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CHARACTERISATION OF *STAPHYLOCOCCUS AUREUS* STRAIN CAUSING SEVERE RESPIRATORY DISEASE IN RABBITS

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Abstract: *Staphylococcus aureus* is acknowledged as one of the important pathogens isolated from humans and animals. However, the *S. aureus* causing severe respiratory diseases in rabbits have not been well characterised. A *S. aureus* named FZHW001, isolated from the lungs of dead rabbits with severe respiratory disease, was characterised by artificial infection of rabbits, detection of virulence factors, multi-locus sequencing typing and antimicrobial susceptibility test. The FZHW001 infected rabbits showed identical respiratory symptoms to those of naturally infected ones, and the isolate could spread through directed contact among rabbits. The isolate was typed into clonal complex 121 and carried 7 of 13 tested virulence factors. Furthermore, the isolate was identified to be methicillin-susceptible *S. aureus* and was susceptible to 7 of 12 tested antibiotics. This study first describes the characteristics of *S. aureus* isolated from rabbits causing severe respiratory disease, which will help in further understanding the pathogenic mechanisms of *S. aureus* in rabbits.

Key Words: *Staphylococcus aureus*, rabbit, respiratory disease, virulence factors, multi-locus sequencing typing.

INTRODUCTION

S. aureus is recognised as one of the important pathogens isolated from humans and animals (Sato *et al.,* 2017; Brady *et al.,* 2018). Owing to the ability to express various virulence factors, the infection of *S. aureus* results in a broad variety of diseases including bacteraemia, sepsis, mastitis and pneumonia (Denayer *et al.,* 2017; Schmidt *et al.,* 2017; Hecker *et al.,* 2018). In recent years, the prevalence and diversity of *S. aureus* in animals has been cause for concern due to animals potentially acting as reservoirs of human infections (Haenni *et al.,* 2017; Schmidt *et al.,* 2017). It has been reported that *S. aureus* from animals, notably the livestock-associated methicillin-resistant clonal complex (CC) 398 (Verkade *et al.,* 2014), could be transmitted to humans and induce serious infection (Angen *et al.,* 2017).

S. aureus poses a great threat to rabbit farming. The manifestations of infected rabbits are subcutaneous abscesses, mastitis and pododermatitis (Hermans *et al.,* 2003; Vancraeynest *et al.,* 2006). In late August 2017, a severe respiratory disease broke out in a rabbit farm with around 1000 adult female rabbits in Fujian Province in south-eastern China, causing the death of roughly 1000 rabbits in a four-week period. Clinical signs in infected rabbits were weight loss, coughing and purulent nasal discharge. Haemorrhage in trachea and purulent pneumonia were observed in dead rabbits. The severe respiratory disease was presumed to be caused by the infection or co-infection of *Bordetella bronchiseptica*, *Pasteurella multocida* and rabbit haemorrhagic disease virus. However, the results of polymerase chain reaction (PCR) assays for detecting the 3 kinds of pathogen in lung samples were negative. In this study, a *S. aureus* named FZHW001 was isolated from the lungs of the dead rabbits. The aim of this study was to characterize the FZHW001 by artifcial infection of rabbits, detection of virulence factors, multi-locus sequencing

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typing (MLST) and antimicrobial susceptibility test to understand the pathogenicity, genetic diversity and level of antimicrobial resistance of the isolate.

MATERIAL AND METHODS

Ethical statement

The present study was approved by the Laboratory Animal Ethics Committee of Institute of Animal Husbandry and Veterinary, Fujian Academy of Agricultural Sciences (FAAS). The animal experiments were in according with the *Guidelines for the operation of laboratory animals* issued by the Institute of Animal Husbandry and Veterinary, FAAS.

Bacterial isolation and identification

Ten lung samples from dead rabbits were received. The samples were homogenised and 50% suspensions with sterile phosphate buffer saline (PBS) were prepared. One hundred µL of each homogenised sample was spread on sheep blood agar plate and incubated overnight at 37°C. Five bacterial colonies produced on each plate were streaked and purified on sheep blood agar plates and the purified isolates were then identified by sequencing of the *16S rRNA* gene.

