THE INCIDENCE OF ANTIBIOTIC-RESISTANT *STAPHYLOCOCCUS* SPP. COLONIZATION AND INFECTION IN CHILDREN AND ADULTS WITH ATOPIC DERMATITIS

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การศึกษาอุบัติการณ์ของ *Staphylococcus* spp. ที่ดื้อต่อยาปฏิชีวนะจากผู้ป่วยเด็กและผู้ใหญ่ที่เป็นโรคผื่นภูมิแพ้ผิวหนัง

น.ส.มัชฌิมา เลาวัณย์ศิริ

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มัชฌิมา เลาวัณย์ศิริ : การศึกษาอุบัติการณ์ของ *Staphylococcus* spp. ที่ดื้อต่อยาปฏิชีวนะจากผู้ป่วยเด็กและผู้ใหญ่ที่เป็นโรคผื่นภูมิแพ้ผิวหนัง. (THE INCIDENCE OF ANTIBIOTIC-RESISTANT *STAPHYLOCOCCUS* SPP. COLONIZATION AND INFECTION IN CHILDREN AND ADULTS WITH ATOPIC DERMATITIS) อ.ที่ปรึกษาหลัก : นพ. ดร.ดิเรกฤทธิ์ เชี่ยวเชิงชล, อ.ที่ปรึกษาร่วม : ดร.ธนิษฐา ฉัตรสุวรรณ,นพ. ดร.อาสา ธรรมหงส์

โรคผื่นภูมิแพ้ผิวหนัง (Atopic Dermatitis หรือ AD) เป็นโรคผิวหนังที่มีการอักเสบเรื้อรังและมีอาการกำเริบอยู่เรื่อยๆ สามารถเกิดได้ทั้งในผู้ป่วยเด็กและผู้ใหญ่ แต่จะพบบ่อยกว่าในผู้ป่วยเด็ก โดยสาเหตุที่ทำให้ผื่นกำเริบอาจเกิดได้จากการมีเชื้อ *Staphylococcus aureus* (*S. aureus*) บนผิวหนัง แพทย์จึงจำเป็นต้องใช้ยาปฏิชีวนะในการรักษาผู้ป่วยโรคนี้ โดยยาปฏิชีวนะที่ใช้กันทั่วไปมีทั้งชนิดรับประทาน เช่น cloxacillin, cephalexin และชนิดทา เช่น mupirocin, fusidic acid แต่การใช้ยาปฏิชีวนะนั้นก็อาจส่งผลให้เกิดการอุบัติการณ์ของ *S. aureus* ที่ดื้อยาเพิ่มขึ้นได้ด้วย ได้แก่ *S. aureus* ที่ดื้อต่อยา methicillin (Methicillin-resistant *S. aureus* หรือ MRSA) ซึ่งเกิดจากการแสดงออกของยีน *mecA* หรือ เชื้อที่ดื้อต่อยา mupirocin เป็นจากการแสดงออกของยีน *mupA* และ เชื้อที่ดื้อต่อยา fusidic acid ที่เป็นผลจากยีนกลายพันธุ์ *fusA* mutation โดยเชื้อดื้อยาเหล่านี้จะก่อให้เกิดปัญหาในการรักษาผู้ป่วยมากขึ้น

โดยในงานวิจัยครั้งนี้มีวัตถุประสงค์เพื่อศึกษาอุบัติการณ์การดื้อยาปฏิชีวนะของ *S. aureus* ในเด็กและผู้ใหญ่ที่เป็นโรคผื่นภูมิแพ้ผิวหนัง และตรวจหายีนดื้อยาของ *S. aureus* ต่อยาปฏิชีวนะ ซึ่งผลการศึกษาพบว่า มีผู้ป่วยทั้งหมด 65 รายแบ่งออกเป็น ผู้ป่วยเด็ก 35 ราย และ ผู้ใหญ่ 30 ราย ซึ่งผู้ป่วยเด็กจะมีความรุนแรงของโรคมากกว่าผู้ใหญ่จากการประเมินด้วย EASI และ SCORAD โดยผู้ป่วยทั้งหมดจะถูกเก็บเชื้อแบคทีเรียที่ผิวหนังจาก 3 ตำแหน่ง คือ บริเวณรอยโรคผิวหนังปกติ และรูจมูก ซึ่งการศึกษาพบว่าเชื้อ *S. aureus* ที่ถูกตรวจสอบด้วยวิธี selective media, biochemical tests และ วิธี PCR เพื่อยืนยันเชื้อนั้น จะพบเชื้อนี้ได้มากที่สุดตรงบริเวณรอยโรคของผู้ป่วยทั้งเด็กและผู้ใหญ่ โดยที่เชื้อที่ตรวจได้จากบริเวณรอยโรคของผู้ป่วยในเด็กนั้นพบมีเชื้อดื้อต่อยา mupirocin ทั้งหมด 1 เชื้อ ที่มีการแสดงออกของยีน *mup*A และ fusidic acid ที่มีการแสดงออกของยีนกลายพันธุ์ *fus*A mutation ทั้งหมด 4 เชื้อ โดยพบว่า *fus*A เป็นผลให้เกิดการเปลี่ยนแปลงของตำแหน่งกรดอะมิโนในโปรตีน elongation factor G (EF-G) ของเชื้อ ซึ่งยังไม่เคยมีรายงานมาก่อน อย่างไรก็ตามการศึกษานี้ยังไม่พบเชื้อดื้อต่อยาปฏิชีวนะในผู้ใหญ่ และยังไม่พบเชื้อ MRSA ที่บริเวณรอยโรคของผู้ป่วยเด็กและผู้ใหญ่อีกด้วย ซึ่งอาจจะต้องเก็บรวบรวมผู้ป่วยให้มากขึ้นต่อไป การศึกษานี้สรุปว่า ผู้ป่วยเด็กมีอาการของโรครุนแรงมากกว่าผู้ใหญ่ และน่าจะเป็นสาเหตุของการใช้ยาปฏิชีวนะมากขึ้นกว่าผู้ใหญ่เป็นผลทำให้เกิดเชื้อดื้อยามากขึ้น โดยผู้ป่วยเด็กที่มีอาการกำเริบของผื่นเมื่อรักษาด้วยยาทา mupirocin หรือ fusidic acid แล้วไม่ตอบสนองต่อการรักษา แพทย์ควรจะต้องพึงระลึกถึง เชื้อ *S. aureus* ที่ดื้อต่อยาปฏิชีวนะเหล่านี้ด้วย

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Matchima Laowansiri : THE INCIDENCE OF ANTIBIOTIC-RESISTANT *STAPHYLOCOCCUS* SPP. COLONIZATION AND INFECTION IN CHILDREN AND ADULTS WITH ATOPIC DERMATITIS. Advisor: DIREKRIT CHIEWCHENGCHOL, M.D., Ph.D. Co-advisor: TANITTHA CHATSUWAN, Ph.D., ARSA THAMMAHONG, M.D., Ph.D

Atopic dermatitis (AD) is an inflammatory skin disease characterized by chronic and recurrent eczematous rash. AD occurs in both children and adults, but children are more affected. An important aggravating factor is caused by *Staphylococcus aureus* (*S. aureus*) infection. Therefore, topical (beta-lactam, macrolides, clindamycin) and systemic (mupirocin and fusidic acid) antibiotics are essential for treatment of AD. However, frequent use of antibiotics results in an increase in the incidence of antibiotic-resistant *S. aureus*. Methicillin resistance *S. aureus* (MRSA) becomes a problem in the treatment of skin diseases. It's found that the change from penicillin binding protein (PBP) to PBP2a with *mec*A gene controlling, causing the drug to inhibit the bacterial less. In addition, there are many topical antibiotics used in treatment. Mupirocin is a topical antibiotic commonly used to eliminate *S. aureus* and MRSA by inhibiting the isoleucyl-tRNA synthetase, but due to improper drug use, resulting in *S. aureus* resistant to mupirocin (Mupirocin resistance S. aureus) found to be related to *mup*A gene. As for fusidic acid, it binds to the elongation factor G (EF-G) of the bacteria and inhibits translocation ribosome causes the bacterial to be destroyed. However, the widespread use of topical antibiotics causes *S. aureus* to increase resistant to fusidic acid. It can change the drug target site into a mutation point in the *fus*A gene, which controls the EF-G, causing the drug to not function. Therefore, the objective of this research is to investigate the incidence antibiotic-resistant *S. aureus* colonization/infection in both children and adults with AD and to characterize the resistance genes in antibiotic-resistant S. aureus (*mec*A, *mup*A and *fus*A mutation) from children and adults with AD. The results of this study, *S. aureus* is found in the lesions of both children and adults with AD. Mupirocin and Fusidic acid-resistant *S. aureus* from the lesions in children with AD, with the expression of antibiotic-resistant genes (*mup*A, *fus*A mutation). *Fus*A gene is a change in amino acid at EF-G. However, resistance to antibiotics has not been found in adults. In addition, Methicillin resistance *S. aureus* is not found in the lesions of children and adults with AD. Therefore, children with exacerbation of the rash should be careful about topical antibiotics resistant *S. aureus* and should consider selecting proper antibiotics for treatment.

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CHAPTER I

INTRODUCTION

Atopic dermatitis (AD) is an inflammatory skin disease characterized by chronic and recurrent eczematous lesions on the affected areas of the skin (1). This condition is very common in children but it can be found at any age with typical morphology and age-specific patterns (2). There are several factors involved in the pathogenesis of AD such as genetic (e.g. *filaggrin* gene), immunological (T helper 2, type 2 innate lymphoid, and regulatory T cells), and environment factors (e.g. microbes, allergens, and irritants) (3).

Filaggrin gene (*FLG*) is one of the common genetic factors that has been reported in patients with AD (4). It has been found that AD patients who have *FLG* mutations develop impaired skin barrier causing severe and chronic eczematous rashes (5). Moreover, disease activity and severity are much higher than those without *FLG* mutations.

Dysregulation of immunological response in patients with AD has been reported (6). In the study using a model of T-helper-2 (Th2) response, they found that the levels of lipid content (e.g. ceramide) were significantly decreased leading to dryness of the skin in AD patients. Furthermore, acute eczematous lesions of patients with AD were found with an increase in the production of Th2 cytokines (e.g. IL-4, IL-5, and IL-13) (3, 7).

These cytokines are involved in both acute and chronic inflammatory processes of AD. Increased Th2 cytokines also drive the polarization of type 2 innate lymphoid cells (ILCs) which also contribute to local inflammation of AD patients (8). Interestingly, it is believed that T cell is a key player in AD disease and many different T cell subsets are associated with the disease development, such as regulatory T (Treg), Th22, Th17, and Th9 (9).

Skin inflammation in AD patients is worsened by environmental factors, such as allergens and irritants (e.g. soap and detergents) and microbes. Allergens and irritants cause dryness of the skin and induce recurrent eczematous lesions (10). Microbes particularly *Staphylococcus aureus* is associated with disease activity and severity of AD patients (11). *S. aureus* colonization/infection can be found up to 90% of patients with AD especially in children with severe symptoms (12). It has been demonstrated that *S. aureus* produces an enterotoxin, which shows superantigen properties and activates T cells causing skin inflammation. Moreover, the enterotoxin also hinders successful treatment as it induces corticosteroid-resistant in AD (13). Therefore, topical and/or systemic antibiotics are usually considered and administered in AD patients to eliminate bacterial colonization and infection.

Although commonly used antibiotics in a topical or systemic form (e.g. β-lactam, macrolides, clindamycin, mupirocin, and fusidic acid), effectively eliminate *S. aureus*, the incidence of antibiotic-resistant *S. aureus* particularly Methicillin-resistant *S. aureus*

(MRSA) become rising across the world which is now a challenging problem in dermatology and medicine (14).

S. aureus is a facultative aerobe, gram-positive cocci bacteria that normally colonize as normal flora (15). It is capable of protecting themselves from the antibiotics by several resistant mechanisms (16). The β-lactam group (e.g. penicillin, methicillin, cloxacillin, and cephalosporins) is a commonly used systemic antibiotic, the incidence of MRSA is unavoidably increased across the world. One of the most important mechanisms is penicillin-binding protein 2a (PBP2a), encoded by the *mec* gene. The PBP2a produced by the bacteria is attached to the bacterial cell membrane and acts as an inhibitor by binding with methicillin and blocking cell wall synthesis of the bacteria. For this reason, *S. aureus* can survive and continue synthesizing proteins on their cell wall (17).

There are several types of topical antibiotics used to treat *S. aureus* infection. Mupirocin is one of the most common topical antibiotics used for *S. aureus* eradication including MRSA. The bactericidal mechanism of mupirocin is inhibition of the isoleucyl-tRNA synthetase of the bacteria (18). However, emerging evidence shows that strains of mupirocin-resistant *S. aureus* associated with the *mupA* gene have been reported (19, 20).

Fusidic acid another widely used topical antibiotic, reacts with elongation factor G (EF-G) of *S. aureus*, and inhibits ribosome function and cell wall synthesis (21).

Mutations of *fus*A gene causing *S. aureus* resistant to fusidic acid have been reported in many studies (22).

CHAPTER II

HYPOTHESIS AND OBJECTIVES

HYPOTHESIS

1. The incidence of antibiotic-resistant *S. aureus* is high both children and adults with atopic dermatitis
2. *mecA, mupA* genes, *and fusA* gene mutation are detected in both children and adults with atopic dermatitis

OBJECTIVES

1. To investigate the incidence of antibiotic-resistant *S. aureus* colonization /infection in both children and adults with atopic dermatitis
2. To characterize the resistance genes in antibiotic-resistant *S. aureus* (e.g. *mec*A, *mup*A, and *fus*A mutation, etc.) from children and adults with atopic dermatitis

CHAPTER III

LITERATURE REVIEW

1. Atopic dermatitis

Atopic dermatitis (AD) or atopic eczema is a common chronic inflammatory skin disease with a typical clinical feature of eczema and periodic exacerbation (1). This skin condition is noted as intense itchy rash and usually found in infancy and childhood younger than 5 years old; however, AD sometimes persists into adulthood (2). It has been reported that the incidence of AD is increasing particularly in developed or industrial countries (e.g. USA and Europe), probably genetic predisposition and distinct environmental factors (23). In Thailand, it is found that there is a higher prevalence (17-30%) of children with AD than the USA and Europe (7-13%) (24). Also, in the research report of Siriwaradon and group, the incidence of AD in Thailand was high (25).

In the past decade, AD was believed to be the first clinical presentation of an atopic march that usually develops before food allergy, allergic asthma, and rhinitis. For this reason, AD is counted for one of the clinical spectrums of allergic or atopic disease.

1.1 Clinical manifestations

Patients with AD usually present with chronic and relapsing eczematous or pruritic rash at specific locations. The typical distribution of atopic rash is different between age groups. Infants (newborn to 2 years old) with AD often show acute eczema or

erythematous papules or plaques with blisters and serum oozing (26), at the face and extensor surface of both upper and lower extremities (27). Childhood (2-12 years old) with AD develop more subacute eczema or dry scaly erythematous plaques around the flexural areas (e.g. neck, arm folds, knee folds and ankles) whilst the typical lesions in adults (more than 12 years old) show chronic, lichenified eczema at the flexural surface and especially both hands (28) and mouth (29).

1.2 Diagnosis

In a clinical setting, patients with AD are routinely diagnosed using the Hanifin and Rajka clinical criteria (Appendix C, Table 2) (30-32). There are no specific laboratory and histological findings for making a diagnosis so personal history and typical clinical features are exclusively included in the criteria for diagnosis which consists of major and minor criteria. The clinical features of pruritus and eczematous rash (acute, subacute, and chronic) are essential findings with AD. Patients who complete 3 out of 4 major criteria; a) eczema or pruritus, b) typical age-specific morphology and distribution, c) chronic and relapsing skin lesions, and d) personal or family history of atopic diseases are diagnosed with AD. However, patients with 3 or more minor criteria (e.g. xerosis, ichthyosis, increased serum IgE, hand and foot dermatitis, cheilitis, orbital darkening, Pityriasis alba, dermatographism, etc.) are also diagnosed with AD once the major criteria are not found in those patients (33).

There are several methods established by international groups of medical professionals and these methods are used to evaluate disease severity of AD that include symptoms and signs, quality of life, and long-term control. Currently, the two most common severity scores for AD evaluation are the Eczema Area and Severity Index (EASI) (Appendix C, Figure 1, 2 and Table 1) (34), and the Scoring of AD (SCORAD) (Appendix C, Figure 3) (33).

