitated the survey in the Philippines.

Figure 1: Number of invited laboratories and participants.



The survey invitation was emailed to a point-of-contact for each laboratory which varied between a chemical pathologist, a laboratory manager or a senior scientist. A reminder was sent if there was no response within two months. The survey questions varied slightly among the three countries in keeping with the local practices. The medium of communication for the survey was English.

Results

A total of 125 laboratories responded. The response rate was 88%, 82% and 40% for Sri Lanka, Singapore and the Philippines respectively (Table 1).

Table 1: The response rates from laboratories in Sri Lanka, Singapore and the Philippines.

Questions		Sri Lanka	Singapore	Philippines
Response Rate (number of labs/total labs contacted*100%)		88% (35 out of 40)	82% (9 out of 11)	40% (81 out of 203)
1.	Units of Reporting Glucose	n=35	n=9	n=79 [2 labs did not respond to this question]
	mmol/L	8 (23%)	7 (78%)	42 (53%)
	mg/dL	22 (63%)	1 (11%)	32 (41%)
	both	5 (14%)	1 (11%)	5 (6%)
2.	Units of Reporting of HbA1c	n=32	n=9	This question was not included in the Philippines survey
	mmol/mol only	2 (6%)	0 (0%)	
	% only	19 (59%)	3 (33%)	
	both	11 (34%)	6 (67%)	
3.	Is HbA1c used for diagnosis in your lab?	This question was phrased differently in the Sri Lankan survey. [See 4]	n=9	n=79 [2 labs did not respond to this question]
	Yes		3 (33%)	70 (89%)
	No		6 (67%)	9 (11%)
4.	Diagnostic cut-offs for diabetes reported by the laboratory [labs may choose more than 1 answer]	n=34 [1 lab did not respond to this question]	n=9	n=77 [4 labs did not respond to this question]
	HbA1c≥6.5% (48 mmol/mol)	18 (53%)	2 (22%)	69 (90%)
	HbA1c≥7.0% (53 mmol/mol)		1 (11%)	
	Fasting plasma glucose≥ 7.0 mmol/L	20 (59%)	8 (89%)	48 (62%)
	Two-hour OGTT≥ 11.1 mmol/L	15 (44%)	8 (89%)	32 (42%)
	Symptoms of hyperglycaemia and random plasma glucose≥ 11.1 mmol/L	16 (47%)	3 (33%)	30 (39%)
5.	HbA1c cut-off recommended for monitoring control	This question was not included in Sri Lankan survey	n=9	n=64 [17 labs did not respond to this question]
	6.5%		1 (11%)	0 (0%)
	7%		3 (33%)	64 (100%)
	Not reported		5 (56%)	0 (0%)

Questions		Sri Lanka	Singapore	Philippines
6A.	Is OGTT recommended for all pregnant mothers?	n=34 [One lab did not respond to this question]	A slightly different question was asked for Singapore Survey (See 6B)	n=79 [2 labs did not respond to this question]
	Yes	24 (71%)		67 (85%)
	No	10 (29%)		12 (15%)
6B.	Does your lab manual recommend OGTT at 24-28 weeks gestation for ALL women, and what is cut-off?	A slightly different question was asked for Sri Lankan Survey (See 6A)	n=5 [Four labs did not respond to this question]	A slightly different question was asked for Philippines (See 6A)
	Use IADPSG and recommend OGTT for all women at 24-28 weeks		2 (40%)	
	Use IADPSG but does not recommend OGTT for all women at 24-28 weeks		3 (60%)	
7.	Sample recommended for albuminuria testing	n=32 [3 labs did not respond to this question]	n=8 [One lab did not test urine albumin, for Singapore laboratories, percentage does not add to 100% as labs can choose more than 1 answer)	n=65 [16 labs did not respond to this question]
	Early morning spot urine	18 (56%)	6 (75%)	45 (69%)
	Twenty-four-hour urine	5 (16%)	4 (50%)	17 (26%)
	Timed overnight	0 (0%)	0 (0%)	3 (5%)
	Random spot urine	9 (28%)	1 (13%)	0 (0%)
8.	Reporting units for albuminuria	n=32 [3 labs did not respond to this question]	n=8 [One lab did not test urine albumin]	This question was not addressed in Philippines survey
	mg/L only	4 (13%)	0 (0%)	
	mg/mmol creatinine	13 (41%)	4 (50%)	
	mg/g creatinine	13 (41%)	1 (13%)	
	Both mg/mmol & mg/g creatinine	0 (0%)	3 (38%)	
	mg/day	0 (0%)	2 (25%)	
	µg/minute	1 (3%)	0 (0%)	
	mg/dL	1 (3%)	0 (0%)	
9.	What method does your lab use for HbA1c?	This question was not included in Sri Lankan survey	n=9	This question was not included in Philippines survey
	HPLC		2 (22%)	
	Immunoturbidimetry		5 (56%)	
	Capillary Electrophoresis		0 (0%)	
	Enzymatic		2 (22%)	

Questions		Sri Lanka	Singapore	Philippines
10A.	Traceability of glucose and HbA1c calibrators (labs may choose more than one answer)	n=34 [One lab did not respond to this question]	This question for Singapore and the Philippines was asked in a different format. (See 10B)	
	Only glucose calibrator is traceable to CRM/SRM	7 (21%)		
	Only HbA1c calibrator is traceable to CRM/SRM	2 (6%)		
	Both glucose and HbA1c calibrators are traceable to CRM/SRM	25 (74%)		
	Is the method IFCC standardized or NGSP certified or both	8 (24%)		
10B.	Is your laboratory HbA1c method NGSP certified?	This question for Sri Lanka was asked in a different format. (See 10A)	n=9	n=77 [4 labs did not respond to this question]
	Yes		9 (100%)	66 (86%)
	No		0 (0%)	11 (14%)
11A.	Participation in an EQA programme (labs may choose more than 1 answer)	n=34	n=9 [One lab did not do urine albumin]	This question for Philippines was asked in a different format. (See 11B)
	Plasma glucose	30 (88%)	9 (100%)	
	HbA1c	13 (38%)	9 (100%)	
	Urine albumin	7 (21%)	8 (89%)	
	Did not participate in EQA for all of the above analytes	3 (9%)	0 (0%)	
11B.	Is your lab participating in a PT program for Glucose and HbA1c?	This question for Sri Lanka and Singapore was asked in a different format. (See 11A)		n=76
				[5 labs did not respond to this question]
	Glucose Only			33 (43%)
	HbA1c Only			2 (3%)
	Both			29 (38%)
	Neither			12 (16%)
12A.	Type of Laboratory	n=34	See 12B	See 12C
		[One lab did not respond to this question]		
	Teaching Hospital	7 (21%)		
	Provincial General Hospital	1 (3%)		
	District General Hospital	3 (9%)		
	Base Hospital	0 (0%)		
	Private Hospital	16 (47%)		
	Private Stand-Alone	4 (12%)		
	University	2 (6%)		
	Research	0 (0%)		
	Special Children Hospital	1 (3%)		

Questions		Sri Lanka	Singapore	Philippines
12B.	Type of Laboratory	See 12A	n=9	See 12C
	Private		2 (22%)	
	Public		7 (78%)	
12C.	Type of Laboratory	See 12A	See 12B	n=76 [5 labs did not respond to this question]
	Public Hospital			18 (24%)
	Private Hospital			33 (43%)
	Public Stand-Alone			5 (7%)
	Private Stand-Alone			15 (20%)
	University/Academic			4 (5%)
	Research			1 (1%)

Reporting units for HbA1c and glucose

For plasma glucose, 63% Sri Lankan laboratories reported in mg/dL while 78% laboratories in Singapore reported in mmol/L. In the Philippines, 53% of laboratories reported in mmol/L; 41% reported in mg/dL; and 6% reported in both units. Dual reporting of units is observed in Sri Lanka (5%) and Singapore (11%) as well.

For HbA1c, 59% of Sri Lankan laboratories reported solely in National Glycohemoglobin Standardisation Program (NGSP) units [%] compared to 33% in Singapore. Six percent of Sri Lankan laboratories but none of the Singapore laboratories reported solely in International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) unit [mmol/mol]. Dual reporting in NGSP and IFCC units is common in Singapore, where 67% of laboratories practise dual reporting as compared to 34% in Sri Lanka.

Diagnostic cut-off for diabetes

Overall, the majority of survey participants; 74% (89 out of 120) utilized the diagnostic cut-off of ≥ 6.5% (48 mmol/mol) for HbA1c. Of the laboratories surveyed, 53%, 22% and 90% participants indicated a diagnostic cut-off of 6.5% for Sri Lanka, Singapore and the Philippines respectively. Of note, one laboratory in Singapore reported a cut-off of ≥7.0% (53 mmol/mol].

Diagnostic threshold for fasting plasma glucose of ≥7.0 mmol/L (126 mg/dL) were reported by 59%, 89% and 62% of the laboratories in Sri Lanka, Singapore and the Philippines respectively.

The diagnostic cut-off of ≥11.1 mmol/L (200 mg/dL) for post two-hour plasma glucose in an OGTT was reported by 44%, 89% and 42% of the laboratories in Sri Lanka, Singapore and Philippines respectively.

What is considered as adequate control of diabetes?

All participants in Philippines reported HbA1c of <7% [53 mmol/mol] as adequate control. This contrasts with Singapore where only 33% reported <7% as adequate control; while one laboratory reported <6.5% [48 mmol/mol]. More than half of the laboratories in Singapore (56%) did not report HbA1c targets recommended for monitoring of DM. HbA1c targets for control was not assessed in the Sri Lankan survey.

Diabetes screening in pregnancy

Since, not all laboratories provide obstetrics services, only 34, 5 and 79 laboratories responded in Sri Lanka, Singapore and the Philippines respectively. Of the participant laboratories that responded, 71%, 40% and 85% stated that an OGTT is recommended for all pregnant women for Sri Lanka, Singapore and the Philippines respectively.

Urine albumin testing

Fifty six percent, 75% and 69% laboratories recommended early morning spot urine in Sri Lanka, Singapore and the Philippines respectively. Random spot urine is recommended by 28%, and 13% of the laboratories in Sri Lanka and Singapore. The laboratories in the Philippines did not recommend random spot urine. Of the participants, 16%, 50% and 26% laboratories also recommended 24-hour urine albumin measurement in Sri Lanka, Singapore and the Philippines respectively.

For reporting units of spot albumin to creatinine ratio (ACR), it was equally divided between mg/mmol and mg/g, 41% each in Sri Lanka. Fifty percent laboratories in Singapore reported ACR in mg/mmol, 13% reported in mg/g; 38% practise dual reporting. This question was not addressed in the Philippines survey.

Methods for HbA1c and glucose

Traceability to certified reference materials in HbA1c was confirmed by 79% of Sri Lankan laboratories. All laboratories in Singapore and 86% of laboratories in the Philippines use NGSP certified methods. Among the participants in Sri Lanka, 94% declared traceability for the glucose assay. This was not checked in the surveys of the other two countries.

For the assay methodology of HbA1c, 56% of Singapore laboratories use immunoturbidimetry; 22% use enzymatic methods and 22% use high performance liquid chromatography (HPLC). Analytical methodology for HbA1c was not surveyed in the Philippines and Sri Lanka.

EQA Participation for HbA1c and glucose

Thirty eight percent, 100% and 41% of laboratories in Sri Lanka, Singapore and the Philippines respectively, participated in an External Quality Assurance (EQA) programme for HbA1c. The EQA participation rates for HbA1c is lower than that for plasma glucose, for which 88%, 100% and 82% of laboratories in Sri Lanka, Singapore and the Philippines participated respectively.

Types of laboratories

A diverse group of laboratories responded in Sri Lanka, comprising of teaching hospitals (21%), provincial general hospital (3%), district general hospitals (9%), private hospitals (47%), private stand-alone (12%), university (6%) and special children's hospital (3%). One participant did not indicate the type of laboratory.

In Singapore, 78% public laboratories and 22% private laboratories responded.

For the Philippines, the participant laboratories comprised of public hospitals (24%), private hospitals (43%), public stand-alone (7%), private stand-alone (20%), university (5%) and research (1%) respectively. Five respondents from Philippines did not indicate the type of laboratory.

Discussion

Both conventional (mg/dL) and SI units (mmol/L) are used for reporting of glucose results. American journals express glucose in mg/dL while European journals express in mmol/L, reflecting the practice in the respective country and the region. We do not expect confusion over the use of mg/dL or mmol/L as the conversion factor [18 mg/dL = 1 mmol/L] is easily done by a phone calculator. However harmonized reporting in SI Units by all laboratories would make it easier for results interpretation for the end user. A system of dual reporting for a specified period of time, prior to full transition will facilitate this process.

While Europe and New Zealand have adopted sole reporting of HbA1c in mmol/mol, only 6% of laboratories in Sri Lanka, and none of the laboratories in Singapore reported solely in mmol/mol. Thirty four and 67% of laboratories in Sri Lanka and Singapore respectively reported both NGSP% and IFCC mmol/mol, in keeping with the 2007 Consensus statement endorsed by American Diabetes Association (ADA), European Association for Study of Diabetes (EASD), IFCC and International Diabetes Federation (IDF) that results be reported in both IFCC and NGSP units [7].

One advantage of IFCC over NGSP units is that numerical changes of IFCC units appear greater compared to the equivalent NGSP units. For example, a diabetic patient whose HbA1c is improved by NGSP 1% (e.g. from 8% to 7%) may perceive it as insignificant. However, in reality, each 1% HbA1c improvement is associated in relative risk reduction of 14% and 37% for myocardial infarction and microvascular complications respectively as reported in the UKPDS Study [8], and 26% relative reduction in major adverse cardiovascular events in a meta-regression of 18 randomized controlled trials [9]. Conversely, that same diabetic patient whose HbA1c is reported by a laboratory using IFCC unit would have seen the result reduced from 64 mmol/mol to 53 mmol/mol. The reduction in mmol/mol may be perceived to be more significant, may provide motivation for continued medication compliance and sustained lifestyle changes. Secondly, numerical values for NGSP units are similar to values of plasma glucose concentration when expressed in mmol/L which may lead to confusion for some patients. Thirdly, IFCC units are scientifically valid, traceable to SI units and accurately indicate the amount of HbA1c. NGSP units are directly related to clinical outcomes in DCCT and UKPDS trials [10]. To mitigate the confusion, and provide time for adjustment, some countries offered dual reporting for a transitional period of two years before implementing the sole IFCC units [11].

