Identification, susceptibility, and detection of integron-gene cassettes of *Arcanobacterium pyogenes* in bovine endometritis

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ABSTRACT

The present study aimed to identify, determine the susceptibility, and detect gene cassettes of *Arcanobacterium* (*Actinomyces*) *pyogenes* isolates from cows with endometritis. *Arcanobacterium pyogenes* isolates were identified first by using the API Coryne Vit system test, and further through PCR. Minimum inhibitory concentrations of 23 antimicrobial agents against *A. pyogenes* were tested using standard broth microdilution assays according to the protocols of the Clinical and Laboratory Standards Institute. The genes of integrons I and II were amplified by PCR using specific primers. Thirty-two *A. pyogenes* isolates were isolated from 136 endometritic cows in the Hohhot region. Antibiotic susceptibility tests revealed that all isolates were highly sensitive to fluoroquinolones (100%), macrolides (~81.2 to 100%) and florfenicol (90.6%), aminoglycosides (~15.6 to 81.2%), and tetracyclines (~43.7 to 68.7%). However, 53.1% were resistant to clindamycin, ~50 to 65.6% were resistant to penicillins, and ~37.5 to 71.9% were resistant to cephalosporins. One hundred percent were resistant to sulfonamides and bacitracin zinc. The integrons were further confirmed by sequencing. No class II integrons were detected, whereas 50% (n = 16) of the *A. pyogenes* isolates were positive for the presence of the *intI* I gene, but only 13 contained gene cassettes. Sequence analysis of gene cassettes revealed 6 gene cassettes, 4 of which encode resistant determinants of aminoglycosides (*aadA1*, *aadA5*, *aadA24*, and *aadB*) and 1 of which encodes the resistance gene of chloramphenicol (*cmlA6*). The function of the sixth identified cassette, designated ORF1, is unknown. The gene cassette arrays *aadA24*-ORF1, *aadA5*, and *aadA1*-*aadB*-*cmlA6* were found in 46.13% (6/13), 38.46% (5/13), and 38.46% (5/13) of the isolates, respectively. These cassettes segregated according to a consistent pattern, with *aadA5* always alone, ORF1 always with *aadA24*,

and *aadA1*-*aadB* and *cmlA6* always together. Most of the positive integrons existed in the multiresistant isolates (n = ~3 to 7), indicating that the integrons played an important role in the dissemination and spread of antimicrobial resistance. This is the first report of *A. pyogenes* infections in dairy cows in China and of detection of gene cassettes and integrons in *A. pyogenes*.

Key words: *Arcanobacterium* (*Actinomyces*) *pyogenes*, antimicrobial susceptibility, integron, gene cassette

INTRODUCTION

Arcanobacterium (*Actinomyces*) *pyogenes* is one of the important opportunistic pathogens of the upper respiratory and genital tracts of cattle, sheep, swine, and many other species (Billington et al., 2002a; Azawi and Azar, 2003; Gröhn et al., 2004; Ertaş et al., 2005). The prevalence of *A. pyogenes* from dairy cows was 32.9 and 17.6% (n = 85) at d 26 and 40 postpartum, respectively (Bonnett et al., 1991). The isolation of *A. pyogenes* was positively correlated with the isolation of anaerobic bacteria at d 26, which resulted in increased uterine lesions at d 40 (Bonnett et al., 1991), and *A. pyogenes* was found in 70% of the uteri of postparturient dairy cows (Cohen et al., 1996). Novel reports showed that the uterine infection could be classified as puerperal metritis, clinical metritis, clinical endometritis, or subclinical endometritis (Földi et al., 2006; Sheldon et al., 2006); that the prevalence of *A. pyogenes* was higher within 26 d postpartum than at 40 d postpartum; and that the coinfection of *A. pyogenes* and anaerobic bacteria could increase uterine lesions, decreasing the chance of successful treatment in dairy cows. Rutter et al. (1999) reported that at 4 to 7 d postpartum, 40% (26/65) of cows with puerperal metritis were positive for *A. pyogenes* and β -hemolytic *Streptococcus*. *Arcanobacterium pyogenes* and β -hemolytic *Streptococcus* were isolated for periods of at least 24 to 27 d postpartum (Rutter et al., 1999). The uterine coinfection with *A. pyogenes* and other anaerobic or β -hemolytic *Streptococcus* has been shown to result in increased purulent secretion, early embryonic death, and infertility (Nolte et al., 2001; Gilbert et al., 2005).