1.3 Etiology and pathogenesis

The etiology of AD has been well studied for the past few decades in many reports. It has been demonstrated that there are several factors involved in the pathogenesis of AD including genetic predisposition, environmental factors, and immune dysregulation.

The genetic background has been identified in patients with AD. One of the well-known gene mutations found in these patients is the filaggrin (*FLG*) gene. This gene fundamentally encodes the cornified envelope protein of skin epithelial cells (keratinocytes). The mutation of the *FLG* gene therefore skin barrier causes severe and chronic eczematous rashes (35). Moreover, there are numerous identified gene mutations associated with AD pathogenesis as shown in Table1 (36).

As mentioned previously, AD is one of the most important clinical findings in patients with allergic diseases. Therefore, it has been proved that abnormal immune

response is involved in the main pathogenesis of allergic diseases including AD. Table 2 summarizes abnormal immune dysregulation in AD patients. For example, evidence shows that an increase in serine protease activity through pattern recognition receptors (PRRs) causes skin inflammation in AD patients (37). Moreover, allergen activation via Toll-like receptor 2 (TLR2) in these patients induces pro-inflammatory, and Th2 cytokine production such as IL-1α, IL-1β, and thymic stromal lymphopoietin (TSLP) that increase skin inflammation (37).

Current microbiome studies have shown that the pattern of microbial colonization on the skin of AD patients is different from healthy controls (38). It is believed that is an abnormal epidermal barrier of the skin and decreased production of antimicrobial peptides lead to colonization of pathogenic bacteria on the skin of AD patients (39). One of the most important pathogenic bacteria found on the affected skin lesions of AD patients is *S. aureus* (40). This bacteria with numerous virulence factors including toxins induce skin inflammation in AD and cause disease recurrence (11). Other environmental factors aggravate clinical symptoms of AD are certain types of food (e.g. milk, eggs, and seafood, etc.), house dust mite, and irritant (e.g. perspiration, soap, and detergent, etc.) (41).

Table 1. Gene mutations associated with AD pathogenesis.

Gene mutations	Role
CARD11	Production of protein changes that abnormal by changing pathway signaling affecting lymphocytes (T-cells) (42). Skin rashes and allergic disorders in affected individuals (36).
SPINK5	Regulation of proteolysis in epithelia formation and keratinocyte terminal differentiation (43). Cause defects in the skin barrier (43).
DSG1	An important desmosome protein desmoglein 1 causes severe dermatitis (44).
TMEM79	It has a function in the lamellar granule secretory system indicating skin barrier deficiency (45).
FLG	Skin barrier causes severe and chronic eczematous rashes (5).

Table 2. Abnormal immune dysregulation in AD patients.

Factors	Role
Dendritic cells (DC)	These cells in AD lesions express an increased level of surface FcεRI (46, 47).
Keratinocytes	Decrease the production of antimicrobial peptide (48). Increase cytokines IL-4, IL-5, IL-13, and TNF-α (49).
Pattern recognition receptors (PRRs)	Skin inflammation in AD patients (37).
Toll-like receptor 2 (TLR2)	Induce pro-inflammatory and Th2 cytokine production (37).
NOD-like receptor protein (NLRPs)	Receptors form a multiprotein complex called inflammasome that leads to the production of IL-1α and IL-1β by activation of caspase 1 (50, 51).

1.4 Treatments

The recommendation for patients with AD usually begins with moisturizers, skincare, and avoid irritating substances (e.g. strong soap and fragrance) (52, 53). The first-line treatment for skin inflammation in AD is topical corticosteroids and/or immunomodulators based on age and disease severity (54). Mild to moderate potency topical corticosteroids are applied to the acute or subacute eczematous lesions of infants or young children with AD whilst adult patients usually required high or very high potency topical corticosteroids for their lichenified rashes. Topical immunomodulators, such as calcineurin inhibitors, are usually prescribed for treatment and disease prevention in AD patients particularly when the side effects of corticosteroids are concerned. The mechanism of topical calcineurin inhibitors is to block cytoplasmic calcineurin molecule inside T lymphocytes leading to decreased synthesis of inflammatory cytokines (55).

Besides, AD patients with evidence of bacterial skin infection are treated with topical or systemic antibiotics. Topical mupirocin and topical fusidic acid are the two most commonly available agents used in clinical practice, but widespread bacterial infection sometimes occurs in AD patients and these patients need systemic therapy (e.g. cloxacillin, cephalexin, etc.) for the elimination of pathogenic bacterial colonization/infection particularly *S. aureus* (22, 56).

2. Staphylococcus aureus

S. aureus is a clinical pathogenic bacteria found in normal human skin and mucous membranes as normal flora (15). This pathogen usually causes localized skin infections (e.g. folliculitis, impetigo, cellulitis, erysipelas, etc.) in humans particularly when skin barriers or mucous membranes are impaired. However, a severe and life-threatening form of *S. aureus* infection (septicemia) could probably occur once the host immune response is compromised and the bacteria invade into the bloodstream (57).

In general, *S. aureus* infection can be classified as a community- and hospital-acquired based on their phenotypes and genotypes (15, 58). The hospital-acquired *S. aureus* is more virulent and clinically difficult to be treated especially antibiotic-resistant strains (59). Currently, β-lactam antibiotics (e.g. penicillin, cloxacillin, cephalosporins, etc.) are the first-line systemic treatment of *S. aureus* infection (60); however, topical antibiotics (e.g. mupirocin and fusidic acid) are commonly used in *S. aureus* skin infection (61, 62).

Although antibiotics are very potent and effective in the treatment of *S. aureus* infection, it has been reported that the incidence of antibiotic-resistant *S. aureus* has been increasing worldwide. One type of the major antibiotic-resistant *S. aureus* is methicillin-resistant *S. aureus* (MRSA) (63). It has been reported that this strain has been developed for many years after the improper use of β-lactam antibiotics, especially

methicillin and cloxacillin. Although MRSA has become a challenging pathogen in skin infection, highly efficacious antibiotics such as mupirocin, fusidic acid, and vancomycin (for systemic infections) are now widely used for MRSA treatment (20). Nevertheless, previous studies reported that the development of *S. aureus* resistance against mupirocin and fusidic acid is now rising (20, 64, 65).

2.1 Morphology of *S. aureus*

S. aureus is a gram-positive, round-shaped (coccus) bacterium, non-spore-forming, facultatively anaerobic bacteria with capsules (Figure 1A.) (15). The bacteria grow splendidly in culture media such as nutrient agar (NA), tryptic soy agar (TSA), and mannitol salt agar (MSA) at 37°C without oxygen requirement (66). MSA agar is normally used as a selective culture media for *S. aureus* isolation and identification from clinical specimens that allow the growth of certain groups of bacteria. The typical colonies of *S. aureus* on blood agar are white and round with β-hemolysis zone but turbid yellowish colonies on mannitol salt agar are highly specific for *S. aureus* identification as shown in Figure 1B and 1C (67).

The structure of *S. aureus* cell wall is composed of oligopeptides, lipoteichoic acid, N-acetylmuramic acid (NAM) and N-acetylglucosamine (NAG) (68). There is an enzyme called transpeptidase (penicillin-binding protein or PBP) used to link between two N-acetylmuramic acids (NAMs) and this enzyme promotes cell wall formation of the

bacteria (as shown in Figure 3). Importantly, PBP is the main target of β-lactam antibiotics (e.g. penicillin) that inhibit cell wall synthesis of bacteria leading to bacterial cell death (69).

Another crucial enzyme produced by *S. aureus* is catalase which breaks hydrogen peroxide (H_2O_2) into oxygen and water (70). The chemical property of this enzyme is normally tested in the routine laboratory to identify *S. aureus*. (known as catalase test). Also, coagulase enzyme degrading fibrinogen into fibrin, produced by *S. aureus* is used to identify this species (71). Figure 3 demonstrates the cell wall structure, virulence factors, and toxins of *S. aureus*.

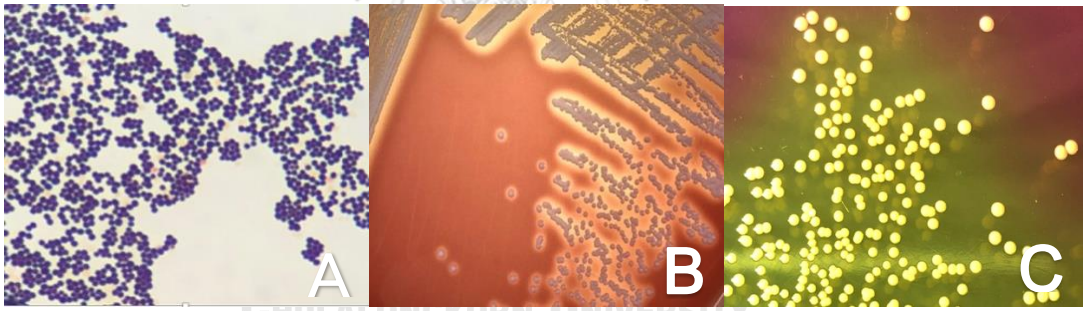


Figure 1. Light microscopic picture shows *S. aureus* stained with Gram staining (100X) (A) Colonies with β-hemolysis zone of *S. aureus* on blood agar plate (B), and yellow colonies of *S. aureus* on mannitol salt agar plate (C).

2.2 *S. aureus* and associated diseases

As mentioned previously, *S. aureus* is one of the clinical pathogenic bacteria and usually colonizes in normal skin such as nasal cavities, axillae, and groins. It has been reported that approximately 30% of bacterial colonization on human skin are *S.*

aureus (as normal flora) (72). However, *S. aureus* is also a pathogen causing many types of infections which are classified as localized and systemic infections. For example, respiratory tract infections (e.g. pneumonia and lung abscess), cardiovascular diseases (e.g. endocarditis), septicemia (e.g. sepsis and toxic shock syndrome) are systemic infection whereas localized infections are organ-specific such as skin infection (folliculitis, impetigo, cellulitis, erysipelas, abscess, etc.) (6, 73).

The localized infection usually occurs when skin epithelial barrier or immune defense mechanism is impaired. Several skin conditions are predisposed to *S. aureus* infection such as skin abrasion, xerosis cutis, insect bite reaction, eczema, trauma, surgical wounds, etc. (74, 75). However, the most severe form of *S. aureus* infection is septicemia and toxic shock syndrome. These conditions occur when the bacteria invade into the bloodstream and spread their toxins into the human system and organs (76, 77).

2.3 Virulence factors of *S. aureus*

Pathogenic strains of *S. aureus* produce plenty of virulence factors such as protein toxins, exotoxins, and exoenzymes, etc. For example, protein A is a component of staphylococcal peptidoglycan inside a bacterial cell wall (78), and it inhibits immunoglobulin binding (IgG) at the Fc region that prevents phagocytosis (79). Exotoxins are a type of toxins released by *S. aureus* and they are categorized into 3 groups; a) exfoliative toxin, b) toxic shock syndrome toxin (TSST) and c) panton-

valentine leukocidin (PVL) (80). The exfoliative toxin is involved in the pathogenesis of staphylococcal scalded skin syndrome (SSSS) due to their protease activity causing skin desquamation (81) whilst TSST is a superantigen that directly activates T lymphocyte and massive cytokine release leading to toxic shock syndrome (fever, erythematous rash, shock, etc.) (81). PVL is resistant to an acid condition in the gastrointestinal tract and causes food poisoning (82), but once the toxin enters into the bloodstream, PVL destroys white blood cells by inducing pore formation (83). Interestingly, it has been found that the expression of PVL on the cell wall of *S. aureus* is associated with community-associated methicillin-resistant *S. aureus* (CA-MRSA) infection in patients (84). Table 3 summarizes the virulence factors of *S. aureus* and Figure 2 shows the structure of *S. aureus* and virulence factors.

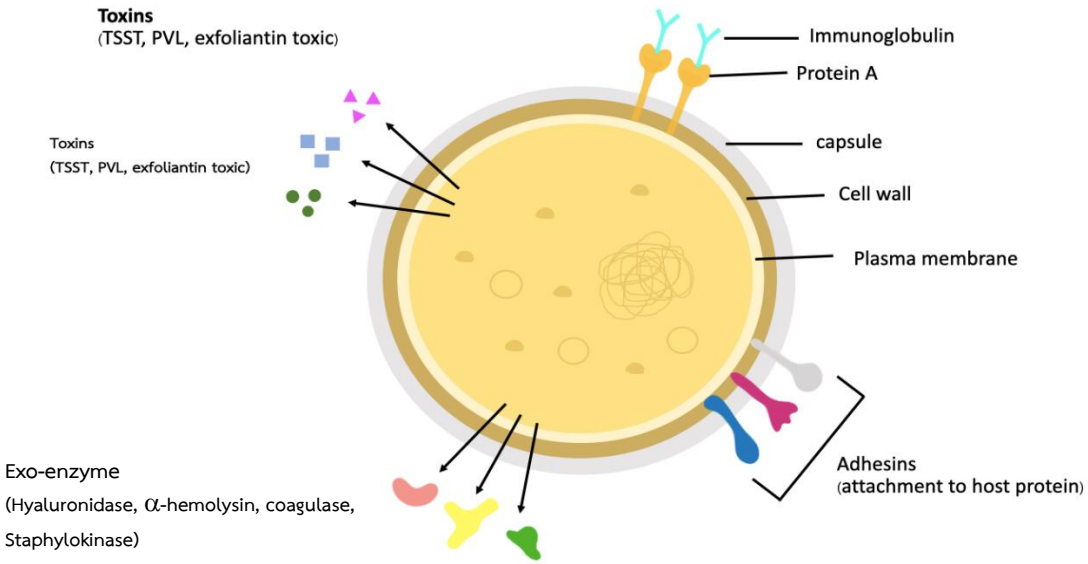


Figure 2. Virulence factors of *S. aureus*

Table 3. Examples of virulence factors produced by *S. aureus*

Factors	Role
1. Protein A	Inhibits immunoglobulin (Fc receptor) binding and phagocytosis (85).
2. Capsule	Prevents phagocytosis of white blood cells (86).
3. Exotoxins Exfoliative toxin (ET) Panton-Valentine leukocidin (PVL) Toxic shock syndrome toxin (TSST)	 Causes epidermal detachment in Staphylococcal scalded skin syndrome (SSSS) (87). Destroys white blood cells by pore forming (83). Superantigen causes T cell activation and cytokine storm in toxic shock syndrome (82).
4. Exoenzymes Hyaluronidase α- hemolysin Coagulase Staphylokinase (SAK)	 Induces hyaluronic acid degradation that allows bacterial spreading in the soft tissues (85). Causes red blood cell damage (88). Degrades fibrinogen to fibrin which prevents phagocytosis of white blood cells (71). Binds to human defensins (89). Degrades plasminogen to plasmin (89).

2.4 *S. aureus* in Atopic dermatitis (AD)

Atopic eczema or AD is a type of skin condition which is prone to bacterial skin infection especially *S. aureus*. It has been reported that one of the most common complications of AD is *S. aureus* cutaneous infection. Moreover, evidence shows that *S. aureus* is not only involved in the pathogenesis of AD but it also aggravates disease severity and recurrence (90). For example, proteases released by *S. aureus* cause epithelial barrier dysfunction (91) that allows exogenous substances including pathogens easily breach through the human skin tissues in AD patients. It has been found that disease exacerbation is usually induced by allergens and irritants in AD (92).

S. aureus colonization or infection is correlated with the expressions of IL-4, IL-13, IL-22, and TSLP in the affected skin tissue of AD patients (92). It is believed that superantigens released by *S. aureus* such as staphylococcal enterotoxin A and B (SEA/B) and toxic shock syndrome toxin-1 (TSST-1) (93-96) stimulate T lymphocytes and polarize into Th2 differentiation causing pathology in AD. Moreover, it has been demonstrated that *S. aureus* is associated with the itch-scratch cycle in the pathogenesis of AD (97). The severity of itch (pruritus) is related to serum levels of IL-31 in AD patients (97). Also, it has been found that increased serum levels of IL-31 adversely affect the production of antimicrobial peptides (e.g. human defensins, and cathelicidins) (98).

Biofilm produced by *S. aureus* is another important aggravating factor in AD. It has a protective property and defends *S. aureus* from host immune systems such as neutrophils and macrophages (99). It has been demonstrated that chronic eczema is related to biofilm development in AD patients, probably due to prolonged *S. aureus* colonization on the affected skin lesions(100).

α-toxin or □-hemolysin released by *S. aureus* is highly cytotoxic to keratinocytes (101). A previous study showed that the levels of this toxin were correlated with disease severity and it was a new drug target for treating patients with AD (102).

