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Cross-resistance to lincosamides, streptogramins A and pleuromutilins in *Streptococcus agalactiae* isolates from the USA

Paulina A. Hawkins[1,2], **Caitlin S. Law**[2], **Benjamin J. Metcalf**[2], **Sopio Chochua**[2], **Delois M. Jackson**[2], **Lars F. Westblade**[3], **Robert Jerris**[4], **Bernard W. Beall**[2], and **Lesley McGee**[2,*]

[1]Rollins School of Public Health, Emory University, Atlanta, GA, USA

[2]Centers for Disease Control and Prevention, Atlanta, GA, USA

[3]Weill Cornell Medical College, New York, NY, USA

[4]Children's Healthcare of Atlanta, Atlanta, GA, USA

Abstract

Background—*Streptococcus agalactiae* (Group B Streptococcus, GBS) is a leading cause of meningitis, sepsis and pneumonia in neonates in the United States. GBS also causes invasive disease in older infants, pregnant women, children and young adults with underlying medical conditions, and older adults. Resistance to lincosamides in the absence of erythromycin resistance is rare in GBS, but has been previously reported in clinical isolates, both on its own or in combination with resistance to streptogramins A and pleuromutilins (L/LSA/LSAP phenotypes).

Objectives—To retrospectively screen the Active Bacterial Core surveillance (ABCs) GBS isolate collection for these phenotypes in order to identify the causal genetic determinants and determine whether their frequency is increasing.

Methods—Based on MIC data, 65 (0.31%) isolates susceptible to erythromycin (MIC ≤0.25 mg/L) and non-susceptible to clindamycin (MIC ≥0.5 mg/L) were identified among 21186 GBS isolates. Genomic DNA was extracted and WGS was performed. The presence of 10 genes previously associated with LSA resistance was investigated by read mapping.

Results—Forty-nine (75%) isolates carried the *lsa*(C) gene and expressed the LSAP phenotype, and 12 (18%) carried both the *lnu*(B) and *lsa*(E) genes and expressed the LSAP phenotype. The four remaining isolates were negative for all determinants investigated.

Conclusions—While the overall observed frequency of these phenotypes among our GBS isolates was quite low (0.31%), this frequency has increased in recent years. To the best of our

*Corresponding author. lmcgee@cdc.gov.

Transparency declarations

None to declare.

Disclaimer

The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the Centers for Disease Control and Prevention.

knowledge, this is the first time the LSAP phenotype has been reported among GBS isolates from the USA.

Introduction

Streptococcus agalactiae (Group B *Streptococcus*, GBS) is a leading cause of meningitis, sepsis and pneumonia in neonates in the United States. In addition to illness in the first week of life, GBS also causes invasive disease in older infants, pregnant women, children and young adults with underlying medical conditions, and older adults.

Penicillin G and ampicillin are the drugs of choice for the prevention or treatment of *S. agalactiae* infections, while erythromycin or clindamycin are the recommended alternatives for patients who are allergic to β-lactams.[1] Although reduced susceptibility to penicillin has been reported,[2] clinical GBS isolates remain susceptible to β-lactams, although the proportion of isolates resistant to erythromycin and clindamycin has increased in recent years.[3–5] Resistance to these antibiotics is usually due to the modification of ribosomal targets, most commonly mediated by an *erm* methylase, which confers cross-resistance to macrolides (e.g. erythromycin, azithromycin), lincosamides (e.g. clindamycin, lincomycin) and streptogramin B antibiotics (e.g. virginiamycin S1, pristinamycin IA, quinupristin), known as the MLS_B phenotype.[6]

In contrast, resistance specific to lincosamides (L phenotype) is rare in GBS, but has been previously reported in clinical isolates from Canada,[7] the United States,[8] Spain,[9] Argentina,[10] Korea[11] and South Africa,[12] due to antibiotic modification mediated by the *lnu*(B) gene. Members of the *lnu* (previously *lin*) gene family encode nucleotidyltransferase enzymes that catalyse the adenylation of lincomycin and clindamycin; *lnu*(B) was first identified in *Enterococcus faecium*,[13] and later in GBS,[7] *Streptococcus uberis*,[14] *Staphylococcus aureus*[15] and *Streptococcus lutetiensis*.[16] Resistance to lincosamides has also been reported in combination with resistance to streptogramin A compounds (e.g. virginiamycin M1, pristinamycin IIA, dalfopristin) and pleuromutilins (e.g. tiamulin, retapamulin, lefamulin) in GBS isolates from Iceland and New Zealand (LSA and LSAP phenotypes), mediated by the *lsa*(C) gene, probably by active efflux, but the exact mechanism remains elusive.[17,18] The *lsa*(E) gene mediates a similar resistance phenotype in *S. aureus*, in combination with *lnu*(B).[19]

