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## Pathogenic characteristics of an unencapsulated *Streptococcus suis* serotype 31 strain isolated from a patient in Thailand

Parichart Boueroy<sup>1</sup>✉, Nattamol Phetburom<sup>1</sup>, Sorujsiri Chareonsudjai<sup>2,3</sup>,  
Peechanika Chopjitt<sup>1</sup>, Rujirat Hatrongjit<sup>4</sup>, Servane Payen<sup>5</sup>, Mariela Segura<sup>5</sup>,  
Marcelo Gottschalk<sup>5</sup> & Anusak Kerdsin<sup>1</sup>

*Streptococcus suis* is a zoonotic pathogen that causes invasive infections in individuals who are in close contact with infected pigs or contaminated pork-derived products. An unencapsulated *S. suis* serotype 31 strain 43640 (sequence type 221) was first isolated from a human in Thailand in 2015; however, the mechanisms underlying host-cell interactions and virulence in animal models of this serotype are still limited. This study aimed to determine the virulence of the human unencapsulated *S. suis* serotype 31 strain 43640 using *in vitro* (A549 epithelial cells) and *in vivo* (C57BL/6 mouse) models. The adhesion rate of the unencapsulated strain 43640 to A549 cells was significantly lower than that of the highly pathogenic *S. suis* serotype 2 strain P1/7 after infection at multiplicity of infection (MOI) 1 ( $p < 0.01$ ) and MOI 10 ( $p < 0.05$ ). The invasion of epithelial cells by *S. suis* serotype 31 strain 43640 was significantly higher than that of serotype 2 strain P1/7 and the reference strain 92-4172 4 h after infection. *S. suis* serotypes 31 and 2 significantly induced cytotoxicity in epithelial cells by more than 50% and 90%, respectively, after 4 h and 18 h of infection. *S. suis* serotype 31 strain 43640 induced apoptosis in A549 cells via a caspase 9-dependent pathway. Using an *in vivo* mouse model, we demonstrated that *S. suis* serotype 31 strain 43640 induced 13.33% and 46.67% mortality due to septicemia after 24 h and 48 h, respectively. Bacterial counts in the blood of mice infected with strain 43640 were significantly lower than those in mice infected with the reference strain 12 h and 24 h after infection ( $p < 0.05$ ). This study provides information on serotype 31-ST221 virulence in *in vitro* and *in vivo* models and proves that ST221 (CC221/234), an emerging human *S. suis* clone in Thailand, is potentially virulent.

**Keywords** *Streptococcus suis* serotype 31, Virulence, Cytotoxicity, Apoptosis, Survival rate

*Streptococcus suis* (*S. suis*) is a zoonotic pathogen that affects individuals in close contact with infected pigs or pork-derived products<sup>1</sup>. Although *S. suis* infection in humans is an occupational disease in industrialized countries, it is more commonly found to be a foodborne infection in Southeast Asia, mainly in Thailand and Vietnam, and the proportion of patients with occupational exposure is lower than that in Europe. The consumption of meals containing raw pork meat, blood, and other related products poses the main risk of transmission of *S. suis* infection in these countries<sup>2</sup>. A study in Thailand showed that *S. suis* human infections are responsible for an estimated USD 11.3 million loss in productivity-adjusted life years in the gross domestic product, which is equivalent to a loss of USD 36,033 per person<sup>3</sup>.

Almost all human *S. suis* cases worldwide are caused by strains of either serotype 2 or 14<sup>4</sup>. In Thailand, human cases of serotypes 1, 4, 5, 9, 14, 24, and 31 have been reported<sup>5–9</sup>. Furthermore, serotype 31, which was isolated from a 55-year-old male alcohol misuser with liver cirrhosis in Thailand<sup>8</sup>, was unexpectedly unencapsulated, and sequence type (ST) 221 belonged to the clonal complex (CC) 221/234<sup>8</sup>. Notably, ST 221 is found exclusively in Thailand<sup>7,8,10–12</sup>. In a previous study, two human cases of *S. suis* serotype 24 ST 221, which belonged to CC

<sup>1</sup>Faculty of Public Health, Kasetsart University Chalermphrakiat Sakon Nakhon Province Campus, Sakon Nakhon, Thailand. <sup>2</sup>Department of Microbiology, Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand. <sup>3</sup>Research and Diagnostic Center for Emerging Infectious Diseases (RCEID), Khon Kaen University, Khon Kaen, Thailand.

<sup>4</sup>Department of General Sciences, Faculty of Science and Engineering, Kasetsart University Chalermphrakiat Sakon Nakhon Province Campus, Sakon Nakhon, Thailand. <sup>5</sup>GREMIP, Faculty of Veterinary Medicine, University of Montreal, Quebec, Canada. ✉email: parichart.bou@ku.th

221/234, were reported<sup>10</sup>. To date, seven cases of human infection with *S. suis* CC221/234 strains have been documented. Of these, five were associated with serotype 24, whereas the remaining two involved serotypes 5 and 31<sup>7,8,10–12</sup>. In addition, several studies on pigs in Thailand and other countries have revealed the isolation of *S. suis* serotype 31 strains from diseased pigs<sup>13–17</sup>. The prevalence of *S. suis* serotype 31 in diseased pigs in the Czech Republic was 3.4% between 2018 and 2022<sup>18</sup>. Another study suggested that the prevalence of serotype 31 strains isolated from diseased pigs in Québec, Canada, was 1.1% and 0.5% in 2009 and 2010, respectively<sup>19</sup>. Most of these show multidrug resistance<sup>13,16,17,20</sup>. Interestingly, 31 *S. suis* serotype strains isolated from one human and 17 clinically asymptomatic pigs in Thailand were multidrug-resistant and resistant to azithromycin (100%; 18/18) and tetracycline (100%; 18/18). Notably, 10 (55.56%) of the serotype 31 strains were resistant to penicillin<sup>12</sup>. Apart from a few epidemiological reports, knowledge regarding the virulence and pathogenesis of *S. suis* serotype 31 remains limited and requires further investigation. Several studies on *S. suis* virulence and pathogenesis have been conducted using serotype 2 strains<sup>21–23</sup>. Knowledge regarding the mechanisms of virulence, including adhesion, invasion, and destruction of host cells, as well as animal models of non-serotype 2 strains, remain limited. In the case of serotype 31, only one study from China has revealed that a porcine *S. suis* serotype 31 (strain 11LB5) was virulent in a mouse model<sup>24</sup>. In this study, we investigated the pathogenic characteristics of the human unencapsulated *S. suis* serotype 31 ST221 strain using in vitro and in vivo models. Our findings will help us understand the mechanisms underlying *S. suis* serotype 31 virulence.

