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Break Through in Clinical Microbiology from Conventional Methods to Molecular assays and Artificial Intelligence

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Abstract
Infectious diseases and Health care associated infections (HAI) account for significant morbidity and mortality in the recent past. Timely diagnosis is the key aspect for effective treatment with the utilization of available resources. Over the decades it has been reported that there is mammoth growth in clinical microbiology from regular traditional methods to novel molecular methods. Artificial intelligence (AI) transforms medical microbiology by replacing slow, manual cultures and identification methods to fast, automated and data driven work flows. This optimistic shift throws a limelight on the accurate diagnosis of many infectious diseases such as HIV-AIDS, HBV, COVID-19, Malaria, Dengue, Tuberculosis, HCV etc. Owing to alarming rise in new vectors and their associated diseases, diagnosis is limited due to lack of infrastructure and expertise. As development is not an overnight process, clinical microbiology evolved from slow, culture based Phenotypic methods to rapid, sensitive molecular methods. With the development of molecular biology several paradigm shifts were observed for disease diagnosis. Moreover, Molecular and genomic approaches have revolutionized many branches of microbiology such as Immunology, Virology, Bacteriology etc. by providing quick and accurate diagnosis there by reducing turnaround time which is not observed in conventional methods. But lack of required facilities is a major problem in some of the laboratories even in developed cities, which still rely on conventional methods. This review article enlightens the optimistic milestones in clinical microbiology from regular conventional techniques to Molecular aspects and the future scope of medical microbiology and its associated branches. Adequate training and education on molecular aspects, artificial intelligence in health sciences is required for the technical team and core fraternity to achieve better results.

Keywords:
HAI, Infectious diseases, Conventional, Molecular methods, Artificial Intelligence, Automation, Sensitive, Phenotypic methods.

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I. Introduction and background

Health sciences play a pivotal role not only in global economy but also in wellbeing of mankind. Of the multiple branches in medical sciences, the laboratory diagnostics are mandatory for disease confirmation of which Clinical Microbiology witnessed an exponential growth from the conventional methods for the isolation and identification of organism to molecular assays further anchored by Artificial Intelligence in the current era. Significant dawn in microbiology started with Leuwenhoek's discovery of "Little animalcules" (1). Louis Pasteur's discovery of Vaccine for Hydrophobia, Edward Jenner's discovery of Vaccine for Small Pox , Koch's work on development of Pure Culture techniques have driven the developmental forces in Microbiology (1). The development of Gram's stain by Sir Christian Joachin Gram in 1884 is considered as one of the microscopic identification tools in bacterial morphology and to initiate empirical chemotherapy (2) The discovery of acid-fast staining by Paul Ehrlich and it's modification by Ziehl and Neelsen is another gold standard in identification of Tubercle bacilli (2). Routine Conventional methods still remain the procedures of choice in terms of diagnosis and are cost effective (3). On the other hand, traditional methods are time consuming especially manual culture methods, regular serological assays etc. Among multiple diagnostic categories, Molecular assays have drawn attention in recent past due to their ability to offer comprehensive insights into diagnosis and therapy modalities (4). Molecular biology has revolutionized the health care system not only by identifying rare pathogen but also

by establishing the genetic correlation and identifying the specific gene responsible for drug resistance (e.g.: rpoB gene in Mycobacterium tuberculosis) in short duration which is masked in conventional methods or traditional manual methods (5). Molecular methods in diagnostics have produced new avenues for studying and implementing treatment by analyzing genetic makeup of the people (6) . But question arises regarding the complete replacement of traditional methods by molecular assays and Artificial Intelligence and day to date update in molecular assays with the development of more sophisticated technology. Artificial Intelligence in present era has become a core part of Medical Microbiology which diminishes the difficulties through automation, by providing more accurate identification of microbes in less time . Inclusion of MALDI-TOF Spectrometry with AI has substantially hastened and enhanced the rate of identification of pathogens (7) Specific diseases and disorders can be interpreted for detailed examination and for treatment implementation in the field of Molecular diagnostics. This method enables to deliver more rigorous and personalized therapies based on individual's genetic profile which is of pivotal use in viral oncology, infectious diseases, serotyping and genotyping of vector borne diseases, analysis of hereditary abnormalities, detection of genes responsible for drug resistance. Molecular diagnostics opened the vistas for two novel therapeutic branches such as Pharmacogenomics, Nutrigenomics. Pharmacogenomics is concerned with examination of a person's genetic variants to determine how they react to specific therapy thereby minimizing negative drug reactions (8,9) Nutrigenomics on other extreme determines the interaction between a person's gene and diet thereby furnishing the nutritional plan posing a profound impact on specific disease management (9).

This review critically evaluates the progress of Medical Microbiology from Conventional, traditional methods to Molecular diagnostics and furnishes the significance and application of Artificial Intelligence.

Epoch of Conventional Methods In Medical Microbiology

The term "Conventional" depicts all culture-based methods which require a pure isolated colony (only one bacteria) for testing in the laboratory for many years. But these methods are not only time consuming but face difficulty in isolation and identification of rare pathogens and sometimes even a common, routine isolate (10) This disadvantage is obvious regarding slow growers or the microbes requiring unique stringent conditions to grow (11) Inspite of availability of novel emerging molecular and non-culture-based techniques, bacterial identification heavily depends on traditional in-vitro procedures (Wilson 2015).