Other genes associated with the LSA phenotype include *vga*(A) and *cfr*. The *vga* genes have been characterized as determinants of streptogramin A resistance; *vga*(A) also confers low-level resistance to lincomycin in *S. aureus* and the variant $vga(A)_{LC}$ has been identified in *Staphylococcus haemolyticus* strains resistant to lincomycin and clindamycin.[20] The *cfr* gene encodes an rRNA methyltransferase that confers resistance to phenicols, lincosamides, oxazolidinones, pleuromutilins and streptogramin A antibiotics.[21]

Active Bacterial Core surveillance (ABCs) is a core component of the CDC's Emerging Infections Programs network (EIP). ABCs is an active surveillance system for invasive bacterial pathogens of public health importance, including GBS. During routine ABCs activities, we identified several GBS isolates that expressed resistance to clindamycin in the

absence of resistance to erythromycin. In order to determine the nature and frequency of these phenotypes in our GBS collection, we screened the ABCs GBS isolate collection retrospectively for all isolates with erythromycin-susceptible, clindamycin-resistant phenotypes, and performed WGS to identify the genetic determinants that confer these unusual phenotypes.

Materials and methods

GBS surveillance areas currently represent over 33 million persons and 400000 live births within 10 states (http://www.cdc.gov/abcs/methodology/index.html). After collection and submission, serotyping of isolates by latex agglutination and antimicrobial testing by broth microdilution was performed at the CDC *Streptococcus* laboratory. At the time of our study, serotype and antimicrobial resistance data were available for 21186 GBS isolates collected from1998 to 2015.

We screened MIC results for clindamycin and erythromycin, in order to identify isolates susceptible to erythromycin (MIC ≤0.25mg/L) and non-susceptible to clindamycin (MIC ≥0.5mg/L). Sixty-five (0.31%) isolates with this resistance phenotype were found. Genomic DNA was extracted from these 65 isolates and WGS was performed using an Illumina MiSeq. The presence of 10 genes previously associated with LSA resistance was investigated by mapping to the full structural gene sequences using the SRST2 bioinformatics tool:[22] *erm*(A) (X03216), *erm*(B) (M11180), *cfr*(NG_047631), *lnu*(A) (J03947), *lnu*(B) (AJ238249), *lnu*(C) (NG_047924), *lnu*(D) (NG_047925), $vga(A)_{LC}$ (DQ823382), *lsa*(C) (NG_047934) and *lsa*(E) (JQ861959). The maximum divergence and minimum coverage thresholds were both set at 80% initially, then lowered to 50% for isolates against which none of the genes mapped at 80%. SRST2 was also used to determine MLST profiles. SplitsTree4[23] was used to build a phylogenetic network with the concatenated sequences of the seven MLST loci.

In order to determine if these isolates were also cross-resistant to streptogramin A or pleuromutilins, we determined the MICs of virginiamycin M1 (Cayman Chemical, Ann Arbor, MI) and tiamulin (Wako Chemicals, Richmond, VA) by broth microdilution, following CLSI guidelines.[24] Since interpretive standards had not been established for these compounds against GBS, we used previously reported values along with MIC distributions of control isolates to determine cut-off values.[25–27] Briefly, we determined broth dilution MIC values of virginiamycin M1 and tiamulin in 45 GBS control isolates displaying three different phenotypes (15 of each): EryS/CliS, EryR/CliS and EryR/CliR; and compared these values with those obtained from the 65 EryS/CliR test isolates.

Results

The EryS/CliR isolates are described in Table 1. The most common serotype among these isolates was serotype III (n = 24, 37%), followed by serotype V (n = 18, 28%), and serotype Ia (n = 16, 25%). Of the 60 isolates for which age group data were available, 11 (18.3%) were from infants, 32 (53.3%) from adults 17–59 years old, and 17 (28.4%) from adults 60+ years old; the most common diagnosis among the 60+ group was cellulitis (41%), while the

largest proportion of adults 17–59 years old (14, 44%) and all infants presented with bacteraemia/sepsis. The majority of infants (7, 64%) were under 7 days old (early-onset disease) and of those, almost all (6/7) carried isolates belonging to serotypes Ia, III or V, which have been previously associated with early-onset disease.[3]

Thirteen sequence types (STs) were identified among the isolates. The most common ST was ST19 (24, 37%), followed by ST23 (13, 20%). The majority of the isolates (81.5%) belong to one of the three previously reported clonal complexes, i.e. CC19, CC23 and CC12,[28] but there was no apparent correlation between ST and serotype or resistance determinant. This is consistent with extensive horizontal transfer and limited clonal expansion (Figure 1).

