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Commentary

Magnetic Capture Hybridisation for Improved PCR Detection of *Nectria galligena* from Lignified Apple Extracts

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Abstract. In order to reduce the effects of inhibitors present in DNA extracts from lignified apple tissues, a magnetic capture-hybridisation PCR (MCH-PCR) technique was developed for *Nectria galligena* using the ITS 1 region of the rRNA gene repeats as target. The trapping reagent used to coat the magnetic beads was an 81 bp single-stranded DNA oligonucleotide biotin-labelled on the 5'-terminal and designed to be complementary to part of the rRNA gene ITS 1 region of *N. galligena.* For specificity, the probe was located from 14 bp downstream from the 3'-terminal nucleotide of the *N. galligena* forward primer Ch1 to the last ITS 1 nucleotide immediately upstream of the 5.8S rRNA gene. Following hybridisation in a total DNA extract of woody tissue, magnetic recovery of the bead-oligomer-template conjugate separated target template from other DNA species and inhibitory compounds. Magnetic capture-hybridisation was followed by PCR amplification with the previously designed species-specific primers, Ch1 and Ch2. Application of the MCH-PCR technique resulted in increased levels of sensitivity and reliability when compared to PCR without MCH when used on total DNA extracts from lignified tissues.

Key words: asymptomatic infection, fungal plant pathogens, *Nectria* canker, PCR inhibitors, woody tissues

Introduction

Inhibition of amplification is a frequent problem for PCR-based systems used to detect plant pathogens in many hosts. It is also often a problem in the detection of soil microorganisms. Despite the development of numerous taxon-specific, or more widely applicable plant DNA extraction procedures designed to minimise co-extraction of inhibitory compounds, inhibition remains a major impediment to many routine applications, compromising both assay sensitivity and reliability. Polysaccharides, secondary plant metabolites (such as polyphenols and phenolic compounds) and ill-defined materials such

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as humic acids are common, PCR-inhibitory contaminants of DNA extracts made from plants and soils. A number of post-extraction procedures (reviewed by Bickley and Hopkins, 1999) designed to overcome inhibition and enhance PCR reliability have become established as standard purification strategies, but these are not always effective (especially where the level of inhibitors is high). Further, they may lead to a loss of target DNA.

Woody tissues contain lignin, a structurally complex cross-linked aromatic biopolymer based on variously substituted *p*-hydroxyphenyl propane units resistant to degradation, linked with a range of materials including polysaccharides, polyphenols, celluloses and polyglucoses, many of which are inhibitory to PCR and relatively easily extracted. For example, inhibition of PCR due to the use of wooden toothpicks to transfer bacterial or yeast colonies for direct PCR analysis of recombinant DNA has been observed (Lee and Cooper, 1995), especially where low amounts of *Taq* DNA polymerase were used. In studies on *Nectria galligena* Bres., the cause of *Nectria* canker in apple and pear, PCR detection using species-specific primers (Langrell, 2000) in clearly infected lignified tissue has often been compromised by the presence of inhibitory compounds. Furthermore, in asymptomatic (or cryptic) infections, which may be implicated in dissemination of the disease (T.R. Swinburne, Wye College, personal communication; Li, 1995; Langrell, 2000), the level of fungus present is usually below the limit of detection by PCR (Langrell, 2000).

Initially developed for the detection of bacterial DNA from soil (Jacobsen, 1995), magnetic capture hybridisation (MCH) is a very useful tool for the separation of specific target DNA from other DNA molecules and interfering compounds. Recently, MCH has been used to improve the PCR-detection of a nucleopolyhedrovirus from field soils (de Moraes et al., 1999) and of verotoxigenic *Escherichia coli* in foods (Chen et al., 1998). MCH uses solution hybridisation with a specific single-stranded biotinylated probe, conjugated to streptavidin coated paramagnetic beads, to immobilize target DNA from a total DNA extract. Beads and bound DNA are collected and washed magnetically, simply and effectively removing non-target DNA and PCR inhibitors (and, if required, allowing concentration of the target molecules). Elution of the DNA is by a simple heating step, which can be incorporated as part of the subsequent PCR reaction. This is shown schematically in Figure 1. The use of MCH to purify template molecules should greatly reduce or exclude the occurrence of false negatives arising from the presence of inhibitory compounds. Because of the problem of inhibition of PCRs associated with DNA extracts from lignified apple tissues (Langrell, 2000), MCH offered the possibility of improved sensitivity and reliability. The objective of this work was to develop an MCH-PCR assay for *N. galligena* and demonstrate the improved detection of this fungus from woody tissue by this technique.

