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A rapid lateral-flow immunoassay for phytosanitary detection of *Erwinia amylovora* and on-site fire blight diagnosis

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ABSTRACT

Fire blight is an invasive disease caused by *Erwinia amylovora* that threatens pome fruit production globally. Effective implementation of phytosanitary control measures depends upon rapid, reliable pathogen detection and disease diagnosis. We developed a lateral-flow immunoassay specific for *E. amylovora* with a detection limit of log 5.7 CFU/ml, typical of pathogen concentrations in symptomatic plant material. The simple assay had comparable sensitivity to standard culture plating, serum agglutination and nested PCR when validated for application in a phytosanitary laboratory as a confirmatory test of cultured isolates and for first-line diagnosis of phytosanitary samples that represent the full range of commercial, ornamental and forestry host species. On-site validation in ring-trials with local plant inspectors demonstrated robust and reliable detection (compared to subsequent plating and PCR analysis). The simplicity, inspector acceptance and facilitation of expedited diagnosis (from 2 days for laboratory submitted samples to 15 min with the immunoassay), offers a valuable tool for improved phytosanitary control of fire blight.

1. Introduction

Erwinia amylovora is a necrotrophic Gram-negative enterobacterium that causes fire blight in over 200 *Rosaceae* species, primarily Pyrinae (Momol and Aldwinckle, 2000). Fire blight is the major economic disease threat to apple, pear, and quince production globally, capable of killing trees and entire orchards within a single season, restricting international trade (Calvin and Krissoff, 1998), and resulting in multi-million EURO expenditures for eradication and quarantine programs (Bonn and van der Zwet, 2000; Duffy et al., 2005; Rodoni et al., 2002). Fire blight is an invasive disease that has spread from the Northeast USA, where it was described as the first bacterial plant disease in the late 1700s (Griffith et al., 2003), across North America following European settler expansion, to Australasia in the early 1900s, and Northern Europe and Mediterranean regions in the late 1950–1960s (Smits et al., 2011). Quarantine efforts have slowed but not prevented disease progression across continental Europe, the Middle East and Northern Africa, with an impending threat to pome fruit germplasm resources in the Central Asian geographic center of origin for pome fruits.

Improving effective implementation of phytosanitary control measures depends upon rapid, reliable diagnostics (López et al., 2003; Pasquer et al., 2010; Ward et al., 2004). In the field, visual symptom ratings by plant inspectors are the basis for phytosanitary control measures and critical samples are sent to official diagnostic laboratories for confirmation. Current fire blight diagnostic methods prescribed in international standards (Anonymous, 2004) are laboratory-based (i.e., semi-selective plating, serum agglutination, IF-microscopy and PCR), labor-intensive, expensive and/or demand specialized training, and necessitate transport of infectious plant samples to a phytosanitary laboratory with a delay of 1–4 days for results (López et al., 2009; Pirc et al., 2009; Stöger et al., 2006). Lateral-flow immunochromatographic assays (dip-stick format) have simplified and expedited end-user available diagnostics in human health, food safety, and more recently in plant protection (Posthuma-Trumpie et al., 2009). Most immunoassays in phytopathology are targeted to detect viral and fungal pathogens (Danks and Barker, 2000; Thornton et al., 2004; Lane et al., 2007). A few have recently been commercialized for bacterial phytopathogen detection but these have provided insufficient specificity or sensitivity, or have not been validated for application in high-throughput diagnostic laboratories or for direct on-site diagnostics (López et al., 2003).

The purpose of our study was to develop and validate a simple, rapid and reliable test to streamline high-throughput sample processing in phytosanitary diagnostic laboratories and enabling on-site fire blight diagnosis in the field. Our objectives were to determine specificity and sensitivity of a novel lateral-flow immunoassay, compare its performance with standard *E. amylovora* detection

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methods and fire blight diagnostic tools according to the EPPO diagnostic protocol for this regulated pathogen (Anonymous, 2004), which is based on pathogen isolation and confirmatory tests including serum agglutination and PCR. Our end goal was to evaluate the reliability and utility of this lateral-flow immunoassay as a rapid tool for in-field diagnostics application by phytosanitary inspectors.

2. Materials and methods

2.1. Ea AgriStrip immunoassay development

The lateral-flow immunoassay (Ea AgriStrip) was developed in a simple dip-stick-format using polyclonal antibodies produced in rabbits against heat-killed whole cells of *E. amylovora* strain C6 and a mixture of strains (*E. amylovora* 01SFR-BO from Italy, ACW56400 from Switzerland, Ea153 from USA, Ea4/82 from Egypt, CFBP1430 from France). While polyclonal antibodies limits the long-term supply of reagent, repeated attempts to obtain a sustainable source of monoclonal antibodies with equivalent detection spectrum were unsuccessful. These target-specific antibodies were immobilized on a test line (Fig. 1) of the nitrocellulose membrane and are conjugated to colloidal gold particles on the sample pad of the test strip. Once the strip is inserted into the sample extract, the liquid migrates upwards and dissolves the antibody–gold conjugate. Antigen present in the sample extract binds to the conjugate forming an antigen–antibody–gold complex, which is then captured by the immobilized antibody in the test line and becomes visible as a red line. Gold antibody conjugate without antigen does not bind to the test line and is subsequently captured by the control line, which contains immobilized goat-anti-rabbit antibodies. Both test and control lines become visible with positive extracts (containing the antigen), whereas negative samples produce the upper control line only (Fig. 1). Red lines start developing after 1–2 min and reach maximum intensity after 10–15 min.

Test analysis was performed by placing the sample side of the strip into 150 µl (approximately four drops) of sample suspension in 1×AgriStrip Extraction buffer B (AEB) [BIOREBA AG, Reinach, Switzerland; Art. No. 110163 (100 ml); Art. No. 110164 (500 ml)] in a cuvette or microcentrifuge tube.

