Prevalence and antimicrobial susceptibility of *Acinetobacter* from raw bulk tank milk in Korea

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ABSTRACT

The objective of this study was to determine the prevalence of *Acinetobacter* spp. in bulk tank milk (BTM) samples from different provinces of Korea and to analyze their antimicrobial susceptibility. Altogether, 2,287 BTM samples were investigated. Among them, *Acinetobacter* spp. were isolated from 176 BTM samples. Out of 176 *Acinetobacter* spp., 57 isolates were identified as *Acinetobacter baumannii*. None of the isolates were resistant to cefepime, imipenem, meropenem, ciprofloxacin, levofloxacin, or colistin. Resistance to amikacin, gentamicin, piperacillin, and cefotaxime was 2.3, 7.4, 2.3, and 4.0%, respectively. *Acinetobacter* spp. were least susceptible to tetracycline (17.6%), followed by trimethoprim-sulfamethoxazole (15.9%), ceftazidime (10.8%), and ampicillin-sulbactam (10.2%). Overall, *A. baumannii* strains were susceptible to most of the antimicrobial agents tested compared with other *Acinetobacter* spp. The *Acinetobacter* isolates showed 17 different patterns of antimicrobial resistance. The most frequent resistance profile observed was ampicillin-sulbactam ($n = 13$), followed by tetracycline ($n = 9$), ceftazidime-tetracycline ($n = 8$), and trimethoprim-sulfamethoxazole-tetracycline ($n = 8$). The results of this study confirmed that *Acinetobacter*, including *A. baumannii* strains, are present in BTM, which clearly showed the importance of examining BTM not only for foodborne pathogens but also for *Acinetobacter* spp., which could be of public health concern. To the best of our knowledge, this is the first report of *Acinetobacter* spp. in BTM samples from Korea.

Key words: *Acinetobacter*, antimicrobial resistance, bulk tank milk

INTRODUCTION

Milk and milk products are important sources of nutrients for both humans (Sharm and Joshi, 1992) and

bacteria (Henry and Newlander, 1997). Therefore, it is mandatory to pasteurize milk for safety. Unfortunately, an increasing number of people consume raw (unpasteurized) milk and products made from raw milk despite the hazards associated with this practice. Consumption of raw milk has been associated with several milkborne disease outbreaks from 2000 to 2008 in the United States (Oliver et al., 2009). In contrast, fewer milkborne disease outbreaks were associated with consumption of pasteurized milk during this period (Oliver et al., 2009). These outbreaks were generally caused by improper pasteurization or postpasteurization contamination.

Bacterial isolates from bulk tank milk (BTM) consist of both pathogenic and commensal bacteria such as *Staphylococcus aureus*, *Streptococcus agalactiae*, *Listeria monocytogenes*, *Enterococcus* spp., *Mycoplasma* spp., *Salmonella* spp., *Campylobacter jejuni*, *Escherichia coli*, *Enterobacter* spp., *Citrobacter* spp., *Klebsiella* spp., and *Yersinia enterocolitica* (Jayarao and Wang, 1999; Jayarao and Henning, 2001; Straley et al., 2006). The study by Jayarao and Wang (1999) identified gram-negative noncoliform bacteria in BTM, including *Acinetobacter*, *Aeromonas*, *Flavobacterium*, *Moraxella*, *Pasteurella*, *Pseudomonas*, and *Xanthobacter*. A further worrying development is that antimicrobial resistant bacteria have been isolated from BTM, which is a matter of public health concern (Straley et al., 2006).

Acinetobacter species are aerobic, gram-negative, nonfermenting bacteria that are ubiquitous in nature (Bergogne-Bérézin and Towner, 1996). The organisms are characterized by their tendency to tolerate drying (Wendt et al., 1997; Webster et al., 1998), resist commonly used disinfectants such as chlorhexidine and phenols (Gallego and Towner, 2001) and heat (50–75°C; Jain and Danziger, 2004), adapt to adverse environmental conditions, and acquire resistance to multiple classes of antibiotics (Bergogne-Bérézin and Towner, 1996), facilitating the organism to survive and spread in the environment. Among them, *Acinetobacter baumannii* has emerged as an important pathogen, causing infections in severely ill patients in intensive care units or immunocompromised individuals (Bergogne-Bérézin

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and Towner, 1996; Villegas and Hartstein, 2003). *Acinetobacter baumannii* also resist the killing action of normal human serum, which was partly thought to be due to the presence of LPS (King et al., 2009).