Materials and Methods

Fungal culture and DNA extraction

Growth of *N. galligena* strain IMI 375721 (isolated from a canker on an apple tree, cv. Carrara Brusca, at the National Fruit Collections, Brogdale, Faversham,

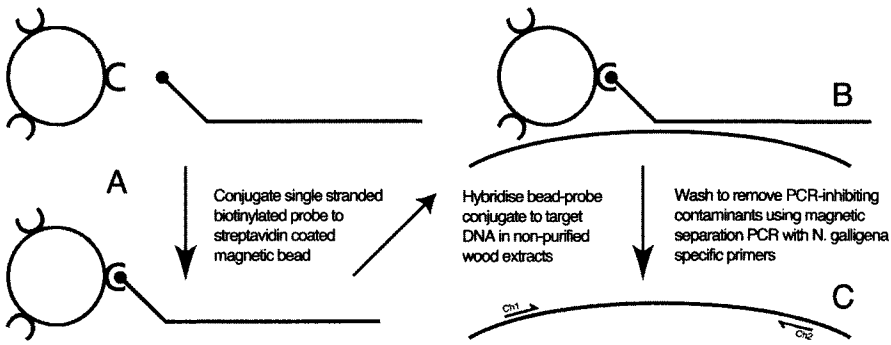

Figure 1. Schematic representation of MCH-PCR assay.

Kent, UK) and DNA extraction was as described previously (Langrell, 2000). The concentration and quality of the DNA were estimated spectrophotometrically (Sambrook et al., 1989).

DNA extraction from lignified tissue

Total DNA extracts from woody apple and pear tissue samples were prepared according to a modified procedure of Torres et al. (1993) as described previously (Langrell, 2000). Milled (or ground) fresh (or freeze dried) wood samples were extracted in 750 µL extraction buffer (20 g L^{-1} CTAB, 10 g L^{-1} polyvinyl pyrrolidone PT-40, 100 mM Tris-HCL (pH 8.0), 20 mM EDTA (pH 8.0), 1.4 M NaCl, 0.5 mM sodium metabisulphite, with the addition of β-mercaptoethanol to 0.4% (v/v) just prior to use) (based on the extraction buffers of Lassner et al. (1989) and Murray and Thompson (1980)) in a 1.5 mL microfuge tube. 100 µL of chloroform:octonol (24:1 v/v)) was added and the mixture was incubated at 65°C for 30 min. Following cooling the mixture was further extracted by the addition of 500 µL chloroform:octonol (24:1 v/v), vortexed and centrifuged (10 min, 11,600 x *g*). The aqueous phase was transferred to a clean microfuge tube and extracted twice with an equal volume of Tris-washed phenol (Fisher Scientific): chloroform: pentan-2-ol (25:24:1), allowing 10 min incubation at room temperature for each extraction. Following centrifugation (10 min, 11,600 x *g*), the supernatant was transferred to a clean microfuge tube and 2/3 volume of water saturated ether added and vortexed briefly. Following another centrifugation (2 min, 11,600 x *g*), the supernatant was discarded and the extraction repeated. An equal volume of cold (-20°C) propan-2-ol (Merck) was added, mixed gently and centrifuged (10 min, 11,600 x *g*). Finally pellets were washed in 70% ethanol, air dried at room temperature, and resuspended in sterile Milli-Q AR grade water. DNA extractions were quantified and checked for quality by electrophoretic comparison with a dilution series of bacteriophage λ DNA (Promega) and by spectrophotometric analysis (Sambrook et al., 1989). DNA concentrations were adjusted to a standard level and the preparations stored at -20°C.

