DOI 10.24425/pjvs.2024.151748

Original article

Pathogenicity and drug resistance characterization of *Streptococcus. agalactiae* isolated from dairy cows

L.J. Jiang[1, 3], H.R. Liu[1], Z.Y. Liu[2, 3], Q. Li[1], Y.C. Wang[1], B.W. Tan[1]

[1] Department of Customs Inspection and Quarantine, Shanghai Customs College, Shanghai, China
[2] College of Animal Science and Technology, Zhejiang A&F University, Lin'an, China
[3] College of Veterinary Medicine (Institute of Comparative Medicine), Yangzhou University, Yangzhou, China

Correspondence to: L.J. Jiang, jianglijie@shcc.edu.cn

Abstract

Streptococcus agalactiae, commonly known as *S. agalactiae*, is a critical zoonotic pathogen that significantly reduces milk yield and product quality and poses a significant risk to public health. Although *S. agalactiae* is increasingly recognised as a principal agent causing milkborne infections, research dedicated to this pathogen in dairy cattle has been less extensive than that of other pathogens. This study aimed to examine the antibiotic resistance profiles of *S. agalactiae* derived from dairy cows and assess its pathogenicity using validated *in vivo* models. The findings contribute essential scientific insights into the realm of environmental antibiotic resistance research. The resistance of *S. agalactiae* isolates to drugs was assessed using the broth microdilution technique. Additionally, PCR analysis was used to identify six important virulence genes. The study revealed that *S. agalactiae* was fully susceptible to streptomycin, meropenem, ciprofloxacin, clindamycin, cefquinome, and cloxacillin in general laboratory settings and within milk samples. However, among the antibiotics tested, tetracycline exhibited the highest level of resistance, with rates reaching 70%. Penicillin showed a resistance level of 50%, followed by doxycycline at 30%. Additionally, the resistance rates for apramycin and cefoxitin were both 20%, whereas florfenicol resistance was observed at a rate of 10%. All isolates of *S. agalactiae* carried the *cfb* gene. However, it is noteworthy that only one isolate possessed this gene exclusively, while the other nine isolates shared a uniform set of four additional virulence genes. The study highlighted the significant impact of these virulence factors on the pathogenic behaviour of *S. agalactiae* from dairy sources. This was demonstrated by the high mortality rates observed in experimental infections using *Galleria mellonella* (*G. mellonella*) larvae and mouse models. These findings contribute to understanding the relationship between the pathogenic properties of *S. agalactiae* and the virulence genes it carries.

Keywords: *Streptococcus. agalactiae*, virulence, pathogenicity, *in vivo* models

Introduction

Group B Streptococcus (GBS), also known as *Streptococcus agalactiae*, significantly impacts bovine health, milk quality, and productivity due to its strong association with mastitis. This highly infectious agent is a significant cause of mastitis in both dairy cattle and buffaloes, with considerable economic implications globally. Despite extensive research on GBS, the specific etiological factors and mechanisms underlying its pathogenicity remain relatively underexplored.

Currently, research has reported a correlation between *S. agalactiae* virulence factors and pathogenicity in mouse and rat models. (Chen et al. 2005). *S. agalactiae* possesses several major virulence factors, including laminin-binding protein (*lmb*), surface immuno-genic protein (*sip*), C5a peptidase (*scpB*), hyaluronate lyase (*hyl*), β-hemolysin/cytolysin(*cylE*), and CAMP factor (*cfb*) (Kannika et al. 2017, Zastempowska et al. 2022). These factors contribute to the pathogenicity of the bacterium. Regarding human-origin *S. agalactiae* strains, there has been extensive characterization of their virulence determinants. In comparison, insights into the properties of animal isolates are less definitive. Therefore, there is a pressing need for broader and deeper investigation into these factors through animal model studies (Shome et al. 2012). Bovine mastitis is the leading cause of antimicrobial use in dairy cows. The antimicrobial resistance patterns of mastitis pathogens have received significant attention since 2005. To effectively treat clinical mastitis and implement dry cow therapy, it is crucial to understand the prevalence and antimicrobial susceptibility of microorganisms isolated from dairy cows.

