Molecular Characterization of *Corynebacterium bovis* causing Clinical Mastitis and Increasing Somatic-Cell Count

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Abstract Bovine mastitis remains the most economically important disease affecting dairy cows. Bacteria of the genus *Corynebacterium* spp., especially *Corynebacterium bovis* (*C. bovis*), are frequently isolated from bovine mastitis although there are divergent reports about the role of this pathogen in bovine mastitis. In the present study we report the presence of new *C. bovis* strains from milk exhibiting increased SCC of 12 cows with subclinical and clinical mastitis. Irregular Gram-positive bacilli isolated in pure cultures underwent conventional biochemical tests which generated indefinite phenotypic profiles (*Corynebacterium mastitidis/C. bovis*). API-Coryne System 3.0 identified all the milk isolates as *C. bovis* (code number 4501014; 99.9% confidence level). Phylogenetic analysis based on 16S rRNA and rpoB sequences confirmed the identification of the strains as *C. bovis*.

Keywords *Corynebacterium bovis*; Mastitis; Sequencing of Genes; 16S rRNA; rpoB

1. Introduction

Bovine mastitis remains the most economically important disease affecting dairy cows. Coryneform bacteria are frequently isolated from bovine mastitis, and lipophilic *Corynebacterium bovis* has been the most frequently isolated microorganism of this group (Watts et al., 2000, 2001). *C. bovis* isolation has been positively correlated to the increase of SCC in milk samples from cows with subclinical mastitis (Brooks and Barnum, 1984; Pankey et al., 1985; Sordillo et al., 1989). The economic loss varies with the degree of alveolar epithelial lesion and the increase in leukocyte infiltration, which decreases milk secretion (Hallberg et al., 1995).
Since there are still some conflicting research results concerning the relevance of *C. bovis* as the etiological agent of mastitis, in the present study we report different cases of clinical and subclinical mastitis due to *C. bovis* during an outbreak in a dairy farm. Phylogenetic analysis, based on 16S rRNA sequences, unambiguously demonstrated that the clinical isolates of different phenotypes belonged to *C. bovis* species.

### 2. Materials and Methods

#### 2.1. Milk Collection

During a mastitis outbreak in a dairy farm located in the central region of São Paulo State, Brazil, screening tests were carried out to detect clinical and subclinical mastitis (positive strip cup test = clinical mastitis; California Mastitis Test-CMT score higher than one cross = subclinical mastitis) among 300 Holstein animals, of different ages and at different lactation stages. Subsequently, 106 milk samples were aseptically collected from mammary quarters showing CMT score +++ and positive strip cup test for microbiological tests and SCC.

#### 2.2. Microbiological Culture Conditions

Milk samples (0.1 mL) were sown in agar base (Difco™) added of 5% ovine blood and incubated at 37°C for 24-72h in aerobiosis. *Corynebacterium*-like colonies were initially identified according to Gram staining, colonial morphology, pigmentation and hemolytic properties. Colonies of irregular Gram-positive rods (IGPR) were re-cultured in brain heart infusion (BHI) added of 1% tween-80 and incubated for 24h. Then, the microorganisms were subjected to phenotypic and genotypic tests for identification as described below.

#### 2.3. Phenotypic Tests for Corynebacteria Identification

Conventional biochemical characterization was done by means of tests of lipophilia, catalysis, O/F metabolism, acid production from glucose, maltose, sucrose, trehalose, ribose, mannitol, lactose, xylose, mannose, arabinose, fructose, galactose and glycogen; hydrolysis tests of esculin, urea, tyrosine, in addition to assays of alkaline phosphatase, pyrazinamidase, nitrate reduction and CAMP reaction using *Staphylococcus aureus* sample (Murray, 2010). ONPG (o-Nitrophenyl-β-D galactopyranoside) hydrolysis test was carried out with the enzyme β-galactosidase produced by *C. bovis*, considered decisive for its differentiation according to Holt et al. (1994).

Microorganisms were also characterized according to the commercially available semi-automated identification API-Coryne System 3.0 (bioMerieux, Lyon, France) by following the manufacturer’s instructions; decoded by the API web system (https://apiweb.biomerieux.com)

