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Role of Methicillin Resistant *Staphylococcus aureus* in rabbits infections

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ABSTRACT

Staphylococcus aureus is a pathogen capable of infecting humans and a wide variety of animals.

A total of 150 samples were collected from 110 diseased and 40 dead rabbits from private farms including (50 abscess swabs, 20 liver samples, 20 lung samples, 30 infected wound swabs and 30 mastitic milk samples) for bacteriological and molecular examinations. *S. aureus* was isolated from 27 cases with a percentage of 18 %, including 7 isolates resistant to methicillin (MRSA) with a percentage of 4.7 %and 20 isolates were sensitive to methicillin with a percentage of 13.3%.

Sixty rabbits were used as laboratory animal models in an experiment designed to study the pathogenicity of S. aureus. rabbits were monitored daily for development of clinical signs in the form of abscess formation, conjunctivitis, keratitis, arthritis, cough, weight loss or lethargy.

MRSA experimentally injected strain could be recovered and confirmed bacteriologically and molecularly form all infected group rabbits.

All *S. aureus* strains were positive for amplification of 756 base fragments specific for 16S rRNA of *S. aureus* using Staph756 F and Staph750 R primers, while 7 MRSA strains showed positive amplification of 450 base pair fragments specific for SCC*mec* subtype IVa genes using SCC*mec* 4a1 and SCC*mec* 4a2 primers.

Four Rep-PCR primers were used for estimating of genetic diversity of MRS A isolates (Rep-1, Rep-2, Rep-12and Rep-18). The four Rep-PCR primers produced about 55 fragments,26 of them consider as monomorphic bands with about 47.5% and other 29 fragments consider as polymorphic bands with about 52.5%.

INTRODUCTION

Staphylococcus aureus is a pathogen capable of infecting humans and a wide variety of animals. This bacterium affects rabbits of different ages, infects dermal lesions and invades subcutaneous tissues , resulting in different pathologies including suppurative dermatitis, mastitis, multisystemic abs cessation and pod

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dermatitis. The ability of *S. aureus* to cause disease is due to a combination of virulence factors. Bacteria are considered major risk factors for diseases of respiratory system , skin , the eye and other septicemia in rabbits. Bacterial infection following ulceration of paws and urine burns was the likely cause to Sore hocks/ chronic ulcerative pododermatitis and arthritis and osteomyelitis **(Jawad et al. 2018).**

Methicillin-resistant *S. aureus* (MRSA) are resistant to all currently available β-lactam antibiotics, including penicillins, cephalosporins, carbapenems, and their derivatives. Resistance to methicillin is mediated by the *mec*A gene which encodes an altered penicillin binding protein, located in the cell wall that has a low affinity for β-lactam antibiotics. The *mec*A gene resides on a large heterogeneous mobile genetic element called the staphylococcal cassette chromosome (SCC) **(Katayama and Hiramatsu, 2000).** This *mec*A encodes for an altered penicillin-binding protein (PBP2a or PBP20). The PBP2a has a lower affinity for beta-lactam antimicrobials than the normal PBP so antimicrobials are ineffective (**Arias and Murray, 2009**) This SCC containing the *mec*A gene can spread horizontally between staphylococcal populations **(Brody et al. 2009)**.

The incidence of pathogenic microorganisms that developed resistance to commonly used antibiotics has become a 21stcentury global issue. The microorganisms of key importance are, above all, those of the Staphylococcus aureus species, especially methicillin resistant strains–MRSA **(Šťástková et al. 2009).**

Methicillin-resistant Staphylococcus aureus (MRSA) is a critically important human pathogen that is also an emerging concern in veterinary medicine and animal agriculture **(Weese, 2010).**

MRSA detection has been reported in cattle, horses, poultry **(Lee, 2003)** and pigs **(Voss et al. 2005).** It has also been found in pet animals such as dogs and cats **(Dequette and Nuttall, 2004**) as well as in some exotic animals **(O'Mahony et al. 2005).**