The aim of this study was to investigate the antimicrobial susceptibility and genotypic profile of *S. agalactiae* strains recovered from dairy cows at a dairy farm in Shanghai, China.

Materials and Methods

Strains

This study evaluated ten *S. agalactiae* strains isolated from dairy cows in Shanghai, using ATCC 29213 and ATCC 25922 as quality controls. The bacteria were grown in LB broth or LB agar at 37°C, unless otherwise specified.

Antimicrobial agents

The antibiotics: cefquinome (CFQ), streptomycin (STR), doxycycline (DOX), meropenem (MEM), ciprofloxacin (CIP), florfenicol (FFC), cefoxitin (FOX), tetracycline (TET), apramycin (APR), clindamycin (CLI), cloxacillin (CLO), and penicillin (PEN) were obtained from Solarbio Life Sciences Co. Ltd. (Beijing, China). The antimicrobial solution freshly prepared before use.

Antimicrobial susceptibility

Susceptibility tests were determined according to the Clinical and Laboratory Standards Institute (CLSI) guidelines, using the following antibiotics: CFQ, STR, DOX, MEM, CIP, FFC, FOX, TET, APR, CLI, CLO, and PEN (CLSI, 2018). The MIC of CFQ in milk was determined using the CLSI microbroth dilution method, with milk as the bacterial medium. The MIC in the milk was measured by sampling a mixture of the drugs and bacteria, then diluting and plating the mixture 10-fold onto MH agar for colony counting. The MIC in milk was defined as the minimum concentration where the bacteria loading is equal to the initial concentration or grows <1 × log10 CFU/mL. *S. aureus* American Type Culture Collection (ATCC) 29213 and *E. coli* ATCC25922 strains were used as quality control. All determinations were performed in triplet.

Genomic DNA extraction

The bacterial strains were inoculated into BHI broth and cultured for 18-24 hours at 36°C. Genomic DNA was then extracted following the instructions provided by the Bacterial DNA Extraction kit manufacturer. (Tiangen BioTech, Beijing, China). The DNA concentration and mass were measured using a Thermo NanoDrop 2000C spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). All DNA samples were stored at -20°C until use.

Animals

ICR mice procured from the experimental animal center of Yangzhou University were bred and maintained with corn cob bedding and fed with a grain-based chow diet, were aged 4-6 weeks and weighed 20 to 25 g. According to the ethics protocol, all surviving mice were euthanized by an overdose of sodium pentobarbital dosed by intravenous route after the experiments. *Galleria mellonella* (*G. mellonella*) larvae were obtained from Huiyude Biotechnology Co., Tianjin, China and stored in the dark at 30°C and 30% RH. Last instar larvae, approximately 300 mg in mass, were used for all experiments described in this study. Larvae displaying signs of death (no movement when touched) were discarded along with those undergoing pupation. The animals were housed in standard conditions with drink and feed supplied *ad libitum*. The animal study

Table 1. Virulence gene primer of *Streptococcus. agalactiae*

Gene	Sequence (5'-3')	Reference
cfb	F: ATGGGATTTGGGATAACTAAGCTAG R: AGCGTGTATTCCAGATTTCCTTAT	(Dmitriev et al. 2002)
cylE	F: TTCTCCTCCTGGCAAAGCCAGC R: CGCCTCCTCCGATGATGCTTG	(Kayansamruaj et al. 2014)
hylB	F: TCTAGTCGATATGGGGCGCGT R: ACCGTCAGCATAGAAGCCTTCAGC	(Kayansamruaj et al. 2014)
lmb	F: TGGCGAGGAGAGGGCTCTTG R: ATTCGTGACGCAACACACGGC	(Kayansamruaj et al. 2014)
scpB	F: CCTGCTAAGACTGCTGATAC R: CATAAGCATAGTCGTAAGCC	(Zastempowska et al. 2022)
Sip	F: TGAAAATGCAGGGCTCCAACCTCA R: GATCTGGCATTGCATTCCAAGTAT	(Zastempowska et al. 2022)

was reviewed and approved by the Jiangsu Administrative Ethics Committee for Laboratory Animals (SYXKSU-2019-0004) on March 11, 2019. All methods were carried out in accordance with relevant guidelines and regulations. All efforts were made to minimize the number of animals and their suffering throughout the experiment.

