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The prevalence, molecular characterization and antimicrobial resistance profiling of *Streptococcus agalactiae* isolated from clinical mastitis cases on large dairy farms in China

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Abstract

This research communication aims to characterize the prevalence, molecular characterization and antimicrobial resistance profiling of *Streptococcus agalactiae* isolated from clinical mastitis in China. A total of 140 *Strep. agalactiae* isolates were identified from 12 out of 201 farms in 6 provinces, overall herd prevalence was 18.6% and the MLST analysis showed clonal complexes (CC) 103 and CC 67 were present in these herds with CC 103 predominant, accounting for 97.9%. Isolates were mostly sensitive to the tested antimicrobials: penicillin, ceftiofur, amoxi/clav, cefquinome, and vancomycin (100%), followed by cefalexin (97.9%), oxacillin (96.4%), enrofloxacin (95.7%), erythromycin (89.3%), and clindamycin (88.6%). Only 19.3 and 0.7% of isolates were sensitive to tetracycline and daptomycin, respectively, and sequence type (ST) 103 was most resistant to antimicrobials. In conclusion, CC 103 was the predominant subgroup of bovine mastitis *Strep. agalactiae* in China, and most antimicrobials apart from tetracycline and daptomycin were effective.

Mastitis is one of the costliest diseases in the dairy industry due to discarded milk and expense of treatments, and may lead to culling of cows (Gussmann *et al.*, 2019). *Streptococcus agalactiae* is one of the major mastitis with a prevalence at herd level as high as 92% in China (Bi *et al.*, 2016).

Multilocus sequence typing (MLST) is a genotype method for characterizing and distinguishing specific clones among *Strep. agalactiae*. It is an unambiguous sequence-based and reliable typing tool, allowing comparison of the gene distribution of different isolates collected from all geographic areas and further investigation of the population structure (Yang *et al.*, 2013). The major sequence type (ST) of *Strep. agalactiae* varies in different regions: ST67 in British dairy herds and ST1 in Danish, Finnish, and Swedish dairy herds (Reyes *et al.*, 2017). However, the authors believe that the studies are deficient for investigating the MLST of *Strep. agalactiae* in North China, which is the main milk production region in China.

Antimicrobials are still the major option for mastitis treatment (Keefe, 2012). However, the abusive use of antimicrobials increases the risks of antimicrobial resistance (AMR) in bacteria, which is of public health concern worldwide (Levy and Marshall, 2004). Investigation of the prevalence, predominant STs and the antimicrobial resistance profiles can contribute to treatment decisions and optimize *Strep. agalactiae* control programs to minimize use of antimicrobials (Kaczorek *et al.*, 2017). Thus, the objectives of this study were: (a) to study the prevalence of *Strep. agalactiae* and characterize the molecular type, (b) to investigate the antibiotic resistance and (c) determine the association between ST and antimicrobial resistance patterns of *Strep. agalactiae*.

Material and methods

Strep. agalactiae *isolation*

Strep. agalactiae were isolated from cows with clinical mastitis from large dairy farms (>500 cows) in China: Milk samples were collected into sterile tubes after teat disenfection and discarding the first three strippings. The volume of 10 µl of milk from each sample was plated onto a blood agar plate (Luqiao, Beijing, China), and plates were incubated at 37 °C for 24 to 48 h. Suspected *Streptococcus* spp. were identified as catalase-negative and Gram-positive cocci. Streptococci were differentiated as esculin-positive (*Strep. uberis* and other esculin-positive cocci) or esculin-negative cocci (*Strep. dysgalactiae* and *Strep. agalactiae*). Christie,

