Outbreak of Subclinical Mastitis in a Flock of Dairy Sheep Associated with *Burkholderia cepacia* Complex Infection

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An outbreak of subclinical mastitis in a flock of 620 milking sheep was investigated. Microbiological and epidemiological analyses identified the causative agent as belonging to the *Burkholderia cepacia* complex (formerly *Pseudomonas cepacia*). Every ewe in the milking flock was individually tested for subclinical mastitis on two separate occasions, 6 weeks apart, by the California (rapid) mastitis test (CMT). The proportion of CMT-positive ewes was 69 of 393 (17.6%) on the first sampling and 27 of 490 (5.5%) on the second sampling. Pure *B. cepacia* cultures identified with the API 20 NE system were grown from 64 of 96 (66.7%) CMT-positive ewes and from 1 of 33 (3.0%) CMT-negative ewes. Statistical analysis confirmed the significant association between a positive CMT result and a positive culture result for *B. cepacia* complex. Additional polyphasic taxonomic analyses of eight isolates showed that seven belonged to *B. cepacia* genomovar III; the remaining isolate was identified as *Burkholderia vietnamiensis* (formerly *B. cepacia* genomovar V). Bacteriological investigation of samples from milking equipment and other environmental sites failed to identify “*B. cepacia*” in any of the samples taken. To our knowledge, this is the first report of an outbreak of natural infection in animals caused by *B. cepacia* complex and the first description of *B. cepacia* complex infection in sheep.

*Burkholderia cepacia* complex isolates are gram-negative, non-spore-forming, aerobic bacilli whose primary habitats include river sediments and soil, particularly the plant rhizosphere (13, 22). Originally described as the cause of soft rot of onions (3), members of this highly adaptable and diverse group of bacteria have emerged as important life-threatening, opportunistic human pathogens, particularly in individuals with cystic fibrosis (CF) (13, 27), patients with chronic granulomatous disease (24, 43), and patients who require intensive care (18). The inherent resistance of most *B. cepacia* complex isolates to the major groups of antibiotics also deprives infected patients of effective antimicrobial therapy. In addition, person-to-person transmission of highly transmissible strains through nosocomial or social contacts (13, 45) has led to the use of stringent infection control measures, including segregation of colonized individuals. Ironically, in contrast to its pathogenic role in plants and humans, interest in *B. cepacia* is also being generated by agricultural applications, in particular, the use of these metabolically active bacteria in bioremediation and biological control (14, 17, 22). As a consequence, government regulatory agencies are presently faced with Solomon-like judgments that require them to balance the biotechnological use of *B. cepacia* with the potential hazards to the CF community, agricultural workers, and the wider human population (50; J. J. LiPuma, *Burkholderia cepacia* Research Laboratory and Repository, International *Burkholderia cepacia* Working Group, http://allserv.rug.ac.be/~tcenyc/). The taxonomy and identification of *Burkholderia*-like organisms are complex and present diagnostic laboratories with problems (2). Integrated genotypic and phenotypic analyses (6, 52) have shown that isolates presumptively identified as *B. cepacia* comprise at least six genomovars (49) which together are referred to as the *B. cepacia* complex (52). All members of the complex have been cultured from humans with infections; however, most epidemic outbreaks are associated with *B. cepacia* genomovar III or *Burkholderia multivorans* (29, 30, 52).

There have been few reports of *B. cepacia* infection in veterinary medicine. Several murine models of *B. cepacia* infection have been developed (8, 42, 44), and *B. cepacia* has been reported in mixed culture with other bacteria in clinical specimens from horses with pneumonia (7) and in specific-pathogen-free piglets (40) and as the sole organism involved in a case of vegetative endocarditis in a horse (47). In this report, we describe an epidemic outbreak of subclinical mastitis associated with infection by the *B. cepacia* complex in a large flock of dairy sheep.

MATERIALS AND METHODS

Study population. The outbreak of subclinical mastitis was detected in the 1999–2000 milking season in a flock of 620 dairy sheep of the lataxa breed run by a full-time professional sheep farmer. Sheep lambed indoors in two separate lambing peaks, with 78% (362 of 467) of young home-reared replacement ewes lambing in February or March 2000. After lambing, the ewes were machine milked twice daily. The milking routine in the flock does not include premilking teat cleaning or disinfection, but the teats are disinfected postmilking with an iodine-based dip solution. Milk from each day’s milking is pooled, stored in a refrigerated bulk milk tank, and used unpasteurized on the same day on the farm to produce mature cheese. Every 2 to 4 weeks from January until the end of lactation, the somatic cell count (SCC) in milk samples from the bulk tank was measured as a flock-level check for mastitis (36) by the local Cheese Producers Association. The SCC measures the number of white blood cells and udder squamous epithelial cells in milk, and individual counts over 500,000 somatic...
cells/ml indicate mastitis (1, 10). At the end of lactation, on average, 5 months after lambing, every ewe is systematically administered an intramammary slow-release antibiotic preparation (“dry-ewe” antibiotic preparation) to treat chronic mastitis and prevent infections during the dry period (36). In the 1998–1999 milking season, the dry-ewe antibiotic preparation used contained ampicillin trihydrate and cloxacillin benzoxate.

