

# Research and Reviews: Journal of Agriculture and Allied Sciences

## Modern Identification Methods of Bacteria.

Raheesa M Khatib<sup>1\*</sup>, Veena<sup>2</sup> and Neelamma Konanavar<sup>1</sup>.

<sup>1</sup>Department of Plant Pathology, UAS, GKVK, Bengaluru - 560065, Karnataka, India.

<sup>2</sup>Department of Plant Pathology UAS, Dharwad- 580 005 Karnataka, India.

### Review Article

Received: 03/03/2014

Revised : 21/05/2014

Accepted: 28/05/2014

#### \*For Correspondence

Department of Plant Pathology, UAS, GKVK, Bengaluru - 560065, Karnataka, India.

**Keywords:** Covered smut, sorghum, botanicals, leaf extract, cattle urine

#### ABSTRACT

The early diagnosis of disease is essential to reduce the diseases from the stage of seedlings to harvest in order to grow healthy crop to get maximum yield. Detection of bacteria is tracing of plant pathogenic bacteria in or on plant material, especially when they occur latently, without causing symptoms. But identification means isolation, characterization and naming of bacteria. It is an important step in the diagnosis of bacterial infection and the taxonomic studies. There is no exclusive or reliably simple method of identifying pathogen and the disease they cause. The traditional identification methods are visual inspection of pathogen *in situ* or *in vitro* in pure culture by microscopic examination stain reaction, colony characters, oxygen requirement, physiological characters biochemical characters and serological methods. The identification by bacteriophage technique can be considered as both traditional as well as modern. Less specificity and less discrimination at species and strain level is the drawback of the conventional identification methods. In recent years completely newer identification techniques have been developed. This is mainly due to the development in molecular biology, unraveling the structure and function of microorganism. Knowledge of the composition and the place of structural elements in microorganism has made the real finger printing methods possible such as special serological methods like Immuno-electrophoresis, Monoclonal antibodies, Immunogold labeling. Identification by separation of bacterial components using chromatographic techniques (FAA Analysis by Gas chromatography), separation of bacterial protein (PAGE). The nucleic acid based identification methods are more important because it is purely on genetic basis. Fluorescent *In situ* Hybridization (FISH), developed by Christoph Lengauer, based on the DNA-DNA hybridization. RFLP based on the Southern hybridization. One of the important development in genomic studies is the discovery of PCR by Kary Mullis in 1985. The PCR based identification methods are RFLP-PCR, PFGE, RAPD, REP-PCR, AFLP, Real Time PCR and BIO-PCR at tracer level infection by the bacteria, especially important for quarantine.

#### INTRODUCTION

Vigilance of the crops required against every possible disease from the stage of seedlings to the harvest in order to grow a healthy crop to its maximum yield. Many diseases are known to reduce the profits: however, some cause total ruin if detected too late. Therefore, early diagnosis of diseases is essential. High quality photographs of important diseases of major crops are advised to be used in the

production of some well-illustrated leaflets and compendia on plant diseases by the plant protection advisory services, phytopathological societies, research centres and universities to guide farmers and others for making correct identification and appropriate managements practices.

Detection of bacteria is tracing of plant pathogenic bacteria in or on plant material, especially when they occur latently, without causing symptoms. But identification means isolation, characterization and naming of bacteria. It is an important step in the diagnosis of bacterial infection and the taxonomic studies. There is no exclusive or reliably simple method of identifying pathogen and the disease they cause. The traditional identification methods are visual inspection of pathogen *in situ* or *in vitro* in pure culture by microscopic examination stain reaction, colony characters, oxygen requirement, physiological characters biochemical characters and serological methods. The identification by bacteriophage technique can be considered as both traditional as well as modern. The main drawback of conventional identification methods are the less specificity and less discrimination at species and strain level.

In recent years completely newer identification techniques have been developed. This is mainly due to the development in molecular biology, unraveling the structure and function of microorganism. Knowledge of the composition and the place of structural elements in microorganism have made the real finger printing methods like special serological methods, protein and fatty acid separation and nucleic acid finger printing [1-5].