Phenol-soluble modulins (PSMs) are part of short peptides (103), secreted by *S. aureus* and it shows cytolytic activity in white blood cells (104) and keratinocyte. These peptides also induce the production of proinflammatory cytokines such as IL-18 and IL-1β in AD (105). Virulence factors of *S. aureus* associated with the pathogenesis of AD are summarized in Table 4 and Figure 4.

Table 4. Role of *S. aureus* and their factors in atopic dermatitis.

Factors	Role
1. Exogenous proteases	Barrier disruption (106). Increased expression of IL-4, IL-13, IL-22 and TSLP (92).
2. Protein A	Mast cell degranulation (104).
3. Staphylococcal superantigens	Staphylococcal enterotoxin A and B (SEA/B) and toxic shock syndrome toxin-1 (TSST-1) induce Th2 differentiation (94, 107).
4. Biofilm	Prevents phagocytosis (99).
5. α- Toxin	Induces skin inflammation and disruption (108).
6. δ-Toxin and other phenol-soluble modulins (PSMs)	Induce proinflammatory cytokine in keratinocytes and cause skin damage (105).

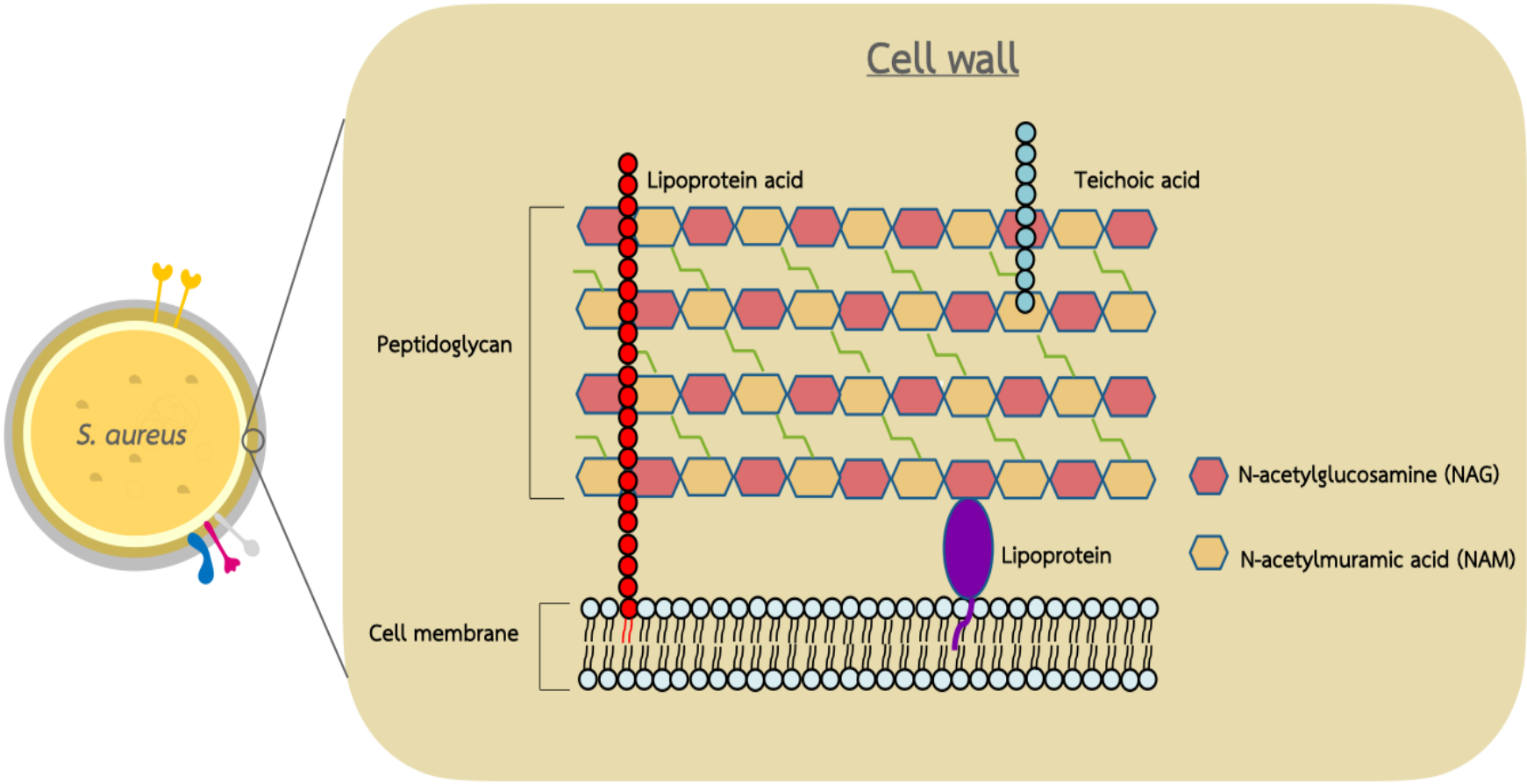


Figure 3. Cell wall structure and virulence factors including toxins of gram-positive bacteria including *S. aureus* (68)

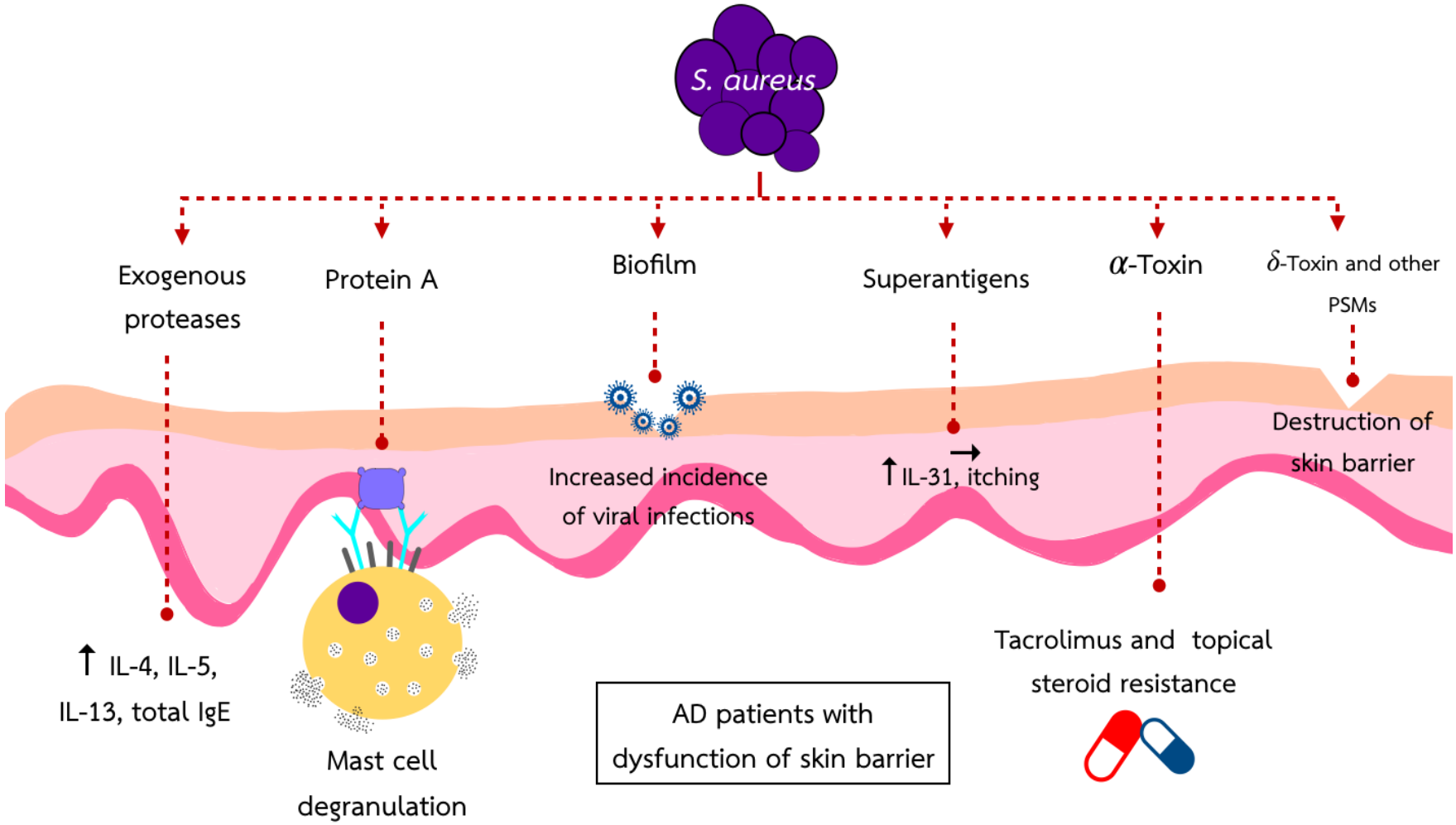


Figure 4. Virulence factors of *S. aureus* in the pathogenesis of AD (109)

3. Antibiotic-resistant *S. aureus*

The outbreak of antibiotic-resistant *S. aureus* has been reported in many studies (110). This is probably because of the improper use of antibiotics particularly the β-lactam group (e.g. penicillin, cloxacillin, cephalosporins, etc.) (60). *S. aureus* produces a penicillinase enzyme encoded by the *blaZ* gene which destroys the β-lactam ring (111). It has been reported that the increased incidence of antibiotic-resistant *S. aureus* becomes a major problem in medicine (112).

Topical antibiotics such as mupirocin and fusidic acid are very effective treatments against antibiotic-resistant *S. aureus* and now widely used for MRSA skin infections. However, it has been found that the resistant strains of *S. aureus* to these topical agents have been evolved. Therefore, the major problem of antibiotic resistance. *S. aureus* includes methicillin-resistant *S. aureus* (MRSA), mupirocin-resistant *S. aureus*, and fusidic acid-resistant *S. aureus*.

3.1 Methicillin-resistant *S. aureus* (MRSA)

The development of semisynthetic penicillin by altering the side chain of penicillin-G leads to a new generation of antibiotics such as methicillin and oxacillin. These two antibiotics are effective treatments for *S. aureus* producing penicillinase (111), and used to treat penicillin-resistant *S. aureus*. The main mechanism of methicillin is to inhibit cell wall synthesis by binding to the enzyme PBP of *S. aureus*. The PBP enzyme performs as

a catalyst for elongation between two N-acetylmuramic acids and forming peptidoglycan of cell wall synthesis (113). Methicillin is found to be used in the clinic in 1959, then the first report of methicillin-resistant *S. aureus* (MRSA) in 1960 from Guildford, UK (114). However, MRSA was evolved after the improper use of these antibiotics by producing PBP2a encoded by *mec* gene (e.g. *mec*A, *mec*I, and *mec*R1). This gene is located at a mobile genetic element called “staphylococcal cassette chromosomal *mec* (SCCmec) (115, 116). *mec*A is responsible for the production of the PBP2a enzyme, which differs from the original PBP as its active site is unable to bind to methicillin or other β-lactam antibiotics (117). Therefore, PBP remains catalyzing the transpeptidase reaction for peptidoglycan cross-linking and cell wall synthesis (118). According to research from 2015-2019, the incidence of MRSA has increased in AD. Research of MY Jung, et al, 2015. is a study of *S. aureus* sensitivity to antibiotics in AD. The results showed that MRSA increased by 22.2% of infants (73). In 2016, the prevalence and relationship of MRSA in AD have been studied by are found that MRSA has increased because of the widespread use of antibiotics (119). M. Alsterholmet al, 2017 is found the incidence of MRSA has increased in AD patients. The study was consistent with previous studies (75). In 2019, a report of E.D. Abad et al showed an increased incidence of MRSA by the severity of AD being correlated with MRSA (120).

3.2 Mupirocin-resistant *S. aureus*

Mupirocin or pseudomonic acid is a topical antibiotic used in the treatment of bacterial skin infections including MRSA. It normally used to eradicate and reduce the number of MRSA in the nasal cavity (121). Mupirocin inhibits bacterial RNA and protein synthesis by binding to bacterial isoleucyl tRNA synthetase, which catalyzes the formation of isoleucyl tRNA from isoleucine and tRNA (122). The epoxide side chain of mupirocin has similar to isoleucine and can bind to isoleucine-specific binding of isoleucyl-tRNA synthase (123). The cause of isoleucyl-tRNA synthase is inhibited, resulting in decreased RNA and causing disruption of bacterial protein and RNA synthesis (124). The use of mupirocin and multiple drugs has resulted in mupirocin resistance among *S. aureus* isolates (125). However, there has been a trend of increasing incidence in mupirocin resistance *S. aureus.* In 2014, an increased incidence of mupirocin resistance *S. aureus* in children with skin infection soft tissue by causing of *mup*A gene (126). According to previous research by Barakat and group, the incidence of 61% high-level mupirocin resistance *S. aureus* is based on the mechanism of the *mup*A gene from surgical site infections in a tertiary center, Egypt (127). Moreover, in 2017, an 98% increase in the incidence of high-level mupirocin resistant *S. aureus* is reported by the mechanism of the *mup*A gene (128). However, previous research from 2014 to the present, there has been an increasing trend of resistance to mupirocin due to the mechanism of the *mup*A gene.

The characteristics of mupirocin-resistant *S. aureus* can be divided into two groups: low-level mupirocin resistance (MIC; 8-256 μg/ml) and high-level mupirocin resistance (MIC; ≥ 512 μg/ml), based on means of minimal inhibitory concentration (MIC) (129, 130). Low-level mupirocin resistance caused point mutation in the isoleucyl-tRNA synthetase gene (*ileS* gene) (131). There has been a change in amino acid from valine to phenylalanine in the mupirocin binding site (132). High-level mupirocin resistance is associated with plasmid-mediated gene; *mup*A (*ileS*2) (123). The *mup*A gene encodes an additionally modified isoleucyl-tRNA synthesis that prevents mupirocin binding so the bacteria protein synthesis can occur (129, 133). In addition, high-level mupirocin resistance can be obtained by MRSA with low-level mupirocin resistance way of the pSK41-like plasmid which family of a multi-resistant conjugative plasmid in *Staphylococcus* (134).

3.3 Fusidic acid-resistant *S. aureus*

Fusidic acid is another topical antibiotics used in the treatment of bacterial skin infections including MRSA. Fusidic acid inhibits protein synthesis of the bacteria cell wall by binding elongation factor G (EF-G), which results in the inhibition of both peptide translocation and ribosome (62). The process of protein synthesis involves elongation, translocation, and release, which are catalyzed by four proteins: IF-2, EF-Tu, EF-G, and ribosome release factor (RRF) (135). EF-G is responsible for shifting the nascent

polypeptide chain from the A site on 30s subunit to P site that a process called peptide translocation and also interacts with RRF (103, 135). It has been shown that two distinct mechanisms of fusidic acid-resistant *S. aureus* have been reported. First, fusidic acid resistance can be high-level resistance (MIC: >64 µg/ml) is the point mutation in the *fus*A gene (136, 137). The research of Castanheira et al., is found high-level resistance resulting in changes of amino acid including L461k (position 461 leucine/lysin) and H457Y (position 457 histidine/tyrosine), etc. (138). It's also found that resistance mediated by the *fus*A gene can be transferred to a new host by a plasmid carrying a *fus*A mutation (138). As, for low-level resistance (MIC: 2-32 µg/ml) is more common and is mediated by *fus*B, *fus*C, and *fus*D gene that code for small proteins that protect EF-G from binding fusidic acid (139). *Fus*B and *fus*C genes encoding proteins that block the binding fusidic acid to EF-G, are induced by horizontal acquisition (136, 140, 141). As for the *fus*D gene, it can only be found in *S. saprophyticus* (139).

As antibiotic-resistant *S. aureus* is very difficult to treat in patients with AD. Therefore, those who are properly treated with increased severity of disease recurrence/relapse or no clinical improvement should be aware of MRSA, mupirocin- and fusidic acid-resistant *S. aureus*.

In summary, *S. aureus* is one of the most common pathogens in both hospitals- and community-acquired infections. The standard treatments of *S. aureus* infections

include topical and/or systemic antibiotics such as β-lactam, mupirocin, fusidic acid, etc. Although topical mupirocin and fusidic acid are the treatment of choice for localized *S. aureus* skin infection, mupirocin-resistant, and fusidic acid-resistant *S. aureus* have been developed and now become a challenging problem in clinical medicine. Therefore, understanding the mechanism of these antibiotic-resistant *S. aureus* help clinicians to choose the proper antibiotics and reduce the rate of antibiotic-resistance in the hospitals.

