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Short Communication

A novel genomic island harbouring *lsa*(E) and *lnu*(B) genes and a defective prophage in a *Streptococcus pyogenes* isolate resistant to lincosamide, streptogramin A and pleuromutilin antibiotics

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ABSTRACT

A lincosamide-resistant and macrolide-susceptible phenotype has not been described to date in *Streptococcus pyogenes* [group A streptococcus (GAS)]. The aim of this study was to characterize a GAS isolate susceptible to macrolides but resistant to lincosamide, streptogramin A and pleuromutilin antibiotics. Antimicrobial susceptibility was tested using the microdilution broth method and the resistance phenotype was tested by D-test. The GAS2887HUB isolate was subjected to whole-genome sequencing. The isolate showed a positive Gots' test (clindamycin inactivation). Whole-genome sequencing revealed that the strain was ST10 and *emm*93, and had five resistance genes [*lnu*(B), *ant*(6)-Ia, *aph*(3′)-III, *tet*(M) and *dfrG*]. The *tet*(M) gene was located in a Tn*916*-like transposon. The *lsa*(E)–*lnu*(B)-containing sequence (inserted downstream of the *rumA* gene) was formed by a 39.6-kb prophage, followed by a gene cluster encoding aminoglycoside–streptothricin resistance [*ant*(6)Ia–*sat4*–*aph*(3′)III] and *lsa*(E)–*lnu*(B) genes. This structure was not transferred by conjugation. This study identified a new genetic element carrying a determinant of lincosamide resistance in a GAS. Further molecular epidemiological surveys are needed to determine the prevalence of this mechanism of resistance in GAS.

1. Introduction

Streptococcus pyogenes [group A streptococcus (GAS)] is a major human pathogen that causes infections such as tonsillitis and skin and soft tissue infections [1]. Penicillins and cephalosporins are widely used to treat GAS infections due to the universal susceptibility of this bacterium to β-lactams. In cases of β-lactam allergy, macrolides or lincosamides are the recommended alternatives. The addition of protein synthesis inhibitors, such as clindamycin, may effectively reduce the synthesis of virulence factors and improve patient outcomes [2].

To date, two transferable mechanisms of macrolide and lincosamide resistance have been described in GAS (ML phenotype). One involves 23S rRNA methylation (*erm* genes), responsible for resistance to macrolides, lincosamides and streptogramins B (MLS_B phenotype). The other involves active efflux (*mef* genes), conferring resistance to 14- and 15-membered macrolides (M phenotype) [3,4]. These genes are widespread in streptococci, mainly through the transfer of mobile genetic elements (MGEs) of the Tn*916* family. Two additional phenotypes have been reported sporadically in other streptococci: lincosamide inactivation by a nucleotidyltransferase (L phenotype, *lnu* genes); and resistance to lincosamide, streptogramin A and pleuromutilin antibiotics due to ribosomal protection (LS_AP phenotype; *lsa* genes) [5,6]. The latter mechanism, also carried by MGEs, has been described in several human and

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animal streptococcal species [7–12], including one *S. pyogenes* isolate [*erm*(B) together with *lsa*(C); MLS_B phenotype] [13].

To the best of the authors' knowledge, this study describes the first *S. pyogenes* isolate with an LS_AP phenotype due to the presence of *lsa*(E) and *lnu*(B) genes. It shows that the genetic element carrying these genes was inserted into the *S. pyogenes* chromosome with a defective prophage similar to those recovered from swine pathogens.

2. Materials and methods

2.1. Bacterial strain and antibiotic susceptibility testing

An *S. pyogenes* exhibiting a LS_AP phenotype (GAS2887HUB) was obtained from a wound sample of a 77-year-old woman with cellulitis who attended the emergency department in September 2009. She consulted for fever (39 °C), headache, vomiting and a confusional state. Physical examination revealed a pretibial leg ulcer with signs of infection and cellulitis. Antipyretic and amoxicillin-clavulanic acid therapy were started after collecting blood cultures and wound samples. The patient presented good evolution after a 15-h observation period and was discharged. The wound resolved after 2 weeks of amoxicillin-clavulanic treatment and 1 month of re-epithelialization therapy.