### Steps in Diagnosis of Bacterial Plant Diseases

- Assessment of symptoms
- Isolation of pathogenic bacteria
- Pure culture of isolated bacteria
- Identification of pure culture
- Pathogenicity test
- Reisolation from inoculated plants
- Reidentification of reisolate
- Diagnosis report

Identification means isolation, characterization and naming of bacteria. It is an important step in the diagnosis of bacterial infection and the taxonomic studies.

### Conventional identification methods

- The disease, host range, and symptoms:
- Morphology of bacterial cells:- shape and flagellar characteristics
- Colony characters:- form, color, and smell of a colony
- Stain reaction: - gram stain, acid fast stain, special stains etc.
- Agar slant or Broth culture: - quality and type of the growth, its surface texture and features.
- Oxygen requirement:-aerobic, anaerobic and facultative aerobic.
- Physiological characteristics: - Fluorescent pigment, Sensitivity to antibiotic and Production of toxin.
- Biochemical characteristics: - fermentation of carbon, cellulose, pectin, fat and starch hydrolysis, production of hydrogen sulphide ammonia indole etc, change of pH, coagulation and production of gases.
- Serological characteristics:-precipitation test, agglutination, immunofluorescence and ELISA.
- Bacteriophage:-are too specific and a strain level identification is possible so it is considered as both conventional as well as the modern method of identification and the method is known as Phage plaque technique.

### Modern identification methods

The modern identification methods are mainly due to the development in molecular biology, unraveling the structure and function of microorganism. Knowledge of the composition and the place of structural elements in microorganism have made the real finger printing methods possible such as:

1. Special serological techniques, eventually in combination with others:
  - Immuno-electrophoresis

- Monoclonal antibodies (monospecific antibodies)
  - Immuno-gold labeling.
2. Separation of bacterial components using chromatography:
- Amino acids and cell wall carbohydrate(Paper chromatography, TLC)
  - Fatty acids, fats (gas chromatography).
3. Separation of bacterial proteins in an electric field:
- SDS polyacrylamide-gel electrophoresis (SDS-PAGE)
4. Nucleic acid based identification (nucleic acid based finger printing)
- Southern and Northern hybridization or DNA/RNA (dot/slot-blot hybridization)
  - Fluorescent in situ hybridization (FISH) using rRNA (rDNA) oligonucleotide probes.
  - Restriction fragment length polymorphism (RFLP) analysis.
  - Pulsed Field Gel Electrophoresis (PFGE).
  - Polymerase chain reaction (PCR) – RFLP.
  - Randomly amplified polymorphic DNA (RAPD) analysis.
  - Repetitive sequence- based finger printing PCR (REP-PCR).
  - Amplified fragment length polymorphism (AFLP) analysis.
  - Real time PCR.
  - BIO-PCR.
  - Microarray.

#### **Special serological techniques, eventually in combination with others**

- a) Immuno-electrophoresis: - Methods for separation and characterization of proteins based on electrophoresis and reaction with antibodies.
- b) Monoclonal antibodies (monospecific antibodies):- Monoclonal antibodies against specific components (e.g. a protein or a polysaccharide) of bacterium. Georges Köhler, César Milstein and Niels Kaj Jerne (1975) who shared the Nobel prize in physiology and medicine in 1984 for the discovery for the production of monoclonal antibodies by hybridoma technique.
- Monoclonal antibodies production; first inject the antigen in to the mouse/rabbit. It will produce antibodies against this antigen. Isolate the specific antibody producing B lymphocyte and hybridize with the tumour cells (myeloma cells).this process is called as hybridoma technique. Screen the hybridoma for the production of desired antibodies. Then clone this hybridoma cells *in vitro* or *in vivo*.
- c) Immuno-gold labeling:- Immunogold labelling was first used in 1971 by Faulk and Taylor to identify *Salmonella antigens*. Mostly used in electron microscopy. Gold labelling means colloidal gold particles attached to secondary antibodies which are in turn attached to primary antibodies designed to bind a specific protein or other cell component. Then take for observation under electron microscope.