Of the 65 isolates, 49 (75%) were positive for *lsa*(C), 12 (18%) were positive for both *lnu*(B) and *lsa*(E), and 4 (6%) were negative for all accessory MLS determinants investigated. The sequences from these four isolates were subjected to further analysis, including variant calling, genomic island prediction (IslandViewer3[29]) and gene detection using several resistance databases (Resfinder,[30] RGI/CARD[31] and ARG-annot[32]), resulting in no determinants being identified.

Among these isolates, *lnu*(B) and *lsa*(E) were carried on a 75.5 kb mobile element, containing genes from two other elements: one described in a GBS isolate (SGB76)[33] and one described in a *Streptococcus suis* isolate (SC070731);[34] this element was invariably integrated into the chromosome at *rum*(A) [23S rRNA(uracil-5-)methyltransferase], in the same position as previously described for *van*(G)-containing elements in GBS and *Streptococcus anginosus*[35] (Figure 2a).

In isolates where *tet*(M) was present along with *lsa*(C), both genes were located in a Tn*916*-like genetic element 23.5 kb long inserted into the chromosome (Figure 2b), while in isolates where *lsa*(C) was present by itself, it was inserted in the same position as previously described for a GBS isolate (UCN70).[17]

The phenotypes and MIC ranges associated with each genotype are shown in Table 2. Based on the bimodal distribution of MIC values, the cut-offs were determined as follows: isolates with MIC values against virginiamycin M1 ≥4mg/L were considered non-susceptible to streptogramins A (Figure 3), while isolates with MIC values against tiamulin ≥1mg/L were considered non-susceptible to pleuromutilins (Figure 4). These values are consistent with reported values for related compounds, dalfopristin (streptogramin A) and retapamulin (pleuromutilin), against GBS.[17,36]

In order to determine whether the frequency of these phenotypes among the GBS isolates in our collection has increased in recent years, the proportion of isolates with any of these three phenotypes (L/LSA/LSAP) for each year was plotted for the 2006–15 period (Figure 5), and a logistic regression model was fitted. The resulting log likelihood ratio (χ^2) confirmed that this upward trend was significant (*P* value <0.001).

Discussion

Resistance to clindamycin in the absence of erythromycin resistance has previously been reported in GBS, both on its own or in combination with resistance to streptogramins A and pleuromutilins. While they still remain relatively rare, the frequency of these resistance phenotypes appears to have increased in the last decade, both in the ABCs collection and in other reports in the literature.[7–12,17,18,37]

The L phenotype mediated by the nucleotidyltransferase expressed by *lnu*(B) has been reported in several species in a number of countries.[7–12,37] Among our isolates, *lnu*(B) is invariably present in combination with *lsa*(E) on the same transposable element, as previously described in *S. aureus*[15] and GBS,[33] and in association with cross-resistance to clindamycin, virginiamycin M1 and tiamulin (LSAP). The MIC values against virginiamycin M1 and tiamulin for these isolates positive for both *lnu*(B) and *lsa*(E) were up to 4-fold and 16-fold higher, respectively. It appears likely that these two genes act synergistically to produce this phenotype.

The first report of the LSAP phenotype described a GBS isolate from New Zealand carrying the *lsa*(C) gene. This isolate was originally identified as expressing the LSA phenotype[36] and later found to be resistant to tiamulin as well.[17] Since then, only 10 additional *lsa*(C)-positive GBS strains have been reported.[18,37] To the best of our knowledge, this study is the first time the LSAP phenotype has been reported among GBS isolates recovered in the USA. As the use of WGS becomes more prevalent in the study of antimicrobial resistance, the number of isolates reported to carry these and other potentially novel determinants will likely increase.

Of the four isolates where no determinant was identified, the three that expressed the LSA phenotype had clindamycin MIC values up to 32-fold higher than those of the *lsa*(C)/*lnu*(B) +*lsa*(E)-positive isolates and the one isolate expressing the L phenotype, which suggests that different mechanisms may be involved. Further investigation is being conducted by our group into the genetic bases for both of these phenotypes, to be presented in a future publication.

Although streptogramins are not as widely used as clindamycin, the presence of genes that confer resistance to streptogramin A in conjunction with widespread MLS determinants that confer resistance to streptogramin B (such as *erm* genes), could lead to the ineffectiveness of the quinupristin (streptogramin A) plus dalfopristin (streptogramin B) drug combination, an important agent for the treatment of complicated skin infections caused by *S. aureus* or *Streptococcus pyogenes*.