Several detection methods based on phenotypic expression of the mecA gene have been evaluated for diagnosing MRSA including antimicrobial susceptibility testing, PBP2a latex agglutination test kit, oxacillin agar screen test, and cefoxitin disk diffusion test **(Diab et al. 2008).** While these tests showed reasonable sensitivity and specificity for detecting MRSA when used in combination with either the tube coagulase test or a commercial biochemical typing system to presumptively identify staphylococci to the species level, these tests rely on phenotypic expression of the *mecA* gene. Molecular detection of the *mecA* gene using polymerase chain reaction (PCR) is considered the gold standard for making a definitive diagnosis of methicillin resistance *S. aureus* **(Counts et al. 2007** and **Francois et al. 2008).**

The genetic variability and relationships among the isolates were established by random amplified polymorphic DNA(RAPD)-PCR analysis **(Salgado-Ruiz et al. 2015).** Repetitive element palindromic-PCR (Rep-PCR) has been identified as a simple PCR-based technique with the following characteristics: (i) low cost, (ii) high discriminatory power, (iii) suitable for a high throughput of strains, and (iv) considered to be a trusty tool for classifying and typing a wide range of Gram-negative and several Gram positive bacteria **(Abdollahi et al. 2016).**

This work designed to study:

1-The incidence of Methicillin-resistant S. *aureus* (MRSA) in diseased rabbits tissue samples and to evaluate the accuracy and the sensitivity of duplex PCR in the diagnosis.

2-Confermation of MRSA strain virulence through experimental design.

3-Detection of diversity of MRSA by repetitive sequence polymerase chain reaction (Rep-PCR).

MATERIALS AND METHODS:

Sample collection:

A total of 150 samples were collected from diseased and dead rabbits from private farms including 50 abscess swabs, 20 liver samples, 20 lung samples, 30 infected wound swabs and 30 mastitis milk samples as shown in table (1).

Table (1) Numbers and types of the collected samples:

Source (No)	Sample (No)
Dead rabbits (40)	Liver (20) Lung (20)
Diseased rabbits (110)	abscess swab (50) infected wound swab (30) mastitic milk (30)
Total (150)	(150)

Bacteriological examination:

It was carried out following standard methods **(Quinn et al. 1994) and (Woo Yong-Ku and Kim Shin 2005).** Briefly, a loopfulof each sample was streaked onto 7% sheep blood agar (Merck). Staphylococci and Micrococci were identified based on their growth characteristics on mannitol salt agar, coagulase production by using Staphylect plus reagent (Oxoid), catalase and oxidase tests.

Oxacillin Screening Agar test (Detection of MRSA strains):

Test was performed according to **Jain et al. (2008)** with some modification. Briefly, plates were prepared with Mueller–Hinton agar supplemented with 4% (w/v) NaCl containing oxacillin at a concentration of 6µg ml-1 (OSA 6µg ml-1). The plates were spot inoculated with a cotton swab dipped into a 0.5 McFarland standard suspension of each isolate, Oxacillin resistance was confirmed by bacterial growth after 24 h incubation at 35ºC aerobically.

Experimental design:

In this study 60 rabbits (Three months old white Newzeland rabbits), with body weight ranged between (2000- 2500gm.) were used. The animals were kept in cages of (20*30*50) cm^3 dimensions one rabbit per each cage, one month before study for acclimatization in optimum conditions of breeding at (22±3) ºC with a (14/10) hours (Light/Dark) cycle.Commercial feed pellets and drinking water were given all the time of experiment.

Inoculum:

S. aureus (MRSA) strain used in this research isolated from mastitic case.

Sixty rabbits were used as laboratory animal models in an experiment designed to study the pathogenicity of S. aureus and divided randomly into infected group (n=30) with a dose of 1 x 10^7 CFU/ml of MRSA *S.aureus* subcutaneously (S/C), and control group (n=30) with sterile distilled water was given by same route.

Clinical assessment:

After inoculation, rabbits were monitored daily for development of clinical signs in the form of abscess formation, conjunctivitis, keratitis, arthritis, cough, weight loss or lethargy

Euthanasia:

On day 15 after inoculation (the end point of the study), rabbits were euthanized and re-isolation of (MRSA) strain was performed and confirmed by bacteriological and molecular methods.