2.2. Analytical specificity and sensitivity

Test specificity was determined using 39 *E. amylovora* strains representing the global and genetic species diversity (Rezzonico et al., 2011), and 61 strains of related *Erwinia* spp. or environmental bacteria and yeasts that are applied in orchards for fire blight biocontrol or are commonly co-isolated from the fire blight diagnostic samples (Table 1). Bacteria were grown overnight at 28 °C on King's medium B (KB) agar plates (Duffy and Défago, 1999). Bacteria were suspended in AgriStrip Extraction buffer B to give approximately 10^8 CFU/ml and 150 µl suspension was placed in a 1.5 ml microcentrifuge tube. An Ea AgriStrip was inserted vertically immersing just the sample side (max 0.5 cm of the strip) and results were recorded after 15 min.

Sensitivity was determined using four *E. amylovora* strains (CFBP1430, ACW35060, ACW42121 and Ea153) at a range of concentrations from to 1×10^2 to 1×10^8 CFU/ml in AgriStrip Extraction buffer B with or without crushed apple shoots (approximately 0.2 g/ml). Concentrations were confirmed by dilution-plating aliquots onto KB agar. Tests with Ea AgriStrip were conducted as described above. After 15 min incubation at 22 °C, the limit of detection (LOD) was determined as the lowest concentration that produced a visible positive test line. In parallel, the LOD for nested PCR (Llop et al., 2000) and plate isolation was determined using dilution series of pure cultures of CFBP 1430. The Ea AgriStrip sensitivity was also evaluated using naturally-infected quince, cotoneaster and pear shoot samples, with *E. amylovora* concentrations determined by dilution-plating. Potential interference from epiphytic bacteria was further evaluated by mixing CFBP1430 dilutions (10^2–10^8 CFU/ml) with either *Pseudomonas syringae* pv. *syringae* strain ACW460 or *Pantoea vagans* strain C9-1 (10^7 CFU/ml) in AEB. Detection of live vs. dead cells was evaluated using a 10^8 CFU/ml suspensions of CFBP1430 and ACW35060 before and after heat-inactivation (95 °C for 30 min) or autoclaving. Specificity and sensitivity assays were performed at least twice.

2.3. Comparison of Ea AgriStrip with serum agglutination

The performance of Ea AgriStrip as a confirmatory test for suspicious colonies recovered after dilution-plating fire blight plant

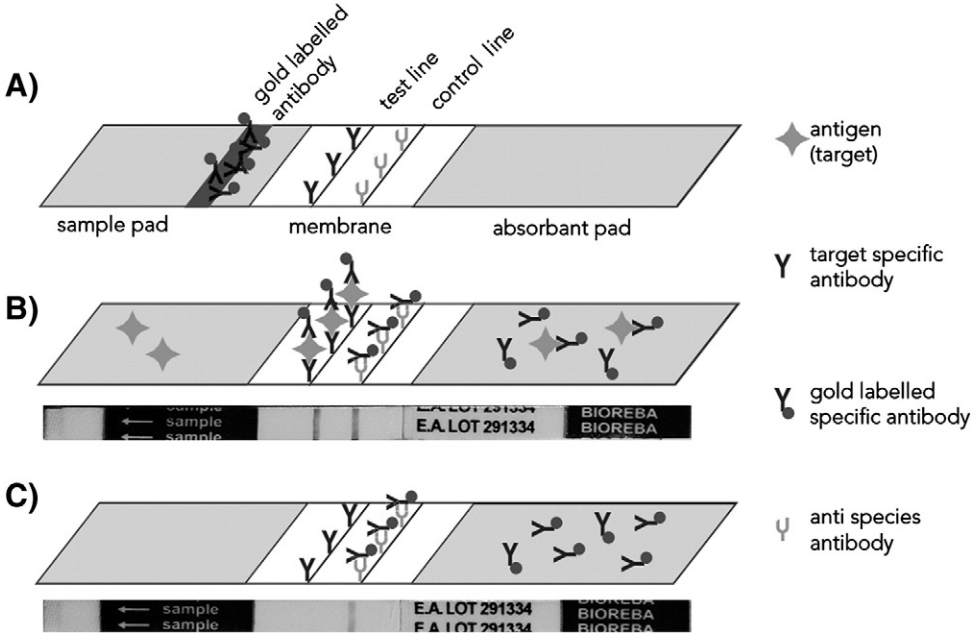

Fig. 1. Design of the Ea AgriStrip lateral-flow immunoassay. (A) Gold labeled target-specific antibodies are applied to the sample pad of the nitrocellulose membrane. Target-specific antibodies without the gold label are immobilized on the test line, while goat-anti-rabbit antibodies (anti-species) are immobilized on the control line. (B) After sample application (immersing the base of the immunoassay strip in sample suspension), both the test and control lines become visible (red) when the antigen target (*Erwinia amylovora*) is present. (C) When samples lack the antigen target, only the control line becomes visible indicating that the assay functioned properly.

Table 1
Analytical specificity of Ea AgriStrip lateral-flow immunoassay against *Erwinia amylovora*, closely related species, biocontrol agents, and environmental bacteria associated with fire blight diagnostic samples.