CHAPTER IV

METERIALS AND METHOD

1. Methodology Scheme

This is an observational cross-sectional study that started from August 2018 to January 2020. Patients diagnosed with AD using Hannifin and Rajka clinical criteria (142), who attended dermatology clinic, pediatric dermatology clinic, and pediatric allergy clinic at the King Chulalongkorn Memorial Hospital were recruited into the study.

Figure 5 shows a schematic diagram of the research methodology. In brief, clinical samples were collected from patients with AD and transferred to the laboratory. *Staphylococcus* spp. were identified using MSA plate and biochemical tests. Antibiotic susceptibilities of each clinical sample were performed using the disk diffusion method. Drug-resistant strains against methicillin, mupirocin, and fusidic acid were confirmed by the identification of resistant gene mutations using the polymerase chain reaction technique (*mecA* and *mupA* genes) and amino acid sequencing (*fusA* gene).

2. Collection of biological samples

Biological samples from the nasal cavity, lesion, and non-lesions were collected from patients with AD with their consent and patient confidentiality (no patient information and identification recorded). *Staphylococcus* spp. Isolates were identified and then kept in tryptic soy broth (TSB) with 20% glycerol at -80^{o}C freezer for later use.

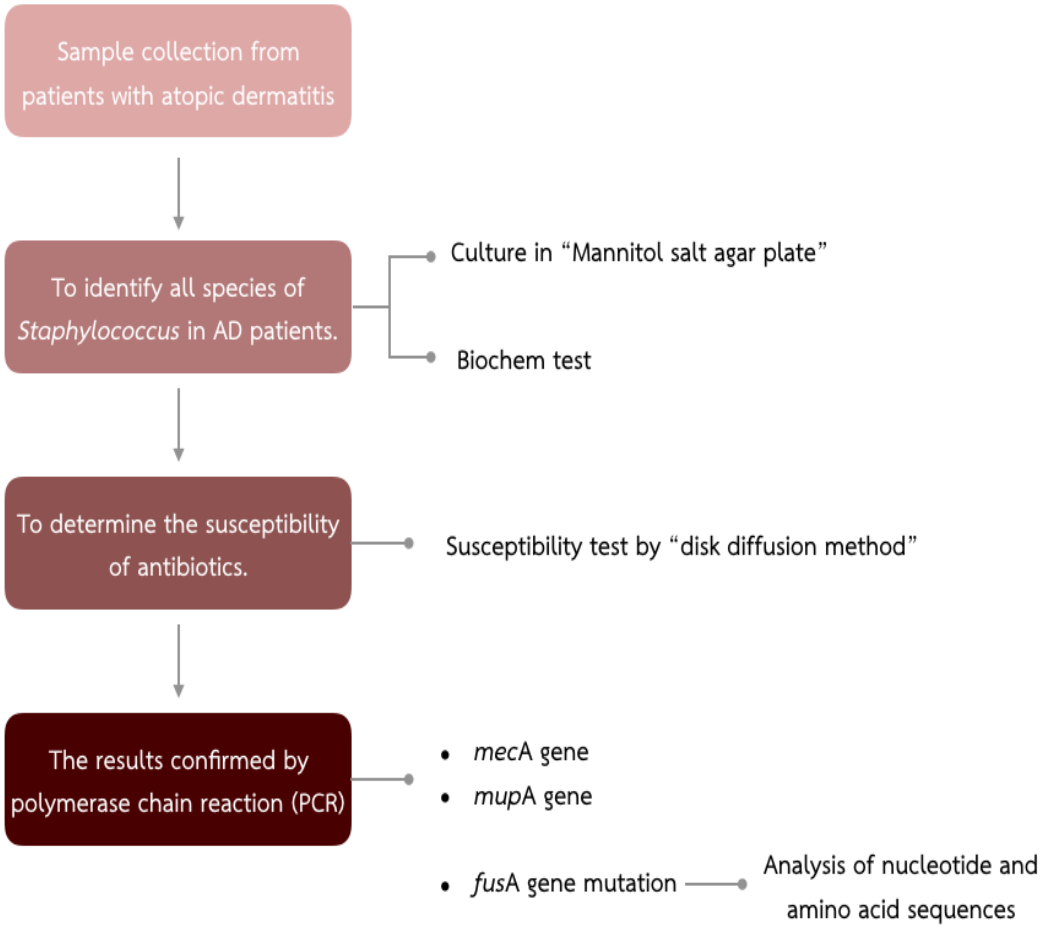


Figure 5. Methodology Scheme

3. Participants

3.1 Sample size

A total of 80 patients (N=80) with AD were recruited into the study. There were 40 children and 40 adult patients using the formula as follow:

$$\text{Sample size} = \frac{2(\square_{1-\square/2}+\square_{1-\square})^2\square^2}{(\text{MCD})^2}$$

$$= \frac{2(1.92+1.28)^2\square\, 4^2}{3^2}$$

= 37.32 patients

Therefore, there were 40 patients from both groups (children VS adult)

By definition:

- α= 0.05, $Z_{1-\alpha/2}$ = 1.96
- Power 90%, β = 0.1, $Z_{1-\beta}$ = 1.28
- MCD value (minimal clinical difference) from clinical experience. Since there is no previous study in a Thai population, the severity scores using both EASI and SCORAD systems over 3 points will be considered as no significant difference. The MCD is equal to 3 in the study.
- Variance or standard deviation is equal to 4 points according to references (73, 75, 119).

3.2 Data collection and case record form (CRF)

- Demographic data including age, gender, nationality, body weight, height, disease onset, comorbidities, family history, history of food/drug allergy, previous laboratory investigations (e.g. skin prick test, serum specific IgE), pets and previous/current treatments were recorded.

- Eczema Area and Severity Index (EASI) (Appendix C, Figure 1,2 and Table 1)(34) and Severity Scoring of Atopic Dermatitis (SCORAD) (Appendix C, Figure 3)(33), which are standard measurements of the severity in the patients with AD were assessed.
- The affected skin lesion, non-lesion, and nasal cavity were swabbed using sterile cotton tips. The samples will be inoculated on mannitol salt agar and blood agar plates before transferring to the laboratory.

3.3 Inclusion criteria

- Patients diagnosed with AD based on Hannifin and Rajka Clinical criteria, (30-32) (Table 5) and written informed consent was obtained from all participants

- Age 2 months to 60 years old

Table 5. Hanifin and Rajka Diagnostic Criteria 1980 for Atopic dermatitis (30-32)
Major criteria: Must have three or more of: 1. Pruritus 2. Typical morphology and distribution ☐ Flexural lichenification or linearity in adults ☐ Facial and extensor involvement in infants and children 3. Chronic or chronically relapsing dermatitis 4. Personal or family history of atopy (asthma, allergic rhinitis, atopic dermatitis)
Minor criteria: Should have three or more of: 1. Xerosis 2. Ichthyosis, palmar hyper linearity, or keratosis pilaris 3. Immediate (type 1) skin-test reactivity 4. Raised serum IgE 5. Early age of onset 6. A tendency toward cutaneous infections (especially *S. aureus* and herpes simplex) or impaired cell-mediated immunity 7. A tendency toward non-specific hand or foot dermatitis **Minor criteria**: Should have three or more of: 8. Nipple eczema 9. Cheilitis 10. Recurrent conjunctivitis

11. Dennie-Morgan infraorbital fold
12. Keratoconus
13. Anterior subcapsular cataracts
14. Orbital darkening
15. Facial pallor or facial erythema
16. Pityriasis alba
17. Anterior neck folds

3.4 Exclusion criteria

- Patients with AD denied being recruited into the study.
- Patients with other allergic skin conditions; allergic contact dermatitis, irritant contact dermatitis, etc.
- Patients treated with topical antibiotics within 2 weeks before.
- Patients treated with oral or intravenous antibiotics within 4 weeks before.
- Patients treated with systemic corticosteroid or immunosuppressive drugs within 4 weeks before.
- Patients admitted to the hospital within 4 weeks before.

3.5 Ethical Consideration

The ethical approval by the Ethics Committee (IRB No.238/2562), Faculty of Medicine, Chulalongkorn University before was required before commencing this study.

3.6 Expected or Anticipated Benefit Gain

- To understand the prevalence of antibiotic-resistant *S. aureus* in AD patients.

- To guide doctors for the proper use of antibiotics in AD patients.

4. Bacterial identification

4.1 *Staphylococcus* spp. isolates

The patient samples from 3 different sites of the skin (nasal cavity, lesion, and non-lesion) were inoculated on the MSA (selective media for *S. aureus*) and blood agar (selective media for *Staphylococcus* spp.) plates. The samples were incubated at 35°C for 18-24 h. The bacterial colonies from MSA plates were counted and further identified by Gram staining, catalase, coagulase, and biochemical tests as shown in Figure 7.

MSA agar is a selective culture media containing high concentrations of sodium chloride (7.5% NaCl) that inhibits gram-negative bacteria (143). Mannitol in MSA is fermented only by pathogenic *Staphylococci* (e.g. *S. aureus* and *S. saprophyticus*) but not by *S. epidermidis*, *S. hominis*, etc. (144) and fermented sugar thereby produces an acid that lowers the pH of MSA. As phenol red in MSA is a pH indicator, it turns to yellow color once the pH is below 6.9 which indicates a positive result for pathogenic Staphylococci (145, 146).

Blood agar is culture media containing 5% sheep blood and it is a common medium used to culture bacteria because of great enrichment media for fastidious

bacteria (147). Hemolysis of sheep red blood cells occurs when α-toxin is produced by certain bacteria (*S. aureus* and *S. haemolyticus*) and disintegrate of the cells completely (complete hemolysis or β-hemolysis) causing clearing zone around bacterial colonies (148, 149). In contrast, other *Staphylococcus* spp. such as *S. epidermidis*, *S. hominis* show incomplete hemolysis (γ-hemolysis), or no clearing zone (149).

4.1.1 Gram staining

The yellow colonies on the MSA plate (Figure 6A) and the white colonies with β-hemolysis on a blood agar plate (Figure 6B) were randomly selected and smeared on the glass slides and fixed by heat. The slide was stained with crystal violet for 1 min and rinsed with tap water. Gram's iodine was added on the slide for 1 min and then washed with tap water. 95% ethyl alcohol was added on the slide for 10 seconds and washed with tap water. Finally, safranin was added on the slide for 30 seconds and then washed with tap water. The bacteria stained by Gram staining were identified under a light microscope.

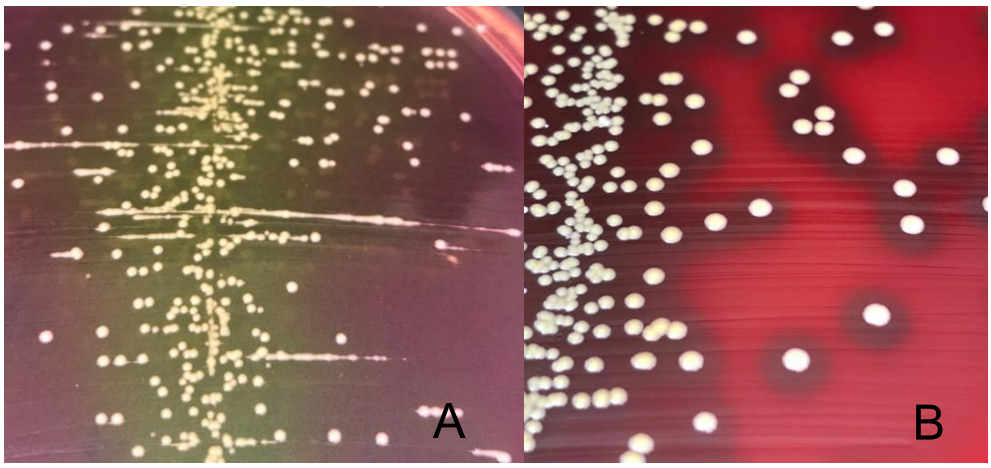


Figure 6. The yellow colonies on MSA (A) and white colonies with β-hemolysis on blood agar (B)

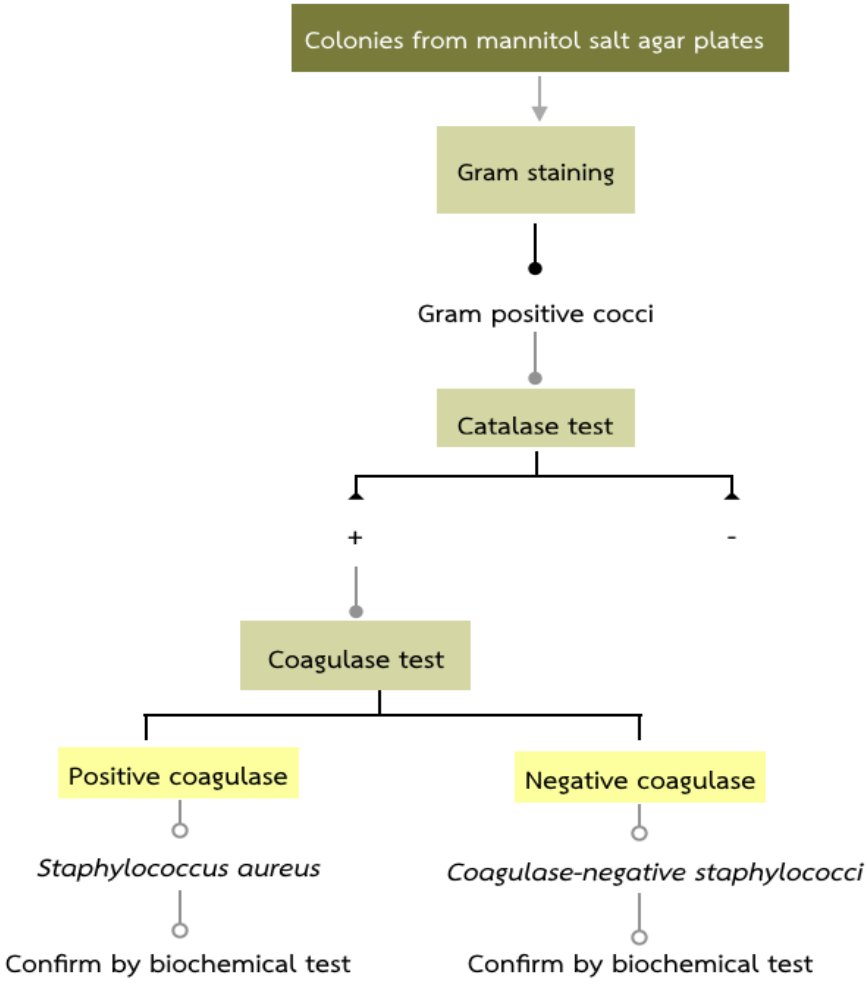


Figure 7. Diagram of bacterial identification

4.1.2 Biochemical testing

- Catalase test

The yellow colonies on the MSA plate were placed onto a glass slide and 3% H_2O_2 was dropped on the colonies. Positive catalase was noted once the evolution of oxygen bubbles occurred (Figure 8A).

- Coagulase test

The yellow colonies on the MSA plate were mixed with 0.5 ml of human plasma and incubated at 35°C for 4 h. Clot formation indicates a positive coagulase (Figure 8B).

- Biochemical conventional methods

Identification of *Staphylococcus* spp., including *S. aureus* was performed by biochemical conventional methods such as Voges–Proskauer (VP), urease production, novobiocin sensitive, produce alkaline phosphatase. Sugar fermentation as shown in Table 6 (15).

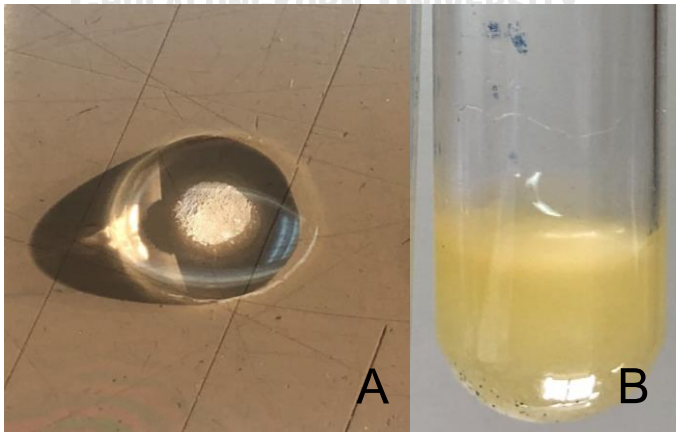


Figure 8. Biochemical test; positive for catalase test (visible bubble) (A) and positive for coagulase test (fibrin formation) (B).

4.1.3 Confirmation method

The presence of the *nuc* gene which is a coagulase-positive *Staphylococci*-targeted locus was detected by PCR to confirm *S. aureus* identification as shown in Table 8 (150).