Infection model

In vivo virulence estimates from two animal experiments included a mouse infection model and a *G. mellonella* infection model.

In both cases, a bacterial suspension consisting of approximately 10^5 to 10^8 bacterial CFU per ml was inoculated intraperitoneally (mice model) or hemocoelly (*G. mellonella* model) with a syringe. For the *G. mellonella* infection model, *G. mellonella* infection assays were performed as previously described (Gaddy et al. 2012). The wax moth *G. mellonella* in the larval stage was stored in the dark and used within 3 days from shipment. Prior to inoculating the larvae, the bacterial pellets were washed with sterile saline and then diluted to three different cell densities. Using a 50 µl Hamilton syringe, 10 µl aliquots of diluted bacterial suspension (10^5, 10^6, 10^7 CFU per ml) were injected into the hemocoel of each larvae, through the rear left pro-leg (Megaw et al. 2015, Tsai et al. 2016, Mikulak et al. 2018, Cutuli et al. 2019, Guevara et al. 2022). A group of 10 larvae were randomly selected to be injected with the vaccine in triplicate. Following injection, the larvae were incubated at 37°C, and the survival of larvae was monitored daily for 3 days. Death was denoted when larvae no longer responded to touch. Results were analyzed using Kaplan-Meier survival curves (GraphPad Prism statistics software). In this experiment, three control groups were used: 10 larvae in the first group were injected with 10 µl sterile saline, the second group included larvae that received a mock injection to ensure death was not caused by physical trauma, and the larvae in the third group had no injection.

For the mouse infection model, according to a previous study (Yang et al. 2014), the mice were grouped into a new environment and adapted to the new environment for 2 days before the start of the experiment. 500 µl aliquots of diluted bacterial suspension (10^6, 10^7, 10^8 CFU per ml) were injected into the intraperitoneal cavity of each mouse. The well-being of each mouse was checked every 24 hours.

Virulence gene detection

Designed oligonucleotide primers were used to target potential virulence factors of *S. agalactiae* (Table 1). The primer sequences for the virulence gene of *S. agalactiae* were synthesized at the Sangon Biotech Co., Ltd. (Shanghai China). *S. agalactiae* isolates were screened for *cfb*, *cylE*, *hylB*, *lmb*, *scpB*, and *sip*. The PCR protocol used and the cycling parameters followed were as described earlier, except for annealing temperatures (Table 1).

Statistical analysis

Statistical analysis was performed using GraphPad Prism, version 8.3.0. All data were presented as the mean ± SD from three biological replicates. P values were determined using an unpaired, two-tailed Student's t-test.

Results

MIC result

The MIC distributions of antimicrobials against *S. agalactiae* (n=10) isolated from dairy cows in Shanghai China are shown in Table 2. *S. agalactiae* was 100% susceptible to STR, MEM, CIP, CLI, CFQ, CLO, and

Table 2. MIC result of 10 *Streptococcus. agalactiae* isolates.

Strains	STR	DOX	MEM	CIP	FFC	FOX	TET	APR	CLI	CFQ	CLO	PEN	CFQ in milk
4007RHP-43.15	1	0.25	0.06	0.002	4	4	0.5	32	1	0.002	0.5	0.5	0.06
5044LHG-23.15	1	0.25	0.06	0.002	2	4	32	16	1	0.002	0.5	0.06	0.125
B4030RH-11.4	32	4	0.25	0.125	4	8	>64	16	1	0.5	0.5	0.5	0.5
1094LQP-23.15	1	0.25	0.06	0.002	1	2	1	32	1	0.002	0.5	0.125	1
B4030RH-11.6	32	4	0.25	0.25	8	8	>64	8	1	1	0.5	0.5	0.06
1094LQP-33.15	1	0.25	0.06	0.002	0.5	1	64	0.5	1	0.002	0.5	0.5	0.06
4007RHP-53.15	4	2	<0.125	1	1	2	2	16	1	0.002	0.5	0.06	1
1094LQP-13.15	1	0.5	0.06	0.06	2	4	1	16	1	0.002	0.5	0.125	0.06
1094LQP-53.15	1	0.25	0.06	0.002	0.5	1	>64	8	1	0.002	0.5	0.5	0.06
4007RHP-33.15	1	0.25	0.06	0.002	1	1	16	4	1	0.002	0.5	0.06	0.25

Table 3. Virulence gene profiles of the 10 *S. agalactiae* isolates.