Strain	Origin	Host	Ea AgriStrip[b]	Source or reference[c]
Erwinia amylovora ACW 35060	Switzerland	*Cotoneaster salicifolius*	+	This study
E. amylovora ACW 35260	Switzerland	*Crataegus* sp.	+	This study
E. amylovora ACW 42288	Switzerland	*Pyrus communis*	+	This study
E. amylovora ACW 26599	Switzerland	*Cydonia oblonga*	+	(Rezzonico and Duffy, 2007)
E. amylovora ACW 42121	Switzerland	*Malus domestica*	+	This study
E. amylovora Ea02	Switzerland	*Cotoneaster* sp.	+	(Molina et al., 2005)
E. amylovora ACW 56400	Switzerland	*P. communis*	+	This study
E. amylovora CFBP 1430	France	*Crataegus* sp.	+	(Smits et al., 2010b)
E. amylovora CFBP 2301	France	*Pyracantha* sp.	+	CFBP
E. amylovora CFBP 1232^T	UK	*P. communis*	+	CFBP
E. amylovora CFBP 3020	Netherlands	*P. communis*	+	CFBP
E. amylovora 1/74	Germany	*Cotoneaster* sp.	+	(Jock et al., 2002)
E. amylovora 1/79 (DSM 17948)	Germany	*Cotoneaster* sp.	+	DSMZ
E. amylovora 7/74	Germany	*Cotoneaster bullatus*	+	K. Geider
E. amylovora UPN 527 (no plasmids)	Spain	*M. domestica*	+	(Llop et al., 2006)
E. amylovora OMP-BO 691.2	Italy	*P. communis*	+	K. Geider
E. amylovora 01SFR-BO	Italy	*Sorbus* sp.	+	(Jock et al., 2002)
E. amylovora Ea 273 (ATCC 49946)	USA	*M. domestica*	+	ATCC
E. amylovora JL 1185	USA	*P. communis*	+	V.O. Stockwell
E. amylovora Ea 153	USA	*M. domestica*	+	(Jock et al., 2002)
E. amylovora OR29/pEU30	USA	*P. communis*	+	(Foster et al., 2004)
E. amylovora Ea 110R	USA	*M. domestica*	+	V.O. Stockwell
E. amylovora LA 476	USA	*P. communis*	+	V.O. Stockwell
E. amylovora UTRJ2/pEU30	USA	*M. domestica*	+	(Foster et al., 2004)
E. amylovora CFBP 3792	USA	*Prunus salicina*	+	CFBP
E. amylovora CFBP 3098	Israel	*M. domestica*	+	CFBP
E. amylovora Ea 209	Israel	*P. communis*	+	(Valinsky et al., 1998)
E. amylovora Ea 263	Germany	*M. domestica*	+	(Jock et al., 2002)
E. amylovora Ea 4/82	Egypt	*P. communis*	+	(Jock et al., 2002)
E. amylovora LebB66/pEL60	Lebanon	*C. oblonga*	+	(Foster et al., 2004)
E. amylovora LebA3/pEL60	Lebanon	*M. domestica*	+	(Foster et al., 2004)
E. amylovora CFBP 3025	NZ	*M. domestica*	+	CFBP
E. amylovora LA025	USA	*P. communis*	+	V.O. Stockwell
E. amylovora LA071	USA	*P. communis*	+	V.O. Stockwell
E. amylovora LA096	USA	*P. communis*	+	V.O. Stockwell
E. amylovora LA102	USA	*P. communis*	+	V.O. Stockwell
E. amylovora JL1168	USA	*P. communis*	+	V.O. Stockwell
E. amylovora JL1170	USA	*P. communis*	+	V.O. Stockwell
E. amylovora LA076	USA	*P. communis*	+	V.O. Stockwell
E. tasmaniensis LA540	USA	*M. domestica*	+	V.O. Stockwell
E. tasmaniensis Et 1/99 (DSM 17950)	Australia	*M. domestica*	+	(Geider et al., 2006)
E. tasmaniensis Et 2/99	Australia	*P. communis*	+	(Geider et al., 2006)
E. tasmaniensis Et 4/99	Australia	*M. domestica*	+	(Geider et al., 2006)
E. tasmaniensis BE57	Australia	*Prunus* sp.	+	R. Powney
E. tasmaniensis BE65	Australia	*Chaenomeles japonia*	+	R. Powney
E. tasmaniensis DAR 61733	New Zealand	*Pyrus pyrifolia*	+	R. Powney
E. pyrifoliae DSMZ 12163^T	South Korea	*P. pyrifolia*	+/−	(Smits et al., 2010a)
E. pyrifoliae CFBP 4174	South Korea	*P. pyrifolia*	+/−	(Kim et al., 1999)
E. pyrifoliae EP 1/96	South Korea	*P. pyrifolia*	+/−	(Kim et al., 1999)
E. piriflorinigrans 2045-T3	Spain	*P. communis*	+/−	(López et al., 2011)
E. piriflorinigrans APA 3958	Spain	*P. communis*	+/−	(López et al., 2011)
E. piriflorinigrans APA 3959	Spain	*P. communis*	+/−	(López et al., 2011)
E. piriflorinigrans APA 3960	Spain	*P. communis*	+/−	(López et al., 2011)
E. piriflorinigrans APA 3967	Spain	*P. communis*	+	(López et al., 2011)
E. piriflorinigrans APA 3978	Spain	*P. communis*	+	(López et al., 2011)
E. billingiae LMG 2613^T	United Kingdom	*P. communis*	–	LMG/BCCM
E. billingiae 23050A	Australia	*P. communis*	–	R. Powney
E. billingiae 23048B	Australia	*P. communis*	–	R. Powney
E. billingiae DAR 72021	Australia	*Cotoneaster* sp.	–	R. Powney
E. billingiae 38#14	Australia	*M. domestica*	–	R. Powney
E. billingiae BE66	Australia	*Chaenomeles* sp.	–	R. Powney
E. persicina CFBP 3622^T	Japan	*Lycopersicon esculentum*	–	CFBP
E. rhapontici CFBP 3163^T	UK	*Rheum rhabarbarum*	–	CFBP
Brenneria rubifaciens LMG 2709^T	USA	*Juglans regia*	–	LMG/BCCM
Pantoea agglomerans Eh239	USA	*Hordeum vulgare*	–	(Braun-Kiewnick et al., 2000)
P. agglomerans ATCC 27155^T	Zimbabwe	Clinical isolate	–	ATCC
P. agglomerans P10c (BlossomBless®)	NZ	*M. domestica*	–	(Rezzonico et al., 2009)
P. agglomerans E325 (Bloomtime FD™E325)	USA	*M. domestica*	–	(Rezzonico et al., 2009)
P. agglomerans CPA-2	Spain	*M. domestica*	–	(Rezzonico et al., 2009)
P. ananatis LMG 2665	Brazil	*Ananas comosus*	–	LMG/BCCM
P. stewartii subsp. *stewartii* CFBP 3517^T	USA	*Zea mays*	–	CFBP
P. vagans C9-1 (Blight Ban C9-1™)	USA	*M. domestica*	–	(Rezzonico et al., 2009)
Escherichia coli DH5α™			–	Invitrogen
Aureobasidium pullulans CF10 (BlossomProtect™)	Germany	*M. domestica*	–	Bio-Protect GmbH

(continued on next page)

Table 1 (*continued*)

