Multi-locus sequence typing and virulence profile in Bacillus cereus sensu lato strains isolated from dairy products

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Abstract

Members of Bacillus cereus group are important food contaminants and they are of relevant interest in food safety and public health due to their ability to cause two distinct forms of food poisoning, emetic and diarrhoeal syndrome. In the present study, 90 strains of B. cereus isolated from dairy products, have been typed using Multi-locus Sequence Typing (MLST) analysis and investigated for the occurrence of 10 enterotoxigenic genes (hblA, hblC, hblD, nheA, nheB, nheC, cytK, entFM, entS and bceT) and one emetogenic gene (ces), to determine their genetic diversity. A total of 58 sequence types were identified and among these 17 were signalled as new profiles. Among the virulence genes, the majority of our strains carried the entS (92%), entFM (86%), nhe (82%) and cytK (72%) genes. All remaining genes were identified in at least one strain with different prevalence, stressing the genetic diversity, how even the different grade of pathogenicity of B. cereus isolated from dairy products.

Introduction

Bacillus cereus (B. cereus) is Gram-positive, rod shaped, endospore-forming, facultative anaerobic organism of genus Bacillus. Several closely related Bacillus species share a significant genetic similarity with B. cereus, thus they are included in a heterogeneous B. cereus group (Chang et al., 2003). B. cereus group is ubiquitous in the environment, particularly soil and water, because of the resistance of its spores to adverse environmental conditions (Brillard et al., 2015). It has been isolated from a variety of foods, including meat, rice, pasta, condiments, raw fruits, spices and vegetables (Harmon and Kautter, 1991), Chinese “take-out foods” (Beuchat Ma-Lin et al., 1980), cooked chicken (López et al., 2015), milk and dairy products (Christiansson, Bertilsson, and Svensson, 1999).

Some members of B. cereus group (i.e. B. cereus and B. thuringiensis) can cause human gastrointestinal diseases, the emetic and diarrhoeal syndromes. The emetic syndrome is characterised by vomiting, followed by diarrhoea (Beecher and Wong, 1994). This syndrome is caused by the ingestion of the cereulide toxin, preformed in the food (Agata et al., 2002). The diarrhoeal syndrome, is characterized by watery diarrhoea associated with abdominal pain (Granum and Lund, 1997) and it is caused by the ingestion of vegetative cells of B. cereus present in food, that colonize the small intestine before starting the enterotoxins production (i.e. non-haemolytic enterotoxin NHE, haemolysin BL, cytotoxin K) (Granum and Lund, 1997). Additionally, enterotoxin FM (EntFM), enterotoxin T (BceT) and enterotoxin S (EntS) have been also described as potential diarrhoeal toxins, although it is not clear the role they play in the pathogenesis of the disease (Hendriksen et al., 2006).

The aim of this study was to identify the virulence genes and to determine the sequence type of B. cereus sensu lato (s.l.) strains isolated from dairy products using Multi-locus Sequence Typing (MLST).

Materials and Methods

Multi-locus sequence typing

A total of 90 strains of B. cereus s.l. were isolated from dairy products: 64% (57/90) from cacicavallo, 25% (22/90) from mozzarella, 2% (2/90) from sheep’s cheese; 2% (2/90) from butter; 2% (2/90) from milk; 1% (1/90) from Bufalo mozzarella; 1% (1/90) from caciocicepta; 1% (1/90) from cream cheese; 1% (1/90) from caciotta; 1% (1/90) from yogurt. All the samples were collected from 2016 to 2018. Genomic DNA was purified using DNAeasy Blood and Tissue Kit (Qiagen, Hilden, Germany), according to manufacturer’s protocol. The MLST analysis was performed as described elsewhere (http://www.pubmlst.org/bcereus/).

DNA sequencing was performed on both strands using ABI PRISM Big Dye™ Terminator Cycle 3.1 sequencing kit cycle (Life Technologies) according to the manufacturer’s instruction and analyzed by ABI Prism 3130 Genetic Analyzer (Applied BiosystemThermoScientific inc.). The sequence of each allele was imported and assembled by BioNumerics v7.5 software (Applied Maths, Belgium), and then compared to those available in the MLST database. New allele sequences were submitted to the B. cereus MLST database.