Animal experiments

Forty-eight rabbits, 6-wk-old, were randomly divided into 4 groups with 6 females and 6 males per group. Rabbits were anesthetised by intravenous injection of ketamine (40 mg/kg). Groups 1 (G1) and 2 (G2) were intranasally inoculated with 1.0×10^{10} and 1.0×10^{6} colony forming units (CFU) of the isolate diluted in 100 µL sterile PBS, respectively. Group 3 (G3) (control group) was intranasally inoculated with 100 µL sterile PBS. Three females and 3 males in group 4 (G4) were intranasally inoculated with 1.0×10^{6} CFU of the isolate diluted in 100 µL sterile PBS, and 6 naïve rabbits were co-housed, direct contact, with infected rabbits. Clinical signs were monitored daily for 4 wk. To further investigate the distribution of inoculated isolate in infected animals, samples from G2 including tracheas, lungs, livers, hearts, spleens, kidneys and blood were harvested. The presence of the inoculated isolate in these specimens was detected by PCR amplification of *16S rRNA* and *nuc* genes. To confirm the identity of isolates, *16S rRNA* and *nuc* genes of the isolates were sequenced.

Detection of genes

The *16S rRNA*, *mecA*, *mecC*, and 13 virulence genes encoding thermonuclease (*nuc*), panton-valentine leukocidin toxin (*pvl*), enterotoxin (*sea* and *seb*), toxic shock syndrome toxin-1 (*tst*), exfoliative (*eta* and *etb*), haemolysin (*hla* and *hlb*), clumping factor (*clfA* and *clfB*) and fibronectin-binding protein (*fnbpA* and *fnbpB*) were screened by PCR. The primers for *16S rRNA*, *mecA*, *mecC*, *nuc*, *pvl*, *sea*, *seb*, *tst*, *eta*, *etb*, *hla* and *hlb* were reported previously (Table 1). The primers for *clfA*, *clfB*, *fnbpA* and *fnbpB* were designed according to the corresponding sequences Z18852, AJ224764, J04151 and AJ629122, respectively (Table 1). The PCR products were separated and sequenced.

MLST analyses

The isolate was analysed by MLST as previously reported (Enright *et al.,* 2000). Briefly, the 7 housekeeping genes (*arcC*, *aroE*, *glpF*, *gmk*, *pta*, *tpi* and *yqiL*) were amplified and sequenced. Each sequence of the 7 housekeeping genes was compared to known alleles in the MLST database (http:// www.mlst.net), and the sequence type (ST) of the isolate was defined according to the allelic profiles.

Antimicrobial susceptibility testing

The antimicrobial susceptibility of the isolate was evaluated by disk diffusion method. Twelve antibiotics were used: ampicillin, penicillin G, florfenicol, streptomycin, gentamycin, kanamycin, doxycycline, neomycin, enrofloxacin, ciprofloxacin, ofloxacin and levofloxacin. *S. aureus* ATCC 29213 was used as control strain. The susceptibility of the isolate to antibiotics was interpreted according to the Clinical and Laboratory Standards Institute (CLSI) criteria (CLSI, 2013).

Table 1: Primers used for *16S rRNA*, *mecA*, *mecC* and 13 virulence genes.