Strains	Virulence gene
4007RHP-43.15	*cylE,hylB,sip,cfb*
5044LHG-23.15	*cylE,hylB,sip,cfb*
B4030RH-11.4	*cylE,hylB,sip,cfb*
1094LQP-23.15	*cfb*
B4030RH-11.6	*cylE,hylB,sip,cfb*
1094LQP-33.15	*cylE,hylB,sip,cfb*
4007RHP-53.15	*cylE,hylB,sip,cfb*
1094LQP-13.15	*cylE,hylB,sip,cfb*
1094LQP-53.15	*cylE,hylB,sip,cfb*
4007RHP-33.15	*cylE,hylB,sip,cfb*

CFQ in milk. Tetracycline exhibited the highest level of resistance at 70%, followed by PEN at 50% and DOX at 30%. APR and FOX showed 20% resistance, while FFC showed 10%. The minimum inhibitory concentration (MIC) range of CFQ in broth was 0.06-0.5, and in milk, it was 0.06-1 μg/mL. The MIC of CFQ in both the broth and milk was less than 1 μg/mL.

Virulence gene profiles

We tested the virulence genes of *S. agalactiae* isolates by PCR. Table 3 shows that all *S. agalactiae* isolates carry the *cfb* gene. One isolate carry only the *cfb* gene, while the remaining nine isolates carry the same four virulence genes.

In vivo infection

Our study found that all ten *S. agalactiae* strains caused severe mortality in the *G. mellonella* infection model, across three different challenge concentrations (as shown in Figs 1 and 2). Notably, the mortality rate was lower in the 10^5 CFU/mL group compared to the other challenge concentrations. In the 10^7 CFU/mL challenge concentration group, the survival rate of all *G. mellonella* infection models were 0%, except for the 1094LQP-23.15 group (Fig 1d). Severe mortality caused by *S. agalactiae* has been observed in mouse models, with most groups showing high mortality rates. However, the 1094LQP-23.15 group displayed lower mortality than the other group, as observed in the *G. mellonella* infection model (Fig 2d).