Pleuromutilins, on the other hand, are almost exclusively used for veterinary applications (retapamulin is the only pleuromutilin approved for topical use in humans), though new drugs in this class are under investigation for human use. In fact, the semi-synthetic pleuromutilin lefamulin is one of the only 11 antimicrobial drugs currently in Phase 3 of the antimicrobial development pipeline.[38] The emergence of resistance to antibiotics that are yet to be approved for human use is concerning and stresses the importance of limiting the use of antibiotics in agriculture, as well as the critical need to develop new agents.

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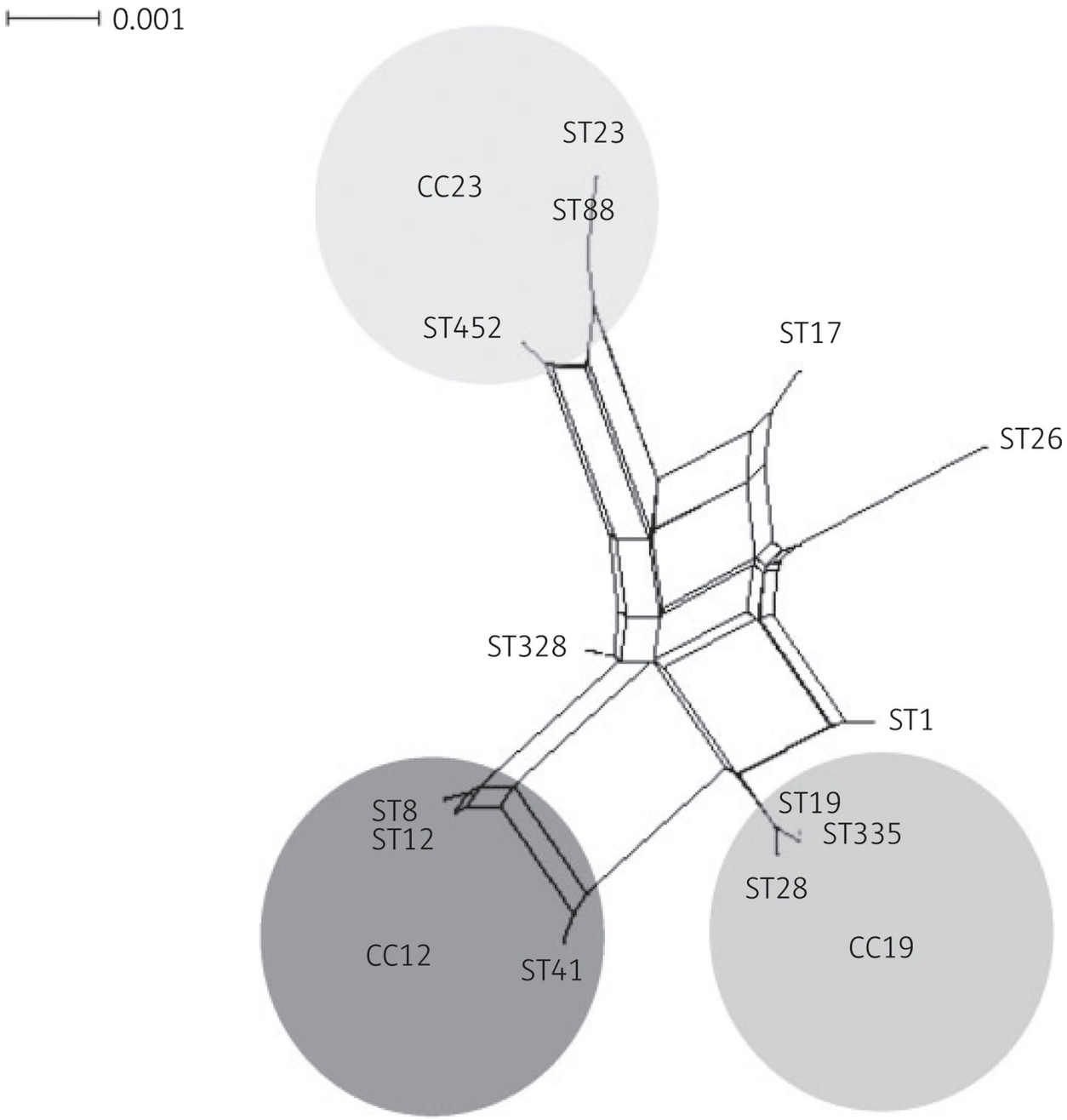

Figure 1.
Phylogenetic network of the 13 sequence types (STs) generated by neighbour-net method using SplitsTree4. The three clonal complexes (CCs) identified are shown in grey circles: CC19 includes 27 isolates in three STs; CC12 includes 5 isolates in three STs; CC23 includes 16 isolates in three STs.

