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Improvement and Evaluation of Culture Media to Detect *Paenibacillus larvae*, the Causative Agent of American Foulbrood (AFB)

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Abstract

American Foulbrood (AFB) is a devastating bee disease, caused by the bacterium Paenibacillus larvae, *and due for notification to responsible authorities. Brood of* Apis mellifera *gets infected after incorporation of low amounts of* P. larvae *spores and die after infection. Veterinarians and beekeepers can identify typical symptoms of diseased colonies. Still, from infection of a colony until the outbreak of AFB several years can remain, which in the bacterium can spread into other colonies. Microbiological methods allow detecting infections before outbreaks and to safe these colonies by beekeeping procedures. However, samples of food or honey are needed as a source to detect the spores of the causative agent. These matrices are native samples, that harbour a lot of ubiquitous contaminants, which may lead to un-analyzable samples. We present an easy microbiological method, using a different commercially available formulation of Columbia sheep blood agar, to decrease the number of un-analyzable samples.*

Keywords — *Paenibacillus larvae, American Foulbrood, Microbiology, Cultivation, Germination, Contamination.*

I. INTRODUCTION

The western honeybee *Apis mellifera* is one of the most important animals for humanity because of its role in pollination [1]. Like all animals, the honeybee can be infected by different pathogens, like mites (i.e. *Varroa destructor*), fungi (i.e. *Ascosphera APIs*), viruses *(*i.e. Deformed Wing Virus) and bacteria. There are still two bacteria, which are known to infect the brood of *A. mellifera*. *Melissococcus plutonius* causes European Foulbrood, a less important brood disease, whereas *Paenibacillus larvae* cause American Foulbrood (AFB) [2]. *P. larvae* can be subdivided into five genotypes (ERIC I-V) [3, 4, 5], that differ in their virulence and pathomechanisms [6, 7, 8, 9]. The genotypes ERIC I and II can be isolated from AFB outbreaks all over the world. In contrast, ERIC III-V are only known from a low number of isolates without knowledge about the colonies status [2, 5]. Only first instar larvae of *A. mellifera* are susceptible to infection with *P. larvae* by the incorporation of low spore amounts [2]. Infected larvae are killed during infection and lysed into a glue-like liquid. After drying out, the remains form a scale on the brood cells bottom, which only consists of *P. larvae*-spores. However, these spores can spread through the colony and harbour the risk of enormous further infections [2]. Because of its high risk to spread between apiaries, AFB is under due for notification in most countries.

The forming of AFB-typical symptoms depends on the responsible genotype and its' LT_{100} [7, 8, 10]. The incubation time of AFB also depends on different factors, like colony strength and condition. Still, in most cases, several years are needed from the infection until clinical symptoms can be detected. Moreover, the optical inspection of a colony represents only a momentary value. State of the art is the detection of *P. larvae*-spores in samples of food or honey [11]. However, this method allows to detect infections of honeybee colonies with *P. larvae* in early stages and to quantify the amounts of spores [11, 12, 13]. By the number of spores, it can be assumed if an AFB hotspot infected the colony, or if this colony may be in the clinical phase and spread AFB to other apiaries.

Samples, like food or honey, out of a colony, represent a heterogeneous kind of matrix, which has to be processed for analysis [11, 12, 13]. These samples may include different fungi (i.e. *Aspergillus* spec.) or bacteria (i.e. *Bacillus licheniformis*), which can persist the sample procession by its spores. Nevertheless, the detection of *P. larvae* spores from heterogeneous matrices includes a cultivation step, to germinate into colonies on complex synthetic media. Cultivation is performed at approximately 37 °C for at least 6 days under aerobic, or microaerophilic conditions. Unfortunately, numerous microorganisms can grow under this condition on complex media. These circumstances lead to the growth of unspecific microorganisms and not available results in the diagnostic detection of *P. larvae*. To decrease the number of un-analyzable samples, we tested different commercial available Columbia sheep blood agar formulations. Finally, we are going to adapt our routine procedure to increase efficiency and explanatory power of our laboratory.

