

CHARACTERISATION OF *STAPHYLOCOCCUS AUREUS* ST3320 CLONE CAUSING FATAL RESPIRATORY INFECTION IN RABBITS

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Abstract: *Staphylococcus aureus* is a well-known pathogen that infects humans and animals. However, information on the fatal respiratory infection in rabbits caused by *S. aureus* is still limited. In the present study, a *S. aureus* isolate designated ND01 was recovered from lung samples of rabbits that died of fatal respiratory infection, and the ND01 was characterised by intranasal infection of rabbits, multi-locus sequencing typing, screening virulence genes and testing antimicrobial susceptibility. Clinical signs of matted forepaws and pathological lesions of haemorrhagic tracheitis and necrotising haemorrhagic pneumonia were observed in the ND01 infected rabbits, which were identical to those of naturally infected ones. The sequence type of the ND01 was defined as ST3320 and the ND01 was further grouped into the clonal complex 398. Notably, the ND01 was *pvl*-positive *S. aureus* and carried the human-associated *scn* gene. Moreover, the ND01 was methicillin-susceptible *S. aureus* and was susceptible to 6 of 10 tested antibiotics. This study described the characteristics of the ND01 causing fatal respiratory infection in rabbits. The results are helpful to further the understanding of the pathogenicity of *S. aureus* ST3320 clone in rabbits. The results also highlighted that operators must be on the alert for the colonisation of *pvl*-positive *S. aureus* in rabbits and potential transmission events between rabbits and humans.

Key Words: *Staphylococcus aureus*, rabbit, fatal respiratory infection, virulence gene, multi-locus sequencing typing.

INTRODUCTION

S. aureus is a well-recognised zoonotic pathogen that infects humans and animals. The infection of *S. aureus* is associated with a variety of diseases such as bacteraemia, pneumonia, sepsis and mastitis (Lozano *et al.*, 2016; Rowe *et al.*, 2020). Moreover, the ability to acquire antibiotic resistance makes *S. aureus* a great threat to public health (Paterson *et al.*, 2012; Algammal *et al.*, 2020; Giulieri *et al.*, 2020).

S. aureus is widespread in important rabbit farming areas (Ferreira *et al.*, 2014; Selva *et al.*, 2015; Wang *et al.*, 2019a). Infection by *S. aureus* in rabbits is associated with subcutaneous abscesses, mastitis, pododermatitis and respiratory diseases (Corpa *et al.*, 2009; Wang *et al.*, 2019b). Our previous studies showed that *S. aureus* belonging to 2 sequence type (ST121 and ST398) were isolated from rabbits in Fujian Province in south-eastern China (Wang *et al.*, 2019a,b). However, the pathogenicity of the strains belonging to the 2 ST was different. The ST121 clone caused severe respiratory diseases in rabbits of different ages. Cough, purulent nasal discharge, haemorrhagic tracheitis, necrotising haemorrhagic pneumonia and purulent pneumonia were observed in the ST121 clone infected rabbits during a 4-wk experiment period (Wang *et al.*, 2019b). The ST398 clone mainly caused chronic lesions such as mastitis and pododermatitis in adult rabbits (Wang *et al.*, 2019a).

In March 2020, a fatal respiratory infection broke out on a rabbit farm with around 400 adult female rabbits in Ningde city of Fujian Province. Around 200 rabbits died of the infection in a 30-d period. Their systematic and

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complete necropsy revealed the presence of matted forepaws, haemorrhagic tracheitis and haemorrhagic, necrotising pneumonia. According to these pathological findings, it was presumed that the fatal respiratory infection was caused by the infection or co-infection of rabbit haemorrhagic disease virus (RHDV), *Pasteurella multocida* (*P. multocida*), *Bordetella bronchiseptica* (*B. bronchiseptica*) and *S. aureus*. The polymerase chain reaction (PCR) assays for the 3 types of pathogens including RHDV, *P. multocida* and *B. bronchiseptica* were negative in the lung samples of dead rabbits, but a *S. aureus* isolate designated ND01 was recovered from the lung samples of dead rabbits. The aim of this study was to evaluate the pathogenicity of the ND01 in rabbits, and characterise the ND01 by multi-locus sequencing typing (MLST), screening virulence genes and testing antimicrobial susceptibility.

