Methods for Detection of *Bacillus* sp., *B. cereus*, and *B. licheniformis* in Raw Milk

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Abstract

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Totally 75 raw milk samples were analysed with the methods employing the media compared – MYPA, PEMBA, Brilliance™ *Bacillus cereus* agar, and HiCrome *Bacillus* agar. The reference method with MYPA seems to be the most suitable for dairy plants laboratories because there is only low risk of mistaken identity. However, the samples containing miscellaneous micro-flora should be heat-inactivated before plating. Both positive and negative strains (totally 132) were isolated. Twelve strains, which could cause problems in the evaluation of the plates, were selected and identified by phenotyping and by PCR methods for *Bacillus* sp., *B. cereus*, and *B. licheniformis*. The PCR methods differed in their selectivity within particular bacilli group, within genera *Bacillus*, and within raw milk microflora.

Keywords: cultivation method; chromogenic medium; genera-specific PCR; species-specific PCR

Genera *Bacillus* are formed by Gram-positive rods able to produce endospores resistant to unfavourable external conditions (Logan & De Vos 2009) that can be distinguished from other spore-formers (*Sporolactobacillus*, *Clostridium*, *Desulfotomaculum*, *Sporosarcina* or *Thermoactinomycetes*) due to their aerobic nature (strict or facultative), rod-shaped cells and catalase synthesis (Slepecky & Hemphill 2006).

Genera *Bacillus* are divided into 11 groups. This work is focused on *B. cereus* (group of *B. anthracis*, *B. cereus*, *B. mycoides*, *B. pseudomycoides*, *B. thuringiensis*, and *B. weihanstephanensis*) and *B. licheniformis* (group of *B. subtilis*, *B. amyloliquefaciens*, *B. atrophaeus*, *B. mojavensis*, *B. licheniformis*, *B. sonorensis*, and *B. vallismortis*) (Ludwig *et al.* 2009). Group of *B. cereus* (so-called *B. cereus sensu lato*) can be distinguished by the formation of intracellular crystallic toxin in *B. thuringiensis*, presence of rhizoids and loss of motility in *B. mycoides*, or sensitivity to penicillin in *B. anthracis* (Standhouders 1992). In 1998, psychrotolerant *B. cereus* strains, able to grow at 4–7°C but not at 40–43°C, were classified as separate species *B. weihanstephanensis* (Lechner *et al.* 1998).

B. cereus as well as *B. subtilis, B. licheniformis* and *B. pumilus* is an important food-borne pathogen (Kotiranta *et al.* 2000; Rowan *et al*. 2003). Another considerable characteristic important in dairy industry is the spoilage potential of these species, especially in manufacturing of pasteurised milk (Zhou *et al*. 2008), condensed sterilised milk

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(Clark 2001), dried baby foods (Becker *et al.* 1994), milk powders, and UHT milk (Chen *et al.* 2004). Thus, suitable methods for the detection and quantification of these micro-organisms in dairy samples are needed.

Most often, *Bacillus* sp. is detected and isolated by methods based on the resistance of spores to heating or ethanol. However, direct isolation of particular species requires a selective medium or other selective conditions that are available only for a few species. The cultivation methods for the determination of *B. cereus* – as tested in this work – are typical examples. These methods detect the failure to utilise mannitol, lecithinase activity or β-glucosidase activity, while other bacteria including some bacilli are inhibited by polymyxin B sulphate or trimethoprim (Logan & de Vos 2009). Other methods for the detection and identification of *B. cereus* are e.g. serotyping, pyrolytic gas chromatography, pyrolytic mass spectrometry, ribotyping, phage typing, plasmid profiles, electrophoresis in pulse electric field and polymerase chain reaction (PCR) using genera-specific and species-specific primers (Te Giffel *et al.* 2000).

The main task of this work is to compare the cultivation methods for the determination of *B. cereus* and PCR methods for *Bacillus* sp., *B. cereus*, and *B. licheniformis* as well as to recommend the suitable ones for the analyses of raw cow milk, especially in dairy plant laboratories.