II. MATERIAL AND METHODS

Bacterial strains and spiked honey samples. Honey samples, without spores of *P. larvae*, were used as a basis for spiking them with spore-containing honey and described elsewhere [14]. Two samples (166 and 746) were spiked with different amounts auf *P. larvae* genotype ERIC I and two samples (348 and 599) were spiked with genotype ERIC II. For each genotype, a sample with lower (ERIC I: 166; ERIC II: 348) and higher (ERIC I: 746; ERIC II: 599) spore amount was prepared. The commercially available standard spore solution "RSK16" (charge RSK 16-03-19) [LAVES - Institute of Apiculture, Germany], representing genotype ERIC II (DSM17237), was used as positive control without any unspecific microorganisms, surviving the typical sample preparation. As a negative control, Ambrosia [Nordzucker AG, Germany] was sued. Additionally, a honey sample without spores of *P. larvae* (259) was analyzed.

Sample procession and cultivation conditions. Three preparations (a-c) of each sample were processed according to authorized methods [11] by four different technicians each. Triplicates of 200 µL suspension of every preparation (a-c) were plated on four different media of Columbia sheep blood agar. Three media were obtained as fix plates from BD [Becton Dickinson GmbH, Germany], representing *BD Columbia Agar with 5% Sheep Blood* (BD-CSA), *BD Columbia CNA Agar with 5% Sheep Blood*, including colistin (10.0 mg/L) and nalidixic acid (10.0 g/L) (BD-CNA) and *BD Columbia III Agar with 5% Sheep Blood*, containing growth activators (BD-CSA-III). A fourth medium was prepared of 35.2 g Columbia agar basis (Oxoid, UK) and 900 mL ddH_2O. After autoclaving, 50 mL of defibrinated sheep blood (WDT, Germany) were added, and plates were cast (Oxoid-CSA). Plates were incubated for 6 days at 36 °C ±1 °C under aerobic conditions.

Analysis of potential *P. larvae* colonies. Plates were inspected after 3 and 6 days of incubation. Colonies, which showed typical morphological characteristics of *P. larvae* were counted [11, 12, 13]. If ≥200 cfu/plate were found, the results were counted as 200. Finally, after six days, colonies were randomly picked for mqPCR to verify and genotype *P. larvae* [15]. If unspecific microorganisms overgrew plates, they were evaluated as un-analyzable.

Analysis of unspecific microbiological growth. Single colonies of unspecific microorganisms were counted. In the case of covering areas ≥10%, their grown area was estimated [%]. Only plates with ≤90% area, grown with unspecific microorganisms were analyzed for cfu of *P. larvae*.

Statistical analysis. Data were digitalized and analyzed by using R (version x64 3.6.1) [R Core Team, Austria]. Significance was tested by unpaired Student *t*-test.

III. RESULTS AND DISCUSSION

The sensitivity of different media for standard spore solution RSK16. The standard spore solution RSK16 was used to define the plate's sensitivity for spores of *P. larvae*. Therefore, the mean of cfu ±SEM was calculated (Fig. 1). According to the results, we classified media with regular sensitivity (t_3: Oxoid-CSA=9.83 ±0.73, BD-CSA-III=19.33 ±1.04; t_6: Oxoid-CSA= 26.03 ±1.05, BD-CSA-III= 29.47 ±0.92) and media with increased sensitivity (t_3: BD-CSA=86.28 ±2.13, BD-CNA=76.58 ±2.77; t_6: BD-CSA=90.50 ±2.01, BD-CNA=84.92 ±2.22). In the case of BD-CSA and BD-CNA, no significant differences could be detected (p=0.0664), but Oxoid-CSA and BD-CSA-II, I showed significant differences (p=0.0159). However, in Lower Saxony and some other states, the absolute number of cfu is only used to generate semi-quantitative information on spores amount, called category [16]. Since we perform the official analysis for Lower Saxony, Oxoid-CSA is used for the detection of *P. larvae* spores. Category 1 ("low") indicates the infection with *P. larvae* (food≤22cfu/plate, honey≤12 cfu/plate), representing the typical incubation time between infection with the pathogen and outbreak of AFB. In case of category 2 ("high"), the cfu reach a level in which normally typical symptoms of AFB can be found in affected colonies (food≥23 cfu/plate, honey≥13 cfu/plate) [16]. The standard spore solution RSK16 is prepared in a spore titer to show the scope between category 1 and 2. To get up with topical quality management systems, commercial ready-to-use available media (BD-CSA-III) should be preferred instead of house-made media (Oxoid-CSA) for reliable official analysis of animal diseases, even in case of complex media. Finally, the cfu level for category 1 and 2 on BD-CSA-III can be adapted by using the standard spore solution RSK16.