#### 2.4. Gene Amplification and Sequencing

*C. bovis* identification was reconfirmed by sequencing 16S rRNA and *rpoB* genes. Each strain was grown in BHI broth under incubation for 24-48h at 30°C and centrifugation for 5 min at 3000 rpm. The pelleted bacteria was suspended in 500 µL sterile water and subsequently boiled during 15 min for DNA extraction. Then, cell extracts were immediately stored at -20°C until used in PCR reactions. 16S rRNA gene was amplified by using the universal primers: pA (5'-AGA GTT TGA TCC TGG CTC AG) and pH (5'-AAG GAG GTG ATC CAG CCG CA), as described by Watts and co-workers (2000). The PCR product was purified in both directions by primer walking with the oligonucleotides using the following primers for sequencing: 1831 (5'- GAG GAA CAC CGA TGG CGA AGG C), 1832 (5'- GCC CCC GTC AAT TCC TTT GAG TT) (Watts et al., 2000), 519r (5'- G(AT)A TTA CCG CGG C(GT)G CTG), and 1242f (5'- CAC ACG TGC TAC AAT GG) (Johnson, 1994). *rpoB* gene was amplified and
sequenced with primers as previously described by Khamis et al., 2004. Sequencing reactions were performed with BigDye Terminator v 3.1 cycle sequencing kit (Applied Biosystems) on an ABI-3730 Automated DNA Sequencer (Applied Biosystems), according to standard protocols. 16S rRNA gene sequences were compared to those available in the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov) using the BLAST algorithm and the Ribosomal Database Project (RDP-II) (http://rdp.cme.msu.edu/html). rpoB gene sequences were only compared in GenBank database.

### 2.5. Phylogenetic Analysis

16S rRNA gene sequences were aligned by CLUSTALX (Thompson et al., 1997). The phylogenetic trees were prepared by using the neighbor-joining genetic distance method and the software MEGA 4.0 package with the option of complete deletion of gaps (Tamura et al., 2007). Kimura two-parameter model was chosen for all NJ tree constructions. The reliability of each tree topology was checked by 1000 bootstrap replications.

### 3. Results

Results related to CMT, SCC/mL milk and mastitis classification are shown in Table 1. Twelve IGPR strains were isolated from different animals, three of which had signs and symptoms of clinical mastitis and nine had subclinical infections. In all opportunities, microorganisms were isolated from milk in pure culture and at concentrations higher than 30 CFU/0.1mL, showing small whitish non-hemolytic colonies of 0.2 to 0.5 mm diameter after 72-h incubation with dried aspect. Bacterioscopy according to Gram’s method indicated Gram-positive cocobacilli forms.

Table 1: California Mastitis Test-CMT and somatic cell count-SCC milk results and type of mastitis of 12 cows during an outbreak in a farm located in Botucatu, São Paulo, Brazil, 2012

| Record | Animal/teat | Mastitis | CMT (score) | SCC (x1000/mL) |
|--------|-------------|----------|-------------|----------------|
| 42/11 – 39 | 1462/PR | Clinical | ND | ND |
| 42/11 – 65 | 1664/PR | Clinical | ND | ND |
| 42/11 – 66 | 139/PR | Clinical | ND | ND |
| 43/11 – 13 | 1589/PL | Subclinical | 3 | 1567 |
| 43/11 – 32 | 1385/PR | Subclinical | 3 | 4765 |
| 43/11 – 88 | 1600/PR | Subclinical | 2 | 4908 |
| 43/11 – 90 | 1367/AL | Subclinical | 3 | 7815 |
| 43/11 – 91 | 1445/PL | Subclinical | 2 | 498 |
| 43/11 – 98 | 1463/PR | Subclinical | 3 | 856 |
| 43/11 – 99 | 1106/PL | Subclinical | 3 | 8631 |
| 43/11 – 100 | 1520/PL | Subclinical | 3 | 6389 |
| 43/11 – 107 | 1591/PR | Subclinical | 3 | 478 |

ND = Not done; SCC = somatic-cell count mL/milk; AR = anterior right teat; AL = anterior left teat; PR = posterior right teat; PL = posterior left teat

All 12 IGPR samples showed lipophilic properties and had negative results for esculin hydrolysis test, nitrate reduction and CAMP; there was no acid production in the presence of the carbohydrates glucose, maltose, sucrose, mannitol, trehalose, xylose, arabinose, ribose, fructose and glycogen, besides weak urea hydrolysis. These results were compatible with the species *Corynebacterium mastitidis* (Holt et al., 1994; Fernandez-Garayzabal et al., 2001; Murray, 2007). On the other hand, the microorganisms showed the following results suggestive of *C. bovis*: growth in the presence of 6.5% NaCl, positive ONPG (β-galactosidase) and negative pyrazinamidase activity. Further analysis by the API-Coryne system identified all 12 strains as *C. bovis* by the code number 4501014 with 99.9% confidence level.

Results of the sequencing of genes 16S rRNA and rpoB from the isolates (Table 2) indicated that the samples had similarity superior to 98.7% for gene 16S rRNA only with the species *C. bovis*. 

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Phylogenetic analysis unambiguously demonstrated that the clinical isolates belonged to *C. bovis* species, as illustrated in Figure 1. The similarity values obtained in the analysis of gene *rpoB* also confirmed the data obtained in the analysis of gene 16S rRNA. The gene sequences of 16S rRNA and *rpoB* were deposited in GenBank under the numbers JX298782 and JX298783 for sample 42/11 - 65; JX298784 and JX298785 for sample 42/11 - 66; JX298786 and JX298787 for sample 43/11 - 99; JX298788 and JX298789 for sample 43/11 - 100.