## Materials and methods

### Cell line and bacterial strain

Human lung adenocarcinoma cell line (A549) (ATCC, CCL-185) was purchased from the American Type Culture Collection (Rockville, MD, USA). A549 cells were cultured in Roswell Park Memorial Institute-1640 medium (Gibco Life Technologies Corporation, Grand Island, NY, USA). Culture media was supplemented with 10% fetal bovine serum, 100 units/mL of penicillin, and 100 µg/mL streptomycin (Gibco) and incubated at 37 °C in a humidified atmosphere of 5% CO<sub>2</sub> until confluence was reached.

The unencapsulated *S. suis* serotype 31 ST221 strain 43640<sup>8</sup>, belonging to CC221/234 and isolated from a human patient in Thailand, was used to investigate virulence in the current study (accession number SAMN46861206). Highly pathogenic *S. suis* serotype 2 ST1, belonging to the CC1 strain P1/7 isolated from pigs, was used as a positive control for comparison with serotype 31 strain 43640 in in vitro studies (accession number SAMEA2298930). *S. suis* serotype 31 ST70 reference strain 92-4172, isolated from pigs, was used as a control in the in vitro and in vivo studies for comparison (accession number AB737835).

### Biofilm formation assay

Biofilm formation by *S. suis* strain 43640, reference strain 92-4172, and P1/7 strains was assessed using Congo red agar (CRA) assay as described previously<sup>25</sup>. Briefly, bacterial strains were cultured on CRA made of brain-heart infusion agar (supplemented with 36 g/L sucrose and Congo red dye 0.8 g/L), incubated, and observed at 24, 48, and 72 h. Biofilm production was characterized based on six color tones of colonies as follows: very black, black, and almost black, which were interpreted as strong, moderate, and weak biofilm producers, respectively. Non-biofilm production was shown on three color tones of colonies as follows: Bordeaux, red, and very red<sup>25</sup>.

To confirm biofilm formation, a crystal violet assay was performed as described elsewhere<sup>26</sup> with some modifications. Briefly, *S. suis* serotype 31 strain 43640, reference strain 92-4172, and serotype 2 strains were cultured on sheep blood agar at 37 °C. A single colony of the *S. suis* strain was diluted to an optical density (OD)<sub>600</sub> of 0.1 in brain heart infusion (BHI) broth (HiMedia, India) and incubated in a 96-well plate (Thermo Scientific, Roskilde, Denmark) for 24, 48, and 72 h at 37 °C. Non-adhering bacteria were removed, aspirated, and washed thrice with phosphate buffer saline (PBS; pH 7.4). The samples were then stained with 0.1% crystal violet for 10 min. Next, the wells were washed thrice with PBS and aspirated. Subsequently, 200 µL of 33% (v/v) glacial acetic acid was added and incubated for 15 min. Biofilm levels were determined using OD<sub>595</sub>. The ODs of these isolates were determined as the mean absorbance and compared with that of the negative control (OD<sub>nc</sub>). Biofilm production was characterized based on the categories of in vitro biofilm formation: no biofilm production (ODs ≤ OD<sub>nc</sub>), weak biofilm production (OD<sub>nc</sub> < ODs ≤ 2 OD<sub>nc</sub>), moderate biofilm production (2 OD<sub>nc</sub> < ODs ≤ 4 OD<sub>nc</sub>), and strong biofilm production (4 OD<sub>nc</sub> < ODs).

### Cell cytotoxicity assay

The cytotoxic effects of *S. suis* serotype 31 (strain 43640) and the highly pathogenic *S. suis* serotype 2 strain P1/7 were examined<sup>27</sup>. Briefly, the A549 cells were infected with either multiplicity of infection (MOI) 1 (~1 × 10<sup>4</sup> colony-forming units (CFU)/mL), 10 (~1 × 10<sup>5</sup> CFU/mL), or 100 (~1 × 10<sup>6</sup> CFU/mL) in culture medium for 2, 4, or 18 h, and subsequently, the effect of *S. suis* serotype 31 strain 43640 and serotype 2 strain P1/7 infection on A549 cells was determined using the cell counting kit-8 (CCK-8) assay (Dojindo, Kumamoto, Japan).

### Adhesion assay

The adhesion of unencapsulated *S. suis* serotype 31 strain 43640, reference strain 92-4172, and serotype 2 strain P1/7 to human lung epithelial (A549) cells was examined<sup>27</sup>. One milliliter of A549 cells was seeded at the density of 1 × 10<sup>4</sup> cells/well in 6-well tissue culture plates (Corning Incorporated, Corning, NY, USA) and incubated until confluence was reached. The mid-log phase culture of bacteria was centrifuged at 2,000 rpm for 5 min, washed twice using 1× PBS, pH 7.5, resuspended in complete media, and co-cultured with A549 cells at an MOI of 1 (1 × 10<sup>4</sup> CFU/mL) or 10 (1 × 10<sup>5</sup> CFU/mL) for 2 h. Non-adherent cells were removed by washing five times with 1× PBS. Bacterial adherence and internalization were assessed after lysing with 0.1% Triton X-100 (Merck, Millipore, MA, USA) for 3 min with agitation at 125 rpm. The number of CFU was determined using the spread plate technique on BHI agar (Becton Dickinson and Company, Le Pont de Claix, France).

### Giems staining

Morphology of the A459 cells after infection with the *S. suis* strain 43640, reference strain 92-4172, and P1/7 was observed using a light microscope<sup>27</sup>. Briefly, a sterile coverslip was placed in each well of a 6-well plate before  $1 \times 10^4$  cells/well. Epithelial cells were co-cultured with *S. suis* strains 43640 and P1/7 at an MOI of 1 ( $1 \times 10^4$  CFU/mL) or 10 ( $1 \times 10^5$  CFU/mL) for 2 h. Thereafter, the coverslip from each well was washed five times with 1× PBS. The samples were then fixed with 1 mL methanol (V.S. Chem House, Bangkok, Thailand) for 1 min and stained with Giemsa (Sigma, St Louis, MO, USA) for 3 min. The coverslip was mounted onto a glass slide and viewed under 100× magnification of a light microscope.