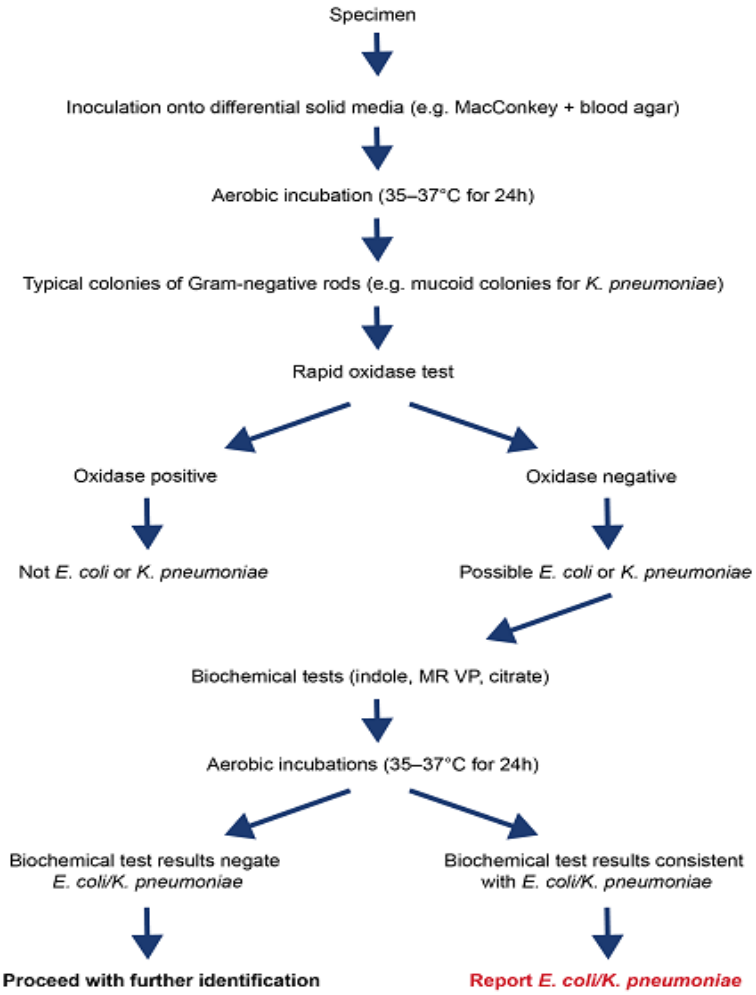
In-vitro methods include Microscopy staining methods, wet mount preparations for fungal elements and parasitic forms, inoculation of collected sample into culture media such as Nutrient agar, Mac-Conkey, Blood agar, special selective media and blood cultures for potential pathogens like Salmonella, Brucella etc.., performance of basic bio-chemical reactions and antibiotic susceptibility testing (12). Pure cultures are vital to study and understand the Virulence mechanisms in bacteria, to interpret drug resistance, bacterial metabolism, gene transfers in bacteria, disease pathogenesis and treatment (13). Identification of proper isolate always depends on proper sample collection in correct time and time consumed for transporting to laboratory. Most common samples received in laboratory not only confined to Urine, Pus, Blood, Sputum, ET secretions, CSF etc. but other vital samples like Throat swab, Naso-Pharyngeal swab, Conjunctival swabs are also sent by the concerned physicians and exemplifying the significance of traditional methods from past to present and the importance of highly sensitive samples like CSF where a new rare microbe (Weinstein 1996) may be identified. In recent past a novel approach in culturing many bacteria simultaneously in a complex environment has emerged, known as "Culturomics" and is pointed on improving cultivation strategies to mimic the complex environmental factors facilitating the natural proliferation of these organisms (14). Culturomics is an innovative branch which targets the isolation and cultivation of organisms where conventional methods fail to identify the bacteria. Culturomics is vested in enhancing the cultivation methods by integrating diverse culture media, with parameters such as temperature adjusted (15). This novel method enhances the growth of broader range of bacteria and facilitates the growth of fastidious bacteria also, which sometimes fail to grow in traditional culture methods due to absence of required growth factors. But more research work is required for the day-to-day application of "Culturomics". Even though more advanced methods are available for bacterial isolation, drug susceptibility testing still depends on regular conventional methods to select proper drug for better treatment and also for antigen production for serological tests. One of the major setbacks in conventional methods usage is prolonged turn around time (TAT) for fastidious isolates, anaerobic bacteria and even sometimes for regular common isolates and in clinical settings culture methods employed often do not deliver immediate results (Nemati. etal 2016).

