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Anne M Alvarez
University of Hawai'i at Mānoa
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INTEGRATED APPROACHES FOR DETECTION OF PLANT PATHOGENIC BACTERIA AND DIAGNOSIS OF BACTERIAL DISEASES

Anne M. Alvarez
Department of Plant and Environmental Protection Sciences, University of Hawaii, Honolulu, Hawaii 96822; email: alvarez@hawaii.edu

Key Words immunodiagnosis, monoclonal antibodies, DNA-based detection methods, genomic probes, polyphasic analysis

■ **Abstract** Disease diagnosis is based on a number of factors, including laboratory tests for pathogen identification. Rapid development of genomic techniques for characterization of bacteria over the past decade has greatly simplified and improved pathogen detection and identification, but DNA-based methods have not yet entirely replaced traditional culture and phenotypic tests in the plant industry. The first section of this review focuses on rapid immunodiagnostic and DNA-based detection methods for known bacterial pathogens in plants or plant products, which often manifest no symptoms of disease. The second section covers the broader topic of disease diagnosis and new methods for identifying and characterizing bacteria.

INTRODUCTION

Why are integrated approaches needed for diagnosing diseases caused by plant pathogenic bacteria? Why don't diagnosticians settle on a few standard, universally accepted methods? Clearly, the variable phenotypic and genotypic characteristics of bacteria, which serve for their identification, often require the use of complementary methods. A simple diagnosis may require only symptom recognition and a rapid test to confirm identity of a known bacterial plant pathogen, but diagnosis of truly unknown pathogens requires field observation, examination of plant tissues, isolation of the pathogen, characterization, and proof of Koch's postulates. Although some bacterial pathogens are relatively easy to identify in mixed populations, others are difficult to distinguish from saprophytic bacteria prevalent in the environment. Moreover, many bacterial species are composed of exceedingly diverse subpopulations, so much so that it is remarkable that we can identify them at all.

Rapid development of genomic techniques for characterization of bacteria over the past decade has greatly simplified and improved pathogen detection and identification. Primer sequences have already been described that can be used with the

polymerase chain reaction (PCR) to identify many common plant pathogenic bacteria (89). Although further advances have been made through the use of real-time PCR, these methods have not been widely adopted for routine screening protocols in USDA, state, or private diagnostic laboratories (88).

Questions as to the universal applicability of a new assay may lead to a general reluctance to abandon traditional methods for reliance on any single molecular test, despite the evidence for its greater sensitivity, specificity, reliability, and enhanced speed in processing large numbers of samples. For some laboratories, the need for chemicals, equipment, and trained personnel for DNA extraction methods is another drawback for using PCR as a standard procedure. Thus, culturing on semiselective agar media or use of immunodiagnostic methods followed by confirmatory tests is still prevalent in the plant industry as well as in clinical diagnosis of human bacterial diseases. Rather than decry the lag in adoption of the latest analytical methods, this review covers some options currently available and focuses on the integration of several complementary methods that have been, or possibly will be, adopted in large-scale screening of agricultural products.

DETECTION METHODS FOR KNOWN PLANT PATHOGENIC BACTERIA

A guiding principle for disease prevention is that when key inoculum sources for a given disease are known, effective measures can be implemented to prevent further spread and subsequent disease outbreaks. A range of new and traditional methods are available for detection of known bacterial pathogens surviving in various habitats, including seed, plant debris, soil, and water that are associated with their wide dissemination and spread. As the majority of bacterial diseases are transmitted through contaminated seed or propagative materials, detection of pathogens in these plant products becomes of paramount importance, especially to international trade.

Quality assurance protocols are often based on the isolation of bacteria from seed or plant extracts by culturing on semiselective media, followed by colony identification by morphological and biochemical characteristics and pathogenicity tests. Such tests require from one to several weeks before a final confirmation is possible. There is a need for rapid, reliable tests to replace the costly and time-consuming culture and plant bioassays. Nevertheless, as few rapid tests are universally acceptable, they must compare favorably with standard methods before they are widely accepted by diagnosticians. Global adoption and acceptance of such rapid tests require periodical revalidation by independent laboratories or institutions and tests with new strains and hosts.

An accurate representation of current trends in detection and identification methodology is somewhat elusive because of a lack of centralized information. To evaluate availability of diagnostic products, websites of 36 commercial companies that offer specific reagents and/or analytical test kits for detection and identification of human, animal and plant bacterial pathogens were examined. Currently

TABLE 1 Commercial test kits for detection and identification of bacterial pathogens

	Number of tests offered in each field		
Type of test	**Plant**	**Animal**	**Human**
Immunodiagnostic[a]	97	5	64
DNA-based[b]		6	10
Metabolic profiling and strip tests	6	6	8
Other tests[c]			3
Totals	103	17	85

[a]Sixty-six immunodiagnostic tests available for bacterial plant pathogens are based on polyclonal antibodies, 21 on monoclonal antibodies, and 10 incorporate both polyclonal and monoclonal antibodies. For animal and human pathogens, this information was not always available, so data are not included.

[b]Companies that produce specific nucleotide sequences for sale to researchers and diagnosticians were not included as it is impossible to estimate which sequences would be used for identification of bacterial pathogens.

[c]Other tests include β-galactosidase tests and an immunochromatographic test for *Helicobacter pylori*.

available diagnostic reagents and kits are summarized in Table 1. Of the 205 products examined, 166 (81%) were immunodiagnostic tests, 16 (8%) were DNA-based tests, 20 (10%) were based on bacterial metabolism, and 2% were other tests. In addition to diagnostic products, several companies offer diagnostic services that use a variety of methods including analysis of fatty acid methyl esters (FAME), 16 S rDNA sequence analysis, immunodiagnostic methods, and phenotypic tests. Approximately 75 websites were examined to evaluate methods used by private companies, state and federal agencies for plant, animal, and human diagnoses worldwide, and 18 websites were found that offered testing services for analysis of plant pathogens or products. These 18 private and governmental laboratories were then interrogated to establish which testing procedures are used for detection and identification of bacterial pathogens in plant samples. Of 23 separate detection tests offered by these laboratories, 7 tests were based on phenotypic tests requiring isolation and culture of bacteria, 10 on immunodiagnostic methods, 3 on DNA-based methods, 1 was a grow-out test and 2 combined several phenotypic tests. On the other hand, when pure cultures of unknown bacteria are submitted for identification, 10 of 24 tests were DNA-based and only 2 were based on bacteriological characteristics. Clearly, the availability of a pure culture shifts the preference toward genotypic analysis.