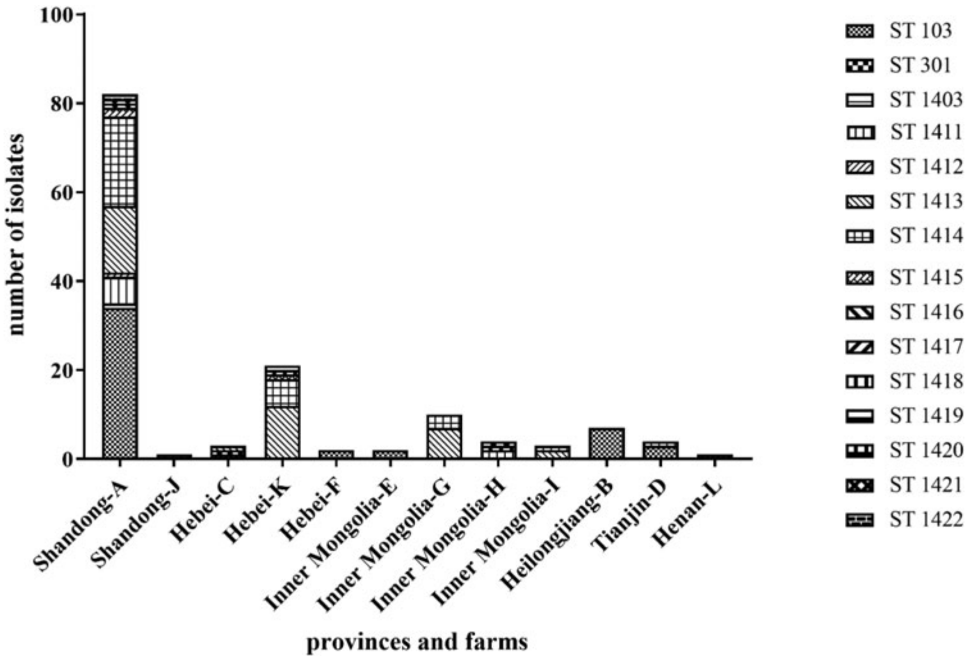

Fig. 1. Sequence types of the 140 *Strep. agalactiae* isolated from 12 farms in 6 provinces.

Atkins and Munch-Petersen tests (CAMP) (Tianhe, Hangzhou, China) were used to distinguish *Strep. dysgalactiae* (CAMP-negative) from *Strep. agalactiae* (CAMP-positive). Finally, the putative isolates were identified using 16S rRNA amplification.

Multilocus sequence typing (MLST)

The seven housekeeping genes (*adh*, *pheS*, *atr*, *glnA*, *sdhA*, *glcK* and *tkt*) were amplified by PCR. For each isolate, the allele number and sequence types (STs) were defined by analysis of the alleles sequence referred to the *Strep. agalactiae* MLST reference database (http://pubmlst.org/sagalactiae/). The allele sequences or previously undescribed ST were assigned new numbers and the data were deposited in the MLST database. CC analysis and phylogenetic analysis of MLST data was performed using Phyloviz (version 2.0a, http://www.phyloviz.net/) using eBURST algorithm.

Antimicrobial resistance testing

Antimicrobial resistance testing was conducted using the broth microdilution method according to the Clinical and Laboratory Standards Institute (CLSI, 2020). *Strep. pneumonia* ATCC 49619 were used as quality control strains. The number of 12 antimicrobials used in practice for bovine mastitis treatment and human medicine (penicillin, cefalexin, ceftiofur, cefquinome, oxacillin, clindamycin, tetracycline, enrofloxacin, amoxicillin/clavulanate, daptomycin, erythromycin, and vancomycin) were selected for antimicrobial resistance testing.

Statistical analysis

In order to make the data representative, $n \geq 10$ farms (A, K, and G) and STs (ST103, ST1413, and ST1414) were selected to research the correlation between AMR and farms and STs. A longitudinal comparison between the MIC and ST changes of isolates collected in 2017 ($n = 29$) and 2019 ($n = 53$) from farm A in Shandong province was conducted. Kruskal–Wallis and Mann–Whitney U test were performed using SPSS 26.0 (IBM Corp, Armonk, NY).

