Original Article

Responses of swine associated methicillin-resistant *Staphylococcus aureus* against human lung carcinoma epithelial cell line A549

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Abstract

Staphylococcus aureus (*S. aureus*) has been shown to adhere, invade, and induce the death of various host cells. The prominent lineage of livestock-associated methicillin-resistant *S. aureus* (LA-MRSA) in Asia is the sequence type (ST) 9 that has emerged as a potential zoonotic pathogen. However, the virulence of LA-MRSA ST9 remains not clear. In this study, sixteen representative Taiwan LA-MRSA isolates were selected for adherence, invasion, and cytotoxicity studies against the human lung carcinoma epithelial cell line A549. The isolates showed 100% of cytotoxicity, 35.2% - 61% of adhesion, and 18% - 52.5% of invasion against A549 cells. Different adherent abilities to A549 cells were found for the isolates from swine nasal group (43.3±8.4%) and the isolates from environmental group (31.4±6.2%) ($p < 0.05$), but no differences were found against A549 cells on invasion of both groups. Differences of cell cycle distribution in the apoptotic (sub-G1) phase of the A549 cells were also noted, with significantly more cells in the swine nasal group than that in environmental group, implying differential gene expression patterns resulting in physiological changes. This study characterized the adhesion and invasion capabilities as well as the cytotoxicity of LA-MRSA ST9 isolates against A549 cells for the first time. These findings reveal the risk for humans and animals to have potential invasion by this novel emerging MRSA lineage. More attention is needed concerning the spread of this bacterium between hospital- and community-associated environments.

Keywords: methicillin-resistant *Staphylococcus aureus*, adhesion, invasion, cytotoxicity, apoptosis

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Introduction

Methicillin-resistant *S. aureus* (MRSA) is an important invasive pathogen that causes infections in humans after surgeries as well as nosocomial infections in hospital and community settings (Deurenberg and Stobberingh, 2008). Over the past decade, livestock-associated MRSA (LA-MRSA) has raised public health concerns regarding its capacity for interspecies transmission (Harper *et al*., 2010; Morgan, 2008). At present, two major lineages of LA-MRSA have been documented, sequence type 398 (ST398) in Europe and ST9 in Asia (Lo *et al*., 2012).

S. aureus is an extracellular pathogen and has the ability of invading cells (Hauck and Ohlsen, 2006). The infection begins with the attachment of the bacteria to the surface of the host cell (Foster and Höök, 1998), and *S. aureus* can form biofilms enabling its survival by evading host defenses and antimicrobial attack (Donlan and Costerton, 2002). In *in vitro* studies, *S. aureus* survives inside the epithelial and endothelial cells and allows its escape from host defenses, particularly in the case of endocarditis (Gordon and Lowy, 2008). In clinical findings, MRSA invaded human endothelial cells and caused various form of cell damages, including apoptosis (Haslinger-Löffler *et al*., 2005; Seidl *et al*., 2011).

Several clinical cases involving LA-MRSA ST398 have consisted of different types of skin and soft tissue infections (Soavi *et al*., 2010). Our previous study showed differences in biofilm formation in colonized and non-colonized LA-MRSA ST9 (Wan and Chou, 2014). However, it is still unclear whether cellular adhesion, invasion, and cytotoxicity are common features of this swine LA-MRSA ST9. This study investigated two sources of LA-MRSA ST9 isolates-swine nasal colonized LA-MRSA and environmental LA-MRSA to (i) determine the abilities of adhering and invading the human lung carcinoma epithelial cell line A549, (ii) evaluate the *in vitro* cytotoxicity they induce over time in A549 cells, and (iii) investigate the potential in causing swine-associated MRSA-induced apoptosis in A549 cells.

Materials and Methods

***Bacterial isolates*:** This study had been approved by the National Taiwan University Hospital Research Ethics Committee. A total of 16 representative LA-MRSA isolates were used in this study, including 8 isolates from asymptomatic MRSA-carrying pigs (the swine nasal group) that were selected from our previous study (Lo *et al*., 2012) and other 8 isolates from swine slaughterhouse wastewater (the environmental group) (Wan and Chou, 2014). *S. aureus* ATCC 29213, a known clinical biofilm-producing and cytotoxic strain, was used as an adhesion and invasive positive control strain (Krut *et al*., 2003; Wan and Chou, 2014). All isolates were stored at -80°C until use.

