Journal of Apicultural Research 52(1): (2013)
DOI 10.3896/IBRA.1.52.1.12

REVIEW ARTICLE

Standard methods for European foulbrood research

Eva Forsgren[1*], Giles E Budge[2], Jean-Daniel Charrière[3] and Michael A Z Hornitzky[4]

[1]Department of Ecology, Swedish University of Agricultural Sciences, PO Box 7044, SE-75007 Uppsala, Sweden.
[2]National Bee Unit, Food and Environment Research Agency, Sand Hutton, York, YO41 1LZ, UK.
[3]Swiss Bee Research Center, Agroscope Liebefeld-Posieux (ALP), Schwarzenburgstr. 161, 3003 Bern, Switzerland.
[4]New South Wales, Department of Primary Industries, Elizabeth Macarthur Agricultural Institute, PB 4008, Narellan NSW 2567, Australia.

Received 30 March 2012, accepted subject to revision 26 April 2012, accepted for publication 12 September 2012.

*Corresponding author: Email: eva.forsgren@slu.se

Summary

European foulbrood (EFB) is a severe bacterial honey bee brood disease caused by the Gram-positive bacterium *Melissocccus plutonius*. The disease is widely distributed worldwide, and is an increasing problem in some areas. Although the causative agent of EFB was described almost a century ago, many basic aspects of its pathogenesis are still unknown. Earlier studies were hampered by insensitive and unspecific methods such as culture based techniques. Recent advances in molecular technology are making it increasingly easy to detect and characterize microbes, and nucleic acid detection technologies are quickly displacing the traditional phenotypic assays in microbiology. This paper presents selected methodologies which focus on EFB and its causative agent *M. plutonius*.

Métodos estándar para la investigación sobre la loque europea

Resumen

La loque europea (LE) es una grave enfermedad bacteriana de la cría de la abeja de la miel causada por la bacteria Gram-positiva *Melissococcus plutonius*. La enfermedad se encuentra ampliamente distribuida en todo el mundo y es un problema creciente en algunas áreas. Aunque el agente causante de la LE fue descrito hace casi un siglo, muchos aspectos básicos de su patogénesis son aún desconocidos. Estudios anteriores se vieron obstaculizados por métodos poco sensibles e inespecíficos, tales como las técnicas basadas en cultivos. Los recientes avances en la tecnología molecular están haciendo cada vez más fácil la detección y caracterización de los microbios, y las tecnologías de detección de ácidos nucleícos están desplazando rápidamente a los ensayos fenotípicos tradicionales de microbiología. Este artículo presenta algunas metodologías seleccionadas que se centran en LE y en su agente causal *M. plutonius*.

欧洲幼虫腐臭病研究的标准方法

欧洲幼虫腐臭病是蜜蜂幼虫病中较为严重的细菌病，由革兰氏阳性细菌Melissocccus plutonium 引起。欧洲幼虫腐臭病在世界范围内广泛分布，成为一些地区日益突出的主要病害。虽然一个世纪前就已有相关病原体的描述，但早期研究受实验技术不灵敏、特异性不好的限制（比如细菌培养的技术），其发病机理至今未能全面揭示。近年来随着分子技术的发展检测微生物日益容易，核酸检测技术快速替换了传统的微生物学表型实验。本文展示了近期开展欧洲幼虫腐臭病及其病原体 M. plutonius方面的常用技术。

Keywords: honey bee, European foulbrood, *Melissococcus plutonius*, brood disease, methodologies, *BEEBOOK,* COLOSS

Footnote: Please cite this paper as: FORSGREN, E; BUDGE, G E; CHARRIÈRE, J-D; HORNITZKY, M A Z (2013) Standard methods for European foulbrood research. In *V Dietemann; J D Ellis, P Neumann (Eds) The COLOSS* BEEBOOK*: Volume II: Standard methods for* Apis mellifera *pest and pathogen research. Journal of Apicultural Research* 52(1): http://dx.doi.org/10.3896/IBRA.1.52.1.12

1. Introduction

1.1. Background

European foulbrood (EFB) is a bacterial brood disease caused by the Gram positive bacterium *Melissococcus plutonius*. EFB is listed in the *OIE Terrestrial Animal Health Code* (2011), but, unlike American foulbrood; it is not notifiable in all countries. The disease occurs in honey bees throughout the world, and may cause serious losses of brood and colony collapse. In many areas, the disease is endemic with occasional seasonal outbreaks, but, in a few countries, the scenario is different. In Switzerland, the incidence of EFB has increased dramatically since the late 1990s; it has become the most widespread bacterial brood disease in the UK, and Norway reported a regional outbreak of EFB during 2010 after a 30 year period of absence. Geographically, the disease appears to vary in severity from being relatively benign in some areas but increasingly severe in others (Wilkins *et al.*, 2007; Dahle *et al.*, 2011; Grangier, 2011; Arai *et al.*, 2012). Virulence tests on individual larvae using exposure bioassays (see section 9), shows that *M. plutonius* strains collected in different geographic European locations vary in their ability to cause larval mortality (Charrière *et al.*, 2011).

1.2. Disease symptoms

The field diagnosis of EFB is based on the visual inspection of brood combs and detection of diseased larvae (see section 4). The general symptoms observed in a colony suffering from EFB are irregular capping of the brood; capped and uncapped cells being found scattered irregularly over the brood frame (known as pepper pot brood). The youngest larvae that die from the infection cover the bottom of the cell and are almost transparent, with visible trachea. Older larvae die malpositioned and flaccid in their cells; twisted around the walls or stretched out lengthways (Fig. 1). The colour of affected larvae changes from pearly white to pale yellow, often accompanied by a loss in segmentation. More advanced symptoms can manifest as further colour changes to brown and greyish black (Fig. 1), sometimes ultimately leaving a dark scale (Fig. 2) that is more malleable than those typically found with American foulbrood (AFB).

Fig. 1. Malpositioned and discoloured larvae in brood with symptoms of European foulbrood. Photo by Eva Forsgren

Fig. 2. The infected larva loses its internal pressure and becomes flaccid, ultimately leaving a dark scale. Photo by Kaspar Ruoff

1.3. Secondary bacteria

Several other bacteria such as: *Enterococcus faecalis; Achromobacter euridice; Paenibacillus alvei* and *Brevibacillus laterosporus* may be associated with EFB (Forsgren, 2010). Although the presence of *P. alvei* - like spores of *E. faecalis* has been considered presumptive evidence of European foulbrood, the role of such secondary bacterial invaders in disease development has been poorly investigated. *A. euridice* is frequently isolated in mixed culture with *M. plutonius* and EFB symptoms in larvae may be more easily induced with inoculate containing *M. plutonius* in combination with *A. euridice* or *P. alvei* (Bailey, 1957). However, a more recent study from Switzerland showed that the simultaneous or 3 days delayed inoculation of *P. alvei* had no influence on the virulence of M. *plutonius* in individual larvae (Charrière *et al.*, 2011; see section 9.2). This paper will focus solely on techniques for diagnosis and research of the causative agent of EFB, *M. plutonius*.

1.4. Diagnosis

Symptoms of EFB may easily be confused with other diseases or abnormalities in the brood, making diagnosis difficult. The diagnosis in the field can be further verified by microscopic examination of brood smear preparations (see section 6; Hornitzky and Wilson, 1989; Hornitzky and Smith, 1998), and a field test kit (see section 7.2) for the detection of *M. plutonius* in larval extracts is also available (Tomkies *et al.*, 2009). Analysing pooled samples of bees from the

Table 1. Reference strains of *M. plutonius*.

species	strain no.	other designation	source
Melissococcus plutonius	LMG 20360	= ATCC 35311	Honey bee larvae, UK
		= CIP 104052	
		= LMG 15058	
		= LMG 19520	
		= LMG 20206	
		= LMG 21267	
		= NCDO 2443	
	NCIMB 702439		Honey bee larvae, India
	NCIMB 702440		Honey bee larvae, Brazil
	NCIMB 702441		Honey bee larvae, Tanzania
	NCIMB 702442		Honey bee larvae, Australia
	NCIMB 702443		Honey bee larvae, UK

brood nest by PCR (see section 8) may be an alternative or complement to visual inspection (Roetschi *et al.*, 2008), although false negatives may sometimes occur (Budge *et al.*, 2010). Sensitive detection methods are required to ensure the absence of the bacterium from bee products and for the confirmation of the visual diagnosis made in the field or for research purposes. Pure isolates of *M. plutonius* may sometimes be desirable for various research purposes (see section 5). There are selective media for the cultivation of *M. plutonius* (Bailey, 1957; Bailey, 1983; Bailey and Collins, 1982; Hornitzky and Wilson, 1989; Hornitzky and Karlovskis, 1989), but to culture the bacterium can be difficult and there is some evidence that *M. plutonius* samples from different regions have a differential response to culturing (Allen and Ball, 1993; Arai *et al.*, 2012). Immunology-based tests such as enzyme linked immuno-sorbent assay (ELISA) (Pinnock and Featherstone, 1984) have been published and used for the detection and quantification of *M. plutonius* (see section 7.1), but DNA amplification using the polymerase chain reaction (PCR) provides lower thresholds of detection than ELISA, and has been successfully used for the detection of *M. plutonius* since the late 1990s (see section 8).