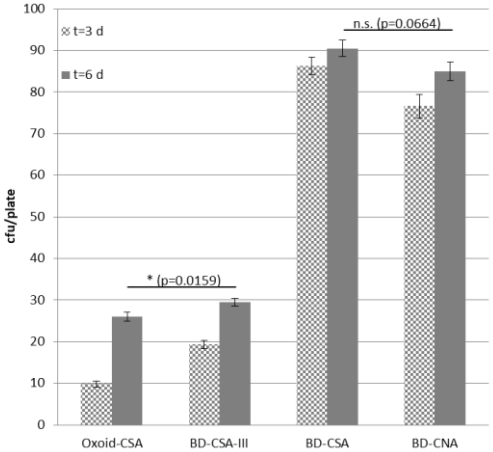

Fig. 1: Germination of standard spore solution RSK16 on different Columbia sheep blood agar.

The sensitivity of different media for spiked honey samples. To validate the results of the RSK16, honey samples were spiked with spores of different *P. larvae* genotypes and germination was evaluated by mean of cfu/plate ±SEM after 3 and 6 days. For

genotype ERIC I isolate 166 with a low spore titer (t_3: BD-CSA=17.97 ±1.87, BD-CNA=8.89 ±1.57, BD-CSA-III=5.44 ±0.46, Oxoid-CSA=5.44 ±0.43; t_6: BD-CSA=20.24 ±1.86, BD-CNA=10.61 ±1.59, BD-CSA-III=7.00 ±0.64, Oxoid-CSA=5.78 ±0.43) and isolate 746 with higher spore amount (t_3: BD-CSA=23.94 ±2.95, BD-CNA=9.97 ±2.37, BD-CSA-III=5.97 ±0.51, Oxoid-CSA=8.34 ±0.77; t_6: BD-CSA=27.33 ±3.01, BD-CNA=11.56 ±2.47, BD-CSA-III=7.41 ±0.61, Oxoid-CSA=10.09 ±0.81) were tested (Fig. 2). Compared to the results of RSK16, significant differences were obtained in the sensitivity between BD-CSA and BD-CNA after 6 days (p_{166}=0.002, p_{746}=0.0001). Moreover, Oxoid-CSA and BD-CSA-II, I differed significantly in their sensitivity after 6 days (p=0.0132), where this difference was not detected for sample 166 (p=0.1215). Taken together, the classified regular and increased sensitivity was also shown for the used honey samples, spiked with genotype ERIC I. Nevertheless, BD-CNA seems to influence the germination of ERIC I, compared to BD-CSA, which only differs in the supplementation of colistin and nalidixic acid. Several strains of *P. larvae* were tested against different antibiotics, including nalidixic acid [17]. *P. larvae* were shown to be not sensitive against this antibiotic [11,17]. Still, less information was found about colistin. Moreover, the concentration of nalidixic acid in BD_CNA is 10.0 g/L, whereas a concentration of 3.0 g/L is recommended for the isolation of *P. larvae* [11].

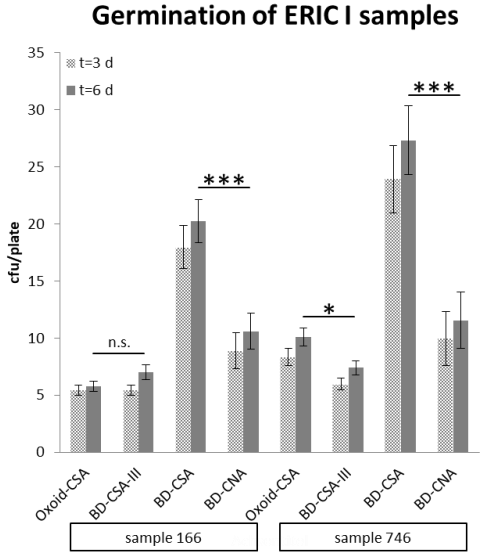

Fig. 2: Germination of used *P. larvae* ERIC I samples on different Columbia sheep blood agar. N.s., no significance (p>0.05); *, significant (0.05>p>0.01); ***, significant (p<0.001).

