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RELIABILITY OF DIAGNOSTIC TECHNIQUES FOR *ERWINIA AMYLOVORA* AND *CLAVIBACTER MICHIGANENSIS* SUBSP. *SEPEDONICUS*

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Abstract

Erwinia amylovora (*Ea*) causing fire blight and *Clavibacter michiganensis* subsp. *sepedonicus (Cms)* causing ring rot of potato belong to the quarantine organisms. Detection and determination of both pathogens using different methods were compared. Commercial MAb were used for *Cms* and PAb for *Ea*. All *Ea* strains were reliably identified by PCR (Bereswill and own primers) after optimisation of PCR protocol and using BIOLOG System. PTA-ELISA and IIF were less reliable because of cross-reacting bacteria associated with *Ea* in host plants. All *Cms* strains were reliably identified by IIF and PCR (Mills and Pastrik primers) regardless of morphological variability. The fluidal *Cms* strains were more reliably identified using DAS-ELISA and BIOLOG System than less fluidal strains.

Key words: *Erwinia amylovora, Clavibacter michiganensis* subsp. *sepedonicus*, detection, determination, PCR, BIOLOG, ELISA, IF.

Introduction

Fire blight, caused by *Erwinia amylovora* (*Ea*), is one of the most serious diseases of pomes-fruit and ornamental plants in the family *Rosaceae* /EPPO/CABI, 1997/. Management measures include surveys of presumptive potato samples and eradication of infected plants confirmed by laboratory tests, and control and regulation of propagative and cultivated plant materials /Vanneste, 2000/.

Ring rot of potato, caused by *Clavibacter michiganensis* subsp. *sepedonicus (Cms),* is an economically important disease for the potato industry, which has been spread in many countries around the world. Management measures based on inspection and the testing of presumptive potato samples include rejection of infected seed lots, and control of potential further dissemination of this dangerous organism /Anonymous, 1993; EPPO/CABI, 1997; Van der Wolf et al., 2005/.

Diagnosis of *Ea* and *Cms* is mostly conducted by means of IF and PCR, but ELISA and biochemical tests are also recommended. Confirmation of *Ea* is carried out by means of bioassay on immature pear fruits and other host plants /Anonymous,

http://www.csl. gov.uk/ and *Cms* by means of bioassay on eggplants /Anonymous, 1993; OEPP/EPPO, 2006/.

ELISA and IF tests are very well suited for routine screening of large numbers of samples taken directly from plant tissues. Evaluation is very rapid and results are accurate when highly specific antibodies are used /De Boer, Hall, 2000; Vanneste, 2000; Pánková, Kokošková, 2002; Van der Wolf et al., 2005/.

High reliability of detection was found when methods based on genetic principle were used. Molecular techniques, such as PCR and others are very helpful in the cases where different cell phenotype directly influences reliability of other diagnostic methods, because they are not dependent on cell physiology. Detection of *Ea* has been usually carried out with primer set designed by Bereswill et al. (1992, 1995) and detection of *Cms* with primer set designed by Mills et al. (1997), Pastrik (2000) and Pastrik and Rainey (1999).

Biochemical identification of *Ea* and *Cms* strains is usually conducted by fatty acid analyses using gas chromatography /Sasser, 1990; Vanneste, 2000/ and/or using the microbial identification system *Biolog Bacteria* based on an extensive spectrum of carbon utilization reaction /Jones et al., 1993; Kokošková et al., 2005/.

The objective of this study was to compare reliability of commonly available polyclonal and monoclonal antibodies for ELISA and IF tests, and PCR with commercially used primers for *Ea* and *Cms*.

Materials and Methods

Bacterial strains. Ea strains and associated bacteria isolated from infected host plants were cultivated on meat peptone agar and incubated at 25° C. Identities of all *Ea* strains tested were confirmed by the immature pear fruit assay. *Cms* strains and associated bacteria obtained from infected tuber samples were cultivated on the medium of Snieszko and Bonde (1943) at 23° C. Identity of all *Cms* strains tested was confirmed by the eggplant bioassay.

ELISA and *immunofluorescence.* Commercial polyclonal antibodies (PAb) (Neogen Europe Ltd., UK) were used for diagnosis of *Ea* by means of PTA-ELISA (indirect enzyme-lined immunosorbent assay) and IIF (indirect immunofluorescence) according to the manufacturer's recommendations. Commercial monoclonal antibodies (MAb) (Agdia, USA) were used for diagnosis of *Cms* by means of DAS-ELISA (double antibody sandwich enzyme-lined immunosorbent assay) and IIF according to the manufacturer's recommendations. Bacteria were prepared in inoculums density OD_{620} 0.1 (10^8 cfu/ml) and after that diluted till to OD 0.0001. Positive/negative reactions were recorded with an ELISA reader. ELISA threshold level was 0.2 at A_{405}. IF slides were observed under a light microscope fitted for epifluorescence at 1000x magnification using a mercury lamp and a suitable filter system.