### Scanning electron microscopy (SEM)

Morphology of the A459 cells after infection with *S. suis* serotype 31 strain 43640, reference strain 92-4172, and reference strain 2 (P1/7) was observed using SEM<sup>27,28</sup>. A459 cells ( $1 \times 10^5$  cells/well) were seeded in 6-well plates with glass slides covering the wells. The epithelial cells were co-cultured with exponential phase *S. suis* at an MOI of 10 ( $1 \times 10^6$  CFU/mL) for 2 h. The glass slides were washed five times with 1× PBS and fixed overnight with 4% formaldehyde. The slides were dehydrated using an ethanol series (10%, 25%, 30%, 35%, 50%, 70%, 80%, 90%, 95%, and 100%), placed on SEM stubs, and coated with a thin layer of gold-palladium before observation using a JSM-IT200 scanning electron microscope (JSM-IT510 SEM, JEOL, Japan).

### Invasion assay

The invasion of A459 cells by unencapsulated *S. suis* serotype 31 strain 43640, reference strain 92-4172, and serotype 2 strain P1/7 was determined<sup>27</sup>. Monolayers of A459 cells were co-cultured with *S. suis* strains 43640 and P1/7 at MOI of 1 and 10, as described previously for the bacterial adhesion assay. At 2–4 h post-infection, the supernatant and extracellular bacteria were gently aspirated. The co-cultured cells in each well were washed three times and treated with 100 µg/mL gentamicin and 5 µg/mL penicillin G to kill residual extracellular *S. suis* for another 2 h. The cell monolayers were then washed and lysed with 0.1% Triton X-100 (MERCK Millipore, MA, USA) for 3 min with shaking at 125 rpm to remove intracellular bacteria. The intracellular bacteria were quantified using the plate count method on BHI agar plates after 24 h of incubation at 37 °C.

### Apoptosis assay

An annexin V-FITC apoptosis kit (Dojindo, Kumamoto, Japan) was used to detect apoptosis or necrosis of A549 cell after infection with *S. suis* isolates<sup>29</sup>. The A549 cells were infected with *S. suis* strains 43640 and P1/7 at MOI 1 ( $\sim 1 \times 10^5$  CFU/mL) or 10 ( $\sim 1 \times 10^6$  CFU/mL) in culture medium for 2 h. Pelleted cells were stained with annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) for 10–15 min at room temperature in the dark prior to analysis using flow cytometry (BD Biosciences San Jose, CA, USA). Annexin V FITC/PI plots from the gated cells showed the populations corresponding to intact cells and non-apoptotic (annexin V-PI-), early apoptotic cells (annexin V + PI-), late apoptotic cells (annexin V + PI+), and necrotic cells (annexin V-/PI+).

### Assessment of caspase activation using Western blot analysis

Caspase expression was measured using western blot analysis. A549 cells were infected with *S. suis* strains 43640 and P1/7 at an MOI of 1 ( $1 \times 10^5$  CFU/mL) or 10 ( $1 \times 10^6$  CFU/mL). The harvested cells were lysed using lysis buffer. Total protein concentration in the cell lysate was determined using the Bradford assay<sup>30</sup>. Cell lysates were separated using 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The membranes were blocked with 5% non-fat milk in 0.1% Tween 20-PBS at 37 °C for 1 h, followed by incubation with the corresponding primary antibodies against β-actin, caspase 9, and caspase 3 overnight at 4 °C. The membranes were then washed with 0.1% Tween 20 in PBS and incubated with the appropriate secondary antibodies (anti-mouse or anti-rabbit; 1:10,000). The bound secondary antibody-horseradish peroxidase (POD) conjugates were visualized using an enhanced chemiluminescence reagent (Pierce; Thermo Fisher Scientific, Waltham, MA, USA), quantified using densitometry (ImageQuant LAS 4000; GE Healthcare Life Sciences, Chalfont, UK), and analyzed using Scion Image (version 4.0.2; Scion Corporation, Frederick, MD, USA). Data for each protein expressed were normalized to β-actin expression.

### In vivo infection studies

A mouse infection model was used to validate the virulence of *S. suis* serotype 31 reference strain 92-4172 and *S. suis* serotype 31 unencapsulated strain 43640. In total, 30 6-week-old female C57BL/6 mice (Charles Rivers) ( $n=15$  per group) were maintained at 22 °C and exposed to 12 h light/dark cycles with free access to food and water. Mice were intraperitoneally injected with  $1 \times 10^8$  CFU. All mice were observed at least three times daily until 72 h post-infection and twice thereafter until the end of the experiment (7 days post-infection) for the development of clinical signs of sepsis, such as depression, swollen eyes, rough hair coat, prostration, and lethargy. For bacteremia studies, 5 µL of blood were collected from the caudal vein of surviving mice at 12 and 24 h post-infection and proper dilutions were plated on Todd Hewitt broth agar (THA, Becton–Dickinson, Mississauga, ON, Canada).

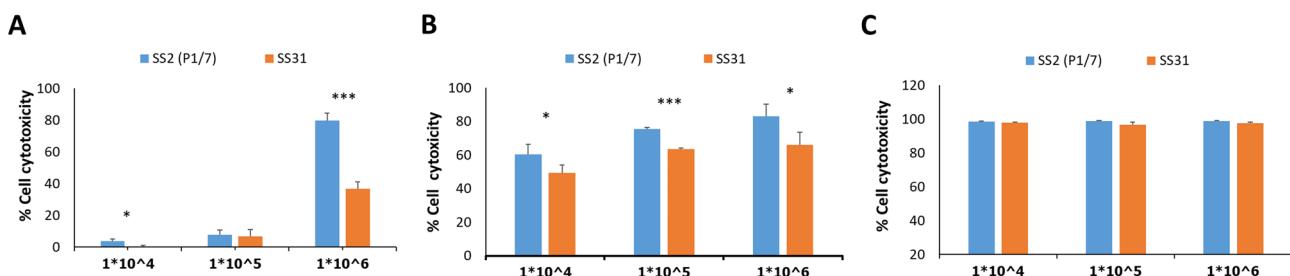
## Results

### Biofilm production by the unencapsulated *S. suis* serotype 31

Polysaccharide production by the biofilm-forming strain was analyzed using the Congo red agar assay. Compared to serotype 2, the 43640 strain produced a strong biofilm after 24 h of incubation; however, serotype 2 showed the strongest biofilm formation at 48 h (Table 1). *S. suis* biofilm formation was assayed using crystal violet staining. As shown in Table 1, the capacity to produce biofilms was higher in *S. suis* serotype 31 than in the