To date, investigations of BTM are limited to established or potential pathogens of animals and humans. Furthermore, several reports exist on the prevalence of *Acinetobacter* from the environment and human clinical isolates, but only a very few studies have investigated the prevalence of *Acinetobacter* from BTM samples worldwide (Jayarao and Wang, 1999). Recently, our group reported the prevalence and antimicrobial susceptibility of gram-negative bacteria (GNB) from milk samples from Korea. However, these studies were limited to mastitic-quarter milk samples (Nam et al., 2009, 2010). Therefore, the present study was undertaken to investigate the prevalence of *Acinetobacter* in BTM samples and determine their antimicrobial susceptibility in Korea.

MATERIALS AND METHODS

Bulk Tank Milk Sampling

Bulk tank milk from 2,287 different dairy farms located in 6 provinces of Korea was collected during November 2011 to April 2012. The milk in the bulk tank was agitated for 5 to 10 min before sampling. Then, the sample was collected from the top of the tank in a sterile snap cap milk collection vial using a sterile dipper from each dairy farm. The BTM samples were brought to the antimicrobial resistance laboratory of the Animal, Plant, and Fisheries Quarantine and Inspection Agency (QIA, Gyeonggi-do, Republic of Korea) in ice-cooled containers and processed within 24 h of collection.

Isolation of *Acinetobacter*

Ten microliters of each milk sample was plated by streaking on CHROMagar *Acinetobacter* (CHROMagar, Paris, France) and incubated at 37°C for 48 h. The suspected red colonies of *Acinetobacter* were further subcultured on MacConkey agar and a single colony from MacConkey agar was further subcultured on Tryptic soy agar for DNA extraction and other experiments.

Identification of *Acinetobacter*

For identification of *Acinetobacter* spp., amplification and sequencing of the RNA polymerase β -subunit (*rpoB*) gene was done by using primers and the protocol as described previously (La Scola et al., 2006).

For *rpoB*-positive isolates, amplification of the 16S-23S rRNA gene intergenic spacer region was done to identify the *Acinetobacter calcoaceticus*-*A. baumannii* complex as described previously (Barry et al., 1991). Finally, *A. baumannii* was differentiated from the *A. calcoaceticus*-*A. baumannii* complex by the DNA gyrase, subunit B (*gyrB*)-based multiplex PCR method as described previously (Higgins et al., 2007).

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility test of *Acinetobacter* was performed by the agar disc diffusion method according to the guidelines of the Clinical Laboratory Standards Institute (CLSI, 2010) using Mueller Hinton agar (Becton Dickinson, Sparks, MD). The antimicrobial drugs tested were amikacin (30 μ g/disc), gentamicin (10 μ g/disc), ampicillin-sulbactam [10 μ g/disc of each (ampicillin and sulbactam)], piperacillin (100 μ g/disc), cefotaxime (30 μ g/disc), cefepime (30 μ g/disc), ceftazidime (30 μ g/disc), imipenem (10 μ g/disc), meropenem (10 μ g/disc), ciprofloxacin (5 μ g/disc), levofloxacin (5 μ g/disc), tetracycline (30 μ g/disc), colistin (10 μ g/disc), and trimethoprim-sulfamethoxazole (1.25–23.75 μ g/disc; Becton Dickinson, Cockeysville, MD). Isolates were categorized as susceptible or resistant based on interpretive criteria according to CLSI guidelines (CLSI, 2010). *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used as a quality control strains.