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Because of the lack of an effective vaccine for uterine infections caused by *A. pyogenes*, a longstanding treatment for this condition is either PGF_{2α} analogs or antibiotics (Steffan et al., 1984). However, the extensive use of antibacterials has resulted in the increasing resistance of pathogenic bacteria (Trinh et al., 2002; Sheldon et al., 2004). The creation of bacterial drug resistance can eventually jeopardize the successful treatment of disease in both humans and animals. For instance, the effect of antibiotic treatment on cow endometritis has been attenuated by emerging resistant strains (Cohen et al., 1996; Ali 2000; Trinh et al., 2002). As Sheldon et al. (2004) reported, many *A. pyogenes* isolated from cattle with clinical metritis, endometritis, or both were resistant to oxytetracycline, which had the highest MIC value (16 mg·L⁻¹), but all were susceptible to 3 cephalosporins (cefquinome, cephalirin, and ceftiofur, MIC <0.6 mg·L⁻¹). Cohen et al. (1996) reported that all *A. pyogenes* isolates from uteri of postparturient dairy cows were susceptible to clindamycin, and most isolates were susceptible to ciprofloxacin. On the other hand, antimicrobial susceptibility of *A. pyogenes* strains isolated from cattle showed that up to 25.9 and 22.2% were resistant to tetracyclines and macrolides, respectively (Trinh et al., 2002). Penicillin was highly effective against *A. pyogenes* (Guerin-Fauble et al., 1993; Ali, 2000).

In resistance mechanism research, Billington et al. (2002b) elucidated that the antimicrobial resistance gene *TetW* mediated the *A. pyogenes* resistance to tetracycline. *ErmX* and *ErmB* were found in tylosin-resistant *A. pyogenes* isolates from animals (Jost et al., 2003, 2004). Integrons are DNA elements that include the gene's determinant group and insertion site for a site-specific recombination system, which enables them to capture mobile gene cassettes (Collis and Hall, 1992). The expression of an integron may induce bacterial multidrug resistance and cause the resistance gene to disseminate in the same and different germs by a conjugative plasmid (Rowe-Magnus et al., 2002). However, gene cassette-integron detection from *A. pyogenes* has not been reported in bovine endometritis.

In this study, 136 cows with endometritis from 15 dairy farms from Hohhot, Inner Mongolia Autonomous Region of China, were sampled for *A. pyogenes*. In addition, MIC of 23 commonly used antibiotics were determined by standard broth microdilution. Eventually, the gene cassettes-integrations were detected by PCR, and the correlation between integrations and resistance was analyzed. The goals of this investigation were 3-fold: 1) to provide data on the prevalence of antimicrobial resistance in strains of *A. pyogenes* isolated from cattle in the Inner Mongolia Autonomous Region of China; 2) to elucidate the resistance mechanisms of these strains;

and 3) to facilitate the correct antibiotic treatment in cases of endometritis in the region.

MATERIALS AND METHODS

Bacteria Isolation and Characterization of the A. pyogenes Reference Strain

The reference *A. pyogenes* strain ATCC 19411 (NCTC 5224, purchased from American Type Culture Collection, Manassas, VA) was cultured on brain heart infusion agar (Difco, Franklin Lakes, NJ) containing 5% defibrinated sheep blood, and incubated for 48 h at 37°C. Glycerol stocks (20%) were made and stored at -80°C until use.