Virulence genes detection

The presence of potential virulence genes hblA, hblC, hblD, nheA, nheB, nheC, cytK, entFM, entS, bceT and ces was evaluated by PCR using primers listed in Table 1 (Asano et al., 1997; Guinebretière et al., 2002; Fagerlund et al., 2004) and followed the procedure described in Ghelardi et al., 2002 and Fricker et al., 2007.

Results

In our study we collected a total of 90 B. cereus s.l. strains isolated from different dairy products (Table 2). The MLST profiles of all isolates are shown in Table 2. A total of 58 STs were identified with ST 1986 as the most frequent. Additionally, 17 STs never described before were identified (Table 2). Thirteen STs belonged to 7 clonal complexes (CC). The major clonal complex was represented by the CC-142, that includes two STs, ST4 and ST2010 (Table 2). The CC-23 included three STs, ST197, ST2013 and ST2033; each CC-111, CC-142 and CC-365 included two STs: ST562 and ST800, ST4 and ST2010, ST1263 and ST2084, respectively. The CC-97 and CC-
205 included each only one ST, 1578 and 255, respectively. The ST1986 was the most frequent ST ad it was detected only in caciocavallo. When we considered the distribution of STs among the samples collected in this study (including samples with two or more strains isolated), we identified 34 different STs in caciocavallo, 15 STs in mozzarella, 2 STs were identified in both butter and milk and sheep’s cheese. Only two STs, ST92 and ST142, were identified in more than one dairy product.

All strains analyzed in this study were tested for the presence or absence of eleven virulence genes; the results were summarized on Table 2. Three genes encoding enterotoxin HBL were identified in 58% (53/90) of strains, with different prevalence: the hblA, hblC and hblD were identified in 67% (60/90), 64% (58/90) and 79% (71/90) of the strains, respectively. Similarly, the cluster of nhe genes was identified in 84% (76/90) of the strains with different prevalence between genes: the nheA, nheB and nheC genes were identified in 92% (83/90), 93% (84/90) and 90% (81/90) of the strains, respectively. The cytK, entFM, bceT and entS genes were identified in 72% (65/90), 86% (77/90), 64% (59/90) and 92% (83/90) of the strains, respectively. Only the 10% (9/90) of the strains carried the ces gene.

### Discussion and Conclusions

*B. cereus s.l.* is a ubiquitous bacterium in the environment that can be present in a wide range of foodstuffs. It is prevalent in soil from cattle grazing fields, raw milk and dairy products. In this study we describe the characterization and the pathogenic potential of a collection of 90 *B. cereus* strains isolated from dairy products. Based on MLST profiles our strains showed a high genetic diversity. Indeed, on a total of 90 isolates 58 MLST profiles were identified, of which 17 were new ST profiles and were submitted in available online database. The ST1986 was the most frequent ST detected and it was found only in the strains isolated from caciocavallo. Interestingly, only the ST92 and ST142 were identified in more than one dairy product; all the other 56STs were detected in a particular dairy product variety. Among previously described STs, ten (ST4, ST12, ST15, ST24, ST26, ST33, ST164, ST205, ST1063 and ST1097) were identified in milk or milk products (Cardazzo et al., 2008; Didelot, Barker, Falush, and Priest, 2009; Yang et al., 2017). The remaining 31 STs were isolated in other foods or in the environment (Didelot et al., 2009; Priest et al., 2004).