#### **FAA by Gas chromatography**

In this technique the total fatty acids profiles of bacteria are compared. Bacteria contain lipid in concentration of 0.2- 50% of the dry weight, usually 5%, mainly in their membranes. The lipids important for the technique are those containing esterified fatty acids, such as

- Phospholipids, present in the cell membrane
- Lipid A in lipopolysaccharide of gram negative bacteria.
- Beta- polyhydroxybutyrate.

#### **Analysis of the total fatty acid profile of bacteria by gas chromatography**

The bacteria are lysed by boiling, the fats saponified to release the fatty acids, the fatty acids methylated to make them more volatile and extracted after gas chromatography the profile obtained can be compared (matched) with a large number of reference profiles present in the database (library) of a

connected computer. The computer identifies the fatty acids, determines deviations from a reference fatty acids, determines deviations from a reference fatty acid mixture and presents the identification of the bacterium and the percentage of similarity.

### In gas chromatography

- The *mobile phase* (or "moving phase") is a carrier gas, usually an inert gas such as helium or nitrogen.
- The *stationary phase* is a microscopic layer of liquid or polymer on an inert solid support, inside a piece of glass or metal tubing called a column.

### Important notes

- Testing bacterial colony should be 48- 72 hrs old pure culture.
- Data bank- including libraries of aerobic, anaerobic clinical bacteria, Actinomycetes, Mycobacteria, are available. Yeast.

### Separation of bacterial proteins in an electric field

SDS polyacrylamide-gel electrophoresis (SDS-PAGE):- With PAGE usually total protein profiles of bacteria are compared. Approximately different protein classes can be made visible. The proteins have first to be extracted and denatured with a detergent (sodium dodecyl sulphate, SDS) and mercaptoethanol. The proteins lose their 3- dimensional structure and are negatively charged. Subsequently the mixture denatured, negatively charged proteins is loaded on a porous polyacrylamide gel in an electric field. The proteins will migrate to the positive pole, where the smaller ones will migrate faster than bigger ones. (less resistance in the gel. After separation the proteins are stained and the protein bands can be compared. Comparison is visual or by computer based analysis.

### Nucleic acid based identification(nucleic acid based finger printing)

Southern and Northern hybridization or DNA/RNA (dot/slot-blot hybridization)

A Southern blot is a method routinely used in molecular biology for detection of a specific DNA sequence in DNA samples. Southern blotting combines transfer of electrophoresis-separated DNA fragments to a filter membrane and subsequent fragment detection by probe hybridization. The method is named after its inventor, the British biologist Edwin Southern.

Northern blotting involves the use of electrophoresis to separate RNA samples by size, and detection with a hybridization probe complementary to part of or the entire target sequence. The term 'northern blot' actually refers specifically to the capillary transfer of RNA from the electrophoresis gel to the blotting membrane, however the entire process is commonly referred to as northern blotting. The northern blot technique was developed in 1977 by James Alwine, David Kemp, and George Stark at Stanford University.

Other blotting methods (i.e., Western blot , Eastern blot, Southwestern blot) are;

The Western blot (alternatively, protein immunoblot) is a widely used analytical technique used to detect specific proteins in the given sample (PAGE).

Eastern blotting is a biochemical technique used to analyze protein post translational modifications (PTM) such as lipids and glycoconjugates. It is most often used to detect carbohydrate epitopes.

Southwestern blotting is a lab technique which involves identifying and characterizing DNA-binding proteins (proteins that bind to DNA)<sup>[2]</sup> by their ability to bind to specific oligonucleotide probes. The proteins are separated by gel electrophoresis and are subsequently transferred to nitrocellulose membranes similar to other types of blotting.

### Fluorescent in situ hybridization (FISH) using rRNA (rDNA) oligonucleotide probes

In this method short (20-30mers) oligonucleotide probes against 16S or 23S r RNA/DNA are used. Fix the plant extract /cells in the microscopic slide. For gram positive bacteria sometime a lysozyme step (the enzyme decomposes the peptidoglycan) is necessary to enhance penetration of the probe in to the cell. The probe will hybridize with the complimentary sequence. When probe is labelled with a fluorescent dye the microorganism can be visualized by incident light( fluorescent) microscopy.