Design and synthesis of biotin-labelled capture probe

An 81 bp oligonucleotide was designed to be complementary to part of the ITS 1 region of the *N. galligena* rRNA genes (Langrell, 2000) with the aid of the computer programme OLIGO version 5 (Molecular Biology Insights, Inc., Cascade, CO, USA). The capture sequence extended from 14 bp downstream of the 3'-terminal nucleotide of the Ch1 primer sequence (to avoid competition during subsequent PCR tests) to the last ITS 1 nucleotide immediately upstream of the 5.8S rRNA gene. Further sequence from the conserved 5.8S rRNA gene was avoided in order to enhance capture-sequence specificity. Sequences and positions of diagnostic primers Ch1 and Ch2 (Langrell, 2000) and the oligonucleotide probe are given in Table 1. The capture oligonucleotide was synthesised by Genosys Biotechnologies Inc., Cambridge, UK, and incorporated a biotin molecule on a twelve-carbon atom spacer arm at the 5'-terminal. The 81 bp biotinylated oligonucleotide was not further purified.

Attachment of capture oligonucleotide to magnetic beads

In all wash/incubation steps the beads were immobilized using a Magnetic Particle Concentrator (MPC-E) (Dynal, Oslo, Norway). 400 µL of a 10 mg mL^{-1} suspension of superparamagnetic M-280 streptavidin (covalently coated) polystyrene beads (Dynal A.S., Oslo, Norway) were transferred to a 1.5 mL microfuge tube and washed three times with 400 µL of 1x PBS (0.05 M phosphate buffer with 9 g L^{-1} NaCl (pH 7.3)) containing 1 g L^{-1} SDS, to remove preservative, followed by a single wash in 400 µL binding buffer (10 mM Tris-HCl, 1 mM EDTA, 1 M NaCl (pH 8.0)). Biotinylated MCH capture oligonucleotide (100 µg) was added in 100 µL and the mixture incubated at 25°C for 60 min. Following incubation, the beads/oligonucleotide conjugate was washed three times with 400 µL of binding buffer and finally resuspended in 400 µL of sterile Milli-Q AR grade water. All manipulations were performed with the use of aerosol barrier tips (Continental Laboratory Products, USA).

Capture hybridisation

Hybridisation of MCH probe to target template from DNA samples essentially followed Jacobsen (1995). Typically, 50 ng of DNA (in 10 µL and denatured by heating at 99°C for 10 min on a Techne PHC-3 thermal cycler (Techne, Cambridge, UK)) from total preparations of woody apple tissues were added to 200 µL of a pre-warmed (to 62°C) hybridisation solution (5x SSC, 10 g L^{-1} low-fat dried milk powder (blocking agent), 1 g L^{-1} N-laurylsarcosine and 0.2 g L^{-1} SDS) followed by 100 µg MCH-capture oligomer/bead conjugate. Samples were incubated for 4 h at 62°C in a Techne HB-1 hybridisation oven. Beads were washed once in sterile Milli-Q AR grade water and resuspended in 25 µL of sterile Milli-Q AR grade water for use in PCR analysis.

PCR

Following hybridisation, samples were subjected to PCR amplification using primers Ch1 and Ch2 (Table 1) which amplify a 412 bp fragment specific to *N.*

Table 1. Sequences and positions within the rRNA gene repeats of primers Ch1 and Ch2 (Langrell, 2000) and 81 bp capture oligonucleotide used for the MCH-PCR detection of *N. galligena* from lignified apple tissue.

Oligonucleotide	Postion in ITS[a]	Sequence
Ch1	20-40	5'-AAC CCC TGT GAA CAT ACC CAT C-3'
Ch2	412-431	5'-GTG GCC GCG CTG CTC TTC CG-3'
Capture oligomer[b]	55-135	5'-GCC CGC TCC GGC GGC CCG CCA GAG GAC CCC CAA ACT CTT GTT TTA TAC AGC ATC TTC TGA GTA ACA CGA TTA AAT AAA TCA-3'

[a]Numbered as for EMBL accession AJ228666, beginning with the 5'-most nucleotide given.
[b]The 81 bp oligonucleotide incorporated a biotin molecule with a twelve-carbon atom spacer arm at the 5'-terminus.