Different virulence genes have been associated with the pathogenic power of *B. cereus*, the Enterotoxins BL (HBL), Non-Hemolytic Enterotoxin (NHE), cytotoxin K, and enterotoxin FM are primarily associated to diarrheal disease (Madeira et al., 2015; Sergeev et al., 2006); evidence suggests that the enterotoxin EntS and BceT also contribute to the severity of diarrheal illness Agata et al., 1995; Castiaux et al., 2016; whereas the ability of *B. cereus s.l.* to synthesize the heat stable cereulide protein is connected to the emetic disease (Ehling-Schulz et al., 2005). In our study we found that the non-emetic strains (90%) were more numerous than the emetic strains (10%). This finding agreed with previous studies, which showed a low prevalence rate of emetic toxin gene ces in *B. cereus s.l.* isolated from milk, dairy product and other various food sources (Yim et al., 2015; Owusu-Kwarteng et al., 2017). All these data seem to suggest that emetic toxin gene is rare among *B. cereus s.l.* strains, but an infection supported by this specific toxin is considered to be the worst-case scenario (Wetterhall et al., 2019). Generally, *B. cereus s.l.* strains related with emetic food poisoning were associated with ST26 (Ehling-Schulz et al., 2005). Based on the genetic profile of our strains, we not only corroborated this thesis, but we identified additionally five STs (ST4, ST33, ST1986, ST2045 and ST2062) as potential emetic strains. Interestingly, among these strains, those belonging to ST26 did not carried HBL gene complexes, as previous reported (Ehling-Schulz et al., 2005); among these, in the BC003 strain we identified only two virulence genes, ces and entS. Similarly, to ST26, both the strains belonged to ST2045 did not carried HBL gene complexes. Among the non-emetic *B. cereus s.l.* strains, we found a high prevalence rate (≥60%) of all enterotoxin genes investigated. The higher prevalence rate was related to entS gene, identified in 93% of all strains, followed by entFM gene (83%), nhe gene complex (82%), cytK gene (72%), bceT gene (66%) and hblACD gene complex.

| Target | Primer name | Primer sequence (5’-3’) | Reference |
|--------|-------------|------------------------|-----------|
| hblA   | hblA_F      | GTGACGATGTTGACGCGTAT   | (Guinebretière et al., 2002) |
|        | HBLA_R      | ATGCCACTGCGTGACATAT    |           |
| hblC   | L2A_F       | AATGGTACCTGGAATCTCTAT  | (Guinebretière et al., 2002) |
|        | L2B_R       | CTCGCTGTCTGCTTTAAT     |           |
| hblD   | L1A_F       | AACAAAGAGGTTCACGAAAT   | (Guinebretière et al., 2002) |
|        | L1B_R       | CACCAATTGACCATGCTAAT   |           |
| nheA   | nheA_F      | TACGCTAAGGAGGACGCGCA   | (Guinebretière et al., 2002) |
|        | nheA_R      | GTTATTTATGGCTTTAGATT   |           |
| nheB   | nheB_F      | CTAATACAGCTAAGGACGCGA  | (Fagerlund et al., 2004) |
|        | nheB_R      | ACCTTACAGGTGTTCTTCC    |           |
| nheC   | nheC_F      | CCGATTGATGTACGTTGCGG   | (Fagerlund et al., 2004) |
|        | nheC_R      | CACCATTCGACTCGCGCAA    |           |
| cytK   | cytK_F      | ACAAGATACCGCGAAAATGCG  | (Ghelardi et al., 2002) |
|        | cytK_R      | TCCACCCGATGTTGTCCTC    |           |
| entFM  | entFM_F     | ATGAAAAATATTGGACGG     | (Asano et al., 1997) |
|        | entFM_R     | TTAGTATGCTTTTGTGACAC   |           |
| bceE   | entT_F      | GCATCGAAAACCGAGTGGTG   | (Agata et al., 1995) |
|        | entR_F      | AATCGTCGGACATGCTGACG   |           |
| Ces    | ces_F       | GTTACGACATTACATATAAGGTCG | (Fricker et al., 2007) |
|        | ces_R       | GTTACGACATTACATATAAGGTCG |           |
Table 2. Source, clonal complex (CC), sequence type (ST) and enterotoxin and emetic toxin genes identified in *B. cereus* s.l. isolated from dairy products. The new STs identified for the first time were indicated with asterisks.