This paper therefore aims to present selected protocols useful for diagnosis and research on the honey bee brood disease European foulbrood.

2. Bio-safety recommendations

It is important to appreciate that there is no internationally accepted biohazard classification for the handling of *M. plutonius* for diagnostic or research purposes. Restrictions on the handling of the bacterium and diseased material vary significantly between countries, and any laboratory should check national policies for guidance before handling material. In those countries where *M. plutonius* is not notifiable, there is still a strong need for precautionary measures to reduce the risk of infection, and further guidance on bio-safety recommendations can be found in the American foulbrood paper of the *BEEBOOK* (de Graaf *et al.*, 2013).

3. Reference strains of *Melissococcus plutonius*

Type or reference strains of *M. plutonius* are available at culture collections, such as the Belgian Co-ordinated Collections of Microorganisms, BCCM/LMG (Table 1). A type strain (or a prototype strain) is a nomenclatural type of a species or a subspecies. Authors who propose new bacterial names are supposed to deposit type strains in two publicly accessible recognized culture collections in two different countries. Type strains are also useful in validation work.

4. Sampling

Methods can be grouped into those looking to confirm the presence of the disease by testing an individual symptomatic larva for *M. plutonius,* and those that hope to confirm the presence of *M. plutonius* in asymptomatic material, such as in bulk samples of adult bees or disease-free colonies in proximity to disease. When considering the latter, it is important to consider the within-hive distribution of the pathogen and also how the sample size may affect the power of the subsequent test. For example, when assuming a hive population of 50,000 individuals, sampling 5 adult bees provides a 95% confidence of detecting a pathogen with a minimum prevalence of 50%, whereas sampling 60 adult bees increases the power of the testing regime to enable a more meaningful minimum pathogen detection of 5% prevalence. No single study has provided the necessary detail to make definitive recommendations on sample location and size, therefore this chapter concentrates on summarizing the sampling methods and

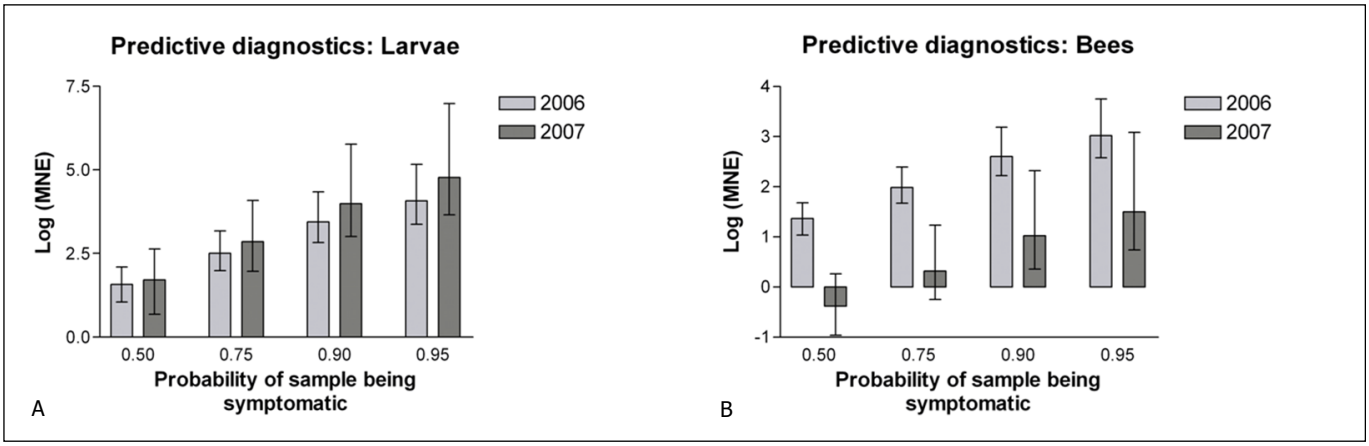

Fig. 3. Estimates of the log amount of *M. plutonius* (MNE) in samples of larvae (A) and adult bees (B) probability of the honey bee colony being symptomatic for EFB. Data from 2006 are from Budge et al. (2010), and those from 2007 previously unpublished findings from follow up work. The plots represent estimates and corresponding 95% confidence limits from a generalized linear model constructed using qPCR data (see Budge *et al.*, 2010).

knowledge to date (See also de Graaf *et al.*, 2013; Human *et al.*, 2013). Storage temperature is not crucial. All sample types can be refrigerated for several hours and stored at -20ºC for longer periods.

4.1. Brood

Upon visual inspection in the field, large pieces of symptomatic brood may be cut out and sent to the laboratory (a piece of 10 x 10 cm cuts through the metal wires of the brood frame) for further examination and confirmation of the diagnosis. Correct sampling of brood is important because even within the same brood frame, *M. plutonius* is mainly found in larvae with visual disease symptoms (Forsgren *et al.*, 2005). Alternatively, and in cases of lower severity, diseased larvae can be smeared on a microscope slide and submitted to the laboratory (see section 6). *M. plutonius* can survive for over three years on such slide preparations (Bailey, 1960), and 6 years within Lateral Flow Devices (Budge, unpublished data), and so culturing often remains a viable option many years after diagnosis.

It is possible to identify the presence of *M. plutonius* in the absence of disease symptoms by collecting bulk samples of 100 larvae, taken at random from across the brood nest and subjecting the samples to qPCR (Budge *et al.*, 2010; see also the molecular methods paper of the *BEEBOOK* (Evans *et al.,* 2013). This method provided robust quantification of *M. plutonius* and is a potentially useful tool to help predict the risk of a colony either prior to disease development or in the absence of an inspection to confirm disease (Fig. 3).

4.2. Adults

Analysis of worker bees indicates that individuals from the brood nest contain more bacteria than bees from flight entrances; therefore, it was suggested that samples of bees are preferably collected from the brood nest (Roetschi *et al.*, 2008). This result has not been replicated by others, where the amount of *M. plutonius* in foragers equalled that found in nurse bees (Budge, unpublished data). Pooled samples of 100 bees have been used for DNA extraction (Roetschi *et al.*, 2008; Budge *et al.*, 2010); however, adult bees show more variation in the amount of *M. plutonius* detected than samples of larvae (Fig. 3).

4.3. Honey and pollen

Brood nest honey, bulk honey and pollen have to some extent been used to confirm the presence of bacteria using culture methods (see section 5) and PCR (see section 8) in both diseased and healthy looking colonies (Hornitzky and Smith, 1998, McKee *et al.*, 2003). For further sampling instructions see the American foulbrood paper of the *BEEBOOK* (De Graaf *et al.*, 2013).

5. Cultivation of *M. plutonius*

For many experiments, it is imperative to use bacterial cultures in which all cells are genetically identical. Since all cells in a colony develop from one single cell, a single isolated colony of *M. plutonius* is an excellent source of a genetically pure bacterial stock. In order to propagate any bacterium, it is necessary to provide the appropriate biochemical and biophysical environment to encourage bacterial growth. The biochemical or nutritional environment is provided as a culture medium based on special needs for particular bacteria, and can be used for isolation and maintenance of bacterial cultures. Bacterial culture media can be classified based on consistency. Liquid media are sometimes referred to as "broths" where bacteria grow uniformly, and tend to be used when a large quantity of bacteria have to be grown. Moreover, liquid media can be used to obtain a viable bacterial count i.e. to physically quantify the amount of organism present (see section 9.1.1). Any liquid media can be solidified by the addition of agar (e.g. Oxoid Technical Agar No. 1) at a concentration of 1-3%. Although *M. plutonius* can be isolated from honey and diseased brood by cultivation, bacterial culture methods seem to be very insensitive detecting less than 0.2% of the bacterial cells (Djordjevic *et al.*, 1998; Hornitzky and Smith, 1998).