Sample Collection

From June to December in 2006, we investigated the prevalence of metritis and endometritis on 15 dairy farms in Hohhot, Inner Mongolia. A total of 136 Holstein cows showing signs of postpartum metritis or endometritis, as diagnosed by veterinarians or herd owners (552 cows postpartum were involved), were sampled, including 4 cases of puerperal metritis (with an abnormally enlarged uterus and a watery red-brown uterine secretion, fever exceeding 39.5°C, decreased milk yield within 15 d postpartum, with no treatment before sampling), 8 cases of clinical metritis (with a large uterus and a purulent uterine discharge within 21 d postpartum), 52 cases of clinical endometritis (with a heavy purulent or mucopurulent discharge after 21 d postpartum), and 72 cases of subclinical endometritis (after 30 to 50 d postpartum).

Some postpartum cows diagnosed with metritis or endometritis by veterinarians or herd owners were treated with antibacterials; however, antibacterials were given to endometritic cows by veterinarians or herd owners only once. There was no abatement of the clinical symptoms of cows with endometritis, and the sample was collected promptly at this time. Penicillin or ampicillin and aminoglycosides were used to treat 18 cows with metritis or endometritis (5 cases of clinical endometritis and 13 cases of subclinical endometritis), oxytetracycline was used to treat 15 cases (7 cases of clinical endometritis and 8 cases of subclinical endometritis), and ceftiofur was used in 31 cases (11 cases of clinical endometritis and 20 cases of subclinical endometritis). The other samples were collected from cows not treated with antibiotics.

Uterine secretions were collected using a chlamydia-semen deposition gun through a sterile procedure. The sample was removed to a sterile vial with buffered glycerol saline, and the vials were placed in a cooler with

ice and immediately delivered to the laboratory within 2 h.

Biochemical Characterization

A 50- μ L uterine secretion sample from each cow was inoculated onto blood agar plates supplemented with 5% defibrinated sheep blood and 100 U/mL of polymyxin B and incubated with 5% CO₂ at 37°C for 48 h to isolate *A. pyogenes*. Smooth, glistening colonies surrounded by a conspicuous β -hemolytic zone were presumed to be *A. pyogenes*. Initial identification of these presumptive *A. pyogenes* was based on conventional methods, including gram stain, colony morphology, and bacterial morphology. These isolates were further identified using the API Coryne Vit system (bioMérieux SA, Marcy l'Etoile, France). The confirmed *A. pyogenes* isolates were stored at -80°C in brain heart infusion agar plus 20% glycerol until resuscitation.

Fifty-microliter aliquots of uterine secretions were inoculated onto different agar plates, such as MacConkey agar, manitol salt agar, cetrinide agar, mannitol-egg yolk-polymyxin B agar, and selective *Streptococcus* agar modified number 2 (Acumedia, Lansing, MI). These plates were subsequently incubated at 37°C for 24 h, and anaerobic CDC (Centers for Disease Control and Prevention) blood agar plates (Sigma, St. Louis, MO) cultured in anaerobic chambers (Bactron III-2E, Shel-lab, Cornelius, OR) at 37°C for 48 h to isolate anaerobic bacteria. Putative pathogenic bacteria, as judged by colony morphology and gram staining, were further identified by API kits (bioMérieux SA): API 20 E, API Staph, API 20 NE, API 50 CHB, API Strep, and API 20 A for *Escherichia coli*, *Staphylococcus aureus*, *Bacillus aeruginosus*, *Bacillus cereus*, *Streptococcus*, and anaerobic bacteria, respectively. Strains ATCC 25922, ATCC 29213, ATCC 27853, ATCC 11778, ATCC 19615, and ATCC 13124 were used as positive controls, respectively.