Genes	Primer sequence (5'-3')	Product size (bp)	Reference
16S rRNA	F: ccgaattcgtcgacaacagagtttgatcctggctcag R: cccgggatccaagcttaaggaggtgatccagcc	1549	(Weisburg *et al.,* 1991)
mecA	F: aaaatcgatggtaaaggttggc R: ttctgcagtaccggatttgc	533	(Murakami *et al.,* 1991)
mecC	F: cattaaaatcagagcgaggc R: tggctgaacccatttttgat	188	(Paterson *et al.,* 2012)
nuc	F: gcgattgatggtgatacggttaaattaa R: agccaagccttgacgaact	279	(Brakstad *et al.,* 1993)
pvl	F: atcattaggtaaaatgtctggacatgatcca R: gcatcaactgtattggatagcaaaagc	433	(Jarraud *et al.,* 2002)
sea	F: tcattgccctaacgttgaca R: gccataaattgatcggcact	432	(Srinivasan *et al.,* 2006)
seb	F: cctaaaccagatgagttgcaca R: accatcttcaaatacccgaaca	405	Srinivasan *et al.,* 2006)
tsst	F: tgcaaaagcatctacaaacga R: tgtggatccgtcattcattg	499	(Xie *et al.,* 2011)
eta	F: actgtaggagctagtgcatttgt R: tggatacttttgtctatctttttcatcaac	190	(Xie *et al.,* 2011)
etb	F: gataaagagctttatacacacattac R: agtgaacttatctttctattgaaaaacactc	612	(Xie *et al.,* 2011)
hla	F: ctgattactatccaagaaattcgattg R: ctttccagcctacttttttatcagt	209	(Jarraud *et al.,* 2002)
hlb	F: gtgcacttactgacaatagtgc R: gttgatgagtagctaccttcagt	309	(Jarraud *et al.,* 2002)
clfA	F: tgaaaatagtgttacgcaatctgatag R: accgcttgattaactacatctttattac	500	this study
clfB	F: tgcaagatcaaactgttcctcaa R: ggtctgtaaataaaggtaatgaaaattg	596	this study
fnbpA	F: cacaaccagcaaatatagaaacagtta R: tacgactgaaccatttttaatttctgg	523	this study
fnbpB	F: gtaacagctaatggtcgaattgatac R: caagttcgataggagtactatgttctat	500	this study

RESULTS

Bacteria isolation and identification

All 10 lung samples from dead rabbits produced the same smooth colonies of the diameter around 1 mm with haemolytic rings (Figure 1A) on blood agar plates, and the purified isolates were Gram-positive cocci (Figure 1B). The *16S rRNA* sequences of these purified isolates were identical and shared the highest identity with that of *S. aureus* (Figure 2), suggesting that these isolates came from the same progenitor. Given the above results, one of the isolates named FZHW001 was used as the representative for the following experiments.

Animal experiments

The pathogenicity of the isolate FZHW001 was evaluated *in vivo*. Rabbits in G1 showed sudden loss of appetite and depression, and all animals died within 24 h post-inoculation (PI). Three out of 12 animals showed nasal cavity

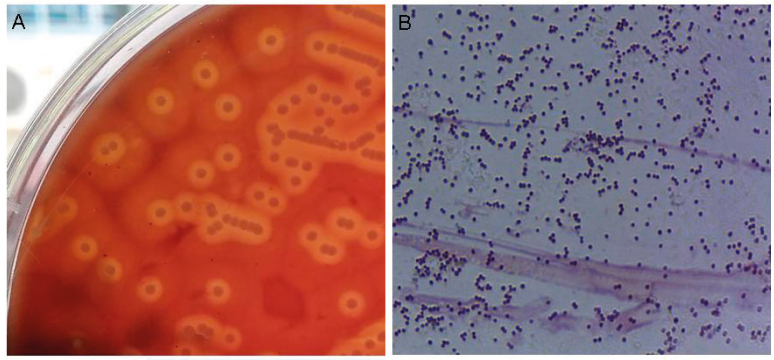

Figure 1: The colonial morphology and Gram-staining of the isolate FZHW001. 1A: The colonial morphology of the isolate FZHW001; 1B: Gram-staining of the isolate FZHW001.