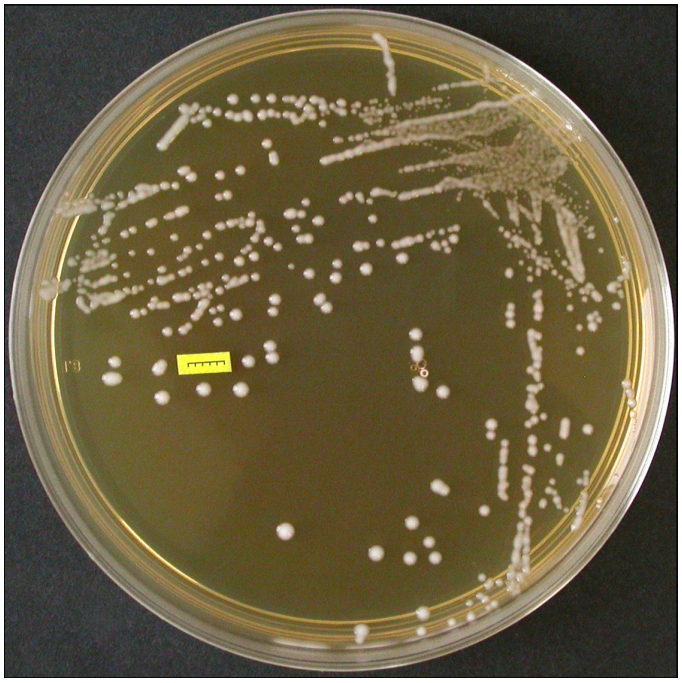

Fig 4a. Agar plate (basal medium) with colonies of *M. plutonius.* The yellow bar represents 5 mm.

Photo by Lena Lundgren and Karl-Erik Johansson

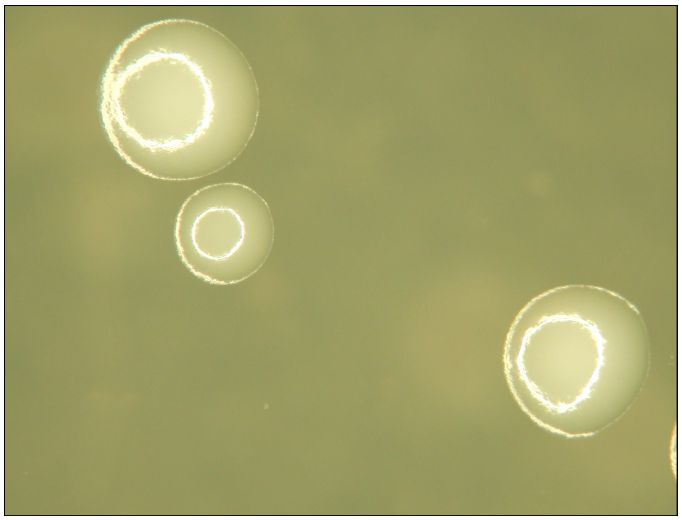

Fig 4b. Colony morphology of *M. plutonius* on basal medium. The bar represents 1 mm. Photo by Eva Forsgren

5.1. Basal medium (modified from Bailey, 1957)

1. Dissolve the following components in approximately 800 ml deionized water:-
   - 10 g of yeast extract
   - 10 g of glucose
   - 10 g of starch
   - 0.25 g L-cysteine
   - 20 g of agar
2. Add 100 ml of 1M KH_2PO_4 (pH 6.7)
3. Adjust the pH to 6.6 using 2.5 M KOH.
4. Adjust the final volume to 1000 ml.
5. Sterilize by autoclaving at 115°C for 15 minutes.

Optional; in order to prevent growth of secondary bacteria, filter-sterilized nalidixic acid (dissolved in 0.1 M NaOH may be added to a final concentration of 3 µg per ml after autoclaving. For liquid cultures, the starch may be replaced with saccharose, making the medium clear. This will facilitate when checking the turbidity or the cloudiness of the cell suspension, e.g. to see if there is any bacterial growth.

6. Incubate the plates for 7 days at 35°C anaerobically. Colonies about 1 mm in diameter will appear after 4-7 days (Figs 4 a, b), and can be further confirmed by staining, LFI or PCR (see sections 6.1, 7.2 and 8). Single bacterial colonies can be screened using real-time PCR / conventional PCR by simply touching a small (10 µl) tip directly onto the colony of interest, touch the tip onto some agar to sub-culture if required, before placing the tip directly into the PCR master-mix (see section 8).

5.2. M110 agar (from BCCM/LMG bacteria catalogue)

5.2.1. Agar base

- 2.5 g of peptone (Oxoid L37)
- 10 g of glucose
- 2 g of soluble starch
- 2.5 g of yeast extract (Oxoid L21)
- 5 g of neopeptone (Difco 0119)
- 2 g of trypticase (BBL211921)
- 50 ml of 1 M phosphate buffer (pH 6.7) = 49.7 ml of 1M K_2HPO_4 + 50.3 ml of 1M KH_2PO_4.

1. Mix all ingredients and make up to 1000 ml with distilled water.
2. Adjust to pH 7.2 with 5M KOH.
3. Add agar (Difco or Oxoid No 1) to a 1.5% final concentration. For 250 ml amounts of agar base, weigh 3.75 g of agar into the 250 ml Duran flasks, for 500 ml, 7.5 gram. Dispense the correct amount of made up and pH corrected broth into the Duran flask.
4. Mix well before dispensing broth into Duran flasks with agar, the soluble starch settles out quite quickly.
5. Sterilize by autoclaving at 115°C for 15 minutes. Higher temperature autoclaving will tend to caramelize the agar (make it darker).
6. Add cysteine hydrochloride H_2O to a final concentration of 0.025%.

NOTE: Cysteine hydrochloride reduces the oxygen content in the agar

more efficiently if added just before the agar is to be used. If the agar is made up in 250 or 500 ml amounts, then autoclaved, this base can be kept for quite a long time (2-3 months or longer), but if the cysteine is added before autoclaving, the base will only keep a week at the most.

5.2.2. Agar plates

1. Melt the agar base in a steamer and cool to 46°C.
2. Add 625 µl of a freshly made 0.2 µm filter sterilized 10% solution of cysteine hydrochloride (does NOT keep at all) to 250 ml of the agar base, or 1.25 ml for 500 ml of base. Make sure the agar is mixed well, but not bubbly as soluble starch settles out.
3. Pour immediately into petri-dishes.
4. Cool and dry in the lamina flow for 20 minutes.
5. Use the plates as soon as possible. It is not advised to keep unused plates to use another day.

5.3. Anaerobic incubation

1. Seal in an anaerobic jar as soon as possible with an anaerobic indicator (Oxoid BR055B) and Oxoid AnaeroGen anaerobic generator sachet (appropriate size for anaerobic jar volume. AnaeroGen AN025A for 2.5 l jar, AnaeroGen AN035A for 3.5 l jar).
2. Incubate for about a week at 35°C. The anaerobic indicator should go colourless from pink after a few hours incubation. If it does not, the jar has failed to achieve anaerobic conditions.

5.4. Medium for long-term storage

Freezing is an effective long-term storage of *M. plutonius* as well as other bacterial isolates. Broth cultures are mixed with chemicals such as glycerol or DMSO to limit damage upon freezing. It is possible to use proprietary cryopreservation kits which can even specialize in the preservation of anaerobic organisms (e.g. PROTECT system from Thermo Fisher Scientific). Using such proprietary kits, pure cultures of *M. plutonius* are added to a tube containing cryopreservation liquid and ceramic beads. After temperature controlled freezing, cultures can be recovered by removing a single ceramic bead and plating directly onto selective media. Results to date indicate 100% successful recovery after 6 months storage at -80°C (Budge, unpublished data).

6. Microscopy

The laboratory diagnosis of European foulbrood is based on the identification of *M. plutonius* in affected brood. One method for the identification *M. plutonius* is the microscopy of smears prepared from diseased brood.

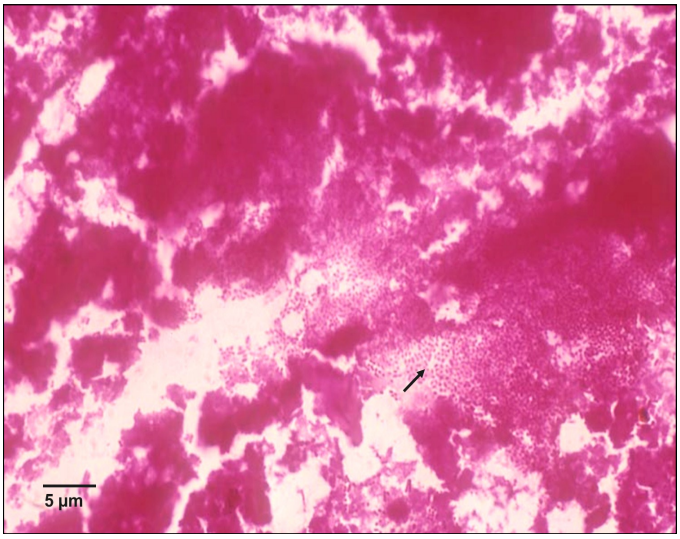

Fig 5a. Early infection- only *Melissococcus plutonius*. Arrow indicates a mass of coccoid/lanceolate *M. plutonius* organisms.

