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# DNA Fingerprinting: Advancements and Future Endeavors

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*Editors*
Hirak Ranjan Dash
DNA Fingerprinting Unit
State Forensic Science Laboratory
Sagar, Madhya Pradesh, India

Pankaj Shrivastava
DNA Fingerprinting Unit
State Forensic Science Laboratory
Sagar, Madhya Pradesh, India

Braja Kishore Mohapatra
Department of Biology and DNA
Fingerprinting Unit
Central Forensic Science Laboratory
New Delhi, Delhi, India

Surajit Das
Department of Life Science, National
Institute of Technology
Rourkela, Odisha, India

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# Contents

# Chapter 9
# Molecular Diagnosis of Enteric Bacterial Pathogens

**Amita Shrivastava, Pradeep K. Singhal, and Pankaj Shrivastava**

**Abstract** Bacterial strains belonging to family *Enterobacteriaceae* are well-established enteric pathogens of humans and animals which are the major cause of mortality worldwide. So to deal with these infections, it is necessary to identify the disease-causing pathogens along with their virulence mechanism. The characterization and detection of these pathogens rely on conventional culturing and biochemical techniques. But these conventional techniques are low sensitive and time intense. This led to search for more rapid, sensitive, and advanced technique for their detection. In the present scenario, the molecular methods are most commonly applied to identify the pathogenic bacterial strains. The molecular methods include DNA-based methods, such as restriction endonuclease analysis of genomic and plasmid DNA, plasmid profiling, chromosomal DNA profiling using pulsed field gel electrophoresis, polymerase chain reaction (PCR)-based methods, 16S rRNA sequencing methods, polyphasic taxonomic approaches, etc. These are the more rapid and more sensitive techniques which overcome the issues of identification by conventional techniques. Hence, these techniques are considered the most promising for bacterial identification.

**Keywords** Conventional identification techniques · Enterobacters · Molecular techniques

A. Shrivastava · P. K. Singhal (✉)
Department of P. G. Studies and Research in Biological Sciences, Rani Durgavati University, Jabalpur, Madhya Pradesh, India

P. Shrivastava
DNA Fingerprinting Unit, State Forensic Science Laboratory, Sagar, Madhya Pradesh, India



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## 9.1 Introduction

Microorganisms are ubiquitous and extraordinarily diverse and accomplish various roles in the environment. They occurred broadly in the biosphere, foods, water, and soils and exogenously as well as endogenously in humans and animals. The microbial population of human intestinal tract comprises of a huge diverse of bacterial, viral, and parasitic forms including aerobic and anaerobic organisms. Majority of these organisms belonging to family *Enterobacteriaceae*, including *Escherichia coli*, *Shigella* sp., and *Salmonella enterica*, but some others such as *Pseudomonas* sp. also inhabit human gastrointestinal tract. Many of these organisms have beneficial effects on their hosts, yet certain conceivably harmful microorganisms can have significant negative consequences on both animals and human. It is accounted for that pathogenic infections cause around 40% of the roughly 50 million aggregate yearly passing around the world [1, 2].

Among enterobacters, widely dispersed species in nature is *Salmonella*. They are gram-negative, rod-shaped, facultative anaerobic, nonspore-forming motile organisms that can grow best at temperature extended from 7 to 48 °C and pH ranged from 6.5 to 7.5. They can dwell as common commensal in the gastrointestinal tracts of animals and humans which may be the reason of several infections such as diarrhea, bacteremia with enteric fever, or invasion of vascular structures, bone, or other localized sites [3]. They are additionally a reason for foodborne sickness around the world and a cause of livestock infections that can be transmitted from animals to humans. Another enterobacter, *Escherichia coli*, is a gram-negative, rod-shaped bacterium which typically resides in the lower intestinal tract of humans and animals. Its pathogenic strains may be the reason of a few diseases such as gastroenteritis, urinary tract infection, meningitis, peritonitis, and septicemia [4].

Enteric contamination-related illness can be caused by live enteric pathogens (such as bacterial dysentery), by toxins which they produced (such as staphylococcal food poisoning), or by combined both toxins and live agents (such as cholera). From these, a few pathogens act primarily through infections at the mucosal surfaces of the gastrointestinal tract (such as *Vibrio cholerae*), whereas others are able to cause systemic infections (such as *S. enterica* subspecies *enterica* serovar *Typhi* or *S. typhi*). Along these lines, the pathogenesis, etiology, and thusly resistance of these diseases are varied. These diseases are transmitted by contaminated food or water and usually lead to symptoms like diarrhea. Many sound peoples will encounter a self-constrained disease that endures a couple of day, yet in others, severe dehydration, bacteremia, chronic symptoms, and serious complications can develop, including malnutrition and death [5].

