Technical Note: Molecular Typing of *Corynebacterium bovis* Isolates by Pulsed-Field Gel Electrophoresis

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ABSTRACT

A typing method for *Corynebacterium bovis* based on pulsed-field gel electrophoresis (PFGE) is described and applied to type 162 strains isolated from mastitic milk from 57 Friesian dairy herds in northern Spain. Isolates were identified to the species level using the API-Coryne system and a polymerase chain reaction-restriction fragment length polymorphism of the 16S rDNA amplicon. Cultures in logarithmic phase were harvested, mixed with agarose, and treated with lysozyme and proteinase K to release intact genomic DNA that was digested with *Xba*I and submitted to PFGE. Seven different pulsotypes were identified; none of them were similar to that of the reference strain (ATCC 7715). The most frequent pulsotype was pattern I (51.9%) followed by pattern II (29.6%). The majority of the herds showed infection with one single pulsotype, but 12 herds yielded 2 pulsotypes, and one herd had 3 pulsotypes. No relationship was found between the geographical location of the herd and the pulsotype. This is the first description of a typing method for *C. bovis*; the technique might prove useful for epidemiological studies on this species. (**Key words:** *Corynebacterium*, *Corynebacterium bovis*, mastitis, pulsed-field gel electrophoresis)

Abbreviation key: PFGE = pulsed-field gel electrophoresis, TE = Tris/HCl-EDTA.

Corynebacterium bovis is the most frequently isolated coryneform from bovine mammary glands (Watts, 1988). Many bacterial pathogens can infect the bovine mammary gland, and *C. bovis* is considered a minor pathogen by many researchers despite its increasing prevalence (Hillerton, 2000). Although some authors describe *C. bovis* as a cause of clinical bovine mastitis (Boyer, 1998), there is much debate about the contribu-

tion of this bacterium to the disease and its virulence properties (Lam et al., 1997; Schukken et al., 1999). *Corynebacterium bovis* is also a sporadic, opportunistic agent of disease in humans (Bernard et al., 2002). Given the controversy surrounding the role of *C. bovis* in the epidemiology of bacterial mastitis, it is important to determine whether there are differences between *C. bovis* isolates that might be related to special virulence properties or protection against other mammary pathogens. Here, we propose a pulsed-field gel electrophoresis (PFGE) method for the typing of *C. bovis* isolates. Some authors consider PFGE as the “gold standard” among molecular typing methods for bacteria (Singleton, 2000), and PFGE has been used to type numerous pathogenic microorganisms including other species of *Corynebacterium* such as *C. diphtheriae* (De Zoysa et al., 1995) and *C. pseudotuberculosis* (Connor et al., 2000).

We used this method to test 162 isolates from bovine milk with high SCC from 57 herds in Northern Spain. All isolates had been identified as *C. bovis* by the API Coryne system (BioMérieux, France) and by endonuclease restriction analysis of the 16S rRNA gene (Huxley et al., 2004). None of the 162 isolates showed a distinct restriction pattern from the reference strain *C. bovis* ATCC 7715.

Bacteria were grown in 2 mL of trypticase soy broth supplemented with 10% horse serum and 0.5% glycine until OD₆₀₀ = 1. Cells were harvested by centrifugation, resuspended in 0.5 mL of Tris/HCl-EDTA (TE) buffer (10 mM Tris/HCl, 1 mM EDTA, pH = 8.0), and then mixed with an equal volume of 1% (wt/vol) low-melting point agarose (Bio-Rad, Hercules, CA) at 40°C. Eighty microliters of the mixture was allowed to solidify in each of 5 plastic plug molds. Plugs were incubated for 24 h at 37°C in 2 mL of lysis solution [1 M NaCl, 10 mM Tris/HCl, 0.5% sodium deoxycholate, 0.25 M EDTA, and 1 mg/mL of lysozyme (Sigma, St. Louis, MO)], followed by 24 h at 50°C in 2 mL of proteinase solution made up of 1 µg/mL of proteinase K (Sigma) in 0.25 M EDTA, 1% Sarkosyl. Plugs were subsequently washed in TE 4

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Table 1. Details of *Corynebacterium bovis* pulsed-field gel electrophoresis *Xba*I pulsotypes.