Code	Congo red agar method			Crystal violet method OD595 (mean $\pm$ SD)		
	24 h	48 h	72 h	24 h	48 h	72 h
SS2 (P1/7)				Weak biofilm ( $0.38 \pm 0.04$ )	Strong biofilm ( $0.81 \pm 0.09$ )	Strong biofilm ( $0.83 \pm 0.05$ )
SS31 (92-4172)				Medium biofilm ( $0.41 \pm 0.02$ )	Strong biofilm ( $0.53 \pm 0.002$ )	Strong biofilm ( $0.59 \pm 0.03$ )
SS31 (43640)				Strong biofilm ( $1.58 \pm 0.22$ )	Strong biofilm ( $1.02 \pm 0.14$ )	Strong biofilm ( $2.47 \pm 0.48$ )

**Table 1.** Biofilm formation by *S. suis* serotype 2 (P1/7), serotype 31 strain 43640, and reference strain 92-4172 assessed using the congo red and crystal Violet assay. \**S. suis* serotype 31 (SS31 (43640)), reference strain 92-4172 (SS31 (92-4172)), and *S. suis* serotype 2 (P1/7) (SS2 (P1/7))



**Fig. 1.** Cytotoxic effect of different concentrations of *S. suis* serotype 31 (SS31) and *S. suis* serotype 2 (SS2 (P1/7)) on cell viability after (A) 2, (B) 4, or (C) 18 h of treatment. Cell cytotoxicity rate (%) is expressed as the mean  $\pm$  SD. \* $p < 0.05$  or \*\*\* $p < 0.001$  indicates significant difference between the cytotoxicity rate of cells after infection with *S. suis* serotype 31 strain compared to that after infection with the *S. suis* serotype 2 (P1/7) strain.

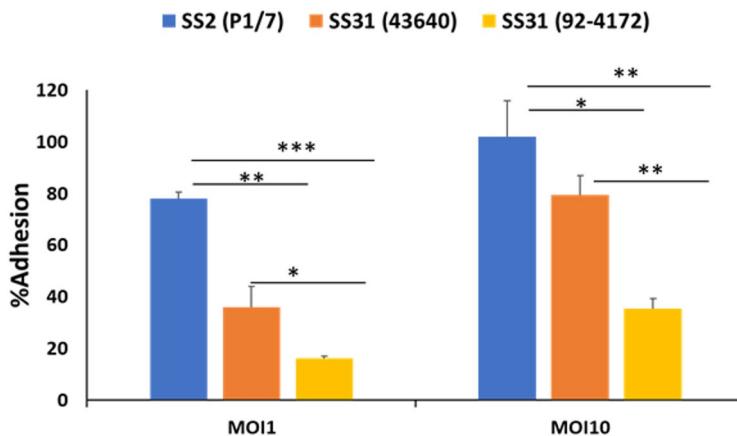
encapsulated serotype 2 (P1/7) and reference strain 92-4172 after 24 h of incubation in both assays (Table 1). All strains showed strong biofilm-forming capacity after 48 h and 72 h of incubation in Congo red agar and crystal violet staining assays.

### Toxicity of the unencapsulated *S. suis* serotype 31

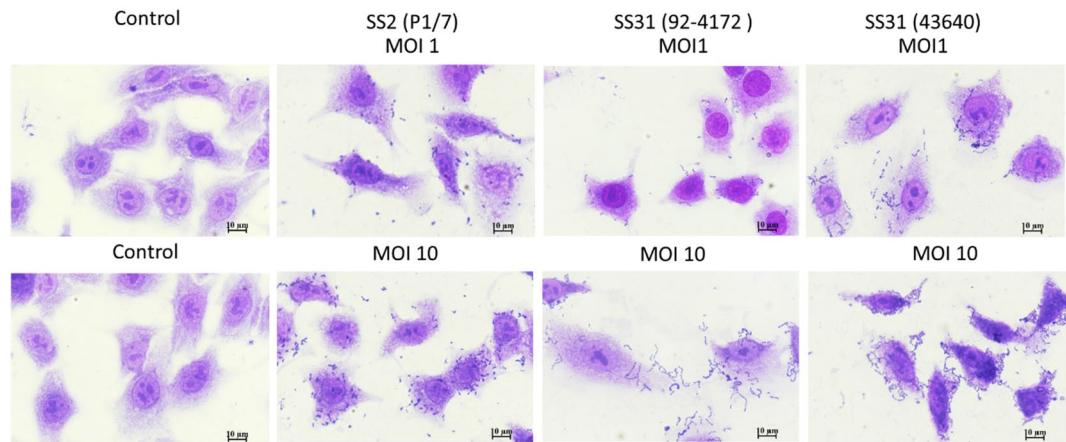
The effects of *S. suis* serotype 31 strain 43640 (ST221) and serotype 2 strain P1/7 (ST1) on A549 cell viability were determined using the CCK-8 assay. A549 epithelial cells were infected with either strain at MOI of 1 ( $1 \times 10^4$  CFU/mL), 10 ( $1 \times 10^5$  CFU/mL), or 100 ( $1 \times 10^6$  CFU/mL) in the culture medium for 2 h (Fig. 1A), 4 h (Fig. 1B), or 18 h (Fig. 1C). The cytotoxic effects of *S. suis* strains 43640 and P1/7 on the epithelial cells increased in a dose- and time-dependent manner (Fig. 1). Both strains showed 100% cytotoxicity in A549 cells (Fig. 1C). In addition, the cytotoxic effect of serotype 2 strain P1/7 was significantly higher than that of *S. suis* strain 43640 after shorter incubation times (2 h and 4 h).

