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Papers

A novel gold nanoparticles-based assay for rapid detection of *Melissococcus plutonius*, the causative agent of European foulbrood

M. Saleh, H. Soliman, H. Sørum, A. K. Fauske, M. El-Matbouli

European foulbrood (EFB) is a severe bacterial brood disease of honey bees (*Apis mellifera*) caused by *Melissococcus plutonius*. Diagnosis of EFB in the field is based on visual inspection of brood-combs and detection of diseased larvae. However, symptoms of EFB may be easily obscured by other diseases or abnormalities in the brood, making definitive diagnosis difficult. Hence, confirmatory laboratory assays, such as PCR and real-time PCR, are used to verify the presence of *M plutonius* in suspected colonies. While these methods are accurate and specific, they are time consuming and labour intensive. Herein, we report development of a label-free colorimetric nanodiagnostic method for direct detection of unamplified *M plutonius* DNA using unmodified gold nanoparticles. Under appropriate conditions, the DNA probes hybridised with their complementary target sequences in the sample DNA, which resulted in aggregation of the gold nanoparticles and a concomitant red to blue colour change, which was observed visually. The assay could detect as few as 25 copies of the *M plutonius* cell wall-associated protease gene within 20 minutes. The assay results were in 100 per cent concordance with real-time PCR-positive and PCR-negative samples. Our study demonstrated that the gold nanoparticles-based assay is a specific and sensitive tool for rapid detection of *M plutonius*.

Introduction

Honey bees (*Apis mellifera*) are not only important for the honey they produce, but also as vital pollinators of agricultural and horticultural crops (Forsgren 2010, Genersch 2010). Colony numbers in Europe decreased from over 21 million in 1970 to about 15.5 million in 2007 (FAO 2009). While this decrease was gradual before 1990, a higher rate of decline was observed thereafter (vanEngelsdorp and Meixner 2009). Reasons for declining honey bee colony numbers include direct affects on broods and adults from a range of pathogens, which include bacteria, viruses, protozoa, fungi and parasitic mites. European foulbrood (EFB) caused by the Gram-positive bacterium *Melissococcus plutonius*, is one of the most economically significant diseases of honey bees (Forsgren 2010). The bacterium is a non-spore-forming, lanceolate coccus, occurring singly, in pairs and in chains, and measuring 0.5–0.7 × 1.0 mm (Alippi 1991, Shimanuki and others 1992). The disease is endemic in North and South America, Europe, Japan, Australia, India and southern Africa (Matheson 1993). EFB is a notifiable disease in several countries (Bailey and Collins 1982, Bailey 1983). In Australia, it has been considered as a serious disease that usually occurs in spring when the brood expands (Graham 1992, Mckee and others 2003). In the UK, the disease is particularly prevalent and increasing dramatically (Wilkins and others 2007, Tomkies and others 2009). In Switzerland, even though clinically diseased colonies are destroyed, the incidence of EFB has constantly risen each year since the late 1990s (Forsgren and others 2005, Belloy and others 2007, Roetschi and others 2008). After a 30-year period without any reported cases, Norway battled a regional outbreak of EFB during 2010, and The Netherlands has also reported an increase over the last 10 years (Forsgren and Genersch 2011).

The clinical symptoms of EFB in honeybee colonies were first described in 1885 by Cheshire and Cheyne (1885). Bee larvae are extremely susceptible to *M plutonius* which kills them at the age of four to five days. When the larvae die, they often turn brown, decompose and give off a foul odour. Usually, a secondary infection with bacteria accelerates death. There is often a well-defined pattern of seasonal outbreaks, mostly in the spring and early summer (Bailey 1961, Graham 1992). Signs of disease usually disappear spontaneously from infected colonies before the end of the active season, but are likely to return in subsequent years (Bailey 1956, 1983, Bailey and Ball 1991).