***Cell culture*:** A549 human lung carcinoma epithelial cells (www.atcc.org) were maintained in RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA, USA) and 1% antibiotic-antimycotic in humidified air (37 °C with 5% CO_2). For adhesion and invasion experiments, A549 cells were cultured in T75 flasks to approximately 70% confluency, detached with trypsin-EDTA, re-suspended in the appropriate culture medium, and aspirate into 24-well plates. Cells were washed once before infection, and suspensions of MRSA were added at a multiplicity of infection of 1:1 (bacteria:cells).

***Cell adhesion and invasion assay*:** The protocol on the adhesion and invasion of MRSA isolates described by Liang and Ji (2007) was performed with some modifications. Briefly, MRSA isolates and the control strain were grown in 5 ml tryptic soy broth (TSB) with shaking at 37 °C for 16 h. After subculture, 500 µl of the bacterial suspension was inoculated in 5 ml TSB at 37 °C for 3 h. After incubation, the suspension was centrifuged at 3,200 *g* for 5 min, and the bacterial pellet was re-suspended in 3 ml phosphate-buffered saline (PBS). Subsequently, the bacteria were adjusted with PBS to an optical density at 600 nm (OD_{600}) = 1.2 ± 0.1, with a density of 1×10^5 bacteria. The adjusted bacterial suspension was centrifuged at 3,200 *g* for 5 min, and the pellet was gently washed twice with 3 ml PBS. The pellet was then re-suspended in 100 µl of RPMI-1640 without FBS, and all of the bacterial suspension was inoculated in 24-well tissue culture plates containing 70% confluent A549 cells (2×10^5 cells each well) at 37 °C for 1 h. After incubation, the cultures were centrifuged at 3,200 *g* for 5 min, and the pellet was washed twice with 3 ml PBS. The pellet was then re-suspended in 1 ml PBS, and the bacteria were quantified by flow cytometry using SYTO-BC stain from the Bacteria Counting Kit (Molecular Probes Inc.).

For invasion assay, the bacterial suspension was removed after the adhesion step, and the 24-well tissue culture plates were washed twice with 3 ml PBS. Subsequently, 200 µl of RPMI-1640 was added to the plates with gentamicin (200 µg/ml) and lysostaphin (5 µg/ml) and incubated at 37 °C for 1 h. After incubation, the 24-well tissue culture plates were washed twice with 3 ml PBS to remove free bacterial cells, and the A549 cells were osmotically shocked for 10 min. Then, the A549 cells were centrifuged at 3,200 *g* for 5 min, and the pellet was washed twice with 3 ml PBS. The final pellet was re-suspended in 1 ml PBS, and the bacteria were quantified by flow cytometry using SYTO-BC stain from the Bacteria Counting Kit (Molecular Probes Inc.). Both adhesion and invasion assays were performed in triplicate in at least three independent experiments.

***Cytotoxicity assay*:** Bacteria were grown as previously mentioned and standardized to an OD_{600} = 1.2 ± 0.1. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed as described by Merghni *et al*. (2015) with modifications. Briefly, confluent monolayers of A549 cells (1×10^5 cells/ml) cultured in 24-well tissue culture plates were washed with PBS and 100 µl of RPMI-1640 medium, and 100 µl of MRSA culture was added to each well for 1, 2, and 3 h incubation at 37 °C. After incubation, the suspension was discarded, and the plates were washed once with 200 µl PBS. Then, 200 µl of RPMI-1640 was added to the plates with gentamicin

(100 µg/ml) and lysostaphin (5 µg/ml) for 1 h incubation at 37 °C. After incubation, the suspension was discarded, and 20 µl of MTT was added for 1 h incubation at 37 °C. Subsequently, the suspension was discarded, and 400 µl of isopropanol was added and vibrated for 15 min. The OD of the cells was determined with a micro-ELISA auto reader (Model 680; Bio-Rad, Laboratories, Hercules, CA, USA) at 540 nm ($OD_{540\ nm}$). Each assay was performed in triplicate. *S. aureus* ATCC 29213 was used as a positive control and medium with no inoculation was used as a negative control to determine background OD.