bleeding (Figure 3A), and haemorrhagic tracheitis and pneumonia were observed in all animals (Figure 3B and 3C). Rabbits from G2 showed obvious clinical signs including loss of body weight (Figure 4), coughing, and purulent nasal discharge (Figure 3D). Three infected animals died on days 7, 10 and 17 PI, respectively. Trachea haemorrhage and purulent pneumonia were observed in the 9 animals remaining at the end of the experiment (Figure 3E). Symptoms including loss of body weight, coughing and purulent nasal discharge were observed in naïve rabbits of G4, and the isolate FZHW001 was detected in tracheas, lungs, livers and spleens of naïve rabbits. The colonies morphology, *16S rRNA* and *nuc* genes sequences of the bacteria isolated from artificially infected rabbits were identical to that of isolate FZHW001, indicating that the isolate FZHW001 was the causative agent of the respiratory disease on the rabbit farm. Moreover, the isolate FZHW001 mainly replicated in tracheas, lungs, livers and spleens of artificially infected rabbits (Table 2).

Detection of genes

The *16S rRNA*, *nuc*, *pvl*, *hla*, *hlb*, *clfA*, *clfB* and *fnbpA* genes were amplified from the isolate FZHW001 (Figure 5). All the amplified genes shared the highest identity with the corresponding genes of *S. aureus*. However, the *mecA*, *mecC*, *sea*, *seb*, *tst*, *eta*, *etb* and *fnbpB* genes were negative in isolate FZHW001.

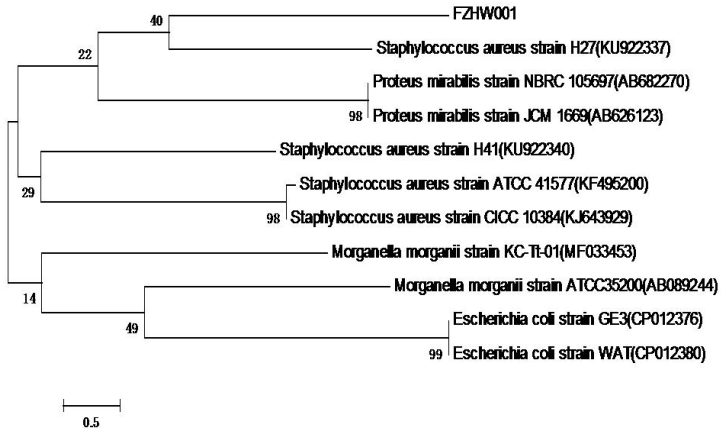

Figure 2: Phylogenetic tree based on *16S rRNA* sequence of the isolate FZHW001.

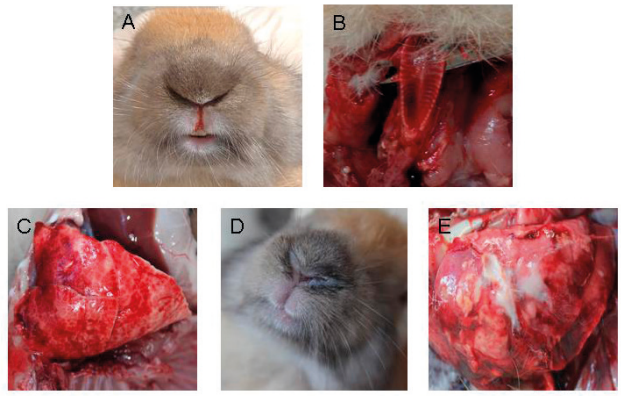

Figure 3: Animal experiments. 3A: Nasal cavity bleeding; 3B: Haemorrhagic tracheitis; 3C: Haemorrhagic pneumonia; 3D: Purulent nasal discharge; 3E: purulent pneumonia.

MLST analyses

The seven housekeeping genes *arcC*, *aroE*, *glpF*, *gmk*, *pta*, *tpi* and *yqiL* were given the allelic profiles of 6, 5, 6, 2, 7, 14 and 5, respectively. The ST of the isolate FZHW001 was defined as ST121 according to the given allelic profiles. The isolate FZHW001 was further grouped into CC121 by using eBURST software (Figure 6).