One-third of laboratories in Singapore, and 89% in Philippines offer HbA1c as a diagnostic test. In Singapore, only 33% of laboratories following a diagnostic cut-off for HbA1c could be explained by the relatively late official adoption of HbA1c for diagnosis in 2019 [12]. The late adoption in Singapore was due to the concerns over high prevalence of beta-thalassemia trait and haemoglobin E variant in the local population [13]. The Singapore Ministry of Health (MOH) Circular 08/2019, [12] recommended a HbA1c diagnostic cut-off of ≥7% (53 mmol/mol), based on a local study [14] while patients with HbA1c of 6.1- 6.9% (43 – 52 mmol/mol) shall proceed to test for fasting glucose or 75 g OGTT.

In 2010, the ADA adopted the diagnostic cut-off of HbA1c ≥ 6.5% (48 mmol/mol). Although ADA acknowledges that HbA1c cut-off of ≥6.5% may identify one-third fewer cases of undiagnosed diabetes than a fasting glucose cut-off of ≥ 7.0 mmol/l (126 mg/dL) based on USA National Health and Nutrition Examination Survey (NHANES) data, it notes that a wider application of a more convenient test (HbA1c) will actually increase the number of diagnoses made [15]. In 2011, the World Health Organisation (WHO) also adopted HbA1c of ≥6.5% as the recommended cut-off for diagnosing diabetes [16]. In this regard, Philippines laboratories with 90% of the laboratories indicating the use of HbA1c, demonstrate concordance with international guidelines. There is significant difference in cost per test for plasma glucose and HbA1c, with the former being more cost effective. This may explain the reason for only 50% of laboratories offering HbA1c as a diagnostic test in Sri Lanka.

For monitoring of diabetes, 56% laboratories in Singapore did not report a recommended target, while 100% laboratories in the Philippines recommended a monitoring target of 7%. This may be attributed to the differences in demographics between Singapore and Philippines. In 2022, 15% and 5% of the population is older

than 65 years in Singapore and Philippines respectively [17, 18]. While the HbA1c monitoring target for non-pregnant adults is ≥7%, older adults may benefit from less intensive glycaemic control [19]. The Action to Control Cardiovascular Risk in Diabetes Study Group (ACCORD) randomized 10,251 patients with mean age of 62.2, and showed that intensive glycaemic control does not significantly reduce major cardiovascular events and may increase mortality [20]. ADA recommends an individualised target of 7.5% to 8.5% (58 to 69 mmol/mol) for older adult based on functional status, cognitive impairment and comorbidities taking into account the risk of hypoglycaemia, fall risk and treatment burden [21]. The need for individualised target for elderly patients may dissuade Singaporean laboratories from following a HbA1c monitoring target.

Seventy one percent laboratories in Sri Lanka and 85% in Philippines recommend OGTT for pregnant women. This contrasts with Singapore, where only 40% of laboratories that served obstetrics patients recommend testing at 24 -28 weeks of gestation, despite all of them using International Association of the Diabetes and Pregnancy Study Groups (IADPSG) criteria for diagnosis of GDM. Variation in practice of GDM screening is not unexpected. For example, United Kingdom National Institute of Clinical Care Excellence (NICE) does not recommend antenatal screening in the absence of risk factors, such as BMI above 30 kg/m2, previous macrosomic baby weighing 4.5 kg or more, and family history of diabetes with or without an ethnicity with a high prevalence of diabetes [22]. Globally the most popular diagnostic criteria are the IADPSG criteria, with cut-offs after a 75 g OGTT of ≥5.1, ≥10.0 and ≥8.5 mmol/L (≥92, ≥180, ≥153 mg/dL) at 0, 1 and 2 hours respectively. These cut-offs were derived from the Hyperglycaemia and Adverse Pregnancy Outcome Study (HAPO) and represent the glucose values at which the odds for birth weight > 90th percentile, cord C-peptide > 90th percentile, and neonatal percent body fat >90th percentile reached 1.75 times the odds of these outcomes at the mean glucose values, based on a fully adjusted logistic regression [23]. IADPSG criteria was noted to increase prevalence of gestational diabetes [24], but may present opportunities for intensive treatment. A Clinical Practice Guideline jointly issued by the Ceylon College of Physicians and Sri Lanka College of Endocrinologists in 2018 endorses universal screening for gestational diabetes at the booking visit based on the IADPSG criteria [25]. In 2022, the Singapore Ministry of Health Agency of Care Effectiveness updated a care guide recommending universal screening at 24 to 28 weeks of gestation using the IADPSG criteria [26]. This updated care guide was timely as only 40% of the Singapore laboratories that served obstetrics patients recommended universal screening prior to the update. In the Philippines, there is no consensus related to the interpretation of results; in addition to the cut-offs recommended by the Philippine Obstetrical and Gynaecological Society, WHO, IADPSG and ADA criteria are also being utilized for interpretation [27]. In view of the wide variation in practice, we recommend professional societies such as IFCC and APFCB to formulate uniform guidelines in consultation with the professional colleges of obstetrics and gynaecology to unify screening strategies for GDM.

Majority of the laboratories in all three countries recommend early morning spot urine or random spot urine for albuminuria, concordant with major professional guidelines [28,29]. The convenience of morning/random spot sampling will enable more patients to be tested, resulting in a higher detection rate. Since effective treatment for albuminuria exists [Angiotensin Converting Enzymes (ACE) inhibitors, Angiotensin Receptor Blocker (ARB) or sodium-glucose cotransporter 2 (SGLT2) inhibitors], early detection and initiation of these drugs will improve renal outcomes [30,31,32].

We note that 78% of laboratories in Singapore currently use immunoturbidimetric assay or enzymatic method for glycated haemoglobin. Only 22% laboratories use HPLC. By automating HbA1c immunoassay or enzymatic method, the requirement to operate a separate instrument is obviated which reduces the manpower requirements and enhances round the clock reporting [33]. Non-HPLC or non-capillary electrophoresis methods (E.g. Immunoassay, enzymatic,) may limit the ability of the laboratory to discern haemoglobin variants [34]. However, HPLC and other chromatographic techniques are also often susceptible to interferences [35,36,37].

While EQA participation rate is high for all participants for glucose, only 38% and 41% of laboratories in Sri Lanka, and the Philippines respectively participated in EQA for HbA1c which may be attributed to high cost and scarce availability of HbA1c EQA programmes.

Our study has several limitations. It has a relatively low response rate of 40% for the Philippines. However, the absolute number of 80, should be representative of the laboratories in Philippines. Validity of our study is contingent on the accuracy of response by the participant laboratories. We did not verify the accuracy of the participant response independently.

The literature reveals wide variation in the cut-offs used for the interpretation of the OGTT in diagnosing GDM in the Philippines [27]. However, we did not verify this observation by a pertinent question related to plasma glucose thresholds in the Philippines survey, which is a limitation.

While we surveyed the reporting units of albuminuria, we did not collate information on the criteria laboratories used to assign albuminuria to different categories. There are various criteria for albuminuria/microalbuminuria. For example, KDIGO guidelines classify spot albuminuria stages into A1 (<30 mg/g or 3 mg/mmol), A2 (30-300 mg/g or 3-30 mg/mmol), and A3 (>300 mg/g or >30 mg/mmol) [38]. In the literature, other guidelines define microalbuminuria as an albumin: creatinine ratio of 2.5 - 25 mg/mmol for men and 3.5 – 35 mg/mmol for women [39]. While we explored the reporting units for albuminuria in question 8, we did not however specify the criteria to assign microalbuminuria.

Our strength includes a diverse group of laboratories (private, public and university) being surveyed in each country, providing a unique perspective of three tropical island-nations in Asia.

Conclusion

Based on the above survey, there are differences in practice across laboratories in the three countries, Sri Lanka, Singapore and the Philippines. Lack of harmonization is evident in reporting units, diabetes monitoring targets, gestational diabetes screening, albuminuria testing and EQA participation. The widespread adoption of SI unit as the sole reporting unit, especially the use of IFCC unit mmol/mol for HbA1c remains difficult for Sri Lanka and Singapore. Scientific bodies and professional associations have an important role in harmonization of laboratory testing related to diabetes. They need to encourage all laboratories to include the use of spot urine albumin to creatinine ratio for albuminuria testing given the convenience, improved access, and actionable renal outcomes through the use of ACE inhibitor, ARB and SGLT2 inhibitor drugs. Overall, the rates for EQA participation for glucose is commendable but can be improved for HbA1c.

Conflicts of interest

Authors have no conflicts of interest to declare.

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Research Article

Revolutionizing DNA Extraction: A Cost-effective Approach for Genomic DNA Retrieval from Dried Blood Spots

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Keywords

Dried blood spots, DNA extraction, CTRC, GAPDH, Polymerase chain reaction

Abstract

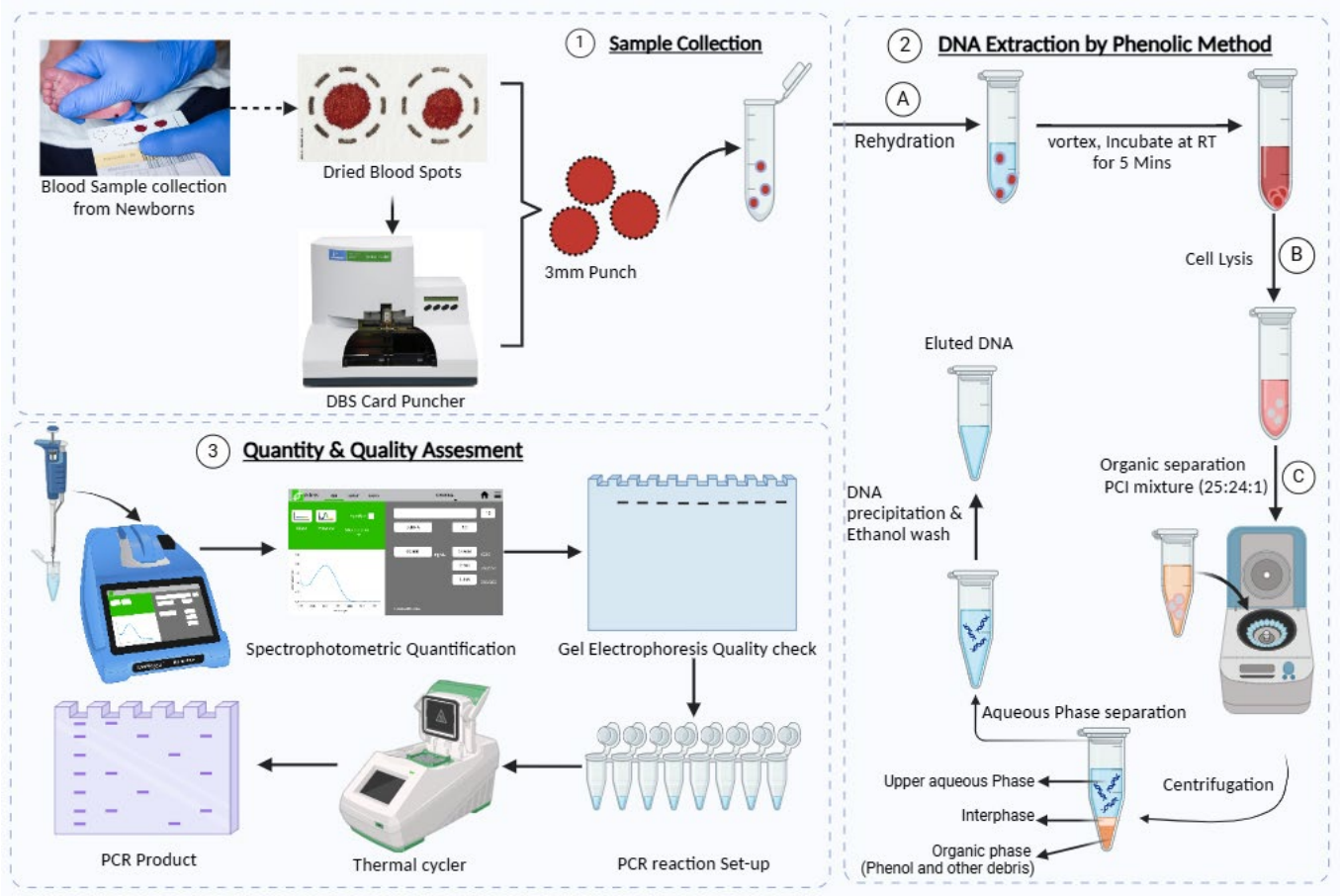
Background: This study introduces an efficient, cost-effective laboratory- derived method for extracting genomic DNA from dried blood spots (DBS) by optimizing the organic separation phenol method.

Methodology: DBS samples, collected via heel prick from 50 neonates as a part of routine newborn screening, were processed using an optimized phenol method that employs lysis buffers with minimal concentrations of proteinase K and phenol:chloroform:isoamyl alcohol (PCI) reagent.

Results: The extracted genomic DNA exhibited a concentration range of 50 to 200ng/µl, with purity levels (A260/280) falling within the range of 1.4 to 1.6, as measured by nanodrop. Gel electrophoresis, post polymerase chain reaction (PCR) amplification, consistently revealed distinct, non-degraded bands for both a 345-bp fragment (Chymotrypsin C, CTRC gene) and a 250-bp fragment (Glyceraldehyde-3-phosphate dehydrogenase, GAPDH gene) across all samples.

Conclusion: The method utilizes routine consumables readily available in basic molecular biology laboratories, circumventing the need for expensive kits. It holds significant promise for genetic diagnostics and research applications, particularly in situations where DBS serves as a means of collecting and preserving samples from individuals in remote areas.

Graphical abstract



Highlights

- Refined phenol-based method that offers cost-effective means of extracting genomic DNA from dried blood spots on filter paper.
- Key attributes of this approach include its simplicity and use of PCI (25:24:1) reagent for superior DNA yield.
- A reliable choice that is economically advantageous for further molecular investigations involving DBS specimens.

Introduction

Molecular diagnostic tests, such as polymerase chain reaction (PCR), restriction enzymatic digestion, and recombinant DNA selection are essential tools in contemporary research and clinical practice [1]. The reliability and accuracy of these tests hinge upon the quality of the starting material: the genomic DNA. Traditionally, whole blood has been the preferred source for DNA extraction. However, this method is not without its challenges, including the need for stringent storage conditions, transportation precautions, and the risk of spillage. In contrast, dried blood spots (DBS) offer a convenient alternative. They are easy to collect, simple to store without the need for specialized facilities, cost-effective to prepare, and do not require the separation of serum and plasma. Moreover, DBS can be treated as non-infectious samples, as most viruses and microorganisms are rendered inactive during the drying process. Their transportability, even from remote areas, makes DBS an optimal choice for sample collection [2]. Furthermore, DBS resolves the issue of obtaining venous blood samples, which can be challenging, especially in neonates.