In contrast to practical application in the public sector, research publications over the past five years indicate rapid movement toward development of DNA-based protocols for diagnostic purposes and etiological and epidemiological studies. Of approximately 200 publications examined, 80% described use of genomic methods, whereas less than 20% papers described use of immunodiagnostic

methods for detecting and/or monitoring pathogen populations in the environment. The rapid development of genomic techniques has resulted in several thorough reviews over the past 10 years (65, 71, 88). In contrast, advances in immunodiagnostic methods for bacterial plant pathogens have not been reviewed since 1985 (43). Thus, this review includes some of the immunodiagnostic methods and their integration with genomic and other approaches for bacterial detection and identification.

Immunodiagnosis

REVISITING THE PRINCIPLES Bacterial cell surfaces display a variety of antigenic molecules, including protein, lipopolysaccharides, and extracellular polysaccharides. Hence, polyclonal antisera raised to bacterial species are mixtures of antibodies with multiple specificities. Although the dominant antibodies may show acceptable specificity for a given pathogen, cross-reactions with unrelated species are common, and the range of specificities of an antiserum is exceedingly difficult to ascertain (66). With the development of hybridoma technology, the direction was set for generation of monoclonal antibodes (MAbs). Immediate improvements in bacterial serology were possible because antibodies secreted by murine myeloma cell lines showed defined specificity to a single epitope, and hybridomas produced consistent antibodies, enabling reproducible results among various laboratories.

Plant pathologists soon adopted hybridoma technology, and numerous papers have described the production, specificity, and epidemiological uses of MAbs for plant pathogenic bacteria (4, 29, 43). Many of these antibodies have been characterized by testing specificities with large numbers of strains of the target pathogen (Table 2). As even distantly related bacteria may share common epitopes, it is important to know which, if any, unrelated bacteria react with the same antibodies. Yet, once a MAb has been thoroughly characterized, panels of MAbs may be combined as synthetic reagents to detect genera, species, subspecies, and pathovars of bacterial pathogens, depending on the level of identification needed (5, 10, 11, 35). Phage-display technology also has been used to select consistent and specific MAbs for identification of *R. solanacearum* (103).

IMPACT OF POPULATION STRUCTURE ON IMMUNODIAGNOSTICS Some bacterial taxa are relatively uniform, that is, they possess common antigenic determinants and one antibody generally reacts with all or nearly all strains of the taxon. Examples are *Clavibacter michiganensis* subsp. *michiganensis* and *Xanthomonas axonopodis* pv. *pelargonii* (8, 16). On the other hand, many plant bacterial pathogens are serologically heterogeneous, so not all members of the population react with a polyclonal antiserum or a taxon-specific MAb. Typical examples are *Erwinia carotovora* subsp. *carotovora* and *E. carotovora* subsp. *atroseptica*. The serological diversity of different plant pathogens belonging to the genus *Xanthomonas* was reviewed in 1993 (111). A number of MAbs defined subpopulations of pathogens that corroborated the clusters made by various genotypic methods, including RFLP

TABLE 2 Taxon-specific monoclonal antibodies produced for plant pathogenic bacteria

Genus species/subspecies or pathovar	MAb designations	No. of target strains tested	Reference(s)
Acidovorax avenae subsp. *citrulli*	3D1F3	26	M. Bandla (personal communication)
Agrobacterium tumefaciens			
Strain 58			(44)
Biovar 3	A6F21-1D3G7C8		(18)
Clavibacter michiganensis			
subsp. *michiganensis*	Cmm1	88	(8)
	Cmm1, Cmm2, Cmm3	231	(9, 55)
subsp. *sepedonicus*	McAb1 to McAb-5	19	(29)
	CS-B-5	3	(67)
Erwinia amylovora			(38a, 61)
E. carotovora			
subsp. *atroseptica*			(28)
	14/18.6, 14/2, 14/8.6	3	(112a)
	1221, 1239	38	(49)
	4G4	82	(38)
E. chrysanthemi	6A6	36	(92)
E. stewartii	C/G7/B2	43	(58)
Pseudomonas			
P. avenae	Pa1 to Pa-5	20	(3)
P. fuscovaginae	Pf-1	21	(3)
P. glumae	Pg1, Pg2	8	(3)
P. syringae	six MAbs[a]	223	(79)
pv. *phaseolicola*	AG-1, AG-2	9	(124)
	Psp1, Psp2, Psp3	24	[b]
pv. *syringae*	Pss-1 to Pss-5	25	[b]
pv. *tomato*	Ps4e[a]	13	(79a)
Ralstonia solanacearum			
Species-specific	Rs1, Rs1a	75	(7)
Species-specific			(34, 85a)
Strain-specific MAb			(39)
Strain-specific MAbs	Rs2–Rs10	75	(7)
Xanthomonas			
Genus-specific MAbs: I	X1, X11	436	(4)
X. albilineans	12 MAbs	1	(98)
	7 MAbs	38	(11)

(*Continued*)

TABLE 2 (*Continued*)