Outbreak investigation. The initial investigation began after the first three consecutive analyses of bulk tank milk had yielded SCCs in excess of 10^9 somatic cells/ml. These counts represented two- to threefold increases from the last three bulk tank SCCs measured during the previous milking season. At the end of February 2000, the farmer examined the udders of all 393 milking ewes and performed a California (rapid) mastitis test (CMT) on individual milk samples collected from each half-udder (right and left side of the mammary gland) of every sheep to detect subclinical mastitis (11, 36). The CMT gives an indirect estimate of the SCCs and it is based upon a gelling reaction between the nucleic acid of the cells and a detergent reagent. Depending on the amount of gel formation, samples are assigned to five categories: negative, a doubtful score, or a reaction is grade 1, 2, or 3. A grade 1 CMT reaction suggests that mastitis is present, and grade 2 and 3 reactions indicate that there is a high probability that infection is present (36). In the present study samples with CMT grade 1, 2, or 3 reactions were considered CMT positive.

A milk sample was collected for microbiological analysis from each of 69 (17.6%) CMT-positive ewes, none of which had macroscopic signs of inflammation of the udder. Initially, no record was kept of the number of CMT-positive half-udders in each ewe, and a CMT-positive ewe was considered an animal whole, regardless of samples, taken from one or both half-udders, gave a positive CMT reaction. For ewes for which milk samples from both half-udders were CMT positive, a mixture of milk from both half-udders was used. At this time, all CMT-positive sheep were clustered and milked after milking of the CMT-negative ewes to reduce the risk of disease transmission during milking. This strategy was associated with a temporary decrease in the bulk tank SCC to 580,000 somatic cells/ml; however, by the end of March the SCC again rose to over 10^9 cells/ml.

In April, 6 weeks after the first milk samples had been collected, CMTs were performed with milk from 490 ewes, including all previously CMT-negative ewes and other ewes that had lambed and joined the milking flock after the first sampling. On this occasion, a further 27 (5.5%) ewes proved to be CMT positive, totaling 40 (4.1%) CMT-positive half-udders. None of the CMT-positive half-udders had visible signs of inflammation. Among the CMT-positive samples, 47.5% (19 of 40) gave a grade 3 CMT reaction, 25% (10 of 40) gave a grade 2 CMT reaction, and 27.5% (11 of 40) gave a grade 1 CMT reaction. Milk samples were collected from each half-udder of all 27 CMT-positive ewes and from each half-udder of 33 randomly selected CMT-negative ewes.

In an attempt to identify possible environmental sources of B. cepacia complex, samples were collected from various sites on the farm. Twenty-six specimens included swabs from the milking machinery, chlorinated and nonchlorinated water supplies, sheep feed (cereal-based concentrates and hay), sheep feces, teat-dip solution, and dry-ewe antibiotic tubes of the same batch as those used in the 1998–1999 milking season.

Bacteriology. All milk samples were cultured fresh on sheep blood agar plates and were incubated aerobically at 35°C for 7 days. Milk samples were stored frozen, and at a later stage, samples from the second sampling were defrosted and similarly cultured under anaerobic conditions. Investigations for the detection of B. cepacia complex in environmental specimens used the enrichment and selective media described previously (4, 5).

Bacterial identification. Cultures were visually examined, and bacteria from all colony types were Gram stained. Presumptive identification of B. cepacia complex was performed with the API 20 NE multistest system (BioMérieux, Marcy l’Étoile, France). Further means of phenotypic and genotypic identification of B. cepacia complex isolates included whole-cell fatty acid analysis, protein analysis, rcd4-based PCR assays, and restriction fragment length polymorphism (RFLP) analyses by procedures described previously (30, 35, 51–53).

Genome macrorestriction analysis. Pulsed-field gel electrophoresis (PFGE) of genomic DNA restricted with XbaI (TCTAGA) was performed as described previously (4).

Epidemic strain markers. The presence of the cable pilus subunit gene (cblA) (37) and the epidemic strain marker BcESM (28) was determined by PCR with established primers.