Identification of these infectious microbes is necessary to evaluate their association and sources of infection for treatment of diseases they cause. There are various types of methods that have been used for this purpose. The conventional methods including antibiotic resistance patterns, biochemical reactions, bacteriocin typing, and phage typing are normally less efficient, time-consuming, and costly. Serotyping may be considered as the reference method for strain characterization [6], but it may not discriminate the organisms originating from diverse regions [7]. These methods

for detection of enteric pathogens have several limitations and require technical expertise and subjective interpretation. A maximum number of pathogenic bacterial strains are identified by culture methods. Routine stool cultures are carry out to identify the existence of *Salmonella* sp., *Shigella* sp., *Campylobacter* sp., and *E. coli* O157, consuming at least 3–4 days. Due to this delayed diagnosis, patients are at risk for untreated infections, and the diseases can be transmitted to others. Further, *Campylobacter* species need specific conditions in the culture medium to regain viability, but non-culturable forms of *Shigella* failed to regain viability in the medium, adding to limitations of sensivity of culture techniques [8]. The closely related pathogenic and nonpathogenic strains of *E. coli* could also not be reasonably differentiated by culture techniques. Generally adopted techniques to detect and identify *E. coli* are culture, fermentation, enzyme-linked immunosorbent assay, and PCR. The conventional culture techniques, therefore, has many disadvantages such as biased, laborious, long and resource-intensive assay protocols, highlighting necessity of quick, relaible and sensitive techniques to identify and manage pathogenic enterobacters [9, 10].

Molecular techniques such as multilocus polymerase chain reaction (PCR), DNA sequencing, enzyme electrophoresis, biotyping, restriction endonucleases analysis, ribotyping, pulsed field gel electrophoresis (PFGE), nucleotide sequence analysis, protein analysis, and plasmid profiling have been successfully adopted in the recent past to supplement culture techniques. These are the most successful techniques that are rapid and sensitive. Among these molecular techniques, plasmid profile analysis [11, 12], random amplified polymorphic DNA analysis (RAPD) [11], repetitive extragenic palindromic sequences analysis by PCR (rep-PCR) [13], and pulsed field gel electrophoresis (PFGE) [10, 14, 15] are generally adopted and considered promising for bacterial identification.

We endeavor to acquire a multidisciplinary approach for the detection of virulent agents belonging to family *Enterobacteriaceae* and control infectious diseases. The main focus of this chapter is to compress the quick and sensitive identification techniques for pathogenic strains which concentrated specially on the identification of species-specific DNA sequences (molecular techniques).

## 9.2 PCR-Based Typing of Enteric Pathogens

It is one of the most commonly used molecular technique that can discriminate microbial strains in the analysis and epidemiological studis of pathogenic diseases. It is an accurate, reliable, and short time taking method to amplify a specific gene or DNA fragment within the genome of a species [16]. This is a powerful molecular strategy as it is able to amplify the smallest amount of particular microbial DNA sequences. PCR offers assortment of techniques for with numerous analytical and epidemiologic investigations such as PCR-RFLP, PCR-ribotyping, random amplification of polymorphic DNA (RAPD), repetitive extragenic palindromic (Rep)-PCR, enterobacterial repetitive intergenic consensus (ERIC)-PCR, PCR sequencing, and

other techniques [17–20]. In PCR, the targeted genes have been increased logarithmically in the number of copies which can be later detected on ethidium bromide-stained gels. On the basis of size (mass) and migration of the PCR product (amplicon), we can detect the expected target in the sample which later confirmed by the sequencing of the amplicon [21].

The advanced methods to detect enteric pathogens are based on specific nucleotide primers designed for PCR. This is a very fast and quick technique to detect and identify the enteropathogens; however, the conventional identification techniques are considered prolonged, too variable, and labor intensive and have several limitations. Previous studies also showed that PCR is quite high sensitive and specific strategy to identify enterobacters in stool sample as compared to that by the other conventional methods [22–24].