MATERIALS AND METHODS

Ethical statement

This study was approved by the Research Ethics Committee of the Institute of Animal Husbandry and Veterinary Medicine, Fujian Academy of Agricultural Sciences (FAAS), and the approval number is FAAS-AHVM2020-0301. All the animal procedures were in accordance with the *Guidelines for the operation of laboratory animals* issued by the Institute of Animal Husbandry and Veterinary Medicine, FAAS.

Sample collection, bacterial isolation and identification

A total of 28 lung samples were collected from naturally infected dead rabbits. Each sample was placed in a sterile tube kept on ice and homogenised to make a 50% suspension in sterile phosphate buffer saline (PBS). One hundred microlitres of suspension was plated evenly on the brain heart infusion (BHI) agar plate containing 5% defibrinated sheep blood, and incubated for 24 to 48 h at 37°C. Five bacterial clones from each plate were selected and purified, and the isolates were identified by amplifying and sequencing of the *16S rRNA* genes.

Animal experiments

The pathogenicity of the isolate was evaluated in 30-d old healthy rabbits obtained from a local rabbit farm. Thirty-two rabbits were randomly divided into 4 groups including 4 males and 4 females in each group. Two rabbits (one male and one female) from the same group were housed in a stainless steel cage. Each group was placed in a separate room. The rabbits had free access to commercial feed and water. Before infection, nasal swabs were collected for bacteriological examination to ensure *S. aureus*-free status of the rabbits.

Rabbits were anaesthetised by intravenous injection with ketamine (40 mg/kg) and challenged with the isolate or sterile PBS. Based on the results of our preliminary experiment, rabbits from groups 1 and 2 were intranasally infected with 1.0×10^{12} and 1.0×10^9 colony forming units (CFU) of the isolate suspended in 100 μ L sterile PBS, respectively. Two males and 2 females from group 3 were intranasally infected with 1.0×10^8 CFU of the isolate suspended in 100 μ L sterile PBS, and the other 2 males and 2 females were co-housed and in direct contact with the infected rabbits. Rabbits from group 4, the control group, were intranasally inoculated with 100 μ L of sterile PBS. The clinical status and signs of the rabbits, including body weight, attitude, appetite, nasal discharge, cough and respiratory distress were monitored daily for 4 wk. During the experimental infection, the appearance of matted forepaws and the reduction of feed intake were considered the main clinical signs consistent with the appearance of infection, which characterise the development of natural infections. The lung samples of the naive rabbits from group 3 were collected to confirm the transmission of the isolate between rabbits by direct contact. Tracheas, lungs, livers, hearts, spleens, kidneys and blood of the rabbits from group 2 were collected to assess the distribution of the isolate in the organs of infected rabbits. Each of the samples was homogenised to make a 50% suspension in sterile PBS. The presence and identity of the isolate in the organs were confirmed by amplifying and sequencing of *16S rRNA* and *nuc* genes.

To prevent or minimise animal pain and distress, challenged rabbits catching up with the endpoint (dyspnoea, weight loss of 15% or inability to access feed or water) were euthanised by bleeding from the jugular vein under ketamine narcosis.

Detection of genes

The genomic DNA of the isolate was prepared using a commercial bacterial genomic DNA extraction kit (Dalian TakaRa Biotechnology Co., Ltd). The *16S rRNA*, *mecA*, *mecC*, *scn*, *tet(M)* and 13 virulence genes including thermonuclease (*nuc*), Panton-Valentine leukocidin (*pvl*), enterotoxin (*sea* and *seb*), toxic shock syndrome toxin-1 (*tsst*), exfoliative (*eta* and *etb*), haemolysin (*hla* and *hlb*), clumping factor (*clfA* and *clfB*) and fibronectin-binding protein (*fnbpA* and *fnbpB*) (Table 1) were determined by PCR assays with the following amplification conditions: initial denaturation at 94°C for

Table 1: Primers used for *16S rRNA*, *mecA*, *mecC*, *scn*, *tet(M)* and 13 virulence genes.

