Short communication: Genetic characterization of antimicrobial resistance in *Acinetobacter* isolates recovered from bulk tank milk

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ABSTRACT

A total of 176 *Acinetobacter* isolates, including 57 *Acinetobacter baumannii* originally obtained from 2,287 bulk tank milk (BTM) samples in Korea was investigated for the genetic basis of antimicrobial resistance using molecular methods. In addition, the occurrence and cassette content of integrons were examined and the genetic diversity of *A. baumannii* strains identified was evaluated. Aminoglycoside-modifying enzyme genes were detected in 15 (88.2%) of the 17 aminoglycoside-resistant *Acinetobacter* isolates tested. The most common aminoglycoside-modifying enzyme gene identified was adenylyltransferase gene *aadB* ($n = 9$), followed by phosphotransferase genes *aphA6* ($n = 7$) and *aphA1* ($n = 5$). Of the 31 isolates resistant to tetracycline, *tet*(39) was detected in 20 of them. The genetic basis of resistance to sulfonamide was identified in 15 (53.6%) of 28 trimethoprim-sulfamethoxazole-resistant isolates and 9 (32.1%) of them carried both *sul1* and *sul2* genes. A *bla*_{ADC-7-like} gene was detected in 1 β -lactam-resistant *A. baumannii*. Furthermore, class 1 integron was identified in 11 *Acinetobacter* isolates. Two gene cassettes *dfrA15*, conferring resistance to trimethoprim, and *aadA2*, conferring resistance to aminoglycosides, were identified in 8 *Acinetobacter* isolates. None of the isolates was positive for class 2 or class 3 integrons. Pulsed-field gel electrophoresis revealed that most of the *A. baumannii* strains from BTM samples were genetically diverse, indicating that the occurrence of *A. baumannii* strains in BTM was not the result of dissemination of a single clone. Elucidation of resistance mechanisms associated with the resistance phenotype and a better understanding of resistance genes may help in the development of strategies to control infections, such as mastitis, and to prevent further dissemination of antibiotic resistance genes. To the best of our knowledge, this is the first report of molecular characterization of antimicrobial-resistant *Acinetobacter* spp. from milk.

Key words: bulk tank milk, *Acinetobacter*, tetracycline resistance gene *tet*(39), class 1 integron

Short Communication

Milk is not only an important source of nutrients for humans (Sharma and Joshi, 1992) but also an ideal growth medium for bacteria (Henry and Newlander, 1997). A wide variety of pathogenic as well as commensal bacteria have been isolated from bulk tank milk (BTM), which can become contaminated with bacteria from various sources, including teat and udder surfaces, infected mammary glands, milking machines, transporting systems, contaminated water, and the dairy farm environment (Thomas and Thomas, 1973; Jayarao and Wang, 1999; Straley et al., 2006). Ironically, the use of antimicrobials in dairy herds, such as for mastitis treatment, has led to an increase in the isolation rate of antimicrobial-resistant bacteria from raw BTM, which is a matter of public health concern (Straley et al., 2006).

Acinetobacter species are strictly aerobic, gram-negative, nonfermenting bacteria that are ubiquitous in nature (Bergogne-Bérézín and Towner, 1996). Among the currently known genomic species, *Acinetobacter baumannii* is the most prevalent species in clinical specimens (Dijkshoorn et al., 2007). Other *Acinetobacter* spp., including *Acinetobacter pittii* (formerly *Acinetobacter* genomic species 3), *Acinetobacter nosocomialis* (formerly *Acinetobacter* genomic species 13TU), *Acinetobacter lwoffii* (genomic species 9), *Acinetobacter ursingii*, *Acinetobacter johnsonii*, and *Acinetobacter parvus*, have also been found to be clinically relevant (Dijkshoorn et al., 2007; Nemec et al., 2011). *Acinetobacter baumannii* causes a significant proportion of nosocomial infections in severely ill patients in intensive care units or immunocompromised individuals (Bergogne-Bérézín and Towner, 1996; Villegas and Hartstein, 2003; Peleg et al., 2008). The organism has emerged as an important cause of community-acquired pneumonia and other infections (Chen et al., 2001). Furthermore, clinical roles of non-*A. baumannii* species are increasingly being recognized worldwide (Karah et al., 2011).