PCR and gel electrophoresis. Bacterial suspensions were used in the same rate of concentrations as for immunochemical tests. If *Ea* was detected, one µl of each dilution of bacterial suspension was directly added to the PCR reaction mix. When infected plant samples were used, it was necessary to use a Dneasy Plant Mini Kit (QIAGEN). The DNA isolation was performed according to the manufacturer's protocol for each dilution of homogenized infected plant sample. Primers according to Bereswill et al. (1995) and our

own primers for detection and determination of *Ea* were used /Kokošková et al., 2007/. Primers were synthesised by *Generi Biotech* (Hradec Králové, Czech Republic). PCR was performed with a Mini Cycler (MJ Research, Watertown, MA, USA).

The pathogen – specific primer set PSA-1/PSA-R and Mills primers /Mills et al., 1997/ were used for the specific amplification of *Cms* strains. Reaction mix and amplification protocol of Pastrik primers were performed according to Pastrik (2000). Amplified PCR products (3 µl) were mixed with 2 µl of loading buffer containing the Sybr Green (*Sigma-Aldrich*) stain and run on 1% agarose gel.

BIOLOG system. Ea strains and bacteria associating with *Ea* in symptomatic plant samples and *Cms* strains and bacteria associating with *Cms* in tuber samples were identified using the microbial identification system *Biolog Bacteria.* Strains were characterised biochemically using the *Biolog* GN and GP MicroPlate System™ (Biolog Inc. USA) following manufacturer's instructions. Evaluation was performed with the naked eye after 4 h and 24 h of incubation. Cultures were identified using the MicroLog™ 2 database for gram-negative and gram-positive bacteria.

Results and Discussion

The fire blight pathogen is usually isolated easily from symptomatic plant material by dilution plating, followed by laboratory tests that aid in reliable identification. The ring rot pathogen is usually detected from latently infected plant material without isolation the bacterium, because of very precise diagnostic tests are required.

Sensitivity and specificity of immunochemical tests depend on the type of used method and on quality of used antibodies. For identification of *Cms* were used MAb, that were more sensitive than PAb used for identification *Ea* (Tables 1 and 2).

In our experiments with *Ea*, PTA-ELISA was sensitive as a value of 10^{6-5} CFU/ml, whereas IIF was able to detect *Ea* at 10^{5-4} CFU/ml for some strains. Zielke et al. (1993) detected *Ea* at a concentration of 10^5 CFU/ml when used PAb in DAS-ELISA and IF tests. In our experiments with *Cms*, where MAb were used, DAS-ELISA was sensitive as a value of 10^5 CFU/ml, whereas IIF was able to detect *Cms* at 10^4 CFU/ml for some of strains. Similar results presented De Boer and Hall (2000).

Table 1. Sensitivity of diagnostic techniques for *Erwinia amylovora*

Method	Identified strains (%)			
	Optical density$_{620nm}$ (CFU/ml)			
	10^7	10^6	10^5	10^4
PTA-ELISA	100	74	30	0
IIF	100	100	61	31
PCR (B)	100	100	80	51
PCR (K)	100	100	85	48

B – Bereswill
K – Kokošková

In our experiments, the sensitivity of PCR for *Ea* was as low as 10^4 CFU/ml for some strains. Based on results both PCR tests were comparably sensitive, PCR with

primers of Bereswill et al. (1995) and PCR with own primers /Kokošková et al., 2007/. A consistent detection level of 10^2 CFU/ml of *Ea* was obtained when Taylor et al. (2001) detected *Ea* directly from plant tissue using PCR, where tissue inhibitors were eliminated and bacterial DNA extracted at the same time.

Table 2. Sensitivity of diagnostic techniques for *Clavibacter michiganensis* subsp. *sepedonicus*

Method	Morphology	Identified strains (%)			
		Optical density$_{620nm}$ (CFU/ml)			
		10^7	10^6	10^5	10^4
DAS-ELISA	fluidal	100	100	90	25
	nonfluidal	20	20	10	/
IIF	fluidal	100	100	100	85
	nonfluidal	100	100	91	/
PCR	fluidal	100	100	100	90
	nonfluidal	100	100	100	/

Of immunochemical methods only IIF was capable to detect both fluidal and rough *Cms* strains with equal sensitivity /Kokošková et al., 2005/. DAS-ELISA uses antibodies produced mostly to surface antigens, which are usually EPS /Baer, Gudmestad, 1993/. EPS are usually missing at rough *Cms* strains. That is why DAS-ELISA fails in detection of rough *Cms* strains. The sensitivity of PCR for *Cms* (primers PSA-1/PSA-R) was as low as 10^4 CFU/ml (Table 2). The sensitivity of PCR for *Cms* appeared not to be affected by *Cms* colony morphology, because fluidal and rough strains of the bacterium were identified with equal sensitivity, which agrees with results of Slack et al. (1996). PCR have shown to be rapid and reliable method for *Cms* identification, suitable for routine screening.