### Restriction fragment length polymorphism (RFLP)

Genomic DNA is digested with a restriction enzyme and the fragments are resolved by gel electrophoresis through an agarose gel. The separated fragments are transferred to nylon or nitrocellulose membrane by southern blotting. The membrane bound nucleic acid is then hybridized to labelled nucleic acid probes homologous to regions of the genome of the organism studied. The probe used may either multicopy or single or low copy.

- Multi copy probes commonly used include the rRNA operon in which the procedure is called ribotyping.eg; studied in case of *X. campestris* and *P. syringae* pathogens.
- Low copy number probes used to assess the genetic diversity of *R solanacearum*.

### Polymerase chain reaction (PCR) – RFLP

PCR is technique used to detect the presence of a specific segment or sequence of nucleic acid, which could be diagnostic for infectious agents such as bacteria. The technique is based on the logarithmic amplification of the specific nucleic acid segment so that even minute trace amounts of a particular infectious agent can become detectable.

### Principles in PCR

- a. Selection of primers: - short segment of nucleic acid often 20- 30 nucleotides in length. The primers "kick start the polymerization process.
- b. Extraction of DNA from test sample: - phenol extraction.
- c. Denaturation of DNA: - primers always attached to the dsDNA so that dsDNA split apart from each other to produce ssDNA. This performed by heating the material to 90-95degree C a process termed denaturation.
- d. Annealing of primers: attachment of primers to the particular region takes place at a temperature of 37-50 degree C (avg: 45 degree C).
- e. Polymerization: elongation takes place by the presence of enzyme called Taq polymerase (it is derived from an organism called *Thermus aquaticus* an archea can, hence the name) .So this process takes place at high temperature, 72 degree C.
- f. Amplification: the cycle is repeated a number of times, each cycle resulting in a logarithmic increase in the amount of DNA which is amplified.
- g. Detection of the target DNA sequence:

### Polymerase chain reaction (PCR)–RFLP

Genetic loci of bacteria are amplified with specific oligonucleotide primers and the amplified product subjected to RFLP analysis; difference in the molecular weight of the fragments produced is identified by gel electrophoresis.

e.g.; *Erwinia spp.*, *P syringae* and used in the taxonomic study of the phytoplasma.

### Pulsed Field Gel Electrophoresis (PFGE)

It is genomic DNA finger printing method, which employs rare cutting restriction endonucleases to digest the genomic DNA of bacteria which is then subjected to electrophoresis using specialized conditions for the separation of large fragments of DNA.

e.g.; used in the epidemiological studies of *Erwinia amylovora*.

### Randomly amplified polymorphic DNA (RAPD) analysis

RAPD assay are based upon the use of short random-sequence primers generally of 10 bp in length, which hybridize to genomic DNA in conditions of low stringency and initiate the amplification of random areas of the genome. The amplification products are then resolved on an agarose gel. E.g. studied in case of *X. campestris*, *X. oryzae* and *R. solanacearum*.

### Repetitive sequence- based finger printing PCR (REP-PCR)

Rep-PCR is quickly becoming the most widely used method for the assessment of genetic diversity of bacteria (identification), particularly plant pathogenic bacteria. This genomic fingerprinting technique employs primers designed to hybridize to repetitive elements (ERIC, REP and BOX) within the genomes of bacteria and amplifies the intervening regions between these elements. These repetitive elements may play an important role in the organization of the bacterial genome.

e.g. in the study of *X. oryzae pv oryzae*.

### Amplified fragment length polymorphism (AFLP) analysis

The AFLP technique involves restriction of genomic DNA using two restriction endonucleases followed by ligation of double-stranded adaptors specific for each restriction endonuclease used and then amplification using the primers specific for the adaptors. The primers used for amplification include additional (to the adaptor sequence) nucleotides at the 3' end of the primer and therefore they amplify a subset of the bacterial genome.

e.g. Employed to assess genetic diversity of *E. carotovora* and *E. chrysanthemi*.