Photo by Michael Hornitzky

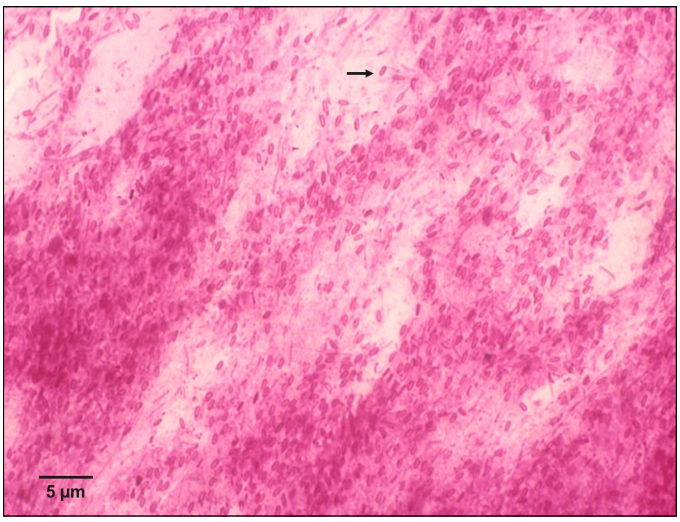

Fig 5b. Infiltration of secondary invader *Paenibacillus alvei*. Arrow indicates one of the many vegetative *P. alvei* cells.

Photo by Michael Hornitzky

6.1. Carbol fuchsin staining

Prepare the carbol fuchsin stain by mixing the following 2 solutions.
Solution A: 0.2 g basic fuchsin and 10 ml 95% ethanol.
Solution B: 5 g phenol and 90 ml distilled water.
Procedure:

1. Select larvae and/or pupae showing signs of European foulbrood and place them on a microscope slide.
2. Using a swab or stick, pulp the larvae together and spread over the slide pushing any excess off one end, to leave a thin smear. Allow the smears to dry before processing.
3. Heat fix by flaming the slide over a burner a few times.
4. Flood with 0.2% carbol fuchsin for 30 seconds.
5. Wash off the stain and either air dry or gently blot dry before microscopic examination at 1000 times magnification.

A diagnosis of European foulbrood is made if examination revealed *M.*

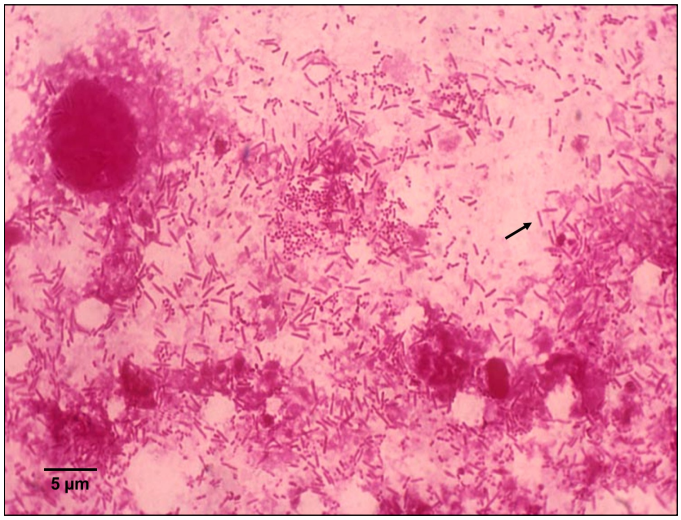

Fig 5c. Proliferation of *P. alvei* spores to the virtual exclusion of *M. plutonius*. Arrow indicates one of the many *P. alvei* spores.

Photo by Michael Hornitzky

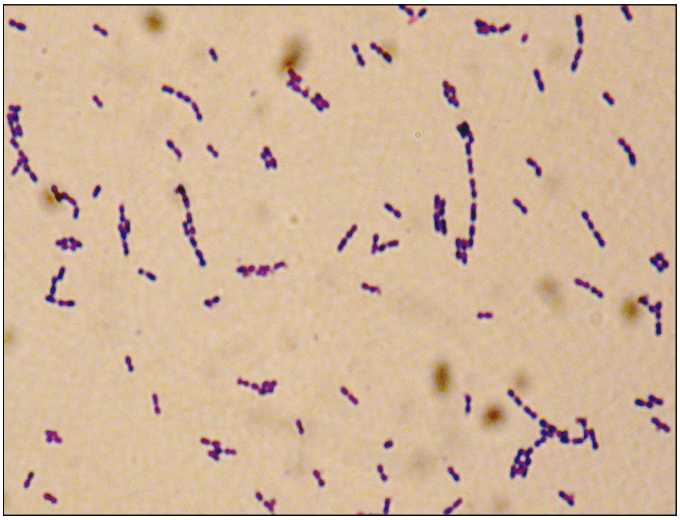

Fig. 6. Gram staining of *Melissococcus plutonius*. The coccoid-shaped bacteria forming pairs or even chains are clearly visible.

Photo by Lena Lundgren and Karl-Erik Johansson

plutonius-like organisms. Organisms are considered to be *M. plutonius* if they are lanceolate cocci, approximately 0.5 x 1.0 µm. *E. faecalis* is very like *M. plutonius* in appearance and has frequently been confused as being the causative agent (Bailey and Gibbs, 1962; Hornitzky and Wilson, 1989) (Figs 5 a, b, c).

An alternative to the 0.2% carbol fuchsin stain is the Gram stain, useful mainly when the Gram positive feature of *M. plutonius* needs to be confirmed.

6.2. Gram staining

Gram-staining is a four part procedure which uses certain dyes to make a bacterial cell stand out against its background. The reagents you will need are:

- Crystal violet (the primary stain)
- Iodine solution (the mordant)
- Decolourizer (ethanol + acetone)
- Safranin (the counter stain)

Procedure:

1. Mount and heat fix the specimen (about 6 times through the flame).
2. Flood (cover completely) the entire slide with crystal violet.
3. Let the crystal violet stand for about 60 seconds.
4. Flood your slide with the iodine solution.
5. Let it stand for 60 seconds.
6. Rinse the slide with water for 5 seconds and immediately proceed to next step.
7. Rinse the slide with decolourizer for 20-60 seconds.
8. Rinse the slide carefully with water for about 5 seconds.
9. Apply the counter stain, safranin, by flooding the slide with the dye.
10. Let it stand for about 10-15 seconds.
11. Rinse with water for 5 seconds.
12. Dry the slide with paper or allow it to air dry.
13. View it under the microscope at 1,000 times magnification (Fig. 6).

7. Immunology-based methods

Various laboratory techniques based on the use of antibodies to visualize or distinguish between microorganisms exist. The key component in any of the vast array of methods used is the antibody. Polyclonal antibodies against *M. plutonius* can be prepared by injection of washed cultures of *M. plutonius* into rabbits either by intravenous injections (Bailey and Gibbs, 1962) or by a single intramuscular injection of 1ml *M. plutonius* suspension mixed with an equal volume of Freund´s incomplete adjuvant (OIE, 2008). Monoclonal antibodies can be prepared by injecting mice as described by Tomkies *et al.* (2009).

7.1. ELISA (Enzyme Linked Immuno Sorbent Assay)

The enzyme-linked immunosorbent assay (ELISA) is a common serological test for particular antigens or antibodies. There are two forms of the test: i. the direct ELISA employs antibodies to detect presence of a particular antigen in a samples and; ii. the indirect ELISA is usually used to detect specific antibodies in a specimen such as blood serum. However, the indirect ELISA method can also be applied for detection of antigens as described in section 7.1.3. The ELISA method described by Pinnock and Featherstone (1984) is

unable to detect bacterial levels less than 10^5 cells per ml.

7.1.2. Sample processing

Individual or pooled samples of bees, larvae or pupae (sampled and stored at -20ºC) can be crushed in phosphate buffered saline, PBS, pH 7.4 (for the recipe, see Table 1 of the cell culture paper of the *BEEBOOK* (Genersch *et al.*, 2013)) , the homogenate centrifuged for 10 sec at 10,000 g and the supernatant stored at -20°C or used directly in an ELISA.