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Acinetobacter spp. have rarely been reported from food animals or animal products for human consumption (Hamouda et al., 2011). However, infections due to *A. baumannii* have been reported in pets and horses from Switzerland in which the majority of the infections were hospital acquired and in animals with predisposing conditions. Moreover, those authors found that these *A. baumannii* strains shared the sequence types and molecular background of resistance with those described in humans (Endimiani et al., 2011). Thus, the animals or their products, or both, could serve as reservoirs of antimicrobial-resistant *A. baumannii* for humans. A better understanding of the basis of antibiotic resistance mechanisms of antimicrobial-resistant *Acinetobacter* associated with food animals or their product may help in the development of therapy regimens and treatment of the disease.

Although much has been reported regarding the genetic characteristics of *A. baumannii* or *Acinetobacter* spp. isolated from nosocomial or community-acquired infections, little is known about *Acinetobacter* isolates from milk. Recently, we reported the prevalence and antimicrobial susceptibility of *Acinetobacter* spp. from raw BTM samples from Korea (Gurung et al., 2013). Because a detailed genetic analysis of these isolates was not performed, data regarding *Acinetobacter* spp. from BTM are still rare. Therefore, the present study was undertaken to investigate the genetic basis of antimicrobial resistance in *Acinetobacter* isolates recovered from BTM samples and to determine the clonal relatedness of the *A. baumannii* strains identified.

A total of 176 *Acinetobacter* isolates, including 57 *A. baumannii* strains, originally recovered from 2,287 BTM samples from various dairy farms located in 6 provinces of Korea during the period between November 2011 and April 2012 (Gurung et al., 2013) were included in this study. The collection of BTM samples and isolation, identification, and phenotypic characterization of *Acinetobacter* isolates were described extensively in our previous report (Gurung et al., 2013).

The presence of the following genes was determined by PCR amplification: the genes encoding aminoglycoside-modifying enzymes *aacC1*, *aacC2*, *aacA4*, *aphA1*, *aphA6*, *aadB*, and *aadA1* (Clark et al., 1999; Noppe-Leclercq et al., 1999; Vila et al., 1999); the genes encoding 16S rRNA methylase *armA*, *rmtA*, *rmtB*, *rmtC*, and *rmtD* (Doi et al., 2004; Yan et al., 2004; Wachino et al., 2006; Doi et al., 2007); tetracycline resistance genes *tet(A)*, *tet(B)*, *tet(C)*, *tet(H)*, *tet(M)*, and *tet(39)* common in *Acinetobacter* (Sengeløv et al., 2003; Agersø and Petersen, 2007); the genes encoding sulfonamide resistance *sul1*, *sul2*, and *sul3* (Agersø and Petersen, 2007); the genes encoding β -lactamases *bla*_{TEM}, *bla*_{SHV}, *bla*_{CTX-M}, and *bla*_{PER-1} (Batchelor et al., 2005; Lee et

al., 2008; Rayamajhi et al., 2008); the *Acinetobacter*-derived cephalosporinase *bla*_{ADC-7-like} gene encoding class C β -lactamase (Hujer et al., 2005); and the genes encoding class D β -lactamases OXA-23-like, OXA-24-like, OXA-51-like, and OXA-58-like (Woodford et al., 2006).

Polymerase chain reaction amplification of class 1 (*intI1*), class 2 (*intI2*), and class 3 (*intI3*) integrase genes was performed using primer sets and conditions, as described previously (Machado et al., 2005). Class 1 integron cassette regions were amplified using primer pairs Att-1-F and 3'CS-R, as described previously (Agersø and Petersen, 2007). To analyze the sequences of the gene cassette regions of the integrons, the purified PCR products were directly sequenced in both directions by the dideoxy-chain termination method using an automated ABI Prism 3700 analyzer (Applied Biosystems Inc., Foster City, CA). The sequences thus analyzed were compared with the sequences in GenBank using the BLAST program at the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/BLAST>).