Total Laboratory Automation

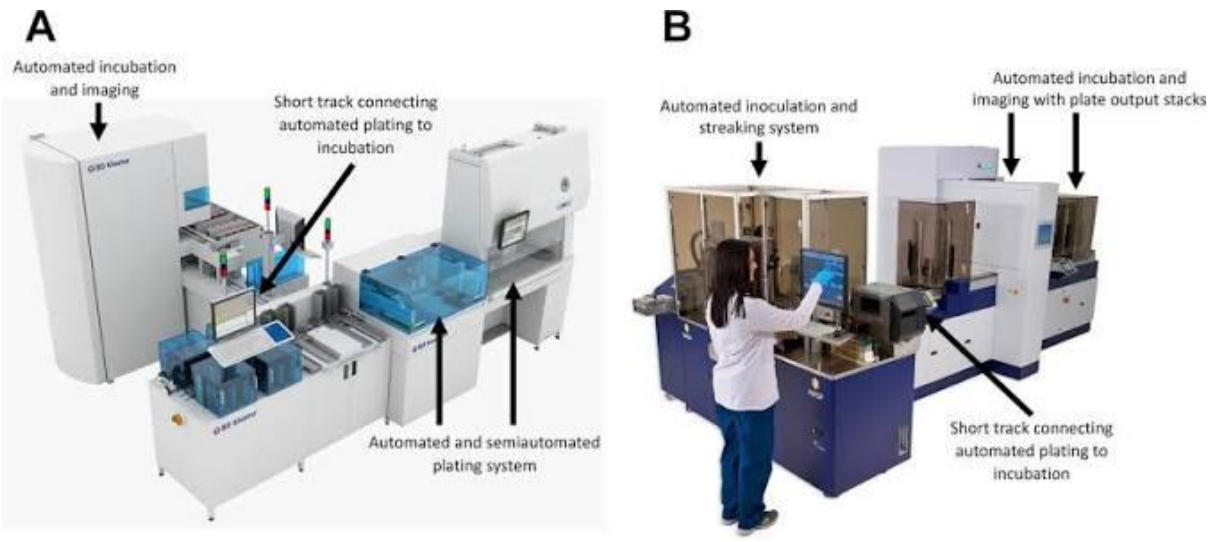
Total laboratory Automation (TLA) in clinical microbiology can overcome these hurdles and is made easier in presence of novel automated instruments such as BAC T ALERT, VITEK-2, etc. These systems cover majority of the steps of culture-based work flow from the point of receiving clinical sample to reporting of results (16). Microbiology value chain includes sample collection, transportation either manually or through pipe line system, sample acceptance and rejection criteria (pre-analytical phase and NABL norms) followed by test performance, validation of results, critical alert remainder and communication to the concerned personnel (post-

analytical phase) (e.g.: Strep.agalactiae in CSF of Neonates, reactive serology report for HBsAg….) and clinical decision support from the clinicians or surgeons. Laboratory Information System (LIS) & Advanced expert system (AES) are critical in delivering electronic based reports to physicians thereby reducing the burden of Manual report dispatch methods.

Sequential work flow through Conventional Methods:



Total Laboratory Automation (TLA) Work flow:



Molecular Diagnostics

Over the decades Molecular diagnostics exhibited promising growth in Medical Microbiology and has become a corner stone in Microbiology for disease diagnosis, confirmation and identification of a rare isolate also. It was in 1949 the introduction of the term "Molecular disease" by Pauling etal. linking a single amino acid change to Sickle Cell Anemia was described (17). Moving forward to 1980s Prenatal genetic testing in "Thalassemia" patients based on Restriction enzymes cutting DNA at specific sequences was conceived. Since then, Molecular diagnostics developed at a rapid pace providing more faster, accurate results aiding the clinicians to deliver timely treatment. Molecular and Genomic approaches are involved in detecting multi drug resistant bacteria and also the specific genes responsible for drug resistance. Molecular and Genomic approaches which are composed of PCR, WGS (Whole Genome Sequencing), Bio-informatics, Molecular biotechnology facilitate the isolation of nucleic acid from clinical specimens such as blood, pus, tissue sections etc. Whole Genome sequencing delivers complete detection of all bacteria present in a clinical specimen where as PCR and modifications of PCR can analyze and interpret the root cause of infection, microbe associated, gene involved in a span of few hours or within a day.

Discovery of PCR

One of the greatest endeavors of humanity is to understand and tackle or harness the forces which pervade the universe and to support this statement, the ability to control high energy nuclear chain reactions in the laboratory is a prime example (18) which is useful to generate immense power coupled with production of Radio Isotopes used in Cancer detection, but also can create hazardous weapons capable of destroying humans and nature .

PCR is another kind of Chain reaction invented several years ago used to exponentially amplify the biologic material (DNA).

PCR Timeline- Discovery and Evolution

PCR was invented in 1983 by American Biochemist Kary B. Mullis when working at Cetus Corporation which fetched him Nobel Prize in 1993 in Chemistry.

Early Conceptual Foundation: 1969-1971

While working in the laboratory of Sir HarGobind Khorana, **"Kjell Kleppe"** for the first time described the basic principles of "Repair Replication" by using Primers with DNA Polymerase. But certain technical glitches and limitations prevented complete realization at the time.

Invention by Kary B Mullis

Sir Kary B Mullis was the master brain behind the invention of PCR in 1983, who conceptualized Thermal Cycling Process to amplify short DNA fragments in an exponential manner and first published the method in 1985 in *"Science"*

Taq Polymerase Breakthrough (1987)

After every heat step ranging to high temperature, fresh DNA Polymerase addition is required and this problem was resolved by adding Taq Polymerase – a heat stable enzyme extracted from Thermophilic bacteria "Thermus aquaticus" present in hot springs.

Pfu Polymerase (1991)

Discovery of Pfu Polymerase from Pyococcus furious provided proof reading capabilities to diminish error rates during DNA Replication.

The subtlety of novel diagnostic methods and interpretation of results required mutual collaboration and in-depth collaborative work among clinicians, microbiologists, department of Bio-informatics (Thom & Farrell, 2017; Renjithetal 2021). Analysis, understanding of molecular assays reports required ample training and experience for both microbiologists and physicians.