### Adhesion of the unencapsulated *S. suis* serotype 31

A549 cells were infected with *S. suis* strain 43640, reference strain 92-4172, or P1/7 at an MOI of 1 ( $1 \times 10^4$  CFU/mL) and 10 ( $1 \times 10^5$  CFU/mL) in culture medium for 2 h under non-toxic conditions. The percentage of A549 cell adhesion after exposure to the strain 43640 at MOI 1 was  $36.11 \pm 7.88\%$ , while it increased to  $79.44 \pm 7.52$  after exposure at MOI 10 (Fig. 2). Figure 2 shows that the adhesion rate of *S. suis* strain 92-4172 at MOI 1 and MOI 10 were  $16.28 \pm 0.86\%$  and  $35.56 \pm 3.85\%$ , respectively. The percentages of A549 cell adhesion after exposure to strain P1/7 at MOI 1 or MOI 10 for 2 h were  $77.93 \pm 2.61\%$  and  $101.89 \pm 13.93\%$ , respectively (Fig. 2). Strain P1/7 adhered significantly more to epithelial cells than strains 43640 and reference strain 92-4172 at both MOI 1 and MOI 10. Bacterial adherence was determined using Giemsa staining and assessed using SEM after



**Fig. 2.** Effect of *S. suis* serotype 31 strain 43640 (SS31 (43640)), reference strain 92-4172 (SS31 (92-4172)), and *S. suis* serotype 2 (P1/7) (SS2 (P1/7)) on A549 cell adhesion. Cell adhesion rate (%) is expressed as the mean  $\pm$  SD. \* $p < 0.05$  or \*\* $p < 0.01$  indicates significant difference between cell adhesion rate of A549 cells infected with SS31 strain compared to that of cells infected with reference strain 92-4172 and SS2 (P1/7) strain.

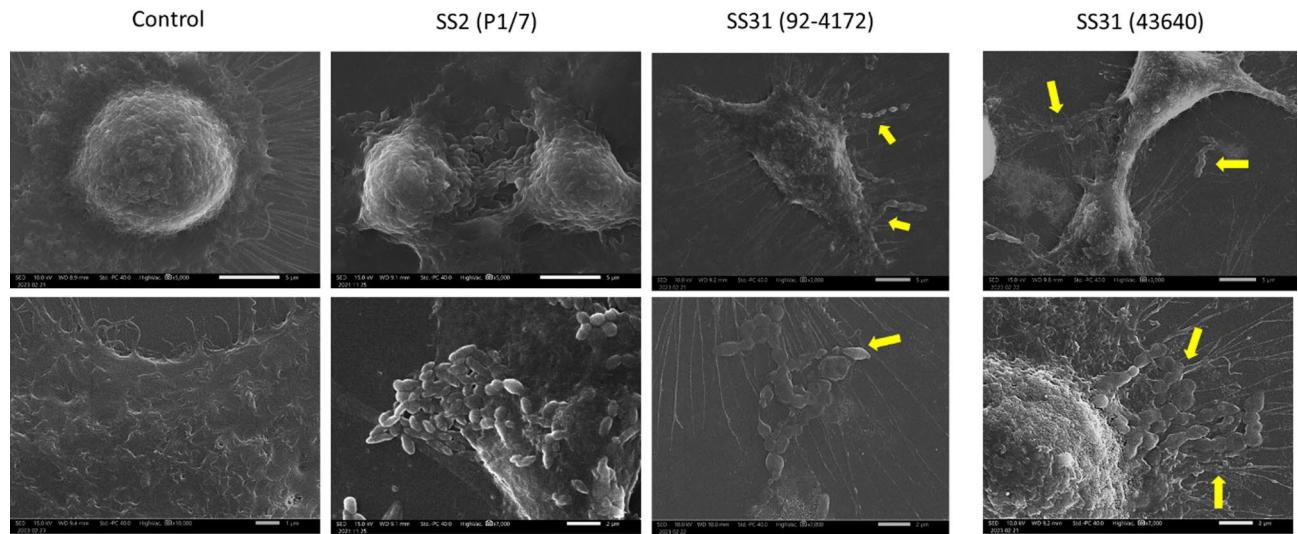


**Fig. 3.** Effect of *S. suis* serotype 31 (SS31 (43640)), reference strain 92-4172 (SS31 (92-4172)), and *S. suis* serotype 2 (P1/7) on A549 cell adhesion. Images of cells at each concentration were captured at 100 $\times$  magnification. Uninfected A549 cells were used as the control.

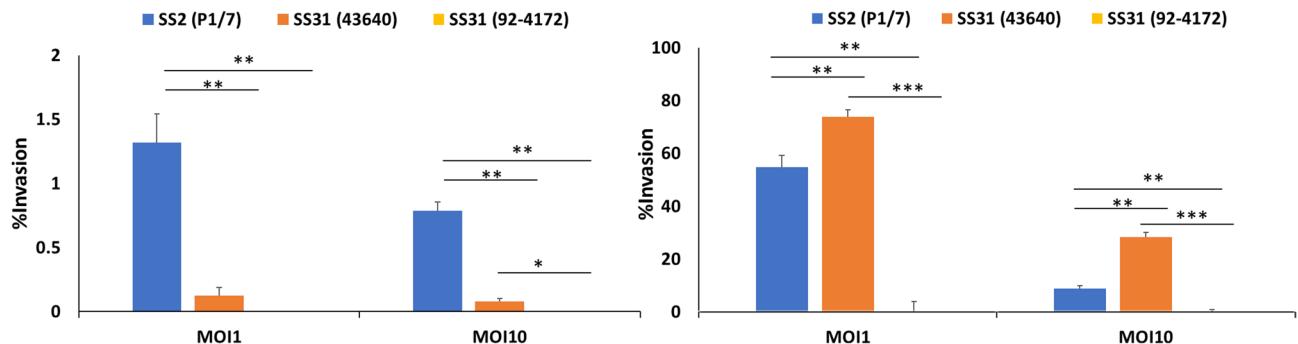
exposure to both strains at MOI 1 or MOI 10 for 2 h. The results showed that both strains adhered to A549 cells, consistent with the results of the adhesion assay (Figs. 3 and 4). However, *S. suis* serotype 2 strain P1/7 showed higher adhesion to A549 cells than strain 43640 and reference strain 92-4172 (Figs. 3 and 4). Morphological observations using SEM indicated membrane reorganization and protrusion of the epithelial cell surface after A549 cells were infected with *S. suis* serotype 31 strain 43640 and reference strain 92-4172 (Fig. 4).