Strain	Origin	Host	Ea AgriStrip[b]	Source or reference[c]
Bacillus subtilis QST713 (Serenade®)	USA	Peach orchard soil	–	AgraQuest
B. subtilis var. *amyloliquefacien* FZB24	Germany	Soil	–	ABiTEP GmbH
Metschnikowia pulcherrima MSK1	Germany	*M. domestica*	–	Bio-Protect GmbH
Pseudomonas fluorescens CHA0	Switzerland	Soil	–	(Duffy and Défago, 1999)
P. fluorescens A506 (Blight Ban A506™)	USA		–	Nufarm Limited
P. corrugata NCPPB 2445		*Solanum lycopersicum*	–	NCPPB
P. syringae pv. *papulans* BBL	Germany	*M. domestica*	–	E. Moltmann
P. syringae pv. *papulans* ACW 38201	Switzerland	*M. domestica*	–	This study
P. syringae pv. *syringae* ACW 460	Switzerland	*M. domestica*	–	This study
P. syringae pv. *syringae* NCPPB 281	United Kingdom	*Syringa vulgaris*	–	This study
P. syringae pv. *savastonoi* ACW 230	Switzerland	*Nerium oleander*	–	This study
Xanthomonas arboricola pv. *pruni* ATCC 19316	New Zealand	*P. salicina*	–	ATCC
X. arboricola pv. *juglandis* NCPPB 411	United Kingdom	*Juglans regia*	–	NCPPB
X. campestris pv. *campestris* ACW 133	Switzerland	*Brassica oleracea*	–	This study
X. hortorum pv. *pelargonii* NCPPB 1615	United Kingdom	*Pelargonium zonale*	–	NCPPB
Fire blight coisolate (v5-66) *P. syringae*[a]	Switzerland	*C. oblonga*	–	This study
Fire blight coisolate (v5–67) *P. syringae*[a]	Switzerland	*M. domestica*	–	This study
Fire blight coisolate (v5–68) *P. syringae*[a]	Switzerland	*C. oblonga*	–	This study
Fire blight coisolate (v5-69) *Enterobacter* sp.[a]	Switzerland	*C. oblonga*	–	This study
Fire blight coisolate (v5–70) *Pseudomonas* sp.[a]	Switzerland	*P. communis*	–	This study
Fire blight coisolate (v5–71) *Erwinia* sp.[a]	Switzerland	*C. japonica*	–	This study
Fire blight coisolate (v5–72) *P. agglomerans*[a]	Switzerland	*C. oblonga*	–	This study
Fire blight coisolate (v5–73) *P. agglomerans*[a]	Switzerland	*Crataegus* sp.	–	This study
Fire blight coisolate (v5–74) *P.agglomerans*[a]	Switzerland	*M. domestica*	–	This study
Fire blight coisolate (v5–75) *Pseudomonas* sp.[a]	Switzerland	*P. communis*	–	This study

[a] Environmental strains co-isolated from the fire blight phyllosphere were sequenced by their 16S rDNA gene and identified as the listed bacteria by similarities ≥ 98% using BLASTN.

[b] Ea AgriStrip results after 15 min: + indicates positive detection; – indicates negative detection; +/– indicates weakly positive or unreliable detection.

[c] ACW = Agroscope Changins-Wädenswil, Switzerland; ATCC = American type culture collection, USA; CFBP = Collection Francaise de Bacteries Phytopathogenique, France; DSMZ = Deutsche Sammlung für Mikroorganismen und Zellkulturen, Germany; LMG/BCCM = Laboratorium voor Microbiologie Gent, Belgium; NCPPB = National collection of plant pathogenic bacteria, United Kingdom.

samples on KB or NSA (nutrient sucrose agar) was compared with a standard serum agglutination test (Anonymous, 2004). Serum agglutination was performed by placing a 20 µl drop of polyclonal antiserum (rabbit K-RAC 13/4 2001, 1:70 diluted in PBS buffer) on a glass slide and gently mixing with a loop of test bacteria to give approximately 10^9 CFU/ml. After 50 min incubation at 22 °C, observation of clear drops with bacteria precipitated in the center indicated positive reactions and uniformly turbid drops indicated negative reactions. The same bacterial colonies were suspended into AgriStrip Extraction buffer B and tested with Ea AgriStrip in parallel as described above.

2.4. Laboratory comparison of Ea AgriStrip with plating for detection of *E. amylovora* in naturally-infected plant samples

Plant samples were submitted to the federal diagnostics laboratory by phytosanitary inspectors from throughout Switzerland in 2006–2008 and triaged using a visual rating scale of 1–4 based on symptoms. Such rating is routinely done for providing feedback to improve inspector training. Visual rating VR1 indicated unlikely fire blight (e.g., undefined or only superficial tissue necrosis, defined margin between necrotic and healthy tissues). Visual rating VR2 indicated uncertain fire blight (e.g., water-soaked flower, immature fruit, shoot tissues, red-brown striation in subcortical woody tissue beneath bark). Visual rating VR3 indicated likely fire blight (e.g., wilted or shriveledvegetative tissues with darkened lesions, scorched leaves without black necrosis, dark cankers or discoloration of vascular tissues in wood). Visual rating VR4 indicated probable fire blight (e.g., leaf necrosis along veins, shoot recurvature, blackened flowers, dark vascular necrosis without clear margin and presence of bacterial ooze).

Symptomatic tissue samples were excised according to the EPPO protocol (Anonymous, 2004)(three to four pieces, approximately 3-cm length, 0.1–1.0 g) and extracted in 14 ml volume plastic tubes containing 4 ml PBS with shaking at 22 °C for 15–30 min, and then tested in parallel with plating and Ea AgriStrip. Plate isolation, used as a 'gold standard', was done by dilution streaking 10 µl of plant extract onto KB and NSA agar (Anonymous, 2004). After 48 h incubation at 28 °C, plates were observed for presence of *E. amylovora* typical white, non-fluorescent, mucoid (KB) and transluscent, levan, domed (NSA) colonies. The Ea AgriStrip was used as described above by mixing 120 µl of plant extract with 24 µl of 5 × AgriStrip Extraction buffer B in a 1.5 ml microcentrifuge tube.