Pulsed-field gel electrophoresis (PFGE) was performed as previously described, with slight modification (Seifert et al., 2005). For PFGE pattern analysis, BioNumerics software (version 4.0; Applied Maths NV, Sint-Martens-Latem, Belgium), with the unweighted-pair group method using average linkages (UPGMA) and the Dice setting for clustering analysis was applied to produce a dendrogram.

Aminoglycoside resistance in *Acinetobacter* spp. mostly involves production of aminoglycoside-modifying enzymes (AME; phosphotransferase, adenyltransferase, and acetyltransferase) and all 3 classes of AME are widespread in *Acinetobacter* spp. (Hujer et al., 2006). In the current study, investigation of 17 aminoglycoside-resistant isolates for underlying resistance mechanisms revealed 6 different AME genes in various combinations in the 15 isolates. Eight (47.1%), 4 (23.5%), and 3 (17.6%) isolates had 1, 2, and 3 AME genes, respectively. The most common AME gene identified was adenyltransferase gene *aadB* (n = 9), followed by phosphotransferase genes *aphA6* (n = 7) and *aphA1* (n = 5). Other genes encoding AME included the acetyltransferase genes *aacC1* (n = 2), *aacC2* (n = 1), and adenyltransferase gene *aadA1* (n = 1). The 16S rRNA methylase genes *armA*, *rmtA*, *rmtB*, and *rmtC* were not detected in any *Acinetobacter* isolate tested. Our results are consistent with earlier reports showing that the AME genes identified in this work are common in *Acinetobacter* resistant to aminoglycosides (Hujer et al., 2006). Moreover, Cho et al. (2009) reported high prevalence of *armA*, *aadA1*, *aphA1*, *aacC1*, *aacA4*, and *aphA6* determinants among human clinical

Acinetobacter spp. in Korea. The resistance phenotypes and genotypes were correlated in all but 2 aminoglycoside-resistant *Acinetobacter* isolates in the current study. These 2 isolates were negative for any type of the studied 16S rRNA methylase and AME genes and may carry other resistance mechanisms against aminoglycosides, such as impermeability of the drugs, efflux pumps, or other rare types of AME.

Of the total 31 isolates resistant to tetracycline, *tet*(39) was detected in 20 (64.5%) of them. Other tetracycline resistance determinants *tet*(A), *tet*(B), *tet*(C), *tet*(H), and *tet*(M) sought were not identified in any isolate. So far, *tet*(A), *tet*(B), *tet*(H), *tet*(M), and *tet*(39) determinants have been described for *Acinetobacter* spp. (Agersø and Petersen, 2007; Peleg et al., 2008). However, none of the targeted determinants was found in the remaining 38.7% of the tetracycline-resistant isolates. These isolates may harbor unknown tetracycline resistance determinants or genes that were not examined in this study. The *tet*(39) gene was first reported among unrelated *Acinetobacter* strains isolated from freshwater trout farms and sewage from Denmark, and from a clinical specimen in the Netherlands (Agersø and Guardabassi, 2005), followed by identification of *tet*(39) in 75% of 222 oxytetracycline-resistant *Acinetobacter* spp. isolated from integrated fish farms in Thailand (Agersø and Petersen, 2007). Similarly, *tet*(39) genes were found in 34 of 89 single-patient tetracycline-resistant *A. baumannii*-*Acinetobacter calcoaceticus* complex isolates from a US military hospital (Akers et al., 2009). Thus, our findings and those of others suggest that the *tet*(39) gene is widespread among *Acinetobacter* strains.