Nucleic Acid Amplification Tests (NAATs)

Molecular diagnostic tests remained as gold standard in the diagnosis, confirmation and estimation of total microbial load (E.g.: Total Viral load) of many infectious diseases which is attributed to their high sensitivity, specificity and shortened turnaround time (TAT) as advocated by Valones et.al 2009; Zhu et.al. 2020. Logarithmic or exponential amplification of nucleic acids either DNA or RNA by PCR enables the detection of Pathogens under three vital clinical situations;

a. When Pathogens are not viable

b. When the causative organism fails to grow under the conditions provided which do not favor its growth

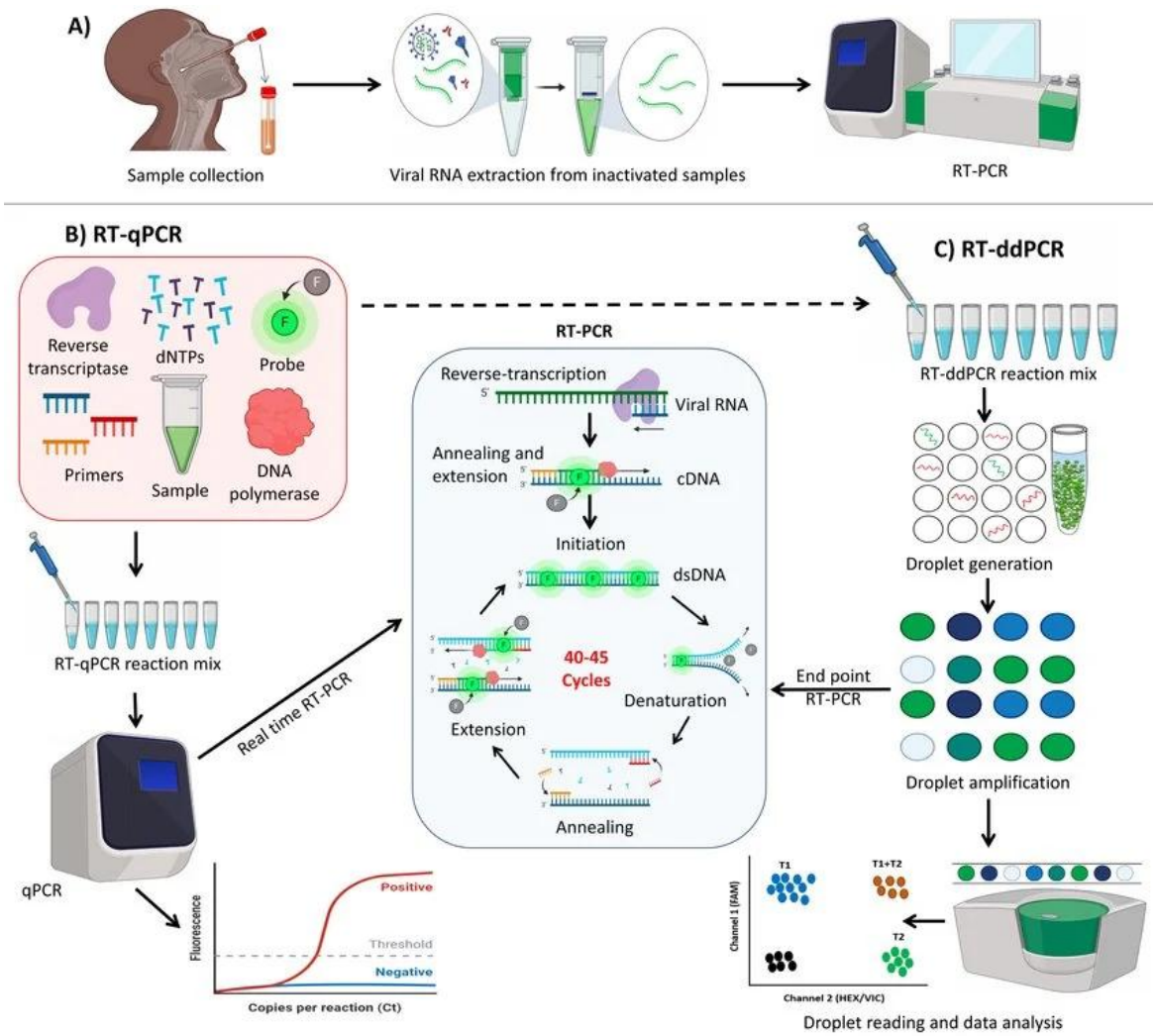
c. Prior initiation of antimicrobial treatment which reduces the growth of the microbes

Underlying principles behind PCR paved the way for molecular science to decipher the genomes of many species and is the basis for many molecular assays. FDA approved molecular tests which were introduced into the clinical microbiology were single target tests that employ PCR based amplification techniques. To quote as examples Transcription Mediated Amplification (TMA), Loop Mediated Iso-thermal Amplification (LAMP), Helicase dependent Amplification which are based on original Hybridization and amplification (19).

Real Time PCR(RT-PCR)

Real time PCR systems depend on detection and quantifying fluorescent reporter whose signal increases in direct proportion to the quantity of PCR product in the reaction. dsDNA specific dye SYBR Green on excitation emits light. Main drawback in using SYBR Green dye is its ability to bind to any dsDNA in the reaction (20). Quantitative PCR (q PCR) provides real time detection and quantification of pathogens through Fluorescence based detection methods. q PCR reduces the risk of contamination by elimination of post amplification processing. Clinical application vests with the detection of pathogens, drug resistant genes and studying the mechanics of Virulence factors (21). Studies conducted by "*Krishnakummariamma etal in 2023* elaborated the application of q PCR in CSF samples of patients with Tuberculosis.

Advancements and innovations in PCR such as d PCR (Digital PCR), Multiplex PCR, Nested PCR further augment the diagnostic precision by enabling absolute quantification and multi target analysis.