Sequencing of the 16S rRNA Gene

To further identify and classify the isolated strains and analyze their evolution status, the 16S rRNA gene of *A. pyogenes* isolates was sequenced. Genomic DNA of *A. pyogenes* isolates was extracted using the MiniBEST Bacterial Genomic DNA Extraction Kit, version 2.0 (TaKaRa, Dalian, China). The 16S rRNA gene of *A. pyogenes* was amplified by PCR using universal primers (forward: 5'-CCGAATTCGTCGACAACAGAGTTT-GATCCTGGCTCAG-3'; reverse: 5'-CCCGGGATC-CAAGCTTACGGCTACCTTGTTACGACTT-3') as described previously (Weisburg et al., 1991; Ramos, et al., 1997; Narayanan et al., 1998; Jost et al., 2002). Poly-

merase chain reaction products were purified and cloned for sequencing. Nucleotide sequencing was performed with an ABI Prism Big Dye Primer Cycle Sequencing Ready Reaction Kit and ABI 377 DNA autosequencing machine using T7 and SP6 primers (TaKaRa), and analyzed with DNASTAR software (DNASTAR Inc., Madison, WI) and the program NCBI-BLAST (<http://www.ncbi.nlm.nih.gov>).

Antibacterial Susceptibility Assay

The following antimicrobial agents were provided by The Chinese Institute of Veterinary Drug Control (Beijing, China): ciprofloxacin, ofloxacin, enrofloxacin, gatifloxacin, streptomycin, gentamicin, amikacin, oxytetracycline, tetracycline, doxycycline, penicillin G, amoxicillin, oxacillin, cefazolin, ceftiofur, sulfadiazine, sulfamethoxydiazine, florfenicol, erythromycin, tilmi-cosin, azithromycin, clindamycin, and bacitracin zinc. The 23 antimicrobial agents in this study included 10 classes of antibiotics, with 4 in the fluoroquinolones, 3 in the aminoglycosides, 3 in the tetracyclines, 3 in the penicillins, 2 in the cephalosporins, 2 in the sulfonamides, 3 in the macrolides, and 1 each in the chloramphenicols, lincosamides, and polypeptide antibiotics, respectively.

Antibacterial MIC against *A. pyogenes* were assayed using a standard broth dilution method, with final inocula of 4×10^5 cfu/mL on Müller-Hinton medium supplemented with 5% fetal calf serum as outlined by the Clinical and Laboratory Standards Institute (2007). The reference strain ATCC 19411 was used as a control on each plate, as described previously (Guerin-Fauble et al., 1993; Lehtolainen et al., 2003; Pitkälä et al., 2004). Minimum inhibitory concentration values were analyzed by MIC₅₀ and MIC₉₀ (MIC₅₀ = MIC, 50% growth inhibition; MIC₉₀ = MIC, 90% growth inhibition).

Detection of the Integrase Gene and Gene Cassettes

The template DNA for PCR was prepared by the MiniBEST Bacterial Genomic DNA Extraction Kit, version 2.0 (TaKaRa). The general strategies for PCR detection of various types of integrons and gene cassettes were according to the methods described by Hall and Collis (1995) and Bass et al. (1999). Specific primers used for PCR detection of the integrase gene (*intI* I and *intI* II) and gene cassette region are shown in Table 1. All positive amplicons were purified and cloned for sequencing. The sequencing and data analysis of the integrase gene and gene cassettes were similar to that of the 16S rRNA gene.

Figure 1. Agarose gel electrophoresis of PCR products of the 16S rRNA gene obtained from *Arcanobacterium pyogenes*. 1: DL2000 DNA marker (TaKaRa, Dalian, China); 2: ATCC 19411 positive control; 3 to 14: isolation strains; 15: negative control.

RESULTS

Isolation and Characterization of Bacteria from Bovine Endometritis

Thirty-two *A. pyogenes* colonies presumed by gram stain, colony morphology, and bacterial morphology compared with ATCC 19411 were further identified by the API Coryne Vit system (bioMérieux SA); the positive rate of *A. pyogenes* was 23.5% (32/136). In addition, other pathogenic bacteria from dairy cow endometritis were identified through API: 57 *E. coli*, 7 *Staph. aureus*, 15 *B. cereus*, 47 *Streptococcus* [*Aerococcus viridans* (15/47), *Streptococcus bovis*II (7/47), *Enterococcus faecium* (11/47)], and 14 anaerobic bacteria (3 *Clostridium naeslundii*, 3 *Clostridium bifermentans*, 4 *Clostridium perfringens*, 2 *Clostridium beijerinckii*, and 2 *Prevotella intermedia*). *Bacillus aeruginosus* was not obtained. Fifteen cases were coinfections of *A. pyogenes* with *E. coli*, and 4 cases were coinfection of *A. pyogenes*, *E. coli*, and anaerobic bacteria.