Genes encoding resistance to sulfonamide, such as *sul1*, are highly prevalent in *A. baumannii* and other *Acinetobacter* spp. This may be attributed to strong association of the *sul1* gene with class 1 integrons, which are themselves highly prevalent in *Acinetobacter* spp. (Poirel et al., 2011). In the current study, the genetic basis of resistance to sulfonamide was identified in 15 (53.6%) of 28 trimethoprim-sulfamethoxazole-resistant isolates and 9 (32.1%) of them carried both *sul1* and *sul2* genes. The *sul1* gene alone and *sul2* alone were found in 3 isolates each. No *sul3* gene was detected. Consistent with our observation, a combination of *sul1* and *sul2* genes in *Acinetobacter* spp. have been reported from manured agricultural soils and pig slurry in the United Kingdom (Byrne-Bailey et al., 2009). Similarly, the *sul2* gene was found widely distributed among clonally distinct *Acinetobacter* spp. isolated from both fish ponds and manure from integrated fish farms in Thailand (Agersø and Petersen, 2007).

Among the 42 *Acinetobacter* isolates resistant to β -lactam antibiotics, *Acinetobacter*-derived *AmpC* cephalosporinase *bla*_{ADC-7-like} gene was detected in a

single *A. baumannii* isolate. However, 3 distinct class D OXA enzyme genes were identified, irrespective of resistance to β -lactams. Although OXA-51-like gene was identified in all *A. baumannii* isolates, OXA-23-like gene and OXA-24-like genes were identified only in 1 and 2 *Acinetobacter* spp., respectively. *Acinetobacter* strains are naturally resistant to β -lactams, due to intrinsic AmpC-type cephalosporinase production at a low level (Poirel et al., 2011). Although a wide range of class A extended-spectrum β -lactamases, including those of the TEM, SHV, CTX-M, and PER families, has been described, particularly among *A. baumannii* isolates (Gordon and Wareham, 2010), such resistant genes sought were not detected in any of the β -lactam-resistant *Acinetobacter* in the current study. Our findings suggest that other resistance mechanisms, such as porins loss, active drug efflux, and modification of penicillin-binding proteins, among others, may be implicated for their β -lactam resistance.

Integrons, particularly class 1 integrons, have been reported in clinical isolates of *Acinetobacter* spp. from humans and in isolates from diverse environments all over the world (Agersø and Petersen, 2007; Byrne-Bailey et al., 2009; Lin et al., 2010). Among the 176 *Acinetobacter* isolates tested, a class 1 integron was identified in the 11 (6.25%) isolates in the present study. None of the isolates was positive for class 2 integrons (*intI2*) or class 3 integrons (*intI3*). All class 1 integron-positive isolates were resistant to trimethoprim-sulfamethoxazole and carried *sul1* as a part of the integron. Two antibiotic resistance gene cassettes *dfrA15* and *aadA2* were identified in 8 of the 11 class 1 integrase-positive isolates. The *dfrA15* gene cassette encodes for dihydrofolate reductase, conferring resistance to trimethoprim, and its presence as a gene cassette explained the trimethoprim resistance phenotype in 8 (28.6%) of the 28 trimethoprim-sulfamethoxazole-resistant isolates. The genes coding for trimethoprim resistance (*dfr*) have also been previously reported within integron structures in *A. baumannii* (Peleg et al., 2008). Similarly, the *aadA2* gene encodes a variant of aminoglycoside adenyltransferase, conferring resistance to spectinomycin and streptomycin. Importantly, the *dfrA15-aadA2* cassette array found in the current study has not been reported previously, to our knowledge. Thus, this finding indicates the important role of this gene combination in the extension and spread of resistance to trimethoprim and aminoglycosides.