RESULTS AND DISCUSSION

In this study, 2,287 BTM samples were investigated. Among them, *Acinetobacter* spp. were isolated from 176 of 2,287 (7.7%) of the BTM samples. Out of 176, 57 isolates (32.4%) were identified as *A. baumannii*. A study conducted by Jayarao and Wang, (1999) in eastern South Dakota and western Minnesota detected coliform and noncoliform bacteria in 62.3 and 76.3% of BTM samples, respectively. Although, they found several GNB in BTM samples, the isolation rate for *Acinetobacter* spp. was only 1.3%. *Acinetobacter* spp. also have been reported from dairy goat milk samples in Kenya where the isolation rate was 5% (Ndegwa et al., 2001). Similarly, our group investigated the prevalence and antimicrobial susceptibility of GNB from mastitic milk samples from Korea (Nam et al., 2009) in which the isolation rate of *Acinetobacter* spp. was 0.3%. However, the isolation rates of *Acinetobacter* spp. in those studies were lower than that of the current study. The reason for this discrepancy is unclear. It is possible that there could be inhibition of *Acinetobacter* spp. from other bacteria or compounds in the mastitic milk that were

absent in the current study, but it should be confirmed by further study. Nevertheless, the use of selective methods for the isolation of *Acinetobacter* spp. in BTM samples in the current study may, in part, explain the higher prevalence of *Acinetobacter* spp. as compared with those previous studies. Although our group previously reported the prevalence of *Acinetobacter* spp. from Korea from mastitic-quarter milk samples (Nam et al., 2009), to the best of our knowledge, this is the first report of prevalence of *Acinetobacter* spp. in BTM samples from Korea. *Acinetobacter* spp. were graded as the third most recurrently found pathogen in humans in intensive care units of hospitals in Korea (Kim et al., 2006). Furthermore, *A. baumannii* isolates were reported in pets and horses from Switzerland in which the majority of the infections were hospital acquired and in animals with predisposing conditions (Endimiani et al., 2011). This shows that *Acinetobacter* spp. were not only important nosocomial pathogens in humans, but also in animals.

Acinetobacter spp. are ubiquitous organisms that are widely distributed in nature. Thus, the presence of *Acinetobacter* in BTM could be due to mere contamination. Bulk tank milk can become contaminated with *Acinetobacter* present on teats, udder surfaces, infected mammary glands, milking machines, transporting systems, and contaminated water used to clean the milking machines, or from the dairy farm environment (Thomas and Thomas, 1973; Bramley and McKinnon, 1990; Straley et al., 2006). Although *Acinetobacter* spp. are virtually avirulent for healthy humans and cause infections almost exclusively in debilitated patients in hospitals and immunocompromised individuals (Bergogne-Bérézin and Towner, 1996; Villegas and Hartstein, 2003), antimicrobial resistant *Acinetobacter* spp. in BTM could be potential reservoirs of resistance genes, as they may survive pasteurization due to their ability to resist heat (Jain and Danziger, 2004). Furthermore, milk gets contaminated with a variety of microbes even after pasteurization (Brisabois et al., 1997; Oliver et al., 2005) and results in rapid multiplication of the microbes, which may change the quality of milk, leading to issues of food safety (Frazier and Westhoff, 1986). Thus, the presence of *Acinetobacter* resistant to multiple antibiotics in BTM is a matter of public health concern.

Acinetobacter spp. are intrinsically resistant to many antibiotics and possesses remarkable ability to acquire resistance determinants to various kinds of antimicrobial agents (Perilli et al., 1996). The antimicrobial susceptibility of *A. baumannii* ($n = 57$) and other *Acinetobacter* spp. ($n = 119$) to 14 antimicrobial agents is compared in Table 1. None of the other *Acinetobacter* spp. was resistant to cefepime, imipenem, meropenem, ciprofloxacin,

levofloxacin, or colistin. The resistance to amikacin, gentamicin, piperacillin, and cefotaxime against *Acinetobacter* spp. was 2.3, 7.4, 2.3, and 4.0%, respectively. Overall, both *A. baumannii* and *Acinetobacter* spp. were least susceptible to tetracycline (17.6%), followed by trimethoprim-sulfamethoxazole (15.9%), ceftazidime (10.8%), and ampicillin-sulbactam (10.2%). Further, our results showed that carbapenem, fluoroquinolone, cefepime, and colistin were found to be the most active agents against all *Acinetobacter* including *A. baumannii*. Although no comparable data are available for *Acinetobacter* isolates from BTM samples worldwide, the resistance of human clinical *Acinetobacter* isolates from the United States for fluoroquinolone, amikacin, ceftazidime, and imipenem were very high (Lee et al., 2011). The clinical *Acinetobacter* isolates from humans from Korea also showed high resistance against fluoroquinolone, aminoglycosides, cephalosporin, and carbapenem (Lee et al., 2011). Compared with the human *Acinetobacter* isolates, the majority of *Acinetobacter* isolates from BTM samples in the current study were found to be susceptible to most of the antibiotics tested. This could be explained by the fact that the cattle from which they were isolated, being healthy cattle, were not exposed to antimicrobial agents.