The 16S rRNA genes of 32 isolates were amplified, cloned, and sequenced using universal PCR primers. Approximate 1.5-kb fragments were obtained from the PCR assays (Figure 1). Sequence alignment analysis by using the GenBank database revealed that the 16S rRNA sequences of all these isolates were highly homologous to the ATCC 19411 (X79225, NCTC 5224; homology rate ~99 to 100%). Seventeen strains' sequence were identical to ATCC 19411 (homology rate 100%).

Therefore, all 32 isolates were identified as *A. pyogenes*. The 16S rRNA gene sequences of some isolates in this study have been submitted to the GenBank database, with accession numbers EU268194 (RY10-1), EU268193 (HC-H10), EU268191 (HC-H13-2), EU268192 (RY04-2), EU267794 (BM-H07-1), EU267795 (RY11-3), and EU308588 (TZQ 01), respectively.

Antimicrobial Susceptibility

As shown in Table 2, all *A. pyogenes* isolates were highly susceptible to fluoroquinolones, such as ciprofloxacin, gatifloxacin, enrofloxacin, and ofloxacin, with MIC₉₀ at 2.0, 2.0, 1.0, and 0.5 µg/mL for ciprofloxacin, gatifloxacin, enrofloxacin, and ofloxacin, respectively. Similarly, all isolates were susceptible to tilmicosin and azithromycin, and 82.2% isolates were susceptible to erythromycin, with MIC in the range of 0.0625 to ≥32 µg/mL. Some *A. pyogenes* isolates were resistant to florfenicol (9.4%), gentamicin (18.8%), cefazolin (37.5%), and amikacin (40.6%). However, high resistance rates were observed in penicillins, including penicillin G (65.6%), amoxicillin (50%), and oxacillin (56.3%), with MIC₉₀ of 4 to 8 µg/mL, and cephalosporins, such as ceftiofur (71.9%) and cefazolin (37.5%). The same tendency was discovered in oxytetracycline (53.1%), tetracycline (68.8%), and doxycycline (62.5%), with relatively high MIC₉₀ of 16 to 32 µg/mL. Moreover, all isolates were resistant to sulfadiazine, sulfamethoxydiazine, and bacitracin zinc.

Detection and Characterization of the Integrase Gene and Gene Cassettes

As shown in Table 3, class II integrons was not detected, whereas 50% (n = 16/32) of the isolates were positive for the *intI* I, which was the marker for class I integrons (Figure 2). Consequently, the gene cassettes were cloned and sequenced. The integrons were found to harbor 1 or 2 gene cassettes, and combinations of these gene cassettes are shown in Table 3. These gene cassettes existed in multiresistant (n = ~3 to 7) isolates and included genes encoding aminoglycoside resistance

Table 1. Polymerase chain reaction primers and the results of detection

Gene	PCR primer	Expected size of PCR product (bp)	Reference	No. positive (n = 32)
<i>intI</i> I	Forward: CCTCCGACGATGATC Reverse: TCCACGCATCGTCAGGC	280	Bass et al. (1999)	16/32
<i>intI</i> II	Forward: TTATTGCTGGGATTAGGC Reverse: ACGGCTACCCCTCTGTTATC	233	Hall and Collis (1995)	0/32
Cassette	Forward: GGCATCCAAGCAGCAAG Reverse: AAGCAGACTTGACCTGA	Unpredictable	Bass et al. (1999)	13/32

Table 2. In vitro susceptibilities of 32 *Arcanobacterium pyogenes* strains isolated from cow endometritis