Among the 176 *Acinetobacter* strains isolated from BTM samples from different provinces of Korea (Gurung et al., 2013), none of them were resistant to cefepime, carbapenems, fluoroquinolones, or polymyxins. Resistance to aminoglycosides, tetracycline, trimethoprim-sulfamethoxazole, and β -lactams was 9.7, 17.6,

Figure 1. Dendrogram showing the cluster analysis of pulsed-field gel electrophoresis (PFGE) patterns of 47 *Acinetobacter baumannii* strains isolated from bulk tank milk samples. The cluster analysis was performed by using the Dice coefficient and the unweighted-pair group method with arithmetic averages. Details given (in the first through seventh columns from the left) are percentage similarity, PFGE banding patterns, strain number, province, date of isolation (year.month.day), resistance phenotype, and pulsotype for each strain, respectively.

15.9, and 23.9%, respectively. In general, the carriage of the AME gene and *tet*(39) gene was largely responsible for resistance to aminoglycosides and tetracycline, respectively. Similarly, the presence of *sul1*, *sul2*, or both, and the *dfrA15* gene cassette was mostly responsible for resistance to trimethoprim-sulfamethoxazole, whereas resistance to extended spectrum β -lactams in 1 *A. baumannii* isolate was due to the *bla*_{ADC-7-like} gene. Because bacteria bearing resistance genes in BTM could be important reservoirs of these genes, the presence of *Acinetobacter* strains carrying various resistance genes in BTM is a matter of public health concern, particularly when an increasing number of people consume unpasteurized milk or products made from unpasteurized milk (Oliver et al., 2009).

Pulsed-field gel electrophoresis was performed to determine the clonal relatedness of 47 of the 57 *A. baumannii* isolates. No DNA banding patterns were obtained from the 10 *A. baumannii* strains due to constant autodigestion of the genomic DNA during agarose plug preparation and were, thus, excluded. Based on a similarity value of 0.85, the 47 isolates represented 35 arbitrary (designated as I through XXXV) pulsotypes (Figure 1), including 29 singleton types and 6 different clusters consisting of 2 to 6 indistinguishable and (or) highly related isolates. Overall, a large number of *A. baumannii* strains were genetically heterogeneous, indicating that they were not derived from a single clone of *A. baumannii*. Unexpectedly, PFGE results also revealed that *A. baumannii* strains obtained from distantly located farms within a province but mostly from different provinces shared identical PFGE profiles. Although it is difficult to explain precisely, it can be speculated that a few independent clones of *A. baumannii* strains might have clonally disseminated or persisted among various farms, not only within a province but also between different adjoining provinces. However, such speculation should be confirmed by further study, such as by multiple-locus variable number tandem repeat (VNTR) analysis of 7 loci (MLVA-7), which has been reported to have greater discrimination power than PFGE in some instance (Hu et al., 2013).

A few limitations existed in this study. The genetic basis of resistance in some of the antimicrobial-resistant *Acinetobacter* strains could not be established in spite of repeated attempts. Similarly, clonal diversity between the *A. baumannii* isolates was assessed only by PFGE and no other genotyping method was used to confirm PFGE findings. Furthermore, we did not investigate the transferability of plasmids carrying antimicrobial resistance genes. As *tet*(39) is often associated with horizontally transferable plasmids (Agersø and Guardabassi, 2005; Agersø and Petersen, 2007), further studies are needed to determine the transferability of

these plasmids because of the possibility of horizontal transfer of this gene to various bacteria in dairy farms and other ecosystems, including to humans via the food chain.

In conclusion, we determined the genetic basis for aminoglycoside, tetracycline, and sulfamethoxazole resistance in most of the *A. baumannii* and *Acinetobacter* spp. isolated from BTM samples. As the presence of antimicrobial-resistant *Acinetobacter* in BTM, which is one of the agents of mastitis (Malinowski et al., 2006), has obvious implications for the treatment of infected animals, the elucidation of their resistance mechanism associated with the resistance phenotype and a better understanding of resistance genes may help in the development of strategies to control infections such as mastitis and to prevent further dissemination of antibiotic resistance genes. To the best of our knowledge, this is the first report of molecular characterization of antimicrobial-resistant *Acinetobacter* spp. from milk.

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