**Table 2:** Sequence analysis based on *Corynebacterium* spp. sequences of 16S rRNA and *rpoB* genes

| Strain number     | bp   | 16S rRNA similarity (%) | bp   | *rpoB* similarity (%) | GenBank Accession Numbers |
|-------------------|------|--------------------------|------|-----------------------|---------------------------|
| 42/11 - 65BR      | 1507 | 100.00                   | 421  | 98.57                 | *Corynebacterium bovis*   |
| 42/11 - 66BR      | 1500 | 100.00                   | 388  | 98.45                 | *Corynebacterium bovis*   |
| 43/11 - 99BR      | 1506 | 100.00                   | 393  | 98.47                 | *Corynebacterium bovis*   |
| 43/11 - 100BR     | 1506 | 99.93                    | 400  | 98.50                 | *Corynebacterium bovis*   |

![Phylogenetic tree based on the neighbor-joining method using 16S rRNA gene sequences. Distances were estimated by using the Kimura two-parameter model. Bootstrap percentages after 1,000 simulations are shown. The Actinomyces bovis (T) X81061 sequence was used as outgroup.](image-url)
4. Discussion

Based on the assessment of the dynamics of intramammary infection by *C. bovis*, Honkanen-Buzalski et al. (1984) assumed that there is no protection against major pathogens and concluded that *C. bovis* is relevant in the pathogenesis of bovine mastitis. Some authors highlight its effects regarding decreased milk production and increased SCC/mL milk (Costa et al., 1986; Viseslava and Vera, 1989; Hallberg et al., 1995; Wilson, 2001). Mettifogo et al. (1991) evidenced the importance of *C. bovis* in subclinical mastitis by isolating it from 25.77% examined milk samples with mean microscopic SCC of 1,032,429 cells/mL milk. In the same region, Beloti et al. (1997) found *C. bovis*, among other pathogens, in 18.98% subclinical cases of mastitis, with mean SCC of 979 x 10^3 cells/mL milk, showing cell response in intramammary infection.

However, *C. bovis* is considered, by some authors, a pathogen of less importance in the etiology of bovine mastitis, or even a commensal of the udder, located especially in the teat duct (Pankey et al., 1985; Bexiga et al., 2011) and capable of protecting it during the invasion of other microorganisms (Bramley et al., 1976; Huxley, 2003) such as *Staphylococcus aureus*, which is considered a major pathogen.

The occurrence as outbreaks or not shows the relationship between *C. bovis* infectivity and pathogenicity characteristics. In addition to these aspects, there is decreased production, as demonstrated by the comparative analysis of the production of teats infected with *C. bovis* with that of their negative homologues (Domingues et al., 1998). Data showed 23.5% reduction in the production of infected teats, which evidenced the risk represented by this agent in the development of dairy farming.

Reasserting the participation of this microorganism in mammary pathology, Costa et al. (1986) recovered it from 32.06% and 22.74% cases of clinical mastitis and subclinical mastitis, respectively, and Langoni et al. (1998) from 12.08% and 15.94% of the 850 studied cases of clinical mastitis and 7902 cases of subclinical mastitis, respectively.

Another aspect that reinforces its pathogenicity injuring the mammary alveolar tissue is the decrease in milk production, as previously reported (Langenegger et al., 1981; Domingues et al., 1998). Association of *C. bovis* with bovine mastitis was also reported by Ferreiro et al. (1981), and Costa et al. (1985) argued that, besides the high prevalence (32.5%) indicating its infectivity, the agent was isolated in pure culture of cases of clinical mastitis (28.18%) and subclinical mastitis (27.5%).

The present results reinforce the potential of *C. bovis* as mastitis agent also due to its pure and exuberant isolation in both cases of clinical mastitis and cases of subclinical mastitis with high SCC, in some cases reaching values superior to 1000 x 10^3 SCC/ml milk, which agrees with Mettifogo et al. (1991), Beloti et al. (1997), Harmon (1998) and Wilson (2001).

Molecular diagnosis has brought great advances, constituting a fundamental tool for molecular epidemiology studies but requiring the use of reagents and specific primers, besides previous standardization, appropriate laboratories and professional experience (Bexiga et al., 2011). The present results indicated the use of the API-Coryne System as an alternative tool for the identification of *C. bovis* in cases of bovine mastitis outbreaks.

5. Conclusion

In conclusion, the differentiation and the diagnosis of *C. bovis* must be grounded in well conducted studies that meet the assessment criteria related to milk production by the affected quarters, as well as in SCC/mL milk, and fundamentally characterization must be appropriate by means of genotypic
studies and genetic sequencing for adequate interpretation of results, leading to more pertinent conclusions as to its role in bovine intramammary pathogenicity.

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**Conflict of Interest**

The authors have declared that no conflict of interest exists.

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