Among the 57 *A. baumannii* isolates, none of the isolates were resistant to amikacin, piperacillin, cefotaxime, cefepime, ceftazidime, imipenem, meropenem, ciprofloxacin, levofloxacin, or colistin. Resistance to gentamicin, ampicillin-sulbactam, tetracycline, and trimethoprim-sulfamethoxazole in *A. baumannii* isolates was 8.8, 1.8, 7.0, and 5.3%, respectively. In contrast, Nam et al. (2010), reported much higher resistance for amikacin (5.9%), piperacillin (5.9%), and tetracycline (18%) among the 17 *A. baumannii* isolated from mastitic-quarter milk samples in Korea. The higher resistance in that study may be attributed to selective pressure caused by the use of these antimicrobial agents for treatment of mastitis in the infected cattle. Similarly, human clinical *A. baumannii* isolates from Korean hospitals also exhibited a high frequency of resistance to various classes of antimicrobial agents, including aminoglycosides, extended-spectrum β -lactams, fluoroquinolone, and carbapenem (Lee and Moon, 2005). As described above, nonexposure to antimicrobial agents may be the reason for low level resistance of *A. baumannii* isolates in the current study compared with human isolates.

In the present study, the most frequently observed antimicrobial resistance in *Acinetobacter* isolates was to tetracycline (17.6%), which is similar to findings in *E. coli* isolates from cattle and pigs in Korea (Lim et al., 2007). This result reflects the use of antimicrobial agents in livestock in Korea, as tetracycline has long

Table 1. Antimicrobial susceptibility of *Acinetobacter* spp. isolated from raw bulk tank milk in Korea

Class	Antimicrobial	Breakpoint zone (mm)	No. of resistant isolates (%) ¹		
			<i>Acinetobacter</i> spp. (n = 119)	<i>Acinetobacter baumannii</i> (n = 57)	Total (n = 176)
Aminoglycosides	Amikacin	≤14	4 (3.4)	0 (0)	4 (2.3)
	Gentamicin	≤12	8 (6.7)	5 (8.8)	13 (7.4)
Penicillins	Ampicillin-sulbactam	≤11	17 (14.3)	1 (1.8)	18 (10.2)
	Piperacillin	≤17	4 (3.4)	0 (0)	4 (2.3)
Cephalosporins	Cefotaxime	≤14	7 (5.9)	0 (0)	7 (4)
	Cefepime	≤14	0 (0)	0 (0)	0 (0)
	Ceftazidime	≤14	19 (16)	0 (0)	19 (10.8)
Carbapenems	Imipenem	≤13	0 (0)	0 (0)	0 (0)
	Meropenem	≤13	0 (0)	0 (0)	0 (0)
Fluoroquinolones	Ciprofloxacin	≤15	0 (0)	0 (0)	0 (0)
	Levofloxacin	≤13	0 (0)	0 (0)	0 (0)
Tetracyclines	Tetracycline	≤11	27 (22.7)	4 (7.0)	31 (17.6)
Polymyxins	Colistin	≤11	0 (0)	0 (0)	0 (0)
Folate pathway inhibitors	Trimethoprim-sulfamethoxazole	≤10	25 (21.0)	3 (5.3)	28 (15.9)

¹The numbers in parentheses indicate percentages of resistant isolates.

been the antimicrobial most commonly used with Koran livestock, accounting for over 30% of the total amount of antimicrobial consumption (Animal, Plant, and Fisheries Quarantine and Inspection Agency, 2011). As tetracycline resistance genes such as *tet*(39) have been found associated with horizontally transferable plasmids (Agersø and Guardabassi, 2005; Agersø and Petersen, 2007), these plasmids may spread among *Acinetobacter* spp. in dairy farms. Furthermore, resistance to tetracycline in *Acinetobacter* is also mediated by overexpression of chromosomally encoded efflux pumps (Coyne et al., 2011).