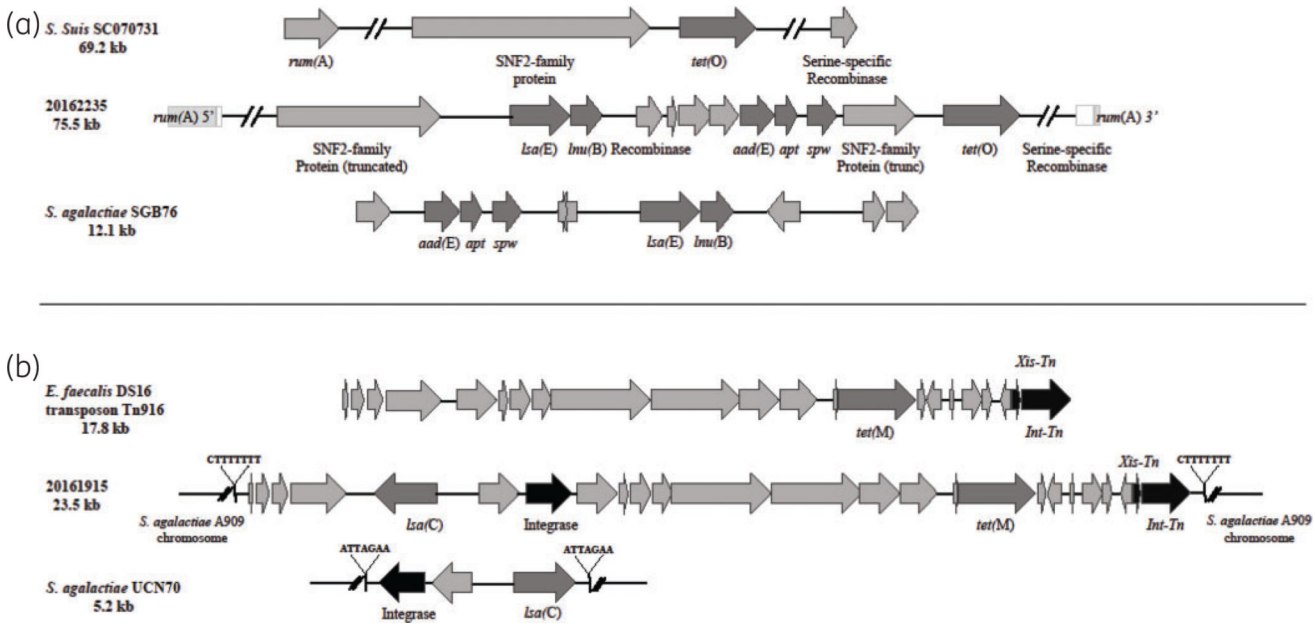

Figure 2.

(a) Genetic element carrying *lsa*(E) and *lnu*(B) in isolate 20162235 and structural comparison with the corresponding regions in *S. suis* SC070731 and *S. agalactiae* SGB76 (accession numbers CP003922 and KF772204); nucleotide sequence identity was at least 98% in these regions. The last 42 bases of the 3′ end of *rum*(A) and the 3′ end of the serine recombinase were exchanged. (b) Genetic element carrying *lsa*(C) in isolate 20161915 and structural comparison with the corresponding regions in *Enterococcus faecalis* DS16 Tn*916* and *S. agalactiae* UCN70 (accession numbers U09422 and HM990671); nucleotide sequence identity between 20161915 and DS16 Tn*916* was 99%, while identity between regions in 20161915 and UCN70 was 92%. Target site duplications flanking the element are shown. The element was inserted in-frame into a GtrA-like protein with a 99.9% identity to the *S. agalactiae* A909 sequence (accession number CP000114), at position 924880 of the A909 genome, 22 kb upstream of the MLST locus *phe*(S). Dark grey arrows indicate resistance genes; black arrows indicate integrases. *aad*(E), aminoglycoside adenyltransferase E; *apt*, adenine phosphoribosyltransferase; *spw*, spectinomycin resistance gene; *tet*(O) and *tet*(M), tetracycline resistance genes; *rum*(A), 23S rRNA(uracil-5-)methyltransferase; *Xis-Tn* and *Int-Tn*, transposases.