| ID sample | Source                  | CC                           | ST             | Virulence genes |
|-----------|-------------------------|------------------------------|----------------|-----------------|
|           |                         |                              | hblA | hblC | hblD | nheA | nheB | nheC | cytK | entFM | bceT | entS | ces |
| BC001     | Caciocavallo            | 1967*                        | +    | +    | +     | +    | +    | +    | +    | +    | +    | +    |    |
| BC002     | Mozzarella              | 26                           | -    | -    | -     | +    | +    | -    | +    | -    | +    |    |
| BC003     | Mozzarella              | 26                           | -    | -    | -     | -    | -    | -    | -    | -    | +    |    |
| BC004     | Caciocavallo            | 26                           | -    | -    | -     | +    | +    | -    | +    | -    | +    |    |
| BC005     | Caciocavallo            | 34                           | -    | -    | -     | +    | -    | +    | +    | +    | -    |    |
| BC006     | Caciocavallo            | 33                           | +    | +    | +     | +    | +    | +    | +    | +    | +    |    |
| BC007     | Caciocavallo            | 509                          | -    | -    | +     | +    | +    | +    | -    | +    | +    |    |
| BC026     | Caciocavallo            | ST-111 complex               | 800  | +    | +    | +    | +    | +    | +    | +    | +    |    |
| BC009     | Caciocavallo            | 34                           | -    | -    | -     | +    | +    | +    | +    | +    | +    |    |
| BC010     | Caciocavallo            | ST-8 complex                 | 8    | +    | +    | -     | +    | +    | +    | +    | +    | +    |    |
| BC011     | Caciocavallo            | ST-142 complex               | 2010*| +    | +    | +     | +    | +    | +    | +    | +    | +    |    |
| BC012     | Caciocavallo            | 2002*                        | +    | +    | +     | +    | +    | -    | +    | +    | -    |    |
| BC013     | Caciocavallo            | 500                          | +    | +    | +     | +    | +    | +    | -    | +    |    |
| BC014     | Caciocavallo            | ST-23 complex                | 2015*| +    | +    | +     | +    | +    | +    | +    | +    |    |
| BC015     | Caciocavallo            | 2026*                        | +    | +    | +     | +    | +    | +    | -    | +    |    |
| BC016     | Caciocavallo            | 2028*                        | -    | -    | +     | +    | +    | +    | -    | +    |    |
| BC017     | Caciocavallo            | ST-365 complex               | 1263 | -    | -    | +     | +    | -    | -    | +    |    |
| BC018     | Caciocavallo            | ST-8 complex                 | 15   | +    | +    | +     | -    | +    | +    | +    |  |
| BC019     | Caciocavallo            | ST-142 complex               | 4    | +    | +    | +     | +    | +    | +    | +    | +    |    |
| BC020     | Caciocavallo            | ST-142 complex               | 4    | +    | +    | +     | +    | +    | +    | +    | +    |    |
| BC021     | Caciocavallo            | 2031*                        | +    | +    | +     | +    | +    | +    | -    | +    |    |
| BC022     | Caciocavallo            | 34                           | -    | -    | +     | +    | +    | +    | -    | +    |    |
| BC023     | Caciocavallo            | ST-23 complex                | 2033*| +    | -    | +     | +    | +    | -    | +    |    |
| BC025     | Caciocavallo            | 2038*                        | +    | +    | +     | +    | +    | -    | -    | +    |    |
| BC024     | Cream cheese            | 996                          | -    | -    | -     | +    | -    | -    | +    | +    |    |
| BC027     | Caciocavallo            | ST-142 complex               | 4    | +    | +    | +     | +    | +    | +    | +    |    |
| BC028     | Caciocavallo            | 59                           | +    | -    | +     | +    | +    | +    | +    | +    |    |
| BC029     | Caciocavallo            | 2041*                        | +    | +    | +     | +    | +    | +    | +    | +    |    |
| BC030     | Mozzarella              | 369                          | -    | -    | -     | +    | +    | +    | +    | +    |    |
| BC031     | Caciocavallo            | 2045*                        | -    | -    | +     | +    | +    | -    | -    | +    |    |
| BC032     | Caciocavallo            | 2045*                        | -    | -    | -     | +    | +    | +    | -    | +    |    |
| BC033     | Mozzarella              | 12                           | +    | +    | +     | +    | +    | +    | -    | +    |    |
| BC034     | Caciocavallo            | 1986*                        | +    | +    | +     | +    | +    | +    | -    | +    |    |
| BC035     | Mozzarella              | 1420                         | +    | +    | +     | +    | +    | +    | +    | +    |    |
| BC036     | Buffalo mozzarella      | 278                          | -    | -    | +     | +    | +    | +    | -    | +    |    |
| BC037     | Caciocavallo            | 1986*                        | +    | +    | +     | +    | +    | +    | -    | +    |    |
| BC038     | Caciocavallo            | 1986*                        | +    | +    | +     | +    | +    | +    | -    | +    |    |
| BC040     | Caciocavallo            | 1986*                        | +    | +    | +     | +    | +    | +    | -    | +    |    |
| BC041     | Caciocavallo            | ST-23 complex                | 197  | +    | +    | +     | +    | +    | +    | +    |    |
| BC042     | Caciocavallo            | 2662*                        | -    | -    | -     | +    | -    | +    | -    | +    |    |
| BC043     | Caciocavallo            | 1986*                        | +    | +    | +     | +    | +    | +    | -    | +    |    |
| BC044     | Caciocavallo            | 262                          | +    | +    | +     | +    | +    | +    | +    | +    |    |
| BC045     | Caciocavallo            | 1980*                        | +    | +    | -     | +    | +    | +    | +    | +    |    |
| BC046     | Caciocavallo            | 2116                         | -    | -    | -     | -    | -    | -    | +    | -    |    |
| BC047     | Caciocavallo            | 1986*                        | +    | +    | +     | +    | +    | +    | -    | +    |    |
| BC048     | Caciocavallo            | ST-205 complex               | 205  | +    | +    | +     | +    | +    | +    | +    |    |
| BC049     | Caciociovitta           | 164                          | -    | -    | +     | +    | +    | +    | +    | +    |    |
| BC050     | Mozzarella              | 818                          | -    | -    | +     | +    | +    | +    | +    | +    |    |
| BC051     | Mozzarella              | 1097                         | -    | -    | +     | +    | +    | +    | -    | +    |    |
| BC052     | Mozzarella              | 2038                         | +    | +    | +     | +    | +    | +    | +    | +    |    |