Overall, *Acinetobacter* isolates showed 17 different patterns of antimicrobial resistance in this study. The most frequent resistance profile observed was to ampicillin-sulbactam-trimethoprim-sulfamethoxazole (n = 13), followed by tetracycline (n = 9), ceftazidime-tetracycline (n = 8), and trimethoprim-sulfamethoxazole-tetracycline (n = 8). Altogether, 33 isolates showed resistance to at least 1 antimicrobial agent tested, whereas 42 and 3 isolates showed simultaneous resistance to 2 and 3 antimicrobial agents, respectively. The resistance profiles of these isolates are shown in Table 2. Multidrug-resistant (**MDR**) *Acinetobacter*

Table 2. Antimicrobial resistance profiles of *Acinetobacter* spp. isolated from raw bulk tank milk in Korea

No. of resistant antimicrobials	Resistance pattern ¹	No. of resistant isolates (%) ²		
		<i>Acinetobacter</i> spp. (n = 119)	<i>Acinetobacter baumannii</i> (n = 57)	Total (n = 176)
0 (n = 98)	—	50 (42.0)	48 (84.2)	98 (55.7)
1 (n = 33)	AMK	4 (3.4)	0 (0)	4 (2.3)
	AMS	4 (3.4)	0 (0)	4 (2.3)
	CAZ	3 (2.5)	0 (0)	3 (1.7)
	GEN	3 (2.5)	2	5 (2.8)
	PIP	5 (4.2)	0 (0)	5 (2.8)
	SXT	2 (1.7)	1 (1.8)	3 (1.7)
	TET	7 (5.9)	2 (3.5)	9 (5.1)
2 (n = 42)	AMS-SXT	12 (10.0)	1 (1.8)	13 (7.4)
	CAZ-CTX	6 (5.0)	0 (0)	6 (3.4)
	CAZ-GEN	1 (0.8)	0 (0)	1 (0.6)
	CAZ-TET	8 (6.7)	0 (0)	8 (4.5)
	GEN-TET	2 (1.7)	1 (1.8)	3 (1.7)
	SXT-GEN	2 (1.7)	1 (1.8)	3 (1.7)
	SXT-TET	8 (6.7)	0 (0)	8 (4.5)
3 (n = 3)	AMS-SXT-TET	1 (0.8)	0 (0)	1 (0.6)
	CAZ-CTX-TET	1 (0.8)	0 (0)	1 (0.6)
	SXT-GEN-TET	0 (0)	1 (1.8)	1 (0.6)

¹AMK = amikacin; AMS = ampicillin-sulbactam; CAZ = ceftazidime; CTX = cefotaxime; GEN = gentamicin; PIP = piperacillin; SXT = trimethoprim-sulfamethoxazole; TET = tetracycline.

²The numbers in parentheses indicate percentages of resistant isolates.

strains have emerged as a substantial clinical problem worldwide (Lee et al., 2011). Different definitions of the terms MDR and pandrug-resistant *A. baumannii* have been used in the literature. In this study, MDR *Acinetobacter* spp. were defined as the isolates resistant to at least 3 different classes of antimicrobial agents (Magiorakos et al., 2012). By this definition, only 2 MDR *Acinetobacter* strains were detected in this work. In contrast, several outbreaks of MDR *Acinetobacter* spp., particularly MDR *A. baumannii* have been reported worldwide, including Korea (Lee et al., 2011).

CONCLUSIONS

We determined the prevalence of *Acinetobacter* spp. in BTM samples from different provinces of Korea and analyzed their antimicrobial susceptibility. *Acinetobacter* isolates were detected in a relatively high number of BTM samples, which clearly showed the importance of examining BTM not only for foodborne pathogens but also for *Acinetobacter* spp., as this could also be a potential problem associated with milk quality as well as public health.

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