Genotype ERIC II was presented by isolate 348 with low spore amounts (t_3: BD-CSA=161.23 ±6.87, BD-CNA=187.22 ±3.94, BD-CSA-III=47.69 ±3.26, Oxoid-CSA=21.11 ±1.65; t_6: BD-CSA=187.91 ±5.09 [28x >200 cfu/plate], BD-CNA=200.00 ±0.00 [42x >200 cfu/plate], BD-CSA-III=58.23 ±3.40, Oxoid-CSA=34.11 ±3.31; 200 cfu/plate) and isolate 299 with higher titer (t_3: BD-CSA=195.71 ±3.16 [25x >200 cfu/plate], BD-CNA=200.00 ±0.00 [29x >200 cfu/plate], BD-CSA-III=131.39 ±6.94, Oxoid-CSA=68.35 ±2.97; t_6: BD-CSA-III=142.36 ±7.20 [4x >200 cfu/plate], Oxoid-CSA=90.29 ±3.82) (Fig. 3). Also for genotype ERIC II of *P. larvae* the different sensitivity between the used media was obtained. In contrast to ERIC I the Oxoid-CSA and BD-CSA-III differed significantly in the cfu of *P. larvae* (p_{348}<0.001, p_{599}=0.0031), while BD-CSA-III showed a higher cfu data. BD-CSA and BD-CNA only differed significantly in sample 348 (p_{348}=0.0393, p_{599}=0.0855).

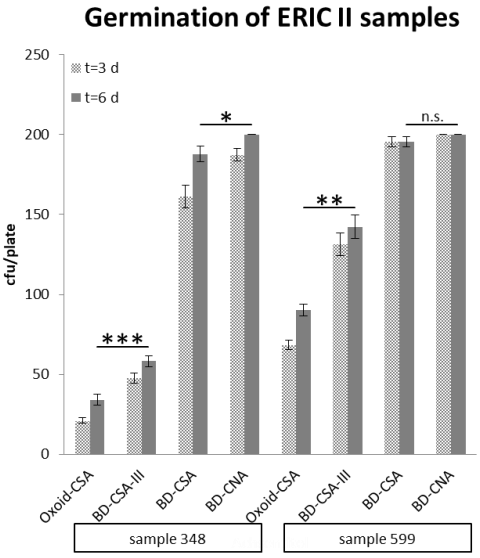

Fig. 3: Germination of used *P. larvae* ERIC II samples on different Columbia sheep blood agar. N.s., no significance (p>0.05); *, significant (0.05>p>0.01); **, 0.01<p<0.001; ***, significant (p<0.001).

Only one isolate was used for each ERIC genotype in the analyzed honey samples. Statistical analysis showed significant differences between BD-CSA and BD-CNA in ERIC II sample 599. Because of its origin [14], this difference cannot be caused by the *P. larvae* isolate or the used honey. Because of co-cultivated unspecific microorganisms, an influence of its quorum sensing [18] and secondary metabolites could be an explanation. In case of the used isolate, representing genotype ERIC I, a decrease of cfu was recorded, when using BD-CNA. However, besides the influence of colistin, strain-specific growth and germination conditions should be reassessed [17]. According to the genotypes differing pathomechanisms [7, 8], the ERIC genotypes can be subdivided into more detailed MLVA or MLST genotypes [19, 20], which could differ in their sensitivity and minimal inhibitory concentration of different antibiotics [17, 21, 22].

Influence on different media on ubiquitous microorganisms. Honey and food samples represent a heterogeneous matrix of environmental samples, which harbours numerous ubiquitous microorganisms.

These organisms have to be reduced efficiently to increase the analyzable number of samples. To characterize the different Columbia sheep blood agar, we analyzed the growth of unspecific microorganisms and calculated the ratio of un-analyzable plates (NA). Sample 259 resulted in 22.22% ±11.05% NA (BD-CSA=16.67%, BD-CNA=0.00%, BD-CSA-III=52.78%, Oxoid-CSA=19.44%). ERIC I sample 166 showed 2.08% ±1.33% NA (BD-CSA=2.78%, BD-CNA=0.00%, BD-CSA-III=0.00%, Oxoid-CSA=5.56%) and sample 746 5.56% ±3.76% NA (BD-CSA=2.78%, BD-CNA=0.00%, BD-CSA-III=16.67%, Oxoid-CSA=2.78%). Sample 348 resulted in 8.33% ±3.00% NA (BD-CSA=8.33, BD-CNA=0.00%, BD-CSA-III=13.89%, Oxoid-CSA=11.11%) and 599 in 13.19% ±5.24% NA (BD-CSA=16.67%, BD-CNA=0.00%, BD-CSA-III=25.00%, Oxoid-CSA=11.11%), while representing samples with spores of genotype ERIC II.