The adoption of DBS for clinical and research purposes has a notable history, dating back to Dr. Robert Guthrie's pioneering use of DBS samples in 1963 to analyze phenylalanine levels in infants, aiding in the identification of phenylketonuria. Since then, DBS has found diverse applications in disease screening [3,4], drug monitoring [5], genetic analyses [6], and epidemiological studies, particularly in resource-constrained settings with suboptimal storage and transportation conditions. Notably, DBS has been instrumental in DNA methylome analysis [7] and the identification of congenital cytomegalovirus (CMV) infections [8,9], which can lead to hearing impairments.

While alternative methods, such as salt extraction, exist for genomic DNA retrieval, they may not provide the high yields and purity necessary for low-DNA content samples such as DBS. . Methods relying on substances such as Chelex [10-12] and InstaGene Matrix [13-16], though effective, may not be the most cost-efficient solutions. Commercial kits, including QIAamp DNA Mini Kit [16-18], provide convenience but can be expensive and yield low recovery rates. Additionally, when collecting blood samples on filter paper, limitations arise from the marginal amount of DNA obtained from a single blood drop, further compounded by the presence of cellulose fibers from the filter paper itself.

In light of these considerations, we have developed a simple yet highly efficient method for genomic DNA extraction from DBS. Built upon the foundational phenolic method, our approach has been meticulously optimized to ensure both quality and quantity. The phenol extraction method, though more complex, was chosen due to its higher efficacy in removing protein and lipids, yielding higher-quality DNA suitable for downstream applications like PCR. We have assessed the integrity of the extracted DNA through PCR amplification, confirming its suitability for downstream molecular analyses. In this study, we present our methodology and results, demonstrating the robustness of our optimized phenol-based DBS DNA extraction method. To validate our approach, we utilized DBS samples collected from newborns as a part of routine screening. We believe that our streamlined method addresses several of the limitations associated with DNA extraction from DBS and provides a valuable tool for researchers and clinicians alike.

Materials and Methods

Ethics

The Institutional Ethical Committee approval was obtained for the present study (Ref. No: JSS/MC/PG/1098/2021- 22 dated 22/04/2021)

Collection and storage of samples

The DBS were collected from 50 newborns within 48 to 72 hours of birth using the heel prick method on Whatman 903 filter paper as a part of routine newborn screening. Subsequently, these DBS samples were stored in a clean box at 4°C following the biochemical screening and were later utilized for DNA extraction. Additionally, whole blood was collected from normal healthy individuals using an EDTA vacutainer, which served as a positive control.

DBS DNA extraction

1. DBS Puncher *(DBS Puncher®, Revvity)* was used to punch out three 3mm diameter circles from the DBS samples into a 2 ml microcentrifuge tube *(QSP 2.0 ml MCT tube, Thermo Fisher Scientific)*.
2. The DBS spots were rehydrated by adding 300µl of solution A [155mM NH4Cl (SRL), 0.1mM EDTA (SRL), 1mM KHCO3 (SRL)] with 2 minutes of vigorous vortex (DLAB). This mixture was then incubated at room temperature for 5 minutes and the subsequent removal of supernatant using a pipette after a short spin in a mini centrifuge *(D1008E Mini centrifuge, DLAB)* for 30 seconds.
3. To the remaining mixture, 210µl of solution B [200µl of lysis buffer (20mM Tris *(SRL)*, 40mM EDTA *(SRL)*, 300mM NaCl *(SRL)*, 2.5%SDS *(SRL)*), 10µl of Proteinase K (20mg/ml) *(HiMedia)*] was added followed by invert mix 4-5 times and incubated at 56°C for 30 minutes.
4. The Genomic DNA was then extracted from the mixture by adding 300µl of phenol:chloroform:isoamyl alcohol mixture (25:24:1) *(SRL)* and gently invert mix the tube 5-6 times. The mixture was centrifuged *(Refrigerated Centrifuge, Hettich)* at 10000rpm for 5 minutes at room temperature and the obtained aqueous phase was transferred to a fresh 1.5 ml microcentrifuge tube *(QSP 1.5 ml MCT tube, Thermo Fisher Scientific)*.
5. The nucleic acid was recovered by standard precipitation, 40µl of 1.5M sodium acetate (pH 5.2) *(HiMedia)*, an equal volume of ice-cold isopropyl alcohol *(SRL)*, mixed well and centrifuged at 12000 rpm for 15 minutes at 4°C.
6. The clear supernatant was discarded, and the pellet was washed with 500µl of 70% ice-cold ethanol *(DNA Diluent, HiMedia)* two times. The obtained pellet was dried at room temperature until there was no trace of ethanol, and the genomic DNA was resuspended in 25µl of 1X-TE *(SRL)* buffer by gentle finger flicking and stored at -20°C *(Dixell, Antech Instruments)*.

The use of chloroform and isoamyl alcohol in conjunction with phenol ensured proper separation of the aqueous (DNA-containing) phase from the organic phase. Chloroform aids the extraction of proteins and lipids, pulling them into the organic phase, while isoamyl alcohol minimizes foaming, enhancing the efficiency of phase separation. This solvent combination helps reduce phenolic contamination in the aqueous phase, which contains the DNA. Residual phenol and other contaminants were removed by washing the extracted DNA with 70% ethanol.

DBS DNA extraction by the Kit method

DNA extraction from the DBS samples was also carried out using a magnetic bead-based kit, Mag genome dried blood spot DNA extraction kit (Xpress DNA Dried blood spots, MagGenome Technologies Pvt Ltd. India) following the manufacturer's protocol.

Table 1 clearly compares and contrasts the method optimised in this study with the previously established methods [19,20].

Table 1: Comparison of Phenol baseline protocol and optimized phenol protocol for DBS DNA extraction.

Phenol baseline protocol	Optimized phenol protocol
Rehydration	
300µl of rehydration solution, vortex for 1-2 minutes & incubation at R.T for 5 mins.	300µl of rehydration solution, vortex for 2 minutes & incubation at R.T. for 5 mins.
Protein digestion	
160µl of double distilled H2O,	***None***
2X Proteinase K lysis buffer containing 20mg/ml, vortex for 20 seconds.	200µl of lysis buffer, 10 µl of Proteinase-K (20mg/ml), ***invert mix 4-5 times.***
Incubate at 56^{o}C for 1hr (with intermittent vortex for 10 seconds).	incubate at ***65^{o}C for 30 mins.***
Genomic DNA extraction	
200µl of buffered phenol and 200 µl of chloroform and isoamyl alcohol mixture (24:1), with subsequent vortex for 30 seconds. The mixture is centrifuged at 10,000 rpm for 4 minutes at room temperature.	***250µl of phenol: chloroform: isoamylalcohol (25:24:1) reagent and gently invert mix the tube 5-6 times.*** The mixture was centrifuged at 10,000 rpm for 5 minutes at room temperature.
DNA precipitation	
To the aqueous phase, 40µl of 3.0 M sodium acetate (pH-5.2) and 400µl isopropyl alcohol is added, mixed, and centrifuged at 10,000 rpm for 4mins at R.T. The supernatant is removed, and the pellet is washed with 70% ethanol, air dried & dissolved in 50µl of 1X TE buffer.	To the aqueous phase, add 40µl of 3.0 M sodium acetate (pH-5.2) and an ***equal volume of isopropanol.*** The solution was centrifuged at ***12,000 rpm for 15mins at 4^{o}C.*** The supernatant was removed, and the pellet was washed with 70% ethanol, air-dried & dissolved in ***25µl of 1X TE buffer.***

Estimation of DNA concentration and quality

The concentration of the extracted DNA samples was determined using a Nanodrop spectrophotometer (DeNovix 11). The absolute concentration was calculated by measuring the absorbance at 260nm (A260), with an assumption that an A260 of 1.0 corresponds to 50ng/µl of DNA. Additionally, the DNA quality was assessed by determining the A260/280 ratio and performing gel electrophoresis to evaluate its integrity.

PCR amplification of *CTRC and GAPDH* genes

A PCR amplification was conducted to assess the quality of the extracted DNA, determined by the successful generation of PCR products visible as bands on an agarose gel. For this purpose, a pair of endogenous control primers were synthesized (Bioserve Biotechnologies, India Pvt. Ltd) (Table 2). These primers targeted the *CTRC* and *GAPDH* genes and were employed to assess the suitability of extracted DNA from dried blood spots. The *CTRC* primer set yielded a 345-bp PCR product, while the *GAPDH* primer set produced a 250-bp PCR product, both of which were visualized on an agarose gel.

Table 2: The primers sequence of an endogenous control gene.

Sl.No.	Gene	Primer sequence
1	*CTRC* Forward primer	5'AAGGACAATGGGAACACTCTCT3'
2	*CTRC* Reverse primer	5'TCAGGTATGGGGTGCGACAG3'
3	*GAPDH* Forward primer	5'CCACTCCTCCACCTTTGACG3'
4	*GAPDH* Reverse primer	5'CCACCACCCTGTTGCTGTAG3'

The PCR reactions were conducted using the Takara Bio Inc. Thermocycler machine. Each reaction had a total volume of 20µl, comprising 1µl of genomic DNA (50ng), 10pmol of each primer, 10mM of dNTPs (TaKaRa), 0.75unit of Taq DNA Polymerase (TaKaRa), 1X buffer with 1.5mM MgCl2 (TaKaRa). For positive control, 50ng of the extracted genomic DNA from whole blood collected in an EDTA vacutainer was used, without the need for an enhancer and additional MgCl2. The PCR cycling conditions consisted of an initial denaturation at 94°C for 5 minutes, followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 59°C for 30 seconds, extension at 72°C for 30 seconds, and a final extension at 72°C for 5 minutes. Approximately 5µl of the obtained PCR product was subjected to electrophoresis on a 2% agarose (HiMedia) gel stained with ethidium bromide (HiMedia). Electrophoresis was conducted in Tris-acetate-EDTA buffer (TAE) (HiMedia) and the resulting bands were compared to a 100bp DNA ladder (Gene Direx).

Results

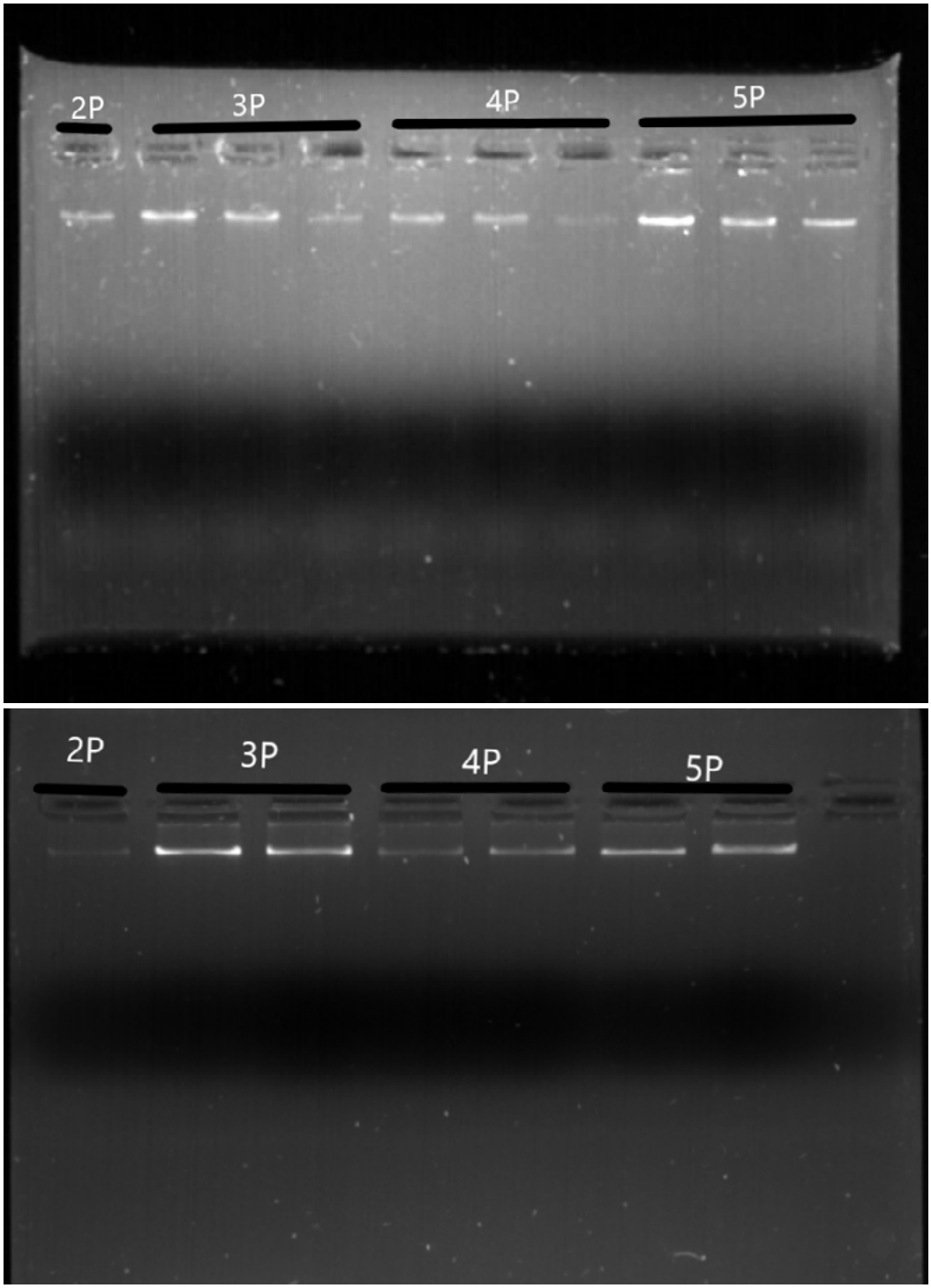
Comparison of DBS DNA isolation methods (Quantity and Quality)

The genomic DNA isolated from DBS samples using the optimized phenol method exhibited superior quality compared to the kit-based method (Table 3). The concentration of DNA ranged between 50-200ng/µl and the purity (A260/280) ranged from 1.4 to1.6 (Table 3, Fig.1). The integrity of DNA was assessed by resolving it in a 1% agarose gel electrophoresis, stained with ethidium bromide. In all DBS samples isolated using the phenol method, a distinct, bright band without any degradation was consistently observed (Figure 1).