Antimicrobial susceptibility was tested by the broth microdilution reference method following EUCAST recommendations. For antibiotics lacking recommendations, the Antibiogram Committee of the French Society for Microbiology breakpoints were used (CA-SFM: www.sfm-microbiologie.org/). The ML phenotype was assessed by the D-test disk-diffusion method [3]. The following antimicrobials were tested: ampicillin, penicillin, cefotaxime, erythromycin, azithromycin, spiramycin, clindamycin, lincomycin, pristinamycin, quinupristin, dalfopristin, quinupristin-dalfopristin, tiamulin, tetracycline, chloramphenicol, ciprofloxacin, trimethoprim/sulfamethoxazole, amikacin, gentamicin, kanamycin, streptomycin and tobramycin. Lincosamide inactivation was screened using Gots' test, as described previously [14].

2.2. Whole-genome sequencing analysis

Genomic DNA was extracted using a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany), quantified with a QuantiFluor dsDNA System (Promega Corp., Madison, WI, USA) and was adjusted to 0.2 ng/μL. The genome was sequenced using a 150 bp paired-end read protocol (NexteraXT kit and MiSeq, Illumina, Los Angeles, CA, USA) at Macrogen, Inc. (Seoul, Republic of Korea). The quality of sequencing was assessed with FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Afterwards, reads were trimmed, duplicated reads were removed and errors were corrected. Finally, reads were assembled with Geneious 9.1.7 (Biomatters, Auckland, New Zealand).

The *emm* type and multi-locus sequence type (MLST) were deduced using the *emm* CDC databases for whole-genome sequencing (https://www2a.cdc.gov/ncidod/biotech/strepblast.asp; https://pubmlst.org/spyogenes/). Additional sequence analysis was performed using different available online tools (ResFinder 3.0, ICEberg and Phaster) to explore the presence of acquired resistance mechanisms and different MGEs, such as integrative conjugative elements and prophages. The genetic environment of *lsa*(E)–*lnu*(B) was studied through comparison with previously described sequences present in public databases. For the putative prophage sequences predicted by Phaster, further characterization was achieved using BlastX. Comparisons between GAS2887HUB, sequences found to be similar based on nucleotide sequence alignments, and *lsa*(E)–*lnu*(B) structures described previously in streptococci were displayed using Easyfig program.

2.3. Mating experiments

S. pyogenes GAS3493HUB and *Streptococcus agalactiae* GBS4777HUB (rifampin-resistant minimum inhibitory concentration: 32 mg/L and clindamycin susceptible) were used as recipient strains for conjugation experiments performed on membrane filter, as described [15]. Transconjugants were selected using blood agar plates containing 20mg/L of rifampicin and 4 mg/L of clindamycin. Mating experiments were repeated three times.

3. Results and discussion

3.1. Antibiotic resistance genes, susceptibility testing and molecular typing

The GAS2887HUB isolate was resistant to clindamycin, lincomycin, dalfopristin, tiamulin, tetracycline, amikacin, kanamycin, streptomycin and trimethoprim/sulfamethoxazole, but was susceptible to the remaining antibiotics tested (Table S1, see online supplementary material). Gots' test was positive, proving lincosamide inactivation, whereas the D-test failed to reveal either synergy or induction events. Consistent with this, the analysis of acquired genes for antibiotic resistance (ResFinder 3.0) demonstrated the presence of *lnu*(B), *ant*(6)-Ia, *aph*(3′)-III, *tet*(M) and *dfrG*. *lsa*(E) was not detected because it is not included in the ResFinder database. Besides this, the GAS2887HUB strain harboured a Tn*916* element [*tet*(M) gene], two prophage-like sequences (P1 and P2) and a partly deleted prophage (P3) (Fig. S1, see online supplementary material).

The GAS2887HUB strain was *emm93* and the sequence type was ST10, a rare association. In fact, this was the only isolate with *emm93* among nearly 500 GAS collected over a 20-year period. Furthermore, the GAS MLST database contains only 14 ST10 isolates, of which only three contain *emm93* (two isolates from India and one from Egypt). However, clindamycin susceptibility was not reported for any of these isolates, which meant that the authors could not clarify the putative clonal association of these resistance mechanisms with other resistance mechanisms in GAS [3].