Genus species/subspecies or pathovar	MAb designations	No. of target strains tested	Reference(s)
X. axonopodis			
pv. *begoniae*	Xcb-1	26	(16)
pv. *citri*	A1, B1, B3, C1	48	(6)
	X-4600	30	(80)
pv. *citrumelo*	CBS1, CBS1a	225	(6)
pv. *dieffenbachiae*	Xcd1, Xcd3, Xcd7	329	(62)
	Xcd108	302	(78)
pv. *vesicatoria*[c]	Xv1 to Xv21	159	(19)
X. *campestris*			
pv. *armoraciae*	XcaB35[d]	24	(5)
pv. *campestris*	X9, X13, X17	200	(4)
	above plus X21, A11	1023	(5)
	10C5, 20H6, 16B5, 17C12, 10H12, 11B6	37	(35)
X. *hortorum*			
pv. *pelargonii*	Xcp-1	76	(16)
pv. *phaseoli*	XP2	18	(124)
	Xcp1 to Xcp 10	49	[b]
X. oryzae			
pv. *oryzae*	Xco1, Xco2, Xco5	178	(15)
pv. *oryzicola*	Xcola	8	(15)
X. translucens			
pv. *undulosa*	AB3-B6	44	(22, 32)
X. campestris			
Pathovars from	Xct	10	(6)
Ti (*Cordyline terminalis*)			
Mango (*Mangifera indica*)	XCM-1–XCM-6	4	(87)
Xylophilus ampelinus		63	(38b)

[a]Two MAbs (Ps core-1 and Ps core-2), specific to core lipopolysaccharide and four O-chain-specific MAbs (Ps-O:2–1, Ps-O:2–2, Ps-O:2–3, and PsO-:3–1) were used to classify 223 strains belonging to 19 pathovars of *P. syringae*. MAb Ps4e was generated against *P. syringae* pv. tomato IPGR 140, but also reacted with strains of nine other tested pathovars.

[b]Unpublished data from A.M. Alvarez & A.A. Benedict.

[c]When MAbs were generated, the pathogen was called *X. campestris* pv. *vesicatoria*. The study included 109 strains in Group A and 50 strains in Group B. The pathogen has since been renamed. A strains are *X. axonopodis* pv. *vesicatoria*; the B strains are *X. vesicatoria*.

[d]This antibody also reacted with some strains of *X. campestris* pv. *campestris*.

analysis (5, 6, 15). Nevertheless, subtyping into serovars often has limited value as correlations with virulence, pathogenicity, or geographical origin are rarely found. However, MAbs are useful for tracking movement of defined strains originating from different inoculum sources in epidemiological studies or for comparing epidemiological fitness of different strains with relatively little expense (75, 91).

WHAT HAS HYBRIDOMA TECHNOLOGY REVEALED ABOUT SUBSPECIFIC VARIATION OF BACTERIAL PATHOGENS? The need to purify antigens prior to immunization is less important when producing MAbs than polyclonal antibodies because the procedure itself involves extensive screening of hundreds of hybridoma cell lines to identify unique cell lines that secrete antibodies of the desired specificities. In effect, antigens that differentiate one pathovar from another were first identified by their reactivities with respective MAbs, and only later were the antigens chemically characterized as proteins, lipopolysaccharides (LPS), or other compounds. Differences in LPS composition among xanthomonads were studied long before MAbs were available, but it was unclear whether such differences were related to the pathovar status and host-specificity (114). In early studies, many of the MAbs that differentiated pathovars of *Xanthomonas campestris* were targeted toward different bacterial LPS components (4, 16). Likewise, pathotype A strains of *X. axonopodis* pv. *citri* associated with the severe form of citrus canker were distinguished from B and C types by MAbs directed toward different LPS molecules (6).

Epitopes on bacterial LPS were found on certain strains of *Agrobacterium tumefaciens*, and MAbs were later generated to identify biovar 3 (18). However, other biovars are serologically heterogeneous, and MAbs do not distinguish *A. tumefaciens* from *A. radiobacter* (20, 44). This is an example where the serological approach is unsuitable for pathogen detection.

Ovod et al. (79) examined the relationships among pathovars of *Pseudomonas syringae* focusing on an analysis of the LPS fractions of 223 strains of *P. syringae* of six murine myeloma cell lines selected; two were specific for outer-core oligosaccharide epitopes and four were directed toward O-antigen side chains of LPS. The core polysaccharide MAbs reacted with all but two pathovars (pv. *coriandricola* and pv. *lachrymans*) and reacted with 99% of the 202 strains representing 17 other pathovars of *P. syringae*. The O-antigen-specific MAbs reacted only with certain pathovars, enabling them to propose a classification scheme of strains of *P. syringae* based on use of MAbs that improved and clarified previous classification schemes based on reactivity of strains with polyclonal antisera (79). Extensive chemical and immunochemical studies of lipopolysaccharides of *P. syringae* were undertaken by these researchers to determine the molecular basis of immunospecificity as related to the taxonomy and classification of *P. syringae* pathovars (55a, 79a, 79b, 79c, 125). The role of LPS in early stages of host pathogen interactions has since become much better defined for several pathovars of *X. campestris*, *P. syringae*, and other genera of bacteria and has been the subject of a recent review (30).

USES OF POLYCLONAL ANTISERA AND MAbs IN DETECTION ASSAYS Both MAbs and polyclonal antisera are available for use in various formats, including

agglutination assays, ELISA, Western blot, immunofluorecence (IF) or immunofluorescence colony-staining (IFC), and lateral flow devices. The utility of such antibodies in these assay formats depend on several factors among which antibody affinity and avidity in relation to the target anigenic determinants or epitopes are important. Pathogen-specific MAbs that react with heat-stable LPS enable development of robust detection kits for use in rapid field and laboratory disease diagnosis. Likewise, MAbs directed toward capsule and/or extracellular polysaccharides in pathogens, such as *C. michiganensis* subsp. *michiganensis* and *Ralstonia solanacearum*, are useful in a number of immunodiagnostic formats (7, 34, 39, 72, 85a).