7.1.3. Indirect ELISA

The reagents needed to perform the ELISA are:

- Bicarbonate / carbonate coating buffer, 100 mM, pH 9.6.
- Phosphate buffered saline, PBS, pH 7.4.
- Blocking solution (PBS with 1-2% BSA).
- Washing buffer (PBS with 0.05% Tween 20).
- Primary antibody (rabbit, chicken, mouse).
- Peroxidase or alkaline phosphatase-conjugated secondary antibody (anti-rabbit, anti-chicken; anti-mouse).
- Substrate for peroxidase alkaline phosphatase-conjugated secondary antibody (e.g. TMB (3,3´,5,5´- tetramethylbenzidine)).
- Stop solution (0.5 M H_2SO_4).
- Microtiter plates.
- Microtiter plate reader.

Many different types of enzymes can be used for detection. Peroxidase-conjugated secondary antibodies and TMB (3,3´,5,5´-tetramethylbenzidine) are commonly used and accessible.

Procedure:

1. Dilute the bee homogenates in coating buffer.
   The total protein concentration should not exceed 20 µg per ml.
2. Coat the wells of a microtiter plate with 100 µl per well of the antigen dilution.
3. Cover the plate using an adhesive plastic.
4. Incubate for 2 hours at room temperature or at 4°C over night.
5. Remove the coating buffer.
6. Wash the plates two times filling the wells with washing buffer.
7. Block the remaining protein-binding sites by adding 200 µl blocking solution to the wells.
8. Incubate for 2 hours at room temperature or at 4°C over night.
9. Wash the plate two times with washing solution.
10. Add 100 µl of the *M. plutonius* specific antibody diluted in blocking solution.
11. The optimal dilution should be determined using a dilution assay.
12. Cover the plate and incubate for 2 hours at room temperature.
13. Wash the plate three times with PBS.
14. Add 100 µl of the secondary, conjugated antibody diluted according to the manufacturer´s instruction.
15. Cover the plate.
16. Incubate for 1 hour at room temperature.
17. Wash four times with washing solution.
18. Dispense 100 µl per well of the substrate solution.
19. Incubate for 15 min in room temperature (dark).
20. Add equal volume of the stop solution (2 M H_2SO_4).
21. Read the optical density at 450 nm using a plate reader. Compare the density reads of unknown samples against standards (*e.g.* suspensions of known concentrations of *M. plutonius*). To ensure accuracy, include standards and at least one blank sample to each plate.

7.2. Lateral flow immunoassay (LFI)

A commercially available lateral flow device for the detection of *M. plutonius* using specific monoclonal antibodies is available. The kit was designed primarily for the confirmation of disease symptoms in the field, but may also be used in the laboratory (Tomkies *et al.,* 2009). The kit is produced by Vita (Europe) Ltd and the protocols available at: http://www.vita-europe.com. Using LFIs gives an instant result (meaning no time delay between disease suspicion and treatment), no expensive equipment required and is relatively cheap compared to posting the samples to the laboratory for diagnosis. However, it works only on single larvae and requires field knowledge to select the correct/infected larvae in a brood sample (see section 1.2).

8. PCR-based methods

Detection of infectious microorganisms has been revolutionized by the polymerase chain reaction (PCR), and has increasingly been described as the "gold standard" for detecting some microbes. Theoretically, a single target DNA molecule is sufficient for detection, making PCR one of the most sensitive biological techniques ever described. For a more generic overview on PCR-based methods and other molecular biology methodologies used in *A. mellifera* research see the molecular methods paper of the *BEEBOOK* (Evans *et al.*, 2013).

8.1. Processing

Samples can be homogenized with glass beads in mechanical 'bead mills', in mesh bags (*e.g.* Bioreba, Neogen) using a grinding pestle, a stomacher (e.g. Seward Ltd UK) or in microfuge tubes with a micropestle. The choice depends on sample size and type. Individuals can be extracted in the manufacturer´s buffer directly, but for bulk samples a primary extract may be necessary (see section 8.2.1).

Table 2. PCR-based methods for the detection of *M. plutonius*.

publication	primers	Sequence (5'-3')	Size (bp)	method and target
Govan et al., 1998	Primer 1 Primer 2	GAAGAGGAGTTAAAAGGCGC TTATCTCTAAGGCGTTCAAAGG	831	PCR, 16S rRNA gene
Djordjevic et al., 1998	MP1 MP2 MP3	CTTTGAACGCCTTAGAGA ATCATCTGTCCCACCTTA TTAACCTCGCGGTCTTGCGTCTCTC	486 276	hemi-nested PCR, 16S rRNA gene
Roetschi et al., 2008	MelissoF MelissoR Probe	CAGCTAGTCGGTTTGGTTCC TTGGCTGTAGATAGAATTGACAAT FAM-CTTGGTTGGTCGTTGAC-MBGNFQ	79	real-time PCR, sodA gene
Budge et al., 2010	EFBFor EFBRev2 Probe	TGTTGTTAGAGAAGAATAGGGGAA CGTGGCTTTCTGGTTAGA FAM-AGAGTAACTGTTTTCCTCGTGACGGT-TAMRA	69	real-time PCR, 16S rRNA gene

8.2. DNA extraction

Cellulose-based affinity columns such as QIAGEN, or generic equivalents are most practical for obtaining clean DNA preparations. They are reliable and yield good quality DNA. Magnetic bead-based purification also works well (e.g. Budge *et al.,* 2010). Since samples of adult bees contain more secondary metabolites and phenolics than larvae, including a QiaShredder in the protocol will yield purer nucleic acid (DNeasy® Plant Mini Kit) and prevent inhibition of the PCR reaction. This is also recommended when extracting bacterial DNA from honey. The columns can be used for manual DNA extraction or in a QiaCube® (QIAGEN) for automated extraction. There are two options when considering extraction controls for the quantification of *M. plutonius* in honey bee samples. First, it is possible to monitor extraction efficiency using a honey bee reference gene (e.g. 18S; Budge *et al.*, 2010). Alternatively extraction failures or PCR amplification inhibition can be monitored by amending the sample with a known amount of *Staphylococcus aureus* before extraction (Grangier, 2011). It is also recommended to include a negative extraction control (e.g. water) to check for possible contamination during the extraction process (Bustin *et al.*, 2009). For further information on nucleic acid extraction see the molecular methods paper of the *BEEBOOK* (Evans *et al.,* 2013).

8.2.1. Adults

Procedure:

1. Place adult bees in filter grinding bag (Neogen™, Bioreba).
2. Add 0.5 ml grinding buffer (e.g. GITC[1]) per bee.
3. Crush the bees.
4. Transfer 1.5 ml of the supernatant to a 2 ml Eppendorf tube. OR; include a "crude" centrifugation step for bigger volumes.
5. Centrifuge at 2,000 g for 10 minutes.
6. Transfer 1.5 ml to an Eppendorf tube.
7. Centrifuge at 20,000 g for 2 minutes.
8. Discard the supernatant.
9. Resuspend the pellet in the manufacturer's lysis buffer (DNeasy® Plant Mini Kit, QIAGEN).
10. For manual DNA-extraction: Use DNeasy® Plant Mini Kit (QIAGEN). Follow the protocol for plant tissue (Mini Protocol). For automated DNA extraction using a QiaCube® (QIAGEN); follow the purification of total DNA from plant tissue standard protocol.
11. Use the DNA templates directly in a PCR or store in –20ºC until needed.

[1]GITC = for 100 ml, add 50 g guanidine thiocyanate, 50 ml nuclease free water, 5.3 ml 1M Tris-Cl (pH 7.6), 5.3 ml 0.2 M EDTA. Stir until completely solved and store at 4ºC.

8.2.2. Larvae / pupae

Procedure:

1. Place the larva / pupa in an Eppendorf tube.
2. Add 0.5 ml grinding buffer (e.g. GITC).
3. Ground with a micropestle.
4. Centrifuge for 10 min at 7,500 g.
5. Discard the supernatant.
6. Resuspend the pellet in 180 µl enzymatic lysis buffer (DNeasy® Blood and Tissue kit, QIAGEN).
7. Use the Qiacube and the DNeasy® Blood and Tissue kit protocol for enzymatic lysis of Gram + bacteria for automated purification.
8. Use the DNA templates directly in a PCR or store in –20ºC until needed.

8.2.3. Honey

Procedure:

1. Heat 5 ml of honey to 40ºC.
2. Mix thoroughly with an equal volume of PBS.
3. Centrifuge at 27,000 g for 20 minutes.
4. Discard the supernatant.
5. Resuspend the pellet in the manufacturer`s lysis buffer (DNeasy® Plant Mini Kit, QIAGEN).
   Follow the protocol for plant tissue (Mini Protocol).
6. Use the DNA templates directly in a PCR or store in -20ºC until needed.