Genes	Primer sequence (5'-3')	Product size (bp)	Reference
<i>16S rRNA</i>	F: ccgaattcgtcgacaacagagtttgatcctggctcag R: cccgggatccaagcttaaggaggtgatccagcc	1549	(Weisburg <i>et al.</i> , 1991)
<i>mecA</i>	F: aaaatcgatgtaaaaggttggc R: ttctgcagtaccggattgc	533	(Murakami <i>et al.</i> , 1991)
<i>mecC</i>	F: cattaanaatcagagcgggc R: tggctgaaccattttgat	188	(Paterson <i>et al.</i> , 2012)
<i>scn</i>	F: agcacaagcttgccaacatcg R: ttaataattacttttagtc	258	(van Wamel <i>et al.</i> , 2006)
<i>tet(M)</i>	F: gtggacaagggtacaacgag R: cggtaaagttcgtcacacac	405	(Warsa <i>et al.</i> , 1996)
<i>nuc</i>	F: gcgattgatggtgatacggttaaattaa R: agccaagccttgacgaact	279	(Brakstad <i>et al.</i> , 1992)
<i>pvl</i>	F: atcattaggtaaaatgtctggacatgatcca R: gcatcaactgtattggatagcaaaagc	433	(Jarraud <i>et al.</i> , 2002)
<i>sea</i>	F: tcattgccctaactgtagca R: gccataaattgatcgccact	432	(Srinivasan <i>et al.</i> , 2006)
<i>seb</i>	F: cctaaaccagatgagttgcaca R: accatctcaaataccccaaca	405	(Srinivasan <i>et al.</i> , 2006)
<i>tsst</i>	F: tgcaaaagcatctacaacga R: tggatccgctcattcattg	499	(Xie <i>et al.</i> , 2011)
<i>eta</i>	F: actgtaggagctagtgcattgt R: tggatactttgtctatcttttcatcaac	190	(Xie <i>et al.</i> , 2011)
<i>etb</i>	F: gataaagagctttatacacacattac R: agtgaacttatcttctattgaaaaacactc	612	(Xie <i>et al.</i> , 2011)
<i>hla</i>	F: ctgattactatccaagaattcgattg R: cttccagcctactttttatcagt	209	(Jarraud <i>et al.</i> , 2002)
<i>hlb</i>	F: gtgcacttactgacaatagtgc R: gttgatgagtactaccttcagt	309	(Jarraud <i>et al.</i> , 2002)
<i>clfA</i>	F: tgaaaatagtgttacgcaatctgatag R: accgcttgattaactacatctttattac	500	(Wang <i>et al.</i> , 2019b)
<i>clfB</i>	F: tgcaagatcaaatgctctctcaa R: ggtctgtaataaaggaatgaaaattg	596	(Wang <i>et al.</i> , 2019b)
<i>fnbpA</i>	F: cacaaccagcaaatatagaacagtta R: tacgactgaaccatttttaatttctgg	523	(Wang <i>et al.</i> , 2019b)
<i>fnbpB</i>	F: gtaacagctaattggtcgaattgatac R: caagttcgataggagtactatgttctat	500	(Wang <i>et al.</i> , 2019b)

5 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s (58°C for *clfA*, *clfB*, *fnbpA* and *fnbpB*), extension at 72°C for 1 min, with a final extension at 72°C for 10 min. The PCR products were purified and sequenced.

MLST analyses

The MLST analysis of the isolate was conducted as described in the PubMLST (<https://pubmlst.org/saureus/>). Briefly, the 7 house-keeping genes of *arcC*, *aroE*, *glpF*, *gmk*, *pta*, *tpi* and *yqi* from the isolate were amplified and sequenced. The 7 allelic numbers were given by comparing the sequences of the 7 house-keeping genes of the isolate to the known sequences in the PubMLST database, and the ST of the isolate was defined according to the 7 allelic numbers.

Antimicrobial susceptibility testing

The antimicrobial susceptibility of the isolate was performed according to the Clinical and Laboratory Standards Institute (CLSI) standards using disc diffusion method (CLSI, 2018). Ten antibiotics were used: penicillin, ceftaroline, gentamicin, azithromycin, tetracycline, doxycycline, levofloxacin, enoxacin, norfloxacin and ofloxacin. *S. aureus* ATCC 29213 was used as the quality control. The results of antimicrobial susceptibility testing were interpreted according to the breakpoints used for *Staphylococcus spp.* (CLSI, 2018).