Antimicrobial agent	MIC (mg/L)			MIC of ATCC 19411 (mg/L)	Break point (mg/L)	Resistant isolates (%) (no.; n = 32)
	MIC ₅₀	MIC ₉₀	Range			
Ciprofloxacin	2	2	2 to 4	2	≥16	0 (0/32)
Ofloxacin	2	2	0.5 to 4	2	≥16	0 (0/32)
Gatifloxacin	0.5	0.5	0.5	0.5	≥4	0 (0/32)
Enrofloxacin	0.25	1	0.25 to 1	0.5	≥4	0 (0/32)
Streptomycin	≥64	≥64	1 to ≥64	≥64		84.4 (27/32)
Gentamicin	0.5	8	0.25 to ≥32	0.25	≥2	18.8 (6/32)
Amikacin	4	≥64	0.5 to ≥64	1	≥8	40.6 (13/32)
Oxytetracycline	8	32	4 to ≥64	8	≥64	53.1 (17/32)
Tetracycline	1	32	0.5 to 32	0.5	≥4	68.8 (22/32)
Doxycycline	0.5	16	0.0625 to 32	1	≥8	62.5 (20/32)
Penicillin G	2	4	0.125 to 16	0.25	≥2	65.6 (21/32)
Amoxicillin	1	4	0.125 to 16	0.25	≥2	50 (16/32)
Oxacillin	2	8	0.125 to 32	0.25	≥2	56.3 (18/32)
Cefazolin	1	16	0.125 to 15	2	≥16	37.5 (12/32)
Ceftiofur	8	16	0.5 to ≥32	0.5	4	71.9 (23/32)
Florfenicol	1	4	0.5 to 8	1	8	9.4 (3/32)
Sulfadiazine	≥128	≥128	≥128	≥128		100 (32/32)
Sulfamethoxydiazine	≥128	≥128	≥128	≥128		100 (32/32)
Erythromycin	0.125	2	0.0625 to ≥32	0.125	1	18.8 (6/32)
Tilmicosin	0.25	0.25	0.25	0.25	2	0 (0/32)
Azithromycin	1	2	0.5 to 2	1	≥8	0 (0/32)
Clindamycin	0.25	32	0.125 to ≥32	0.25	≥2	53.1 (17/32)
Bacitracin zinc	≥32	≥32	≥32	≥32		100 (32/32)

(*aadA1*, *aadA5*, *aadA24*, and *aadB*), chloramphenicol resistance (*cmlA6*), and ORF1, respectively. The 1,048- and 1,608-bp amplicons were revealed to contain *aadA5* and *aadA24*-ORF1 gene cassettes, respectively, and the 3,149-bp amplicon included *aadA1*-*aadB*-*cmlA6* gene cassettes (Figure 3).

DISCUSSION

The presence of pathogenic bacteria, including *E. coli* and *A. pyogenes*, disrupts ovarian follicle growth and function (LeBlanc, 2008; Sheldon et al., 2008). *Arcanobacterium pyogenes* was the predominant aerobic species; it was found previously in 40% of the samples (Rutter et al., 1999). The isolation rate of *A. pyogenes* from dairy cows was higher at d 26 and 40 postpartum (32.9 and 17.6%, respectively), and the coinfection of *A. pyogenes* and anaerobic bacteria could increase uterine lesions (Bonnett et al., 1991).

In this paper, the cows sampled were classified as having puerperal metritis, clinical metritis, clinical endometritis, or subclinical endometritis, according to the reports of Sheldon et al. (2008) and Földi et al. (2006). This study showed that the positive rate of *A. pyogenes* from the uteri of bovines with metritis or endometritis was 23.5%; 65.6% (21/32) *A. pyogenes* were isolated from cows with metritis and clinical endometritis. Meanwhile, 12.5% (4/32) of the cases infected with *A. pyogenes* were coinfections of *A. pyogenes*, *E. coli*, and anaerobic bacteria, 2 cases of which were within