PCR techniques has a few constraints, for example, it is not capable to distinguish between viable and nonviable microorganisms, and the PCR inhibitors can lead to false-negative outcomes [25]. Other than the development of direct hybridization methods, there are several non-PCR-based techniques to amplify DNA like nucleic acid sequence-based amplification (NASBA) and strand displacement amplification [26, 27].

## 9.3 Gel Based Typing of Enteric Pathogens

The method of pulsed field gel electrophoresis (PFGE) had been introduced in 1984 [28] and has since been considered as a gold standard method to identify microorganisms and epidemiological investigations [29]. Among molecular techniques, it is the most reliable, precise, and reproducible method as well as presently being utilized as a part of international surveillance programs. It is a DNA-based subtyping method that creates DNA banding patterns. Initially, some rare restriction enzymes cut the DNA into short segments, generating 8–25 large bands. Later, the DNA segments are subjected to electrophoresis on agarose gel for their separation on the basis of their size, and to diminish sheering of large DNA segments, the current have been applied at alternating angles. The selection of restriction enzymes ensures the generation of adequate size DNA segment for electrophoretic separation (1000–15,000 base pairs) along with suitable number of bands. Comparative study of multiple enzyme patterns explains the existence of new species, thus enhanced the power of discrimination of this method [30].

PFGE has been used successfully for characterizing *E. coli* [31, 32], *Salmonella*, *Listeria*, and other pathogens. In PFGE, the restriction pattern concerning the entire bacterial genome is studied barring the use over probes. However, point mutations, deletions, insertions, and loss or acquisition of plasmids may elucidate minute variations in profiles inside a subtype or between epidemiologically homologous strains. These progressions for the most part result in a few fragment contrasts in PFGE banding patterns. PFGE is a very successful method to identify enteric pathogens besides the fact that it has some disadvantages too. It is distinctly expensive and

needs more than 3 days getting the outcome. The level of separation likewise relies upon the selection of restriction enzymes. A further drawback of this technique is poor portability. Specialized issues with PFGE change in their unpredictability and simplicity of analysis. They can incorporate the feeble power of banding designs because of low cell concentration, art factual bands because of inadequate processing of DNA, skewed lanes because of faulty electrodes or uneven gel thickness, and additionally buffer height because of uneven surfaces utilized for gel casting or electrophoresis. Some serotypes, especially those with certain distinct phage types, can be so genetically homogeneous that multiple genotypic techniques fail to discriminate outbreak from non-outbreak strains [33].

## 9.4 Ribotyping

For the bacterial identification, the most commonly used technique is PCR, and it can be utilized successfully by focusing on signature sequences of the target DNA. In any case, it is typically hard to discover particular primers that have a solitary target and do not yield any non-specific amplification. This technical issue can be overwhelmed by another technique termed as PCR-ribotyping which is generally an adopted technique to characterized bacterial strains. The diverse bacterial species comprises highly conserved ribosomal gene in respect to other bacterial strains. This feature is very beneficial to describe the genetic relationship of diverse bacteria species. The rRNA coding sequence fingerprinting defines about the hybridization of restriction-digested DNA fragments along with probes specific for rDNA [34]. In this method, a multiple band pattern is generated which is extremely prejudicial and can be utilized for affirmation of microorganisms up to species level as well as in many cases even beyond.

rRNA gene sequencing has been an exceptionally authentic technique to characterize bacterial species, yet it has insufficient heterogeneity for more advance classification. PCR-ribotyping relies upon the spacer regions or IV (intervening sequence) amplification between 16S and 23S RNA genes. The inconstancy in length and copy number may facilitate classification of several bacterial strains and mycoplasma [35, 36]. For instance, in a previous report, PCR-ribotyping had identified seven serovars of *Salmonella* sp. [37]. This method has replaced the conventional PCR for affirmation of divergent species as it subsequently convoluted by non-specific amplifications and hence required supporting morphological and biochemical information.

Ribotyping is actually capable to subtype a few microbial isolates that fall inside some basic serotypes and phage types [38]. Lin et al. [39] distinguished seven diverse ribotypes among *S. enteritidis* isolates when chromosomal DNA was treated with SphI. Fernandes et al. [40] demonstrated that ribotyping is more appropriate for following the diversity of *S. enteritidis* and the restriction endonuclease SphI distinguished finest within subtypes of this serotype. Dambaugh et al. [41] obtained discrete patterns for the most widely recognized serovars of Salmonella species by using PvuII (restriction enzyme).