***Cell cycle analysis by fluorescence-activated cell sorting*:** To determine cell cycle arrest following MRSA infection, fluorescence-activated cell sorting (FACS) was performed as described previously by Merghni *et al*. (2015) with modifications. Briefly, A549 cells (10^3 cells) were grown in 6-well tissue culture plates up to 80% confluency. Cells were then exposed to MRSA (10^5 bacteria) for 2 h. The wells were washed with PBS after 2 h post-infection and incubated in RPMI-1640-gentamicin (100 mg/ml) for 1 h. Detached cells were then combined with adherent cells, washed with PBS, and fixed in 70% ethanol overnight. Subsequently, fixed cells were washed with PBS and stained with propidium iodide (PI) (10 mg/ml) in PBS containing 2 mg/ml RNase A for 30 min at 37 °C in the dark. Cells were analyzed by FACS (CantoII; Becton Dickinson, France) using an excitation wavelength of 488 nm and emission wavelength of 585 nm. Data were collected from 10,000 cells.

***Statistical analyses*:** Statistical analysis was performed using STATISTICA 8.0 (StatSoft Inc., Tulsa, OK, USA). Differences in the degree of adhesion, invasion, and cytotoxicity between the swine nasal and the environmental isolates were assessed by Student's *t*-test. A *P* value of < 0.05 was considered significant.

Results

***Adhesion and invasion of swine and environmental LA-MRSA to A549 cells*:** The results on the ability of representative LA-MRSA isolates to adhere to the A549 epithelial cell line are given in Fig. 1. Overall, all of the isolates exhibited adhesion ability ranging from 23.1% to 61%. Quantitative analysis revealed that the adhesion ability of different MRSA groups was different. The ATCC 29213 human cytotoxicity *S. aureus* strain (55.2 ± 16%) had better adhesion ability compared with the swine nasal group (43.3 ± 8.4%) and the environmental group (31.4 ± 6.2%) ($p < 0.05$, Fig. 1). The adhesion ability of the swine nasal LA-MRSA isolates (range 35.2–61%, 3.6×10^5–4.3×10^5 cells/ml) was significantly higher than that of the environmental LA-MRSA isolates (range 23.1–41.5%, 2.2×10^5–2.9×10^5 cells/ml) ($p < 0.05$, Fig. 1). The results also showed that all isolates exhibited invasive ability to A549 cells ranging from 18.3% (1.6×10^5 cells/ml) to 52.5% (2.3×10^5 cells/ml) (Fig. 1). In contrast to adhesion ability, there was no significant difference in invasive ability among the environmental group (33.3 ± 8.7%), the swine nasal group (28.9 ± 18.5%), and the ATCC 29213 strain (29.7 ± 21.3%) ($p > 0.05$, Fig. 1).

***Cytotoxicity assay*:** Cytotoxicity assay was performed on all isolates. All tested isolates demonstrated cytotoxic activity after 1, 2, and 3 h of A549 cell infection compared with the control group (100%) ($p < 0.05$, Fig. 2). No significant difference in cytotoxicity ability was observed among the human clinical strain, the swine nasal group, and the environmental group ($p > 0.05$, Fig. 2).