ELISA Most of the MAbs generated for a wide number of bacterial plant pathogens (Table 2) have been initially screened using an ELISA format and later checked for applications with other immunodiagnostic assays. ELISA is a well-established method for testing large numbers of samples in quality assurance programs worldwide. The procedure is readily adapted to automation and produces relatively reproducible results for large-scale testing. To date, ELISA procedures using both poly- and monoclonal antibodies are available for numerous taxa of phytopathogenic bacteria, and rapid detection kits are commercially available. The sensitivity of ELISA assays (10^5–10^6 CFU ml^{-1}) is adequate for identification of bacterial pathogens from symptomatic plants and colonies on selective media. The sensitivity of ELISA can be increased tenfold by using an extraction buffer containing EDTA (ethylenediaminetetraacetic acid) and lysozome, which released LPS into solution, thereby enhancing the antibody-antigen reaction without increasing background readings (54). Similarly, boiling the bacterial samples to destroy proteins or use of commercial extraction buffers like BEB (Agdia Inc., Elkhart, Indiana) improves the sensitivity by increasing the signal:noise ratio. Enrichment techniques also increase sensitivity (discussed below), but this results in a qualitative (nonquantitative) method, unless the most probable number method is used (12, 78, 85). Multitarget or multiplex ELISA are developed by Agdia, Inc, where more than one species is detected in the same ELISA plate well using different enzyme labels. A multiple ELISA for detection of *C. michiganensis* subsp. *michiganensis* and *X. axonopodis* pv. *vesicatoria* is currently available.

LATERAL FLOW DEVICES The principles used for rapid lateral flow devices are primarily those of ELISA, but various types of filters are used as the solid support for the initial binding reaction (27b, 33). A lateral flow device test kit developed by Central Science Laboratory, U.K., permits detection of *R. solanacearum* in a 3-minute, single step (27b). Kits also are available for *X. hortorum* pv. *pelargonii* and *E. amylovora* (J. Elphinstone, pers. comm.) Rapid ImmunoStrip® tests for *R. solanacearum* and *C. michiganensis* subsp. *michiganensis*, and *X. hortorum* pv. *pelargonii* are available from Agdia, Inc.

IMMUNOFLUORESCENCE IF is widely used in Europe for detection of bacterial pathogens in seed and propagative materials. In the Netherlands, IF is used to screen 60,000 potato seed pieces annually for the presence of *R. solanacearum*, the causal agent of brown rot (103). Potato seed pieces are also screened by IF for the ring rot pathogen, *C. michiganensis* subsp. *sepedonicus*, and in France, IF is used to screen tomato seed lots for the bacterial canker pathogen, *C. michiganensis* subsp. *michiganensis*.

FLOW CYTOMETRY Immunodiagnostic detection has been enhanced by the development of flow cytometry. This is a technique for rapid identification and quantification of cells or other particles as they pass individually through a sensor in a liquid stream. Cells are identified by fluorescent dyes conjugated to specific antibodies, and multiple cellular parameters are determined simultaneously based on the cell's fluorescence and its ability to scatter light. The cells may be sorted electronically, permitting purification and/or culture of subpopulations of selected cells for further confirmatory tests (1, 2). Sample preparation is simple: Cell suspensions are filtered to remove large particles then stained with fluorochrome-labeled antibodies. Fluorescent markers for viability (vital stains, such as propidium and hexidium iodide for red fluorescent staining of dead cells and carboxy fluorescein diacetate and calcein AM for green fluorescent staining of viable cells can be used to differentiate live from dead cells (103). Flow cytometry has been applied for detection of *C. michiganensis* subsp. *michiganensis* in tomato seed extracts (2), detection of *X. axonopodis* pv. *dieffenbachiae*, causal agent of anthurium blight (2), detection of *X. campestris* pv. *campestris* in seed extracts of *Brassica* sp. (26), and to determine viability of *R. solanacearum* in seed potatoes (104).

IMMUNOMAGNETIC SEPARATIONS Target cells can be isolated from a mixed solution using paramagnetic polystyrene beads coated with specific antibodies. Following a washing step, bound cells can be used for PCR, or as they are still viable, they can be recovered on semiselective media. The use of immuno-magnetic separations followed by culturing and/or PCR are covered later under integrated methods (102).

Genotypic Approaches

A vast array of genotypic approaches for detection of known pathogens in plant samples have been developed over the past 10 years, and have been the subject of several thorough reviews (65, 71, 88, 108). The majority of papers report the use of PCR in some form for detection of bacterial pathogens in heterogeneous mixtures. Since a review in 1999 (65), numerous publications have reported the development and/or application of pathogen-specific primers for detection of bacterial plant pathogens in heterogeneous mixtures. Most of these involve amplification by PCR coupled with one or more other techniques (Table 3). Multiplex PCR is used to identify several pathogens simultaneously (17a,b). Use of internal PCR controls

TABLE 3 Advances in genomic probes and protocol development for rapid detection of known bacterial plant pathogens and related bacteria (1999–2003)[a]

Genomic assay	Targeted bacterial taxa[a]	Reference(s)
DNA-based approaches		
PCR (rRNA operon)	*Acidovorax avenae* subsp. *citrulli*	(115)
	Stenotrophomonas maltophilia	(120)**
	Burkholderia cepacia[a]	(121)
	B. cepacia	(74)
	B. cepacia	(73)
	B. cepacia	(31)
	B. cepacia	(77)
	R. solanacearum	(79e)
	X. axonopodis pv. *citri*	(27)
PCR (*pthA* gene)	All citrus canker bacteria	(27)
PCR (*fimA* gene)	*X. hyacinthi*	(106)
PCR (*syrD* gene)	*P. syringae* pv. *syringae*	(23)
PCR (several genes)	*P. herbicola* pv. *gypsophilae*	(69)
	X. campestris pv. *pelargonii*	(69)
	A. tumefaciens	(27a, 69)
	X. campestris pv. *campestris*	(69)
	R. solanacearum	(82)
PCR (using competitor DNA template from *E. coli*)[b]	*E. carotovora* subsp. *atroseptica* (all serogroups)	(48)
PCR of *hrp* gene sequence[c]	*R. solanacearum*	(83, 84)
PCR of *recA* gene sequence[c]	*Erwinia* sp.	(116)
PCR of a pathogenicity locus	Soft-rot erwinias	(95, 96)
	Xanthomonas sp.	(37)
Nested PCR	*E. amylovora*	(63)
Cooperational PCR	*R. solanacearum*	(17a,b)
PCR of insertion sequences	*R. solanacearum*	(59)
	X. oryzae pv. *oryzae*	(86)
PCR of short-sequence DNA repeats	*E. amylovora*	(53)
PCR-RFLP	*R. solanacearum*	(83, 84)
	B. cepacia	(68)
	B. cepacia	(73)
	Soft-rot erwinias	(95)
	Erwinia sp.	(116)
	B. cepacia	(99)
	E. amylovora/ Pantoea agglomerans	(51)