Table 3: The genomic DNA concentration of Dried blood spot samples measured by DeNovix DS-11 Spectrophotometer.

DBS DNA Samples – Optimized Phenolic Method					DBS DNA Samples – Kit Method				
Sl.No.	Concentration (ng/µl)	Absorbance A260	A260/280 ratio	A230/260 ratio	**Sl.No.**	Concentration (ng/µl)	Absorbance A260	A260/280 ratio	A230/260 ratio
1	92.91	1.858	1.47	1.07	**1**	48.48	0.977	1.30	0.82
2	127.73	1.554	1.63	1.39	**2**	50.03	1.000	1.40	0.78
3	167.00	1.340	1.45	1.05	**3**	51.75	1.035	1.42	0.77
4	193.82	1.028	1.45	1.47	**4**	54.66	1.093	1.39	0.72

Figure 1: Representative of 1% Agarose Gel Electrophoresis of isolated DBS DNA samples by manual Phenolic method and the Xpress DBS DNA MagGenome Kit method.

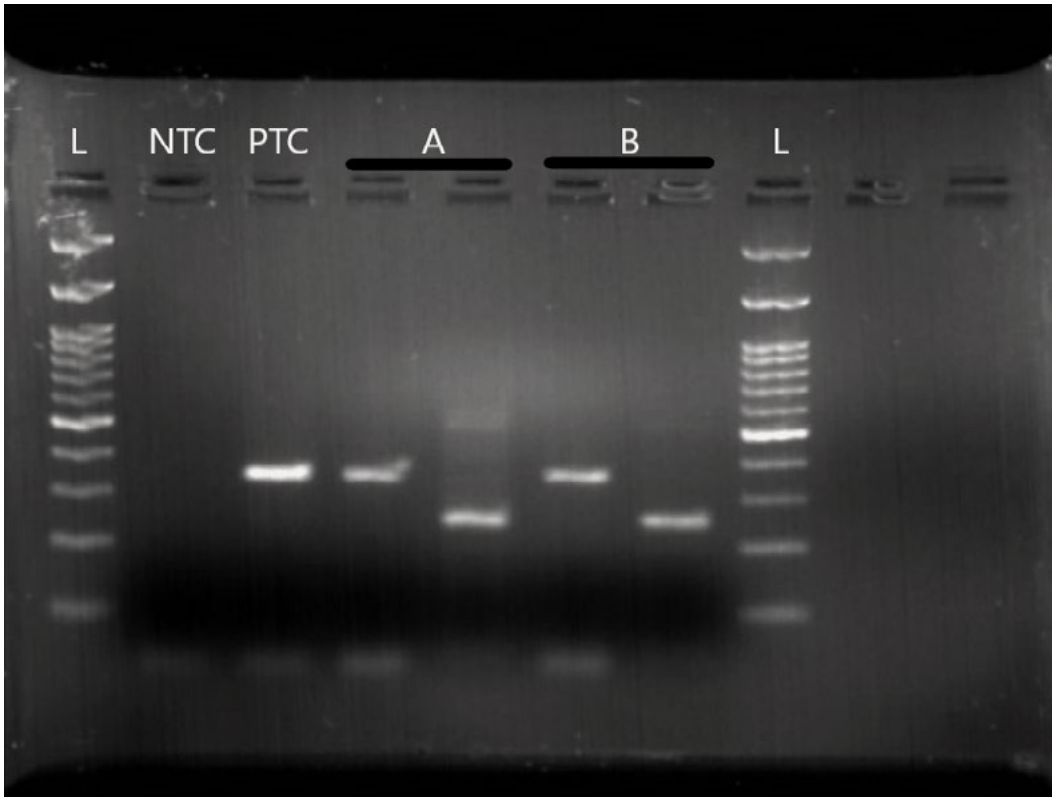


A (optimized Phenolic method)- Lane 1: 2punches, Lane 2-4: 3punches, Lane 5-7: 4punches, Lane 8-10: 5punches of DBS (3mm).; B (Kit method) Lane 1: 2punches, Lane 2-3: 3punches, Lane 4-5: 4punches, Lane 5-6: 5punches of DBS (3mm). Distinct bright bands without any degradation were observed in all the samples.

PCR Product Gel-Electrophoresis

To assess the presence of PCR inhibitors in the genomic DNA obtained from DBS samples, we subjected the isolated DNA to PCR amplification targeting the *CTRC & GAPDH* endogenous genes. As a positive control, we used genomic DNA extracted from the whole blood of a normal healthy human. The results demonstrated the presence of PCR products of the expected sizes: a 345-bp fragment of the *CTRC* gene and a 250-bp fragment of the *GAPDH* gene in all the DBS-extracted DNA samples by both kit and manual methods. Notably, no bands were observed in the negative control and positive control showed *CTRC & GAPDH* gene amplification (Figure 2).

Figure 2: Representative of 2% Agarose Gel Electrophoresis of PCR products of two different targeted genes.



Lane 1-DNA Ladder (100bp); Lane 2-Negative control; Lane 3-Positive control; A: Kit method Lane 4-CTRC gene (345bp), Lane 5-GAPDH gene (250bp); B: Manual method Lane 6-CTRC gene, Lane 7-GAPDH gene.

Discussion

This study aimed to develop a cost-effective in-house protocol for extracting genomic DNA from the dried blood samples collected on Whatman 903 filter paper. DBS sampling is most frequently used to assess small-molecule analytes in newborn screening for metabolic disorders, epidemiological studies, and biobanking due to its ease of collection, transportation, and long-term storage stability in clinical laboratories [21,22]. Given the historical reliance on DBS for over a century and its use in metabolic disease screening for the past five decades, it is crucial to optimize DNA extraction methods that are both efficient and economically viable [23].

Previous studies have explored various DNA extraction methods from DBS, emphasizing the need for methods that ensure high purity and yield while minimizing cost and complexity. For instance, Kumar et al. evaluated the efficacy of column-based and magnetic bead-based methods. While these methods provide sufficient DNA for genetic studies, they were associated with significant drawbacks, including a 5-10% loss of magnetic beads and consequently, genomic DNA [24]. Such losses are particularly problematic when working with limited samples or in resource-limited settings. Storm and colleagues compared commercially available kits, including the Chelex-100, QIAamp DNA Mini Kit, and InstaGene Matrix, with a TE buffer-based method. Although Chelex-100 showed fewer PCR inhibitors and was effective in chelating polyvalent metal ions, the cost and availability of these kits, particularly in remote areas, present significant challenges [25]. Moreover, methods such as the QIAamp DNA Mini Kit, despite being widely used, still involve multiple steps that could introduce variability and reduce DNA recovery, particularly when combined with the inherent variability of DBS samples [25]. Ghantous et al. further highlighted the variability in DNA yield across different methods, noting that even optimized protocols, such as the combination of GenSolve and Qiagen kits, resulted in DNA yields ranging from 3-40 ng/µl [7]. Such variability is concerning, especially for applications requiring high DNA concentrations or when working with samples stored over extended periods, where the integrity of the DNA may be compromised.

Similarly, Barsa Baisalini Panda and colleagues explored several extraction methods, including Methonol-based and PBS-based methods, in addition to Chelex-100 and TE buffer-based protocols. Methanol-based extraction, while cost-effective due to the low price of methanol, involves time-consuming steps with repeated drying and overnight incubation. The PBS-based method, which is similar to the conventional phenol-chloroform extraction technique, also requires prolonged overnight treatment with lysis buffer and proteinase K. While the Chelex-100 method is rapid, its high cost and limited availability in remote areas make it less feasible in certain settings. Importantly, the PCR

results using the optimized phenolic method were comparable to those obtained with commercial kits, with the phenolic method being particularly advantageous for large sample sizes due to its cost-effectiveness, approximately one-third of the cost of kit-based methods [26].

Phenol-based methods are often criticized for the potential risk of contamination, our protocol minimizes this issue through the use of phenol: chloroform: isoamyl alcohol mixture. Chloroform facilitates the separation of proteins and other organic contaminants into the organic phase, while isoamyl alcohol prevents foaming and aids in more efficient phase separation. Together, these components ensure a clean separation of the DNA-containing aqueous phase, thereby reducing the risk of phenolic contamination. Furthermore, multiple ethanol wash steps were performed to remove any remaining phenol residues.

While non-toxic methods such as salt extraction offer simplicity, our optimized phenol: chloroform: isoamyl alcohol protocol demonstrates several advantages, particularly when working with DBS. Organic solvent extraction consistently yielded higher DNA concentrations (50-200 ng/µl) and improved purity compared to salt extraction, which may not perform as well with limited sample volumes such as those found in DBS. Additionally the phenol method effectively removes proteins and cellular debris, which can interfere with downstream applications, making it a more robust solution for these challenging sample types. Salt extraction, while non-toxic, is better suited for larger sample volumes where DNA yield is not as much of a constraint. The higher yield and purity obtained through our optimized method justify its use over salt extraction in this context, particularly for laboratories working with limited or low-volume samples like DBS. The optimized phenolic method demonstrated several advantages over commercial kits, including a significantly lower cost and fewer steps, which reduces the likelihood of human error. While phenol is a toxic reagent, its benefits in terms of yield and purity in genomic DNA extraction from challenging samples like DBS justify its use. Importantly, the method proved effective in amplifying targeted genes using conventional PCR technique. This suggests that the method is suitable for mutation studies, which require precise and reliable DNA amplification. Further refinement of non-toxic alternatives, such as salt extraction, may provide safer but equally effective methods in the future. However, at present, the phenol-based approach offers a significant advantage in terms of both quality and quantity of DNA retrieval.

Limitations

Despite its advantages, the phenolic method does present some limitations. The purity of the extracted DNA was lower compared to commercial kits, potentially due to the presence of cellulose fibres and phenol residues, which could interfere with downstream applications. Additionally, the uneven distribution of blood on the filter paper may result in variable DNA yields, a factor that warrants further investigation. The methods effectiveness across different storage conditions and durations also remains to be validated, which is critical for ensuring its applicability in diverse research and clinical settings.

Future Directions

Further studies should focus on optimizing the phenolic method further to enhance DNA purity, perhaps by incorporating additional purification steps to remove contaminants such as cellulose fibers. It is also essential to validate the method's performance in highly demanding diagnostic techniques, such as microarray analysis and next-generation sequencing (NGS), to confirm its broader applicability. Additionally, exploring the effects of storage conditions, such as temperature and duration, on DNA integrity and yield from DBS will provide critical insights into the methods robustness and reliability in biobanking and long-term epidemiological studies.

Conclusion

In conclusion, our DNA extraction method holds significant promise for genetic diagnostics and research applications, particularly in situations where DBS serves as a successful means of collecting and preserving samples from individuals in remote areas. While further optimization is required to address its limitations, the method provides a valuable solution in regions where access to freezers is limited. Moreover, DBS emerges as a potential sample source for biobanking in epidemiological studies.

Ethics statements

The study was approved by the Institutional Ethical Committee of JSS Medical College, Mysuru (India) via approval letter number JSS/MC/PG/1098/2021-22 dated 22/04/2021. Informed consent was obtained from the human subjects.

Author contributions

Akila Prashant: Conceptualization, Methodology, Writing-Reviewing and Editing. Haripriya S Kundapura: Writing-Original draft preparation, Investigation. Anju Srinivas: Method Validity tests, Data curation. Manju Hosuru Chikkalingaih & Anshu Kumar Yadav: Visualization, investigation. Prashant Vishwanath and Suma M. N: Supervision.

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Case report

A rare pure calcite urolithiasis confirmed by infrared spectroscopy

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Keywords

calcite, urolithiasis, kidney stone, infrared spectroscopy

This case report describes a 41-year-old woman with no significant medical history and a normal body mass index (BMI), who presented with ureterohydronephrosis due to a 5.5mm x 9mm calculus composed primarily of calcite ($CaCO_3$) at the ureterovesical junction. The kidney stone, associated with cystitis and perirenal fat infiltration, was spontaneously expelled and subsequently analyzed. Optical microscopy revealed a grey homogeneous stone with a rough surface and white crystals upon examination. Fourier-transform infrared spectroscopy (FTIR-ATR) confirmed the stone's composition as pure calcite, displaying characteristic absorption bands indicative of its crystalline structure. The patient reported long-term use of multiple vitamins and plant-based supplements, possibly contributing to stone formation. The discussion includes insights on calcite urolithiasis, highlighting factors such as alkaline urine pH and calcium metabolism that can influence stone formation, underscoring the complexity of managing kidney stone risk in supplement users.

Introduction

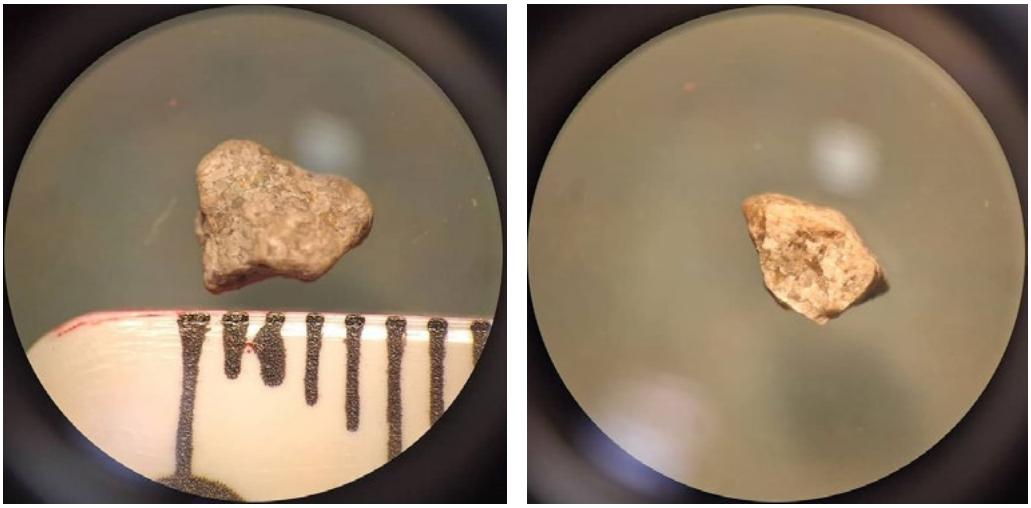
The majority of renal stones are of mixed composition. In humans, the most frequent stone components include calcium oxalate (whewellite [COM] and weddellite [COD]) and calcium phosphate (apatite and brushite) [1,2]. Calcite is rarely considered a true component of a kidney calculus, being present in only 0.01% to 0.25% of all stones, and usually mixed with other components. Calcite, the predominant form of natural calcium carbonate ($CaCO_3$), is renowned for its diverse crystal formations and widespread occurrence in nature. It exhibits polymorphism, with some specimens being nearly pure $CaCO_3$ while others contain varying proportions of additional cations such as Mg, Mn, Fe, B, Br, Sr, and/or Y substituting for calcium [3]. Metastable magnesian calcites, containing approximately 5 to 18% $MgCO_3$, are widespread in biogenic skeletal structures and serve as cement in some modern marine sediments. Calcite is a common earth mineral and the principal constituent in limestone and marble, common in human pancreatic and salivary lithiasis and in equine urolithiasis, but rare in human urolithiasis [4]. Calcite stones presented by patients as spontaneously passed are often artifactual or factitious. The aim of this case study is to outline an uncommon instance of a patient who exhibited a calcite urolithiasis confirmed by infrared spectroscopy.

