A scientific note on improved isolation methods for *Melissococcus plutonius* from diseased *Apis mellifera* larvae

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European foulbrood (EFB) caused by *Melissococcus plutonius* is a significant infectious disease of honey bee brood. EFB is typically diagnosed based on the visual inspection of brood combs and detection of diseased larvae; however, since symptoms of EFB may easily be confused with other diseases or abnormalities in larvae, verification of the presence of *M. plutonius* in diseased larvae is required for a reliable diagnosis of EFB in many cases. The isolation and storage of *M. plutonius* strains are also important for future EFB studies. However, difficulties are sometimes associated with the isolation of *M. plutonius* from diseased larvae by culture methods due to the growth of secondary bacteria associated with EFB.

In an inspection of foulbrood on May 16, 2017, an abnormal *Apis mellifera* colony was detected in an apiary in Saitama prefecture, Japan. The colony showed irregular capping, and dead larvae were observed in uncapped cells. Gram-positive cocci were detected in dead larvae by microscopy. Furthermore, an *M. plutonius*-specific DNA fragment was found in dead larvae by PCR (Arai et al. 2014); however, due to the vigorous growth of

Enterococcus faecalis, a common secondary invader of EFB, on agar medium in anaerobic conditions, it was not possible to isolate *M. plutonius* from larvae using culture methods in the first examination in the laboratory. To prevent the growth of some secondary bacteria from larvae samples, the addition of nalidixic acid to agar medium at a final concentration of 3 µg/ml has been suggested (Forsgren et al. 2013); however, since nalidixic acid does not work on *E. faecalis* (Hornitzky and Smith 1998), it was not suitable for the selective isolation of *M. plutonius* in this case. Thus, in order to eliminate secondary bacteria from the samples, we spread out homogenates of dead larvae on glass slides, allowed the smears to dry, and stored them for 1 month in the laboratory (temperature, approximately 20–35 °C) by referring to the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.02.03_EUROPEAN_FOULBROOD.pdf). Viable *E. faecalis* cells decreased during storage, and *M. plutonius* was isolated from the smears (Online Resource 1). These results confirmed the usefulness of this method for the isolation of *M. plutonius* from larvae contaminated with secondary bacteria; however, since it was a time-consuming method, we herein conducted two experiments using the same larvae samples and devised time-saving and time-efficient methods to isolate *M. plutonius* from enterococci-contaminated samples.

In the first experiment to investigate the minimum storage time required to eliminate *E. faecalis*, we prepared dried smears of dead larvae as described above

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and stored them for 1, 3, 7, 10, 14, and 21 days in the laboratory (temperature, approximately 25–35 °C). After storage, each smear was mixed with 50 µl of sterilized saline on glass slides, and bacteria that survived in the smears were isolated by streaking 10 µl of the saline and smear mixture on KSBHI agar plates (Arai et al. 2012) and incubating the plates at 37 °C under anaerobic conditions. Isolated bacteria were identified using *M. plutonius*-specific PCR (Arai et al. 2014). As summarized in Table 1, *Enterococcus*-like bacteria from the smear stored for only 1 day vigorously grew on KSBHI agar, and *M. plutonius*-like colonies were not observed. However, viable *Enterococcus*-like bacteria markedly decreased in samples stored for 3 or more days, and *M. plutonius* was predominantly or purely isolated from the samples (Table 1 and Online Resource 2), suggesting that storage for 3 days is sufficient to reduce *Enterococcus* from dried smears of larvae samples.

Takamatsu et al. (2017) recently showed that *M. plutonius* strains were more resistant to the antimicrobial activity of royal jelly (RJ) than other bacterial strains including an *E. faecalis* strain. In the previous study,

most of the *M. plutonius* strains tested were not killed completely within 24 h, even in 50% RJ-containing broth, whereas viable *E. faecalis* cells became undetectable in 6.25–12.5% RJ-containing broth after a 24-h incubation at 35 °C. These findings prompted us to employ the antimicrobial activity of RJ to eliminate secondary invaders, and in the second experiment, larval homogenates were incubated in 0, 6.25, 12.5, 25, 50, and 75% RJ-containing KSBHI broth at 37 °C for 24 h. After the incubation, bacteria that survived in the RJ-containing broth were isolated by spreading 200 µl of the broth on KSBHI agar plates and incubating the plates at 37 °C under anaerobic conditions.

Enterococcus-like bacteria vigorously grew from samples incubated in broth without RJ (0%) and that containing 6.25 and 12.5% RJ, and *M. plutonius* was not isolated from the samples (Table 1). Conversely, *M. plutonius* was predominantly or purely isolated from samples incubated in 25, 50, and 75% RJ-containing broth (Table 1

Table 1. Isolation of *M. plutonius* from secondary invader-contaminated larvae

Pretreatment method for samples ^a		Isolation ^b	
		<i>M. plutonius</i> ^c	<i>Enterococcus</i> -like bacteria
Storage of dried larval smears for	1 day	–	+
	3 days	+ ^s	+ ^s
	7 days	+	–
	10 days	+	+ ^s
	14 days	+	+ ^s
	21 days	+	–
24-h incubation of larval samples at 37 °C in KSBHI broth	without RJ	–	+
	with 6.25% RJ	–	+
	with 12.5% RJ	–	+
	with 25% RJ	+	+ ^s
	with 50% RJ	+	–
	with 75% RJ	+	–
Inoculation of larval homogenates on KSBHI agar with 3 µg/ml NA		–	+