### Real time PCR

It is a quantitative procedure, which helps to detect, accumulation PCR products during the PCR reaction. Real time quantitative PCR instrument (Taqman R) is used in which the ABI prism 7900 continuously measures PCR product accumulation using dual labeled fluorogenic oligonucleotide probe called a Taqman probe. This probe is labelled with two different fluorescence dyes the 5' terminus reporter dye and the 3' terminus quenching dye.

#### Advantage:

- Quantification is possible.
- No need for running in the agarose gel.
- Helps to detect the PCR accumulation during reaction by connecting to computer.

### BIO-PCR

BIO-PCR is a method can be used in combination with isolation of media, where viable cells of the target bacterium can be enriched in liquid or solid media and detected in extremely low levels in seeds and other propagative materials.

In a BIO-PCR assay, the plant extract is plated onto agar or added liquid media and enriched for 15-72 hrs, depending upon the organism, and the resulting cell growth used for direct PCR. No DNA extraction is needed for bacteria since the cells will lyse during the initial denaturation step of the amplification. BIO-PCR protocols have been developed for several bacteria including *P. syringae pv. phaseolicola*, *Cavibacter michigensis* subsp. *sepedonicus*, *R. solanacearum*, *Acidovorax avenae* subsp. *avenae*.etc

### Microarray

A microarray is a multiplex lab-on-a-chip. It is a 2D array on a solid substrate (usually a glass slide or silicon thin-film cell) that assays large amounts of biological material using high-throughput screening methods. Types of microarrays include:

- DNA microarrays, such as cDNA microarrays, oligonucleotide microarrays and SNP microarrays

- MMChips, for surveillance of microRNA populations
- Protein microarrays
- Tissue microarrays
- Cellular microarrays (also called transfection microarrays)
- Chemical compound microarrays
- Antibody microarrays
- Carbohydrate arrays (glycoarrays)

A DNA microarray is a multiplex technology used in molecular biology. It consists of an arrayed series of thousands of microscopic spots of DNA oligonucleotides, called features, each containing picomoles ( $10^{-12}$  moles) of a specific DNA sequence, known as *probes* (or *reporters*). These can be a short section of a gene or other DNA element that are used to hybridize a cDNA or cRNA sample (called *target*) under high-stringency conditions. Probe-target hybridization is usually detected and quantified by detection of fluorophore-, silver-, or chemiluminescence-labeled targets to determine relative abundance of nucleic acid sequences in the target. Since an array can contain tens of thousands of probes, a microarray experiment can accomplish many genetic tests in parallel. Therefore arrays have dramatically accelerated many types of investigation.

#### **Advantage of Modern molecular methods**

- Rapid, sensitive, cost-effective.
- Integration to certification and inspection.
- Commercially available standardized kits.
- Non- culturable phytoplasma can be analyzed.
- Less sensitive to mutation and variation.
- Discrimination at low taxonomic level i.e. strains level.

#### **Disadvantage**

- Sensitivity and reproducibility unknown.
- Experimental errors and sampling errors.
- Impossibility to discriminate viable and non- viable cells.
- False negative and false positive difficult to verify.
- Changing probes/primers/enzymes/methods/chemicals/ may yield different.
- Expensive.

#### **CONCLUSION**

It is sometimes suggested that the molecular methods being genetic, are of a higher and better than conventional method, which are phenotypic. However there is no such contradiction .after purifying bacteria and isolating their nucleic acids, these nucleic acids no longer function. They are immobilized in gels, cut in to pieces. etc. and their analysis purely phenotypic one too. Living organisms are able to switch genes on and off and where they seem to be similar in the lab, they behave totally different in the field. So integrated approach of identification i.e. both conventional and modern methods give good and accurate results.

#### **REFERENCES**

1. Fegan M, Hayward C. 2004, Genetic diversity of bacterial plant pathogens, In "Plant Microbiology", eds; Gallings, M. and Holmes, A., BIOS Scientific Publishers, London, and pp: 181-204
2. Janse JD. 2005, Phytobacteriology-principles and practice, CABI Publishing, Cambridge, pp; 43-75.
3. Tripathi DP. 2008, "Introductory Plant Bacteriology", Kalyani publishers, New Delhi., pp: 34-36.
4. <http://www.studentsguide.in>,
5. <http://en.wikipedia.org>,