Case report

A 41-year-old woman with no medical history, having a BMI of 24,91 (height: 170 cm, weight: 72 kg), is considered to be healthy weight. Blood samples tests revealed normal levels of calcium, and normal potassium, sodium, magnesium, phosphate, creatinine, and urea. The patient's history revealed that she took multiple vitamins during pregnancy and for more than three years afterward. She also took plant-based dietary supplements to promote vasodilation, aiming to enhance oxygen and nutrient delivery to the placenta and the developing foetus. The uro-CT scan shows the left kidney with dilation of all excretory cavities up to its terminal portion upstream of a 5.5mm x 9mm calculus (ureterohydronephrosis) with a density of 930 Hounsfield Units, lodged at the ureterovesical junction. It is associated with cystitis and a slight infiltration of the perirenal fat. The kidney stone is spontaneously expelled.

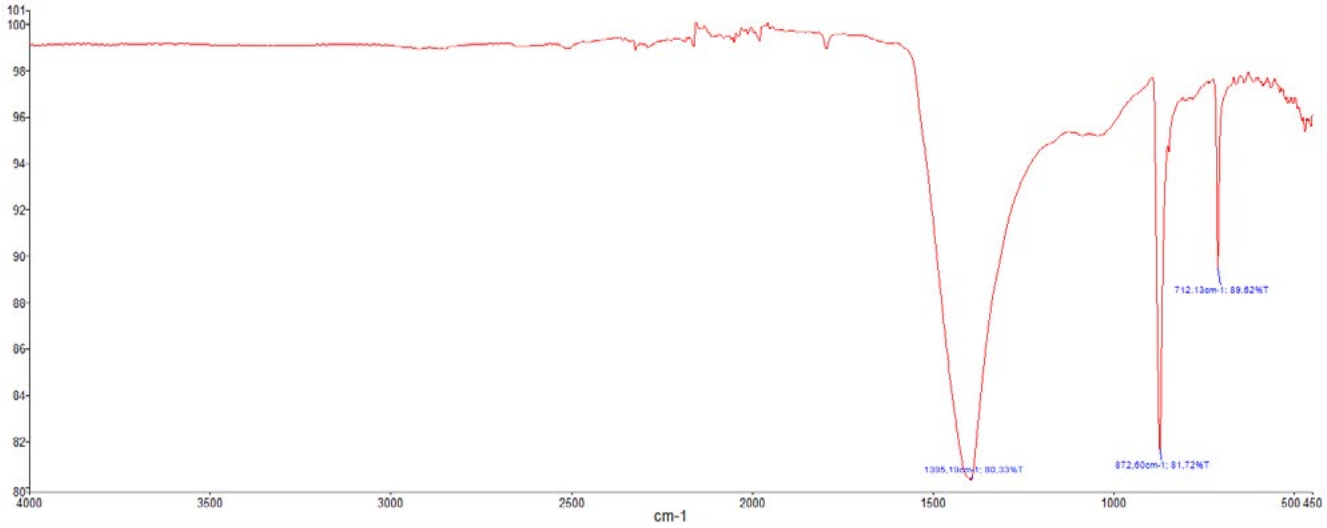
The stone weighed of 0,05g and was observed under an optical microscope (Gx10x40) to examine the morphology of the surface and section. The stone appeared grey and homogeneous with a rough surface containing white crystals. The cross-section was found to be crystalline, compact, and dense (Figure 1).

Figure 1: Surface and section of the calculi under optical microscope (10*40).



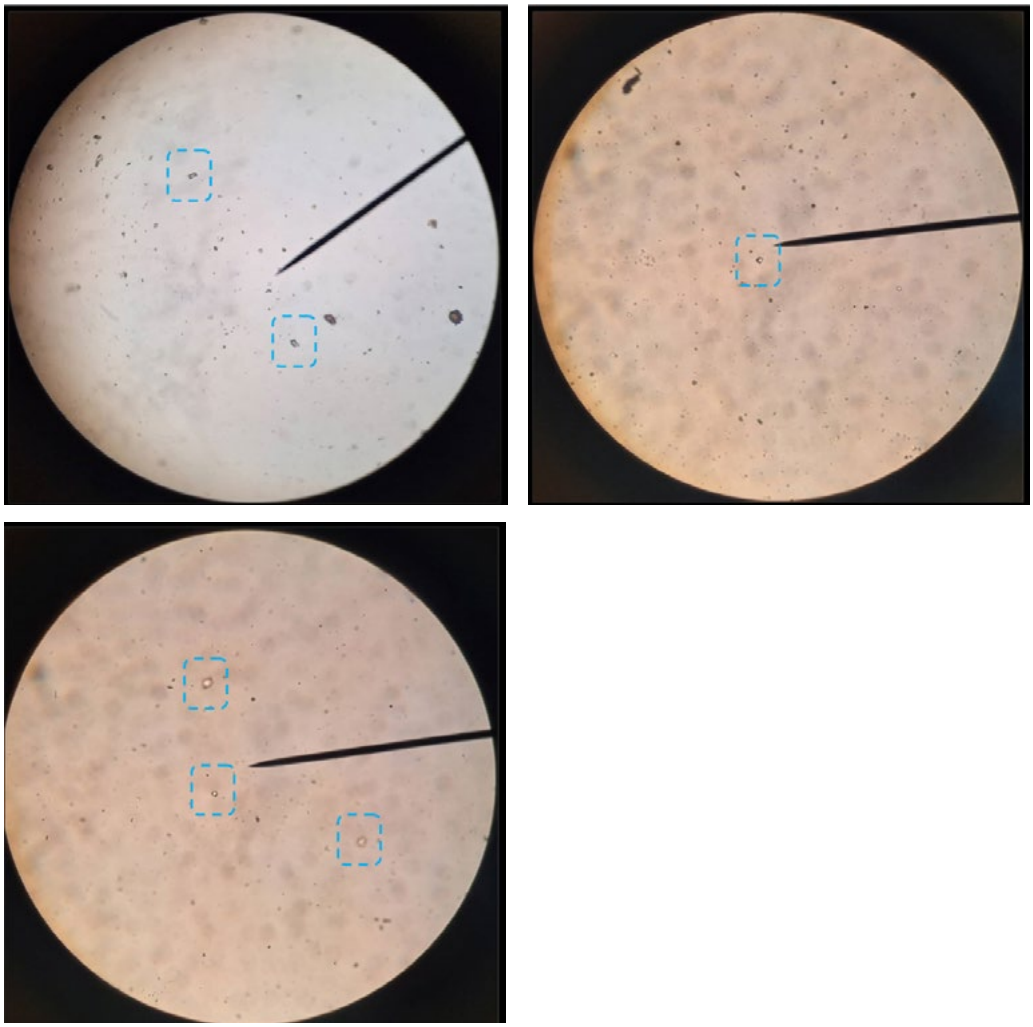
The infrared spectra were obtained using Fourier transform infrared spectroscopy coupled with attenuated total reflection (FTIR-ATR) (Perkin Elmer, Shelton, CT, USA) within the range of 450–4000 cm^{-1}. The absorption spectrum acquired is indicative of the molecular composition and potential crystalline structure of the sample (Figure 2). A strong absorption band was observed around 1395 cm^{-1}, corresponding to C=O stretching. Medium to strong absorption bands were noted near 872 cm^{-1} and 712 cm^{-1}, attributed to C-O stretching and CO_3 out-of-plane bending. Additionally, low-frequency bands below 700 cm^{1} were detected, associated with lattice vibrations of the calcite crystal structure. In summary, the precise positions and intensities of these bands confirm the stone's composition and purity as calcite, primarily composed of calcium carbonate ($CaCO_3$).

Figure 2: FTIR spectrums of the kidney stone.



Some of the calculi were dissolved in pure water and examined under a microscope to search for crystals (Figure 3). The crystals were observed as needle-like or prismatic structures, as well as envelope-shaped or elongated structures with pointed ends and a light brown color.

Figure 3: Crystals under optic microscope (G*100).



Discussion

Often, when calcite is found as a component of urinary calculi, it is considered false calculi or artifacts [5]. In this case, renal calculi were obtained following spontaneous elimination, after which a second computed tomography (CT) scan revealed left kidney pyelocaliceal cavity dilation and absence of the calculi. Similar to a case previously reported in a 42-year-old woman with anorexia since adolescence and a 5-year history of recurrent nephrolithiasis [6]. In that case, stone analysis from both sides using IRS revealed pure calcite ($CaCO_3$). In an Egyptian study of pediatric nephrolithiasis, calcite was found in 10% of cases; however, the underlying causes of calcite stone formation were not discussed [7]. The underlying cause of calcite urolithiasis is unclear. Calcium carbonate is frequently identified as a constituent of "milk of calcium" stones, occasionally forming as partially calcified fluid accumulations within obstructed areas of the upper urinary collecting system, including cases of hydronephrosis [8,9]. In Gault's study, a number of patients were diagnosed with medullary sponge kidney, prompting his hypothesis that elevated urine concentrations of bicarbonate, carbonate, or calcium in an alkaline pH environment were significant factors. Additionally, Gault conducted experiments demonstrating that calcite stones dissolved in vitro at a pH of 5.0 but remained stable at 6.5, indicating their susceptibility to dissolution in an acidic environment [10]. CT imaging and direct visualization of this patient's kidney demonstrated no evidence of medullary sponge kidney; however, her initial CT demonstrated ureterohydronephrosis to the obstructing stone. According to a Swedish cohort study, the risk of kidney stones would double in men taking dietary supplements of vitamin C. Without questioning the health benefits of this vitamin, these findings underscore the importance of adhering to recommended daily doses to limit the risk of overdose. The ascorbic acid found in urine corresponds to the unmetabolized portion, but it can also be excreted in urine in the form of oxalate and, to a lesser extent, in the respiratory system as CO_2. In this case, the overconsumption of multivitamins over the years may have caused the kidney stone formation. Herbal supplements can impact the formation of calcium carbonate kidney stones in several ways [10,11]. Certain herbs possess alkalizing properties that elevate urinary pH levels, potentially promoting the precipitation of calcium carbonate in urine, which is particularly relevant for individuals prone to alkaline stone formation. Additionally, some herbal supplements can interact with calcium metabolism, altering its absorption, excretion, or utilization in the body. These interactions may indirectly influence the risk of calcium stone formation by affecting the balance of calcium ions available for crystallization in the urinary tract. Therefore, caution is advised when considering herbal supplementation, especially for individuals at risk of calcium carbonate kidney stones, to mitigate potential effects on stone formation [12].

Conclusion

This case underscores the complexity of kidney stone formation and the potential role of long-term vitamin and herbal supplement use in predisposing individuals to calcite urolithiasis. The spontaneous expulsion of a calcite stone in this otherwise healthy woman, coupled with the absence of medullary sponge kidney, suggests a multifaceted etiology potentially influenced by dietary habits. The presence of alkaline urine pH and interactions with calcium metabolism are critical considerations in understanding stone formation mechanisms. Clinicians should remain vigilant in assessing dietary supplement histories and educating patients on adhering to recommended doses to mitigate the risk of stone recurrence. Further research is warranted to elucidate specific risk factors and preventive strategies tailored to individuals susceptible to calcium carbonate kidney stones.

Declarations

Ethical approval and Consent to Participate

It were not required for this kidney stone analysis as it involved the use of previously collected and anonymized clinical data. The research that involves the analysis of existing, de-identified data, without any direct patient interaction or intervention, does not necessitate ethical approval.

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Author contributions

AAB, MZ, SH, and AA carried out the study, designed and conducted all laboratory analyses, interpreted experimental results, and prepared the manuscript. HT supervised the study.

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Availability of data and materials

NA.

Consent for publication

All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Declaration

During the preparation of this work, the authors used OpenAI's language model (Chat GPT and WordVice) in order to enhance the clarity and coherence of the manuscript. After using this tool, the authors reviewed and edited the content as needed and takes full responsibility for the content of the publication.

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Case Report

The missing M band: Is it really Non Secretory Multiple Myeloma?

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Keywords

Non- secretory Multiple myeloma, Oligo-secretory multiple myeloma, light chain multiple myeloma, serum -free light chain assay, case report

Background: Non-secretory multiple myeloma (NSMM) is defined as clonal bone marrow plasma cells ≥10% or biopsy proven plasmacytoma, evidence of end-organ damage due to underlying plasma cell dyscrasia, namely hypercalcemia, renal insufficiency, anaemia, bone lesions and lack of serum and urinary monoclonal protein on electrophoresis and immunofixation. They represent 3-5% of multiple myeloma (MM). With the advent of serum free light chain (s FLC) measurement, most of NSMMs have been classified as Light chain Multiple myeloma (LCMM). Thus, the proportion of true NSMM, meaning MM that secretes no monoclonal protein (complete immunoglobulin, heavy or light chain) is close to 1-2% of all myelomas. There is a need to distinguish between the true non-secretory from the other forms of oligo-secretory (OSMM) and secretory form of myeloma like LCMM with use of advanced diagnostic tools such as s FLC assay as the former has a good prognosis.

Case Presentation: We discuss a case of a 65-years-old female who presented with chronic chest pain since one year. Cardiac and musculoskeletal involvement were ruled out. Monoclonal gammopathy was suspected in view of imaging abnormalities. Surprisingly, SPE and IFE reported absence of M band. A provisional diagnosis of NSMM was made based on biopsy features. However, diagnosis of NSMM was later changed to LCMM in view of a positive sFLC ratio.