Role of Multiplex PCR

Multiplex PCR is mainly employed in clinical Virology where primers are designed and used to detect different types of viruses from clinical specimens. Major advantage of Multiplex PCR lies in its ability to amplify many targets of interest in one reaction by using more than one primer and results can be obtained with in 4hrs , turnaround time being 1-4hrs. Sophisticated companies such as Qiagen, BioMérieux, etc. use different type of panels enabling the detection of pathogens from different specimens such as respiratory, CNS, GIT. Multiplex PCR also facilitates species differentiation e.g.: differentiation and identification of Campylobacter coli from Campy.jejuni from feces, differentiating pathogenic Entamoeba. histolytica from nonpathogenic, harmless E. dispar, E. moshkovskii using species specific reverse primers, for differential diagnosis of Taeniasis and Cysticercosis (22)

Nested PCR

Nested PCR was developed to enhance both sensitivity and specificity of PCR, which uses two pairs of amplification primers and two rounds of PCR. As mentioned by ***Chelly etal.*** in 1989, Nested PCR allows amplification of every cDNA from any desired body tissue, as each cell of the body expresses most Genes, even though only to an extremely slight degree – a process known as "Illegitimate Transcription". This variant of PCR minimizes the chances of nonspecific amplification of target DNA thereby increasing specificity of the reaction. It involves consecutive 2 PCR reactions of 25 cycles, in which 1st PCR uses primers external to the sequence of interest and 2nd PCR uses the product of 1st PCR in conjunction with one or more nested primers .

Table showing Merits, demerits, clinical applications of some of the Molecular diagnostic methods:

Molecular Assay	Merits	Demerits	Applications
PCR	High Sensitivity	Requires Thermal cycler, chances of false positives /negatives	Infectious disease diagnosis (Viral, bacterial ..)
qPCR	Quantitative analysis, detection of multiple pathogens	Requires Thermal cycling, high cost	Viral load estimation, estimating infection prognosis
Multiplex PCR	Identifying multiple pathogens in one step	Costly primers, requires optimization	Diagnosis of Respiratory, CNS, GIT Pathogens
LAMP	Fast results, portable, low price, isothermal operation	Targets a single pathogen, complex design system in primers	Field diagnosis, diagnosis of tropical infectious diseases.

Digital PCR(d PCR)

It is more advanced form of PCR in which clinical specimen is fragmented into more than thousands of micro reactions to achieve accuracy quantitatively. This method can trace out the presence of even low levels of pathogens, can estimate rare mutations (23).