Staphylococcus aureus culture for DNA extraction:

Bacterial growth for the purpose of DNA extraction was prepared as follows: 20 µl of stock solution was streaked onto a Brain Heart Infusion (BHI) agar plate (prepared as specified, Oxoid Australia Pty Ltd, Adelaide) and cultured overnight at 37ºC. the following day a single colony was selected and suspended in a 5 ml falcon tube (Becton Dickinson, New South Wales, Australia) containing BHI broth (prepared as specific, Oxoid Australia Pty Ltd, Adelaide) and cultured overnight at 37ºC, with

shaking.

DNA Extraction:

According to Stephens (2008), from the overnight BHI broth culture, 1 ml was extracted using the Qiagen DNA extraction Kit (Qiagen, Victoria, Australia), as per manufacturer's instructions, including lysostaphin at 200 µg/ml for the lysis step. Purified DNA samples were eluted using dd H_2O and stored at -20ºC.

Quantification of DNA:

Concentration and purity of DNA samples from the Qiagen DNA extraction kit were determined by measuring the absorbance at 260 and 280 nm using an Bio-Rad biophotometer with each DNA sample diluted 1/100.

DNA amplification and Analysis:

Polymerase Chain Reaction (PCR)

Standard PCR amplifications were performed using a Bio-Rad Research Thermocycler in 0.2 ml PCR tubes.

Multiplex Polymerase Chain Reaction:

Multiplex Polymerase Chain Reaction for detection of *Staphylococcus aureus* species specific 16S rRNA and (SCCmec) type IV genes (responsible for methicillin resistance): Two sets of primer pairs were used, the first one was Staph756F (5'-AACTCTGTTATTAGGGAAGAAC-3') and Staph750R (5'-CCACCTTCCTCCGGTTTGTCACC-3') primers which could amplify 756 base pair fragments specific for 16S rRNA of *S. aureus*; the second one was SCC*mec* 4a1(5'-TTTAATGCCCATGAATAAAAT-3') and SCC*mec* 4a2 (5'-AGAAAAGATAGAAGTTCGAAAGA-3') primers which could amplify 450 base pair fragments specific for SCC*mec* subtype IVa gene according to **Ryffel et al. (1990).**

The reaction mixtures consisted of 5 µl of the extracted DNA template of the bacterial isolates, 5 µl 10× PCR buffer (75 mMTris-HCl, pH 9.0, 2 mM $MgCl_2$, 50 mMKCl, 20 mM (NH_4) $2SO_4$), 1 µl dNTPs (40 µM), 1 µl (1U AmpliTaq DNA polymerase), 1 µl (50 pmol) from the forward and reverse primers. The two sets of primer pairs were used in each reaction mixture and the volume of the reaction mixture was completed to 50 µl using DDW. 40 µl paraffin oil was added and the thermal cycler was adjusted as follows: 94°C for 5 min, followed by 10 cycles of 94°C for 1 min, 55°C for 1 min., and 72°C for 1.5 min, and 25 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 1.5 min, followed by final extension at 72° C for 1.5 min, and the PCR products were stored in the thermal cycler at 4°C until they were collected.

PCR product were stained with ethidium bromide and visualized on 1.5% agarose gel with a UV light transilluminator.

Rep-PCR analysis:

For repetitive sequence analysis, PCR conditions for Staphylococcus isolates in the present investigations were standardized. Four repetitive sequence primers (Rep-1, Rep-2, Rep-12and Rep-18) were used to amplify genomic DNA of the *S. aureus*isolates according to (**Woo Yong-Ku and Kim Shin 2005).** Following the experiments for optimization of component concentrations, PCR amplification of repetitive sequence primers were carried out in 25 µl volume containing 1 µl (20 ng) of genomic DNA, 12.5 µl of Go Taq® Green Master Mix, Promega, USA. 1 µl of primer (20 p.mol), deionizer D distilled water (up to a total volume of 25 µl). For DNA amplification, the C1000TM Thermo Cycler Bio-Rad, Germany, was programmed under the conditions involving denaturation at 94°C for 5 min; 30 cycles of denaturation at 94°C for 1 min, primer annealing at 52°C for 45 Sec and primer extension at 72°C for 2.5 min; final extension step at 72°C for 10 min. Data analysis In order to determine the genetic relationship among studied bacteria, Rep-PCR data were scored for presence (1) or absence (0) of the bands using Gene Tools software from Syngene. A simple matching coefficient was estimated by means of the Jaccard's coefficient to construct a similarity matrix. Cluster analysis and dendrogram were produced on the basis of the un weighted average pair group method (UPGMA) using the NTSYS-PC Statistical Package (Rohlf, 2000. Control marker with molecular mass of 100 bp was used (fermentase, Lithuania).