Conclusions: It is well-known that the sequence of diagnostic investigations plays a crucial role in the timely diagnosis and management of patients. However, in this case it was a faulty sequence of ordering investigations which prolonged the hospital stay and delayed therapeutic intervention for the patient concerned. Serum Protein Electrophoresis (SPE), Immunofixation electrophoresis (IFE) and sFLC are simple blood-based tests which can help diagnose a majority of cases of monoclonal gammopathies. They need to be included as first line tests in our approach to evaluating a suspected case of monoclonal gammopathy.

Introduction

Multiple Myeloma (MM) is the second most common hematological malignancy in the United States of America (USA), with ~30,000 new cases/year accounting for 10% of all hematologic cancers and 1% of malignant diseases in USA [1]. It is a neoplasm of the bone marrow's well differentiated plasma cells (PCs), which usually contribute towards humoral response. MM is characterised by clonal proliferation of plasma cells of the bone marrow, and presence of CRAB features (hypercalcemia, renal involvement, anaemia, bone pain,) as well as three defined components. These three include: clonal bone marrow plasma cells ≥60 %, Serum free light chain (SFLC)ratio ≥100 (when involved FLC level is ≥100 mg/L) and greater than one focal lesion on magnetic resonance imaging (MRI) [2,3]. In India, MM accounted for 1.19% of all cancers in females and 1.36% in males during 2012-2014[4]. However, the age of onset is almost a decade earlier than the Western countries [4].

MM is characterised by the unchecked production of clonal immunoglobulin (Ig) from the plasma cells. They may be complete (heavy and light chain) or incomplete immunoglobulins (Igs) (either heavy chain or light chain). The invasive neoplastic plasma cell growth in the bone and bone marrow and the production of aberrant immunoglobulin leads to manifestations which include osteolytic lesions manifesting as bone pain, increased serum calcium, anaemias, cytopenias, neuropathy and renal injury [5]. Presence of the clonal aberrant immunoglobulin in the blood or urine is quantified to diagnose and monitor plasma cell dyscrasias using tests like serum and urine protein electrophoresis (SPEP and UPEP), serum and urine immunofixation (IFE), total immunoglobulin quantification and the serum free light chain assay [6]. Monoclonal component (MC) or protein (MP) can be detected in serum and urine as [7]:

a. a complete immunoglobulin molecule made of heavy and light chains bound together;
b. increased concentrations of complete Immunoglobulin molecule along with raised levels of light chains not bound to heavy chain (free light chains [FLCs]);
c. mainly FLC with low levels or absence of complete Immunoglobulin: LCMM
d. a fourth entity characterised by the presence of only free heavy chain with no bound light chain (very rare): heavy chain disease and
e. a fifth subclass characterised by MC not detectable either in the serum or the urine by SPE/IFE: the non-secretory variant of multiple myeloma.

The incidence of NSMM ranges from 3% to 5% of the total MM cases [8]. However, with technological advancements, FLCs can be detected by the serum FLC assay. Many previously classified NSMMs are now reclassified as oligo -secretors, producing FLC solely minus the heavy chain. Hence, actual proportion of true NSMM i.e MM secreting neither monoclonal heavy nor light chains is only 1–2% of all MMs [9,10]. The International Myeloma Working Group presently defines NSMM as MM devoid of MP detection by serum or urine immunofixation, which may include light-chain MM. This definition needs review since LCMM indeed actively secretes a component of the immunoglobulin monoclonal FLCs capable of detection by the SFLC assay [2,3,5,11,12]. This case highlights the diagnostic challenges faced in identification of NSMM and the importance of sFLC test in differentiating NSMM from LCMM.

Case history

A 65-years-old female presented to the hospital with complaints of chest pain that had lasted for one year. A cardiac consultation revealed no abnormality.

Investigations

A spine MRI done during early 2021 revealed the presence of numerous ill-defined lesions, replacement of the normal marrow and altered marrow signal intensity involving multiple thoracic and lumbar vertebral bodies. Spine MRI was done again six months later which revealed numerous ill-defined mixed lytic-sclerotic lesions in multiple thoracic and lumbar vertebrae. The MRI results suggested haematological malignancy most likely MM or metastasis. Compared to the earlier MRI scan there was a reduction in the height of multiple vertebral bodies in the second MRI and few of the vertebral bodies were partially collapsed. A heterogenous lesion measuring 1.8 x 1.9 cm in size was seen in left iliac blade and biopsy of the same was suggested. A Computed Tomography (CT) guided biopsy from left iliac blade revealed features suggestive of plasma cell dyscrasia (Figure 1 shows detailed radiological findings). A bone marrow aspirate revealed 26% plasma cells. She was then referred to the radiotherapy unit for further treatment. Serum protein electrophoresis and immunofixation using gel-based methodology (Sebia Diagnostics, France) failed to reveal the M band (Figure 2). A 24- hour urine specimen immunofixation (Sebia Diagnostics, France) using gel based methodology also did not reveal any M band. The fluorescent in situ hybridisation (FISH) for multiple myeloma panel performed on peripheral blood after CD 138 + cells collection was positive for chromosomal translocation t(11;14) (CCND1/IGH).The serum sample value for β2 microglobulin was raised at 3.66mg/l. Immunohistochemistry(IHC) marker CD138 was diffuse and strongly positive in tumour cells on biopsy. Kappa restriction was positive while tumor cells were negative for lambda light chain. sFLC assay by nephelometry (ATTELICA NEPH 630, Siemens) revealed an increased kappa light chain concentration of 57.5mg/l and lambda light chain concentration of 8.85mg/l. Kappa / lambda ratio was elevated at 6.49. Table 1 shows details of laboratory investigations done on fully automated clinical chemistry analyser (Beckman Coulter, USA) and five part hematology analyser (Transasia) which revealed anaemia, borderline hypercalcemia with normal urea, creatinine and normal A:G ratio with low protein and albumin levels. In view of discrepancy between bone marrow biopsy findings and immunohistochemistry results vis-à-vis SPE and IFE findings,a SFLC was finally done which revealed an elevated κ/λ ratio. Immunoglobulin quantification of IgD could

not be performed due to unavailability of facility for the same in house. Unavailability of anti- sera to IgE and IgD during serum and urine immunofixation as well as IHC posed a limitation in ruling out IgD and IgE multiple myeloma. However, Serum IgE quantification by nephelometry revealed values below detection limits. We then made a provisional diagnosis of Light chain multiple myeloma with osteolytic lesions of the vertebra and anaemia as Myeloma defining events (MDE) without significant hypercalcemia and renal involvement. The elevated β2 microglobulin indicated a poor prognosis. Presence of chromosomal translocation (t 11;14) was noted in our case as has been observed in many previous studies on MM.

Figure 1: Radiological findings A) lateral skull radiograph showing multiple punched out lytic lesions of variable sizes in the calvarium as well the mandible B) frontal knee radiograph showing lytic lesions in both femur and tibia C) Sagittal reformatted CT thoracic spine image showing diffuse osteopenia and lytic lesions of the entire visible spine as well as the sternum, wedging and collapse of multiple vertebrae is evident D) CT guided bone biopsy procedure from a lesion of the left ilium E) Sagittal T1W image of the lumabosacral spine, F) Sagittal whole spine T2W image, and G) Sagittal post contrast fat-suppressed T1W image showing replacement of the vertebral marrow with poorly defined lesions, some of them showing enhancement, and the multi-level vertebral collapse H) Sagittal T2W, and I) Sagittal post contrast fat suppressed T1W images six-months after the first MRI, showing further progression of the disease, with extensive marrow replacement, enhancement and vertebral collapse.

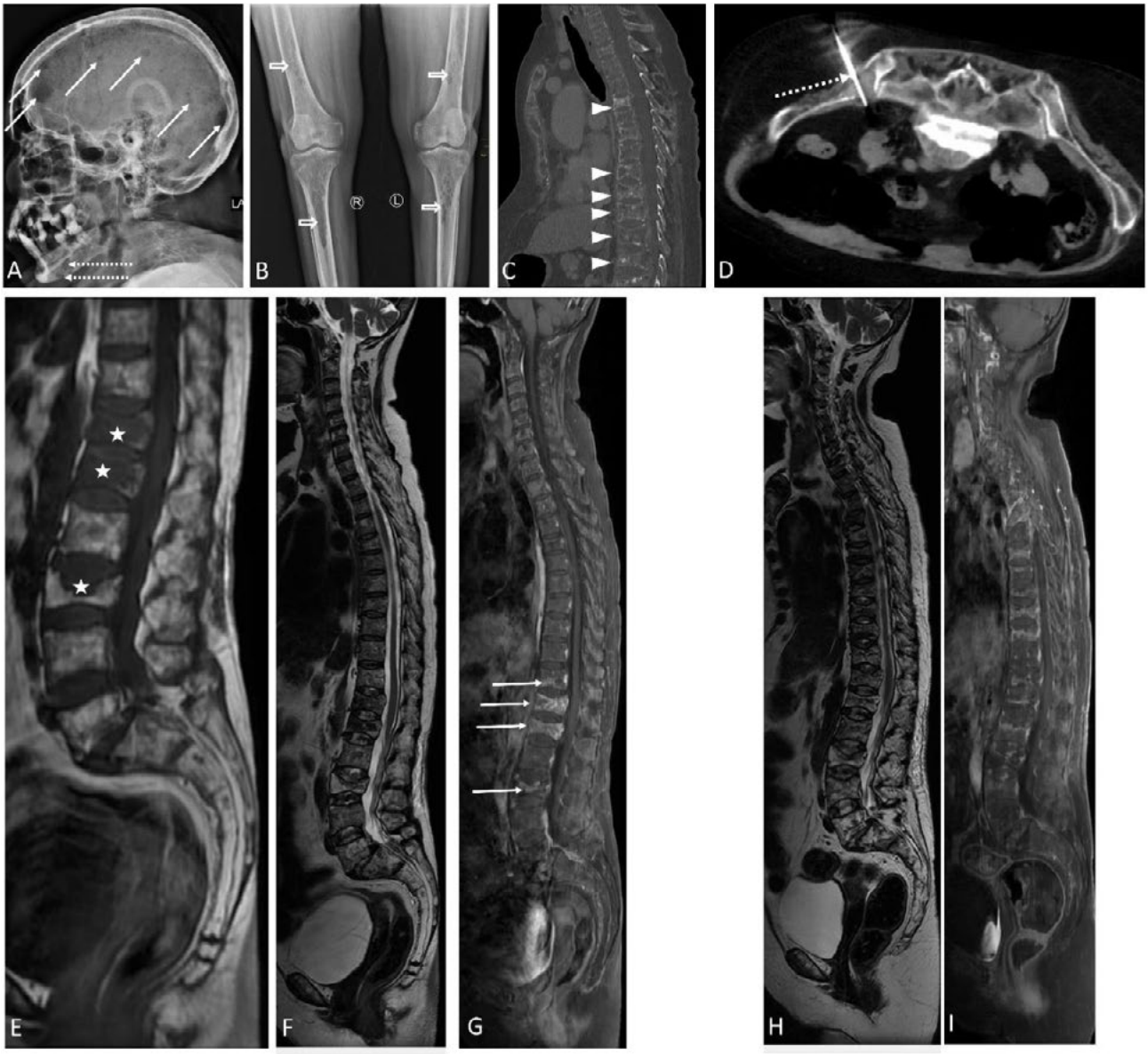
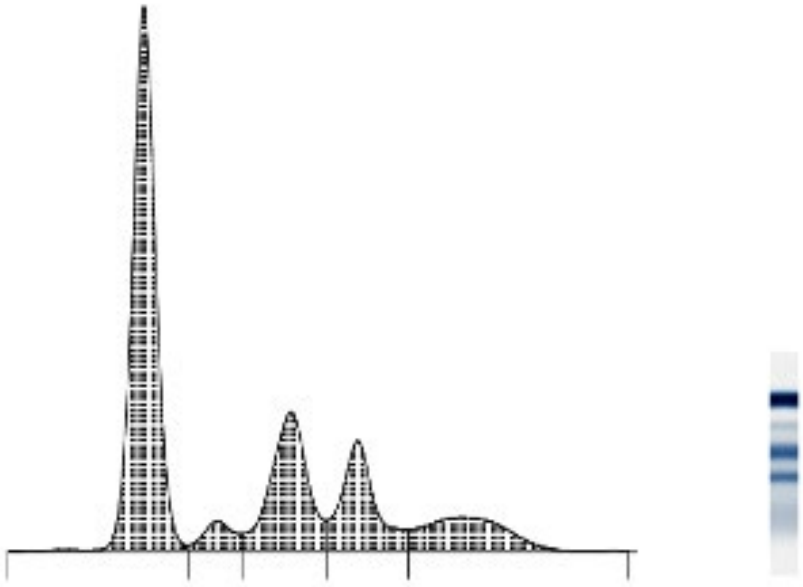


Figure 2: Serum protein electrophoresis reveals absence of M band.



Serum protein electrophoresis

Fractions	%		Ref. %	Conc.	Ref. Conc.
Albumin	**50.3**	<	59.8 - 72.4	**3.5**	
Alpha 1	**3.6**	>	1.0 - 3.2	**0.2**	
Alpha 2	**20.3**	>	7.4 - 12.6	**1.4**	
Beta	**14.5**	>	7.5 - 12.9	**1.0**	
Gamma	**11.3**		8.0 - 15.8	**0.8**	

Table 1: Routine and special laboratory investigations.

Parameters	Result	Unit	Reference Range
Serum urea	5.81	mmol/l	2.1-7.1
Serum creatinine	42.44	µmol/l	40-66
Serum uric acid	0.277	mmol/l	0.15-0.35
Serum calcium	2.58	mmol/l	2.15-2.57
Serum phosphorus	0.946	mmol/l	0.81-1.45
Sodium	138.37	meq/l	136-145
Potassium	4.10	meq/l	3.5-5.1
Chloride	103.42	meq/l	98-107
Total bilirubin	12.48	µmol/l	0-34
Indirect bilirubin	7.87	µmol/l	0.0-30.6
Direct bilirubin	4.62	µmol/l	0.0-3.4
Alanine Amino Transferase(ALT)	25	U/L	<34
Aspartate Aminotransferase (AST)	30.1	U/L	<31
Alkaline phosphatase(ALP)	118.6	U/L	33-96
Total Protein	4.50	gm/dl	6.4-8.3
Albumin	2.79	gm/dl	3.5-5.5
Globulin	1.71	gm/dl	2.0-3.5
A:G ratio	1.63	---	1.5-2.5
Hemoglobin	10.9	gm/dl	12-15.8
White Blood Cells	7.81	$10^3/\mu L$	3.54-9.06
Platelet	153	$10^3/\mu L$	165-415
Beta 2 microglobulin	3.66	mg/l	1.09-2.53
SPE findings	M band absent	--	--
IFE findings	No MP detected	--	--
SFLC(Kappa)	57.5	mg/l	6.7-22.4
SFLC (Lambda)	8.85	mg/l	8.3-27
κ/λ ratio	6.49	---	0.31-1.56
Urine IFE	No MP detected	--	--
Bone marrow biopsy	Plasma cells 26%	--	--

Diagnosis and Management

Our patient presented to the hospital's radiology wing for an MRI of the spine due to progressive increase in pain in chest wall. She was initially diagnosed as a case of MM stage II based on laboratory and radiological findings and chemotherapy started. She was put on VTD (bortezomib, thalidomide, and dexamethasone) regimen.