Reliability of detection of *Ea* using immunochemical methods is presented in Table 3. Out of 50 older samples of host plants with typical and atypical symptoms of fire blight disease, using IIF *Ea* was detected in 100 and 90% of samples at 1:100 and 1:1000 dilution, respectively meantime using PTA-ELISA *Ea* was detected only in 95 and 40% of samples at 1:10 and 1:100 dilution, respectively (Table 3). In our tests, IIF appeared more sensitive than PTA-ELISA similarly as in experiments of Lin et al. (1987).

Table 3. Reliability of detection of *Erwinia amylovora*, and specificity of PAb in immunochemical methods

Method	Detected strains (%)				Specificity
	Dilution of plant extract				False positives
	1:1	1:10	1:100	1:1000	
PTA-ELISA	70	95	40	/	20
IIF	/	80	100	90	15

Reliability of detection of *Cms* using immunochemical methods is presented in Table 4. Out of 42 tuber extracts of potato, using IIF *Cms* was detected in 100% of samples at 1:10, 1:100 and 1:1000 dilution, respectively meantime using DAS-ELISA *Cms* was detected in 98, 96 and 70% of samples at 1:10 and 1:100 and 1:1000 dilution, respectively (Table 4). In our tests, IIF appeared more sensitive than DAS-ELISA.

Table 4. Reliability of detection of *Clavibacter michiganensis* subsp. *sepedonicus*, and specificity of MAb in immunochemical methods

Method	Detected strains (%)				Specificity
	Dilution of extract				False positives
	1:1	1:10	1:100	1:1000	
DAS-ELISA	98	98	96	70	2
IIF	/	100	100	100	2

In specificity of MAb for *Cms* and PAb for *Ea* there were found high differences (Tables 3 and 4). When PAb were used for associated bacteria with *Ea*, about 15% cross-reactions were found in IIF and 20% in PTA-ELISA. Various enterobacteria were found associated with *Ea* in plant samples as *Pantoea agglomerans*, *P. dispersa*, *Enterobacter cloaceae*, *E. gergoviae*, *Rahnella aquatilis* and *Klebsiella pneumoniae.* Some of these strains cross-reacted with PAb for *Ea.* The majority of workers achieved better results using MAb or mixture of MAbs than with PAb /Hutschemackers et al., 1990/.

When MAb were used for associated bacteria with *Cms*, 2% cross-reactions only were found in IIF and DAS-ELISA. There were 2 cross-reacting isolates in each methods, one of them was the same in both methods. These strains were not identified using Biolog Bacteria system. Low number of cross-reactions with bacteria associated with *Cms* in tuber samples was recorded also by other authors De Boer et al. (1996). Excellent results were obtained in comparable experiments conducted in eight diagnostic laboratories parallel within four years, where specificity of IIF was 100 % because none false positives occurred and specificity of DAS-ELISA was almost 100 % because only about 0.5 % false positives were recorded /De Boer, Hall, 2000/.

When we detected *Ea*, for PCR from infected plant samples it was necessary to use the bacterial DNA isolated using a DNeasy Plant Mini Kit (QIAGEN). This procedure was more laborious and time consuming. We found that the capability of *Ea* detection might be increased using an optimised /Kokošková, Mráz, 2005/ PCR technique according to Bereswill et al. (1995), eliminating false positives. PCR was also much more specific than both immunochemical techniques, PTA-ELISA and IIF, when our primers (EaF72 and EaR560) designed for *Ea* detection were used, because no false positives were observed.

All European and USA *Cms* isolates amplified by Pastrik primers PSA-1/PSA-R used in PCR showed positive reaction on the 1% agarose gel. PCR appeared not to be affected by *Cms* colony morphology, as we were able to identify fluidal and rough strains of the bacterium with equal sensitivity, this is in harmony with results of Slack et al. (1996). PCR have shown to be rapid and reliable method for *Cms* identification.

All *Ea* strains tested were reliably identified using Biolog Bacteria method, GN MicroPlate System. For *Cms* strains Biolog Bacteria method GP MicroPlate System did not appear reliable, because rough strains usually were not identified and fluidal *Cms* strains were identified only to the subspecies level. Our results are in harmony with results of other authors /Jones et al., 1993/

Conclusions

The use of more than one screening tests based on different biological principles is recommended for diagnosis of both pathogens. If *Ea* is detected from older or asymptomatic samples, detection of pathogen could be complicated. Based on results of our tests, when only PAb are used we recommend as screening tests IIF and PCR, because ELISA is less reliable. For detection of *Cms* we recommend also IIF and PCR tests, because ELISA is not capable of detecting less fluidal strains of *Cms* and is less sensitive when *Cms* is detected from plant samples.

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