RESULTS

Bacteriological findings:

S. aureus was isolated from 27 cases with a percentage of 18 % , including 7 isolates resistant to methicillin with a percentage of4.7% and 20 isolates were sensitive to methicillin (MSSA) with a percentage of13.3%, both were differentiated by the Oxacillin Screening Agar test.

Table (2) Occurrence of MSSA and MRSA in examined samples:

Source (No)	Sample (No)	MSSA	MRSA
Dead rabbits (40)	Liver (20)	2	1
	Lung (20)	2	-
Diseased rabbits (110)	abscess swab (50)	8	2
	infected wound swab (30)	4	2
	mastitic milk (30)	4	2
Total (150)	(150)	20	7

Experimental infection observations

- Clinical Findings were monitored daily for development of clinical signs on the experimentally infected rabbits as well as control group.

-Control rabbits had no clinical signs, where as infected rabbits displayed abscess formation, conjunctivitis, keratitis, arthritis, cough, weight loss, lethargy and weight loss with different degrees as shown in table (3).

Figure (1): experimentally infected rabbit showing conjunctivitis, keratitis and facial abscess.

Table (3) Clinical manifestations developed in experimentally infected rabbits:

Group	Development of clinical signs /days of infection			
	3rd day	7th day	10th day	15th day
Infected	Lethargy (15)*	Lethargy (25)* Abscess(10)* Conjunctivitis (3)*	Lethargy (30)* Abscess(15)* Conjunctivitis (10)* Keratitis (5)*	Lethargy (30)* Abscess(20)* Conjunctivitis (15)* Keratitis (10)*
Control	-	-	-	-

*=N^{o} of rabbits affected
-Weight loss was noticed in all rabbits of the infected group.
-MASA experimentally injected strain could be recovered and confirmed bacteriologic ally and molecularly form all infected group rabbits.

Multiplex Polymerase Chain Reaction results:

Multiplex PCR for detection of *S. aureus* species specific 16S rRNA and (SCC*mec*) type IVa genes were performed.

Strains previously identified phenotypic ally as *S. aureus* with bacteriological examination were used in PCR run. All strains are positive for amplification of 756 base fragments specific for 16S rRNA of *S. aureus* using Staph756 F and Staph 750 R primers, while 7 strains (known from antimicrobial sensitivity assay as methecillin resistant) showed positive amplification of 450 base pair fragments specific for SCC*mec* subtype IVa genes using SCC*mec* 4a1 and SCC*mec* 4a2 primers, as shown in Figure (2).

Figure (2). Agarose gel electrophoresis showing:
Lane M :100bp ladder.
Lanes 1, 2, 3, 4, 5, 6, 7 and 8 showing amplification of 756 bp fragments of 16S rRNA.
Lanes 1, 2, 3, 4, 5, 6 and 7 showing amplification of 450 bp fragments of SCC *mec*IVa gene.
+ve :control positive.
-ve :control negative.

Rep-PCR analysis:

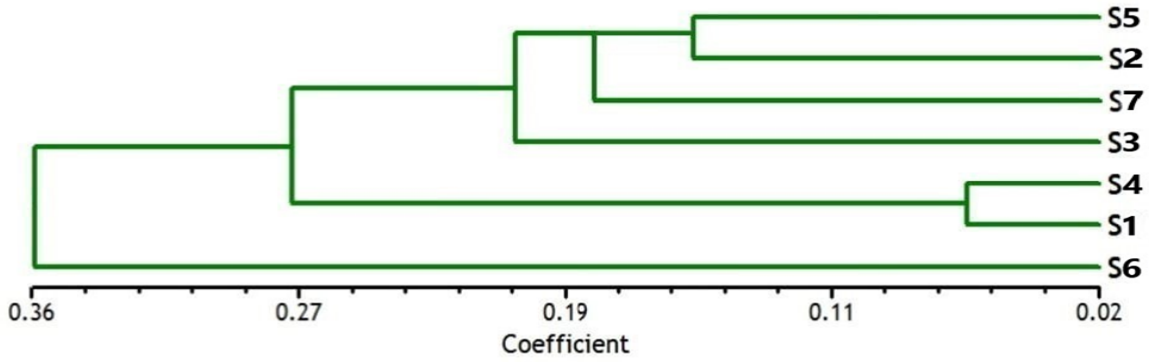

Figure (3): Rep-PCR profile of 7 (MRSA) isolates generated with 4 repetitive sequence primers. First lane on each panel is 100 bp molecular weight markers.