8.3. PCR

When PCR is used solely for detecting the presence or absence of a specific DNA signature, it is referred to as qualitative PCR (yes or no answer). The qualitative PCR detects only the end product whereas the real-time PCR detects the amplicon as it accumulates and determines the number of new DNA molecules formed in each reaction. The amount of the target molecule can be quantified (qPCR) either relatively or as absolute values or numbers (for further general information see the molecular methods paper of the *BEEBOOK* (Evans *et al.*, 2013)).

Four protocols for the detection and quantification of *M. plutonius* using PCR have been published to date (Table 2). Two protocols for qualitative PCR; one for detection in diseased larvae (Govan *et al.*, 1998) and a hemi-nested PCR assay (Djordjevic *et al.*, 1998).The latter method was further developed for the detection of *M. plutonius* in larvae, adult bees, honey and pollen (McKee *et al.*, 2003; see section 8.3.1). The results obtained indicate: 1. that the PCR assay is far more sensitive than culture; 2. that not all the *M. plutonius* detected is viable or amenable to culturing; and 3. that honey samples may be a useful tool for detecting sources of *M. plutonius.*

Real-time PCR assays for the quantification (qPCR) of *M. plutonius* (Roetschi *et al.*, 2008; Budge *et al.*, 2010) have been used to analyse pooled samples of brood nest workers from several colonies within an apiary as a suggested alternative to routine visual brood control (Roetschi *et al.*, 2008). However, more recent results suggest the amount of *M. plutonius* in adult bees provides a less stable estimate of the likelihood of finding disease than using larvae (Budge *et al.*, 2011). The qPCR method can also be used to attribute a risk of EFB infection to collected samples measured as probability of the sample showing clinical symptoms and providing a trigger for later inspection of apiaries at risk (Budge *et al.*, 2010; Grangier, 2010). This may provide a definitive diagnosis of EFB, based on a combination of the presence of clinical disease and the confirmed presence of *M. plutonius*. However, in some territories, the costs of such preliminary screening using real-time PCR may not be economically viable (Grangier, 2011).

8.3.1. Qualitative PCR

Procedure (after McKee *et al.*, 2003):

1. Genomic DNA (5-30 ng) is amplified using a thermal cycler in a 50 µl reaction comprising:
   - 4 mM $MgCl_2$,
   - 200 µM of each deoxyribonucleotide triphosphate,
   - 100 ng of primers MP1 and MP2 (Table 2),
   - 5 µl of 10 x PCR buffer (100 mM tris-HCl, pH 8.3; 500 mM KCL),
   - 2-5 µg of *Taq* DNA polymerase.
2. Conditions for amplification consist of:
   - initial denaturation at 95° C for 2 min,
   - 40 cycles of denaturation (95° C, 30 s),
   - primer annealing (61° C, 15 s),
   - primer extension (72° C, 60 s),
   - final extension cycle (72° C, 5 min).
3. Amplification products are analysed by electrophoresis (55 V, 1.5 h) through 1.0-1.5% (wt / vol) agarose containing ethidium bromide. A 486 bp PCR product is produced from primers MP1 and MP2. To ensure test specificity; a second PCR following the same protocol (using primers MP1 and MP3) is conducted, and a specific 276 bp hemi-nested product is amplified from the 486 bp template.

8.3.2. Quantitative PCR, qPCR

Procedure (after Budge *et al.,* 2010):

1. Genomic DNA is amplified in a 25 µl reaction comprising:
   - 1 x buffer A (Applied Biosystems),
   - 0.025 U/µl AmpliTaq Gold,
   - 0.2 mM each dNTP,
   - 5.5 mM $MgCl_2$,
   - 300 nM of each primer,
   - 100 nM probe,
   - 10 µl of nucleic acid extract.
2. PCR reactions are carried out in duplicate or triplicate wells and plates cycled using generic system conditions:
   - 95ºC for 10 min,
   - 40 cycles of 60ºC for 1 min,
   - 95ºC for 15 sec.

   in a 7900 Sequence Detection System (Applied Biosystems; Branchburg, New Jersey, USA) or equivalent with real-time data collection.
3. Quantification of *M. plutonius* in each sample can be achieved using the standard curve method (Anon, 1997) with assay EFBFor/EFBRev2/EFBProbe (*M. plutonius* 16S; Budge *et al.*, 2010; Table 2) as the target and assay AJ307465-955F/1016R/975T (*A. mellifera* 18S; Budge *et al.*, 2010) as the reference assay.
4. As fluorescence increases in the presence of the target, the change in fluorescence (DRn) enters an exponential phase. The quantification cycle (Cq) is defined as midway through the exponential phase of this amplification curve (Bustin *et al.*, 2009). It is often required to manually move the threshold of measurement manually to intercept midway through the

exponential phase of the amplification curve and obtain an appropriate Cq.

5. To account for variation in extraction efficiency between samples, the result can be expressed as a ratio of the number of *M. plutonius* and *A. mellifera* cells.

9. Exposure bioassays using *in vitro* rearing of larvae

Bioassays can be used to determine the biological activity of a substance by its effects on a test organism. Differences in virulence of a pathogen are best analysed in exposure bioassays, and such methods involving *in vitro* rearing of honey bee larvae (see the *in vitro* rearing paper of the *BEEBOOK* (Crailsheim *et al.*, 2013)) have been used for both *Paenibacillus larvae* and *M. plutonius* (McKee *et al.*, 2004; Genersch *et al.*, 2005; Giersch *et al.*, 2009). Virulence tests using this technique show that *M. plutonius* strains collected in different geographic places in Europe present important variations in the mortality rate and how fast the larvae die (Charrière *et al.*, 2011).

Three common measurement results can be obtained from exposure bioassays: the dose (LD_{50}) or concentration (LC_{50}) of the pathogen it takes to kill 50% of the hosts tested, and the time (LT_{50}) required for killing 50% of infected individuals. For the purpose of determining the LD_{50} or $LC_{50,}$ a reliable estimation of the concentration of bacterial cells used in the exposure bioassay is crucial.

9.1. Estimating the concentration of bacteria

The plate (viable) count method is an indirect measurement of bacterial cell density as it only detects live (or cultivable) bacteria whereas the microscopic (total) count includes all bacterial cells, cultivable or not (see the miscellaneous methods paper of the *BEEBOOK* (Human *et al.*, 2013)).

9.1.1. Plate count

The plate count method means diluting bacteria with a diluent solution (*e.g.* sterile saline) until the bacteria are dilute enough to count accurately when spread on a plate. The assumption is that each viable bacterial cell will develop into a single colony. Bacterial cell numbers need to be reduced by dilution, because more than 200 colonies on a standard 9 cm plate are likely to produce colonies too close to each other to be distinguished as distinct colony-forming units (CFUs).

The materials needed to perform a plate count are:

- Sterile 0.9% NaCl (sterile saline)
- Sterile tubes, tips and spreaders
- Agar plates (three per sample)

Procedure:

1. Make a ten-fold dilution serial dilution of your bacterial culture (broth). Dilute the suspension to a dilution factor of 10^{-6} (a million-fold dilution).
2. Spread out aliquots using a sterile bacterial spreader (0.1 ml) of each dilution onto 3 agar plates.
3. Incubate the plates for 4-7 days as previously described in section 5.3.
4. Count the number of bacterial colonies that appear on each of the plates that has between 30 and 200 colonies.
   Any plate which has more than 200 colonies is designated as "too numerous to count". Plates with fewer than 30 colonies do not have enough individuals to be statistically acceptable.
5. To compute the estimated number of bacteria on the surface that you tested, use the following formula: B = N/d
   where: B = number of bacteria; N = average number of colonies counted on three plates; d = dilution factor.

Example: Plate 1: 56 CFU; Plate 2: 75 CFU; Plate 3: 63 CFU; Average (N) = 64.7; Dilution (d) = 1/1,000; B = (64.7 x 1,000) = 64,700 bacteria in 0.1 ml, 647,000 bacteria per ml.

9.1.2. Total or microscopic count

A direct microscopic or total count is the enumeration of bacteria found within a demarcated region of a slide, a counting chamber. The slide is placed under a microscope, preferably with phase contrast. For counting bacteria, an oil immersion lens is usually required (1000 x magnification). For the procedure description refer to the section on hemocytometer counting in the miscellaneous methods paper of the *BEEBOOK* (Human *et al.*, 2013).