3.2. Whole-genome sequencing analysis: genetic context of *lsa(E)-lnu(B)* genes

To the best of the authors' knowledge, the genetic environment of *lsa*(E)–*lnu*(B) genes in the study strain was different to that reported previously [7,8,13,16,17]. The *lsa*(E)–*lnu*(B) genes resided together with the 39.6-kb P2 that was inserted downstream of the *rumA* gene (Fig. 1A). This sequence was formed by a 39.6-kb prophage, followed by an aminoglycoside–streptothricin resistance cluster [*ant*(6)Ia–*sat4*–*aph*(3′)III] and the *lsa*(E)–*lnu*(B) genes. The extremes of this composite structure were highly similar to the *Streptococcus porci* DSM 23759 prophage (67–100% identity, average 85.8%, Acc. No. AUIP01000001, positions 1–57586) and to the *lsa*(E)–*lnu*(B) genes (99.7% identity, Acc. No. AUIP01000004, positions 2690–7146). Moreover, the GAS2887HUB sequence from *ant*(6)Ia–*sat4*–*aph*(3′)III to *lsa*(E)–*lnu*(B) was found in a different arrangement in *Erysipelothrix rhusiopathiae* Ery-11 [16] (Acc. No. KP339868, 99.1% identity, positions 11622–17508; 99.9% identity, positions 4868–9307). The *ant*(6)Ia–*sat4*–*aph*(3′)III cluster shared 98.1% identity with the *Streptococcus suis* TZ080501 genome (Acc. No. KX077897, positions 51296–56201) (Fig. 1B).

Among streptococci (Fig. 1C), the MGE harbouring *lsa*(E)–*lnu*(B) genes were first reported in an *S. agalactiae* structure (SGB76) also containing a multi-drug resistance cluster (*aadE*–*apt*–*spw*) [8]. This combination was also described with a different arrangement in *S. suis* [17,18]. A second *lsa*(E)–*lnu*(B)-containing element was