^a RJ, royal jelly; NA, nalidixic acid. Fresh RJ used in this study was purchased from Yamada Bee Farm (Tomata, Okayama, Japan) and stored in a freezer at ≤ – 80 °C until used. The absence of *M. plutonius* in RJ used in this study was confirmed by culturing RJ on KSBHI agar under anaerobic conditions. Each pretreated sample was inoculated onto a single KSBHI agar plate for the isolation of bacteria

^b +, Innumerable colonies of the corresponding bacteria appeared on KSBHI agar; +^s, Less than 200 colonies appeared on KSBHI agar; –, No colonies of the corresponding bacteria were observed on KSBHI agar (Online Resources 2 and 3)

^c *M. plutonius*-like colonies grown on KSBHI agar were identified as *M. plutonius* by species-specific PCR (Arai et al. 2014)

and Online Resource 3). These results demonstrated for the first time that the incubation of larval homogenates in high-concentration RJ-containing broth is effective for the selective isolation of *M. plutonius* from enterococci-contaminated larvae.

In the present study, we found that a 3-day storage at room temperature was sufficient to reduce enterococci from dried larval smears and that the antimicrobial activity of RJ was useful for the selection of *M. plutonius* from larvae containing enterococci. RJ only needed 24 h to select *M. plutonius*. In this case, the growth of *Enterococcus*-like bacteria was not inhibited on 3 µg/ml nalidixic acid-containing KSBHI agar (Table 1) which confirms the report of Hornitzky and Smith (1998). However, since this antibiotic is expected to be effective against other secondary bacteria like *Paenibacillus alvei*, the incubation of larval homogenates in RJ-containing broth and subsequent cultivation of samples on nalidixic acid-containing agar media may be more effective for the isolation of *M. plutonius* from EFB cases.

It is important to note that the *M. plutonius* strains analyzed to date have been grouped into three clonal complexes (CC3, CC12, and CC13) by multilocus sequence typing (Haynes et al. 2013; Budge et al. 2014; Takamatsu et al. 2014). Among these groups, CC3 strains exhibited the strongest resistance to the antimicrobial activity of RJ (Takamatsu et al. 2017). Since the causative *M. plutonius* strain in this EFB case was sequence type 3 of CC3, the strain may be efficiently selected by RJ in this study. In addition, the degree of antimicrobial activity of RJ may be different according to the origins (Takamatsu et al. 2017). Therefore, further studies using larvae samples of other EFB cases and RJ of different origins are needed in order to verify the usefulness of the selective isolation method for *M. plutonius* proposed in this study.

AUTHORS' CONTRIBUTIONS

YN and MI: Diagnosed the EFB case described in this study and performed experiments; RA: Designed the first experiment; DT: Designed the second experiment and contributed to writing the manuscript. All authors read and approved the final manuscript.

COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interest The authors declare that they have no conflict of interest.

Une note scientifique sur l'amélioration des méthodes d'isolement pour *Melissococcus plutonius* de larves malades d'*Apis mellifera*

Eine wissenschaftliche Notiz über eine verbesserte Methode zur Isolierung von *Melissococcus plutonius* aus kranken *Apis mellifera* Larven

REFERENCES

- 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 honeybee larvae in Japan and experimental reproduction of European foulbrood with cultured atypical isolates. *PLoS One* 7 (3), e33708
- Arai, R., Miyoshi-Akiyama, T., Okumura, K., Morinaga, Y., Wu, M., Sugimura, Y., Yoshiyama, M., Okura, M., Kirikae, T., Takamatsu, D. (2014) Development of duplex PCR assay for detection and differentiation of typical and atypical *Melissococcus plutonius* strains. *J. Vet. Med. Sci.* 76 (4), 491–498
- Budge, G. E., Shirley, M. D. F., Jones, B., Quill, E., Tomkies, V., Feil, E. J., Brown, M. A., Haynes, E. G. (2014) Molecular epidemiology and population structure of the honey bee brood pathogen *Melissococcus plutonius*. *ISME J.* 8 (8), 1588–1597
- Forsgren, E., Budge, G. E., Charrière J.-D., Hornitzky, M. A. Z. (2013) Standard methods for European foulbrood research. *J. Apic. Res.* 52 (1), 1–14
- Haynes, E., Helgason, T., Young, J. P. W., Thwaites, R., Budge, G. E. (2013) A typing scheme for the honeybee pathogen *Melissococcus plutonius* allows detection of disease transmission events and a study of the distribution of variants. *Environ. Microbiol. Rep.* 5 (4), 525–529
- Hornitzky, M. A. Z., Smith, L. (1998) Procedures for the culture of *Melissococcus plutonius* from diseased brood and bulk honey samples. *J. Apic. Res.* 37 (4): 293–294
- Takamatsu, D., Morinishi, K., Arai, R., Sakamoto, A., Okura, M., Osaki, M. (2014) Typing of *Melissococcus plutonius* isolated from European and Japanese honeybees suggests spread of sequence types across borders and between different *Apis* species. *Vet. Microbiol.* 171 (1–2), 221–226
- Takamatsu, D., Osawa, A., Nakamura, K., Yoshiyama, M., Okura, M. (2017) High-level resistance of *Melissococcus plutonius* clonal complex 3 strains to antimicrobial activity of royal jelly. *Environ. Microbiol. Rep.* 9 (5), 562–570