### Invasion of unencapsulated *S. suis* serotype 31

The percentage of A549 cell invasion after exposure to *S. suis* serotype 31 strain 43640 at a MOI of 1 was  $0.13 \pm 0.06\%$ , while it decreased to  $0.08 \pm 0.02\%$  after exposure to a MOI of 10 (Fig. 5A). The percentages of A549 cell invasion after exposure to *S. suis* serotype 2 strain P1/7 at MOI 1 or MOI 10 were  $1.32 \pm 0.23\%$  and  $0.79 \pm 0.07\%$ , respectively after 2 h of infection (Fig. 5A). Figure 5A shows that *S. suis* strain 92-4172 did not invade the epithelial cells 2 h post-infection. After 4 h of infection, the percentages of A549 cell invasion after exposure to *S. suis* serotype 31 strain 43640 at MOI 1 or MOI 10 were  $73.89 \pm 2.55\%$  and  $28.33 \pm 1.67\%$ , respectively, while the percentages of A549 cell invasion after exposure to *S. suis* serotype 2 strain P1/7 at MOI 1 or MOI 10 were  $54.78 \pm 4.30\%$  and  $8.75 \pm 1.25\%$ , respectively. Figure 5B shows that the invasion rate of *S. suis* strain 92-4172 at MOI 1 and MOI 10 were  $0.04 \pm 0.01\%$ , and  $0.01 \pm 0.001\%$ , respectively, after 4 h of infection. The invasion of epithelial cells by the unencapsulated strain was significantly higher than that of the serotype 2 strain P1/7 and reference strain 92-4172 after 4 h (but not after 2 h) of incubation (Fig. 5B). Our results showed that the invasion of epithelial cells may not be an essential step in the pathogenesis of infection caused by the human unencapsulated *S. suis* serotype 31.



**Fig. 4.** Scanning electron microscopy showing *S. suis* serotype 31 (SS31 (43640)), reference strain 92-4172 (SS31 (92-4172)), and *S. suis* serotype 2 (P1/7) adhered to A549 cells; bars: 1, 2, or 5  $\mu$ m. The yellow arrow indicates membrane reorganization of the cell surface after *S. suis* serotype 31 infection of A549 cells. Uninfected A549 cells were used as controls.



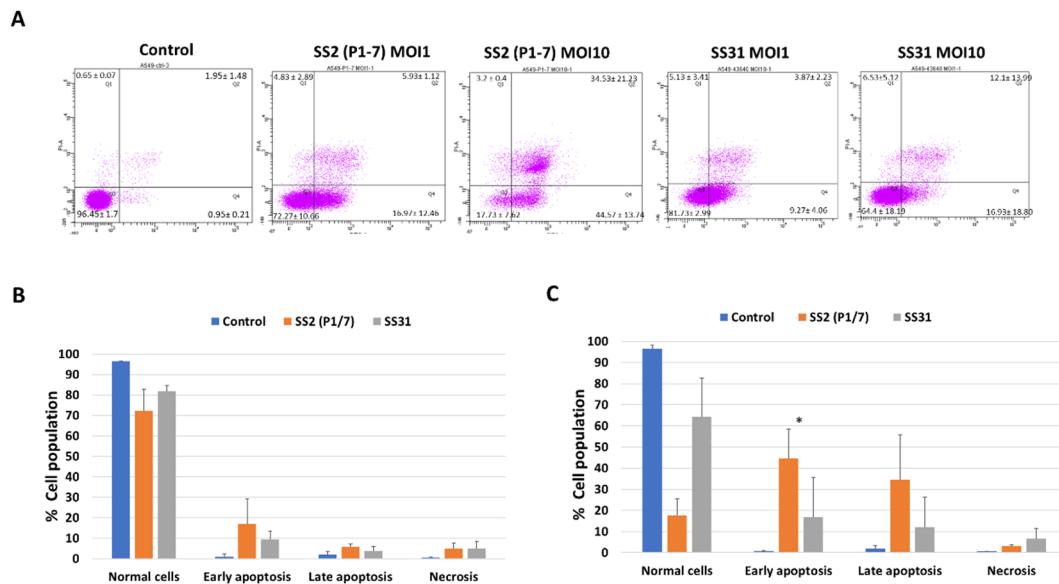
**Fig. 5.** Effect of *S. suis* serotype 31 (SS31 (43640)), reference strain 92-4172 (SS31 (92-4172)), and *S. suis* serotype 2 (P1/7) (SS2 (P1/7)) on A549 cell invasion after (A) 2 h or (B) 4 h. Cell invasion rate (%) is expressed as the mean  $\pm$  SD. \* $p$  < 0.05 or \*\* $p$  < 0.01 or \*\*\* $p$  < 0.001 indicates a significant difference between cell adhesion rate of A549 cells infected with SS31 strain 43640 compared to that of cells infected with the reference strain 92-4172 and SS2 (P1/7) strain.

### Induction of apoptosis by unencapsulated *S. suis* serotype 31

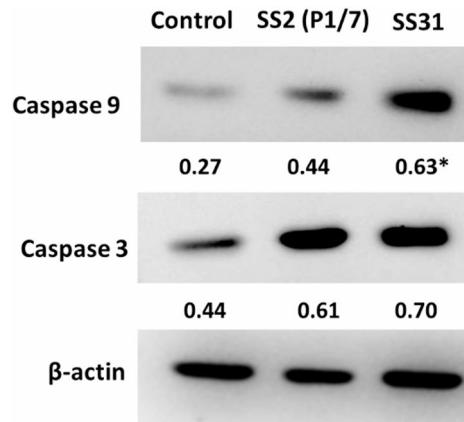
The percentage of apoptotic cells increased when cells were infected with *S. suis* serotype 31 strain 43640 (Fig. 6). Strain 43640 increased the percentage of apoptotic cells to  $13.13 \pm 6.29\%$  and  $29.03 \pm 19.98\%$  at MOI 1 (Fig. 6A, B) and MOI 10 (Fig. 6A, C), respectively, compared to the control ( $2.9 \pm 1.69\%$ ). In contrast, infection with *S. suis* serotype 2 strain P1/7 increased the proportion of apoptotic cells to  $22.9 \pm 13.45\%$  and  $79.1 \pm 7.58\%$  at MOI 1 (Fig. 6A, B) and MOI 10 (Fig. 6A, C), respectively, compared to the control. The early apoptotic rate of *S. suis* serotype 2 strain P1/7 was significantly higher than that of strain 43640 at an MOI 10 (Fig. 6C), confirming that the human unencapsulated strain 43640 induces epithelial cell apoptosis.

### Expression of apoptosis regulators by the unencapsulated *S. suis* serotype 31

To determine the signaling pathways that participate in *S. suis* serotype 31-induced apoptosis in A549 cells, proteins related to the caspase pathway were analyzed. Results of flow cytometry using annexin V/PI staining showed that infection with *S. suis* serotype 31 significantly increased caspase 9 expression compared to that in the control cells, although the expression of caspase 3 did not differ significantly (Fig. 7). In contrast, *S. suis* serotype 2 strain P1/7 increased the expression of both caspase 3 and caspase 9 although they did not differ significantly from that in uninfected cells (Fig. 7).