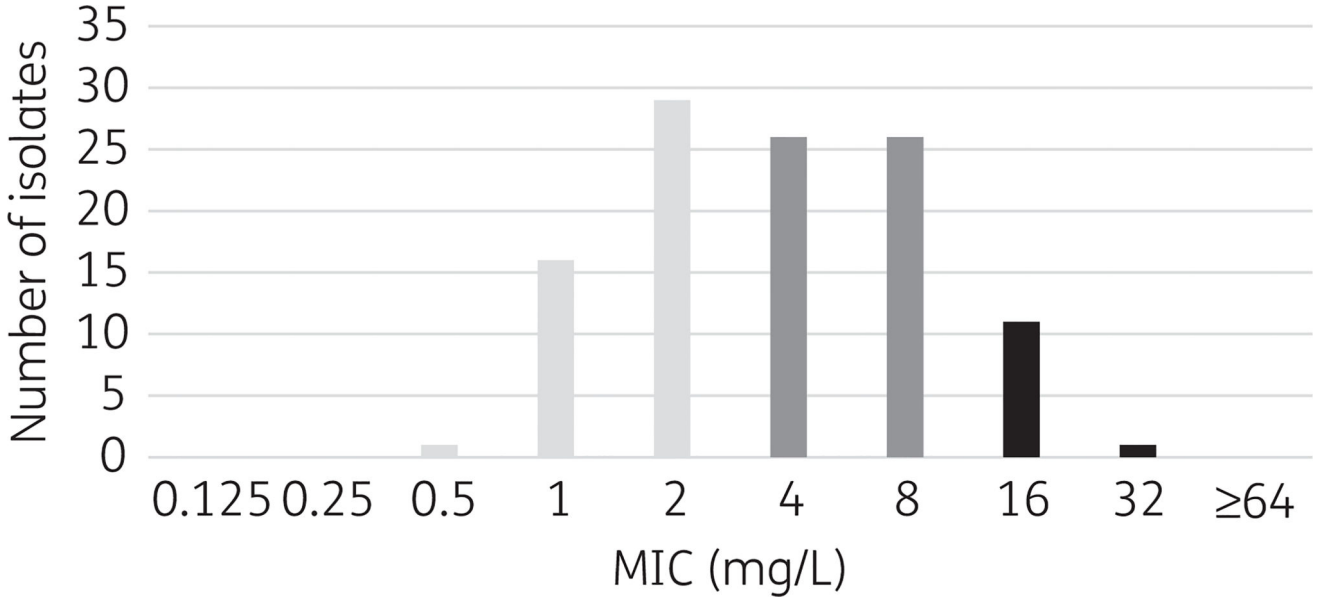

Figure 3.

MIC distribution for virginiamycin M1. Light grey bars represent control isolates, plus one isolate with L phenotype; dark grey bars represent isolates carrying *lsa*(C), plus three isolates with LSA phenotype; black bars represent isolates carrying *lnu*(B)+*lsa*(E).

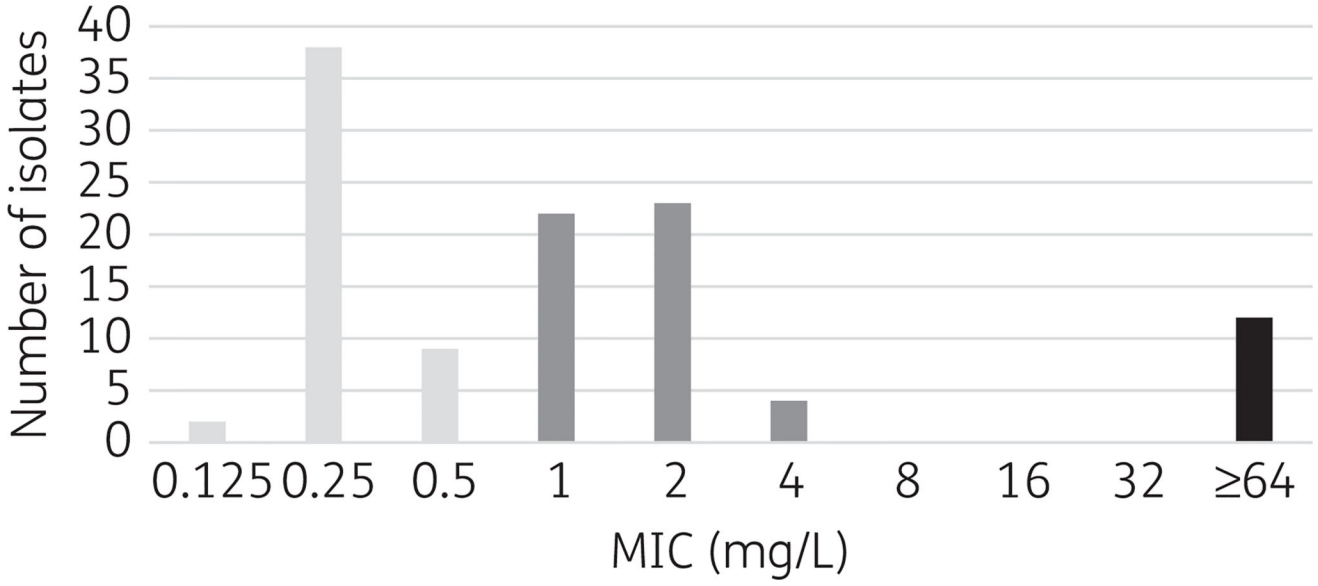

Figure 4.
MIC distribution for tiamulin. Light grey bars represent control isolates, plus isolates with L and LSA phenotypes; dark grey bars represent isolates carrying *lsa*(C); black bars represent isolates carrying *lnu*(B)+*lsa*(E).