MATERIAL AND METHODS

Micro-organisms. *B. subtilis* DMF 2006, *B. cereus* DMF 2007 and DMF 2008, *B. pumilus* DMF 2009, *B. mycoides* DMF 2010, *Lactobacilus sakei* DMF 2017 (Aplin & Barrett, Beaminster, UK), *Lb. rhamnosus* VT1 (Department of Dairy and Fat Technology, Institute of Chemical Technology, Prague, Czech Republic), *B. licheniformis* 1044-B, SPA 9 and 1646-B, and *B. cereus* 1867-C and SPA 12 (MILCOM Company Limited, Prague, Czech Republic) were used as positive and negative control strains for the PCR methods.

Analysis of raw milk. Raw milk samples and the same samples inactivated by heating at 85°C/10 min were inoculated on the reference medium mannitol-egg yolk-polymyxine agar (MYPA; Merck, Darmstadt, Germany) and on the media tested: polymyxine-egg yolk-mannitol-*Bacillus* agar (PEMBA; Oxoid, Basingstoke, UK), Brilliance™ *Bacillus cereus* agar (Oxoid, Basingstoke, UK) and HiCrome *Bacillus* agar (Himedia, Bombay, India). Cultivation took place at 30°C/24–48 h (EN ISO 2004). Both positive and negative strains were isolated and grown on the other media tested. The selected strains were identified by the genera-specific and the species-specific PCR methods and by the phenotyping (Czech Collection of Microorganisms, Brno, Czech Republic).

The PCR methods. DNA was isolated by GenElute™ Bacterial Genomic DNA Kit (Sigma Aldrich, St. Louis, USA). The genera-specific PCR for *Bacillus* sp. was performed with the designed primers BAC-F and BAC-R. The species-specific PCR for *B. cereus* was performed both according to Manzano *et al.* (2003) with the primers BCFW1 and BCrevnew, and with the designed primers BCER-F and BCER-R. The species-specific PCR for *B. licheniformis* was performed with the designed primers BLICH-F and BLICH-R. Primers BAC-F, BAC-R, BCER-F, BCER-R, BLICH-F and BLICH-R were designed by Němečková *et al.* (2010) using the sequences of genes encoding 16S and 23S rRNA. These primers were prepared

Table 1. Principle of cultivation media for determination of *B. cereus*

Characteristics to be detected	MYPA	PEMBA	Brilliance™ *Bacillus cereus* agar	HiCrome *Bacillus* agar
Absence of mannitol fermentation	Y (by phenol red)	Y (by bromthymol blue)	N	Y (by phenol red)
Lecithinase activity	Y	Y	N	N
β-Glucosidase activity	N	N	Y	Y
Resistance to polymyxin B	Y	Y	Y	Y
Resistance to trimethoprim	N	N	Y	N

Y – medium enables detection of the characteristic; N –medium does not enable detection of the characteristic

by Generi Biotech s.r.o. (Hradec Králové, Czech Republic).

Statistics. The repeatability of the reference method (EN ISO 2004) is 0.29 log N. Because *B. cereus* occurred in a relatively low density (< 1.6 ± 0.5 log CFU/ml) and as there were about 0–4 typical colonies grown on particular plates, quantitative comparison could not be performed.

RESULTS AND DISCUSSION

The principle of the media tested is summarised in Table 1. MYPA and PEMBA enable the detection of the same characteristics (fermentation of mannitol and lecithinase activity) but they differ in the acidobasic indicator and base composition. While MYPA is commonly used in USA and Canada, PEMBA is frequent in the U.K. (Bennett & Belay 2001). Brilliance™ *Bacillus cereus* agar and HiCrome *Bacillus* agar are chromogenic media detecting β-glucosidase activity. While most of the Gram-negative bacteria are inhibited by polymyxin B, diverse species of the Gram-positive bacteria (including certain bacilli) are inhibited by both polymyxin B and trimethoprim as selective agents (Fricker *et al*. 2008). Typical colonies of *B. cereus* and *B. licheniformis* are described in Table 2.