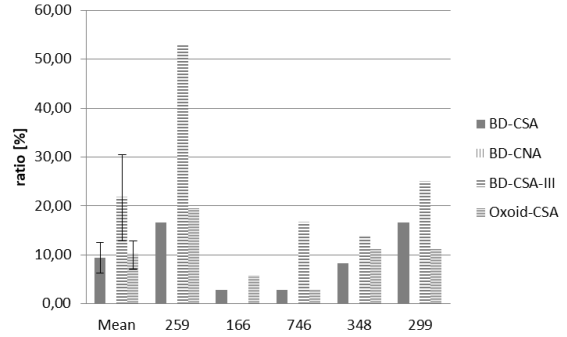

Fig. 4: Ratio of un-analyzable plates from used honey samples on different Columbia sheep blood agar.

BD-CSA and Oxoid-CSA showed a moderate number of un-analyzable plates, whereas the most NA was detected on BD-CSA-III. By the use of BD-CNA, all plates were analyzable. BD-CSA and Oxoid-CSA are based on the same complex medium, which leads to comparable growth conditions of *P. larvae* and unspecific bacteria that can be cultivated under the chosen conditions. BD-CSA-III is also based on Columbia sheep blood agar, but growth activators are added. These activators may increase the growth of unspecific microorganisms. *P. larvae* need a relatively long incubation time until spores germinate and bacterial colonies can be detected. If the mentioned growth factors increase the proliferation of other, less discerning microorganisms, it may lead to un-analyzable results. BD-CNA is based on Columbia medium and supplemented with two antibiotics, at which at least nalidixic acid is in a relatively high concentration. This may result in reduced growth of several microorganisms, resulting in 100% analyzable plates in this study. Nevertheless, growth of unspecific microorganisms was detected on all four used media, but the covered plate area differed (BD-CSA=48.95% ±0.36%, BD-CNA=22.22% ±0.72%, BD-CSA-III=60.17% ±0.35%, Oxoid-CSA=50.57% ±0.17%).

However, samples for *P. larvae* spiked honey were prepared from the same honey as basic, but they differed in the number of un-analyzable plates. Potential reasons for these differences might be founded in sample heterogeneity, as well as in quorum sensing and secondary metabolites of cultured unspecific microorganisms or by *P. larvae* itself.

IV. CONCLUSIONS

Four different formulations of Columbia sheep blood agar were tested, to increase the number of analyzable samples and to keep up with actual quality management guidelines. The tested media were divided into those with regular (BD-CSA-III, Oxoid-CSA) and increased (BD-CSA, BD-CNA) sensitivity for *P. larvae*.

In Lower Saxony and some other states, the spore amount is correlated with categories [12, 13, 16]. Oxoid-CSA is used to prepare the standard spore solution RSK16 and to differentiate between “low” and “high” spore titer. The use of BD-CSA-III results in quantifiable cfu/plate. Therefore, BD-CSA-III should be used as a ready-to-use alternative to Oxoid-CSA, while the cfu level to determine category 1 and 2 have to be adapted.

An increased sensitivity of culture media to detect discerning microorganisms (BD-CSA, BD-CNA) could be used to prevent false-negative results if samples with the very low amount of spores were analyzed only by media with regular sensitivity. Moreover, BD-CNA did not lead to any un-analyzable plates in this study. About 5% of samples cannot be analyzed because of unspecific microorganisms’ growth [23]. The use of BD-CNA could increase this number.

Taken together, we will improve our analysis for the detection of *P. larvae* in honey and food samples at the Lower Saxony State Office for Consumer Protection and Food Safety, Institute of Apiculture. BD-CSA-III will be used for the first triplicate to quantify the *P. larvae* spores’ category and to detect those isolates of target organism that might be sensitive against nalidixic acid or colistin. BD-CNA will be used for the second and third replicate to detect *P. larvae* in samples with very low spore titer and to reduce the number of un-analyzable samples. Moreover, both media will be supplied by one company (Becton Dickinson), which can supervise the production process under more standardized conditions.

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