- DNA extraction

Five colonies were randomly picked and added into an eppendorf tube with 100 µl sterile water. The tube was heated at 100°C for 10 min and was centrifuged at 13000 rpm for 5 min and the supernatant containing bacterial DNA was collected for further use.

- Identification of *nuc* gene by PCR

The presence of the *nuc* gene was amplified using specific primers as reported previously (80). The PCR mixtures (100 µl) contain 5 U of *Taq* DNA polymerase, 2 mM $MgCl_2$, 0.5 mM deoxyribonucleoside triphosphate (dNTP), 0.5 pmol of each primer and 2 µl of DNA template. Distilled water was used as a negative control. The parameters of the amplifications were used with the following conditions: 30 cycles of 95°C for 2 min; 52°C for 30 sec and 72°C for 30 sec; and then once at 72°C for 2 min.

5. The antibiotics susceptibility testing

A susceptibility test of *S. aureus* with different antibiotics was performed using the disk diffusion method. Four or five colonies of *S. aureus* were suspended in 5 ml normal saline solution. The turbidity of this suspension was adjusted to a 0.5 McFarland

standard. A sterile swab was dipped into the suspension and inoculated onto Muller-Hinton agar (MHA). Disks of cefoxitin (FOX), clindamycin (DA), erythromycin (E), trimethoprim/sulfamethoxazole (SXT), ciprofloxacin (CIP), tetracycline (TE), fusidic acid (FD), and mupirocin (MUP) were mounted on the agar plate and incubated at 35°C for 18-24 hrs. The inhibition zones of each drug were measured by Vernier caliper. Susceptible (S), intermediate (I), and resistant (R) to each antibiotic was interpreted using the Clinical and Laboratory Standards Institute (CLSI) and European Committee on Antimicrobial Susceptibility Testing (EUCAST) (Table 7).

6. Detection of antibiotic resistance genes by PCR, and nucleotide with amino acid sequencing

Methicillin- and Mupirocin-resistant genes of confirmed *S. aureus* clinical isolates (*mec*A and *mup*A genes) were detected using PCR. The fusidic acid-resistant gene of confirmed *S. aureus* clinical isolates (*fusA* gene) was detected by nucleotide with amino acid sequencing. DNA extraction and PCR were mentioned previously in 4.1.3, and amino acid sequencing as described below

The presence of *mec*A, *mupA,* and *fusA* genes was amplified from DNA products of Methicillin-, Mupirocin- and Fusidic acid-resistant *S. aureus* using specific primers as reported previously (151-153). The PCR mixtures were similar to the previous experiment (in 4.1.3). The parameters of the amplification for Methicillin-resistant *S. aureus* were used with the following conditions: 30 cycles of 94°C for 30 sec; 55°C for

30 sec and 72°C for 4 min. The parameters of the amplification for Mupirocin- and Fusidic acid-resistant *S. aureus* were used with the following conditions: 30 cycles of 95°C for 30 sec; 57°C (*mup*A and *fus*A) for 30 sec and 72°C for 4 min. The PCR product of the *fus*A gene was purified and sequenced using the QIAquick PCR purification kit. Automated DNA sequencing was done at the 1st BASE Inc, Malaysia. Table 8 shows oligonucleotide primers for each gene of *S. aureus.*

6.1 Analysis of PCR products

The 5 µl amplified PCR products were analyzed on 1.5% agarose gel electrophoresis in 0.5X Tris-Borate-EDTA buffer (TBE) and 0.5 µg/mL of SYBR green was added to the solution before pouring the gel into a casting tray. The 6X loading dry buffer was mixed with PCR products and loaded into the gel in the electrophoresis chamber containing 0.5X TBE. Electrophoresis was run for 50 min at 100 volts/cm and the gel was visualized using the gel documentation system (Bio-Rad, USA).

6.2 Analysis of nucleotide sequences

Nucleotide sequences and deduced amino acid sequences were analyzed using Online Software available at the National Center for Biotechnology Information (NCBI) (https://blast.ncbi.nlm.nih.gov), SnapGene (version 5.0.7) and ExPASy (www.expasy.org). Multiple sequence alignment was analyzed using Multilin (http://multalin.toulouse.inra.fr/multalin). The *fus*A sequences from confirmed *S. aureus* clinical isolates identified in this study were compared with the *fus*A sequence from

S. aureus NCTC 8325 (GenBank accession no. NC_007795), using Mega 4 software (Biodesign Institute, Tempe, AZ, USA).

7. Data analysis

Data were analyzed using SPSS and GraphPad Prism 8

- Quantitative data has summarized the information in the average or median, and standard deviation (SD).
- Qualitative data have summarized the information in percent and the proportion.
- A comparison of the association between *S. aureus* colonization and the severity scores of AD patients using the Chi-square test and Pearson's correlation and statistically significant was considered at p-value < 0.05.

Table 6. Biochemical tests for confirm all species of *Staphylococcus*

Species	Characteristic										
	Expression of		Novobiocin resistance	Sugar fermentation from:							
	Alkaline phospha-tase	Urease		Trehalose	Mannitol	Mannose	Arabinose	Maltose	Lactose	Sucrose	Voges–Proskauer (VP)
S. aureus	+	d	-	+	+	+	-	+	+	+	+
S. epidermidis	+[m]	+	-	-	-	+	-	+	d	+	+
S. haemolyticus	-	-	-	+	d	-	-	+	d	+	+
S. hominis	-	+	-	d	-	-	-	+	d	+	+
S. xylosus	d	+	+	+	+	+	d	+	d	+	+

[m], approximately 6-15% of strains of *S. epidermidis* are negative for alkaline phosphatase activity, depending on the population sampled.

d, 11-89% of strain positive

Table 7. Interpretive categories and zone diameter breakpoints for *S. aureus*

Antimicrobial agent	Disk content	Interpretive categories and zone diameter breakpoints, near whole mm			Reference
		S	I	R	
Cefoxitin	30 µg	≥ 22	-	≤ 21	CLSI (2019)
Trimethoprim/ sulfamethoxazole	5 µg	≥ 16	11-15	≤10	CLSI (2019)
Fusidic acid	10 µg	≥ 24	-	< 24	EUCAST (2019)
Mupirocin	200 µg	any zone	-	no zone	CLSI (2019)
Tetracycline	30 µg	≥ 19	15-18	≤ 14	CLSI (2019)
Ciprofloxacin	5 µg	≥ 21	16-20	≤ 15	CLSI (2019)
Clindamycin	2 µg	≥ 21	15-20	≤ 14	CLSI (2019)
Erythromycin	15 µg	≥ 23	16-18	≤ 13	CLSI (2019)

Table 8. Oligonucleotide primers used in this study

Gene	Primer	Sequence 5'to 3'	Amplicon size (bp)	Reference
Nuc	Nuc-alF1 Nuc-alR	CCNAAYACNCCNGTNCARCCN NADCCANACRTANGCNARNGT	359	(150)
mecA	mecA-F mecA-R	AAAATCGATGGTAAAGGTTGGC AGTTCTGCAGTACCGGATTTGC	532	(151)
mupA	mupA-F mupB-R	TATATTATGCGATGGAAGGTTGG AATAAAATCAGCTGGAAAGTGTTG	458	(152)
fusA	fusA-F fusA-R	CGGTATCATGGCTCACATTG GTACCGCGACCTTGAGTGTT	1962	(153)

CHAPTER V

RESULT

1. Demographic data

1.1 The characteristics of AD patients

A total of 65 patients with AD was recruited in this study. There were 33 children (18 boys and 15 girls) and 23 adults (6 males and 17 females) with 9 incomplete/missing data. The mean ages of children and adults with AD were 3±3.97 and 24.94±7.24 years old, respectively. There were 17 children (51.51%) and 17 adults (73.91%) were diagnosed with allergic rhinitis whilst 2 children (6.06%) and 2 adults (8.70%) had allergic asthma. The history of drug or food allergy was noted in 9 children (27.27%) and 5 adults (21.74%). Other parameters are demonstrated in Table 9, and a family history of other allergic or autoimmune diseases in family members of children and adults with AD is shown in Table 10.

1.2 Hannifin and Rajka clinical criteria

According to Hannifin and Rajka clinical criteria, the results showed that all children and adults completed at least 3 out of 4 major criteria, and were diagnosed with AD. Moreover, the most common minor criteria found in children with AD were xerosis (93.94%), early age of onset (78.79%), and orbital darkening/itch when sweating (60.61%), respectively. Similarly, the most common minor criteria found in adults with AD

were xerosis (86.96%), orbital darkening/itch when sweating (82.61%), and early age of onset (78.26%) (Table 11).

1.3 Eczema area and severity index score (EASI) and severity scoring of AD (SCORAD)

The result showed that the average EASI score was higher in children (15.98±14.4) when compared with adults (5.21±6.15). Also, the average SCORAD showed in the same trend as children with AD had a higher score (60.38±22.99) than adults (30.78±11.13). Notably, moderate to severe symptoms were observed in most children with AD whilst mild symptom was commonly found in adult patients (Table 12).

Table 9. The characteristics of children and adults with AD

Parameters	Children (N=33) (%)	Adults (N=23) (%)
Sex		
□ Male	18 (54.55)	6 (26.09)
□ Female	15 (45.45)	17 (73.91)
Age (years)		
□ Mean ± SD	3 ± 3.97	24.94 ± 7.24
□ Median (Min-Max)	1 (0 - 13)	23 (16 - 47)
Weight		
□ Mean ± SD	33.91± 26.21	61.43 ± 11.1
□ Median (Min-Max)	25.2 (6 - 89)	60 (48 - 94)

Parameters	Children (N=33) (%)	Adults (N=23) (%)
Height		
Mean ± SD	123.15 ± 38.93	163 ± 7.11
Median (Min-Max)	128.5 (58 - 180)	163 (151 - 179)
Comorbidities		
Allergic rhinitis	17 (51.51)	17 (73.91)
Allergic asthma	2 (6.06)	2 (8.70)
Other*	8 (24.24)	1 (4.35)
Drug/ Food allergy	9 (27.27)	5 (21.74)
Smoking in family	7 (21.21)	8 (34.78)

*Others: ADHD, obesity, acne, anaphylaxis, urticaria, congenital heart disease, delayed speech, depression, hypothyroidism, hypertension, metabolic syndrome, PCOS, Noonan syndrome, OCD, OSA and obesity.

Table 10. Family history of other allergic or autoimmune diseases in any family members of children and adults with AD

Family history	Children (N=24) (%)	Adults (N=15) (%)
Allergic rhinitis	17 (70.83)	10 (66.67)
Asthma	9 (37.50)	3 (20.00)
AD	8 (33.33)	4 (26.67)
SLE	1 (4.17)	-

Table 11. Patients diagnosed with AD based on Hannifin and Rajka clinical criteria

Parameters	Children (N=33) (%)	Adults (N=23) (%)
Major criteria		
□ Pruritus	33 (100)	23 (100)
□ Typical morphology and distribution	32 (96.97)	18 (78.26)
□ Flexural lichenification or linearity in adults	24 (72.73)	17 (73.91)
□ Facial and extensor involvement in infants and children	8 (24.24)	1 (4.35)
□ Chronic or chronically relapsing dermatitis	33 (100)	22 (95.65)
□ Personal or family history of atopy	26 (78.79)	19 (82.61)
Minor criteria		
□ Xerosis	31 (93.94)	20 (86.96)
□ Ichthyosis/ palmar hyperlinearity/ keratosis pilaris	11 (33.33)	12 (52.17)
□ Immediate (type 1) skin test reactivity	2 (6.06)	1 (4.35)
□ Elevated serum IgE	4 (12.12)	-
Minor criteria		

Parameters	Children (N=33) (%)	Adults (N=23) (%)
Early age of onset	26 (78.79)	18 (78.26)
Tendency toward cutaneous infections	12 (36.36)	6 (26.09)
Tendency toward on-specific hand or foot dermatitis	4 (12.12)	9 (39.13)
Nipple eczema	2 (6.06)	5 (21.74)
Cheilitis	11 (33.33)	10 (43.48)
Recurrent conjunctivitis	3 (9.09)	8 (34.78)
Dennie-Morgan infraorbital fold	18 (54.55)	14 (60.87)
Keratoconus	-	-
Anterior sub capsular cataracts	-	-
Orbital darkening	20 (60.61)	19 (82.61)
Facial pallor/ facial erythema	8 (24.24)	1 (4.35)
Pityriasis alba	8 (24.24)	2 (8.70)
Anterior neck folds	4 (12.12)	10 (43.48)

Parameters	Children (N=33) (%)	Adults (N=23) (%)
Minor criteria	20 (60.61)	19 (82.61)
□ Itch when sweating	5 (15.15)	6 (26.09)
□ Intolerance to wool and lipid solvents	9 (27.27)	8 (34.78)
□ Perifollicular accentuation	5 (15.15)	5 (21.74)
□ Food intolerance	11 (33.33)	17 (73.91)
□ Course influenced by environmental/ Emotional factors	5 (15.15)	4 (17.39)
□ White dermographism/ Delayed blanch		

Table 12. Eczema area and severity index score (EASI) and severity scoring of AD (SCORAD)

Parameters	Children (N=33) (%)	Adults (N=23) (%)
EASI score		
Mean ± SD	15.98 ± 14.4	5.21 ± 6.15
Median (Min-Max)	12.2 (1 – 56.4)	2.9 (0.6 – 27.2)
EASI score severity		
Mild	9 (27.27)	18 (78.26)
Moderate	15 (45.45)	4 (17.39)
Severe	9 (27.27)	1 (4.35)
SCORAD		
Mean ± SD	60.38 ± 22.99	30.78 ± 11.13
Median (Min-Max)	60.6 (14.1 – 110.5)	32.36 (12.7 – 52)
SCORAD severity		
Mild	1 (3.03)	7 (30.43)
Moderate	10 (30.3)	15 (65.22)
Severe	22 (66.67)	1 (4.35)

2. Bacterial identification in patients with AD

Clinical samples were swabbed and collected from different sites of children (N=35) and adults (N=30) diagnosed with AD at the Dermatology, Pediatric Dermatology, and Pediatric Allergy and Immunology clinics at King Chulalongkorn Memorial Hospital, Thailand during 2018 to 2020. All samples were inoculated on two types of agar plates (as described in the Methods) and the results showed that bacterial colonies were found on the plates in 29 samples (83%), 16 samples (46%) and 25 samples (71%) of lesions, non-lesions, and nasal swabs in children, respectively. Bacterial colonies were found in 16 samples (53%) of lesions swabs, 6 samples (20%) of non-lesion swabs, and 26 samples (87%) of nasal swabs in adult patients (Table 13).

Table 13. Bacterial colonies from different sites of 65 AD patients

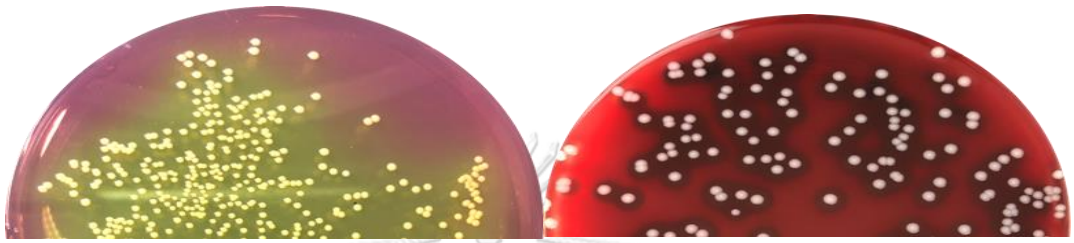
Sites of bacterial colonies	Children (N=35)(%)	Adults (N=30)(%)	Total (N=65)(%)
Lesion swabs	29 (83%)	16 (53%)	45 (69%)
Non-lesion swabs	16 (46%)	6 (20%)	22 (34%)
Nasal swabs	25 (71%)	26 (87%)	51 (78%)

2.1 Identification of bacterial isolates on selective media

2.1.1 Yellowish halo around colonies and white colonies with complete hemolytic ring (β-hemolysis)

The results showed that yellowish halo around colonies on MSA agar and white colonies with complete hemolytic ring (β-hemolysis) on blood agar were found in 20

samples (69%), 6 samples (37.5%) and 15 samples (60%) of lesions, non-lesions and nasal swabs in children, respectively. In contrast, these colonies were found in only 8 samples (50%) of lesions swabs, 2 samples (33%) of non-lesion swabs, and 6 samples (23%) of nasal swabs in adult patients (Figure 9).