RESULTS

Bacterial isolation and identification

The bacterial colonies recovered from the 28 lung samples of naturally infected dead rabbits were round, smooth, golden-yellow and surrounded by haemolytic rings on BHI agar plate containing 5% defibrinated sheep blood (Figure 1A), and the isolates were Gram-positive cocci (Figure 1B). The sequences of the *16S rRNA* genes of the isolates were identical and shared the highest identity (ranged from 99 to 100%) with that of *S. aureus* (Figure 2), which suggested that all these isolates were *S. aureus*. Moreover, the STs and virulence gene profiles of the isolates were also identical, indicating that all the isolates were derived from the same progenitor. In this study, an isolate designated ND01 was selected as the representative, and the ND01 was characterised in the following experiments.

Animal experiments

The pathogenicity of the ND01 was evaluated by intranasal infection of rabbits. Rabbits from group 1 became seriously ill and all of the 8 rabbits died within 24 h post-infection (PI). Haemorrhagic tracheitis (Figure 3A) and haemorrhagic pneumonia (Figure 3B) were observed in the all dead rabbits from group 1. The clinical sign of matted forepaws (Figure 3C) was observed in all rabbits from group 2. Three rabbits from group 2 died on days 20, 22 and 27 PI, respectively. Haemorrhagic tracheitis and necrotising haemorrhagic pneumonia (Figure 3D) were observed in the 3 dead rabbits, and these pathological lesions were also observed in the remaining 5 rabbits from group 2 at the end of the experiment. Clinical sign of matted forepaws and pathological lesions of haemorrhagic tracheitis and necrotising haemorrhagic pneumonia were also observed in the 4 naïve rabbits from group 3. The presence of the ND01 in the lungs of the 4 naïve rabbits from group 3 was detected. The presence of the ND01 was mainly restricted to the tracheas, lungs and livers of infected rabbits from group 2. Moreover, the clinical sign and pathological lesions of the challenged male and female rabbits were identical. Taken together, the ND01 was the causative agent of the fatal respiratory infection on the rabbit farm.

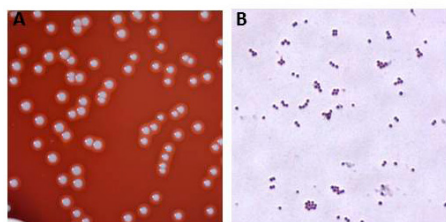


Figure 1: Colonial morphology and Gram-staining of ND01. 1A: The colonial morphology of ND01 on the BHI agar plate containing 5% defibrinated sheep blood; 1B: Gram-staining of ND01.

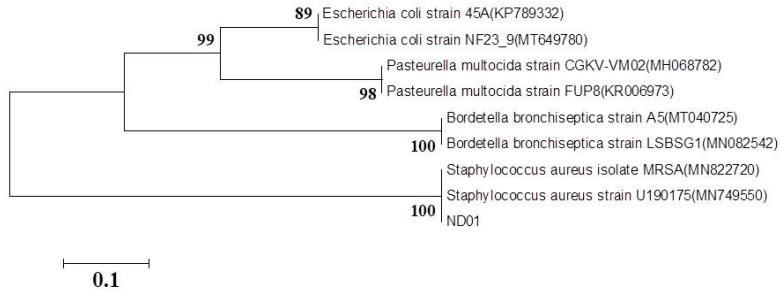


Figure 2: Phylogenetic tree based on the sequence of 16S rRNA gene of ND01.

Detection of genes

The 6 virulence genes of *nuc*, *pvl*, *hla*, *clfB*, *fnbpA* and *fnbpB* were positive in the ND01 (Figure 4), whereas the other 7 virulence genes of *sea*, *seb*, *tsst*, *eta*, *etb*, *hly* and *clfA* were negative. Moreover, the methicillin-resistant genes *mecA* and *mecC* were negative. Interestingly, the *scn* gene that strongly associated with the *S. aureus* strains isolated from humans was positive in the ND01 (Figure 4).

MLST analyses

The sequences of the 7 house-keeping genes of *arcC*, *aroE*, *glpF*, *gmk*, *pta*, *tpi* and *yqi* of the ND01 were analysed in the PubMLST database, and a panel of allelic numbers of 3-35-19-249-20-26-39 was assigned. According to the 7 allelic numbers, the sequence type of the ND01 was defined as ST3320. By using goeBURST (<http://www.phyloviz.net/goeburst/>), the ND01 was grouped into clonal complex CC398 (Figure 5).