Antimicrobial susceptibility testing

The isolate FZHW001 was *mecA* and *mecC* negative, indicating that it was a methicillin-susceptible *S. aureus* (MSSA). The isolate was resistant to ampicillin and streptomycin, intermediately susceptible to penicillin G, kanamycin and gentamycin, and susceptible to florfenicol, doxycycline, neomycin, enrofloxacin, ciprofloxacin, ofloxacin and levofloxacin.

DISCUSSION

Rabbits are important natural hosts of *S. aureus*. The strains in rabbits can be grouped into low-virulence and high-virulence. The infection of low-virulence strains is limited to a few animals, whereas the infection of high-virulence strains shows an epidemic spread among the entire flock causing significant economic losses (Hermans *et al.,* 2003). However, the pathogenicity of *S. aureus* to rabbits was not well elucidated. This study describes a *S. aureus* strain

Table 2: Dissemination of the FZHW001 in artificially infected rabbits intranasally inoculated with 1.0×10^6 colony forming units of FZHW001 diluted in 100 µL sterile phosphate buffer saline (G2).

Rabbits	Tissues						
number	Trachea	Lung	Liver	Heart	Spleen	Kidneys	Blood
1	+	+	+	–	+	–	–
2	+	+	+	–	+	–	–
3	+	+	+	+	+	+	–
4	+	+	+	–	+	–	–
5	+	+	+	–	–	–	+
6	+	+	+	–	+	–	–
7	+	+	+	–	+	–	–
8	+	+	+	–	+	+	–
9	+	+	+	+	–	–	–

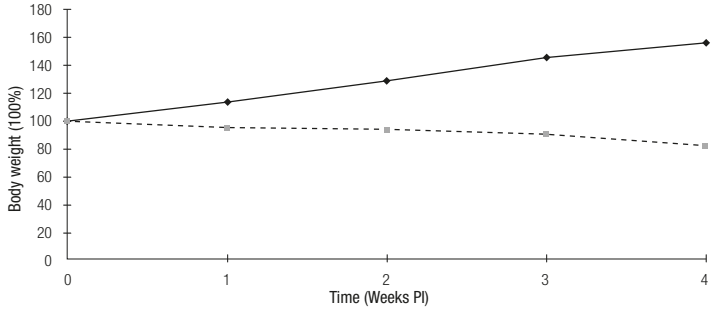

Figure 4: Body weight changes of rabbits. Rabbits in G2 were intranasally inoculated with 1.0×10^6 colony forming units of FZHW001 diluted in 100 µL sterile phosphate buffer saline (PBS); Rabbits in G3 were intranasally inoculated with 100 µL sterile PBS. G2: —■— ; G3: -◆- .

called FZHW001, isolated from a rabbit farm with severe respiratory disease. The ST of the isolate FZHW001 (ST121) was different from the strains that we had isolated previously (all of them were typed as ST398) and the infection of FZHW001 resulted in high mortality, indicating it as a strain of clinical importance. Interestingly, the symptoms of FZHW001 infected rabbits were identical to those of naturally infected rabbits. Moreover, the isolate FZHW001 could spread from infected rabbits to naïve ones. Finally, the strain could break through the barriers between organs and be replicated in liver and spleen.

Possessing a number of virulence factors is the most prominent feature of *S. aureus*, and the virulence factors facilitate the colonisation, dissemination and transmission of the bacterium (Edwards *et al.,* 2010). *S. aureus* strains isolated from rabbits have variable combinations of virulence factors (Viana *et al.,* 2015). The isolate FZHW001 carries a panel of virulence factors including *nuc*, *pvl*, *hla*, *hlb*, *clfA*, *clfB* and *fnbpA*, which were thought to contribute to the pathogenicity of the bacterium. It was revealed that the panton-valentine leukocidin (PVL) is related to necrotising pneumonia (Lina *et al.,* 1999) and infection of both PVL-positive MSSA and methicillin-resistant *S. aureus* (MRSA) could result in necrotising pneumonia (Sicot *et al.,* 2013). The FZHW001 is a PVL-positive MSSA, the PVL might contribute to the severe respiratory diseases of infected rabbits. It was reported that the dissemination of *S. aureus in vivo* is mediated by the interaction of fibronectin-binding protein A with host cell receptors (Que *et al.,* 2005; Edwards *et al.,* 2010). The isolate FZHW001 harboured the *fnbpA* gene. However, further studies are still needed to understand the transmission and dissemination of FZHW001 in rabbits.