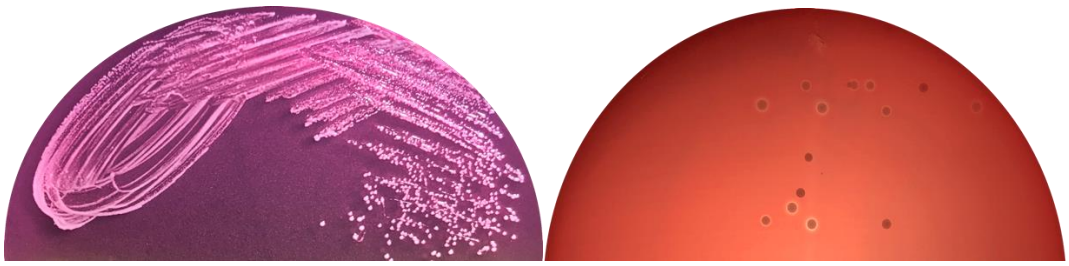


(A) (B)

Figure 9. Identification of bacterial colonies on selective media.
(A) The yellowish halo around colonies on MSA agar and (B) β-hemolysis (complete hemolytic ring) on blood agar.

2.1.2 Pink or red colonies and white colonies with complete hemolytic ring (β-hemolysis)

The results showed that pink or red colonies on MSA agar and white smooth skin with complete hemolytic ring (β-hemolysis) on blood agar were found in 1 sample (3%) of lesion swabs, 2 samples (12.5%) of non-lesion swabs in children. No bacterial colonies with these phenotypes were found in nasal swabs of children with AD. In adult patients, 2 samples (12.5%), 2 samples (33%), and 5 samples (19%) were found with these bacterial colonies as shown in Figure 10.



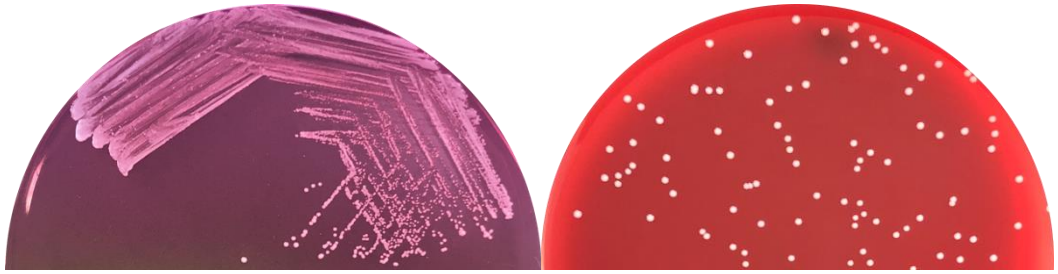
(A) (B)

Figure 10. Identification of bacterial colonies on selective media.

(A) Pink or red colonies on MSA agar and (B) white colonies with complete hemolytic ring (β-hemolysis) on blood agar.

2.1.3 Pink or red colonies and white colonies without a hemolytic ring (γ-hemolysis)

Pink or red smooth surface colonies on MSA agar and white turbid colonies with γ-hemolysis (non-hemolysis) on blood agar were found in 8 samples (28%, 50%) of both lesion and non-lesion swabs, and 10 samples (40%) of nasal swabs in children, respectively. In adult patients, 6 samples (37.5%) of lesion swabs, 2 samples (33%) of the non-lesion swabs, and 15 samples (58%) of nasal swabs were found with these bacterial colonies (Figure 11).



(A) (B)

Figure 11. Identification of bacterial colonies on selective media.

(A) Pink or red colonies on MSA agar and (B) white colonies with incomplete hemolytic (γ-hemolysis) on blood agar.

Table 14 summarizes all bacterial phenotypes on selective media found in both children and adults with AD.

2.2 Gram staining and biochemical test for *S. aureus* identification

Yellowish halo around colonies on MSA plates and white colonies with complete hemolytic ring (β-hemolysis) on blood agar plates isolated from both children (N=41) and adults (N=16) with AD were tested for gram staining and biochemical test. The results showed that all samples (N=57) were gram-positive cocci (grape-like clusters) (Figure 12A). The biochemical test for *S. aureus* identification of all samples showed catalase-positive (visible bubble) (Figure 12B), coagulase-positive (fibrin formation) (Figure 12C), alkaline phosphatase positive and urease positive. Moreover, all samples showed sugar fermentation from trehalose, mannitol, mannose, maltose, lactose, sucrose, and Voges–Proskauer positive (Table 15 and Appendix D, Figure 5C). APISTAPH kit was used for further discrimination of *S. aureus* from other species of *Staphylococcus*. The characteristics of *S. aureus* from clinical isolates in the APISTAPH kit were shown in Figure13.

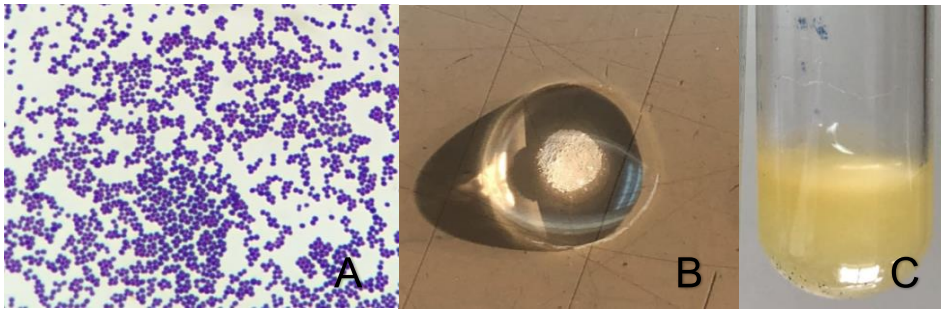


Figure 12. Identification of *Staphylococcal* species.
Gram positive cocci in clusters (*S. aureus*) under light microscope (magnification 100X) (A), and biochemical test; catalase positive (B) and coagulase positive (C)

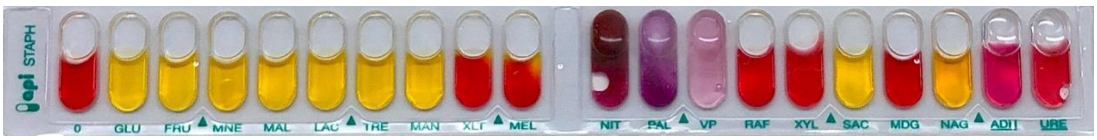


Figure 13. APISTAPH kit for identification of *S. aureus*

0: No substrate, GLU: D-Glucose, FRU: D-Fructose, MNE: D-Mannose, MAL: Maltose, LAC: Lactose, TRE: D-Trehalose, MAN: D-Mannitol, XLT: Xylitol, MEL: D-Melibiose, NIT: Potassium nitrate, PAL: β-naphthyl-acid phosphate, VP: Sodium pyruvate, RAF: Raffinose, XYL: Xylose, SAC: Sucrose, MDG: α-methyl-D-glucoside, NAG: N-acetyl-glucosamine, ADH: Arginine dihydrolase, URE: Urea

2.3 Confirmation of *S. aureus* in clinical samples

The presence of the *nuc* gene detected by the PCR technique was used to confirm the identification of *S. aureus*. The results showed that all *S. aureus* clinical isolates (N=57) from both children and adults with AD carried the *nuc* gene as shown in Figure 14.

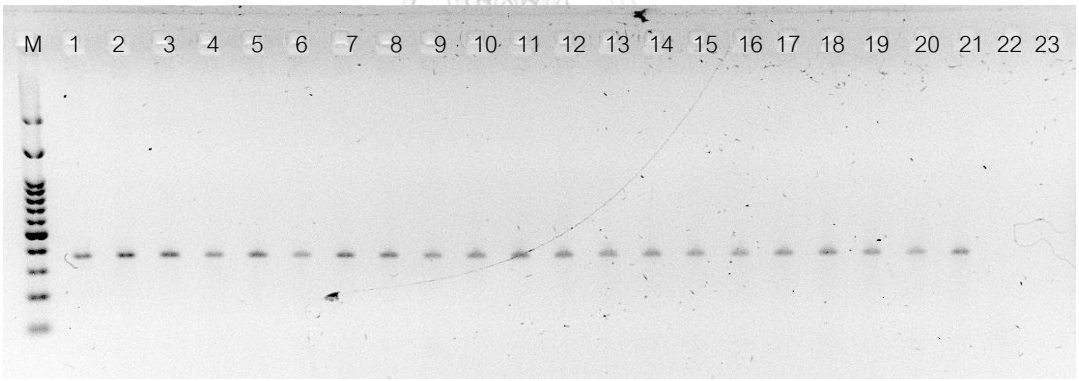


Figure 14. The representative PCR analysis for *nuc* gene of *S. aureus*
(10 children and 10 adults with AD) M, 100-bp plus DNA ladder; Lanes 1, Templates, *nuc*-like *(*359 bp*)* of S. aureus ATCC 25923; Lanes 2-21, S. aureus isolates harboring the *nuc*-like gene; Lanes 22-23, *S. epidermidis* isolates

S. epidermidis was found in 6 samples (20%), 4 samples (25%), and 5 samples (20%) of lesions, non-lesions, and nasal swabs in children, respectively. In adult patients, *S. epidermidis* was found in 2 samples (12.5%) of lesion swabs and 10 samples (38%) of nasal swabs, but they were not found in non-lesion swabs.

S. hominis was found in 2 samples (6.8%), 4 samples (25%), and 4 samples (16%) of lesions, non-lesions, and nasal swabs in children, respectively. In adult patients, *S. hominis* was found in 3 samples (18.8%), 2 samples (33%), and 3 samples (12%) of lesions, non-lesions, and nasal swabs, respectively.

S. haemolyticus was found in 1 sample (3%) and 2 samples (12.5%) of lesions and non-lesion swabs, respectively. However, they were not found in nasal swabs in children. In adult patients, *S. haemolyticus* was found in 2 samples (12.5%) of lesion swabs, 2 samples (33%) of non-lesion swabs, and 5 samples (19%) of nasal swabs.

S. xylosus were found in 1 sample (4%), 2 samples (7.7%) of nasal swabs in children and adult patients whereas *S. sciuri* were found only in 1 sample (6%) of lesion swabs in adults.

Coagulase-negative *Staphylococcus* spp. found in children and adults were shown in Figure 15, 16 and Table 16

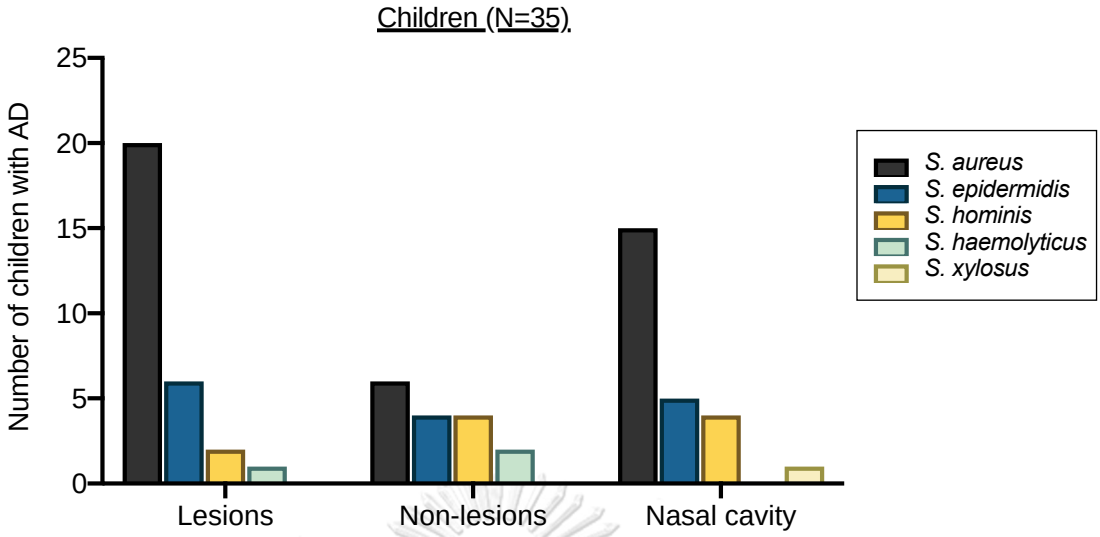


Figure 15. *Staphylococcus* spp. including *S. aureus* in children with AD.

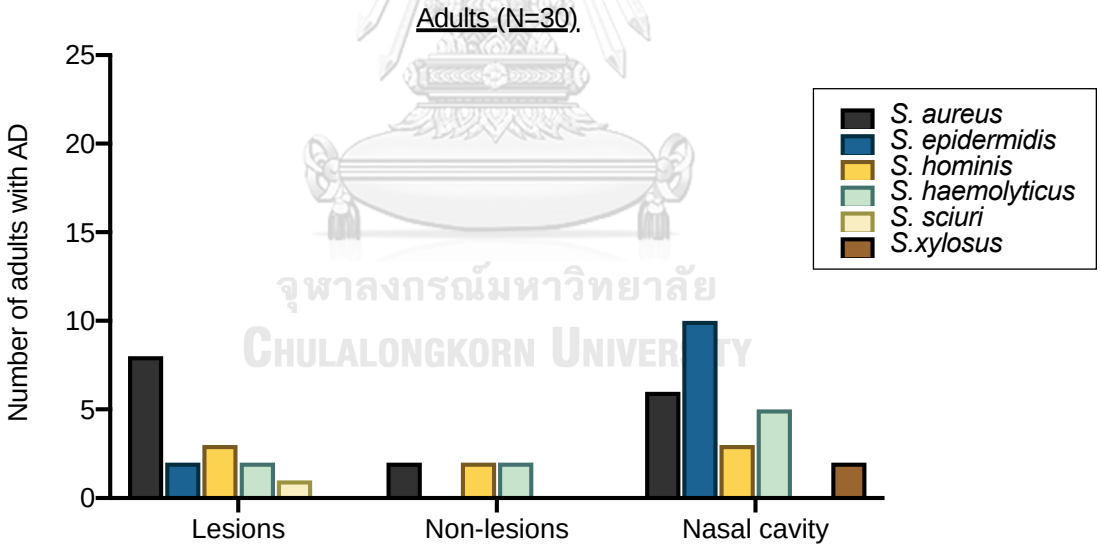


Figure 16. *Staphylococcus* spp. including *S. aureus* in adults with AD

Table 14. Screening for bacterial colonies from children and adults with AD by MSA and blood agar.

Other tests	Children (N=35)			Adults (N=30)		
	LS (N=29) (%)	NLS (N=16) (%)	NS (N=25) (%)	LS (N=16) (%)	NLS (N=6) (%)	NS (N=26) (%)
MSA, BA (+, +)	20 (69%)	6 (37.5%)	15 (60%)	8 (50%)	2 (33%)	6 (23%)
MSA, BA (-, +)	1 (3.44%)	2 (12.5%)	0 (0%)	2 (12.5%)	2 (33%)	5 (19%)
MSA, BA (-, -)	8 (28%)	8 (50%)	10 (40%)	6 (37.5%)	2 (33%)	15 (58%)

MSA = Mannitol salt agar, BA = Blood agar

* MSA, BA: +, + = yellow colony, β-hemolysis; -, + = pink or red colony, β-hemolysis; -, - = pink or red colony, γ-hemolysis

** LS = lesion swabs, NLS = non-lesion swabs and NS = nasal swabs

Table 15. Biochemical conventional method for identification of *S. aureus*

Species	Characteristics										
	Expression of		Novo	Sugar fermentation							
	Alk	Ure		Tre	Man	Mann	Ara	Mal	Lac	Suc	VP
S. aureus	+	d	-	+	+	+	-	+	+	+	+

d; 11-89% of strain positive., Alk: Alkaline phosphatase, Ure: Urease, Novo: Novobiocin resistance, Tre: Trehalose, Man: Mannitol, Mann: Mannose, A: Arabinose, Mal: Maltose, Lac: Lactose, Suc: Sucrose and VP: Voges–Proskauer.