(*Continued*)

TABLE 3 (*Continued*)

Genomic assay	Targeted bacterial taxa[a]	Reference(s)
PCR-MERFLP	*Pseudomonas* sp.	(81)
PCR-FISH	*Pseudomonas* sp.	(41)
PCR-DGGE	*R. solanacearum*	(107)
	Clavibacter michiganensis subsp. *michiganensis*	(113)
Multiplex PCR	*R. solanacearum*	(17a,d,e)
Real-time PCR	*R. solanacearum*	(92a, 118, 118a)
	C. michiganensis (several subspecies)	(14, 87a)
DNA-DNA hybridization (dot blot, Southern blot)		
Southern blot	*Xanthomonas*	(119)
Hybridization probes	*X. albilineans*	(50)
	Streptomyces saraceticus	(57)
	P. syringae pv. *actinidiae*	(56)
RNA-based approaches		
NASBA	*R. solanacearum*	(17)
	R. solanacearum	(105)
	Clavibacter michiganensis subsp. *sepedonicus*	(100)
Other approaches		
Identification of novel β lactamase	*Xanthomonas* sp.	(119)
Laser-induced fluorescence	*E. carotovora*	(25)

[a]Detection methods for *Burkholderia cepacia* are included. Although they were developed for strains associated with cystic fibrosis and other human diseases, the methods could be useful for bacteria in the same genus. *Burkholderia gladiolii* and *B. andropogonis* are plant pathogens and some *B. cepacia* strains are used for biological control. References to *Stenotrophomonas maltophilia* are included because it is prevalent in environmental samples and is potentially confused with plant pathogens.

[b]Also incorporated competitor DNA into the extraction protocol.

[c]See also PCR-RFLP.

(co-amplification of host DNA) is a major advantage when using PCR in routine diagnostics (79d,e). A new cooperational PCR (Co-PCR) also has been developed to detect *R. solanacearum* in water (23a). Real-time PCR, which provides accurate and rapid detection of bacterial pathogens and has several other advantages, has been recently reviewed (88). Other genotypic methods include DNA-hybridization, dot-blots, and nucleic acid sequence-based amplification (NASBA) (103).

Development of specific probes for detection of target pathogens has been the research focus over the past decade. Manulis et al. (69, 70) developed pathovar-specific probes and PCR tests for *X. campestris* pv. *pelargonii*, which could detect between 10 and 50 colony-forming units (CFU) per sample. Thus, these tests

should be able to identify a potential pathogen in asymptomatic tissues. Likewise, Koh & Nou (56) developed primers and a probe for detection of *P. syringae* pv. *actinidae* in asymptomatic kiwi fruit. Cubero & Graham (27) developed primer sets for *X. axonopodis* pv. *citri* that can distinguish pathotype A from *X. aurantifolii* pathotypes B and C. Primers were based on sequence differences in the ITS region and the *pthA* gene. Primer sets based on ribosomal sequences had high-level specificity for *X. axonopodis* pv. *citri* A, whereas those based on the *pthA* gene were universal for all types of citrus bacterial canker. In addition, rep-PCR using ERIC and BOX primers have been used to identify the geographic origin of strains introduced into Florida (27).

Pathogens in the genus *Erwinia* can be distinguished from enteric saprophytes, but once the genus is known, bacteriological tests and serology are not always consistent for identification of *E. carotovora* subsp. *carotovora*, *E. carotovora*. subsp. *atroseptica*, and *E. chrysanthemi*. Avrova et al. (13) found that AFLP analysis of strains revealed subclusters that coincided well with subspecies designations. Cluster 1 contained *E. carotovora* subsp. *carotovora* and *E. carotovora* subsp. *odorifera*, cluster 2 contained *E. carotovora*. subsp. *atroseptica* and *E. carotovora* subsp. *betavasculorum*, cluster 3 contained *E. carotovora* subsp. *wasabiae*, and cluster 4 contained *E. chrysanthemi*. Although AFLP analysis cannot be performed on mixed cultures, such studies of pathogen diversity using pure cultures of representative strains will eventually permit rapid identification of unknown isolates and potentially be useful in diagnostics.

Other Approaches

A novel approach using laser-induced fluorescence was developed as a noninvasive tool for early detection of disease in agave plants (25). A He-Ne laser was used as an excitation source, and in vivo fluorescence emission spectra were recorded in the 660–790-range. Infected plants showed an increase in fluorescence intensity within three days after inoculation and decreased again after the fifth day, whereas noninfected and bactericide-treated plants showed no significant change in fluorescence. Fluorescence intensity ratios were indicators of disease progress (25).

Integrating Several Methods for Detection and Identification

New methodologies have consistently been compared to basic culturing methods for sensitivity and validation of results. For example, primers directed to 16S rRNA loci of pseudomonads were more specific than those amplifying the 16S to 23S spacer region of these organisms in comparison to cultural techniques (122). PCR using the specific primers showed 95% concordance with detection assays using culture, but PCR also identified seven positive samples that were negative by culture.

ENRICHMENT ASSAYS A short culturing step preceding an immunodiagnostic or DNA-based assay has frequently been used to increase the sensitivity of IF, ELISA,

and/or PCR. An enrichment-ELISA assay using pathogen-specific MAbs was used to detect *X. axonopodis* pv. *dieffenbachiae* in leaf samples, and *E. carotovora* subsp. *atroseptica* or *R. solanacearum* in potato seed pieces (24, 38, 78). Even a limited culturing step, which is still insufficient for colony identification, increases the sensitivity of ELISA about 10,000 fold (10). Enrichment assays have the added advantage that only viable cells multiply to give a positive signal. The IFC technique combines an enrichment step in pour plates for colony detection with fluorochrome-labelled antibodies and also increases the sensitivity of IF by approximately 10^4 (109, 112). Viable cells multiply to form microcolonies observed at 40-60X with an epifluorescence stereomicroscope, whereas individual (dead) cells are not visible at these low magnifications. IFC is used mostly for epidemiological studies and for validation of results achieved by other detection methods that do not distinguish between live and dead cells (107). Optimization of buffers and conjugates improves test results (112). Antibodies in subclass IgG are most suitable for IFC formats, as molecules are smaller than IgAs (dimers) and IgMs (pentamers) and diffuse more readily through agarose gel (10).