15 d postpartum and 2 cases of which were within 21 d postpartum. The finding that 46.9% (15/32) of the cases were coinfections of *A. pyogenes* and *E. coli* was consistent with the report of Cohen et al. (1996), who found aerobic and anaerobic bacterial coinfections in 55% of the samples. There was a strong association between isolation of *A. pyogenes* and *Streptococcus* within 21 d postpartum. Further study of the effect of coinfection on uterine lesions and cow reproductive capacity should be carried out. Because antibacterials were given to endometritic cows by veterinarians or herd owners only once, there was hardly any abatement of the clinical symptoms of cows with endometritis. Samples were collected promptly at this time, when the resistance might not be developed. Of the 32 *A. pyogenes* isolates, only 6 were from cows treated with antibacterials before sampling. For example, although 4 of the 6 *A. pyogenes* isolates were resistant to penicillin G, the resistance level was not obviously different from that of untreated cows.

The antimicrobials examined in this study are available for food-producing animals in China, and their withdrawal times have been defined. The actions of some antibacterial agents were not affected by purulent materials, such as β -lactam antibacterial and aminoglycoside agents, but the action of sulfonamides was largely affected by the intrauterine discharge. In addition, antimicrobial agents are administrated intrauterine after the uterus has been washed with sodium chloride, during which large amounts of intrauterine liquid were

Table 3. Antibiotic resistance patterns of 32 *Arcanobacterium pyogenes* strains to 10 kinds of antimicrobial agents and its correlation with occurrence of the integrons

Resistance patterns ¹	No. of resistant strains (n = 32)	Rate of resistance patterns (%)	Detection rate of class I integrons ² (%) (no.; n = 32)	Inserted gene cassettes (order in the resistant strains)
sb	1	3.1	— ³	—
sbc	5	15.6	6.3 (2)	<i>aadA1-addB-cmlA6</i> (1)
sbp	3	9.4	3.1 (1)	<i>aadA5</i> (1)
sbpc	2	6.3	—	—
sbcl	2	6.3	—	—
sbcg	1	3.1	3.1 (1)	<i>aadA24-ORF1</i> (1)
sbce	1	3.1	—	—
sbpl	1	3.1	—	—
sbcep	1	3.1	3.1 (1)	<i>aadA1-addB-cmlA6</i> (1)
sbple	1	3.1	3.1 (1)	<i>aadA24-ORF1</i> (1)
sbcfl	2	6.3	6.3 (2)	<i>aadA5</i> (1)
sbcpo	1	3.1	3.1 (1)	<i>aadA5, aadA1-addB-cmlA6</i> (1)
sbcpl	3	9.4	3.1 (1)	<i>aadA24-ORF1</i> (1)
sbcgp	1	3.1	—	—
sbcgpl	2	6.3	3.1 (1)	<i>aadA24-ORF1</i> (1)
sbfgpl	2	6.3	6.3 (2)	<i>aadA1-addB-cmlA6</i> (2)
sbcepl	1	3.1	3.1 (1)	<i>aadA24-ORF1</i> (1)
sbceopl	2	6.3	6.3 (2)	<i>aadA5, aadA24-ORF1</i> (1)

¹Abbreviations for antimicrobial agents: s = sulfadiazine; b = bacitracin zinc; p = penicillin G; c = ceftiofur; e = erythromycin; f = florfenicol; g = gentamicin; l = clindamycin; o = oxytetracycline.

²Class I integrons were detected in 16 *A. pyogenes* isolates, but only 13 contained gene cassettes. Class II integrons were not detected in any *A. pyogenes* isolate.

³A dash indicates not detected.

discarded; thus, the factors that affected the drug action had been reduced as much as possible.