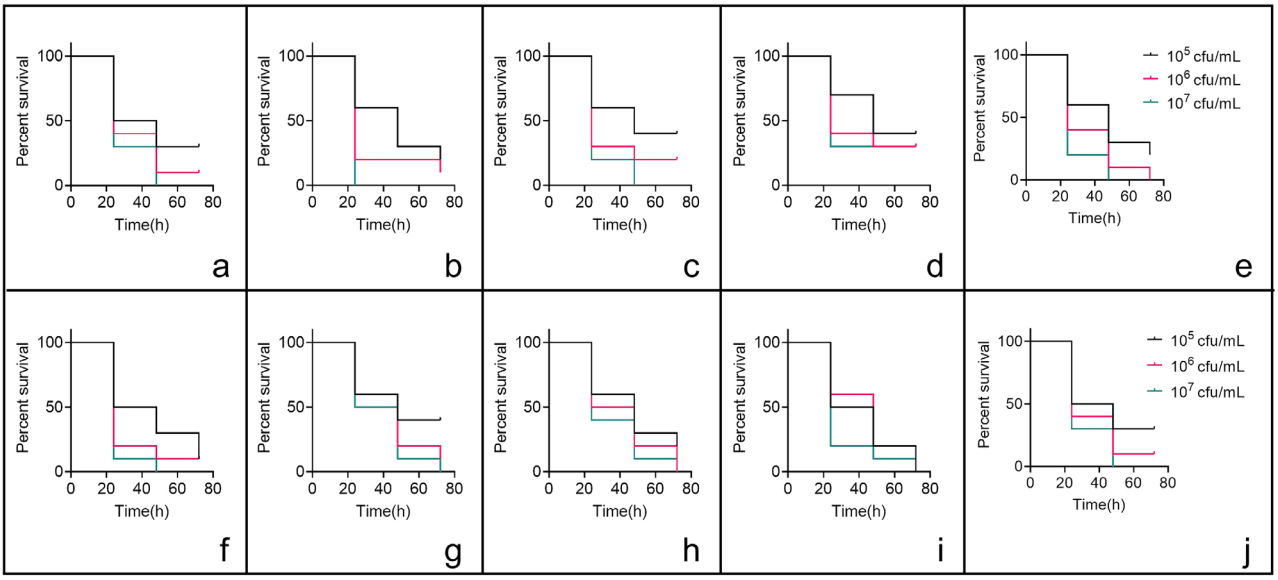

Fig. 1. *Galleria* mellonella were injected with $10^{5\text{-}7}$ CFU/mL *S. agalactiae*. Percent survival of *G. mellonella* over 72 h post infection with 10 *S. agalactiae* isolates (4007RHP-43.15 [a], 5044LHG-23.15 [b], B4030RH-11.4 [c], 1094LQP-23.15 [d], B4030RH-11.6 [e], 1094LQP-33.15 [f], 4007RHP-53.15 [g], 1094LQP-13.15 [h], 1094LQP-53.15 [i], 4007RHP-33.15 [j]).

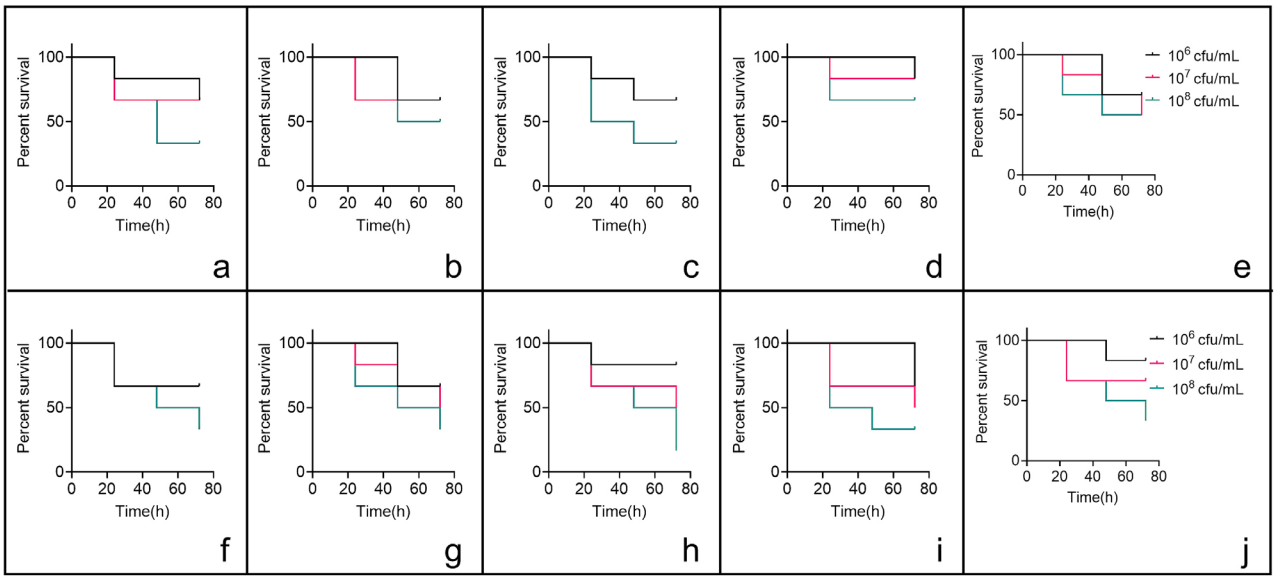

Fig. 2. *in vivo* mouse model was injected with $10^{6\text{-}8}$ CFU/mL *S. agalactiae*. Percent survival of mice over 72 h post infection with 10 *S. agalactiae* isolates (4007RHP-43.15 [a], 5044LHG-23.15 [b], B4030RH-11.4 [c], 1094LQP-23.15 [d], B4030RH-11.6 [e], 1094LQP-33.15 [f], 4007RHP-53.15 [g], 1094LQP-13.15 [h], 1094LQP-53.15 [i], 4007RHP-33.15 [j]).