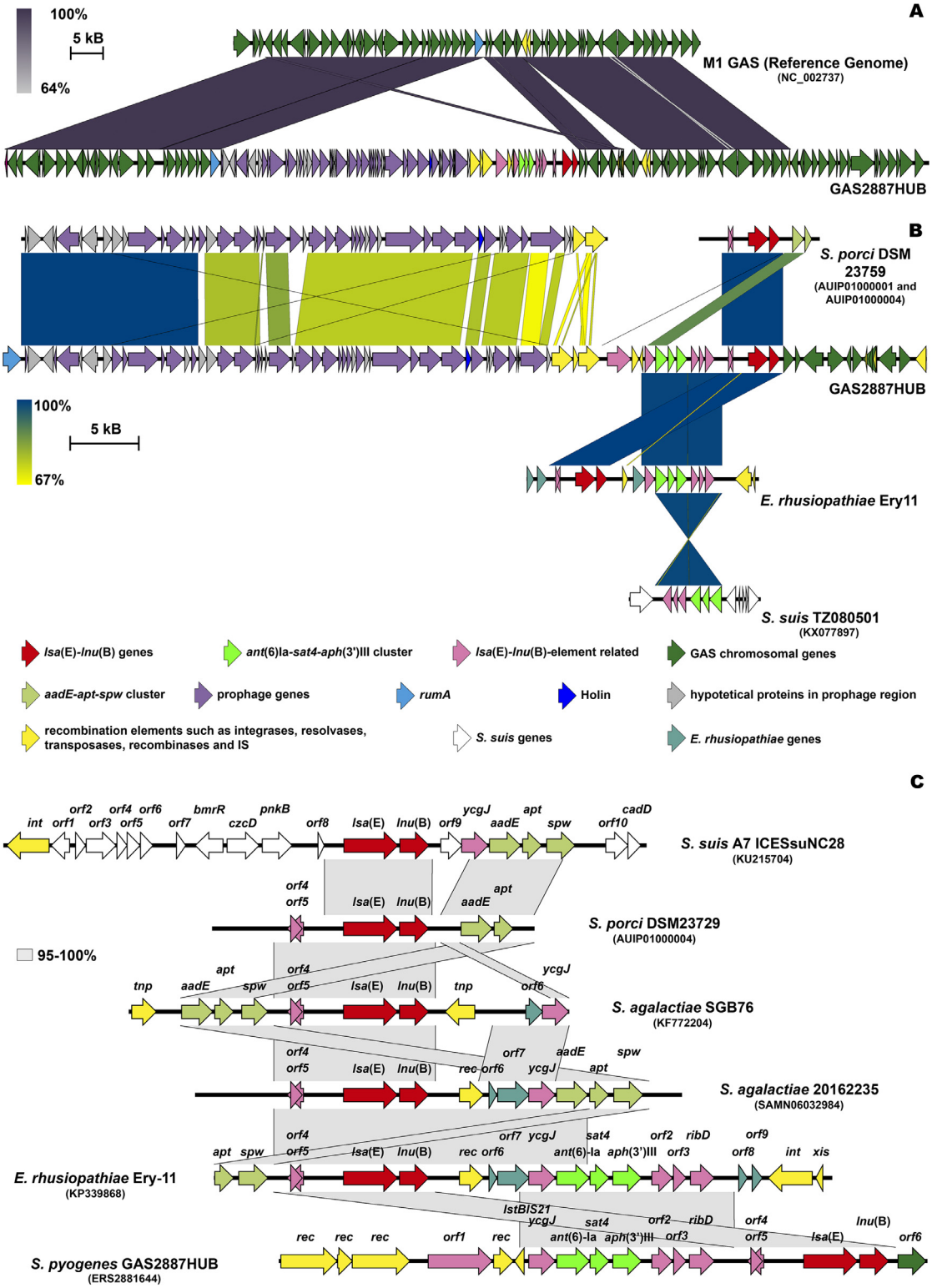

Fig. 1. Schematic and comparative of the sequence containing *lsa*(E)–*lnu*(B). (A) Comparison of GAS2887HUB to the M1 group A streptococcus strain (reference GAS genome: Acc. No. NC_002737 positions 1080518–1152718) showing the insertion of the sequence containing *lsa*(E)–*lnu*(B) genes together with the prophage 39.6-kb P2 downstream of *rumA* [23S rRNA (uracil-5)-methyltransferase]. The purple-gradated regions show 64–100% sequence identity, and the red line highlights the part of the GAS2887HUB sequence shown in more detail in Fig. 1B. (B) Representation of the sequence containing *lsa*(E)–*lnu*(B) genes together with the prophage 39.6-kb P2, showing the relatedness of the prophage sequence and *lsa*(E)–*lnu*(B) genes with *Streptococcus porci* DSM 23759 [19] genome (Acc. No. AUIP01000001 positions 1–57586 and Acc. No. AUIP01000004 positions 1–8831), the different arrangement of the *ant*(6)Ia–*sat4*–*aph*(3′)III cluster and *lsa*(E)–*lnu*(B) genes in *Erysipelothrix rhusiopathiae* Ery-11 [16] (Acc. No. KP339868; positions 11622–17508 and 4868–9307), and the presence of the *ant*(6)Ia–*sat4*–*aph*(3′)III cluster in *Streptococcus suis* TZ080501 [17] (Acc. No. KX077897 positions 46803–68299). The yellow-to-blue shaded regions show 67–100% sequence identity. The green line emphasizes the GAS2887HUB sequence shown in more detail in Fig. 1C. (C) Distribution of the *lsa*(E)–*lnu*(B) genes, the *ant*(6)Ia–*sat4*–*aph*(3′)III cluster and the *aadE*–*apt*–*spw* cluster among the previously described structures in streptococci containing *lsa*(E)–*lnu*(B) genes [ICESsuNC28 of *S. suis* A7 [18] (Acc. No. KU2115704); *S. porci* DSM23759; *Streptococcus agalactiae* SGB76 [8] (Acc. No. KF772204); *S. agalactiae* 20162235 (SRA number SAMN06032954) [13] and *E. rhusiopathiae* Ery-11. There is a different arrangement to that found in GAS2887HUB. The grey regions indicate that there is up to 95% sequence identity.