Results and discussion

Distribution of Strep. agalactiae

A total of 140 *Strep. agalactiae* isolates were identified from 12 out of 201 farms in 6 provinces from 2017 to 2019. Overall herd prevalence was 18.6% (95% CI 16%_21.6%), the most high prevalence region of *Strep. agalactiae* was Shandong province ($n = 83$, prevalence = 19.5%), followed by Tianjin ($n = 4$, prevalence = 4.3%) and Hebei province ($n = 26$, prevalence = 3%). (online Supplementary Tables 1 and 2; Supplementary Fig. 1). Only a few samples and isolates were collected in several provinces (Henan, Tianjin, and Heilongjiang), so more samples should be collected from these regions in further research to make the samples more representative. As a contagious mastitis pathogen, *Strep. agalactiae* can transfect among cows and persist for a long period. Strict biosecurity and sterile standards should be taken to eliminate *Strep. agalactiae* from herds (Jørgensen *et al*., 2016). The high prevalence of *Strep. agalactiae* was observed in a few herds in the main milk production regions in north China (Herd A in Shandong and herd K in Hebei, online Supplementary Table 2), which indicates the infection of *Strep. agalactiae* was not under proper control in these herds.

MLST analysis

The MLST analysis showed that two main clonal complexes (CC), CC 103 and CC 67, were present in these herds (online Supplementary Fig. 2). The isolates of CC 103 were predominant, accounting for 97.9% (95% CI 93.89%_99.27%; Supplementary Table 3). This finding is consistent with studies in Denmark

Table 1. Antimicrobial resistance profile of the 140 *Streptococcus agalactiae* isolated from clinical mastitis cases on dairy farms in north China. Minimum concentration of an antimicrobial that inhibited the visible growth of 50% (MIC$_{50}$) and 90% of microorganisms (MIC$_{90}$)

	Isolates at each MIC interval (µg/ml)																
Antimicrobial	<0.015	0.015	0.03	0.06	0.125	0.25	0.5	1	2	4	8	16	32	>32	MIC$_{50}$	MIC$_{90}$	Resistance rate
Penicillin	1	3	22	36	42	32	1	–	–	3	–	–	–	–	0.06	0.125	0
Cefalexin	–	–	–	–	–	–	2	1	57	77	1	1	–	1	2	4	2.1%
Ceftiofor	2	48	82	1	2	3	–	–	1	1	–	–	–	–	0.015	0.03	0
Cefquinome	–	4	74	56	2	2	–	–	1	1	–	–	–	–	0.03	0.06	0
Oxacillin	–	–	–	2	–	12	117	3	1	1	3	1	–	–	0.5	0.5	3.6%
Clindamycin	–	42	41	39	1	–	1	1	3	–	2	–	10	–	0.03	0.06	11.4%
Tetracycline	–	–	–	1	3	2	–	2	19	–	5	53	54	1	16	32	80.7%
Enrofloxacin	–	–	–	–	–	23	90	21	1	–	–	–	5	–	0.5	0.5	4.3%
Amoxi/clav[a]	–	–	–	–	–	19	112	5	1	–	–	3	–	–	0.5	0.5	0
Daptomycin	–	–	–	–	–	–	–	1	2	36	79	22	–	–	8	8	99.3%
Erythromycin	75	–	40	3	2	3	2	1	–	–	–	–	–	14	<0.015	0.03	10.7%
Vancomycin	–	–	–	2	5	116	17	–	–	–	–	–	–	–	0.25	0.25	0

MIC, minimum inhibitory concentration.

[a] Amoxicillin/clavulanate potassium.

(Zadoks *et al.*, 2011) and eastern China (Yang *et al.*, 2013). CC 67, including ST 301 and 1422, only accounted for 1.4% of isolates in our study, but was extensively distributed in Europe in mastitis cases (Reyes *et al.*, 2017), indicating the predominant subgroup in China was CC103. A total of 13 new STs were found in this study, which clarified the prevalence and the predominant STs of *Strep. agalactiae* in Chinese dairy herds, and is important for tailor-made *Strep. agalactiae* mastitis control programs (Yang *et al.*, 2013).