**Fig. 6.** Apoptotic effect in A549 cells following treatment with *S. suis* serotype 31 (SS31) and *S. suis* serotype 2 (P1/7) (SS2 (P1/7)). (A) Flow cytometric analysis of the induction of apoptosis in A549 cells following treatment with *S. suis* serotype 31 and *S. suis* serotype 2 (P1/7) for 2 h. Graph showing the percentage of apoptotic cells for the indicated concentrations at (B) MOI 1 and (C) MOI 10. Uninfected A549 cells were used as the control. \* $p < 0.05$  indicates a significant difference between the cell adhesion rate of A549 cells infected with the SS 31 strain compared to that of cells infected with the SS 2 (P1/7) strains.

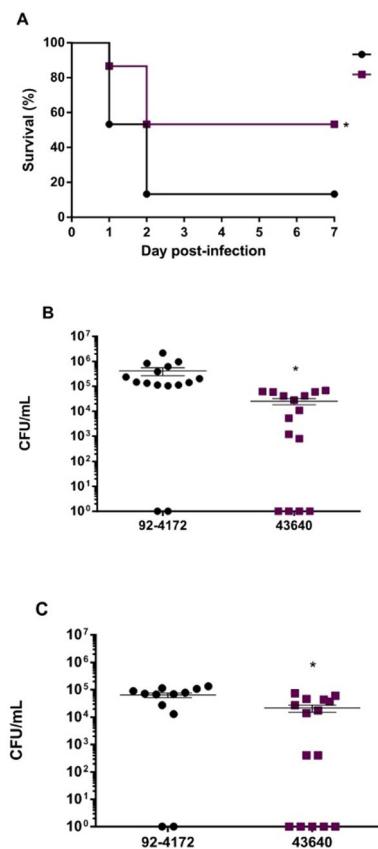


**Fig. 7.** Changes in the expression of proteins related to apoptosis in A549 cells. A549 cells were infected with *S. suis* serotype 31 (SS31) and *S. suis* serotype 2 (P1/7) (SS2 (P1/7)). The expression of apoptosis-related molecules, caspase 3 and caspase 9, were normalized that of  $\beta$ -actin. Data are presented as mean  $\pm$  SD of three separate experiments. \* $p < 0.05$ . The original blots are presented in Supplementary Fig. 1.

### Virulence of the unencapsulated *S. suis* serotype 31 in mice model

The virulence of the unencapsulated *S. suis* serotype 31-ST221 strain 43640 compared to that of the reference strain 92-4172 was evaluated using a C57BL/6 mouse model of infection. Seven days post-infection, severe clinical signs, such as depression, swollen eyes, weakness, and prostration, were observed in mice infected with the reference strain 92-4172 and the unencapsulated strain 43640. Only two mice in the unencapsulated strain 43640-infected group died from septicemia 24 h post-inoculation (13.33% mortality) (Fig. 8). In contrast, seven mice died from septicemia in the reference strain 92-4172 group at 24 h post-inoculation, with 46.67% mortality (Fig. 8). At 48 h post-inoculation, 13 mice died from septicemia in the reference strain 92-4172 group (86.67% mortality), whereas seven mice died after infection with strain 43640 (46.67% mortality). In general, the reference strain 92-4172 showed significantly higher mortality rates ( $p < 0.05$ ) than the isolated strain (Fig. 8). This suggests that the unencapsulated strain has lower virulence than the encapsulated reference strain.

Live bacterial loads were recovered from blood at 12 h and 24 h. Bacterial counts from the blood of the reference strain 92-4172- and the unencapsulated strain 43640-infected mice were  $4.09 \times 10^5$  CFU/mL and



**Fig. 8.** Survival (A) and blood bacterial burden at 12 h (B) and 24 h post-infection (C) of C57BL/6 mice following intraperitoneal inoculation of the *S. suis* serotype 31 reference strain 92-4172 (black) and the *S. suis* serotype 31 unencapsulated strain 43640 (purple). Data represent survival curves (A) ( $n=15$ ) or geometric mean (B, C) ( $n$  = number of surviving mice at each time point). \*  $p < 0.05$  indicates a significant difference between survival or blood bacterial burden of mice infected with unencapsulated strain compared to those of mice infected with the reference strain.

$2.51 \times 10^4$  CFU/mL, respectively, at 12 h ( $p < 0.05$ ) and  $5.13 \times 10^4$  CFU/mL and  $2.13 \times 10^4$  CFU/mL, respectively, at 24 h ( $p < 0.05$ ) (Fig. 8A, B). The bacterial counts in the blood of mice infected with the unencapsulated strain 43640 were significantly lower at 12 h and 24 h post-infection ( $p < 0.05$ ) than in those infected with the reference strain 92-4172.

## Discussion

Our study demonstrated the in vitro interactions with host cells and in vivo virulence of the human unencapsulated serotype 31-ST221 strain 43640. A previous report has shown that this strain contained 13 virulence-associated genes (VAG: *IgA protease*, *arcA*, *luxS*, *glnA*, *apuA*, *eno*, *sspA*, *srtA*, *covR*, *zur*, *fur*, *adcR*, and *feoB*) that are usually found in serotype 2 strains<sup>8</sup>. This strain belongs to ST221 of CC221/234, which is an emerging clone associated with human infection in Thailand<sup>31</sup>. A previous study has revealed that CC221/234 strains contained 56 of the 80 VAGs present in virulent serotype 2, suggesting that it is potentially virulent<sup>32</sup>.

Although *S. suis* adheres to different epithelial cell lines, including A549, human cervical carcinoma (HeLa), kidney of a normal adult dog (MDCK), kidney of an adult pig (PK (15)), and kidney of a normal juvenile pig (LLC-PK1), HEp-2 epithelial cell invasion may depend on strain genotypes/phenotypes, uptake mechanisms, persistence in acidified phagolysosomal compartments, or environmental signals<sup>21,33,34</sup>. Benga et al. (2004) showed that some strains persisted in HEp-2 cells for at least 24 h, whereas others were significantly eliminated<sup>33</sup>. Intracellular survival may be a key factor determining the invasion mechanisms. The mechanism used by *S. suis* serotype 31 in host-cell interactions may be associated with adhesion activity, which is an essential step in bacterial pathogenesis or infection<sup>1</sup>. The adhesion rate of *S. suis* serotype 31 increased after infection at MOI 10 compared to that at MOI 1, although it was significantly lower than that of strain P1/7. This was unexpected as it has been reported that the absence of capsular polysaccharides increases the surface exposure of adhesins<sup>35</sup>. Interestingly, the invasion capacity of serotype 31 was significantly higher than that of the serotype 2 strain P1/7, indicating that higher adhesion is not necessarily followed by increased bacterial internalization<sup>36</sup>. As described previously, unencapsulated strains normally exhibit higher invasion capacities<sup>33</sup>. We can hypothesize that for the serotype 31 strain, the absence of a capsule increases the surface exposure to invasins but not adhesins. However, this finding remains to be confirmed. A previous study has reported that 106 putative virulence-associated genes