As mentioned above, the media tested could be compared only qualitatively because of the relatively low density of *B. cereus* spores in the analysed samples – less than 1 spore/ml. These findings agree with the occurrence of *B. cereus* described in literature (Prakash *et al*. 2007; Bartoszewicz *et al*. 2008).

During the first stage of experiments MYPA and PEMBA were compared using 40 milk samples. Totally 86 strains were isolated from the selected colonies grown from heat-inactivated samples. Both media provided the same results for 77% of strains, of which 14 were identified as *B. cereus sensu lato* and 52 as other species. The remaining 23% of the strains (3 strains identified as *B. cereus sensu lato* and 17 strains as other species) required cultivation at 30°C/48 h on PEMBA. Evaluation was facilitated by subsequent incubation of the plates at 6°C/24 h (changes in colour were more pronounced after this treatment) and required skilled laboratory personnel. On the contrary, there was no doubt concerning the evaluation of plates with MYPA.

There are the results of the identification of six strains that could cause difficulties in the evaluation on PEMBA in Table 3. The results obtained by PCR with *Bacillus* sp. agreed with phenotypical identification – all bacilli were positive while *Streptococcus haemolyticus* and an unidentified non-spore-forming strain were negative.

On the contrary, PCR with *B. cereus* according to Manzano *et al.* (2003) provided false-positive results for each of the bacilli strains tested (both isolated strains and negative control strains). The reason could be that Manzano *et al*. (2003) designed their method for distinguishing of species in terms of *B. cereus* group and did not focus on other groups of bacilli.

PCR gave negative results with *B. licheniformis* in all six strains tested. Thus, there was a difference between the designed PCR method and phenotypical identification that proved two *B. licheniformis* strains. These two strains did not exhibit the growth typical for *B. licheniformis* on MYPA and PEMBA. However, further identification methods should be employed to find out which method is more reliable.

Table 2. Features of typical colonies of *B. cereus* and *B. licheniformis* on selective media

	B. cereus	*B. licheniformis*
MYPA	Large (4–8 mm in diameter) shapely pink colonies with pink precipitate and surroundings without changes in colour	Middle (max. 5 mm in diameter) irregular or creeping yellow colonies with surroundings slightly changed to yellow
PEMBA	Large (4–8 mm in diameter) shapely blue colonies with white margin, blue-green precipitate and surroundings without changes in colour	Middle (max. 5 mm in diameter) irregular or creeping green-yellow colonies with surroundings without changes in colour
Brilliance™ *Bacillus cereus* agar	Small (1–3 mm in diameter) shapely green-blue colonies that may have white margine	Does not grow
HiCrome *Bacillus* agar	Middle (max. 5 mm in diameter) shapely blue colonies with white margine and surroundings slightly changed to deep pink	Middle (max. 5 mm in diameter) irregular or creeping yellow colonies with surroundings slightly changed to yellow

Table 3. Identification of selected strains isolated from heat-inactivated samples on MYPA and PEMBA

Strain	Growth on MYPA*	Phenotypical identification	PCR for *Bacillus* sp. (designed method)	PCR for *B. cereus* (method according to Manzano *et al.* (2003))	PCR for *B. licheniformis* (designed method)
A	OS	*B. licheniformis*	+	+	–
B	OS	unidentified	–	–	–
C	OS	*B. licheniformis*	+	+	–
D	OS	*B. pumilus*	+	+	–
E	OS	*Staphylococcus haemolyticus*	–	–	–
F	OS	*B. pumilus*	+	+	–

*OS – species other than *B. cereus sensu lato* or *B. licheniformis;* + the specific product present; – the specific product absent

During the second stage MYPA and the chromogenic media were compared using 35 milk samples. Totally 46 strains were isolated from the selected colonies grown from both raw milk samples and heat-inactivated samples. The strains isolated from the heat-inactivated samples did not represent the risk of mistaken identity when using the chromogenic media. However, five strains isolated from raw milk exhibited this characteristic. Moreover, Fricker *et al.* (2008) mentioned a higher risk of atypical *B. cereus* colonies (false-negative results) on Brilliance™ *Bacillus cereus* agar in comparison with other media. The results of the identification of these strains, as well as those obtained for the strain with the appearance of *B. cereus* but not precipitating yolk emulsion, are summarised in Table 4.