Figure 1 shows the distribution of STs amongst the 12 farms. Several STs were widely distributed on different farms, and heterogeneous STs were also found within herds in the current study, which agrees with a study on dairy farms in east China (Yang *et al.*, 2013). CC67 is considered to be contagious (Jørgensen *et al.*, 2016), while CC103 is an environmental pathogen (Cobo-Ángel *et al.*, 2018), which indicated parallel transmission routes (cow to cow and environmental reservoir to cow transmission) within herds.

Antimicrobial resistance testing

Isolates were mostly sensitive to the tested antimicrobials: penicillin, ceftiofur, amoxi/clav, cefquinome, and vancomycin (100%), followed by cefalexin (97.9%), oxacillin (96.4%), enrofloxacin (95.7%), erythromycin (89.3%), and clindamycin (88.6%). However, only 19.3 and 0.7% of isolates were sensitive to tetracycline and daptomycin, respectively (Table 1). The MIC of the isolates against eight antimicrobials in 2019 was significantly lower than that in 2017 (online Supplementary Table 4). A total of 10 antimicrobials were effective to most of the isolates, and the multi-resistant rate is rare (13.6%), which was lower than the earlier report of Tian *et al.* (2019). The antimicrobial resistance situation was also remarkably improved compared with Gao *et al.* (2012). Such an improvement is due to well-organized large dairy farms and the employment of on-farm veterinarians and pharmacists working together to optimize udder health.

The percentage of tetracycline resistance was 80% in our research, which was consistent with Gao *et al.* (2012) and Tomazi *et al.* (2018), who reported resistance percentages of 72.5 and 68.6% in China and Brazil, respectively. The low efficacy of tetracycline in mastitis treatment was reported worldwide due to its abuse in treatment and growth promotion (Kaczorek *et al.*, 2017).

A total of 16 isolates exhibited resistance to clindamycin, accounting for 11.4%. The resistant gene of clindamycin was located on a plasmid (Bozdogan *et al.*, 1999). Hence, the interspecies transmission of resistant genes of clindamycin within bacterial populations mediated by plasmid is a risk to public health.

The MIC of seven drugs (penicillin, cefalexin, ceftiofor, tetracycline, enrofloxacin, daptomycin, and erythromycin) against isolates from three farms (A, K, and G) is also heterogeneous. Overall, the MIC of drugs against isolates from A was the highest, followed by G and K. This finding may indicate that farm A has antimicrobial abuse problems because ST103 was a predominant subtype in farm A and MIC of ST103 was relatively higher than other STs (online Supplementary Table 5).

Association between antimicrobials resistance and MLST

Tomazi *et al.* (2018) found through random amplified polymorphic DNA analysis that isolates of cluster Ib were the most resistant to tetracycline, erythromycin, and pirmycin due to the presence of resistant genes in exact sub-types of isolates. Yang *et al.* (2013) also found isolates assigned to capsular genotype II associated with alpha-like proteins 1 and 4. In our research, the association between STs and antimicrobial resistance profile patterns was determined. The ST103 had higher MIC in the 12 tested antimicrobials compared with the two remaining major STs (online Supplementary Table 6), and exhibited increasing MIC under antimicrobial pressure as time went by.

In conclusion, overall herd prevalence of *Strep. agalactiae* was 18.6% and 15 STs were found. The majority of the isolates were ST103, and 13 STs were newly reported in these Chinese dairy herds. The *Strep. agalactiae* isolates were susceptible to the commonly used antimicrobials in practice, except for resistance to clindamycin, erythromycin, tetracycline, and daptomycin. ST103 was significantly associated with antimicrobial resistance in this study. The current study suggests that *Strep. agalactiae* mastitis control programmes should mainly focus on ST103 type in Chinese dairy herds and awareness of the prudent use of antimicrobials should be improved on farms.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0022029922000152.

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