and 58 genes are present in the *S. suis* serotype 31 human strain<sup>12</sup>. The 1910HK/RR two-component system is essential for adhesion and invasion of *S. suis* serotype 2<sup>37</sup>. The IgA1 protease is a surface-protective antigen that helps bacteria counter the mucosal immune system<sup>38</sup>. The surface-associated protein, SspA, is a conserved virulence factor in *S. suis* serotype 2 and is involved in the pathogenesis of *S. suis*<sup>39</sup>. d-Alanylation of lipoteichoic acid dltA contributes to the virulence of *S. suis* by allowing it to escape immune clearance mechanisms and move across host barriers<sup>40</sup>. Additionally, luxS plays a vital role in biofilm formation of *S. suis*<sup>41</sup>. All virulence-associated genes were identified in *S. suis* strain 43640<sup>12</sup>.

Previous studies have shown that unencapsulated strains form biofilms more efficiently than encapsulated strains<sup>33,42,43</sup>. Both strains in the current study showed strong biofilm formation. The capacity of *S. suis* to produce biofilms is associated with greater resistance to antibiotics and longer periods of antibiotic therapy, persistent colonization, exchange of genetic materials, and tolerance to host immune system clearance, and it may contribute to the establishment of infections, such as meningitis and endocarditis<sup>44,45</sup>.

The cytotoxicity of serotype 31 was significantly lower than that of the highly virulent serotype 2 strain P1/7, mostly during the early incubation period. The exact virulence factors of serotype 31 that are involved in cytotoxicity are not known because this strain lacks the suilysin-coding gene<sup>8</sup>. Suilysin is an important factor for cell cytotoxicity<sup>1</sup>. A study in Thailand demonstrated that suilysin-negative strains were as cytotoxic to peripheral white blood cells as a suilysin-positive strain<sup>46</sup>. However, whether other unidentified factors contribute to the cytotoxicity remains unknown.

Apoptosis is an important pathogenic mechanism that helps to eliminate key immune cells or evade host defenses that limit infection<sup>47,48</sup>. Several underlying pathogenic mechanisms can induce apoptosis in host cells. *S. suis*-induced caspase 3 and caspase 9 activity

promotes apoptosis of porcine choroid plexus epithelial cell<sup>29</sup>. Consistent with the results of the above study, *S. suis* serotype 31 induced apoptosis in A549 cells, which may be associated with caspase 9 activation. This may indicate that the cytotoxic activity in host epithelial cells via apoptotic activation may be an important mechanism used by the unencapsulated *S. suis* serotype 31 to damage cells, similar to that observed with the virulent serotype 2 strain. A previous study showed that the expression of *S. suis* serotype 2 endolase induced apoptosis of porcine brain microvascular endothelial cells by promoting the expression of host heat shock protein family D member 1 and activation of caspase 8, followed by that of caspase 3, which increased blood-brain barrier (BBB) permeability and promoted bacterial penetration through the BBB<sup>22</sup>.

An animal model revealed that our unencapsulated serotype 31 strain 43640 generally presented an intermediate virulence capacity in a mouse model of infection, with 13% mortality within 24 h. The survival rate of mice infected with strain 43640 was approximately 53% at 7 days post-infection. This result is similar to that reported in a previous study in which an encapsulated serotype 31 strain 11LB5 (ST421) isolated from a diseased pig also presented an intermediate level of virulence in CD1 mice<sup>24</sup>. In this study, 91% mice showed clinical signs and high levels of bacteremia ( $4.09 \times 10^5$  CFU/mL), and 37.5% of the animals died after four days of infection with strain 11LB5<sup>24</sup>. Our study also showed that the blood bacterial burden in mice infected with the unencapsulated strain was significantly lower than that of the reference strain. These results confirmed that the presence of capsular polysaccharides increases resistance to bacterial killing via phagocytic cells<sup>1</sup>. A previous study has shown that 34% of the *S. suis* isolates from porcine endocarditis were unencapsulated, indicating that the loss of capsular production is beneficial for adherence to porcine or human platelets in infective endocarditis<sup>42</sup>.

A previous study has suggested that serotypes 21 and 31 (ST750 and ST821, respectively) may be associated with the commensal pathotype, which indicates avirulence or very low virulence<sup>49</sup>. However, the current study showed that serotype 31-ST221 (CC221/234) recovered from a human patient harbored virulence potential based on in vitro and in vivo models. Based on the schematic systems of pathotyping and virulence-associated gene profiles, CC221/234 was hypothesized to belong to the virulence group or intermediate/weak virulence group, as described elsewhere<sup>32</sup>. The findings of the current study support those of a previous study<sup>32</sup> that showed that the unencapsulated serotype 31 strain 43640 in CC221/234 is a pathogenic pathotype with intermediate virulence.

## Conclusions

The current study revealed that the human unencapsulated *S. suis* serotype 31 strain 43640 was cytotoxic for an epithelial cell line. Notably, this strain displayed strong biofilm formation after 24 h, 48 h, and 72 h. Strain 43640 induced apoptosis of epithelial cells via a caspase 9-dependent pathway. Although the strain adhered effectively to epithelial cells, it did not exhibit invasive capabilities. In addition, this strain was found to be pathogenic in a mouse infection model. Therefore, *S. suis* serotype 31 is a potential threat to public health and awareness regarding its surveillance and prevention should be increased.

## Data availability

The datasets used and/or analyzed in the current study are available from the corresponding author upon reasonable request.

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## Author contributions

P.B. conceptualization, performed experiment, designed the study, formal analysis, and wrote the original manuscript; N. P., and S. P. performed the experiments; P.C., R.H., A. K., methodology, investigation, formal analysis, conceptualization; S.C., M.S., and M.G. performed the validation and data analysis; all authors have read, agreed, and approved the final version of the manuscript.

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## Declarations

### Competing interests

The authors declare no competing interests.

### Additional information

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**Correspondence** and requests for materials should be addressed to P.B.

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