Diagnosis of EFB in the field is based on visual inspection of brood-combs and detection of diseased larvae. However, confirmatory laboratory methods, such as isolation of bacteria from infected larvae or honey (Hornitzky and Smith 1998), and microscopic examination of carbol fuchsin-stained smears of diseased larvae (Hornitzky and Wilson 1989) are recommended to verify the presence of *M plutonius*. The bacterium may also be identified by scanning electron microscopy, ELISA or by the recently presented Lateral Flow Device for detection in extracts of infected tissue (Pinnock and

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Featherstone 1984, Alippi 1991, Tomkies and others 2009). PCR assays, both semi-nested and real-time, have been developed for detection of *M plutonius* (Djordjevic and others 1998, Govan and others 1998, McKee and others 2003, Roetschi and others 2008). All these confirmatory methods require a laboratory setting, and have high associated costs that prevent their wide-scale use at points-of-care and in developing nations (Kaittanis and others 2010).

A powerful emerging technology based on the unique properties of nanoscale materials presents a great opportunity to develop fast, accurate and cost-effective diagnostics for the detection of pathogenic infectious agents (Jain 2005, Rosi and Mirkin 2005, Kaittanis and others 2010). The optical transduction by noble metal nanoparticles is based upon the phenomenon of nanoparticle surface plasmon resonance (Kreibig and Genzel 1985, Quinten and Kreibig 1986, Link and El-Sayed 2000). Surface plasmon resonance is the collective oscillations of surface electrons induced by visible light, and is responsible for the intense colours exhibited by colloidal solutions of noble metals, such as gold and silver (Nath and Chilkoti 2004, Jain and others 2006). The colours obtained by nanoparticles of these metals come about through interaction of incident light with the free electrons that promote resonant excitation of an oscillating dipole (Yguerabide and Yguerabide 1998a, b, Nath and Chilkoti 2004). Because of the unique size, shape and composition-dependent optical, physical and chemical properties that gold nanoparticles display, they are suitable for a wide range of biological applications (Pissuwan and others 2010). Gold nanoparticles (AuNPs) in solution are particularly stable due to adsorbed negatively charged citrate ions on their surfaces, whose repulsion prevents the strong van der Waals attraction between gold particles from causing them to aggregate (Shaw 1991, Hunter 2001). An aqueous solution of gold particles modified with synthetic surface oligonucleotides appears initially brilliant red, but changes to blue after aggregation of the particles upon hybridisation with the target sequence, after salt addition (Mirkin and others 1996, Elghanian and others 1997, Li and Rothberg 2004, Storhoff and others 2004).

Li and Rothberg (2004) used the different electrostatic properties of single- and double-stranded oligonucleotides (ssDNA and dsDNA) to signal oligonucleotide hybridisation. They document that the adsorption of ssDNA on gold nanoparticles is selective, and show that this process stabilises the gold nanoparticles against aggregation at concentrations of salt that would ordinarily screen the repulsive interactions of the citrate ions in the absence of a complementary target sequence. Consequently, this phenomenon has been used to design various colorimetric hybridisation assays (Li and Rothberg 2004, Li and Rothberg 2005, Shawky and others 2010).

In this study, we present a label-free gold nanoparticle-based assay for direct detection of unamplified *M plutonius* DNA. The colorimetric assay is specific, sensitive, rapid and cost-effective.

Materials and methods

Bacteria

M plutonius-type strain ATCC 35311 was grown anaerobically at 35°C on EFB-agar/broth media (Bailey 1957). Prior to use, the media were supplemented with 3 µg/ml of nalidixic acid (Hornitzky and Smith 1998). *Enterococcus faecalis*, *Actinobacillus pleuropneumoniae, Enterococcus faecium, Erysipelothrix rhusiopathiae* and *Paenibacillus larvae* strains were grown on their specific media (Alippi 1991).

DNA extraction from bacterial cultures

Bacterial colonies were harvested from cultures of *M plutonius, E faecalis, A pleuropneumoniae, E faecium, E rhusiopathiae* and *P larvae*, and pelleted by centrifugation. Bacterial genomic DNA was extracted from each pellet with a GeneMole DNA extraction machine (Mole Genetics, Norway) as per the manufacturer's instructions.