described in a human *S. agalactiae* isolate (Fig. 1C; 20162235) [7]. Besides this, the *lnu*(B) gene has also been found in streptococci and staphylococci with L and LS_AP phenotypes isolated from animals and some food products [18], indicating that some streptococci serve as a reservoir for antimicrobial resistance genes [17]. When comparing the characterized structures harbouring *lsa*(E)–*lnu*(B) genes with GAS2887HUB (Fig. 1C), three findings were notable [8,16,18,19]. First, the *orf4*–*orf5*–*lsa*(E)–*lnu*(B) structure was highly conserved (95% identity) among them. Second, in the previously described streptococcal structures, the *lsa*(E)–*lnu*(B) genes went together with a multi-drug resistance cluster (*aadE*–*apt*–*spw*). Third, the aminoglycoside–streptothricin resistance cluster [*ant*(6)Ia–*sat4*–*aph*(3′)III] detected in the study strain (GAS2887HUB) was found in a different arrangement in *E. rhusiopathiae*.

The presence of a prophage highly similar to one found previously in animal isolates of *S. porci* DSM 23759 [19] and *E. rhusiopathiae* [16,20] could suggest a putative animal source for this element. It is likely that the widespread use of lincosamides to treat (or prevent) infections in animals could have favoured the selection and spread of such mechanisms of resistance. In the study strain, the genomic island located downstream of the defective prophage, which contains the *ant*(6)Ia–*sat4*–*aph*(3′)III–*cluster and lsa*(E)–*lnu*(B) resistance genes, could be a disrupted integrative mobilizable element. Nonetheless, it contains integration- and excision-like genes and one putative insertion sequence, but no genes required for MGE conjugation. However, no transconjugants were obtained after mating experiments.

Clindamycin is frequently used, combined with a β-lactam, for the treatment of severe skin and soft tissue infections as it prevents the ribosomal synthesis of virulence factors. Furthermore, the World Health Organization (WHO) has included lincosamides and streptogramins in the list of critically important antimicrobials for human medicine. To date, clindamycin resistance in GAS has been associated with *erm* genes responsible for the MLS_B phenotype. The description of this new genomic island encoding lincosamide resistance in macrolide-susceptible GAS is a cause for concern, and surveillance is needed of this rare resistance mechanism that could be underestimated if clindamycin is not tested routinely. Moreover, *lnu*(B)-*lsa*(E) confers resistance to pleuromutilins. Among them, lefamulin is one of the 11 phase III antimicrobials included in the WHO 2017 priority list for antibacterial agents.

4. Conclusion

In conclusion, this study described a genomic island conferring a LS_AP phenotype in a GAS similar to those found in *S. porci* and *E. rhusiopahiae*. In-vitro mobilization of this element by conjugation was not achieved. The presence of a lincosamide inactivation gene precludes the use of lincosamides in the treatment of severe GAS infections from this pathogen. Further studies are needed to understand if this mechanism of resistance is spread among GAS.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijantimicag.2019.08.019.