Antibiotic susceptibility. The B. cepacia complex isolates were tested for their susceptibilities to ampicillin and oxacillin by disk tests to examine the potential resistance of isolates to the dry-ewe antibiotic therapy used in the 1998–1999 milking season.

<table>
<thead>
<tr>
<th>Sampling</th>
<th>Ewe or sample</th>
<th>CMT positive and B. cepacia:</th>
<th>CMT negative and B. cepacia:</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>First</td>
<td>Ewes</td>
<td>41</td>
<td>28</td>
<td>69</td>
</tr>
<tr>
<td></td>
<td>Samples</td>
<td>41</td>
<td>28</td>
<td>69</td>
</tr>
<tr>
<td>Second</td>
<td>Ewes</td>
<td>23</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Samples</td>
<td>33</td>
<td>7</td>
<td>2</td>
</tr>
<tr>
<td>Total</td>
<td>Ewes</td>
<td>64</td>
<td>32</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Samples</td>
<td>74</td>
<td>35</td>
<td>2</td>
</tr>
</tbody>
</table>

Phytopathogenic analysis. A phytopathogenicity test was performed with the B. cepacia complex isolates to determine if the bacteria possessed the characteristic ability to induce onion soft rot. Briefly, onion segments were inoculated with approximately 10^5 CFU, incubated in a moist chamber, and observed for tissue maceration as described previously (13).

Statistical analysis. The bivariate relationship between the results of the CMT and those of culture of the B. cepacia complex was investigated by calculating Yates chi-square test and the odds ratio (OR) and 95% confidence intervals (CI) with the epidemiological software EpiInfo (version 6.04; Centers for Disease Control and Prevention, Atlanta, Ga.). Unconditional logistic regression in the statistical software EGRET was then used to adjust the estimated OR for confounding effects by extraneous variables (23). In these analyses, CMT status was the response variable, with two possible outcomes (negative or positive), and the explanatory variables considered to be fixed effects were the presence or absence of B. cepacia complex and other bacteria in the milk sample and ewe-related variables including month of lambing (four levels: November, December, January–February and March–April) and number of lactation periods (three intervals: one, two to four, and five or more). Variables that changed the estimate of the regression coefficient of the variable “B. cepacia complex” by more than 5% were considered important confounders and were included in the models. In addition, to account for the potential correlation between measurements taken from half-udders belonging to the same animal (26, 32), the variable “ewe,” with one level for each ewe, was included in the logistic models as a random effect. For all comparisons made in this study, the critical probability was taken as 5% (P < 0.05) for a two-sided test.

RESULTS

Bacterial cultures and biochemical identification of B. cepacia complex. Only slowly growing, bright yellow colonies were observed in cultures from 76 of 189 (40.2%) milk samples from 65 ewes (Table 1). The bacteria were negative by Gram staining. Biochemical tests (analysis with the API 20 NE system) carried out with 63 isolates from 63 of 76 (82.9%) samples containing similar gram-negative cells identified the bacteria in all cases as B. cepacia (discrimination level, >99.0%). Isolates from the remaining 13 of 76 samples were not collected and tested with the API 20 NE system. Among the B. cepacia isolates tested, eight were selected for further biochemical and molecular taxonomic analyses. These included five isolates from five samples for which colonies looked slightly paler than those on other samples or for which colonies had irregular edges. In addition, three isolates were randomly selected from the remaining samples. Fatty acid methyl ester analysis with the MIDI system identified seven isolates as B. cepacia, with identification scores ranging between 0.629 and 0.875; one isolate remained unidentified (its highest identification score was 0.051, toward Burkholderia gladioli). Whole-cell protein electrophoresis generated identical protein profiles for seven
One isolate had a clearly distinct profile (data not shown) and was identified as *Burkholderia vietnamiensis* by using a database comprising over 2,000 protein profiles representing all presently known *Burkholderia*, *Ralstonia*, and *Pandoraea* species. The profile that was common to the remaining seven isolates resembled those of *B. cepacia* genomovar I and III strains, but discrimination at the genomovar level was equivocal. Subsequent analyses of the eight isolates by *recA*-based PCR tests and RFLP analyses confirmed the identification of the one isolate as *B. vietnamiensis* and indicated that the remaining isolates belonged to *B. cepacia* genomovar III.

For the first sampling of milk samples from CMT-positive ewes, bacteria other than *B. cepacia* complex were cultured from 11 milk samples; these included coagulase-negative staphylococci (eight samples), *Staphylococcus aureus* (two samples), and streptococcal species (one sample). None of these samples produced growth of *B. cepacia* complex bacteria.