9.2. Protocol for inducing EFB infection in honey bee larvae reared in vitro

A protocol for inducing EFB involves grafting an individual larva (less than 24 hours old) into a single well in a micro-titer plate (for detailed protocols see the *in vitro* rearing paper of the *BEEBOOK* (Crailsheim *et al.*, 2013)). Older larvae may also become infected but are less susceptible. Each larva is fed 10 µl of larval diet (Crailsheim *et al.,* 2013) containing a defined number of *M. plutonius* cells (*e.g.* 500,000; see section 9.1.2). From 72 hours post grafting, the larvae are examined for mortality and fed uninfected feed daily, following the feeding regime recommended in Crailsheim *et al.* (2012). The mortality of the larvae can be evaluated using a microscope or by eye. Dead larvae are distinguished by the lack of respiration and loss of body elasticity.

The ability of *M. plutonius* to produce symptoms in the absence of secondary bacteria such as *P. alvei* seems to differ regionally. In Australia, feeding only *M. plutonius* has been demonstrated not to produce the typical clinical signs of EFB, but in Europe, *M. plutonius* was capable of inducing significant mortality in isolation (Charrière *et al.*, 2011). When infecting with *M. plutonius* in combination with *P. alvei*, the larval colour changes to a greyish brown rather than a yellowish colour and the gut content of infected larvae turns watery

rather than pasty. Infecting larvae with *M. plutonius* and subsequently feeding *P. alvei* (*e.g.* 60,000 spores in 10 µl larval diet) after 72 hours may produce signs typical of that seen in field cases of EFB (Giersch *et al.,* 2010). The simultaneous or 3 days delayed inoculation of *P. alvei* has, however, been demonstrated not to influence the virulence of some European strains of *M. plutonius* (Charrière *et al.*, 2011). The feeding of *P. alvei* in addition to *M. plutonius* has no influence on larval mortality as such, but may be important for the presence of all the typical EFB-symptoms, and the saprophyte *P. alvei* is probably important for the presence of some of the clinical symptoms in the field.

10. Measuring susceptibility / resistance to antibiotics of *Melissococcus plutonius*

Due to the fastidious culture requirements and slow growing nature of *M. plutonius*, measuring antibiotic susceptibility of this organism using traditional techniques such as a disc diffusion assay, which is a test that uses antibiotic - impregnated discs to determine whether particular bacteria are susceptible to specific antibiotics, is not possible. Oxytetracycline hydrochloride (OTC) is the antibiotic of choice for the treatment of EFB. However, only two reports of the sensitivity of *M. plutonius* to this antibiotic have been published (Waite *et al.*, 2003; Hornitzky and Smith, 1999) and both these studies indicated that all strains tested were sensitive to OTC. In both studies an agar plate method was used. This involves incorporating antibiotic at decreasing concentrations into culture plates (see section 5) of EFB culture medium, to determine the lowest concentration at which growth would occur. This methodology would be suitable for testing the susceptibility of *M. plutonius* to other antibiotics.

11. Conclusions

The pathogenic mechanisms of EFB are poorly understood, and the factors and timescales leading to overt symptomatology remain enigmatic. Molecular tools will open new possibilities for the identification of putative virulence factors in both the bacterium as well as the host in order to unravel some of the pathogenic mechanisms. To date, there are no published methods for genotyping and molecular differentiation of *M. plutonius* strains, but the nucleotide sequence of the bacterial genome was recently deposited in the DNA Database of Japan under accession no. AP012200 and AP012201 (Okomura *et al.*, 2011), and it is likely that new molecular methods such as genotyping will be developed in the near future. Moreover, research fields and methods already in use for research on *P. larvae* such as selection of reference genes, quantifying and knocking down gene expression (see designated parts in the American foulbrood and molecular protocols papers of the *BEEBOOK* (deGraaf *et al.*, 2013; Evans *et al.*, 2013)) could be adapted to *M. plutonius* and EFB research. Moreover, new technologies may also be useful tools to study interactions between secondary bacteria and the causative agent and to fully understand their role in symptomatology.

Molecular diagnostic methods such as PCR are also widely employed for EFB diagnosis. The PCR method is user-friendly and theoretically, a single target DNA molecule is sufficient for detection, making it one of the most sensitive biological techniques ever described. Considering this, we might ask whether a positive PCR result is always biologically relevant. Low levels of *M. plutonius* can be found in apiaries where no symptoms of disease are present and the PCR will also detect non-viable bacterial cells. However, it is clear that *M. plutonius* is still below the level of detection in honey bee colonies located in some geographical areas (Budge *et al.*, 2010). Future work should help understand whether this observation is due to the genetics of the honey bees from these areas, unfavourable meteorological conditions, lower apiary density, gut microbiota unfavourable to disease development, or simply down to an absence of movement of the causative organism.

Infectivity tests causing disease at the colony level using both cultured *M. plutonius* and extracts from diseased larvae were carried out during the 1930s (Tarr, 1936) and the 1960s (Bailey, 1960; Bailey, 1963; Bailey and Locher, 1968), but not much has been published since. This is an area of research where new information can be obtained by a combination of colony level infection experiments and modern diagnostic methods. Such advances would benefit from cross country collaborations, where advanced diagnostics from one country may complement field trials in another country where there may be less stringent rules governing EFB control.

12. References

ANON (2012) *User Bulletin #2: ABI PRISM 7700 Sequence Detection*, December 11, updated 10/2001:1-36.

ARAI, R; TOMINAGA, K; WU, M; OKURA, M; ITO, K; OKAMURA, N; ONISHI, H; OSAKI, M; SUGIMURA, Y; YOSHIYAMA, M; TAKAMATSU, D (2012) Diversity of *Melissococcus plutonius* from honey bee larvae in Japan and experimental reproduction of European foulbrood with cultured atypical isolates. *PLoS ONE* 7 (3): e33708. http://dx.doi.org/10.1371/journal.pone.0033708

BAILEY, L (1957) The isolation and cultural characteristics of *Streptococcus pluton* and further observations on "*Bacterium eurydice*". *Journal of General Microbiology* 17(2): 39-48.

BAILEY, L (1960) The epizootiology of European foulbrood of the larval honey bee, *Apis mellifera* Linneaus. *Journal of Insect Pathology* 2: 67-83.

BAILEY, L (1963) The pathogenicity for honey bee larvae of microorganisms associated with European foulbrood. *Journal of Insect Pathology* 5: 198-205.

BAILEY, L (1983) *Melissococcus pluton*, the cause of European foulbrood of honey bees (*Apis* spp.). *Journal of Applied Bacteriology* 55(1): 65-69.

BAILEY, L; COLLINS, M D (1982) Reclassification of '*Streptococcus pluton*' (White) in a new genus *Melissococcus,* as *Melissococcus pluton* nom. rev.; comb. nov. *Journal of Applied Bacteriology* 53 (2): 215-217.

BAILEY, L; GIBBS, A J (1962) Cultural characters of *Streptococcus pluton* and its differentiation from associated *Enterococci*. *Journal of General Microbiology* 28: 385-391.

BAILEY, L; LOCHER, N (1968) Experiments on the etiology of European foulbrood of the honey bee. *Journal of Apicultural Research* 7: 103-107.

BUDGE, G E; JONES, B; POWELL, M; ANDERSON, L; LAURENSSON, L; PIETRAVALLE, S; MARRIS, G; HAYNES, E; THWAITES, R; BEW, J; WILKINS, S; BROWN, M A (2011) Recent advances in our understanding of European Foulbrood in England and Wales. In *Proceedings of the COLOSS Workshop; The future of brood disease research – guidelines, methods and development, Copenhagen, Denmark, 10-12 April, 2011*, p 7.

BUDGE, G E; BARRETT, B; JONES, B; PIETRAVALLE, S; MARRIS, G; CHANTAWANNAKUL, P; THWAITES, R; HALL, J; CUTHBERTSON, A G S; BROWN, M A (2010) The occurrence of *Melissococcus plutonius* in healthy colonies of *Apis mellifera* and the efficacy of European foulbrood control measures. *Journal of Invertebrate Pathology* 105: 164-170. http://dx.doi.org/10.1016/j.jip.2010.06.004

BUSTIN, S A; BENES, V; GARSON, J A; HELLEMANS, J; HUGGETT, J; KUBISTA, M; MUELLER, R; NOLAN, T; PFAFFL, M W; SHIPLEY, G L; VANDESOMPELE, J; WITTWER, C T (2009) The MIQE guidelines: Minimum information for publication of quantitative real-time PCR experiments. *Clinical Chemistry* 55: 611-622. http://dx.doi.org/10.1373/clinchem.2008.112797

CHARRIÈRE, J-D; KILCHENMANN, V; ROETSCHI, A (2011) Virulence of different *Melissococcus plutonius* strains on larvae tested by an *in vitro* larval test; In *Proceedings of the 42nd International Apicultural Congress, Buenos Aires 2011*: p 158.