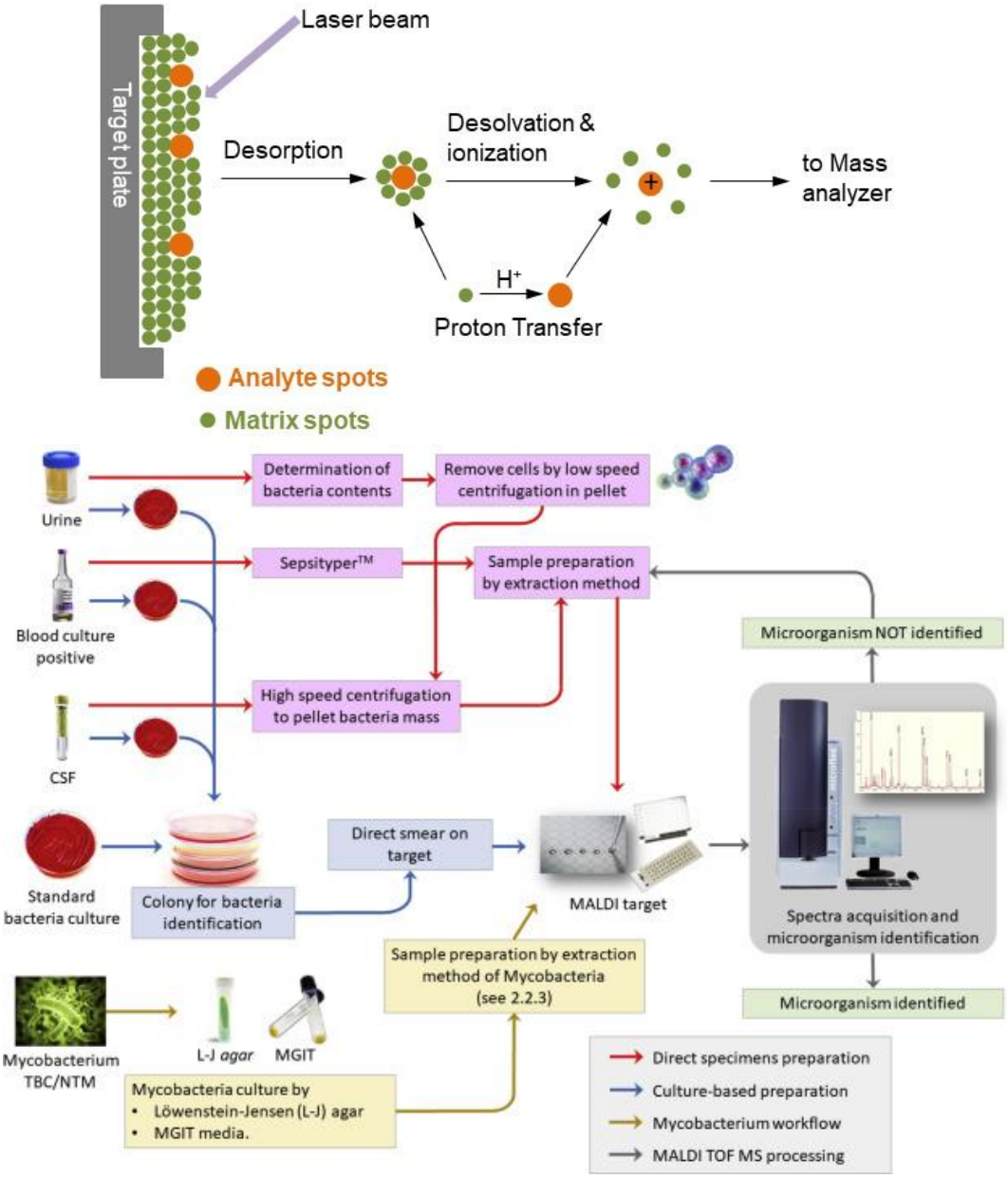
COVID-19 VS PCR

Significant importance of PCR was witnessed during COVID-19 Pandemic for the detection of SARS-CoV2 and for viral load estimation. Amplification of RNA from Nasopharyngeal swabs and from other respiratory specimens using Reverse Transcriptase PCR (RT-PCR) provided reliable results both in symptomatic and asymptomatic carriers, playing a significant role in infection diagnosis (24) In diagnostic SARS-CoV-2 assays, RT-PCR is based on detection of number of distinct genetic fragments of virus in an individual. Number of gene fragments is determined semi quantitatively through Cycle Threshold (Ct) value. Ct value increases with a decreasing viral load and contrary to that a low Ct value indicates high viral load (Velavan and Meyer 2020). An interlink exists between PCR results and possibility of virus culture with respect to Ct values and certain guidelines need to be implemented to execute recommendations such as inclusion of antigen tests, determination of RNA copies on the self-isolation of health care workers, people in quarantine zones.

In a nutshell NAATs and PCR and its modifications have redefined the current diagnostic era providing unparalleled accuracy and efficiency. But its optimal use requires trained personnel, integration with clinical data and continuous cyclical upgrade is essential to overcome the existing limitations.

Role of MALDI-TOF

Invented by "Franz Hillen Kamp" and "Michael Karas" in 1985, while the coupling of "Time of Flight" Mass Analyzer for large macro molecules/ proteins and their analysis was pioneered by "Koichi Tamaka" in 1987. MALDI-TOF MS is a novel technique in clinical microbiology for the isolation and identification of microbes (Lay Jr, 2001). Its underlying principle is based on the application of Nitrogen Laser beam and separation based on m/z (mass: charge) ratio and time taken for ions to reach detector at the end of TOF tube will be measured. Major positive aspect in using MALDI-TOF MS is its ability to detect microbes directly from the bodily fluids/ clinical specimens, its capacity to detect and measure any biomolecule which is ionized (Patel etal 2015). Owing to its high sensitivity, speed, versatility, MALDI-TOF is cornerstone of Mass spectrometry and is essential in modern research in microbiology.



MALDI-TOF coupled with Artificial Intelligence has hastened and enhanced the precision of pathogen detection in clinical microbiology . In present scenario MALDI-TOF is accurate, cost effective way to identify and interpret cultured bacteria and fungus , better than traditional bio-chemical methods. This technology has emerged as a novel method to detect anaerobic bacteria, Mycobacteria also. But it does have its own constraints with respect to Species identification.