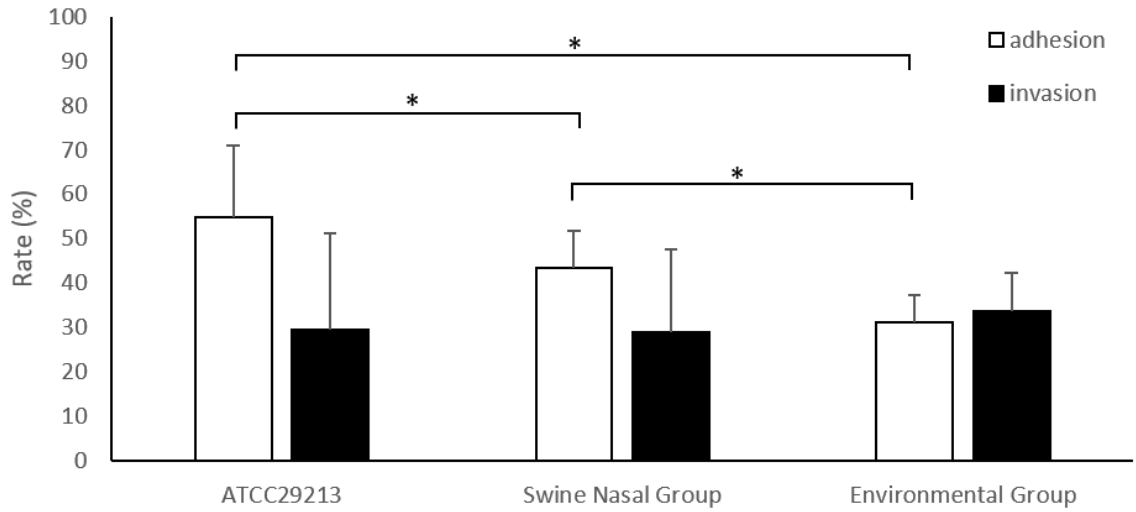

Figure 1 Evaluation of the adhesion and invasion ability by representative swine nasal and environmental LA-MRSA isolate groups. White bar: adhesion rate of ATCC 29213- a human clinical cytotoxicity MSSA strain, Swine nasal MRSA isolate group, and Environmental MRSA isolate group against A549 cells; Black bar: invasion rate of ATCC 29213, Swine nasal isolate group, and Environmental isolate group against A549 cells. Error bars represent standard deviation. Asterisk indicates comparison with ATCC 29213: *, Significantly different ($p < 0.05$).