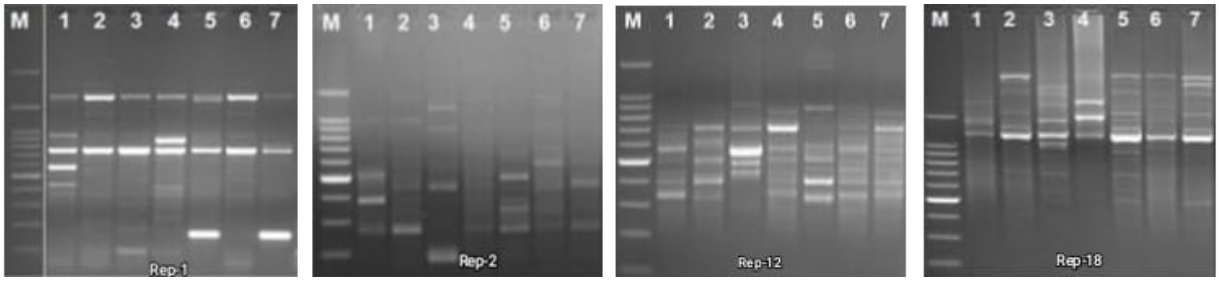

Figure (4): Dendrogram analysis among the 7 MRSA based on the 4 repetitive sequence primers.

Table (4): Polymorphic bands of Rep-PCR primers and percentage of polymorphism in Staph lococcus aureus strains isolated from rabbits:

Primers	Total Bands	No. of Monomorphic Bands	No. Polymorphic Bands	% Monomorphic bands	% Polymorphic bands
Rep-1	13	7	6	53.8	46.2
Rep-2	14	5	9	35.7	64.3
Rep-12	13	7	6	53.8	46.2
Rep-18	15	7	8	46.7	53.3
Total	55	26	29	47.5	52.5

DISCUSSION:

Staphylococcus aureus is a pathogen capable of infecting humans and a wide variety of animals. Commonly rabbits affected by this bacterium resulting in different pathologies including suppurative dermatitis, mastitis, multisystemicabscessation and pododermatitis .

Bacteriological examination of 150 samples collected from diseased and dead rabbits (table (1)). Results revealed the isolation of *Staphylococcus aureus* from 18% of examined samples. (MRSA) present at a percentage of4.7% while (MSSA) at a percentage of 13.3% of total *S. aureus* isolates as shown in table (2). This percentage considered high and because of the organisms potensity to acquire antimicrobial resistance, whereas most infections can be treated or prophylacted with antibiotics; antimicrobial resistance of *S. aureus* especially methicillin resistant *S. aureus* (MRSA) contin-

ues to be a problem for clinicians worldwide (**Shittu and Lin 2006) .**

Experimentally injected rabbit group showed clinical signs of infection including lethargy, weight loss, abscess formation. Conjunctivitis and Keratitis progressed along the period of the study as mentioned in table (3).

The body weight along the period of the study which showed a significant decrease ($P<0.05$)was due to systemic reaction against the bacterial infection and it toxins on enteralorgans which confirmed by positive isolation of bacteria and the effect of bacterial toxin on the digestive system which effect the absorption of food from intestine **(Jawad et al. 2018).**

MRSA experimentally injected strain could be recovered and confirmed bacteriologically and molecularly form all infected group rabbits.

To determine susceptibility to methicillin, following CLSI recommendations, oxacillin was used, as it is more stable thanmethicillin in the lab conditions and is able to recognize cross-resistance. In addition, agar screen method was used which is preferable to the disk diffusion method **CLSI (2021)**.