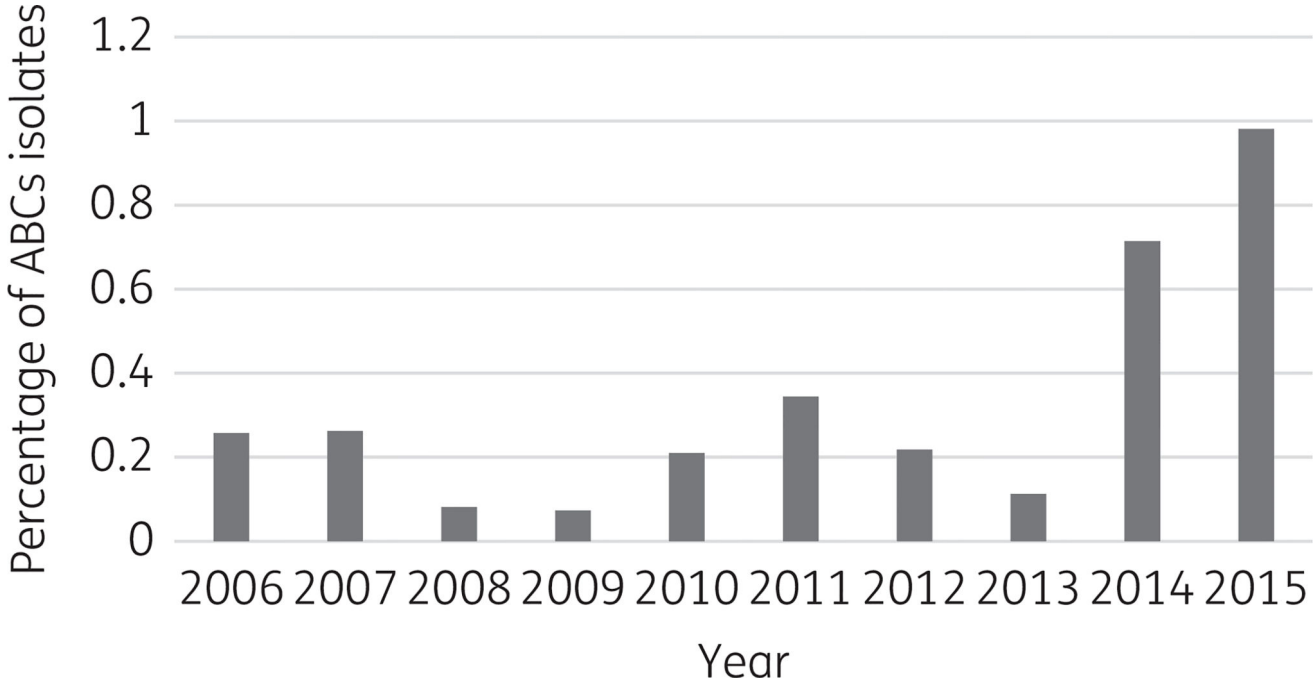

Figure 5.
Percentage of ABCs isolates with L/LSA/LSAP phenotypes by year, 2006–15. Logistic regression results: slope = 0.176, OR = 1.19, $R^2 = 0.35$, $\chi^2 = 16.62$, P value <0.001.