For comparing results of two diagnostic methods used in this study and for the purpose of evaluating the immunoassay as a new diagnostic method, contingency tables were prepared and diagnostic sensitivity and specificity, false negative and positive ratings (FNR, FPR), positive and negative predictive values (PPV, NPV), prevalence, and likelihood ratios (LR) were calculated (Chakraborty et al., 2009; Grange and Lazlo, 1990).

2.5. On-site field comparison of Ea AgriStrip with plating and nested PCR

Field plant material was first rated visually for symptoms of fire blight and then cut and placed into extraction bags (BIOREBA AG) with 4 ml of 1 × AgriStrip Extraction buffer B. Plants were macerated in the extraction bags using a small rubber hammer. Four drops of the sample extract (120–150 µl) were placed in a plastic cuvette. One strip was inserted into the cuvette with the sample end immersed in the extract and the reaction was read after 15 min. The rest of the sample liquid was decanted into sterile plastic tubes, kept on ice during transport and at 4 °C overnight until further investigation by plate isolation the next day and then frozen at −20 °C until DNA extraction and nested PCR. Plate isolation was done as described above. Nested PCR was used as second'gold standard' according to the EPPO protocol for diagnosis of regulated pests (Anonymous, 2004) following Llop et al. (2000) after DNA extraction of 1 ml plant extracts (Llop et al., 1999). After extraction, DNA pellets were resuspended in 100 µl Milli-Q ultra-pure water and 1 µl used in nested PCR reactions. Since preliminary experiments demonstrated PCR inhibition after DNA

extraction, samples were diluted 1:10 prior to nested PCR. Results were rated positive when an *E. amyolovora*-specific amplicon was detected in samples but not in negative controls (ultra-pure water, healthy plant extract). Nested PCR was rated negative if an *E. amylovora*-specific amplicon of expected size was not detected for the sample but was detected for all positive controls (CFBP 1430, 10^4 CFU/ml; aliquot of healthy plant extract spiked with 10^6 CFU/ml of CFBP 1430). *E. amylovora* specific PCR amplicons (391–447 bp) of a subset of six samples of different plant origin were further confirmed by sequencing on an ABI Prism Apparatus, 3130 x/Genetic Analyser (Applied Biosystems, Rotkreuz, Switzerland). This was done to make sure the amplified DNA sequence belonged to *E. amylovora* isolates since there is natural variation in target DNA size. The target DNA sequence on plasmid pEA29 is based on a short sequence DNA repeat SSR first described by (Kim et al., 1999). The copy number of plasmid pEA29 is 0–5 copies per cell/genome. All six samples were identified as *E. amylovora* by 99–100% sequence identity using sequence alignments with the recently sequenced CFBP1430 genome (Smits et al., 2010b).

Mathematical comparison of results for two diagnostic methods was performed using contingency tables as described above. Correlation analyses were performed using the Spearmann Rank Order Correlation and Sigma STAT 3.1 (Systat Software Inc., UK), where the pair of variables with positive correlation coefficients (r) and P values below 0.05 tend to increase together. For pairs with P values greater than 0.05, there is no significant relationship between the two variables. Method indices were calculated for every single method by giving values of 'zero' to negative test 'one' to positive test results.

3. Results and discussion

3.1. Analytical specificity

Out of 100 bacterial strains tested, the lateral-flow immunoassay specifically reacted with 39 *E. amylovora* strains out of a worldwide collection (Table 1). The only cross-reactions observed were with genetically closely related *E. pyrifoliae*, *E. tasmaniensis*, and *E. piriflorinigrans*. *E. pyrifoliae* is the cause of fire blight on Asian pear (*Pyrus pyrifoliae*) (Smits et al., 2010a), thus far only reported from East Asia (Kim et al., 1999, 2001). *E. tasmaniensis* is a pome fruit epiphyte with features suggesting pathogenicity on unknown hosts (Smits et al., 2010a, 2010b, 2011) and *E. piriflorinigrans* is a recently described necrotic pathogen of pear flowers in Spain (López et al., 2011). These cross-reactions have limited impact since these bacteria have only been reported from restricted geographic regions, and their detection would be of plant protection interest as potential emerging pathogens. The implications of such an extended detection potential of the Ea AgriStrip should nevertheless be considered by phytosanitary authorities since *E. pyrifoliae*, *E. piriflorinigrans*, and *E. tasmaniensis* are not currently regulated. None of the other tested bacteria, including common fire blight co-isolates and commercially used biocontrol agents, reacted with the lateral-flow immunoassay confirming its specificity in detecting only fire blight pathogens.

3.2. Analytical sensitivity

Sensitivity of the lateral-flow immunoassay was tested using dilution series of several *E. amylovora* strains in pure culture and spiked plant extracts. Over all strains tested, dilution 1:256 still showed a weak positive test line corresponding to an LOD of log 5.7 (CFBP 1430) to 5.9 (ACW42121) CFU/ml (Fig. 2A). In spiked apple twig extracts the LOD of strain CFBP1430 was determined at log 5.7 CFU/ml (Fig. 2B), similar to the pure culture demonstrating no significant influence of plant material on the detection limit of the lateral-flow immunoassay. *E. amylovora* concentrations in diluted plant samples (quince, cotoneaster, and pear) that still showed a weak positive test line were log 5.2 CFU/ml for quince, log 5.5 CFU/ml for cotoneaster and log 5.6 CFU/ml for pear indicating no significant differences to the sensitivity of pure cultures and only minor differences in sensitivity between host plant species. In mixed cultures with *P. syringae* pv. *syringae* and *P. vagans* C9-1 the LOD of strain CFBP1430 increased slightly from log 5.7 to log 6.0 CFU/ml indicating a slight decrease in sensitivity of the lateral-flow immunoassay when a lot of other bacteria are present in the plant sample. As expected, using pure culures, the LODs for plate isolation and for nested PCR were lower compared to the lateral-flow immunoassay. The LOD for plate isolation of strain CFBP1430 was log 3.3 CFU/ml and for nested PCR log 3.2 CFU/ml. Heat-killing or autoclaving of bacteria still showed positive test line development demonstrating heat-stability of epitopes on outer membranes of bacterial cells detected by the antibodies in the lateral-flow immunoassay.