DNA extraction from samples of infected and healthy bee colonies

M plutonius-infected and uninfected larvae were mixed in lysis buffer (20 mM Tris-HCl, pH 8.0, 2 mM EDTA, 1.2 per cent Triton X-100), supplemented with 20 mg/ml lysozyme and incubated at 37°C for 30 minutes. Subsequently, 15 µl Proteinase K (Qiagen, Germany) was added to each sample, and incubated at 56°C for 30 minutes. DNA was then extracted from the homogenate (200 µl) using the GeneMole DNA extraction machine (Mole Genetics, Norway) as per the manufacturer's instructions.

Real-time PCR of *Mplutonius*

The primers, Melisso-F, Melisso-R and the minor groove binding TaqMan probe (Table 1), were used to amplify a 79-bp fragment of the *M plutonius sodA* gene according to Roetschi and others 2008. The real-time PCR was carried out in a final reaction volume of 12 µl containing 0.3 µM of each primer, 0.1 µM probe, 2 × reaction buffer and 2 µl of DNA. The qPCR MasterMix No Rox (Eurogentec, Belgium) was used for all measurements. The cycling profile was: 50°C for two minutes, 95°C for 10 minutes, and 40 cycles of 95°C for 15 seconds and 60°C for one minute. Fluorescent data were collected during the 60°C step.

Preparation of gold nanoparticles

Gold nanoparticles (13 nm diameter) were synthesised by reduction of $HAuCl_4$ by sodium citrate, following the protocol of Storhoff and others (1998). Briefly, an aqueous solution of $HAuCl_4 \cdot 3H_2O$ was brought to boil under reflux while being stirred, then 10 ml of 1 per cent trisodium citrate was added quickly. The colour of the solution changed from yellow to deep red. The solution was refluxed for an additional 15 minutes and then allowed to cool to room temperature. The solution was then filtered through a 0.45 µm acetate filter and stored at 4°C.

Probe design for the *M plutonius* gold nanoparticles-based assay

Two specific probes, EFB- AuNPs- P1 & EFB- AuNPs -P2 (Table 1), were designed to target the *M plutonius* cell wall-associated protease gene (GeneBank accession number NC_015516, Okumura and others 2011). The specificity of the probes against DNA sequences of related bacteria available in GenBank was assessed by BLAST search.

Colorimetric detection of *M plutonius*-DNA from bacterial isolates and infected samples

To determine the optimum concentrations sufficient for visual detection of the colour change, and at the same time, appropriate for proper annealing of the probe to its target, different concentrations of NaCl (0.01M–1.0M) and probe (0.1 µM–3 µM) were tested.

Extracted DNA (5 µl) was mixed well in a sterile PCR tube with 3 µl hybridisation buffer (10 mM phosphate buffer saline, pH 7.0, containing 0.5M NaCl), 2 µM probe, with sterile distilled water to a total volume of 10 µl. The assay was conducted at different annealing temperatures and incubation times to determine optimal conditions (annealing temperature and time). The mixture was denatured at 95°C for five minutes, annealed at 50°C for further five minutes, and then cooled to room temperature for 10 minutes. The change in the solution colour was observed visually within one minute after addition of 10 µl of colloidal AuNPs to the reaction mixture.

Specificity and sensitivity of the *M plutonius* gold nanoparticles-based assay

After optimisation of the reaction, the gold nanoparticles-based assay was tested with DNA from a range of bacterial species (Table 2) to test the specificity of the designed probes.