There was a significant delay in the final diagnosis; more so as the patient's workup began with radiological tests followed by SPE, immunofixation and FISH. The bone marrow biopsy and IHC were done at a much later stage. SFLC was done much later due to its unavailability at the hospital. She is now regularly on follow up treatment receiving chemotherapy cycles in our hospital.

Discussion

NSMM is defined as clonal plasma cells in the bone marrow to the extent of ≥10% or plasmacytoma proven on biopsy with evidence of end-organ involvement due to the underlying plasma cell neoplasm. Manifestations include raised serum calcium, renal impairment, anaemia, skeletal lesions and undetectable serum and urinary MP on electrophoresis and immunofixation [13].

NSMM patients are categorised into different groups. True NSMM consists of non-producers of immunoglobulin chains with a defect in immunoglobulin synthesis leading to undetectable protein in the blood or urine despite significant plasmacytosis in the bone marrow. The s FLC assay will not detect any MP and hence no measurable disease can be identified in such patients [13]. In the second category of NSMM patients,

neoplastic plasma cells produce non-functional MC with defects in secretion. It is believed that true NSMMs arise from a loss of heavy chain secretion first followed by the loss of light chain secretion [14,15]. LCMM patients produce only light chain component of immunoglobulin. They are hypothesised to have never displayed a legitimate heavy chain recombination. Non-functional IgH recombinations at the DNA level indicative of abnormalities in the IgH gene re-arrangements during B-cell maturation allows secretion of only light chains in the abnormal plasma cells [16]. A study in 2002 observed that 11 amongst 14 NSMM patients had a t(11 ; 14)(q13;q32) rearrangement, probably giving the cells a more "lymphoplasmacytic morphology" with a decreased secreting capacity [17]. All these are suggestive that the progression of NSMM cells may be sequentially from fully secretory MM to MM devoid of heavy chain production and then ultimately the light chain.

A group of patients with impaired secretion producing only low levels of light chains also exists. Such patients are classified as oligo-secretory "free light only" myeloma as their protein secretion is not as high as classical myeloma, but still measurable using sFLC assay. Oligo-secretory multiple myeloma is often characterized by serum MP concentration less than1.0 g/dL, urinary protein less than 200 mg/day and sFLC values less than 100 mg/L [12].

The identification of NSMM poses a significant challenge to physicians and laboratory specialists. A combination of biochemical, pathological and radiological investigations is required to identify non- secretory MM including SPEP, UPEP, and sFLC assay and an imaging survey apart from the routine tests. Bone marrow aspiration and biopsy of the suspected lesion for plasma cell morphology and count is suggested in all patients with suspected NSMM. Flow cytometry may sort CD138-enriched cells for FISH testing to identify associated translocations. Samples should also be stained for intracellular immunoglobulin if there is a suspicion of true NSMM. The diagnosis of NSMM requires the presence of MM-mediated end-organ damage (CRAB). Patients with LCMM may have only detectable serum/urine free light chain but they should not be classified as NSMM. The group of true NSMM has disease manifestations with no serum/urine MC, or presence of free light chain. A skeletal survey is recommended in such patients by use of positron emission tomography (PET)/CT scan. Bone survey and marrow plasmacytosis, assess disease extension at presentation and the level of response to treatment. PET/CT imaging can help identify sites of bone involvement and differentiate between active and dormant lesions at treatment completion and during follow-up.

The inability to use serum and urine immunoglobulin tests by clinicians as dependable markers for tumour load assessment presents a dilemma for clinical decision making in NSMM. Serially performed bone marrow studies permit direct assessment of tumour burden and are the gold standard. However, the cost, time, and patient discomfort involved with frequent bone marrow aspirations and biopsies are practically unconvincing and hence the preference for imaging studies. Conventional X-rays don't correlate with tumor response. Advanced imaging modality such as PET/CT offers an advantage over MRI for monitoring response to treatment. Current treatment modality for NSMM is the same as other variants of MM. However as chromosomal translocation t(11 ; 14) is more frequently found in NSMM, there may be a change in treatment modality in near future. As NSMM variant is less frequent and difficulties are encountered in monitoring response to treatment, very less data is available either in the form of retrospective or prospective studies on NSMM [18].

The sensitivity of sFLC assays has been particularly useful in detection of M protein in patients previously described non-secretory as per electrophoresis findings. In a study of 28 patients with NSMM, it has been found that sFLC measurement at diagnosis for many NSMM patients has proved beneficial for them. It helps avoid diagnostic delays and is therefore recommended by the International Myeloma Working Group for patients with NSMM [19]. In another study involving 74 patients diagnosed with monoclonal gammopathy, sFLC assays were carried out using two different methods i.e Freelite assays and N latex FLC assay [20]. The data obtained in this study suggested the superiority of the polyclonal antibody reagent used in Freelite assay over the monoclonal antibody reagent used in N Latex FLC resulting in better clinical senstivity. The authors speculated that the Freelite assays could identify majority of the polymorphic monoclonal FLC but the N Latex FLC based on monoclonal antibodies with limited epitope specificity was unable to recognize all monoclonal FLC clones. More studies are needed in future to establish the rates of agreement between different sFLC assays available in the market. In patients with renal failure, reticuloendothelial pinocytosis is the main mechanism for sFLC clearance. This leads to increased half-life of sFLC and serum levels 20–30 times normal. The sFLC assay can become potentially unreliable as the differential ability to clear κ and λ LCs by the kidney is lost in such cases, leading to the change in sFLC ratio. About 500 mg/day of FLCs are produced normally with a κ/λ ratio of about 2:1. Due to dimeric nature of λ FLC, resulting in slower renal clearance compared with κ LCs leading to a κ/λ ratio in the serum of about 0.58 (range 0.26–1.75). FLCs have a serum half-life of 2–6 hours as they are rapidly filtered by the glomeruli followed by metabolism in the proximal tubules. When FLCs are produced in excess of the reabsorptive capacity of the tubules they can lead to accumulation of sFLC. A different normal range with κ/λ ratio increased to 0.37–3.1for patients with renal failure may result in an increased sensitivity of the assay. The treatment of renal myeloma aims to rapidly eliminate nephrotoxic light chains from the serum. Plasma exchange or high cut-off hemodialysis, is suggested as a supplement to dexamethasone ± bortezomib-based regimens in these settings. sFLC assays could also be helpful in these situations to monitor the effectiveness of the dialytic procedures intended to selectively remove the SFLC(21). Table 2 shows a comparative analysis of NSMM and LCMM. Authors have suggested an algorithm for workup of patients clinically suspected to be probable cases of monoclonal gammopathy (Figure 3).

Figure 3: Algorithm suggested for evaluation of suspected cases of Monoclonal Gammopathy.

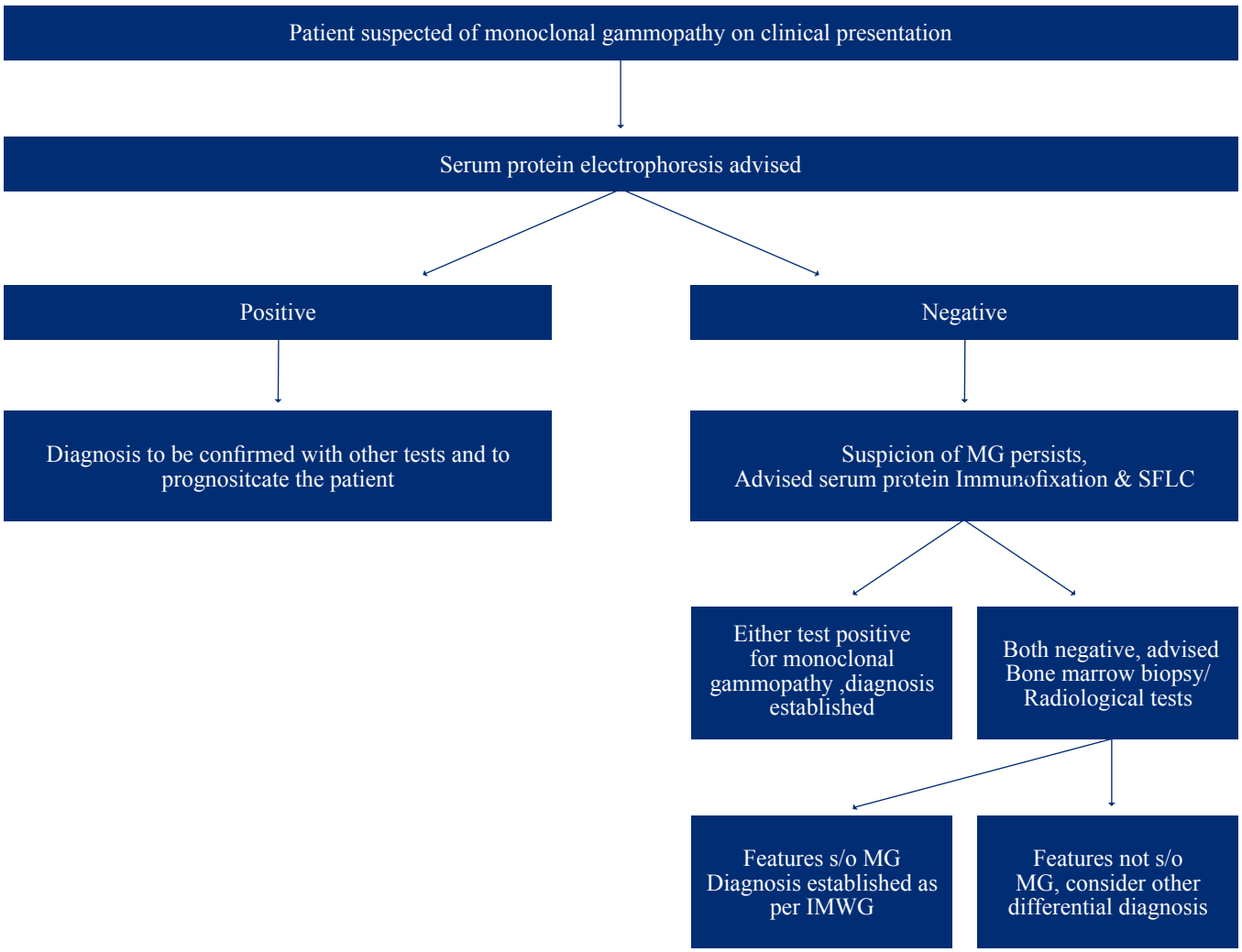


Table 2: A comparative analysis of NSMM and LCMM.

Non secretory multiple myeloma (NSMM)	Light Chain multiple myeloma (LCMM)
a) Accounts for upto 2-3% of all multiple myeloma cases	a) Accounts for up to 15% of all multiple myeloma cases
b) No M protein detectable in serum /urine protein electrophoresis and immunofixation	b) Clonal plasma cells are unable to produce heavy chains resulting in exclusive production of light chains
c) 85% of these will have M protein detectable in the cytoplasm of neoplastic plasma cells ,15% will have no immunoglobulin detectable in plasma cells	c) kappa or lambda light chain will be constituting the M protein
d) sFLC assays can be used to detect monoclonal protein in the absence of M protein by the above mentioned techniques	d) SPE and IFE may be able to detect the M protein depending on tumour load
e) sFLC assays will also fail to detect M protein	e) However cases which escape detection will be eventually detected by s FLC assays.
f) Better prognosis, Not at risk for developing myeloma kidney	f) Poorer prognosis, has more aggressive course, poses high risk for development of myeloma kidney.

Conclusion

Paraprotein absence on serum protein electrophoresis or serum immunofixation does not exclude a multiple myeloma diagnosis. sFLC assays can differentiate non-secretory/oligo-secretory type MM from light chain MM which may go undetected by SPE, Serum IF or Urine IF in case of less tumor burden. Additionally, a pre-treatment FLC assay should be performed because serial FLC values are helpful in monitoring treatment effects. For further differentiation of non-secretory /oligo-secretory from other cases of MM, a bone marrow aspiration/biopsy or osteolytic lesion biopsy is helpful. Moreover, we would suggest an algorithm involving initial workup of suspected multiple myeloma cases to include sFLC along with serum immunofixation.

Author disclosures

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Ethical considerations

Ethical clearance is waived off for case reports from our institute.

Consent for publication

Informed consent has been taken from husband of the patient concerned to go ahead with possible publication in a medical journal.

Competing interests

None.

Funding

None.

Author's contributions

All authors fulfil the criteria for authorship as set out by the ICMJE and as recommended by the Committee on Publication Ethics (COPE).

Data availability

All data related to the case has been submitted along with the manuscript.

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Brief Report

Abnormal Urine Drug Screens in Pregnancy- Opportunity for Laboratory Stewardship

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Background: Clinical testing for drugs of abuse typically involves initial screening followed by confirmatory testing. Due to limited evidence-based guidelines, the healthcare provider makes the decision to confirm abnormal screens based on the clinical context. This two-step approach proved to be inadequate in scenarios like maternal substance abuse and subsequent fetal/ newborn exposure. The goal of this study is to assess and improve the confirmatory testing rate of abnormal screens among pregnant patients at our women's center.

Methods: A retrospective chart review was conducted to assess the confirmation rates among positively screened pregnant patients, and a lab stewardship initiative was implemented to remind ordering physicians about the importance of confirmatory drug tests. Abnormal screens were classified as expected positives based on the medication-related interference, social history and self-reported substance use from the provider notes.

Results: Only 28% of pregnant patients with unexpected positive drug screens underwent confirmatory testing during the pre- intervention period, which rose significantly to 67% during the post-intervention period. Furthermore, outcome analysis revealed that 50% of patients with concordant confirmatory test results were referred to social work and psychiatry in the post-intervention period.