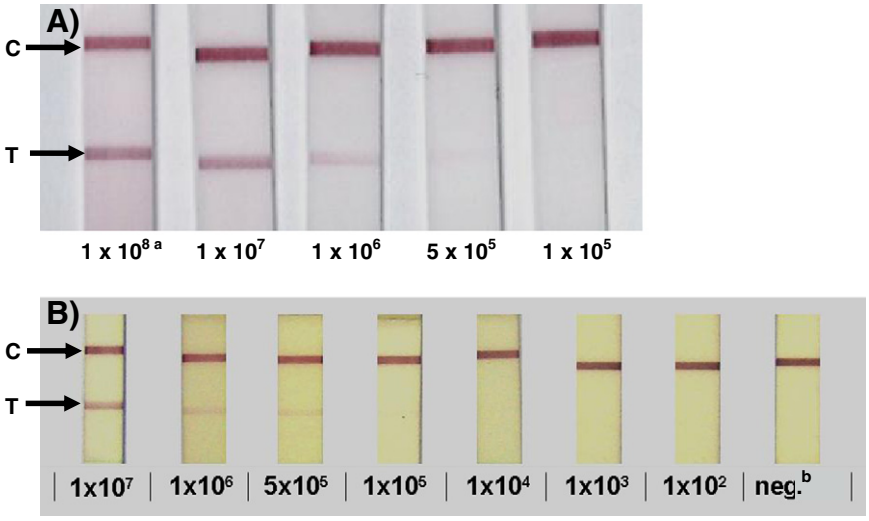

Fig. 2. Sensitivity of the immunoassay (Ea AgriStrip) for pathogen detection in dilution series of pure cultures of *Erwinia amylovora* CFBP 1430 (A) and spiked plant extracts (B) in AgriStrip buffer with bacterial concentrations indicated as CFU/ml. A non-spiked negative control is indicated as 'neg.' The assay control line is indicated as 'C' and the fire blight specific test line as 'T'.

Table 2
Results of direct colony testing from nutrient sucrose agar (NSA) plates or King's B (KB) plates comparing the standard method (serum agglutination) with the immunoassay Ea AgriStrip (Strip) with respect to *Erwinia amylovora* (*Ea*) colony confirmation.

	2006					2007			
	Ea-positive		*Ea*-negative			*Ea*-positive		*Ea*-negative	
Colony no.	Strip	Serum	Strip	Serum	Colony no.	Strip	Serum	Strip	Serum
NSA 179	86	87	92	92	NSA 134	108	100	26	26
KB 173	47	51	122	122	KB 4	2	2	2	2
Total 352	133	138	214	214	Total 138	110	102	28	28

3.3. Comparison of Ea AgriStrip with serum agglutination

When using suspicious single *E. amylovora* colonies from either NSA or KB plates, respectively, results of the standard serum agglutination test and Ea AgriStrip were significantly correlated. In 2006, out of 352 colonies tested 347 (99%) were similarly detectable with different methods (Table 2). From a total of 138 colonies from culture plating in 2007, only eight putative *E. amylovora* isolates (6%) reacted differently in both confirmatory tests, showing a significant correlation (94%) between the lateral-flow immunoassay and serum agglutination tests (Table 2). The differences in 2007 might be explained by obstruction or masking of pathogen cells in mixed microbial isolations common on diagnostic plates. Agglutination tests required pure colony material to obtain conclusive results, whereas Ea AgriStrip was less stringent and detected minority *E. amylovora* in complex isolation mixtures. The major advantage of using Ea AgriStrip is the time-saving it offers compared to all current detection and confirmatory methods. The lateral-flow immunoassay delivered clear results within 2–15 min, compared to serum agglutination requiring approximately 50 min and PCR requiring considerably longer sample processing steps. This represents a practical critical advantage for routine diagnostic laboratories charged with processing fire blight suspicious samples and returning timely results to phytosanitary authorities in the field.

3.4. Laboratory comparison of Ea AgriStrip with plating for detection of *E. amylovora* in naturally-infected plant samples

From 2006 to 2008, a total of 525 fire blight suspected samples sent to the disease diagnostic clinic by plant inspectors (Table 3) were tested in parallel with the 'gold standard' plate isolation (Anonymous, 2004) and the lateral-flow immunoassay. Out of 525 samples processed, 55.6% (292/525) were positive by plate isolation and 53.5% (281/525) were positive using the Ea AgriStrip (Table 4). Thus, the tests demonstrated high diagnostic sensitivity (95.2%) and specificity (98.7%) with only low rates of false positives (1.3%) and false negatives (4.8%) but high likelihood ratios for positive (73.2) and negative (0.05) test results. The latter provided convincing diagnostic conformity between the two methods with values of LR+ >10 and LR − <0.1 (Schwarzer et al., 2002) despite their difference in analytical sensitivity. Considering that different host plant species (Table 3) and different plant tissues (i.e., woody cankers, flowers, leaves and fruits) were used, these values are significant and demonstrate an overall strong correlation between the lateral-flow immunoassay and plate isolation results (96.3% over 3 years), indicating its potential in replacing time-consuming plate isolation for faster fire blight diagnosis.

3.5. On-site testing of naturally infested plant samples by Ea AgriStrip and plating and nested PCR in the laboratory

Ea AgriStrip was compared with the 'gold standards' of plate isolation and nested PCR methods (Anonymous, 2004) as diagnostic tools for fire blight detection in naturally infected field samples. A total of 201 suspected fire blight plant samples were tested by the lateral-flow immunoassay directly in the field in a ring-trial with Swiss cantonal plant inspectors. Samples were tested in parallel with plate isolation and nested PCR in the laboratory using the same plant samples demonstrating the relative reliability of results returned (Table 5). While detection rates for *E. amylovora* were 70% with nested PCR and comparable with the novel immunoassay (65%), they were only 50% for plate isolation over all samples. This lower efficiency of detection obtained with culture plating was particularly evident with pear samples (36%; Table 5). This could be due to antibacterial substances or enzymatic reactions with crushed pear plant samples (Rudolph, 1990). It should also be noted that Ea AgriStrip and nested PCR both detect dead and putative viable but not culturable (VBNC) bacteria based on their different test principles (England et al., 1997; Ordax et al., 2009; Schena et al., 2004), while plate isolation only detects living bacteria. This extended detection capacity offers maximum confidence in phytosanitary decision making since even dead or VBNC cells in samples may indicate historical exposure and potential contamination of inspection objects (i.e., orchards, nurseries and import/export plant material). The antibodies that form the basis of the lateral-flow immunoassay detect epitopes on outer membranes of bacterial cells that have a degree of stability (Feodorova et al., 2003). The PCR assay based on nucleic acid detection, has greater

Table 3
Naturally infested plant samples investigated in the fire blight disease clinic from 2006 to 2008 by Ea AgriStrip and plate isolation.