As shown in Table 2, most of the *A. pyogenes* isolates were susceptible to gentamicin but were highly resistant to streptomycin, clindamycin, and tetracyclines. Similar findings were reported by Guerin-Faublee et al. (1993), who found that despite *A. pyogenes* being isolated from diverse animals and samples, only 4 were recovered from uterine discharges. However, Cohen et al. (1996) reported that all isolates from the uteri of dairy cows were susceptible to clindamycin and that most isolates were resistant to tetracycline. Trinh et al. (2002) showed that *A. pyogenes* isolated from bovines had different resistance rates to tetracyclines (25.9%) and macrolides (22.2%), respectively. In addition, penicillin has been shown to be highly effective against *A. pyogenes* (Guerin-Faublee et al., 1993; Ali, 2000). In this study, 65.6% of the isolates were resistant to penicillin and 56.3% (18/32) were resistant to oxytetracycline; this is higher than in the previous reports of Trinh et al. (2002) and Sheldon et al. (2004; MIC₅₀ of 16 µg/mL). The difference may be due to penicillins and oxytetracycline being used to treat bovine endometritis in China. No isolate was resistant to quinolones, which may have been caused by overuse of penicillin for endometritis therapy, with quinolones being used less in veterinary medicine.

In the multiresistant isolates, the resistance rates to penicillin G, amoxicillin, and oxacillin were very similar

to each other, suggesting high cross-resistance rates in penicillins. However, 50.0% of these isolates were resistant to more than 5 antibiotics, with very high MIC values. The different resistance patterns were probably due to the different aims and kinds of antimicrobial agents used on these dairy farms.

According to prior reports, the rate of gene cassettes-integrons in gram-negative bacteria was higher than that in gram-positive bacteria, and these integrons also played significant roles in transfer of the antimicrobial resistance gene. Keyes et al. (2000) reported that the positive-integron incidence rate in *E. coli* isolates from chickens was 82%. Wang et al. (2008) reported that it

Figure 2. Agarose gel electrophoresis of the *intI1* gene amplification fragment. 1 to 6: *intI1* gene positive isolates; 7: DL2000 DNA marker (TaKaRa, Dalian, China).

Figure 3. Agarose gel electrophoresis of gene cassette amplification products. Left) 1,048 bp; Center) 3,149 bp; Right) 1,608 bp.

was 56.90% from bovine mastitis. Antunes et al. (2006) found that there were 1 or 2 class I integrons among *Salmonella*. However, no gene cassettes-integrons were reported among *A. pyogenes*. In the present study, the positive integron rate was 50% (16/32) from *A. pyogenes* isolates, but the rate of gene cassettes among these integrons was 81.25% (13/16), and integrons in the other 3 isolates were empty. This is the first report of the gene cassettes-integrons in *A. pyogenes*. It has been indicated that the detection rate of integrons is related to the antimicrobial resistance of *A. pyogenes* (Rowe-Magnus et al., 2002).

The discovery of antimicrobial resistance gene cassettes in this study revealed that aminoglycoside-resistance determinants (*aadA5*, *aadA24*, and *aadB*), chloramphenicol-resistance determinants (*cmlA6*), and ORF1 (whose function is not known) were prevalent among *A. pyogenes* strains isolated from cow endometritis, and these resistance genes were carried by I integrons in genomic DNA. The gene cassette arrays were different from those of other resistant bacteria. Although *aadA5*, *aadB*, and *cmlA6* had been detected in *E. coli* and *Salmonella*, the cassette arrangements were different in these bacteria: gene cassettes of *dfrA17-aadA5* and *aadB-aadA1* or *aadB-cmlA1* were frequently discovered in *E. coli* (Kang et al., 2005; Chang et al., 2007; Wang et al., 2008), whereas *dfrA1-aadA24* was found in *Salmonella* (Rodríguez et al., 2008). In this study, the *aadB-cmlA6* array was a novel type of cassette arrangement. These cassettes segregated according to a consistent pattern, with *aadA5* always alone, ORF1 always with *aadA24*, and *aadA1-aadB* and *cmlA6* always together. This may be explained by antibiotic selective pressure and resistance transfer between different bacterial species.

However, the class I integron examined in this study did not account for the total resistance phenotype observed among the *A. pyogenes* isolates from cow

endometritis, such as that there was high resistance of *A. pyogenes* isolates to β -lactams, macrolides, and tetracyclines, which could possibly be attributed to the presence of other mobile DNA elements or some undetectable resistance genes. Further investigations are needed to assess determinants of antimicrobial resistance other than integron-mediated resistance in these isolates.

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