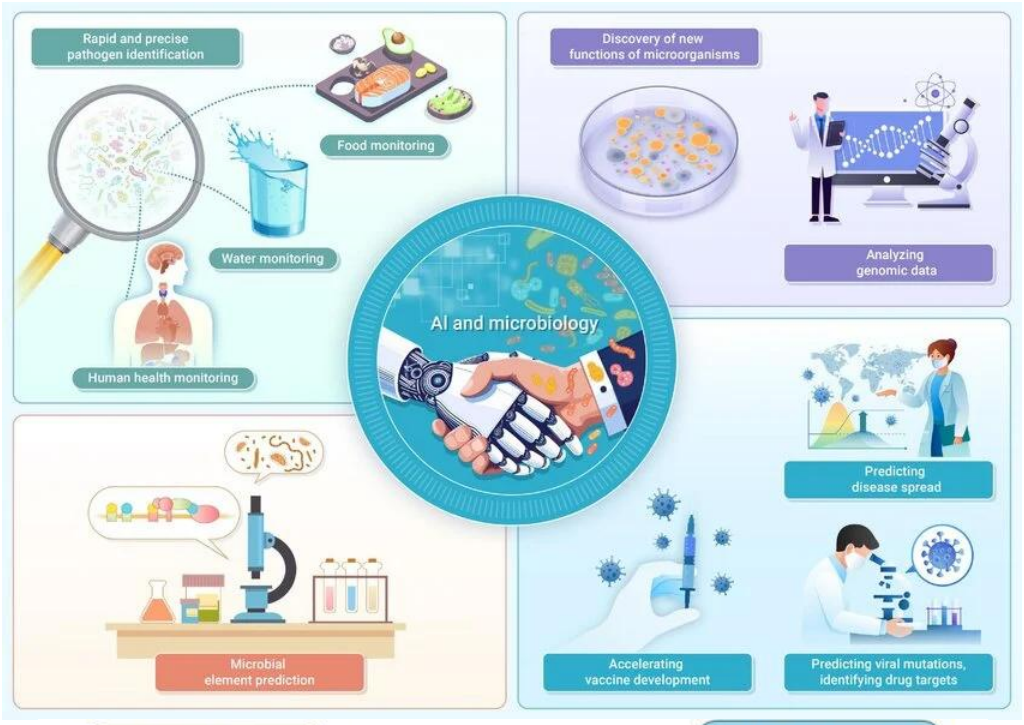
Artificial Intelligence in Microbiology

AI emerged as a pivotal tool in clinical microbiology focused mainly on the interpretation of image. Based on digital images captured by Automated machines bacterial colony classification would be done by Convoluted Neural Networks (CNN)(25). The ability of AI to analyze extensive information provides new avenues for microbiological diagnoses and research. Because of its potential to address public health challenges including diagnosis of infectious diseases, sepsis, vector borne diseases, AI assists not only in diagnosis , but also in monitoring prognosis and implementation of treatment strategies (26). Artificial Intelligence facilitates the identification of possible treatment targets through the analysis of microbial genomes, Proteomes etc. AI enhances the drug development process by providing the binding of compounds to microbial targets and enhancing the selection of candidate drugs for clinical experimental evaluation (27). Automated systems such as "Automated Plate Assessment", PhenoMatrix have augmented the examination of cultures in Urine specimens and facilitating the identification of MRSA strains in health care associated infection outbreaks (28).

AI based algorithms can detect the genes responsible for anti-microbial resistance (AMR) by genetic sequence analysis of a specific pathogen. AI subsets mainly Machine Learning (ML) and Deep Learning (DL) are useful to handle the challenges posed in AMR domain through in depth analysis of large amount of data in hospital based settings . AI opened new vistas in reducing time for disease diagnosis from days to hours and detecting the novel genes responsible for Anti-Microbial Resistance (AMR) and also in tracing out Mutations (29).

AI based Infection Prevention and Control Measures

Application of analyzing complicated datasets from Electronic health records is of significant importance to monitor infection trends and evaluating infection surveillance. AI has profound impact in diagnosing Tuberculosis by Deep Learning application on Chest Radiographs. Many advanced laboratories utilize AI coupled microscopy and Machine Learning algorithms to deliver accurate diagnosis and precise treatment (30)



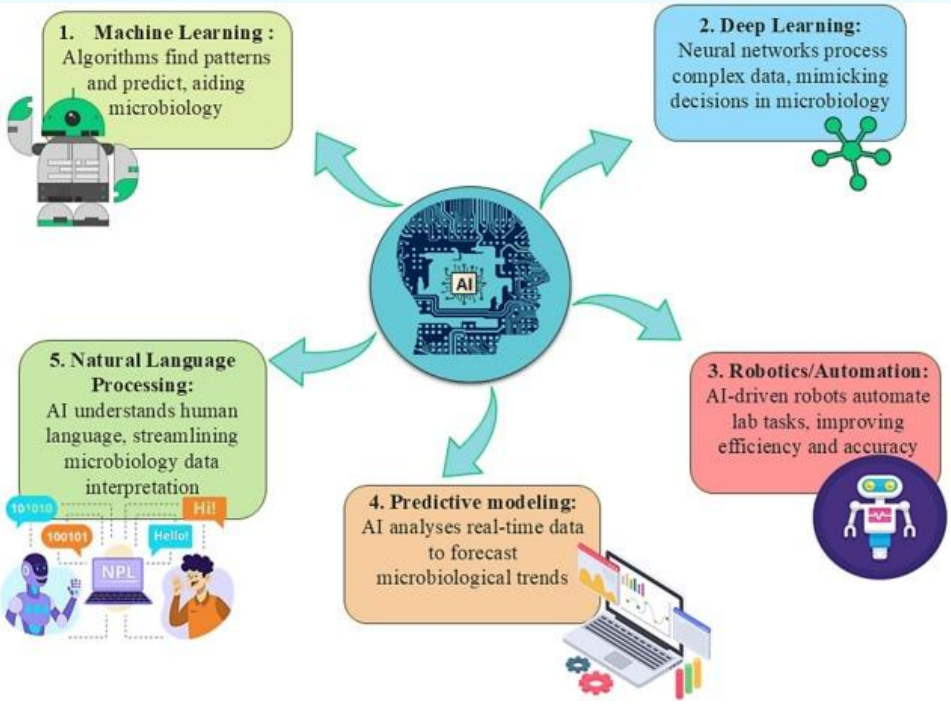
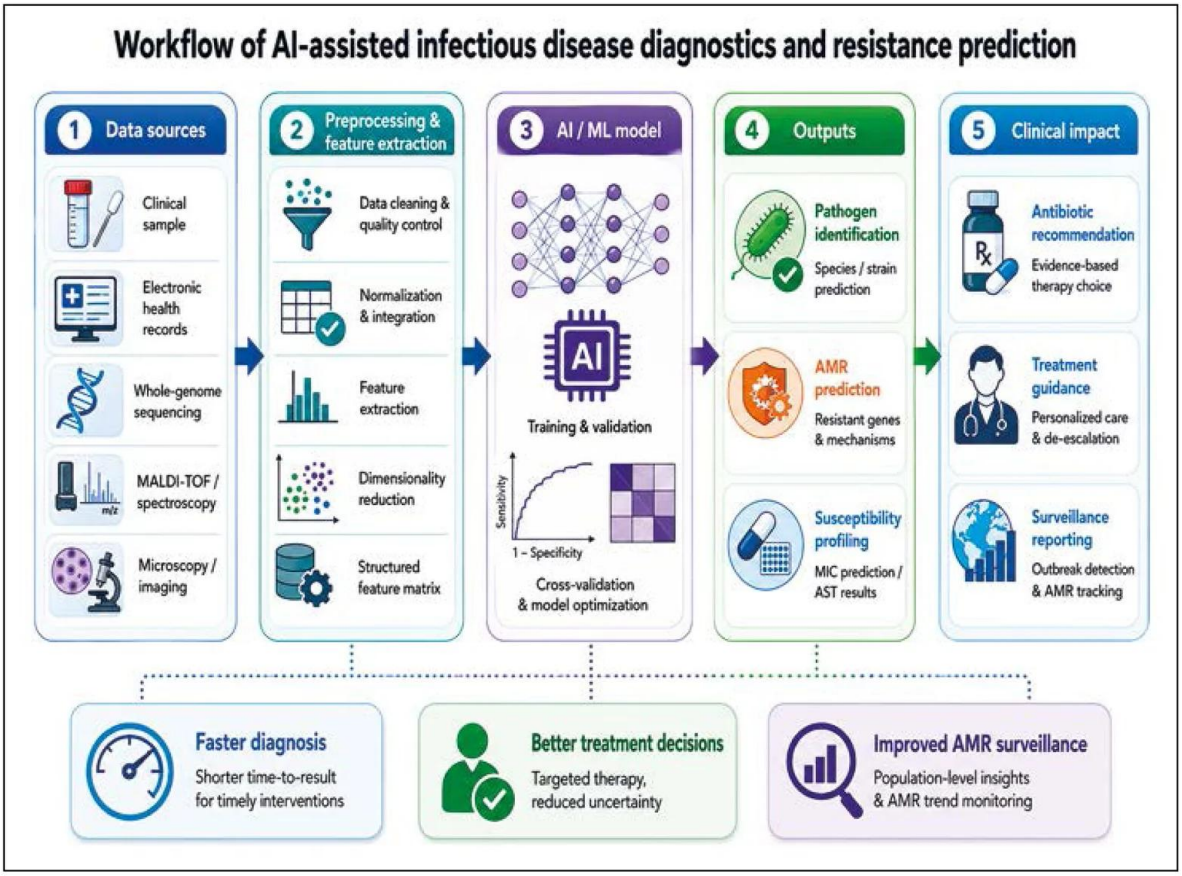