Plant species	Year			
	2006	2007	2008	Total
Pyrus	95	49	13	157
Malus	72	63	42	177
Cydonia	17	56	11	84
Crataegus	14	17	–	31
Cotoneaster dammeri	10	20	–	30
Other[a]	6	37	4	47
Total	214	242	70	525

[a] Other plants tested include *Chaenomeles* spp., *Sorbus* spp., *Pyracantha* spp. and *Cotoneaster salicifolius*.

Table 4
Contingency table comparing immunoassay (Ea AgriStrip) results with plate isolation for detection of *E. amylovora* in plant samples in a federal diagnostic laboratory.

Plate isolation				
Immunoassay		Positive	Negative	Total
	Positive	278 (A)	3 (B)	281
	Negative	14 (C)	230 (D)	244
	Total	292	233	525
Sensitivity (true positive rate; [A/(A+C)])		95.2%		
Specificity (true negative rate; [D/(D+B)])		98.7%		
Positive predictive value		98.9%		
Negative predictive value		94.3%		
False positive rate		1.3%		
False negative rate		4.8%		
Prevalence rate		55.6%		
Likelihood ratio for positive results		73.2		
Likelihood ratio for negative results		0.05		

Table 5
Comparison of immunoassay (Ea AgriStrip), plate isolation and nested PCR for detection of *Erwinia amylovora* in naturally-infested plant samples directly in the field.

Positive samples/Total samples			
Host species	Plate isolation	Nested PCR	Immunoassay
Total	105/201	142/201	131/201
Apple	79/141	98/141	95/141
Pear	18/49	36/49	28/49
Quince	3/3	3/3	3/3
Other[a]	5/8	5/8	5/8
Rapidity of test	48–72 h	24 h	2–15 min

[a] Other host species include *Crataegus* spp., *Sorbus aria*, *Pyracantha* spp. and *Chaenomeles* spp.

sensitivity for detecting not only infectious cells but also non-infectious DNA or VBNC of uncertain epidemiological significance and thus has a potential drawback for delivering diagnostic results that lead to unwarranted implementation of phytosanitary and/or regulatory measures. We found in growth chamber assays that heat-killed *E. amylovora* were detectable with Ea AgriStrip for up to 4 months from artificially inoculated apple bark tissue, and that this level of detection was similar to that obtained with nested PCR.

Furthermore, equivalent detection of *E. amylovora* positive samples was observed for Ea AgriStrip and nested PCR in samples from plants rated VR3 and VR4, respectively by visual ratings (Fig. 3). In the lower categories of visual ratings (VR2 and VR1) *E. amylovora* could be detected more frequently by the immunoassay than by plate isolation, but less frequently than by nested PCR (Fig. 3). Most positive *E.amylovora* containing samples were detected by nested PCR over all visual ratings (Fig. 3), followed by Ea AgriStrip and plate isolation. The differences in detecting positive samples over all methods tested were significant when analyzed over all visual ratings (Friedman test; $X^2 = 6.533$, df = 2, $P = 0.038$). However, there was no statistical difference in detecting positive samples when comparing Ea AgriStrip and PCR results within visual ratings VR3 and VR4 (Chi-square test; $X^2 = 0.00382$, df = 1, $P = 0.951$). Since most samples sent to the fire blight disease clinic are rated VR2 (54%; Fig. 1S supplemental) the lateral-flow immunoassay provides a rapid diagnostics tool to reliably detect *E. amylovora* in suspicious plant samples, especially with uncertain symptoms, without delayed results obtained with cumbersome culture plating.

Looking at the contingency table for the evaluation of a diagnostic method, we found a diagnostic sensitivity of 100% and specificity of 72.9% comparing the lateral-flow immunoassay with plate isolation, resulting in a false positive rate of 27%, which is quite high and

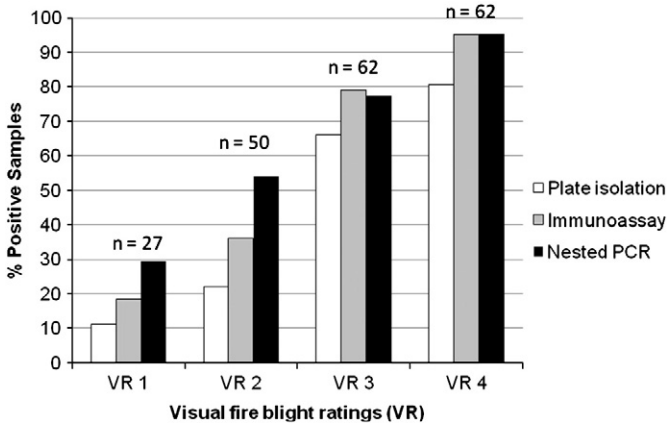

Fig. 3. Detection of *Erwinia amylovora* by plate isolation, immunoassay and nested PCR in plant samples (% positive samples) after visual rating based on increasing presence of fire blight symptoms (VR1–VR4).

Table 6
Contingency table comparing immunoassay (Ea AgriStrip) used in the field with plate isolation for detection of *Erwinia amylovora* in plant samples.