Table 16. Identification of different species of *Staphylococcus* in children and adults with AD using other biochemical tests

Staphylococcus spp.	Children (N=35)			Adults (N=30)		
	LS (N=29) (%)	NLS (N=16) (%)	NS (N=25) (%)	LS (N=16) (%)	NLS (N=6) (%)	NS (N=26) (%)
S. aureus	20 (69%)	6 (37.5%)	15 (60%)	8 (50%)	2 (33%)	6 (23%)
S. epidermidis	6 (20%)	4 (25%)	5 (20%)	2 (12.5%)	0 (0%)	10 (38%)
S. hominis	2 (6.8%)	4 (25%)	4 (16%)	3 (18.8%)	2 (33%)	3 (12%)
S. haemolyticus	1 (3%)	2 (12.5%)	0 (0%)	2 (12.5%)	2 (33%)	5 (19%)
S. xylosus	0 (0%)	0 (0%)	1 (4%)	0 (0%)	0 (0%)	2 (7.7%)
S. sciuri	0 (0%)	0 (0%)	0 (0%)	1 (6%)	0 (0%)	0 (0%)

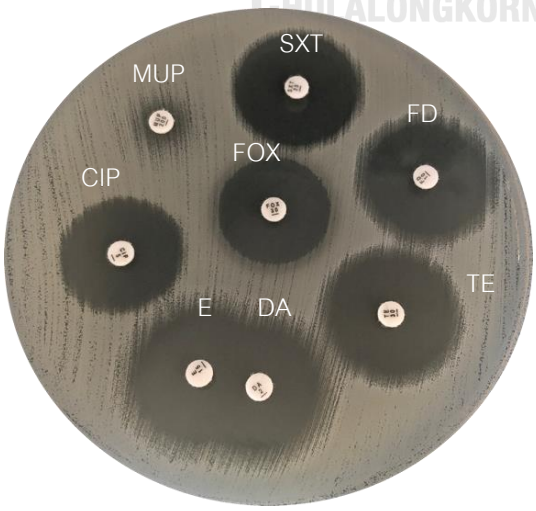
LS = lesion swabs, NLS = non-lesion swabs and NS = nasal swabs

3. The antibiotic susceptibility testing of *S. aureus* isolated from lesion swabs

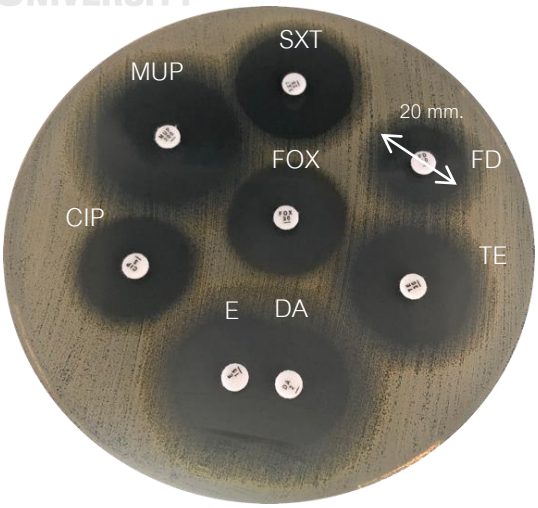
The susceptibility of *S. aureus* isolated from lesions of children (N=20) and adults (N=8) with AD was tested for different antibiotics using the disk diffusion method. The results showed that mupirocin-resistant *S. aureus* was found in 1 sample (5%) of lesion swabs in children (Figure 17A). There were 4 samples (20%) of lesion swabs in children that showed fusidic acid-resistant (Figure 17B). Unexpectedly, no methicillin-resistant *S. aureus* was found in children with AD in this study.

Interestingly, all *S. aureus* isolated from lesion swabs of adult patients (N=8) were sensitive to methicillin, mupirocin, and fusidic acid (as shown in Table 18).

Furthermore, *S. aureus* isolated from lesion swabs of children with AD were found resistance to tetracycline in 6 samples (30%), to ciprofloxacin in 1 sample (5%), clindamycin in 3 samples (15%), and erythromycin in 5 samples (25%). In adult patients, only 2 samples (25%) were resistant to tetracycline (shown in Table 17 and 18).



(A)



(B)

Figure 17. The antibiotic susceptibility testing of mupirocin-resistant and fusidic acid-resistant *S. aureus* in children using disk diffusion method.

(A) Inhibitory zone with mupirocin-resistant *S. aureus* (MUP: 0 mm.), and (B) inhibitory zone with fusidic acid-resistant *S. aureus* (FD: 20 mm.); Cefoxitin (FOX), clindamycin (DA), erythromycin (E), trimethoprim/sulfamethoxazole (SXT), ciprofloxacin (CIP), and tetracycline (TE), were interpreted using CLSI and EUCAST

4. Detection of antibiotic-resistant genes by PCR

4.1 Mupirocin-resistant S. aureus

As mentioned previously, mupirocin-resistant *S. aureus* was found in one sample from lesion swab in a child with AD, PCR and gel electrophoresis was used to detect the resistant gene (*mupA*) in this clinical isolated. The result demonstrated that *mupA* gene (*ileS-2*) was found as shown in Figure 18.

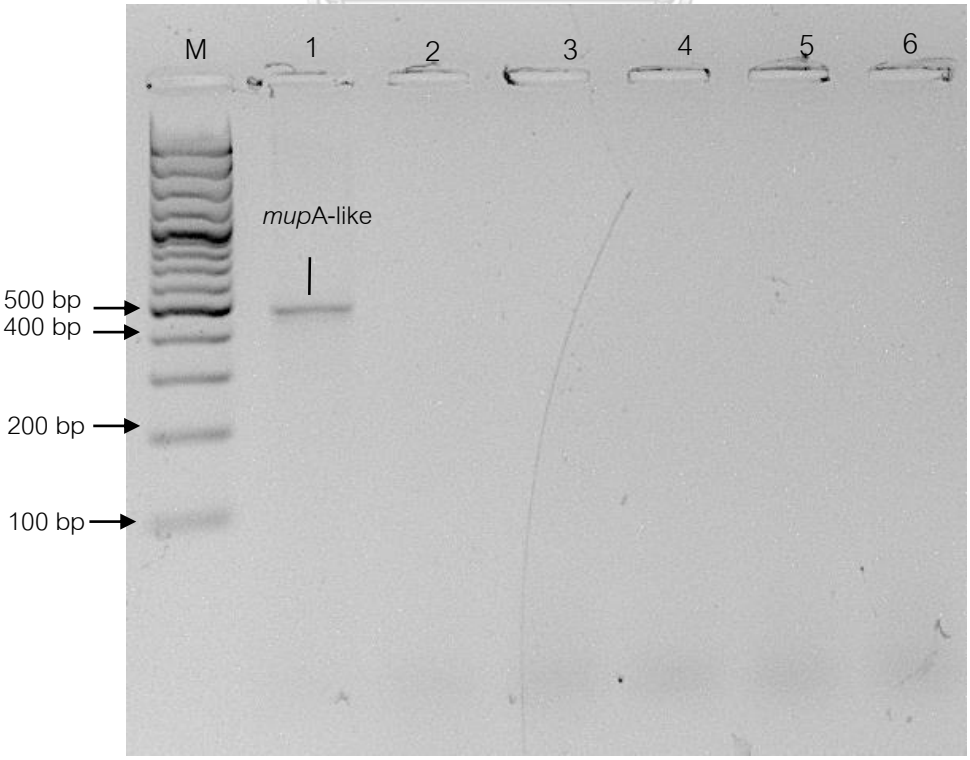


Figure 18. The PCR analysis of *mup*A gene

M, 100-bp plus DNA ladder; Lanes 1, Templates, mupirocin-resistant *S. aureus* harboring *mup*A-like (458 bp) gene; Lanes 2-6, mupirocin-susceptible *S. aureus*

4.2 Fusidic acid-resistant *S. aureus*

Fusidic acid-resistant *S. aureus* was found in 4 samples of lesion swabs in children with AD. PCR and gel electrophoresis was used to detect the *fus*A gene (Figure 19) and the mutated gene (*fus*A mutation) was then identified using nucleotide and amino acid sequencing.

The alignments of the amino acid sequence of EF-G patterns from four *S. aureus* samples of lesion swabs in children with AD (*fus*A number 4, 5, 7 and 20) were compared with wild-type EF-G pattern of *S. aureus* NCTC 8325 (GenBank accession no. NC_007795) using MultAlin program (Figure 20). The results showed that amino acid sequences of EF-G from four fusidic acid-resistant *S. aureus* were different from the sequence of wild-type. Surprisingly, novel EF-G patterns belonged to *fus*A numbers 4, 5, 7, and 20 were observed in this study. Two isolates possessed a mutation P404Q (404 proline/glutamine) and Q505H (505 glutamine/histidine) substitution, one isolate harboured mutation in S238A (238 serine/alanine) and C258W (258 cysteine/tryptophan), and one isolate showed a H457Y (457 histidine/tyrosine) mutation (Table 19).

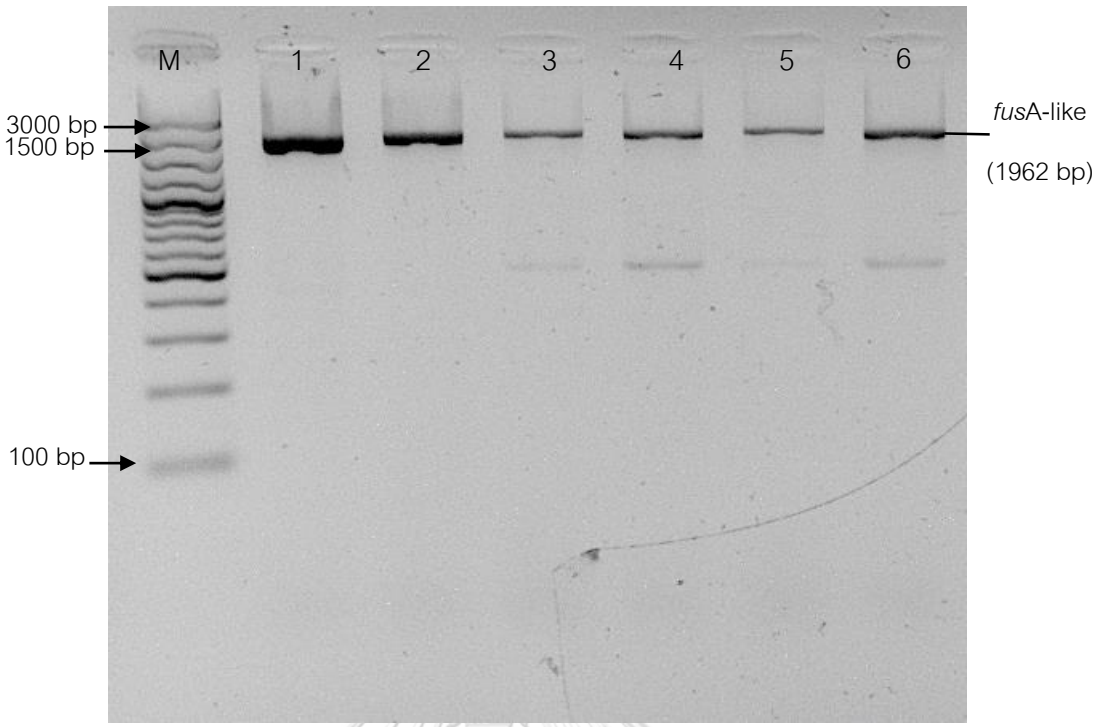


Figure 19. The PCR analysis of *fus*A gene

M, 100-bp plus DNA ladder; Lanes 1-2, Templates, *fus*A-like (1962 bp) of *S. aureus* ATCC 25923 and ATCC 2923; Lanes 3-6, fusidic acid-resistant *S. aureus* with *fus*A-like gene.

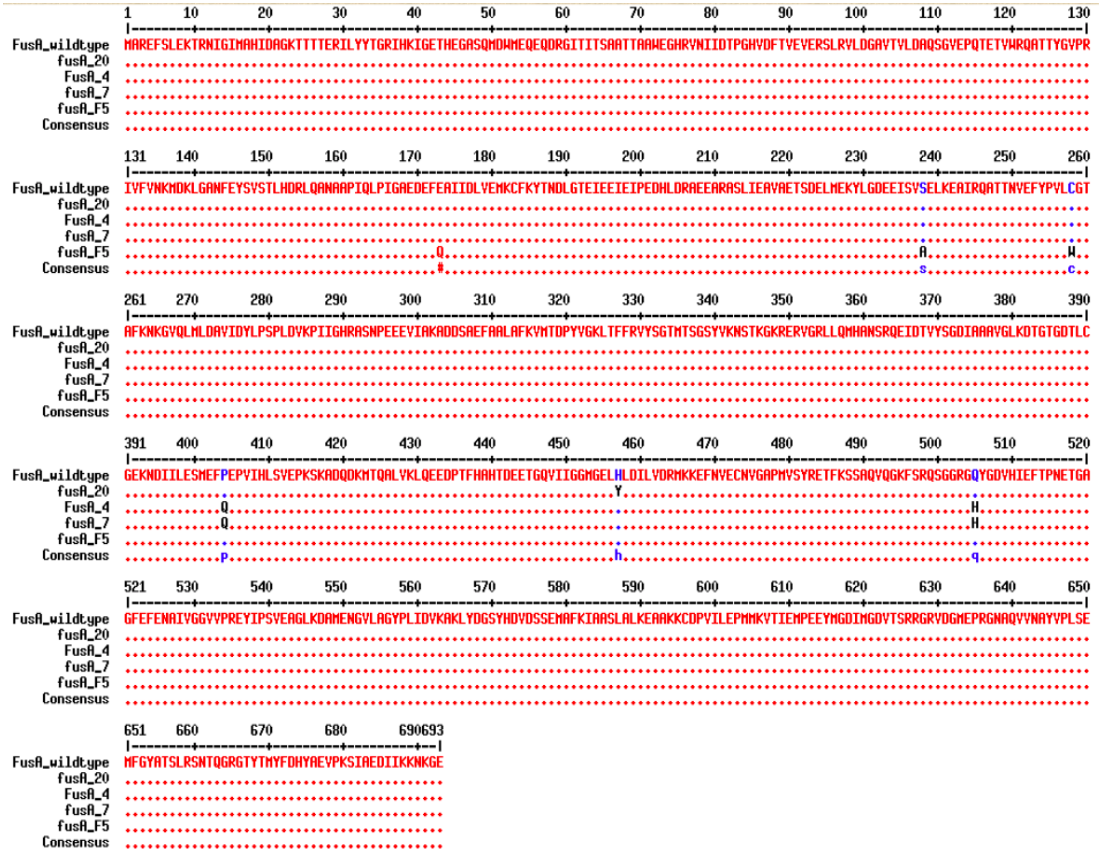


Figure 20. The alignments of amino acid sequences of EF-G patterns from four fusidic acid-resistant *S. aureus* (*fus*A number 4, 5, 7 and 20) isolated from lesions of children with AD and, *S. aureus* strain NCTC 8325 *(fus*A wildtype*)* (GenBank accession no. NC_007795). These were changes in H457Y of *fus*A_20, and in S238A and C258W of *fus*A_5. *fus*A_4 and _7 harboured mutation in P404Q and Q505H

* Consensus: indicate of the changed position

** P: pro (Proline), Q: Gin (Glutamine), S: Ser (Serine), A:Ala (Alanine), C: Cys (Cysteine), W: Trp (Tryptophan), H: His (Histidine), and Y: Tyr (Tyrosine)

Table 17. Antimicrobial susceptibility test of *S. aureus* from lesions of children with AD (N=20)

Antibiotics	Disk content	Number of isolates (N=20)		
		Sensitive N (%)	Intermediate N (%)	Resistant N (%)
Cefoxitin (FOX)	30 µg	20 (100)	0 (0)	0 (0)
Mupirocin (MUP)	200 µg	19 (95)	0 (0)	1 (5)
Fusidic acid (FD)	10 µg	16 (80)	0 (0)	4 (20)
Trimethoprim/sulfamethoxazole (SXT)	5 µg	20 (100)	0 (0)	0 (0)
Tetracycline (TE)	30 µg	14 (70)	0 (0)	6 (30)
Ciprofloxacin (CIP)	5 µg	19 (95)	0 (0)	1 (5)
Clindamycin (DA)	2 µg	17 (85)	0 (0)	3 (15)
Erythromycin (E)	15 µg	15 (75)	0 (0)	5 (25)

* Zone Diameter interpretive criteria ; FOX (S: ≥ 22, R:≤ 21 mm), MUP (S: any zone, R: no zone), FD (S: ≥ 24, R: < 24 mm), SXT (S: ≥ 16, I: 11-15, R: ≤ 10 mm) TE (S: ≥ 19, I: 15-18, R: ≤ 14 mm) , CIP (S: ≥ 21, I: 16-20, R: ≤ 15 mm), DA (S: ≥ 21, I: 15-20, R: ≤ 14 mm) and E (S: ≥ 23, I: 16-18, R: ≤ 13 mm)

**S: Sensitive, I: Intermediate, R: Resistant

Table 18. Antimicrobial susceptibility test of *S. aureus* from lesions of adults with AD (N=8)