Pulsotype	Fragments (no.)	Approximate genome size (kb) ¹	Number of strains (%)	Number of herds (%)	Other pulsotypes in same herd ²
I	11	2902	84 (51.9)	36 (63.2)	II (5), III (1), IV (4), V (1)
II	12	3099	48 (29.6)	13 (22.8)	I (5), IV (2)
III	14	3535	5 (3.1)	5 (8.8)	I (1), VI (2)
IV	9	2991	13 (8.0)	10 (17.5)	I (4), II (2)
V	9	2217	3 (1.9)	1 (1.8)	I (1)
VI	10	3015	8 (4.9)	5 (8.8)	III (2)
VII	9	2102	1 (0.6)	1 (1.8)	None

¹Approximate genome sizes for each pulsotype were obtained by adding up the size of all its pulsed-field gel electrophoresis fragments.

²Twelve herds had 2 different pulsotypes, and one herd had 3 pulsotypes (I, II, and IV).

times for 30 min each and kept at 4°C in TE until further analysis. One-half of each plug was preincubated in 100 μ L of enzyme buffer for 1 h at room temperature and then digested with 30 units of *Xba*I (Roche, Switzerland) at 37°C for 4 h in a final volume of 100 μ L. Digested DNA was subjected to electrophoresis using a CHEF-DR II apparatus (Bio-Rad) at 15°C for 24 h at 6 V/cm with pulse times of 10 to 40 s (linear ramp). Concatamers of bacteriophage lambda DNA were used as molecular size standards. The gels were stained with 0.5 μ g/mL of ethidium bromide and photographed under ultraviolet light. The reproducibility of the PFGE protocol was verified by processing multiple batches of some *C. bovis* isolates and the type strain ATCC 7715. Numerical analysis of banding patterns was performed using the Quantity One software (Bio-Rad). The similarity between all pairs was assessed using the Dice coefficient correlation; UPGMA (unweighted pair group method) clustering was used to construct a dendrogram.

Figure 1. Dendrogram illustrating the genetic relatedness of the 7 pulsotypes found in 162 *Corynebacterium bovis* isolates and the type strain of *C. bovis* ATCC 7715. The scale indicates similarity. PFGE = Pulsed-field gel electrophoresis.

Pulsed-field gel electrophoresis of *Xba*I-restricted isolates generated 7 pulsotypes, each containing 9 to 14 restriction fragments ranging from 20 to 600 kb in size (Table 1; Figure 1). The most frequent pulsotype was pattern I (51.9%; 84 isolates) followed by pulsotype II (29.6%; 48 isolates). The rest of the pulsotypes (III to VII) contained between 1 and 13 isolates each. None of the pulsotypes resembled that of *C. bovis* ATCC 7715. This could be explained by the different origin of the reference strain (American) and this study's isolates (European). However, no relationship was found between the geographical location of the herd and pulsotype among our isolates. Isolates from only 12 of the 57 herds yielded 2 different pulsotypes, whereas just 1 herd had 3 pulsotypes (I, II, and IV) isolated (Table 1). The finding that mixed infections are relatively rare in dairy herds suggests that pulsotype information can be a valuable epidemiological tool that deserves further investigation to clarify the real implications of *C. bovis* infection in mammary disease.

The average genome size of *C. bovis* was estimated to be 3 Mbp by adding up the sizes of the linear fragments in each pulsotype. This is similar to the genome sizes of other *Corynebacterium* species such as *C. glutamicum* (3.31 Mbp; Correia et al., 1994) and *C. diphtheriae* (2.49 Mbp; Cerdano-Tarraga et al., 2003). However, further analysis using more restriction enzymes is necessary to confirm this estimation of the *C. bovis* genomic size and to increase the discriminatory power of the PFGE technique.

In summary, we propose a method to type *C. bovis* isolates. This PFGE-based protocol clearly classified 162 *C. bovis* isolates from 57 dairy herds into 7 pulsotypes. This method provides information that may be very useful in the study of the epidemiology of *C. bovis* mastitis and other pathological conditions involving this species.

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