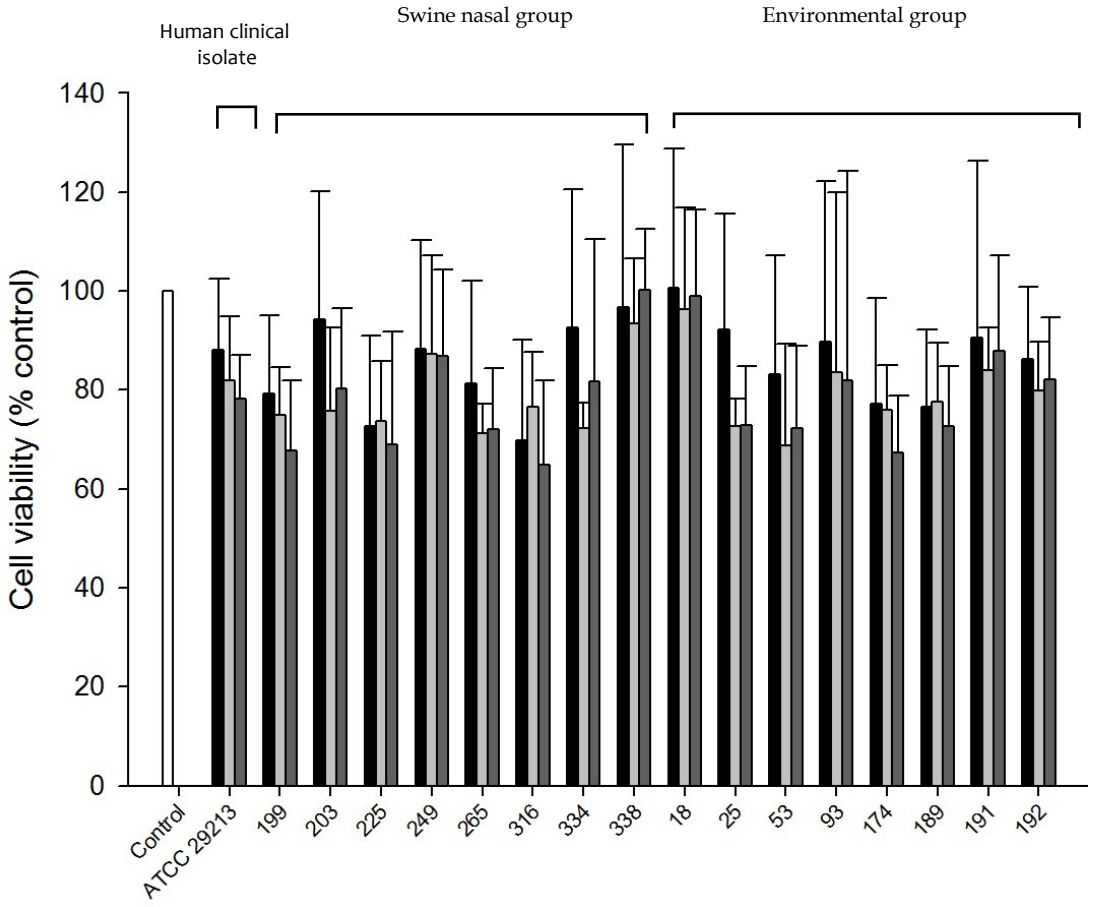

Figure 2 Cell viability was determined by a modified MTT assay. Bars represent standard deviation. Black bar: 1 h bacterial treatment; Gray bar: 2 h bacterial treatment; Dark Gray bar: 3 h bacterial treatment.

***Effect of LA-MRSA on cell cycle*:** To investigate the cytotoxicity effects of LA-MRSA from the swine nasal and the environmental group on A549 cells, four representative isolates (VPH-174 and VPH-265: lower invasive ability isolates; VPH-189 and VPH-334: higher invasive ability isolates) were selected. During the cell cycle, interphase is broken down into the G1 (GAP 1) phase, S (Synthesis) phase, G2 (GAP 2) phase and the mitotic (M) phase which in turn is broken down into mitosis and cytokinesis. Analysis results of cell DNA content after 2 h infection with swine nasal isolates (VPH-265 and VPH-334) revealed a significant increase of cells in the sub-G1 phase compared with the control group. In contrast, no significant change in sub-G1 phase cells was observed with the environmental isolates (VPH-174 and VPH-189) ($p > 0.05$, Table 1). The lower and higher invasive ability isolates from swine nasal exhibited a significantly different distribution of sub-G1 phase cells ($p < 0.05$); however, those from the environment were not different (Table 1). Neither swine nasal nor wastewater isolates resulted in a significant increase in G1, S, and G2/M phase cells compared with the control group.

Table 1 Cell cycle distribution of A549 cells treated with or without control LA-MRSA from swine nasal (VPH-265 and VPH-334) and swine slaughterhouse wastewater (VPH-174 and VPH-189)

Phase	Control (%)	VPH-174	VPH-189	VPH-265	VPH-334
Sub-G1	0.11 ± 0.05	0.16 ± 0.04	0.13 ± 0.04	0.26 ± 0.07*	5.84 ± 3.83*
G1[a]	50.17 ± 5.69	53.69 ± 6.46	49.58 ± 6.69	45.84 ± 9.83	46.7 ± 4.16
S[b]	31.23 ± 4.14	30.79 ± 7.46	35.44 ± 6.42	39.0 ± 10.75	34.59 ± 4.04
G2/M[c]	18.49 ± 4.35	15.36 ± 2.43	14.85 ± 1.72	14.9 ± 1.85	12.87 ± 3.15

The data were obtained from three representative repeats. *$p < 0.05$ when compared to control group. [a]: GAP 1 phase; [b]: Synthesis phase; [c]: GAP 2/Mitotic phase.

Discussion

This study revealed the initial feature of two different sources of swine-associated MRSA in infecting cell line A549. First, all of the isolates exhibited a wide range of adhesion and invasive abilities. Second, the adhesion ability of the swine nasal group was significantly higher than that of the environmental group. Third, all tested isolates demonstrated cytotoxic activity in A549 cell infection over time. Lastly, swine nasal isolates resulted in a

significant increase in sub-G1 (apoptotic) phase cells compared with the environmental isolates.

MRSA CC398 infection in humans has been reported in several countries (Rasigade *et al*., 2010; Valentin-Domelier *et al*., 2011; Mediavilla *et al*., 2012). The majority of reported infections were at skin and soft tissue and very few were severe infections and death (Smith, 2015). LA-MRSA CC398 isolates adhered less to human cells compared with human community-associated (CA) MRSA and hospital-acquired (HA) MRSA isolates (Ballhausen *et al*., 2014). In contrast to LA-MRSA CC398, our previous studies showed that Taiwan LA-MRSA ST9, a unique clone of LA-MRSA in Asia, harbors various virulence factors and microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) (Wan *et al*., 2013; Wan and Chou, 2014). In the present study, all of the swine-associated MRSA isolates demonstrated adhesion and invasive ability. These results are in agreement with our previous suggestion that these surface proteins may play a key role in promoting the host adhesion and invasive ability of LA-MRSA ST9 strains. Furthermore, the higher frequencies of certain MSCRAMMs in human patients with invasive diseases than in healthy human blood donors have been documented (Peacock *et al*., 2002). By combining all the above study results, Taiwan LA-MRSA has the ability to initiate the infectious processes.