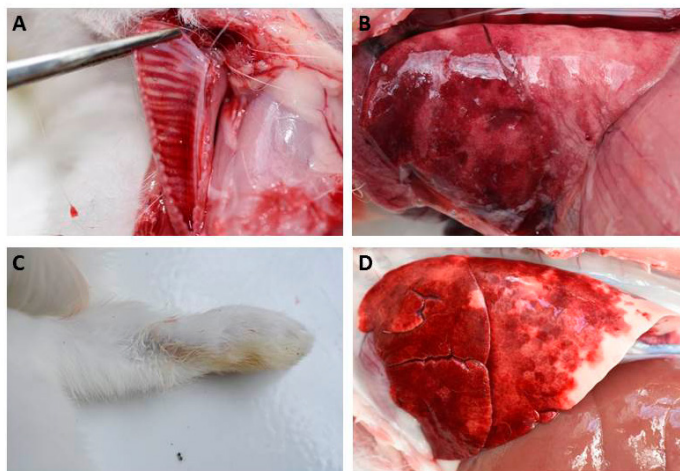


Figure 3: Animal experiments. 3A: Severe haemorrhagic tracheitis of the rabbits intranasally infected with 1.0×10^{12} CFU of ND01; 3B: Diffuse haemorrhagic pneumonia of the rabbits intranasally infected with 1.0×10^{12} CFU of ND01; 3C: Matted forepaws of the rabbits intranasally infected with 1.0×10^8 CFU of ND01; 3D: Severe necrotising haemorrhagic pneumonia of the rabbits intranasally infected with 1.0×10^8 CFU of ND01.

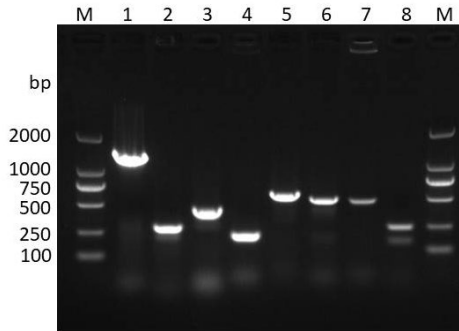


Figure 4: Gel electrophoresis of genes of the isolate ND01. M: DNA marker; 1: *16S rRNA* (1549 bp); 2: *nuc* (279 bp); 3: *pvl* (433 bp); 4: *hla* (209 bp); 5: *clfB* (596 bp); 6: *fnbpA* (523 bp); 7: *fnbpB* (500 bp); 8: *scn* (258 bp).

Antimicrobial susceptibility testing

The results of antimicrobial susceptibility testing showed that the ND01 was resistant to norfloxacin, intermediately susceptible to evofloxacin, enoxacin and ofloxacin, and susceptible to penicillin, cefaroline, gentamicin, azithromycin, tetracycline and doxycycline. Moreover, the methicillin-resistant genes of *mecA* and *mecC* were negative. The results suggested that the ND01 was not multidrug-resistant and that the ND01 was MSSA.

DISCUSSION

S. aureus is an important pathogen that infects rabbits in Fujian Province (Wang *et al.*, 2019a). Our previous study showed that the *S. aureus* ST121 clone caused severe respiratory disease in rabbits (Wang *et al.*, 2019b). In the

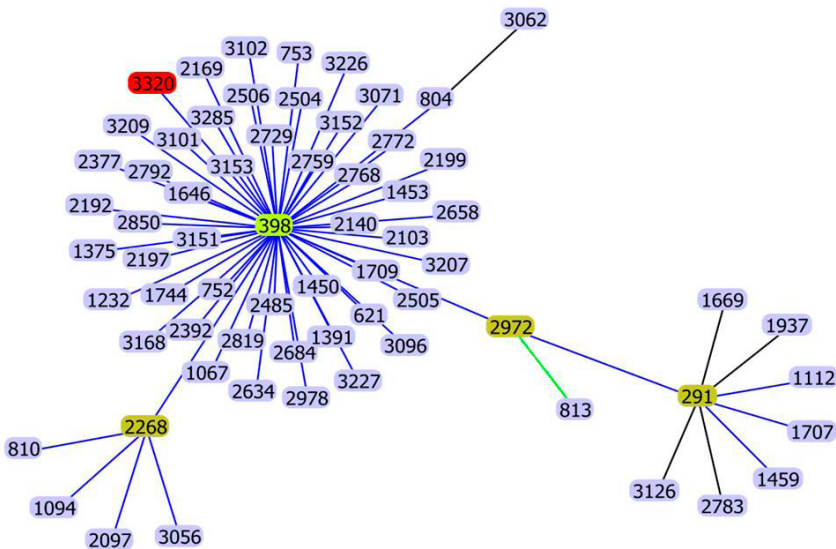


Figure 5: *S. aureus* strains belonging to CC398. *S. aureus* ST398 clone (coloured green) is the putative founder. *S. aureus* ST3320 clone (coloured red) is a single locus variant of the ST398.