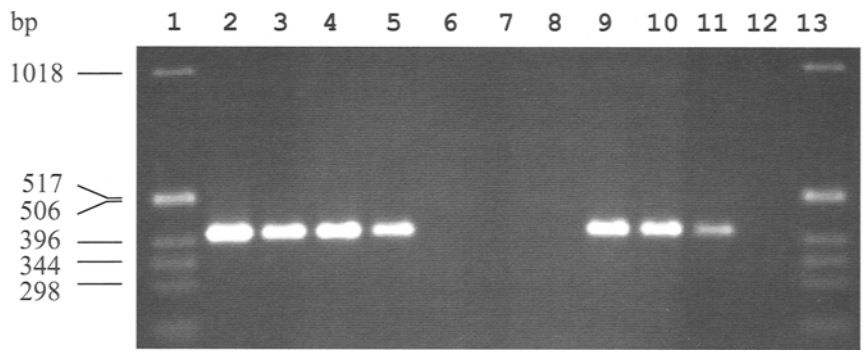

Figure 2. Improved sensitivity and reliability of MCH-PCR for detection of *N. galligena* in DNA preparations from lignified tissue. Lanes 1 and 13, 1 kb size marker (Life Technologies). Lane 2, DNA extract from *N. galligena* (IMI 375721) in culture. Lanes 3-8, PCR without MCH; lanes 3-5 are extracts from young cankers on apple cv. Discovery, apple cv. Royal Gala and pear cv. Conference; lane 6, extract from old canker on apple cv. Bramley Seedling; lane 7, extract from apple rootstock (M9) suspected of being infected asymptomatically; 8, extract from wood without canker but distal to an active canker on apple cv. Queen Cox. Lanes 9-11, samples as lanes 6-8 but tested using MCH-PCR as described in text. Lane 12, negative control.

galligena (Langrell, 2000). Following PCR, typically 10 µL of each reaction mixture was analysed by gel electrophoresis in 10 g L^{-1} agarose gel (Biogene, UK) in 1x TBE (89 mM Tris-borate, 2 mM EDTA (pH 8.0)) at 4 V/cm for 2 h. A 1 kbp ladder (Gibco BRL) was used as a size marker. Following ethidium bromide staining, bands were visualised and photographed over UV light.

Results and Discussion

Intra-specific homogeneity within the *N. galligena* rRNA gene ITS regions, the repetitive nature of these genes, and the fact that species-specific primers have been developed (Langrell, 2000) made this the locus of choice for use in MCH-PCR. A specific complementary hybridisation probe was designed to the ITS 1 region, shown to be less variable than ITS 2 when compared with other

closely related *Nectria* species including *N. radicicola* (Langrell, 2000). Only 95 bases occur between the end of the forward primer, Ch1, and the highly conserved 5.8S rRNA gene. As MCH requires a capture molecule nested between the diagnostic primers and of sufficient length and GC content to ensure efficient hybridisation, an 81 bp biotinylated single-stranded oligomer was designed and synthesised (Table 1) (excluding the conserved 5.8S rRNA gene and the Ch1 primer recognition site, to avoid reduction in specificity or compromise in PCR efficiency through competition for the recognition site). Other than desalting, no purification of the biotinylated probe after synthesis was performed because of potentially large losses of oligonucleotide during HPLC or PAGE purification. Although purification is recommended to remove unbound biotin molecules from biotinylated oligonucleotide (since free biotin may potentially compete for binding sites on the streptavidin-coated magnetic beads, reducing the effective binding capacity of the biotinylated hybridisation conjugate), spectrophotometric analysis of oligomer-bead conjugate stocks and wash supernatants from experiments indicated high levels of probe-bead binding (>80%) (data not shown). To evaluate the sensitivity of the MCH-PCR technique, 10-fold serial dilutions from 1 ng to 1 fg, of genomic DNA from *N. galligena*, strain IMI 375721, in sterile Milli-Q AR grade water were tested by MCH-PCR with primers Ch1 and Ch2. The lower limit of visual detection on ethidium bromide stained agarose gels was approximately 10-100 fg genomic DNA (data not shown), an approximate 10-100 fold increase in sensitivity over that achieved by PCR without MCH (Langrell, 2000).