LA-MRSA isolates from the swine nasal group were more likely to have stronger adhesion ability than those from the environmental group (Fig. 1). It was found that environmental LA-MRSA harbored lower levels of two MSCRAMMs- clumping factor A (*clfA*) and fibrinogen (*fib*) than those of the swine nasal group (Wan and Chou, 2014). Clumping factor (or fibrinogen-binding protein) is one of the three types of surface determinants in *S. aureus* that might mediate adhesion to damaged valves and promote endocarditis (Moreillon *et al*., 1995). Therefore, we hypothesized that with or without *clfA* and *fib* genes may contribute to different adhesion ability of LA-MRSA between the swine nasal group and the environmental group.

The LA-MRSA CC398 isolates have been reported to cause host epithelial cell damage that showed a relative higher cytotoxic potential than the HA-MRSA and CA-MRSA isolates (Ballhausen *et al*., 2014). In current study, an increasing in cytotoxicity over time was found for all tested isolates (Fig. 2). The findings indicated the potential threat of this novel pathogenic LA-MRSA lineage in causing opportunistic human infections. Furthermore, there was no significant difference in cytotoxicity among the human clinical strain, the swine nasal group, and the environmental group. Several studies have documented results on pathogenicity; for example, cytotoxicity of *S. aureus* including MRSA strains may vary depending on isolation source, bacterial strains, genetic background of the host cells, and other parameters (El-Housseiny *et al*., 2010; Oosthuysen *et al*., 2014; Merghni *et al*., 2015).

Little is known about the apoptosis of host cells induced by LA-MRSA. In the present study, we elucidated the cell cycle distribution of A549 cells resulting from swine nasal colonizing LA-MRSA and environmental LA-MRSA. The results clearly indicated that the cell cycle distribution of the sub-G1 phase was significant difference, and swine nasal LA-MRSA isolates had higher levels of inducing apoptotic ability than environmental LA-MRSA isolates did. *S. aureus* produces a wide array of cell surface and extracellular proteins involved in virulence expression (Saïd-Salim *et al*., 2003). For example, *S. aureus* α-toxin is a critical pore-forming cytotoxin that targets a broad range of host cell types (Xiong *et al*., 2006) and is differentially expressed in the growth phase under the regulation of *agr* system (Caiazza and O'Toole, 2003; Koohsari *et al*., 2016). In addition, clinical and environmental isolates had a differential gene expression pattern because of different physiological status (Sharafi *et al*., 2016). All of the above may contribute different cell cycle expression patterns of the swine nasal isolates and environmental isolates.

There are two limitations in this study. First, we did not compare adhesion, invasion, and cytotoxicity between human clinical MRSA ST9 and our isolates because of limited available samples of human MRSA ST9. Further analyses on the pathogenesis between human clinical MRSA ST9 and swine-associated MRSA ST9 are warranted. Second, these findings were the results of short-term and stressful experiments; thus, further confirmation via long-term *in vitro* and animal model studies are suggested. Nevertheless, this study describes the initial pathogenesis of LA-MRSA from swine nasal and the environment that swine MRSA isolates were able to adhere to and invade A549 cells in varying degrees, and these isolates could exhibit different levels of cytotoxicity and apoptotic-inducing ability in epithelial cells.

Acknowledgements

This study was supported by the Ministry of Science and Technology with grant number of MOST 102-2313-B-002-029-MY3. We would like to acknowledge the Taoyuan County Meat Market Co., Ltd, for allowing wastewater sampling. The authors acknowledge Miss Jo Ting Tang, Miss Sing Shan Wu and Miss Wei Chuanga for assistance in cell culture maintenances and experimental testing. We thank Dr. Tsai Ling Lauderdale from National Health Research Institutes of Taiwan for expert consultation and also Joint Center for Instruments and Researches, College of Bioresources and Agriculture, National Taiwan University for flow cytometry analysis.

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