Table 1

Characteristics of study isolates

Collection year	Isolate ID	Age group (years)	Diagnosis	Serotype	Sequence type	Resistance phenotype[a]
1998	6057-99	<1	bacteraemia	V	335	LSAP
1999	4310-99	<1	bacteraemia	III	1	LSAP
2000	2860-00	<1	bacteraemia	III	23	LSAP
2002	5422-03	<1	bacteraemia	Ia	19	LSAP
2004	3019-05	17–59	bacteraemia	Ia	19	LSAP
2004	9044-04	17–59	cellulitis	Ia	19	LSAP
2005	8001-05	17–59	septic arthritis	Ib	19	LSAP
2006	3574-07	<1	bacteraemia	Ia	19	LSAP
2006	6575-06	60+	cellulitis	Ia	23	LSAP
2006	8678-06	60+	cellulitis	III	88	LSAP
2007	4408-08	17–59	bacteraemia	Ia	19	LSAP
2007	4428-08	17–59	bacteraemia	II	28	LSA
2007	6919-07	<1	sepsis	Ia	19	LSAP
2008	4943-08	17–59	bacteraemia	III	8	LSAP
2009	2010200279	17–59	bacteraemia	V	8	LSAP
2010	2010225665	60+	cellulitis	V	23	LSAP
2010	2010227572	17–59	septic abortion	Ia	23	LSAP
2010	2011207735	17–59	bacteraemia	Ib	12	LSA
2011	2011216702	60+	cellulitis	Ia	88	LSA
2011	2011217685	<1	blood	III	1	LSAP
2011	2012202087	60+	bacteraemia	III	23	LSAP
2011	2012205532	17–59	chorioamnionitis	Ib	19	LSAP
2011	2012208851	60+	bacteraemia	III	23	LSAP
2011	2012208908	17–59	bacteraemia	V	19	LSAP
2012	2012211493	60+	septic arthritis	III	19	LSAP
2012	2012220214	60+	septic arthritis	V	23	LSAP
2012	2013206339	17–59	bacteraemia	Ia	19	LSAP
2012	2013213001	17–59	bacteraemia	V	1	LSAP

J Antimicrob Chemother. Author manuscript; available in PMC 2017 December 18.

Collection year	Isolate ID	Age group (years)	Diagnosis	Serotype	Sequence type	Resistance phenotype[a]
2013	2014205249	60+	cellulitis	V	41	LSAP
2013	2014211676	17–59	septic arthritis	Ia	23	LSAP
2014	20140516	17–59	osteomyelitis	III	19	LSAP
2014	20150265	60+	pneumonia	III	452	LSAP
2014	20150266	60+	cellulitis	Ia	23	LSAP
2014	20150644	17–59	septic arthritis	III	335	LSAP
2014	20150724	17–59	pneumonia	V	1	LSAP
2014	20151063	60+	pneumonia	III	19	LSAP
2014	20152059	17–59	pneumonia	III	19	LSAP
2014	20152077	NA	NA	Ia	23	LSAP
2014	20153795	60+	pneumonia	Ia	23	L
2014	2014210955	<1	bacteraemia	III	17	LSAP
2014	2014212363	17–59	endometritis	III	335	LSAP
2014	2014213333	NA	NA	V	19	LSAP
2014	2014215425	60+	osteomyelitis	Ia	23	LSAP
2014	2014215482	17–59	bacteraemia	III	19	LSAP
2015	20154642	17–59	bacteraemia	V	1	LSAP
2015	20154852	17–59	cellulitis	III	19	LSAP
2015	20155096	17–59	meningitis	III	19	LSAP
2015	20155213	NA	NA	V	1	LSAP
2015	20155222	17–59	chorioamnionitis	V	19	LSAP
2015	20155245	<1	bacteraemia	III	328	LSAP
2015	20155699	17–59	necrotizing fasciitis	V	1	LSAP
2015	20155703	17–59	bacteraemia	IV	452	LSAP
2015	20155802	17–59	cellulitis	III	335	LSAP
2015	20155815	NA	NA	V	19	LSAP
2015	20155859	17–59	septic arthritis	V	26	LSAP
2015	20156201	<1	bacteraemia	III	19	LSAP
2015	20156818	60+	cellulitis	III	335	LSAP
2015	20156857	17–59	bacteraemia	IV	452	LSAP
2015	20156878	60+	bacteraemia	V	26	LSAP

Collection year	Isolate ID	Age group (years)	Diagnosis	Serotype	Sequence type	Resistance phenotype[a]
2015	20160219	17–59	bacteraemia	Ia	23	LSAP
2015	20160255	NA	NA	V	1	LSAP
2015	20160336	17–59	osteomyelitis	III	19	LSAP
2015	20160967	60+	bacteraemia	III	19	LSAP
2015	20161915	<1	bacteraemia	Ib	8	LSAP
2015	20162235	17–59	septic arthritis	V	19	LSAP

NA, data not available.

[a]L, lincosamides; SA, streptogramins A; P, pleuromutilins.

Table 2

Phenotypes and resistance genes observed with associated MIC values

			MIC range (mg/L)			
Phenotype	**Gene**	**Isolates (*n*)**	**ERY**	**CLI**	**VM1**	**TIA**
L	—	1	0.12	1	1	0.5
LSA	—	3	0.06–0.25	8–32	4	0.25
	lnuB/lsaE	11	0.03–0.25	4–8	16–32	64+
	lsaC	50	0.03–0.12	0.5–4	4–8	1–4

ERY, erythromycin; CLI, clindamycin; VM1, virginiamycin M1; TIA, tiamulin.