Enrichment of target bacteria on semiselective media also has been used to enhance the sensitivity of PCR reactions. Such tests, often termed "BIO-PCR" (88, 90), favor detection of living cells as colonies are harvested from culture plates prior to the PCR reaction. Although small amplicons from dead bacteria also may be amplified, the major bands result from amplification of the DNA from living cells. Sakthivel et al. reported detection of 55 fg of DNA per reaction tube (equivalent to about 7 cells of *X. oryzae* pv. *oryzae* per reaction tube or 70 cfu ml^{-1} in the original sample) (86). In a "BIO-PCR" designed to detect *P. cepacia*, contaminated samples were incubated only 24 h in broth, DNA was extracted, and added to "Ready to Go" PCR beads with specific primers. The entire assay was completed in 27 h, whereas standard methods required 5–6 days for isolation and identification of target bacteria (52).

IMMUNOMAGNETIC SEPARATION FOLLOWED BY CULTURE OR PCR Compounds that interfere with DNA amplification have limited the use of direct PCR for detection of target bacteria from natural samples. Immunocapture or immunomagnetic separation (IMS) has been used to overcome this problem in a number of cases (42, 46, 82, 122). The optimum time for immunocapture of *P. syringae* pv. *syringae* with Advanced Magnetics™ (AM) beads coated with a polyclonal antiserum was just one-hour incubation. The detection limit for immunocapture followed by reaction with a chromophore was only 10^6 cfu ml^{-1}; however, when immunocapture was followed by a specific PCR test, detection limits were significantly improved. Enhanced recovery of *E. carotovora* subsp. *carotovora* from potato peel extract was achieved through immunocapture with AM beads and a pathogen-specific polyclonal antiserum. Particle concentrations ranging from 12 to 200 μg ml^{-1} show a linear relationship to recovery, and 66% of the target cells were recovered per μg of particles. Coupled with PCR, the method can detect 50 cells per reaction tube (2×10^3 cfu ml^{-1}). IMS-PCR has shown a 100-fold enhancement of sensitivity

over PCR without IMS, which was positive only when target cells were present at 10^5 cfu ml^{-1} of potato peel extract (101). IMS plus PCR resulted in a 100-fold increase in detection of *Acidovorax avenae* subsp. *citrulli* over direct PCR (115).

IF AND FISH Fluorescent in situ hybridization (FISH) with probes targeted to 23S rRNA have been used to detect *R. solanacearum* race 3 biovar 2 from potato peels (103). A probe specific for *R. solanacearum* was used to confirm identity of the pathogen in potato samples that were simultaneously and independently tested by a pathogen-specific IF assay. Use of two independent assays in a rapid (1-day) test immediately increases the confidence limits for a positive result, and this is especially important for large-scale screening of propagative materials for major crops.

POLYPHASIC APPROACHES FOR PATHOGEN DETECTION Van Overbeek et al. (107) employed multiple tests to study behavior of a known strain of *R. solanacearum* in bulk soil and in soil from the tomato rhizosphere. FISH assays were used to demonstrate differences in root colonization by the pathogen following treatment with strains of a potential biocontrol agent, *P. corrugata*. PCR was also coupled with density gradient gel electrophoresis (DGGE) analysis to support evidence for antagonism between *P. corrugata* and *R. solanacearum*. Strong bands were seen in soil profiles from systems containing only the pathogen, whereas weak bands of *R. solanacearum* were detected in profiles from mixed systems. For further confirmation, the authors also used viable counts, *gfp* as a genetic marker, and serological techniques (IFC) to trace the spread of the known strain of *R. solanacearum*. This use of multiple detection methods confirmed and validated results obtained by the molecular assays (107).

DIAGNOSIS OF DISEASE

Often the purpose of a rapid diagnosis is to confirm the presence of a suspected pathogen in infected tissues, and in this case, the detection methods described earlier can be applied. However, all the general principles of diagnosis must be integrated if a field problem is to be resolved (40, 93, 117). Stowell & Gelernter (93) point out that a diagnosis for turfgrass diseases must be made within 72 h as control measures must be applied immediately. Abiotic factors are considered, but as there is no time to culture the pathogen, biotic diseases caused by fungi and nematodes are diagnosed using a combination of interview, experience, and microscopy. These principles apply to diagnosis of bacterial diseases at early stages of the diagnostic effort. However, microscopy does little to identify the pathogen, and unless nothing more is needed than the general knowledge that bacteria may be involved, culturing and/or use of specific tests to identify suspected known organisms are essential, but these steps will obviously delay the diagnosis.

HOST LISTS AND DESCRIPTIVE SYMPTOMS Many bacterial diseases can be diagnosed quickly and efficiently using established methods and materials that are already widely available. The first step is to consult a comprehensive host list that covers known disease, typical symptoms, and the known potential pathogens for a specific host. Crop hosts and diseases are listed for many bacteria (21), but the lists need updating and pathogen names need revision as bacterial taxonomy may have changed radically since publication. Compendia of crop diseases are excellent resources, and photographs that distinguish symptoms caused by bacteria are often contrasted with symptoms caused by other pathogens or abiotic factors. A list of known pathogens for a given crop greatly reduces the choices to one or two suspect genera for a given symptom. Caution against oversimplification is essential, however, as a number of pathogens may cause similar symptoms under field conditions. When symptoms are confounded by root disease, insect damage, water stress, excess moisture, nutritional deficiency, or high temperature, textbook symptoms may be obscured.