Automated ribotyping has been shown as being useful for the identification of various bacterial spp. [42–44] and successfully applied for the identification of enterobacters. As compared to conventional PCR, this technique is more reliable as it requires a single primer as opposed to setting up reactions for every species. Thus, odds of false-negative outcomes because of non-specific amplification are decreased. Comparison study of ribotyping with PFGE has been to some degree flighty and frequently relies upon the enzymes utilized for digestion and also the idea of the population nature.Various researches have found that PFGE has more discrimination power as compare to that by ribotype analysis [45] whereas others have found these two techniques equivalent [46] or ribotype analysis superior [47]. Moreover, this technique is fast, more reliable, and highly reproducible and can be successfully applied for bacterial characterization. However, it has some disadvantages too. This method has more influence in epidemiological applications yet has low influence in the quick identification of pathogenic microbes [48]; however, ribotyping is considered as not suitable for local epidemiological applications or surveillance studies in a restricted region [49]. Further, important disadvantages of automated ribotyping are the need of expensive reagents per isolate and the high cost of the automated RiboPrinter itself.

## 9.5 DNA Sequence-Based Typing Methods

The DNA sequencing provides data regarding the degree of genetic diversity and population structure of the bacterium of interest. This strategy is considered as reproducible and easy transposable between laboratories [50, 51]. The following are the commonly used DNA sequence-based typing methods.

### *9.5.1 Variable Number of Tandem Repeat (VNTR) Analysis*

Genomes of several bacterial species have regions with repetitive DNA sequence motifs extending from a couple of bases to more than 100 base pairs in length. The repetitions of sequences termed as tandem which means a number of copies of each of the repeat motifs are clustered together and arranged in the similar direction. These repeats in a tandem can be highly variable, even between strains of the same species. It is referred as PCR-based method that depends upon the DNA amplification that includes short tandem repeats (STR) of a DNA sequence. PCR primers are intended to anneal non-repetitive sequences simply outside the repeat region, and amplified products are isolated and estimated to detect the number of repeats present in the amplicon.

In this technique, variations in the number of repeated copies at particular loci were used to differentiate the microbial isolates. As a result of moderately high mutation rate, strains can consolidate specific patterns in a comparatively small

duration of time [52]. Several regions of repeated motif may be investigated in the meantime to increase expanded differentiation to analyze genetic diversity. The most widely recognized strategy utilizing multiple VNTR loci for typing is termed as multiple locus VNTR analysis (MLVA) or MLV fingerprinting (MLVF) [53].

### 9.5.2 Multilocus Sequence Typing (MLST)

The MLST technique, a molecular typing approach, introduced since 15 years is for the most part performed on seven housekeeping genes. It analyzes DNA sequences from regions of housekeeping or virulence genes or potentially rRNA sequences which changes because of mutation or recombination events [54]. In a particular gene, the nucleotide variations are merged and used in the determination of discrimination between the strains [55]. MLST gives information like those got by multilocus enzyme electrophoresis, yet in substantively more noteworthy detail, since it can survey particular nucleotide changes as opposed to screen for changes in the overall charge and expression of the enzyme under study [54]. In MLST, a sequence type (ST) can be defined as sequencing the allelic group including the isolate for each of the genes. Relatedness between STs can be disclosed through various techniques of clustering: (a) the unweighted pair group method with arithmetic mean, using distance matrices containing the pairwise differences of allelic profiles; (b) the minimum spanning tree approach, constructing a tree that interfaces all sections such that the summed distance of all connections of the tree is the shortest, i.e., minimum; and (c) the base upon related sequence types (eBURST or the more recent global optimized version, goeBURST), algorithm, inferring patterns of evolutionary descent among isolates by a model of clonal expansion and diversification and assigning isolates to clonal complexes [56].

This technique is to a great degree valuable for protracted epidemiological or phylogenetic analyses. More than 230 *Salmonella* isolates were lately described by MLST in light of sequences from the 16S RNA, *pduF*, *glnA*, and *manB* genes [57]. These outcomes were contrasted with PFGE and serotype analysis. MLST could discriminate strains superior to others like PFGE; however, not all genes performed similarly. For MLST to be compelling as an epidemiological tool, the criteria for the selection of genes and their number should be satisfactory for the discriminations of the isolates with later genetic diversity. In this case, genes under greater selective pressure, such as virulence genes, might be giving a superior outcome. For this situation the technique is commonly termed as multivirulence-loci sequence typing (MVLST) [58].