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References

[1] Walker MJ, Barnett TC, McArthur JD, Cole JN, Gillen CM, Henningham A, et al. Disease manifestations and pathogenic mechanisms of group A streptococcus. Clin Microbiol Rev 2014;27:264–301.
[2] Andreoni F, Zürcher C, Tarnutzer A, Schilcher K, Neff A, Keller N, et al. Clindamycin affects group A streptococcus virulence factors and improves clinical outcome. J Infect Dis 2017;215:269–77.
[3] Ardanuy C, Domenech A, Rolo D, Calatayud L, Tubau F, Ayats J, et al. Molecular characterization of macrolide- and multidrug-resistant *Streptococcus pyogenes* isolated from adult patients in Barcelona, Spain (1993–2008). J Antimicrob Chemother 2010;65:634–43.
[4] Cattoir V. Mechanisms of antibiotic resistance. In: Ferretti JJ, Stevens DL, Fischetti VA, editors. *Streptococcus pyogenes*: basic biology to clinical manifestations, 6. Oklahoma City, OK: University of Oklahoma Health Sciences Center; 2016. p. 1–34.
[5] Bozdogan B, Berrezouga L, Kuo MS, Yurek DA, Farley KA, Stockman BJ, et al. A new resistance gene, *lin*B, conferring resistance to lincosamides by nucleotidylation in *Enterococcus faecium* HM1025. Antimicrob Agents Chemother 1999;43:925–9.
[6] Sharkey LKR, Edwards TA, O'Neill AJ. ABC-F proteins mediate antibiotic resistance through ribosomal protection. MBio 2016;7:1–10.
[7] Hawkins PA, Law CS, Metcalf BJ, Chochua S, Jackson DM, Westblade LF, et al. Cross-resistance to lincosamides, streptogramins A and pleuromutilins in *Streptococcus agalactiae* isolates from the USA. J Antimicrob Chemother 2017;72:1886–92.
[8] Montilla A, Zavala A, Cáceres Cáceres R, Cittadini R, Vay C, Gutkind G, et al. Genetic environment of the *lnu*(B) gene in a *Streptococcus agalactiae* clinical isolate. Antimicrob Agents Chemother 2014;58:5636–7.
[9] Achard A, Villers C, Pichereau V, Leclercq R. New *lnu*(C) gene conferring resistance to lincomycin by nucleotidylation in *Streptococcus agalactiae* UCN36. Antimicrob Agents Chemother 2005;49:2716–19.
[10] Petinaki E, Guerin-Faublee V, Pichereau V, Villers C, Achard A, Malbruny B, et al. Lincomycin resistance gene *lnu*(D) in *Streptococcus uberis*. Antimicrob Agents Chemother 2008;52:626–30.
[11] Gravey F, Galopin S, Grall N, Auzou M, Andremont A, Leclercq R, et al. Lincosamide resistance mediated by *lnu*(C) (L phenotype) in a *Streptococcus anginosus* clinical isolate. J Antimicrob Chemother 2013;68:2464–7.
[12] Almuzara M, Bonofiglio L, Cittadini R, Vera Ocampo C, Montilla A, del Castillo M, et al. First case of *Streptococcus lutetiensis* bacteremia involving a clindamycin-resistant isolate carrying the *lnu*(B) gene. J Clin Microbiol 2013;51:4259–61.
[13] Chochua S, Metcalf BJ, Li Z, Rivers J, Mathis S, Jackson D, et al. Population and whole genome sequence based characterization of invasive group A streptococci recovered in the United States during 2015. MBio 2017;8:1–19.
[14] Leclercq R, Carlier C, Duval J, Courvalin P. Plasmid-mediated resistance to lincomycin by inactivation in *Staphylococcus haemolyticus*. Antimicrob Agents Chemother 1985;28:421–4.
[15] Giovanetti E, Magi G, Brenciani A, Spinaci C, Lupidi R, Facinelli B, et al. Conjugative transfer of the *erm*(A) gene from erythromycin-resistant *Streptococcus pyogenes* to macrolide-susceptible *S. pyogenes*, *Enterococcus faecalis* and *Listeria innocua*. J Antimicrob Chemother 2002;50:249–52.
[16] Zhang A, Xu C, Wang H, Lei C, Liu B, Guan Z, et al. Presence and new genetic environment of pleuromutilin-lincosamide-streptogramin A resistance gene *lsa*(E) in *Erysipelothrix rhusiopathiae* of swine origin. Vet Microbiol 2015;177:162–7.

[17] Huang J, Ma J, Shang K, Hu X, Liang Y, Li D, et al. Evolution and diversity of the antimicrobial resistance associated mobilome in *Streptococcus suis*: a probable mobile genetic elements reservoir for other streptococci. Front Cell Infect Microbiol 2016;6:118.
[18] Huang K, Zhang Q, Song Y, Zhang Z, Zhang A, Xiao J, et al. Characterization of spectinomycin resistance in *Streptococcus suis* leads to two novel insights into drug resistance formation and dissemination mechanism. Antimicrob Agents Chemother 2016;60:6390–2.
[19] Vela AI, Perez M, Zamora L, Palacios L, Dominguez L, Fernandez-Garayzabal JF. *Streptococcus porci* sp. nov., isolated from swine sources. Int J Syst Evol Microbiol 2010;60:104–8.
[20] Wang Q, Chang BJ, Riley T V. Erysipelothrix rhusiopathiae. Vet Microbiol 2010;140:405–17.