Finally, no growth was observed in the anaerobic cultures, and *B. cepacia* complex was not cultured from any of the environmental samples taken from the farm.

**Genome macrorestriction analysis.** Genomic fingerprinting of the eight random isolates by PFGE showed that all seven genomovar III isolates shared the same DNA fragment pattern after their DNA was cut with the *XbaI* restriction enzyme. The PFGE pattern obtained with the single *B. vietnamiensis* isolate was different.

**Epidemic strain markers.** None of the eight isolates showed evidence of the *cblA* or BCESM markers.

**Antibiotic susceptibility tests.** Standard disk antibiograms carried out with 20 *B. cepacia* complex isolates selected by simple random sampling showed that all isolates were fully resistant to both ampicillin and oxacillin.

**Phytopathogenicity.** The same eight samples used for molecular taxonomic analysis were used for the phytopathogenicity test, and all eight isolates produced maceration of onion samples within 7 days, typical of most taxa within the *B. cepacia* complex (13).

**Relationship between CMT results and culture for *B. cepacia* complex.** Among the 76 samples with *B. cepacia* complex colonial morphotypes, 41 and 35 samples were taken from 65 ewes on the first and second sampling occasions, respectively. The 41 samples taken at the first sampling belonged to 41 of 69 (59.4%) CMT-positive ewes (Table 1). The remaining 35 samples from the second sampling were obtained from 33 of 40 (82.5%) CMT-positive half-udders from 23 ewes and from 2 of 80 (2.5%) CMT-negative half-udders belonging to 1 ewe (Table 1). A CMT-positive result was significantly associated with a positive *B. cepacia* complex culture result (*P < 0.01*). Among the samples taken at the second sampling, which belonged to individual half-udders, the unadjusted OR (exact 95% CI) for *B. cepacia* complex culture was 183.9 (32.8 to 1709) (*Yates chi-square; 78.8; *P < 0.001*). All *B. cepacia* culture-positive samples and all except one of the samples with CMT-positive results were from ewes on their second or later lactation. Among animals that were at least in the second lactation period, *B. cepacia* complex-positive cultures were not associated with the stage and number of lactations or month of lambing (*P > 0.05*). In the multivariable regression analysis of the relationship between the results of CMT and culture of *B. cepacia* complex, the month of lambing was an important confounding variable and was included in the models as a fixed effect. The results of these analyses confirm the association between a positive CMT result and culture of *B. cepacia* complex bacteria. The estimated ORs (95% CIs) for the *B. cepacia* complex after adjusting for month of lambing were 650 (56.2 to 7538) in the fixed-effects model and 59,006 (1.98 × 10^9 to 1.8 × 10^10) in the random-effects model (Table 2).

**DISCUSSION**

This first report of the association of *B. cepacia* complex bacteria with ovine subclinical mastitis has important implications for veterinary and human medicine and draws attention to the potential hazards of bacterial biopesticides and bioremediants based on the *B. cepacia* complex.

The presence of high SCCs in milk is strongly associated with reduced milk quality and reduced levels of production (36). The inherent resistance of isolates of the *B. cepacia* complex to most antibiotics also limits the options for the treatment of infected animals which, in turn, leads to further losses due to culling of infected animals. During the outbreak described here, attempts were made to identify the origin of the infection. Since no animals had recently been introduced into the flock, it is likely that ewes became infected from an environmental source. Furthermore, strains of the *B. cepacia* complex were isolated in milk samples from ewes with at least one previous lactation and not in milk from first-lactation ewes. It is possible that only sheep with two or more lactation periods were originally exposed to infection. Alternatively, all ewes may have been exposed to isolates of the *B. cepacia* complex,

<table>
<thead>
<tr>
<th>Variable</th>
<th>Random-effects model</th>
<th>Fixed-effects model</th>
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<tbody>
<tr>
<td></td>
<td>OR</td>
<td>95% CI</td>
</tr>
<tr>
<td><em>B. cepacia:</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absent</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Present</td>
<td>59,006</td>
<td>1.98–1.8 × 10^9</td>
</tr>
<tr>
<td>Month of lambing:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>November</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>December</td>
<td>0.89</td>
<td>0.01–75.27</td>
</tr>
<tr>
<td>January–February</td>
<td>0.04</td>
<td>&lt;0.01–3.71</td>
</tr>
<tr>
<td>April–March</td>
<td>0.05</td>
<td>&lt;0.01–5.51</td>
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</tbody>
</table>

TABLE 2. ORs and 95% CIs of the relationship between CMT and *B. cepacia* culture results adjusted for month of lambing for models that include ewe as a random effect and that do not include ewe in the model (fixed effect).
but only those with one or more previous lactation periods were susceptible to infection. If original exposure was limited to ewes with at least one previous lactation, a possible source of infection may have been the dry-ewe antibiotic therapy used in 1998–1999, to which the isolates examined were resistant. Indeed, outbreaks of *Pseudomonas aeruginosa* mastitis in cows have been associated with the use of a dry-cow antibiotic infusion suspected of contamination (33). Alternatively, dry-ewe mastitis may not have been the source of infection but could have contributed to the selection of the *B. cepacia* complex in this population.