Continued on the next page.
(60%). Similar results of high prevalence of cytK, entFM and hbl gene complexes in B. cereus isolated from milk and dairy products (Reis et al., 2013) and of nhe gene complex, entS and bceT genes complexes in B. cereus isolated from several food and environmental source (i.e. soil) have been described (Yang et al., 2005; Chaves et al., 2012; Yim et al., 2015). When we considered the virulence genes profile and the source analysed, none correlation was found. Whereas when we correlated the ST profiles to virulence genes detected we found nine STs (ST8, ST509, ST996, ST1097, ST1263, ST2028, ST2033, ST2084 and ST2116) that were associated to an exclusive virulence genes combination. Nevertheless, this genetic profile correlation is questionable, considering that we identified one strain for each of nine STs previously mentioned. Thus, further studies will be necessary for demonstrated really correlation between MLST and virulence genes profiles.

Based on the results and considering the genetic diversity of B. cereus s.l., the MLST approach has proven to be an accurate method for strain genetic typing. Interestingly, our analysis demonstrated the presence of toxin genes in all strains of the B. cereus s.l., stressing the their potentially pathogenic power. Taken together, these data appear to be of significant interest for public health, since highlight potential risk connected to ready-to-eat foods, generally consumed without cooking. In order to prevent contamination by B. cereus s.l., it is very important observe the correct hygiene practices along food chain. In addition, it would be advisable to implement scrupulous control measures of food preservation, to prevent the multiplication of the microorganism and the consequent production of toxins.

Table 2. Continued from previous page.