The results confirmed the conclusion of **Riffon *et al.* (2001)**. Because PCR could detect the *mecA* gene in all 7 strains out of 8 examined strains which appeared methicillin / oxacillin resistant phenotypicaly with Oxacillin screening agar test. Also, **Štastkova et al. (2009)** found that 23 isolates of *S.aureus* were identified phenotypic ally and genotypic ally as resistant to methicillin. And The*mec*A gene was confirmed in all of these MRSA isolates.

All 27*S. Aureus* isolates identified by conventional tests were confirmed as *S. aureus* genotypically by PCR. While 7 of 27 (4.7%) *S. aureus* isolates were found to be methicillin resistant (MRSA) in PCR. This may be explained as conventional susceptibility tests such as agar disc diffusion and broth dilution methods may not give reliable results in detecting MRSA because of heterogenic expression of resistance. Also, the authers classified the *S. aureus* lack *mec*A gene and resist oxacillin as false resistant by the oxacillin disc diffusion method and the authers considered that it may due to another resistance mechanism such as hyper production of beta-lactamase. The discrepancy between results may be attributed to the differences in method of methicillin resistance detection, we use oxacillin screening agar test which more efficacy in detection of resistance.

Rep-PCR is a new typing method that differentiates microbes by using primers complementary to interspersed repetitive consensus sequences that enable amplification ofdiverse-sized DNA fragments consisting of sequences between the repetitive elements, the technique utilizes repetitive sequence oligonucleotides, is particularly a powerful tool for genetic studies and it is useful as ascreening genotyping method and can generate various fingerprint patterns with unlimited number of fragments (**Woo Yong-Ku and Kim Shin 2005).**

Four Rep-PCR primers were used for estimating of genetic diversity of MRSA isolates. Reactions were performed in duplicate and all amplification products were found to be reproducible (Fig. 3 and Table 4).

Results using primer Rep-1 has showed a total of 13 bands sized ranged from 150-2800 bp long in all seven MRSA isolates. Seven common bands were observed in all isolates which exhibited about 53.8 % monomorphism, while the other 6 fragments have showed 46.2% polymorphism (Table 4).

In case of (Rep-12) primer, a total of 6 fragments have showed 46.2% polymorphismamong the 7MRSA isolates (Figure 3). The molecular size of the amplicon products ranged from 150-2300 bp long. Also, this primer recognized different unique fragments at 2200 bp specific to isolate S4 and S7, respectively.

In case of (Rep-2 and Rep-18) primers, about 35.7 % and 46.7% monomorphism, respectively. while 64.3% and 53.3% polymorphism, respectively.

The four primers reduced about 55 fragments, 26 of them consider as monomorphic bands with about 47.5% and other 29 fragments consider as polymorphic bands with about 52.5%. According to genetic similarity and interspecies differentiation, the seven MRSA isolates were grouped into two main different clusters with about 75% genetic similarity. S6 isolate was found to be alone in the first cluster. while, the second cluster was divided to two sub-clusters. The first sub-cluster contained S4 and S1 only, while S2, S3, S5 andS7 were grouped in the second sub-cluster (Figure 4).

Rep-PCR technique was proved to be useful genetic markers used for fingerprinting of S. aureus strains isolated from clinical and subclinical cases. Although major bands from Rep -PCR reactions are highly reproducible, minor bands can difficult to repeat due to repetitive sequence priming nature of this PCR reaction and potential confounding effects associated with co-migration with other markers (**Woo Yong-Ku and Kim Shin 2005).** Because AP-PCR has its limitations for widespread use, another more reproducible PCR method should be considered. The Rep-PCR has been described for the molecular genotyping of S. aureus **(Alharthi et al. 2016).** It also generates strain-specific DNA fragments when *S.aureus* DNA is used as an amplification template **(Hassan et al. 2014).**

CONCLUSSION:

It was concluded that, MRSA infection leads to great economic losses in rabbit industry due to high mortalities and treatment costs.

Multiplex PCR have the same sensitivity and accuracy as isolation and identification of *S. aureus* methicillin resistance, but with less labor and very little time in comparing with the traditional method of isolation and identification. While the PCR is more expensive than traditional methods, but rapid diagnosis of MRSA in rabbit farms help in rapid treatment and rapid cure which means money save.

The multiple primer sets in Rep-PCR analysis can be used as a rapid method for preliminary biotyping of *S. aureus* strains.

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