Plate isolation				
Immunoassay		Positive	Negative	Total
	Positive	105	26	131
	Negative	0	70	70
	Total	105	96	201
Sensitivity (true positive rate)		100%		
Specificity (true negative rate)		72.9%		
Positive predictive value		80.2%		
Negative predictive value		100%		
False positive rate		27%		
False negative rate		–		
Prevalence rate		52.2%		
Likelihood ratio for positive results		3.7		
Likelihood ratio for negative results		–		

demonstrates that Ea AgriStrip detects more positive samples in the field than plate isolation in the lab (Table 6). This is much higher than results obtained in the fire blight disease laboratory in 2006–2008, where there was only a false positive rate of 1.3% and a false negative rate of 4.8% (Table 4). The difference could be attributed to the difference in sample taking and processing. Samples sent to the laboratory were excised and extracted in buffer for 15–30 min at 22 °C to release bacteria from infected material before streaking onto plates, while field samples were macerated, stored at 4 °C and plated onto plates the next day. This might indicate that most viable bacteria did not survive transport and overnight storage in buffered but macerated plant tissue.

Comparing the novel lateral-flow immunoassay with nested PCR (Table 7), we observed a sensitivity for the immunoassay of 88.7% and a specificity of 91.5%, with a Likelihood Ratio for positive results of 10.4 and a Likelihood Ratio for negative results of 0.03. This demonstrates high conformity between the methods for delivering reliable diagnostic results (Schwarzer et al., 2002), although nested PCR had slightly greater sensitivity (16 samples negative with Ea AgriStrip but positive with nested PCR, Table 7), The detection of five positive samples by Ea AgriStrip that were negative with nested PCR might be explained by the isolates either potentially lacking plasmid pEA29 (Llop et al., 2006), upon which the standard nested PCR is designed or by the potential presence of closely related *Erwinia* species in the samples (Smits et al., 2010a; López et al., 2011; Smits et al., 2011). Non-pathogenic *Erwinia* and other common non-pathogenic epiphytic bacteria do not cross-react with either Ea AgriStrip or nested PCR. The cross-reacting species, while not found in Switzerland, are of phytosanitary relevance in terms

Table 7
Contingency table comparing immunoassay (Ea AgriStrip) used in the field with nested PCR analysis for *Erwinia amylovora* detection in plant samples.

Nested PCR				
Immunoassay		Positive	Negative	Total
	Positive	126	5	131
	Negative	16	54	70
	Total	142	59	201
Sensitivity (true positive rate)		88.7%		
Specificity (true negative rate)		91.5%		
Positive predictive value		96.2%		
Negative predictive value		77.1%		
False positive rate		8.5%		
False negative rate		11.3%		
Prevalence rate		70.6%		
Likelihood ratio for positive results		10.4		
Likelihood ratio for negative results		0.03		

Table 8
Contingency table comparing nested PCR with plate isolation for *Erwinia amylovora* detection in field plant samples.

Plate isolation				
Nested PCR		Positive	Negative	Total
	Positive	105	37	142
	Negative	0	59	59
	Total	105	96	201
Sensitivity (true positive rate)		100%		
Specificity (true negative rate)		61.5%		
Positive predictive value		74%		
Negative predictive value		100%		
False positive rate		38.5%		
False negative rate		–		
Prevalence rate		52.2%		
Likelihood ratio for positive results		2.6		
Likelihood ratio for negative results		–		

of pathogenic potential. Thus there may be an advantage in broader detection capacity offered by the lateral-flow immunoassay in monitoring currently restricted pome fruit pathogenic *Erwinia* that may in the future emerge as pathogens with wider distribution. Moreover, while PCR diagnostic assays are limited to laboratory use, the Ea AgriStrip has dual application for laboratory and on-site field diagnostics.

Finally we compared results of nested PCR and plate isolation and found a sensitivity of 100% but a specificity of only 61.5% with the highest false positive rate of 38.5% (Table 8). This could be due to higher sensitivity of nested PCR detecting dead or VBNC cells of limited phytosanitary relevance in field samples. Overall agreement (including positive and negative results) between immunoassay results and culture plate isolation was 89% (178/201) and for immunoassay results and nested PCR 91% (183/201), demonstrating the relative reliability of the simpler and on-site compatible assay. Correlation analyses using Spearman Rank Order Correlation comparing Ea AgriStrip results with plate isolation demonstrated a significant positive correlation ($R^2 = 0.810$, $P \leq 0.001$, $n = 201$) between the two methods comparable to immunoassay and PCR results ($R^2 = 0.834$, $P \leq 0.001$, $n = 201$). However, comparison of plate isolation with PCR results resulted in a significant but moderate positive correlation ($R^2 = 0.698$, $P \leq 0.001$, $n = 201$).

4. Conclusions

In conclusion, the newly developed lateral-flow immunoassay (Ea AgriStrip) is suitable for reliable detection of *E. amylovora* in symptomatic plant tissues in phytosanitary diagnostic laboratories and for on-site diagnosis. Because of its conformity (about 90% in the field, 96% in the laboratory) with standard diagnostic methods for fire blight detection, its high specificity, its simplicity and speed (15 min vs. 2–3 days) (Table 5), it offers a reliable substitute for currently available confirmatory tests of putative plate isolates (e.g. serum agglutination) as well as for plate isolation at least with the majority of plant samples submitted by field inspectors (i.e., having a visual rating ≥VR2).

This saves time in returning diagnoses to cantonal and local plant protection inspection authorities, thereby expediting implementation of phytosanitary control measures to limit epidemic spread and sanitize inoculum reservoirs. Since the test provides fast and reliable on-site diagnostic results and has successfully been tested and validated with end-user cantonal plant protection inspectors, this immunoassay offers a simple tool for decision making in the field with the majority of suspect fire blight cases (VR2-VR3; Fig. 3). While the immunoassay demonstrated ability to detect *E. amylovora* in essentially asymptomatic plant samples (i.e., VR1), it was just as variable as plating and less sensitive than PCR. The phytosanitary and/or epidemiological significance of latent or asymptomatic infections is uncertain, and in most cases detection limit of this immunoassay is sufficient. In fact, the more conservative detection limit is advantageous since the higher the amount of bacteria detected the higher the probability that bacteria are still alive to cause infection (Johnson and Stockwell, 1998; Taylor et al., 2003). More expensive and technically demanding PCR methods may be required to identify asymptomatic plant material at ports of entry (López et al., 2009).

Supplementary materials related to this article can be found online at doi:10.1016/j.mimet.2011.06.015.

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