TO CULTURE OR NOT TO CULTURE? As soon as a new method that appears to be more sensitive or efficient than culture is used to detect the presence of a pathogen, questions arise as to whether the bacterium was actually present. Thus, comparisons with culturing and pathogenicity tests are generally made to validate the assay. The main challenge is to separate the presumptive pathogen from numerous saprophytes that overgrow it on culture media. Familiarity with basic characteristics of saprophytes will help to eliminate the "noise" and locate the pathogen on culture plates. It is far more efficient to make judicious initial isolations from a series of symptoms in various stages of development on a general, nonselective medium than to prejudice the diagnosis using semiselective media. A general-purpose nonselective but differential medium containing tetrazolium chloride (89) is useful for distinguishing a potential pathogen from saprophytes during the initial isolation as long as the tetrazolium is reduced to 0.001% to avoid inhibitory effects on genera such as *Xanthomonas*. If the pathogen is present, it is usually present in sufficient numbers to be isolated on nonselective media. A given pathogen may have various colony morphologies, but experience with colony variants of known strains will assist in distinguishing them from saprophytes. On the other hand, when the purpose is to confirm presence of a suspected pathogen, semiselective media are useful. Semiselective media are needed for isolations from soil but usually not from plant tissues that may be surface sterilized.

PRESUMPTIVE IDENTIFICATION OF KNOWN PATHOGENS Isolation followed by presumptive identification to the genus level can be done rapidly using a minimal number of key diagnostic features (89). Simultaneous use of differential and semiselective media are very useful for the second stage of presumptive identification. Single colonies are removed from the original isolation plates with sterile toothpicks, patched (not streaked) at specific positions on a series of culture media, and growth characteristics are recorded in 24–72 h. For example, seven known

bacterial pathogens of tomato can be rapidly sorted to genus by a few classical tests (gram stain and oxidase test), followed by patch-plating onto a series of culture plates containing differential and semiselective media. The combination of growth characteristics on YDC, KMB, FS, ET, MS, CVP, D1 agar differentiates the genera [names and contents of these media are described in Reference 89]. In 72 h, this battery of tests will readily distinguish between *E. carotovora*, *C. michiganensis*, *X. axonopodis* pv. *vesicatoria*, *P. syringae*, *P. corrugata*, *R. solanacearum*, *A. tumefaciens*, and contaminating saprophytes, such as *Pantoea herbicola*, *Enterobacter agglomerans*, *Micrococcus luteus*, and *Stenotrophomonas maltophilia*. Similar rapid diagnostic protocols can be designed for numerous other known bacterial diseases and can be customized for specific purposes once the diagnostician is familiar with the symptoms of potential pathogens from a given host.

CONFIRMATION WITH BIOASSAYS OR PATHOGENICITY TESTS The ultimate verification of a plant pathogen generally requires a pathogenicity test, but these tests require time, availability of host germplasm, optimal greenhouse conditions, and controls. For some pathogens the hypersensitivity test (HR) on tobacco is useful, but other pathogens give little or no HR response. For leaf spotting pathogens confirmation with pathogenicity tests is often efficient as leaf spot symptoms often can be induced in 3–4 days under appropriate conditions for many pathogens. However, as neither pathogencity tests nor HR will distinguish between closely related pathovars, such as *P. syringae* pv. *tomato* and *P. syringae* pv. *syringae*, a rapid immunodiagnostic assay using MAbs or PCR tests using pathovar-specific primers can provide rapid confirmation (89).

Pathogenicity tests are sometimes impractical for pathogens that require relatively long incubation times to manifest symptoms. For example, highly virulent strains of *C. michiganensis* subsp. *michiganensis* may produce symptoms following stem inoculation of tomato in 3 to 4 days, yet a large proportion of moderately virulent strains require 7 to 10 days, and some strains produce symptoms only after 21 to 28 days.

CONFIRMATION WITH IMMUNODIAGNOSTIC AND DNA-BASED TESTS While waiting for results of pathogenicity tests that require long incubation times, it may be more efficient to use a rapid immunodiagnostic or DNA-based assay when the data base for analysis is available. For example, *C. michiganensis* subsp. *michiganensis* can be identified to species and subspecies using immunodiagnostic test strips and/or rep-PCR fingerprinting analysis (55, 65). A direct PCR using published primers can be used for many strains, but more false positives and false negatives were observed in a worldwide collection of strains than with the immunodiagnostic assay (9, 55).

Immunodiagnostic reagents and PCR protocols are already available for direct identification of specific pathogens (Tables 2 and 3). When pure cultures are available, pathogen identity can be confirmed using DNA fingerprinting methods that

were recently reviewed by Louws et al. (65). Genomic fingerprinting approaches continue to be applied and correlations between genotype and phenotype continue to emerge (64, 96). Commercial kits have been developed for direct identification from plant tissues. These kits are particularly useful for confirming the presence of known pathogens in symptomatic leaf and stem tissues where an abundance of bacterial cells associated with symptoms is sufficient to trigger a positive response in both immunodiagnostic and DNA-based assays. However, such tests often miss detection of the pathogen in asymptomatic tissues and should not be relied upon for diagnosing disease in latent infections. For that matter, culturing methods, enrichment ELISA, and BIO-PCR also have a very high probability of missing the pathogen in latently infected tissues because of the overriding element of scale, which has not been well addressed in sampling bacterial populations in the environment (60). Tests based on molecular methods prescribe very small–volume samples, and the diagnostician has few or no criteria upon which to base a sound selection of potentially infected tissues.

Other Resources

Rapid analytical methods are available for identification of pure bacterial cultures and some are provided as commercial services. These include analysis of fatty acid methyl esters (MIDI, Newark, DE), metabolic profiling (Biolog, Hayward, CA), DNA sequencing analysis (Microbial Identification, Newark, DE and MicroSeq, San Jose, CA). Metabolic tests for bacterial characterization also are widely used (bioMérieux, Marcy L'Etiole, France). Companies offering a range of tests for detection and identification of bacteria are Agdia (Elkhart, IN), Agri-Analysis (Davis, CA), Adgen (Ayr, Scotland), SARDI (South Australia), BioReba (Switzerland), Central Science Laboratory (York, UK), Seed Testing of America (Boulder, CO), Hydros Environmental Diagnostic Corp, Loewe Phytodiagnostica (Sauerland, Germany), and HortiTech (Warwick, UK).