Isolates of the *B. cepacia* complex were not cultured from the dry-ewe antibiotic preparation or the wide range of potential sources of infection examined. This may reflect a lack of sensitivity of the culturing methods used or, alternatively, may indicate that the source of infection was not present at the time that the samples were taken. The former reason seems unlikely since the selective media and culture conditions used had previously been used successfully to culture *B. cepacia* complex isolates from natural environments (4). The failure to culture these organisms from environmental sites during epidemic outbreaks of human infection is well documented (12, 28). In the outbreak described here, initial acquisition from a natural environment is plausible. Clonality between environmental and human isolates of *B. cepacia* genomovar I has been shown (15). Furthermore, *B. vietnamiensis* has been widely reported in soils, and a similar habitat for *B. cepacia* genomovar III has recently been confirmed (15). Whatever the source, once introduced into the flock, it is possible that *Burkholderia* strains were transmitted to other previously noninfected animals through the milking process. These and other epidemiological features of the outbreak, including a more representative study of the frequencies of *B. cepacia* genomovars from a wider selection of *B. cepacia* complex isolates and the relative roles of different genomovars in mastitis, are being investigated.

Given the difficulties in culture and identification of members of the *B. cepacia* complex (16, 39), the role of these opportunistic pathogens in veterinary medicine might be underestimated. As evidence, in a controlled study involving 115 North American CF centers, only 36 (31%) successfully cultured the organism from sputum (46). More than a decade later, among 485 isolates referred to a North American laboratory as “unidentified” or “other species,” 174 (36%) were identified as *B. cepacia* complex isolates (J. J. LiPuma, *Burkholderia cepacia* Research Laboratory and Repository [see above]). It is relevant that this high level of false-negative results was for samples referred from microbiologists and clinicians working with CF patients, namely, individuals who were actively seeking the organism.

The medical significance of *B. cepacia* complex ovine mastitis merits comment. This outbreak confirms previous reports of the detection of *B. cepacia* in raw milk (48) and cheese (41), indicating the possibility of food-borne spread to susceptible humans. The involvement of *B. cepacia* genomovar III in seven of the eight samples analyzed at the genomovar level is particularly interesting. There is much medical and agricultural interest in the pathogenic potential of individual taxa and in individual strains of the *B. cepacia* complex (50). All members of the complex have been cultured from humans with infections; however, most epidemic outbreaks are associated with strains identified as *B. multivorans* or *B. cepacia* genomovar III (29, 30, 52).

The pathogenic mechanisms that could account for the inflammatory reactions and cell desquamation associated with *B. cepacia* infection require investigation. Recent reports have shown that *B. cepacia* J2315, which represents a virulent and transmissible genomovar III lineage, ET12, produces a hemolysin that induces apoptosis and degranulation of mammalian phagocytes (20). This strain has been shown to enter and survive in macrophages and pulmonary epithelial cells (31) and within free-living amoebae (25; E. Berriatu, I. Zihaga, C. Miguel-Virto, P. Uribarren, R. Juste, S. Laevens, P. Vandamme, and J. R. W. Govan, unpublished). Whole cells and lipopolysaccharide (LPS) from *B. cepacia* complex bacteria show potent endotoxocities. For example, they induce proinflammatory cytokines from human monocytes at levels 10 times greater than the levels of proinflammatory cytokines induced by *P. aeruginosa* (9, 21, 38, 54). *B. cepacia* complex LPS also increases neutrophil surface expression of a β2 integrin, complement receptor 3, and primes neutrophil respiratory burst responses to inflammatory stimuli (19). Cell extracts have also been shown to induce interleukin-8 from human epithelial cells (34). It is tempting to speculate that intracellularity and LPS-induced inflammation, exacerbated by mechanical milking, play a role in *Burkholderia*-associated ovine mastitis and the increased level of shedding of somatic cells into milk.

Further investigations of *B. cepacia* complex-associated ovine mastitis could provide further insights into the pathogenic potential of these bacteria and lead to useful in vitro and in vivo models for investigation of the virulence potential of different taxa within the *B. cepacia* complex.

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