The usefulness of the MCH in improving reliability and sensitivity of PCR for *N. galligena* from naturally infected and putatively infected lignified apple material, was demonstrated by applying it to a range of extracts from known infected and suspect samples, some of which had proved problematic in PCR without MCH: (i) two young cankers from apple trees and one from pear; (ii) a mature *Nectria* canker lesion from an 8-year-old branch from an apple cv. Bramley Seedling tree; (iii) an apple (M9) rootstock suspected to be asymptomatically infected from a 2-year-old layer-bed; (iv) wood collected from about 10 cm beyond the leading edge of an active *Nectria* canker on a 4-year-old branch of an apple cv. Queen Cox tree. As expected, PCR without MCH was successful in detecting *N. galligena* in the young cankers (Figure 2, lanes 3-5). However, for the samples from older cankers, or asymptomatic wood, amplification by PCR without MCH was unsuccessful (Figure 2, lanes 6-8), even when post-extraction the samples were passed through Sepharose® CL-6B (Amersham Pharmacia Biotech UK) or insoluble PVP or when the DNA samples were diluted (data not shown). A standard internal positive control (Langrell, 2000) also did not amplify in these experiments, indicating the presence of PCR-inhibitory compounds (data not shown). Following capture-hybridisation with the MCH-oligomer/streptavidin-bead conjugate, but without any other post-extraction treatment, amplification of an approximate 412 bp fragment by PCR using primers Ch1 and Ch2 was successful for these sample extracts (Figure 2, lanes 9-11).

No attempt was made to quantify by PCR the level of *N. galligena* in each of the samples. Also, isolation of *N. galligena* from lignified tissues is not easy, and with old lesions or asymptomatic wood it is often extremely difficult, so we have no independent estimate of the fungal biomass present in the lignified tissue

samples used here. However, as even the limited number of samples presented here clearly show, MCH-PCR was able to detect *N. galligena* in samples where PCR without MCH was unsuccessful, representing an overall qualitative improvement in sensitivity and reliability.

The development and application of a magnetic capture hybridisation procedure employing a biotinylated capture oligonucleotide for improved PCR detection of *N. galligena* from lignified apple extracts has been demonstrated. The results show a 10-100 fold increase in sensitivity for DNA from the fungus in culture and for samples from woody tissues represents a significant improvement in PCR sensitivity and reliability. While routine application of MCH-PCR to the screening of materials with low or cryptic levels of infection may be limited by the additional cost and relative assay complexity, it is an effective research tool for use in studying disease aetiology and asymptomatic spread within the tree. To our knowledge, this is the first report of the detection of a fungal plant pathogen using MCH-PCR.

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References

Bickley J and Hopkins D (1999) Inhibitors and Enhancers of PCR. In: Saunders GC and Parkes HC (eds), Analytical Molecular Biology Quality and Validation, supplement 190: pp 81-102, Redwood Books Ltd., Wiltshire, UK.

Chen J, Johnson R and Griffiths M (1998) Detection of verotoxigenic *Escherichia coli* by magnetic capture-hybridisation PCR. Appl Environ Microbiol 64: 147-152.

de Moraes RR, Maruniak JE and Funderburk JE (1999) Methods for detection of *Anticarsia gemmatalis* nucleopolyhedrovirus DNA in soil. Appl Environ Microbiol 65: 2307-2311.

Jacobsen CS (1995) Microscale detection of specific bacterial DNA in soil with a magnetic capture-hybridisation and PCR amplification assay. Appl Environ Microbiol 61: 3347-3352.

Langrell SRH (2000) Molecular phylogeny, detection and epidemiology of *Nectria galligena* Bres. the incitant of *Nectria* canker on apple. Ph.D. Thesis, University of London, pp 204.

Lassner MW, Peterson P and Yoder JI (1989) Simultaneous amplification of multiple DNA fragments by polymerase chain reaction in the analysis of transgenic plants and their progeny. Plant Mol Biol Reptr 7: 116-128.

Lee AB and Cooper TA (1995) Improved direct PCR screen for bacterial colonies: wooden toothpicks inhibit PCR amplification. BioTechniques 18: 225-226.

Li R (1995) Studies on the infection of apple trees by *Nectria galligena* Bres. M. Phil. Thesis, University of London, pp 136.

Murray MG and Thompson WF (1980) Rapid isolation of high molecular weight plant DNA. Nucleic Acids Res 8: 4321-4325.

Sambrook J, Fritsch EF and Maniatis TA (1989) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, USA.

Torres AM, Weeden NF and Martin A (1993) Linkage among isozyme, RFLP and RAPD markers in *Vicia faba*. Theor Appl Genet 85: 937-945.