MLST demonstrates incredible guarantee for exact strain differentiation with information that can be precisely shared between research labs. Be that as it may, general interest of this approach will be enhanced when automated sequence apparatus turns out to be more moderate and labs can generate familiar environment with complicated DNA sequencing and statistical software sequencing. This technique is very tedious and exorbitant yet can be exceptionally oppressive if the genes are

accurately chosen. It has been effectively utilized worldwide in the study of disease transmission and population genetic studies of many gram-positive and gram-negative bacteria. It is less appropriate for routine typing in outbreak investigations or local surveillance studies on account of its moderately low discriminative power, high cost, and workload.

### *9.5.3 Sequencing of 16S rRNA Gene*

The 16S rRNA gene sequencing, a universal method, has been widely proposed to identify microorganisms and detect new species [59]. In all living cells, ribosomes are present which are involved in protein synthesis mechanism. They consist of two subunits, and each subunit is composed of protein and ribosomal RNA (rRNA). In prokaryotes 70S ribosome is present which consists of 30S subunit (small subunit) and 50S subunit (large subunit). The 30S subunit is made out of 16S rRNA and 21 polynucleotide chains, while 50S subunit is made out of 5S and 23S rRNAs. The 16S rRNA contains 16S rRNA gene which gives a species-specific signature sequences, and this signature sequences are very important in bacterial identification. The signature sequences are unique DNA sequences (five to ten bases long) to numerous major groups of prokaryotes, archaea, and eukarya which are found specifically in the 16S rRNA location in all groups of organisms. The average lengths of the structural rRNA genes are 1522 bp, 2971 bp, and 120 bp, respectively, for 16S, 23S, and 5S rRNAs.

There are numerous focal points of utilizing ribosomal RNA in identification methods as they are present in all cells; RNA genes comprise of highly conserved nucleotide sequences, mixed with variable regions that are genus or species specific, and there is no need to culture the microbial cells [60]. All bacterial species contains 16S rRNA gene [61], so this technique can be used as gold standard for bacterial identification up to species level. This technique may ensure reclassification of bacterial species into into new genera [62, 63]. This method might be likewise valuable in studying the relationship of unidentified species to known ones and in the discovery of new species.

The members of *Enterobacteriaceae* family could be identified up to 80.1–94.4% through the Vitek GNI+ and API 20E systems. Some members of this family like *E. coli* are difficult to distinguish; in such condition 16S rRNA sequencing should be employed which is reported as a significantly better technique compared to that by others [64]. Subsequently, bacterial strains can be characterized accurately by nucleotide sequencing of the PCR product and comparing it with known sequences put away in a database [65]. It can likewise be utilized to distinguish microorganisms that are hard to culture or be applied to samples from post-antibiotic treatment [66–68].

### 9.5.4 Whole Genome Sequencing

The sequencing of the entire genome of a microbe and comparing the sequenced genome with known references are referred as whole genome sequencing (WGS). This is a key approach in molecular methods for genome mapping of novel species, finishing genomes of known organisms, to compare genomes over diverse samples, to generate more precise reference genomes, and to identify microbes and other genomic analyses. Presently, this technique is considered as reference microbial typing method to enhance knowledge and understanding of infectious diseases and clinical microbiology [69].

The members of family *Enterobacteriaceae* are well-known human and animal pathogens including *Escherichia coli*, *Salmonella enterica*, *Yersinia*, *Shigella*, etc. They cause various diseases in humans specially diarrhea which is the second biggest killer of children globally and most common problem in human beings. WGS of multiple strains of these bacterial pathogens has contributed immensely to our understanding of the high level of similarities and differences among closely related organisms at sequence level. By WGS of closely related species, we can distinguish genes, characterize gene structure, highlight fast as well as slow evolutionary change, recognize regulatory elements, and disclose combinatorial limit about gene regulation. Hence, it can be concluded that the WGS approach offers the potential to understand and differentiate the basic pathogenic mechanisms employed by different pathogenic bacteria.