Table depicting Merits and challenges in AI application

Merits	Challenges
Cost effectiveness: AI reduces labor cost, resource utilization and provides cost effective health care	Establishment and installation is a huge task and expensive. Repeated training is essential for people involved in research using AI
Early detection of outbreaks, drug resistant genes and provides timely intervention	Ethical and legal issues are a major concern and maintaining patient privacy, data safety are of immense importance
Consistent, verifiable results by reducing human errors and more safety and successful treatment	Integration with the existing health care infrastructure is a potential challenge
Integration and data analysis from multiple sources	Continuous internet facilities and UPS should be provided to all the staff having keen to learn AI models in health care . But some of the medical colleges, research institutes lack this facility
ML, DL models/ algorithms are useful in detecting drug resistant genes, surveillance studies (e.g: SSI Surveillance)	Some of the health care professionals, medical faculty are reluctant in learning advanced technology which has become a mandatory in the present competitive world.



II. Conclusion

For more than a century traditional culture-based methods were pivotal in diagnostic microbiology of which even today some of the techniques were irreplaceable. As years passed an era of automation seeded in health care field which in a span of time lead to the onset of Molecular diagnostics and at present, we are in the epoch of "Artificial Intelligence" , a technology driven system in medical sciences. To cite as an example, isolation of M. tuberculosis bacilli from clinical specimens usually takes more than 4 weeks through conventional methods of culture on LJ medium. But at present through Gene Xpert Mycobacterium tuberculosis complex and detection of drug resistance gene to Rifampicin (MTB/RIF) PCR assay produces results within 2-3 hours. Detection of broad range of pathogens from stool specimens with in 2 hours by Multiplex PCR outclasses much time-consuming cultures and false reports. These indicate how advanced diagnostic techniques enhanced the efficiency of diagnostic microbiology in patient care and research. A mutually reinforcing approach is essential, as cultures deliver viable isolate which is required for Phenotypic methods like antibiotic sensitivity testing. At present clinical microbiology in health care is at the conjunction of conventional testing, implementation and execution of advanced methods to whole level automation and AI.

From academic pont of view these novel methods pose considerable enigma in both the work force and in classroom. Problem in implementation of novel diagnostic methods with AI also exists apart from multiple benefits, as most of the health care professionals were more broadly exposed to conventional methods and lack

adequate skill and knowledge on mathematical algorithms using AI . The upper hand of novel diagnostic methods lies in their ability to detect key resistance markers such as rpoB, mecA, vanA/B, blakpc, KatG ..directly from clinical specimens enhancing faster, accurate treatment than phenotypic based AST.

In near future, fusion of AI and ML into the analysis of molecular diagnostics will make disease diagnosis more accurate, faster and can deliver fruitful results , especially in the analysis of NGS, Metagenomics which can produce immense quantity of data.

Molecular diagnostics in the present demanding world remain as core components in infectious disease diagnosis, AMR Surveillance and provides improved decision making clinically. Advances in PCR, Mass Spectrometry, LAMP, CRISPR (Clustered regularly interspaced short palindromic repeats), NGS have reduced the turn around time, delivering more sensitive and optimistic results.

To conclude, future will be more excited to visualize Medical microbiology coupled with technology driven methods with much more broad scope for engineering and medicine as dual courses together. Many research institutes in India like IITs, IISER, National level medical universities are offering courses to nurture the knowledge and augmented research in India.

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