TABLE 1: *Melissococcus plutonius* primers and probes used in this study (GeneBank accession number NC_015516)

Primers/probes name	Sequences (5'–3')	References
RT-PCR: Melisso-F*	CAGCTAGTCGGTTTGGTTCC	Roetschi and others 2008
RT-PCR: Melisso-R	TTGGCTGTAGATAGAATTGACAAT	Roetschi and others 2008
RT-PCR: TaqMan Probe	FAM-CTTGGTTGGTCGTTGAC-MGB-NFQ	Roetschi and others 2008
EFB-AuNPs-P1†	AGGGAACTGTCAATTTAGCAGT	This study
EFB-AuNPs-P2	CCAGTTCCCTTGGC AGTT	This study

*EFB European foulbrood, MGB Minor groove binding, RT-PCR, Real-time PCR
† = Probes used for *M plutonius* gold nanoparticles assay

TABLE 2: Bacterial species used to verify the specificity of the *Melissococcus plutonius* gold nanoparticles-based assay

Bacterial species	Strain	Results of the specificity assay
Actinobacillus pleuropneumoniae	NVH164a	Negative
Enterococcus faecalis	DSM20478	Negative
E faecalis	NVH 1103/98	Negative
Enterococcus faecium	CCUG36804	Negative
Erysipelothrix rhusiopathiae	NVH93	Negative
M plutonius	NVH524	Positive
M plutonius	NVH526	Positive
Paenibacillus larvae	CCUG28515	Negative

A 10-fold serial dilution of 100 ng purified *M plutonius* DNA was used to determine the lower detection limit of the gold nanoparticles assay, and was tested also by real-time PCR assay.

Applicability of the *M plutonius* gold nanoparticles-based assay

The ability of the assay to detect *M plutonius* DNA in clinical samples was evaluated by testing eight infected and 18 uninfected bee colonies. All samples were tested by both the gold nanoparticles and real-time PCR assays.

Results

A 0.5M NaCl concentration was sufficient for both aggregation of AuNPs and proper annealing of the probe to its target. The optimum concentration of the *M plutonius* probes was 2 µM. Thus, the hybridisation buffer was prepared using 0.5M NaCl and 2 µM probes in 10 mM PBS at pH 7.0. Optimal reaction conditions were: denaturation at 95°C for five minutes, annealing at 50°C for five minutes, then hold the reaction mixture at room temperature for 10 minutes before addition of 10 µl AuNPs. The designed probes were highly specific to *M plutonius* sequences in GenBank and strain used in this study. Moreover, they were not complementary to any related bacterial DNA sequences indicated by the BLAST search. After addition of the AuNPs, the colour of the reaction mixture changed to blue in the presence of DNA extracted from *M plutonius*; it remained red with the negative control. Neither aggregation nor colour change were observed when DNA extracted from the other bacteria were used, which confirmed the specificity of the assay.

The *M plutonius*-AuNPs assay could detect as little as 50 fg *M plutonius* DNA (Fig 1) which represents 25 copies of *M plutonius* (Staroscik 2004, Okumura and others 2011). The sensitivity of the real-time PCR assay was 5 fg of *M plutonius* DNA. Both assays detected *M plutonius* DNA in the eight infected clinical samples, with no detections in the 18 uninfected samples. The results obtained using the nanoparticle-based assay (Fig 2) were in concordance with those obtained with the real-time PCR system each of the five times the assays were performed.

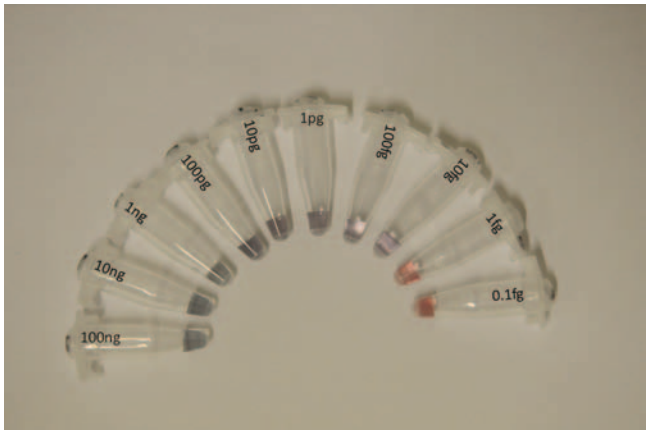

FIG 1: Sensitivity of the *Melissococcus plutonius*-AuNPs assay. The lower detection limit of the *M plutonius*-AuNPs assay was estimated using 10-fold serial dilutions from100 ng to 0.1 fg of *M plutonius* DNA. The colour changes from red to blue in positive samples