DIAGNOSING NEW OR UNKNOWN PATHOGENS AND DISEASES Diagnosing a new disease is obviously more complicated and requires far more experience than confirming the presence of a suspected pathogen on a known host. The entire array of diagnostic principles (40, 93, 117) must be used to narrow the choices. No simple tests are suitable; rather, attention must be focused on microscopic examination and the initial association of a pathogen with the disease syndrome. Once the disease is ascertained to be likely caused by a bacterial agent, potential known pathogens are eliminated from consideration by presumptive tests described in the previous section and in Reference 89.

PHENOTYPIC AND GENOTYPIC TESTS REQUIRING PURE CULTURE When diagnosing the true unknown, dilution streaking to obtain pure cultures and repeated pathogenicity tests under appropriate conditions are essential. Before time is spent on the numerous phenotypic and genotypic tests for pathogen identification, some

basic information about the potential pathogen should be obtained immediately in order to best decide which tests to use for further identification. Minimal tests include gram reaction, the O/F test for oxidative versus fermentative metabolism, presence or absence of spores, and motility. At that point, additional relatively inexpensive tests can be used to further characterize the bacterium. Examples include metabolic tests (API strip tests, bioMérieux, Inc.), metabolic substrate analysis (Microlog TM, Biolog, Inc., Hayward, CA), analysis of fatty methyl esters (MIDI, Newark, DE), or 16S rDNA sequence analysis. Extreme caution must be used in interpreting results for any one test, especially for identification of an unknown disease caused by an as-yet undescribed bacterial pathogen. For example, in attempting to identify bacteria associated with rare crops such as macadamia, orchid, and ginger, pure cultures were analyzed by API strips, Microlog^{TM}, FAME analysis, and 16S rDNA sequencing analysis. In some cases, a single strain was assigned four different species names, depending on the analytical method used. Strains showed less than 97.5% sequence similarity with known species and thus could not be identified by 16S rDNA sequence analysis. Obviously, unknowns may not be well represented in the databases for the respective analytical method. Yet, the examples point out the importance of performing basic bacteriological tests before unequivocally accepting a name that comes from a database, regardless of the refined analytical methods used to generate a similarity index.

INTEGRATION OF VARIOUS DIAGNOSTIC METHODS FOR A POLYPHASIC ANALYSIS A polyphasic analysis still appears to be the most reliable approach for identification of new pathogens (110). Once pathogenicity has been confirmed with controlled tests and the genus is determined with relatively simple basic bacteriological tests, the final identification of the bacterium is best interpreted from the results of various genotypic methods. Comparison of 16S rDNA sequence data with phenotypic data for type strains assists in selection of deteminative tests that may discriminate distinct taxa for simplified laboratory analyses (47). Comparative genomics is now possible with recent advances in sequence analysis of entire bacterial genomes, and this permits improved taxonomic analysis as well as analysis of gene function (108). Polyphasic analysis has been used to identify previously undescribed pathogens of passion fruit and artichoke (36, 37, 97). Concalves & Rosato (36, 37) isolated a pathogen from passion fruit plants (*Passiflora* spp.) using nonselective media and presumptively identified the suspect as *Xanthomonas campestris*. They later isolated 55 xanthomonad strains from passion fruit and examined their genetic diversity using RAPD analysis; rep-PCR with BOX, ERIC, REP primers; RFLP of the 16S-23S rDNA intergenic spacer region; pulsed field gel electrophoresis of genomic DNA digested with rare cutting restriction enzymes; AFLP; and SDS-PAGE of whole-cell proteins. Profiles characteristic of *Xanthomonas* species were generated by these methods but the taxonomic position of the *Xanthomonas* strains from *Passiflora* could not be assigned. DNA-DNA hybridization placed them in *X. axonopodis* pv. *passiflorae*.

CONCLUSIONS

Diagnosis requires astute field and laboratory observations as well as accurate identification of the pathogen. Plant pathogenic bacteria, which are represented by enormously diverse and fluid populations in the environment, often require multiple complementary tests for a definitive identification. As diagnostic tests and reagents are only as reliable as the methods used to characterize them, multiple representative strains of a given taxon must be characterized to ascertain their universal properties. Thus, thorough studies of target pathogens to both characterize their diversity and locate common and stable markers are essential, and the specificity of PCR primers or antibodies must be validated on an international collection of strains of the target bacteria before such tests are used as standards. Often the common taxa can be detected more simply with a few phenotypic features, such as antigenic molecules on cell surfaces. These have enabled development of robust and reliable immunodiagnostic tests that can be accomplished in minutes and followed up with confirmatory tests that require hours or even days. The most reliable tests will eventually be validated after years of use build confidence in their accuracy and robustness in international laboratories. Eventually they will be adopted by diagnosticians, regulatory agencies, and the plant industry.

Meanwhile, the importance of sampling that represents realistic orders of scale must not be ignored, especially when attempting to detect bacteria in symptomless plants, propagative materials or soil, and plant debris. Conclusions drawn from ultrasensitive analytical methods that require only microliters of analyte often misrepresent the real situation because sample volumes and procedures have not been thoroughly addressed. Epidemiological data in relation to the initial inoculum density of pathogens are needed to establish threshold infection levels for certification programs. There is a great need to find efficient and meaningful ways to survey plant products and concentrate analytes into the small volumes required for testing purposes. Otherwise, the extreme sensitivity of novel methods will not provide meaningful insights on the presence of pathogens in enormous shipments of plants and plant products that cross international borders. Accelerated use of biochips (71) and sensor arrays (76, 123) may be applied for pathogen detection and identification. Novel approaches including biosensors or an electronic nose (45, 94) to detect a quarantined pathogen in a large-volume sample, such as an entire seed lot, are interesting prospects for the future.

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