The reference method (EN ISO 2004) admits the occurrence of *B. cereus* strains without lecithinase activity. The phenotypical identification and the PCR methods confirmed that the last strain mentioned above was *B. cereus*.

As concerns the strains with the appearance of *B. cereus* on some of the chromogenic media, they were phenotypically identified as *Chryseobacterium* sp., *Stenotropomonas maltophilia*, and *Elizabethkingia meningoseptica*, while two strains remained unidentified. PCR for *Bacillus* sp. provided false-positive results with each of these strains. Thus, the method seems selective only inside the group of heat-resistant micro-organisms.

On the contrary PCR designed for *B. cereus* provided results in agreement with the phenotypical identification. This method seems to be more selective in the identification of *B. cereus sensu lato* among other bacilli and other bacterial species than the method according to Manzano *et al.* (2003).

The PCR for *B. licheniformis* provided negative results with each of the five strains tested that was in agreement with the results of the other methods.

Another question is whether the micro-organisms isolated from the raw milk samples (without heat-

Table 4. Identification of selected strains isolated from raw milk samples on MYPA and chromogenic media

Strain	Growth on MYPA*	Phenotypical identification	PCR for *Bacillus* sp. (designed method)	PCR for *B. cereus* (designed method)	PCR for *B. licheniformis* (designed method)
G	BC	*B. cereus*	+	+	–
H	OS	unidentified	+	–	–
I	OS	*Chryseobacterium* sp.	+	-	–
J	OS	*Stenotropomonas maltophilia*	+	–	–
K	OS	*Elizabethkingia meningoseptica*	+	–	–
F	OS	unidentified	+	–	–

*OS – species other than *B. cereus sensu lato* or *B. licheniformis*; BC – lecithinase negative *B. cereus sensu lato;* + the specific product present; – the specific product absent

inactivation) that form colonies similar to those of *B. cereus* represent the real risk of mistaken identity. Micro-organisms able to grow in the presence of polymyxine and under other conditions of the methods tested occur in raw milk in a density of 3–5 log CFU/ml, which is significantly higher than the common density of *B. cereus* in this type of sample, see above. Thus, the samples containing miscellaneous mixtures of micro-organisms, such as raw milk, should be strictly heat-inactivated before the cultivation analysis of *B. cereus*.

CONCLUSION

The cultivation methods for the determination of *B. cereus* are simple and widely accessible but there is a risk of false positive or false negative results. Heat-inactivation (e.g. 85°C/10 min) can be at least partial help. Dairy plants laboratories can be advised to use the reference method employing MYPA medium. Chromogenic medium HiCrome *Bacillus* agar seems to be suitable especially for the research purposes because it enables to distinguish the widest spectrum of biochemical activities in one step.

B. cereus was found in 10.6% of raw milk samples. Both positive (suspect *B. cereus*) and negative (other species) strains were isolated. *B. cereus* was represented by 18 strains of the total of 132 strains tested (13.6%). Heat-resistant micro-organisms that could be misidentified as *B. cereus* by the cultivation methods were phenotypically identified as *B. pumilus*, *B. licheniformis*, and *Staphylococcus haemolyticus*, heat-sensitive micro-organisms as *Chryseobacterium* sp., *Stenotropomonas maltophilia*, and *Elizabethkingia meningoseptica*.

The designed PCR method for *Bacillus* sp. enables to distinguish between bacilli and other heat-resistant microflora but it can provide false positive results with some other species.

While the species-specific method for *B. cereus* according to Manzano *et al.* (2003) enables to distinguish the species within the group *B. cereus sensu lato*, the designed method proved to be competent for distinguishing *B. cereus* from other bacilli groups and other bacterial genera.

The method designed for *B. licheniformis* may exhibit a similar limitation for closely related strains that could be mistakenly considered to be *B. licheniformis* due to their phenotypical characteristics.

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