Conclusions: This study highlights the value of laboratory stewardship in optimizing drug testing practices for pregnant patients.

Introduction

Drug testing is indicated in various contexts, such as pain management, medication adherence, monitoring controlled substance abuse, work-up for sudden unexplained symptoms, forensics, workplace safety and athlete compliance [1]. In clinical settings, drug testing typically involves a two-step sequential approach, with an initial screen followed by a confirmatory test [2]. Urine is the preferred matrix for drug testing due to its relatively long analyte detection window and high analyte concentrations compared to blood. Urine drug screens are primarily immunoassay-based qualitative tests and are prone to both false positive and false negative results [3]. Moreover, immunoassay-based screens are unable to identify synthetic analogs [4]. Hence, screening results are considered presumptive until confirmed with a definitive test using liquid chromatography-tandem mass spectrometry (LC-MS/MS) or gas chromatography-mass spectrometry (GC-MS). Given limited evidence-based guidelines, healthcare providers often make the decision to confirm a presumptive positive screen depending on the clinical context as well as the institutional policy [2].

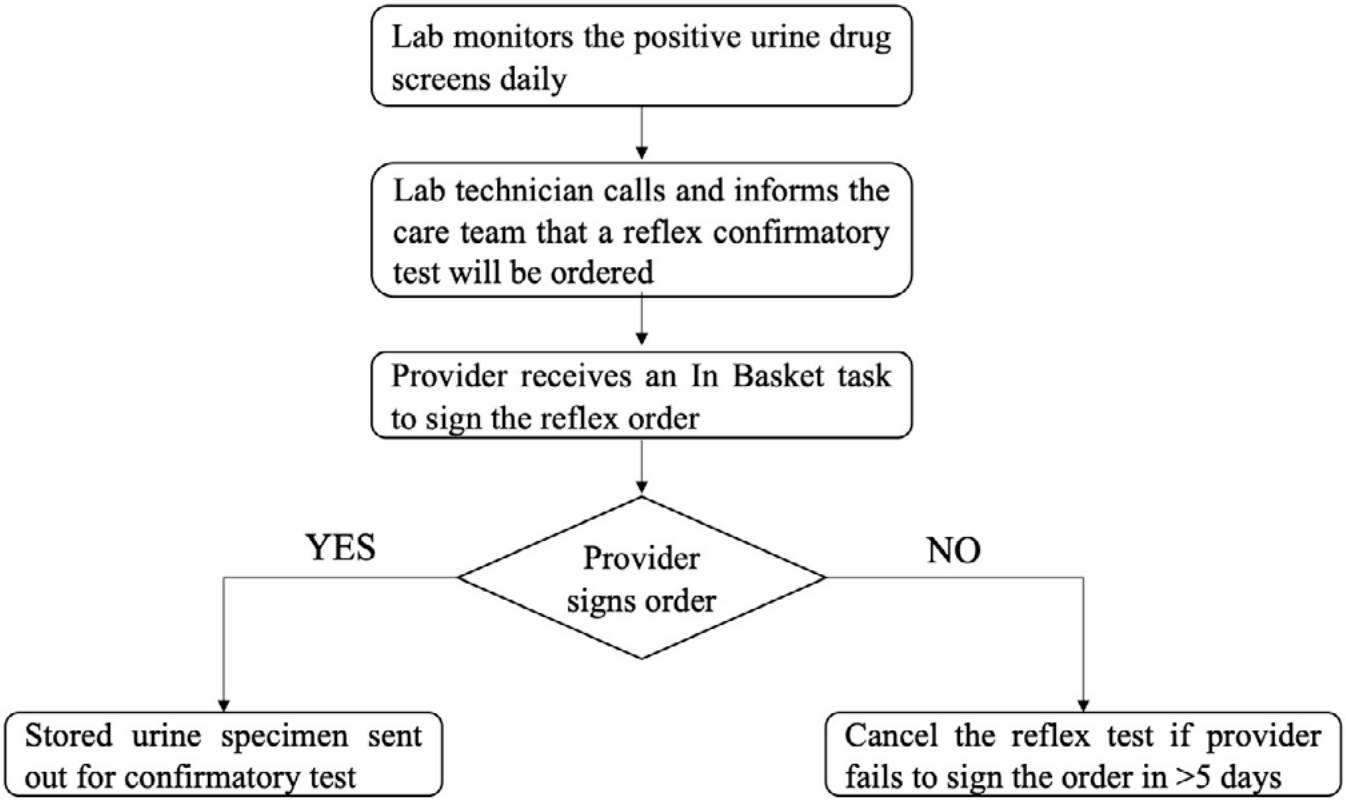
Several studies have underscored the inconsistent and inadequate drug testing practices in children and pregnant women [5-7]. Importantly, timely and accurate interpretation of urine drug tests is critical for clinical decision-making in these populations as it might also have social and legal ramifications. As per the United States National Poison Data Center, ~40% of substance exposure cases correspond to the pediatric population [8]. Additionally, maternal substance use causes debilitating effects on newborns, including cognitive impairment, neonatal abstinence syndrome, respiratory insufficiency and behavioral problems [9]. In 2017, the American College of Obstetrics and Gynecology (ACOG) and the American Society of Addiction Medicine jointly recommended all pregnant women be universally screened for substance use at the first prenatal visit [10]. Pregnant patients are first assessed using verbal questionnaires such as National Institutes of Drug Abuse Quick Screen and high-risk individuals undergo urine drug screening [10]. Confirmatory testing of discordant screens often involves sending specimens to the reference laboratories for LC-MS/MS or GC-MS analysis, further delaying the medical and social interventions in these patient populations. In this study, we launched a laboratory stewardship initiative to improve the drug confirmation rates among abnormally screened pregnant patients at our women's center and highlighted the need for optimal drug testing panels.

Methods

This study was conducted in accordance with the protocol approved by the Baylor College of Medicine Institutional Review Board. We performed a retrospective cohort study involving pregnant patients who have undergone urine drug screens at our women's hospital from May 2022 to May 2023 (pre-intervention period) and November 2023 to May 2024 (post-intervention period). The urine drug screens were performed on the PROFILE-V MEDTOXScan Drugs of Abuse Test System, which employs a one-step, competitive later-flow immunoassay. The panel included the following drug classes with indicated cutoffs: tetrahydrocannabinol (50 ng/mL), phencyclidine (25 ng/mL), cocaine (150 ng/mL), methamphetamine (500 ng/mL), opiates (100 ng/mL), amphetamines (500 ng/mL), benzodiazepines (150 ng/mL), tricyclic antidepressants (300 ng/mL), methadone (200 ng/mL), barbiturates (200 ng/mL), oxycodone (100 ng/mL), propoxyphene (300 ng/mL) and buphenorphine (10 ng/mL). Of note, Fentanyl (1 ng/mL; ARK Diagnostics, Inc.) was added to the panel during the post-intervention period.

In collaboration with Obstetrics and Gynecology physicians and nurses, laboratory implemented a pilot scale stewardship initiative (Figure 1). Our major intervention was having the bench technician call the ordering provider to suggest confirmatory testing for positive urine drug screens. Additionally, a standardized risk assessment strategy devoid of racial and ethnic disparities was implemented during the post-intervention phase. As per our hospital policy, all the presumptive positive urine specimens were stored in the freezer at -20°C for 30 days. Upon the physician's request, presumptive positive urine specimens were sent for confirmatory testing to ARUP reference laboratory (test code: 0092186). In certain cases, alternate specimens, such as maternal serum or meconium from the newborn, were sent for confirmatory testing (test codes: 0092420 and 3004583). A blinded reviewer performed chart reviews for patients with positive screening results to retrieve the status of confirmatory testing, medication history and provider's progress notes. Abnormal screens were classified as expected positives if the patient self-reported the substance use or was on a medication that could potentially interfere with the assay. A 2-tailed Fisher's exact test was used to compare the confirmatory testing rates in the pre- and post-intervention periods. Results with $p<0.05$ were considered statistically significant.

Figure 1: Confirmatory urine drug test stewardship.



Results

During the pre-intervention period, a total of 370 urine drug screens were ordered for pregnant patients at our women's hospital, of which 83 were positive (Table 1). A majority of positive screens were noted for tetrahydrocannabinol, followed by methamphetamine and benzodiazepines (Table 2). Among the 83 positive screens, 25 cases were expected positives due to self-reported substance use or medication-related interference (Table 1). Out of 58 unexpected positive screens, confirmatory testing was pursued in 16 cases, of which 8 showed positive results and 8 showed negative results (Table 1). Hence, the confirmation rate during the pre-intervention period was 19% for all positive screens and 28% for unexpected positive screens.

Table 1: Confirmatory testing status in patients with positive urine drug screen.

Drug Tests	Pre-intervention	Post-intervention
Total Unique Patients	370	143
Positive screens	83	106
Expected positive screens	25	64
Unexpected positive screens	58	42
Total confirmations	16	68
Positive on confirmation	8	30
Negative on confirmation	8	38

Table 2: Distribution of abnormal urine drug screens.

Drug Class	Pre-intervention	Post-intervention
Tetrahydrocannabinol	30	23
Phencyclidine	0	0
Cocaine	7	10
Methamphetamine	12	15
Opiates	9	9
Amphetamines	6	5
Benzodiazepines	12	11
Tricyclics	2	3
Methadone	0	0
Barbiturates	3	1
Oxycodone	2	0
Propoxyphene	0	0
Buprenorphine	0	1
Fentanyl	na	28

During the post-intervention period, a total of 143 urine drug screens were ordered, of which 106 were positive (Table 1). Fentanyl showed the highest proportion of positive screens, followed by tetrahydrocannabinol and methamphetamine (Table 2). However, patients with positive Fentanyl screen were expected cases due to epidural administration for labor pain relief. Among the 106 positive screens, 64 cases were expected positives due to social history or medication-related interference (Table 1). Confirmatory testing was pursued in 68 cases, which included 28 unexpected and 40 expected positive screens (Table 1). Confirmatory testing showed concordant results in 30 cases and discordant results in 38 cases. Hence, the confirmation rate in the post-intervention period was significantly high at 64% ($p<0.00001$) for all the positive screens and 67% ($p=0.002$) for the unexpected positive screens compared to pre-intervention period. Notably, half of the patients with concordant confirmatory testing results were subjected to social work or psychiatry as deemed necessary by the ordering physician. Altogether, this data highlights the role of laboratory stewardship in promoting better patient care for pregnant patients with substance use disorders.

Discussion

Due to a lack of standard guidelines, clinical drug testing practices are highly variable [11]. Factors such as clinical context, medical urgency, individual physician's practice and availability of orderable drug panels influence the drug testing approaches. Our retrospective analysis revealed overall suboptimal drug confirmatory testing rates among the positively screened pregnant population during the pre-intervention phase. Only 28% of pregnant patients with unexpected positive urine drug screens underwent confirmatory testing. This inadequacy led us to launch a pilot-scale lab stewardship initiative, which involved reminding the ordering physician of the importance of confirmatory testing among positively screened pregnant patients. Moreover, we ensured ACOG's universal screening recommendation by integrating a standardized risk assessment questionnaire devoid of racial and ethnic disparities. Our strategy proved to be effective as the confirmation rates increased significantly from 28% in the pre-intervention phase to 67% in the post-intervention phase. Our ultimate goal is to develop a clinical decision support tool to exclude the expected positive screens and perform reflex testing on the rest. This study underscores the importance of developing optimal drug testing panels and standardizing the screening practices in an institute-wide manner.

In contrast to the conventional staged approach, various alternate strategies depending on the clinical need may be implemented, such as 1) reflexive testing panels (positive immunoassay triggers LC-MS/MS testing), 2) direct-to-definitive testing panels (skips immunoassay) and 3) hybrid testing panels (combination of 1 and 2) [6,12]. For instance, a recent study implemented a reflexive testing approach for opioid monitoring, which involved universal drug screening of all pregnant women admitted to the labor and delivery ward with immunoassay followed by rapid confirmation with LC-MS/MS (turnaround time-1 day) [6]. Interestingly, this approach not only improved the early identification of newborns at risk for neonatal opioid withdrawal syndrome, but it also substantially reduced the burden of the neonatal intensive care unit by allowing the discharge of newborns with false positive screens [6]. Moreover, the utility of comprehensive direct-to-definitive testing by liquid chromatography-high-resolution mass spectrometry in pediatric patients presenting to the emergency room with suspected drug exposure was recently demonstrated [5]. A major limitation of direct-to-definitive testing is the need for frequent updates and validation of the panels due to the

constant evolution of designer drugs.

During the pre-intervention phase, 8 positive screens showed negative results upon confirmatory testing. Whereas during the post-intervention phase, 38 positive screens showed negative results upon confirmatory testing. Although mass spectrometry based confirmations are more reliable than immunoassay-based screens, it is important to consider various factors such as the clinical presentation, medication history, drug cross-reactivity, detection window and specimen quality/validity while interpreting these results. The integration of drug screening and confirmatory test results within the electronic medical records, along with a clinical chemist's interpretation, was found to aid clinicians in efficiently initiating medical and social interventions [11]. Furthermore, this approach may also prevent test misinterpretation owing to the provider's limited analytical knowledge related to drug cross-reactivity, drug metabolism, assay cutoffs, and medication interference [13]. However, there are several challenges that need to be addressed in an institute-wide manner before implementing reflexive testing panels like: 1) excluding expected positive screens for confirmatory testing, 2) specimen storage and integrity monitoring and 3) medicare coverage for reflexive panels, 4) obtaining the patient's consent. Therefore, the development of institutional drug testing guidelines requires the active participation of stakeholders from various subspecialties, such as chemistry, toxicology, pain management, legal/risk management, healthcare finance management, social services and laboratory information systems. Local drug surveillance and positivity rates should be factored in while designing orderable reflexive drug panels. Overall, this study highlights the need to standardize drug testing practices in pregnant patients by designing clinical context-specific orderable panels.

Disclosures

None.

CRediT author statement

Anil K. Chokkalla: Conceptualization, Methodology, Investigation, Formal Analysis, Writing -Original Draft. Sahil Malik: Methodology, Investigation. Ridwan Ibrahim: Methodology, Investigation. Sridevi Devaraj: Conceptualization, Investigation, Formal analysis, Writing -Original Draft, Supervision.

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Abbrieviations

LC-MS/MS, liquid chromatography-tandem mass spectrometry; GC-MS, gas chromatography-mass spectrometry, ACOG, American College of Obstetrics and Gynecology.

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