In recent years, the MRSA was highly concerned for the threats to public health (Robinson *et al.,* 2004). Fortunately, the FZHW001 was a MSSA and resistant to only 2 of 12 tested antibiotics. The relatively high sensitivity of FZHW001 to antibiotics is likely because the rabbit is a monogastric herbivore (Crowley *et al.,* 2017). The abuse of antibiotics in rabbit may result in disorder of gastrointestinal microbiota.

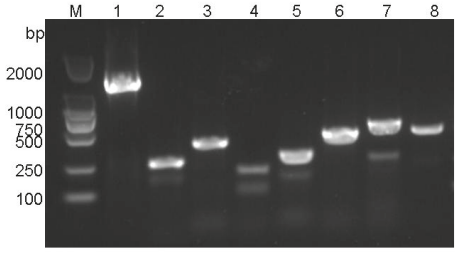

Figure 5: Gel electrophoresis of genes of the isolate FZHW001. M: DL-2000 DNA marker; 1: *16S rRNA* (1549 bp); 2: *nuc* (279 bp); 3: *pvl* (433 bp); 4: *hla* (209 bp); 5: *hlb* (309 bp); 6: *clfA* (500 bp); 7: *clfB* (596 bp); 8: *fnbpA* (523 bp).

The isolate FZHW001 was assigned into CC121, in which the isolate was the predicted ancestor. Strains in CC121 are globally distributed, causing infections both in humans and animals (Argudin *et al.,* 2013; Gomez-Sanz *et al.,* 2013; Merz *et al.,* 2016; Doudoulakakis *et al.,* 2017). Generally, strains in CC121 are MSSA (Kurt *et al.,* 2013; Moreno-Grúa *et al.,* 2018), although one report showed that MRSA had been found in CC121 (Kurt *et al.,* 2013). With the rapid development of rabbit farming in Fujian Province in south-eastern China, operators should be alert to the prevalence of the ST121 strain in rabbit populations, which causes high mortality.

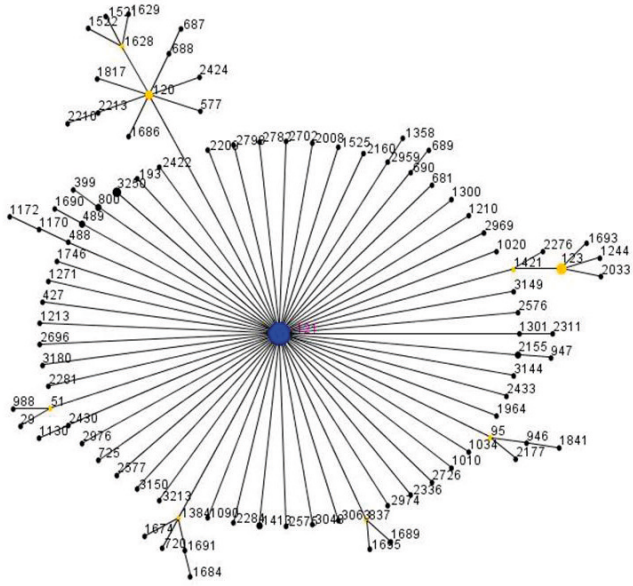

Figure 6: Population snapshot of the isolate FZHW001. The isolate FZHW001 is the putative founder and coloured blue.

This study has contributed to a better understanding the pathogenic mechanisms of high-virulence *S. aureus* in rabbits and proving the methods to control the epidemic of high-virulence *S. aureus* in rabbits.

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