present study, we showed that the *S. aureus* ST3320 clone could also cause fatal respiratory infection in rabbits, as the clinical sign and pathological lesions observed in the rabbits intranasally infected with the ND01 were identical with that of naturally infected rabbits. Moreover, the ND01 could be transmitted between rabbits by direct contact and the presence of ND01 could be detected in the tracheas, lungs and livers of the intranasally infected rabbits.

S. aureus possesses a variety of virulence genes, which facilitates the colonisation and invasion of the bacterium in the infected host (Jenul and Horswill, 2019). Previous reports showed that the profiles of virulence genes were different in *S. aureus* isolates recovered from rabbits (Viana *et al.*, 2015). The ND01 carried a combination of virulence genes of *nuc*, *pvl*, *hla*, *clfB*, *fnbpA* and *fnbpB*. The genes of *fnbpA* and *fnbpB* encode the fibronectin-binding proteins, *fnbpA* and *fnbpB*, respectively. The *fnbpA* and *fnbpB* are multifunctional virulence factors, which facilitates *S. aureus* colonisation by interacting with several host extracellular matrix/plasma proteins such as fibronectin, fibrinogen, histones, elastin and plasminogen (Speziale and Pietrocola, 2020). It was reported that the *fnbpA* and *fnbpB* were the important virulence factors associated with staphylococcal pneumonia (Mongodin *et al.*, 2002). Notably, the *pvl* gene of the ND01 was positive. The *pvl* gene encodes the Pantone-Valentine leukocidin, which is a well-known virulence factor causing necrotising pneumonia (Huang *et al.*, 2020). The infection of *pvl*-positive *S. aureus* was associated with lethal necrotising pneumonia in humans (Gillet *et al.*, 2002; Davies *et al.*, 2011; Sicut *et al.*, 2013). Moreover, the infection of *pvl*-positive *S. aureus* was also associated with fatal respiratory disease in rabbits (Wang *et al.*, 2019b). Taken together, the virulence genes of *pvl*, *fnbpA* and *fnbpB* of ND01 might be the important virulence factors contributing to the fatal respiratory infection in rabbits.

The ND01 was grouped into CC398. *S. aureus* strains belonging to CC398 are widespread in humans and animals, resulting in a spectrum of infections ranging from relatively minor or localised lesions to more serious or invasive pathologies (Chroboczek *et al.*, 2013). *S. aureus* strains belonging to CC398 can be clustered into 2 distinct phylogenetic clades, human-associated clade and livestock-associated clade (Stegger *et al.*, 2013). Human-associated CC398 strains carry the *scn* gene, whereas livestock-associated CC398 strains carry the *tet(M)* gene (Stegger *et al.*, 2013). Unexpectedly, the *scn* gene was positive in the ND01, but the *tet(M)* gene was negative. It is suggested that the ND01 might derive from human-associated *S. aureus* strain, and the ND01 might be introduced into rabbits from humans.

The emergence and resurgence of methicillin-resistant *S. aureus* (MRSA) presents a potential significant challenge to public health (Liu *et al.*, 2021). Fortunately, the ND01 was MSSA. This result is consistent with our previous study, in which all of the 281 *S. aureus* isolates recovered from rabbits in Fujian Province were MSSA (Wang *et al.*, 2019a). However, the colonisation of MRSA in rabbits was detected in Italy and the Iberian Peninsula (Agnoletti *et al.*, 2014; Moreno-Grúa *et al.*, 2018; Silva *et al.*, 2020), and transmission of MRSA from rabbit to human has been previously reported (Agnoletti *et al.*, 2014). Therefore, we should be on the alert for the potential emergence of MRSA in rabbits in Fujian Province.

This study described the characteristics of a *S. aureus* ST3320 clone causing fatal respiratory infection in rabbits. These results may therefore be of help to further the knowledge on the pathogenic mechanisms of *S. aureus* ST3320 strain in rabbits.

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