## 9.6 Polyphasic Taxonomic Approach

In modern technology a new technique has been employed for classifying microorganisms taxonomically and phylogenetically to distinguish bacterial species which is known as polyphasic taxonomy approach. This technique is first proposed by Rita Colwell [70]. The phenotypic, genotypic and chemotypic, technologies to elucidate the taxonomic position of microbes together are termed as polyphasic taxonomy approach. Recently, this is the most prominent technique for identification and classification of microorganisms. The microorganisms as were partitioned beneath invalid taxa have recently been partitioned under new genera and species kind through this strategy. This approach is coupled with various phenotypic, genotypic, and chemotypic characters and is more advanced than other techniques. The main advantage of this approach is that it reduces the burden of misidentification as well as incomplete ID and offers authenticate identification of microbes.

It was observed that the phenotypic characters often among genetically different species and molecular methods alone are not capable to distinguish phylogenetically related species. So this technique is more reliable to discriminate microbes accurately.

## 9.7 Pros and Cons of Molecular Typing Methods

Previously used cultural and biochemical methods for microbial typing are too variable, labor intensive, and time-consuming and provided unreliable results, but the recently used molecular methods for detecting and typing of bacterial pathogens are fast, labor reducing, more touchy, particular, and proficient which provides reliable data for tracing the bacterial pathogens.

In spite of the fact that the utility of molecular approaches will emphatically influence the diagnosis of diseases caused by enterobacters, there are a few impediments. One burden of any non-culture strategy is the unavailability of the microbial isolates for antibiotic susceptibility test or any other tests by public health laboratories. This will frustrate the public health laboratories' capacity to give data concerned subtyping to the studies of epidemiology, thusly restricting their capacity for determining and characterizing outbreaks. A further impediment of this single-stool work process includes the identification of certain ova or parasites that are not accessible on molecular testing menus. Even though these parasites are uncommon, they will show the necessity of microscopy for their identification. One final aspect in utilizing molecular procedures for enteropathogens is the noteworthiness of the discoveries when the approach can't discriminate viable and nonviable pathogens [71]. Obvious indicators of viability have been the isolation of enteropathogens from stool sample. However, the importance of the nucleic acid of enteropathogens presented in stool samples and their concern with infections are not understood so far. Additionally the researches are expected to address the relationship of these discoveries. Molecular approaches can likewise recognize other bacterial species that are not identified with conventional culture techniques. The expanded affectability of this approach leaves open inquiries with regard to the hugeness of the discoveries. Present information about intense gastroenteritis including analysis and the study of disease transmission depends on traditional cultures and microscopic examination. The pattern might change as we push far from culture toward fast non-culture techniques.

## 9.8 Conclusions

Numerous approaches are accessible to distinguish bacterial strains; however, each of them has its focal points and impediments that make it valuable in a few examinations and prohibitive in others. A perfect strategy ought to satisfy the accompanying six criteria are the following: type ability, reproducibility, discriminatory power, simplicity of elucidation, simple to utilize, and minimal effort. It is clear that any technique utilized as of now for the identification of enteric bacterial strains is a perfect strategy alone as far as these criteria, yet all strategies display benefits and furthermore confinements. Clearly it is hard to locate a solitary approach, which is most appropriate to identify and characterize microbial strains.

Amid most recent couple of decades, the quality of molecular science has altered biological sciences. The molecular approaches have opened another part to characterize diverse bacterial strains. These approaches can distinguish to a superior degree than phenotypic techniques and enhance our insight into genetic and epidemiological relationships. Conventional research center testing techniques depending on microbial morphology and growth factors are progressing to a work process structure where molecular testing is fit for distinguishing pathogenic strains. The molecular testing industry is promoting extended menus and instruments that empower research facilities of any size to solidify testing on a solitary stage. The capacity to automate sample preparation, extraction, and amplification on a solitary framework spares time and enhances lab effectiveness while enhancing institutionalization of test outcomes. The decision of a molecular typing method will rely on the expertise level and assets of the research center/laboratory and the point and extent of the investigation.

A change from the conventional research center testing approaches to molecular approaches will substantially affect the lab too. The workload in conventional methods is relentless and tedious. Molecular techniques will decrease workload and material prerequisites as well as dispose of the subjective elucidation concerned with culture methods. Albeit molecular approaches are expensive, their capacity to identify various pathogenic strains with one test board is more savvy than carrying out numerous tests utilizing conventional techniques.

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