Discussion

S. agalactiae is the primary cause of bovine mastitis, with the exception of *E. coli* and *S. aureus*. Currently, there are no studies that have used milk as a medium to determine the MIC value of *S. agalactiae*. CFQ is also one of the primary drugs used to treat bovine mastitis in dairy cattle. This study established the MIC of *S. agalactiae* in milk, providing a scientific foundation for the clinical management of bovine mastitis caused by *S. agalactiae*. The study shows that the MIC value of CFQ in milk is 1 to 500 times higher than that measured in broth, which is consistent with previous research on *S. aureus*. This indicates the possible presence of certain elements in milk that may interact with or affect the drug's effectiveness (Jiang et al. 2022). In summary, the MIC results above indicate that the MIC value is more significant in clinical treatment when tested in milk compared to broth.

The study investigated multiple virulence genes as proxies for the pathogenic potential of the infecting strain and its host. This approach was chosen to accu-

rately represent the interaction dynamics between the two. This approach is consistent with previous research in the field (Leitão, 2020, Paria et al. 2021, San Francisco et al. 2022). These isolates, with the same virulence genes, were initially thought to be from the same clone. However, their MIC results revealed that each strain had different MIC values. It has been discovered that most strains carry these virulence genes. However, the expression of these genes is dependent on various factors. In order to confirm the virulence of these strains in animals, we conducted animal virulence experiments.

In the 10^7 CFU/mL challenge concentration group, the survival rate of all *G. mellonella* infection models was 0%, except for the 1094LQP-23.15 group (Fig 1d). It is possible that 1094LQP-23.15 carries fewer virulence genes than other strains. Severe mortality caused by *S. agalactiae* has been observed in mouse models, with most groups showing high mortality rates. However, the 1094LQP-23.15 group displayed lower mortality than the other group, as observed in the *G. mellonella* infection model (Fig 2d). In the present investigation, is was found that most *S. agalactiae* strains carry multiple virulence genes and exhibit strong pathogenicity in *in vivo* models. However, the 1094LQP-23.15 strain carried fewer virulence genes than the other strains and was found to be less pathogenic. Based on the results, it is suggested that virulence genes may be related to pathogenicity, which is consistent with previous studies (Rodríguez-Andrade et al. 2016, Han et al. 2022). The study did not identify whether the virulence genes of 10 strains of S. agalactiae are located in plasmids, which poses a risk of horizontal transfer. Further investigation is necessary.

Conclusions

Using two *in vivo* infection models, we estimated the pathogenicity of ten strains of *S. agalactiae*. These results demonstrate high-level pathogenesis and indicate that the virulence genes may be the primary cause of bacterial pathogenesis (Figure 1,2). However, the pathogenic mechanism *in vivo* remains unclear and requires further study. Milk is a commonly consumed dairy product that has a close relationship with human health. This study isolated ten strains of *S. agalactiae* from dairy cows, all of which exhibited drug resistance, including multi-drug resistance (Table 2). Therefore, monitoring dairy cows on a daily basis can help prevent the transmission of antibiotic-resistant bacteria to humans. In recent times, there has been a growing focus on preventing and controlling drug-resistant bacteria in the environment (Bengtsson-Palme et al. 2018, Waseem et al. 2018, Schnitt et al. 2021, Gelalcha et al. 2022). Therefore, it is important to also focus on drug-resistant *S. agalactiae* in milk. However, this study only compared the pathogenicity resulting from carrying different virulence genes, and the mechanism of pathogenesis is still unclear and requires further investigation.

Acknowledgements

This project was supported by the research startup foundation of Shanghai Customs College (No. 202305).

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