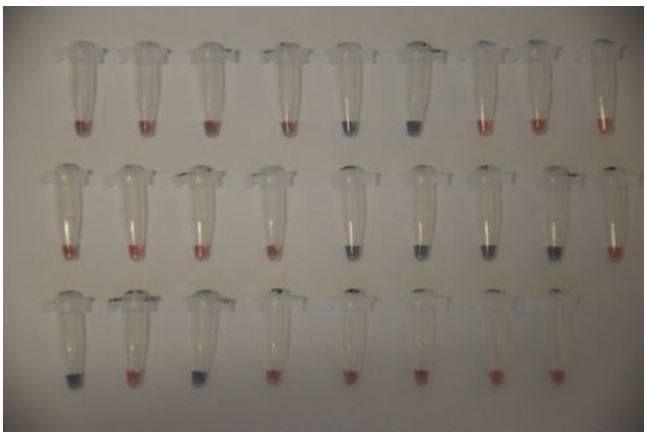

FIG 2: Feasibility of the *Melissococcus plutonius*- AuNPs assay to detect *M plutonius* DNA in clinical samples. The ability of the *M plutonius*-AuNPs assay to detect *M plutonius* DNA in clinical samples was evaluated by testing eight DNA samples extracted from *M plutonius* infected bee-hives, and 18 DNA samples extracted from uninfected samples. The colour changes from red to blue in positive samples.

Discussion

Although selective media can be used to cultivate *M plutonius*, its detection can prove difficult due to the relative complexity of the culture procedure and its fastidious growth requirements (Pinnock and Featherstone 1984, Alippi 1991, Djordjevic and others 1998, Hornitzky and Smith 1998, Forsgren 2010). We employed an alternate strategy: a novel gold nanoparticle-based assay that is rapid, accurate and specific for the detection of *M plutonius* directly from diseased bee larvae. Several gold nanoparticles-based colorimetric detection assays have been designed, the first of them by Elghanian and others (1997), which could detect down to 10 fmol of target DNA. A recent gold nanoparticle method for direct detection of unamplified hepatitis C virus RNA to a detection limit of 50 copies/reaction (Shawky and others 2010). Herein, we introduce a sensitive colorimetric method that could detect at least 50 fg (25 copies/reaction) of *M plutonius* DNA.

The oligonucleotide probes that we designed did not cross-react with related bacterial species, and successfully detected DNA extracted from *M plutonius* in a rapid and highly specific manner. It was essential to optimise the concentration of the ssDNA because too low a concentration can lead to particle aggregation in the absence of target DNA, that is, false positives, whereas in the presence of the target, a very high probe concentration would have prevented aggregation, producing false negative results. We optimised pH, temperature, time and salt concentration conditions for hybridisation, before addition of the AuNPs. A fluorescent probe or a DNA-binding dye was not required for detection. The assay requires only 20 minutes to set up, and positive results could be observed visually within one minute after addition of the AuNPs. Carry-over contamination was reduced and simultaneous testing of many samples was facilitated by performing the assay in a single tube. As neither a DNA amplification step nor post-PCR analyses, such as gel electrophoresis, were needed prior to detection, this method should be widely applicable at points-of-care facilities.

The assay directly detected *M plutonius* DNA extracted from both bacterial isolates and infected bee larvae. The assay's performance on clinical samples was compared with a real-time PCR method, and found to have 100 per cent concordance for both positive and negative samples. Although, the real-time PCR assay was 10 times more sensitive than the gold nanoparticles assay, the nanoparticles-based assay was more rapid to perform, had equivalent specificity, and did not require a real-time PCR machine.

We suggest that the developed gold nanoparticle assay is particularly suited for use in carrier screening programmes for tracking the

EFB agent, *M plutonius*, in field diagnostic scenarios. The test is suitable also for verifying clinical diagnosis of EFB.

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