CRAILSHEIM, K; BRODSCHNEIDER, R; AUPINEL, P; BEHRENS, D; GENERSCH, E; VOLLMANN, J; RIESSBERGER-GALLÉ, U (2013) Standard methods for artificial rearing of *Apis mellifera* larvae. In *V Dietemann; J D Ellis; P Neumann (Eds) The COLOSS* BEEBOOK*, Volume I: standard methods for* Apis mellifera *research*. *Journal of Apicultural Research* 52(1): http://dx.doi.org/10.3896/IBRA.1.52.1.05

DAHLE, B; SØRUM, H; WEIDEMANN, J E (2011) European foulbrood in Norway: How to deal with a major outbreak after 30 years absence. In *Proceedings of the COLOSS Workshop: The future of brood disease research – guidelines, methods and development, Copenhagen, Denmark, 10-12 April, 2011*, p 8.

DE GRAAF, D C; ALIPPI, A M; ANTÚNEZ, K; ARONSTEIN, K A; BUDGE, G; DE KOKER, D; DE SMET, L; DINGMAN, D W; EVANS, J D; FOSTER, L J; FÜNFHAUS, A; GARCIA-GONZALEZ, E; GREGORC, A; HUMAN, H; MURRAY, K D; NGUYEN, B K; POPPINGA, L; SPIVAK, M; VANENGELSDORP, D; WILKINS, S; GENERSCH, E (2013) Standard methods for American foulbrood research. In *V Dietemann; J D Ellis; P Neumann (Eds) The COLOSS* BEEBOOK*, Volume II: standard methods for* Apis mellifera *pest and pathogen research*. *Journal of Apicultural Research* 52(1): http://dx.doi.org/10.3896/IBRA.1.52.1.11

DJORDJEVIC, S P; NOONE, K; SMITH, L; HORNITZKY, M A Z (1998) Development of a hemi-nested PCR assay for the specific detection of *Melissococcus pluton*. *Journal of Apicultural Research* 37: 165-174.

EVANS, J D; CHEN, Y P; CORNMAN, R S; DE LA RUA, P; FORET, S; FOSTER, L; GENERSCH, E; GISDER, S; JAROSCH, A; KUCHARSKI, R; LOPEZ, D; LUN, C M; MORITZ, R F A; MALESZKA, R; MUÑOZ, I; PINTO, M A; SCHWARZ, R S (2013) Standard methodologies for molecular research in *Apis mellifera*. In *V Dietemann; J D Ellis; P Neumann (Eds) The COLOSS* BEEBOOK*, Volume I: standard methods for* Apis mellifera *research*. *Journal of Apicultural Research* 52(4): http://dx.doi.org/10.3896/IBRA.1.52.4.11

FORSGREN, E (2010) European foulbrood in honey bees. *Journal of Invertebrate Pathology* 103(Suppl. 1): S5-S9. http://dx.doi.org/10.1016/j.jip.2009.06.016

FORSGEN, E; LUNDHAGEN, A C; IMDORF, A; FRIES, I (2005) Distribution of *Melissococcus plutonius* in honey bee colonies with and without symptoms of European foulbrood. *Microbial Ecology* 50: 369-374. http://dx.doi.org/10.1007/s00248-004-0188-2

GENERSCH, E; ASHIRALIEVA, A; FRIES, I (2005) Strain and genotype-specific differences in virulence of *Paenibacillus larvae* subsp. *larvae*, a bacterial pathogen causing American foulbrood disease in honey bees. *Applied and Environmental Microbiology* 71: 7551-7555. http://dx.doi.org/10.1128/AEM.71.11.7551-7555.2005

GENERSCH, E; GISDER, S; HEDTKE, K; HUNTER, W B; MÖCKEL, N; MÜLLER, U (2013) Standard methods for cell cultures in *Apis mellifera* research. In *V Dietemann; J D Ellis; P Neumann (Eds) The COLOSS* BEEBOOK*, Volume I: standard methods for* Apis mellifera *research*. *Journal of Apicultural Research* 52(1): http://dx.doi.org/10.3896/IBRA.1.52.1.02

GOVAN, V A; BROZEL, V; ALLSOPP, M H; DAVIDSON, S (1998) A PCR detection method for rapid identification of *Melissococcus pluton* in honey bee larvae *Applied and Environmental Microbiology* 64 (5): 1983-1985.

GRANGIER, V (2011) Early detection of European foulbrood using real-time PCR. PhD thesis, Vetsuisse faculty, University of Bern, Switzerland.

HORNITZKY, M A Z; SMITH, L (1999) The sensitivity of Australian *Melissococcus pluton* isolates to oxytetracycline hydrochloride. *Australian Journal of Experimental Agriculture* 39(7): 881-883.

HORNITZKY, M A Z; WILSON; S (1989) A system for the diagnosis of the major bacterial brood diseases. *Journal of Apicultural Research* 28: 191-195.

HUMAN, H; BRODSCHNEIDER, R; DIETEMANN, V; DIVELY, G; ELLIS, J; FORSGREN, E; FRIES, I; HATJINA, F; HU, F-L; JAFFÉ, R; KÖHLER, A; PIRK, C W W; ROSE, R; STRAUSS, U; TANNER, G; TARPY, D R; VAN DER STEEN, J J M; VEJSNÆS, F; WILLIAMS, G R; ZHENG, H-Q (2013) Miscellaneous standard methods for *Apis mellifera* research. In *V Dietemann; J D Ellis; P Neumann (Eds) The COLOSS* BEEBOOK*, Volume I: standard methods for* Apis mellifera *research*. *Journal of Apicultural Research* 52(4): http://dx.doi.org/10.3896/IBRA.1.52.4.10

McKEE, B A; DJORDJEVIC, S P; GOODMAN, R D; HORNITZKY, M A Z (2003) The detection of *Melissococcus pluton* in honey bees (*Apis mellifera*) and their products using a hemi-nested PCR. *Apidologie* 34: 19-27. http://dx.doi.org/10.1051/apido: 2002047

McKEE, B A; GOODMAN, R D; HORNITZKY, M A Z (2004) The transmission of European foulbrood (*Melissococcus plutonius*) to artificially reared honey bee larvae (*Apis mellifera*). *Journal of Apicultural Research* 43: 93-100.

OIE (2008) Chapter 9.3. European foulbrood of the honey bees. In *OIE Manual of diagnostic tests and vaccines for terrestrial animals (mammals, birds and bees) (6th Ed.), Vol. 1.* OIE; Paris, France. pp 405-409.

OIE (2011) Chapter 9.3. European foulbrood. In *OIE Terrestrial animal health code, vol. 2 (20th Ed.).* OIE; France. pp 507-508.

OKOMURA, K; ARAI, R; OKURA, T; TAKAMATSU, D; OSAKI, M; MIYOSHI-AKIYAMA, T (2011) Complete genome sequence of *Melissococcus plutonius* ATCC 35311. *Journal of Bacteriology* 193: 4029-4030. http://dx.doi.org/10.1128/JB.05151-11

PINNOCK, D E; FEATHERSTONE, N E (1984) Detection and quantification of *Melissococcus pluton* infection in honey bee colonies by means of enzyme-linked immunosorbent assay. *Journal of Apicultural Research* 23: 168-170.

ROETSCHI, A; BERTHOUD, H; KUHN, R; IMDORF, A (2008) Infection rate based on quantitative real-time PCR of *Melissococcus plutonius*, the causal agent of European foulbrood, in honey bee colonies before and after apiary sanitation. *Apidologie* 39: 362-371. http://dx.doi.org/10.1051/apido: 200819

TARR, H L A (1936) Studies on European foulbrood of bees II. The production of the disease experimentally. *Annals of Applied Biology* 23: 558-584.

TOMKIES, V; FLINT, J; JOHNSSON, G; WAITE, R; WILKINS, S; DANKS, C; WATKINS, M; CUTHBERTSON, A G S; CARPANA, E; MARRIS, G; BUDGE, G; BROWN, M A (2009) Development and validation of a novel field test kit for European foulbrood. *Apidologie* 40(1): 63-72. http://dx.doi.org/10.1051/apido:2008060

WAITE, R; JACKSON, S; THOMPSON, H (2003) Preliminary investigations into possible resistance to oxytetracycline in *Melissococcus plutonius*, a pathogen of honey bee larvae. *Letters in Applied Microbiology* 36: 20-24. http://dx.doi.org/10.1046/j.1472-765X.2003.01254.x

WILKINS, S; BROWN, M A, CUTHBERTSON, A G S (2007) The incidence of honey bee pests and diseases in England and Wales. *Pest Management Science* 63: 1062-1068. http://dx.doi.org/10.1002/ps.1461