| ID sample | Source          | CC | ST   | Virulence genes | hblA | hblC | hblD | nheA | nheB | cytK | entFM | bceT | entS | ces |
|-----------|-----------------|----|------|-----------------|------|------|------|------|------|------|-------|------|------|-----|
| BC0053    | Caciocavallo    | 1886*|      | +               | +    | +    | +    | +    | +    | +    | +     | +    | -    | +   |
| BC0054    | Caciocavallo    | 26  |      |                 | +    | +    | +    | +    | +    | +    | +     | +    | +    | +   |
| BC0055    | Caciocavallo    | 1886*|      | +               | +    | +    | +    | +    | +    | +    | +     | +    | +    | +   |
| BC0056    | Butter          | 120 |      | +               | +    | +    | +    | +    | +    | +    | +     | +    | -    | +   |
| BC0057    | Mozzarella      | 1665|      | -               | -    | -    | -    | -    | -    | -    | -     | -    | -    | -   |
| BC0058    | Caciocavallo    | 1886*|      | +               | +    | +    | +    | +    | +    | +    | +     | +    | +    | +   |
| BC0059    | Caciocavallo    | ST-142 complex | 4 | +               | +    | +    | +    | +    | +    | +    | +     | +    | -    | +   |
| BC0060    | Mozzarella      | ST-97 complex | 1578 | +          | +    | +    | +    | +    | +    | +    | +     | +    | -    | -   |
| BC0061    | Caciocavallo    | 1986*|      | +               | +    | +    | +    | +    | +    | +    | +     | +    | +    | +   |
| BC0062    | Caciocavallo    | 500 |      | +               | +    | +    | +    | +    | +    | +    | +     | +    | +    | +   |
| BC0063    | Mozzarella      | 1097|      | +               | +    | +    | +    | +    | +    | +    | +     | +    | -    | +   |
| BC0064    | Sheep’s cheese  | 1063|      | +               | +    | +    | +    | +    | +    | +    | +     | +    | -    | +   |
| BC0065    | Caciocavallo    | 12  |      | -               | -    | -    | -    | -    | -    | -    | -     | -    | -    | -   |
| BC0066    | Mozzarella      | 12  |      | -               | +    | +    | +    | +    | +    | +    | +     | +    | -    | -   |
| BC0067    | Caciocavallo    | 371 |      | +               | +    | +    | +    | +    | +    | +    | +     | +    | -    | -   |
| BC0068    | Mozzarella      | 1007|      | +               | +    | +    | +    | +    | +    | +    | +     | +    | -    | +   |
| BC0069    | Caciocavallo    | 92  |      | +               | +    | +    | +    | +    | +    | +    | +     | +    | -    | +   |
| BC0070    | Caciocavallo    | 1886*|      | +               | +    | +    | +    | +    | +    | +    | +     | +    | +    | +   |
| BC0071    | Mozzarella      | 92  |      | +               | +    | +    | +    | +    | +    | +    | +     | +    | -    | +   |
| BC0072    | Mozzarella      | 2261|      | +               | +    | +    | +    | +    | +    | +    | +     | +    | -    | +   |
| BC0073    | Caciocavallo    | 632 |      | +               | +    | +    | +    | +    | +    | +    | +     | +    | -    | +   |
| BC0074    | Caciocavallo    | ST-111 complex | 582 | +          | +    | +    | +    | +    | +    | +    | +     | +    | -    | +   |
| BC0075    | Mozzarella      | 1223|      | +               | +    | +    | +    | +    | +    | +    | +     | +    | -    | +   |
| BC0076    | Mozzarella      | 24  |      | -               | -    | -    | -    | -    | -    | -    | -     | -    | -    | -   |
| BC0077    | Mozzarella      | 92  |      | +               | +    | +    | +    | +    | +    | +    | +     | +    | +    | +   |
| BC0078    | Caciocavallo    | 2083*|      | +               | +    | +    | +    | +    | +    | +    | +     | +    | -    | +   |
| BC0079    | Caciocavallo    | ST-365 complex | 2084*| +          | +    | +    | +    | +    | +    | +    | +     | +    | -    | +   |
| BC0080    | Milk            | 56  |      | +               | +    | +    | +    | +    | +    | +    | +     | +    | -    | +   |
| BC0081    | Caciocavallo    | 1833*|      | +               | +    | +    | +    | +    | +    | +    | +     | +    | +    | +   |
| BC0082    | Butter          | 1355|      | +               | +    | +    | +    | +    | +    | +    | +     | +    | +    | +   |
| BC0083    | Caciocavallo    | 1867*|      | +               | +    | +    | +    | +    | +    | +    | +     | +    | +    | +   |
| BC0084    | Mozzarella      | 414 |      | -               | -    | -    | -    | -    | -    | -    | -     | -    | -    | -   |
| BC0085    | Caciocavallo    | 142 |      | +               | +    | +    | +    | +    | +    | +    | +     | +    | -    | +   |
| BC0086    | Mozzarella      | 24  |      | +               | +    | +    | +    | +    | +    | +    | +     | +    | -    | +   |
| BC0087    | Caciocavallo    | 1967*|      | +               | +    | +    | +    | +    | +    | +    | +     | +    | -    | +   |
| BC0088    | Milk            | 1810|      | +               | +    | +    | +    | +    | +    | +    | +     | +    | -    | +   |
| BC0089    | Yogurt          | 142 |      | +               | -    | -    | -    | -    | -    | -    | -     | -    | -    | -   |
| BC0090    | Caciocavallo    | 2095*|      | +               | +    | +    | +    | +    | +    | +    | +     | +    | -    | +   |
| BC0094    | Caciocavallo    | 2006*|      | +               | +    | +    | +    | +    | +    | +    | +     | +    | -    | -   |
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