Antibiotics	Disk content	Number of isolates (N=8)		
		Sensitive N (%)	Intermediate N (%)	Resistant N (%)
Cefoxitin (FOX)	30 µg	8 (100)	0 (0)	0 (0)
Mupirocin (MUP)	200 µg	8 (100)	0 (0)	0 (0)
Fusidic acid (FD)	10 µg	8 (100)	0 (0)	0 (0)
Trimethoprim/sulfamethoxazole (SXT)	5 µg	8 (100)	0 (0)	0 (0)
Tetracycline (TE)	30 µg	6 (75)	0 (0)	2 (25)
Ciprofloxacin (CIP)	5 µg	8 (100)	0 (0)	0 (0)
Clindamycin (DA)	2 µg	8 (100)	0 (0)	0 (0)
Erythromycin (E)	15 µg	8 (100)	0 (0)	0 (0)

* Zone Diameter interpretive criteria ; FOX (S: ≥ 22, R:≤ 21 mm), MUP (S: any zone, R: no zone), FD (S: ≥ 24, R: < 24 mm), SXT (S: ≥ 16, I: 11-15, R: ≤ 10 mm) TE (S: ≥ 19, I: 15-18, R: ≤ 14 mm) , CIP (S: ≥ 21, I: 16-20, R: ≤ 15 mm), DA (S: ≥ 21, I: 15-20, R: ≤ 14 mm) and E (S: ≥ 23, I: 16-18, R: ≤ 13 mm)

**S: Sensitive, I: Intermediate, R: Resistant

Table 19. Mutations of fusidic acid-resistant strain of *S. aureus* from lesions of children with AD using nucleotide and amino acid sequencing

Isolates no.	Fusidic acid-resistant *S. aureus*		
	Zone diameter of fusidic acid (mm) (S: ≥ 24, R: < 24)	Nucleotide mutation	Amino acid substitution
*fusA*_4	20 (R)	CAG → AAG	404 Pro → Gin
		AAT → ACT	505 Gin → His
*fusA*_5	20 (R)	TTC → TGC	238 Ser → Ala
		TAA → AAA	258 Cys → Trp
fus*A*_7	20 (R)	CAG → AAG	404 Pro → Gin
		AAT → ACT	505 Gin → His
*fusA*_20	0 (R)	TTC → TTT	457 His → Tyr

* R: Resistant, S: Sensitive, P: pro (proline), Q: Gin (Glutamine), S: Ser (Serine), A: Ala (Alanine), C: Cys (Cysteine), W: Trp (tryptophan), H: His (Histidine), Y: Tyr (Tyrosine)

CHAPTER VI

DISCUSSION

Atopic dermatitis (AD) is a chronic and relapsing inflammatory skin disease which is one of the most common skin problems across the world. This condition is found up to 10-30% of the population especially in developed countries (23). There is no gender preference, but the children are highly affected more than adults. It has been reported that the incidence of AD is approximately 6-13% in Thai children (154), and 2-10% in Thai adults (25). However, the incidence of the disease in both children and adults have been increasing especially in patients with a personal history or family history of allergies such as allergic asthma and allergic rhinitis (155, 156). The etiology of AD is still unclear but evidence shows that genetic predisposition, immune dysregulation, and environmental factors are involved in the immunopathogenesis of AD (157).

One of the most important predisposing and aggravating factors of AD is a skin bacterial infection, particularly caused by *S. aureus*. These gram-positive cocci bacteria not only relate to the pathogenesis of the disease but also trigger disease recurrence and flare. *S. aureus* is facultative anaerobic bacteria and usually found as a part of the microbial colonization of the skin and nasal cavity (158). In AD patients, it's found that abnormal epidermal barrier combined with decreased expressions of antimicrobial

peptides and pattern recognition receptors (PRRs) favored *S. aureus* colonization of the affected skin. Moreover, AD is associated with increased expression of Th2 cytokines (IL-4, IL-13, and IL-31) (159). These cytokines reduce epidermal differentiation and therefore reduce filaggrin expression and AMPs expression (160). For example, IL-31 induces severe itch resulting in an increase in *S. aureus* colonization and infection in AD patients (23). In recent studies, it has been reported that *S. aureus* was found up to 70-90% on the lesions of AD patients (161). Also, it has been proved that bacterial toxin (e.g. exogenous proteases, Staphylococcal superantigens, δ-Toxin, etc.) released by *S. aureus* also induce acute exacerbation of the disease (105).

In this study, children and adults with AD patients who visited at our tertiary care hospital during 2018-2020 were recruited, and demographic data showed that the median ages of children and adults diagnosed with AD were 1 and 23 years old, respectively. The majority of both children and adults with AD had a personal/family history of allergic diseases, particularly allergic rhinitis. Furthermore, all children, and adults completed at least 3 out of 4 major criteria based on the Hannifin and Rajka clinical criteria, and the most common minor criteria found in both groups were xerosis, early age of onset, and orbital darkening/itch when sweating, respectively. These findings are consistent with the previous studies (162), suggesting that AD is still a

common skin condition found in infants and young adults with dry skin (xerosis cutis) and personal/family history of allergy.

According to the severity scores, the average severity scores (EASI and SCORAD) is higher in children when compared with adult patients in this study. This finding is consistent with a previous study by Son JH, *et al*. (163), but the contrast with another study showing that the severity score was higher and moderate symptoms are often observed in adult patients (162). This is probably due to differences between genetic disposition and environmental factors of their population and our study.

As mentioned previously, *S. aureus* is an important aggravating factor of AD, this study, therefore, aimed to identify *S. aureus* colonization in our Thai population. Clinical samples from lesions, non-lesions, and nasal cavity from children and adults with AD are swabbed and collected using MSA and blood agar plates (164). MSA agar containing 7.5% NaCl is a selective media for the identification of *S. aureus* (165), because only a few bacteria (e.g. *S. aureus*), not gram-negative bacteria, can tolerate and grow on the agar with high salt concentration. Moreover, *S. aureus* ferments mannitol in MSA and decreases pH media (pH=6.9) (143). The acidic conditions caused by fermentation turns phenol red (pH indicator) to yellow color as seen as a yellow halo around colonies on the agar plate (166). Like other species of *Staphylococcus*, such as coagulase-negative bacteria; *S. epidermidis*, *S. hominis*, *S. haemolyticus*, *S. xylosus* and *S. sciuri*,

hardly ferment mannitol, the red color of MSA around these bacterial colonies remains unchanged and appears in small pink or red colonies (144).

Besides, blood agar is a commonly used medium for bacterial culture in the laboratories. The hemolysis zone of red blood cells on the agar caused by toxins of certain types of bacteria (e.g. *S. aureus*) helps in bacterial identification (147). For example, the hemolysis zone by *Staphylococcus* spp. is classified into 3 types; β□□α□□and γ hemolysis. Bacteria producing hemolysins (e.g. *S. aureus* and *S. haemolyticus*) induce complete red blood cell hemolysis resulting in a clear zone around bacterial colonies on the blood agar (β-hemolysis or complete hemolysis) (147, 167-169). α-hemolysis or partial hemolysis observed as a green zone surrounding bacterial colonies because hemoglobin is converted to biliverdin (170). γ-hemolysis (non-hemolysis) is found in non-producing hemolysin, coagulase-negative *Staphylococcus* spp. (e.g. *S. epidermidis*, *S. hominis*, *S. xylosus* and *S. sciuri)* and characterized as a bacterial colony without a zone of hemolysis (147).

The conventional methods (e.g. coagulase test, catalase, and biochemical tests) and rapid methods (APISTAPH kit) for the identification of *S. aureus* were performed in this study. However, the PCR technique is used to reconfirm *S. aureus* identification using the *nuc* gene which is a locus for specific identification of coagulase-positive *Staphylococci* (*S. aureus*, *S. intermedius*, *S. Pseudintermedius*, etc.) (150). The result

showed that *S. aureus* is the most common bacteria isolated from lesions, non-lesions, and nasal swabs in patients with AD, followed by *S. epidermidis* and *S. hominis* (Table 10). Noticeably, lesions of AD patients were mostly colonized by *S. aureus* (69% in children and 50% in adults) than non-lesions and nasal cavities which were consistent with previous studies (161, 171). Nonetheless, it appeared that *S. aureus* found in nasal swabs of children with AD (60%) are greater than those found in nasal swabs of adult patients. This is probably because children with AD usually have a higher chance to receive *S. aureus* from mothers as a previous study showed that mothers with *S. aureus* colonization could pass this pathogen from their nasal cavities through their children particularly in a young infant during breastfeeding (172, 173). One limitation of this experiment was that *S. aureus* found in all 3 areas of AD patients may not representative and the phenotype of the bacteria probably needs further investigated.

Systemic antibiotics and topical agents are standard treatments for *S. aureus* colonization and infection in AD patients. β-lactam antibiotics (e.g. penicillin, cloxacillin, first-generation cephalosporin) in oral or intravenous administration are the first-line systemic treatment of *S. aureus* infection (174). β-lactam is an effective bactericidal agent that disturbs covalent binding to penicillin-binding proteins (PBPs) on the bacterial cell wall formation (175). However, the prevalence of methicillin-resistant *S. aureus* (MRSA) is first reported in 1968 (176), and it has been constantly increasing

every year (73). Previous studies showed that MRSA colonization is strongly associated with the disease severity of AD (75). Therefore, this study investigated the prevalence of MRSA and aimed to determine the correlation between the number of colonies and disease severity using EASI and SCORAD. Unexpectedly, MRSA isolates are not found in both children and adults with AD, and this is probably because of an inadequate number of patients (N=65). Our results are consistent with our previous study which showed a decrease in MRSA in patients at the King Chulalongkorn Memorial Hospital (177). Therefore, more patient recruitment is required; however, this study demonstrated that some *S. aureus* isolates found in lesions of both children and adults with AD are resistant to other antibiotics, such as tetracycline, ciprofloxacin, clindamycin, and erythromycin as shown in Table 11 (178). This finding probably suggests that these antibiotics should be avoided in some children and adults with AD.

Topical antibiotics were widely used for cutaneous bacterial infections in AD patients particularly caused by *S. aureus*. Mupirocin in ointment and fusidic acid in cream/ointment are frequently used to treat this bacterial infection. However, the prevalence of mupirocin and fusidic acid-resistant *S. aureus* has been reported (73). In theory, mupirocin resistance is classified into 2 types. Low-level mupirocin resistance (MIC: 8-64 μg/ml) caused by point mutations in isoleucyl RNA synthetase gene (*ileS*) (56, 179), and high-level mupirocin resistance (MIC: ≥ 512 μg/ml) due to acquisition of

plasmid-mediated *mup*A (*ileS*2) that encodes a novel isoleucyl RNA synthetase (180). The low-level mupirocin resistance is not clinically significant as topical mupirocin remains effective in patients infected with this phenotype. Therefore, this study focused on the high-level mupirocin resistance and found that one mupirocin-resistant *S. aureus* clinical isolate is observed in the lesion of a child with AD (Figure 17A). Moreover, *mup*A (*ileS*2) gene is identified and confirmed high-level mupirocin resistant mechanism in this isolate. Although the incidence of mupirocin-resistant *S. aureus* is rare in our population, this study suggests that dermatologists should consider other topical antibiotics in children treated with topical mupirocin without clinical response. However, mupirocin-resistant *S. aureus* is not observed in lesions of adults with AD, probably because of infrequent use of topical mupirocin or less disease severity without the necessity of topical antibiotics used in the adult group.

Fusidic acid-resistant *S. aureus* is caused by point mutations within the chromosomal *fus*A gene encoding elongation factor G (EF-G) (136). It has been demonstrated that mutational changes in the *fus*A gene result in amino acid substitutions in EF-G protein leading to fusidic acid resistance (181). In this study, four *S. aureus* clinical isolates from lesions of children with AD were fusidic acid-resistance (Table 11). To confirm the importance of observed amino acid changes in EF-G in these fusidic acid-resistant *S. aureus* isolates, *fus*A gene mutation was identified using

nucleotide and amino acid sequence analysis (Table 13). The results showed alleles encoding EF-G acquisition with the exchanges of P404Q (404 proline/glutamine), Q505H (505 glutamine/histidine), S238A (238 serine/alanine), C258W (258 cysteine/tryptophan) and H457Y (457 histidine/tyrosine) are constructed by site-directed mutagenesis in these four clinical isolates. Interestingly, these mutations have never been reported elsewhere. A few studies were demonstrating a substitution of the amino acid (H457) in EF-G (22, 138, 181). Therefore, further investigation is required to identify the mechanisms of fusidic acid resistance in these isolates. However, this study found no fusidic acid-resistant *S. aureus* in the lesions of adults with AD, probably because of infrequent use of topical fusidic acid or less disease severity without the necessity of topical antibiotic used in adult patients.

Conclusions

1. *S. aureus* was the most common *Staphylococcus* spp. found at the lesions of both children and adults with AD.
2. Unexpectedly, MRSA was not found in the lesions of AD patients in our population.
3. Mupirocin- and fusidic acid-resistant *S. aureus* were found only in children with AD in our population.

4. The expressions of resistance genes (*mup*A, *fus*A mutation) were identified in mupirocin- and fusidic acid-resistant *S. aureus* isolated from children with AD.
5. Mutations of *fus*A gene change amino acid sequences in EF-G protein of fusidic acid-resistant *S. aureus* were novel and have never been reported elsewhere.
6. Children with AD had clinically more severe than adult patients and these children probably required antibiotic treatment leading to a higher incidence of antibiotic resistance

Future plans

1. To increase clinical samples from children and adults with AD.
2. To investigate the mechanism of *fus*A mutations in those 4 fusidic acid-resistant *S. aureus* clinical isolates.
3. To investigate the interaction between *Staphylococcus* spp. and *S. aureus* in AD patients.

APPENDIX A

REAGENTS AND INSTRUMENTS

Reagents

Agarose	(Vivantis, USA)
Alkaline phosphatase	(Sigma, USA)
Arabinose	(Sigma, USA)
dNTPs	(Invitrogen, Brazil)
EDTA	(Amresco, USA)
FOX, DA, E, SXT, CIP, TE, FD, and MUP disks	(Oxoid, UK)
Glycerol	(Sigma, USA)
Nucleic Acid Gel Stain	(Biotium, USA)
Lactose	(Oxoid, UK)
LB broth	(Pronadisa, Spain)
Maltose	(Sigma, USA)
Mannitol	(Difco, USA)
Mannitol salts agar	(Oxoid, UK)
Mannose	(Sigma, USA)
Mueller-Hinton II agar	(BBL, USA)
NaCl	(Merck, Germany)
NaOH	(Sigma, USA)

Sheep blood agar	(Clinag, UK)
Sucrose	(Sigma, USA)
Taq DNA Polymerase	(Invitrogen, Brazil)
Trehalose	(Sigma, USA)
Trytic soy agar	(BBL, USA)
Trytic soy broth	(BBL, USA)
Urease	(Oxoid, UK)
Voges–Proskauer (VP)	(Sigma, USA)
100 bp DNA ladder	(GeneDirex, Taiwan)

Instruments

Automatic pipette	(Gilson, Lyon, France)
Autoclave	(Hirayama, Japan)
Camera Gel Doc™MZL	(BIO-RAD, USA)
Hot air oven	(Memmert, Germany)
McFarland Densitometer	(Bio-San, USA)
Microcentrifuge	(Eppendrof, Germany)
Proflex Veriti MiniAmp Miniamp Plus SimpliAmp	(Thermo Fisher, USA)
Vortex mixer	(Wiggen-Hauser, USA)

APPENDIX B

MEDIA AND ANTIBIOTIC SOLUTION PREPARATION

1. Mannitol Salt agar (Oxoid, UK)

Mannitol Salt agar (111 g) was suspended in 1000 ml of distilled water and boiled until it dissolved completely. The agar was autoclaved at 121°C for 15 min and mixed well before pouring into the plates.

2. Muller-Hinton agar (BBL, USA)

Muller-Hinton agar (38 g) was suspended in 1000 ml of distilled water and boiled until it dissolved completely. The agar was autoclaved at 121°C for 15 min and mixed well before pouring into the plates.

3. Tryptic soy broth (BBL, USA)

Tryptic soy broth (30 g) of the dehydrated medium was suspended in 1000 ml of distilled water. The broth was autoclaved at 121°C for 15 min and stored at 4°C.

4. Tryptic soy agar (BBL, USA)

Tryptic soy agar (40 g) of the dehydrated medium was suspended in 1000 ml of distilled water. The agar was autoclaved at 121°C for 15 min and mixed well before pouring into the plates and stored at 4°C.

5. 1.5% agarose gel

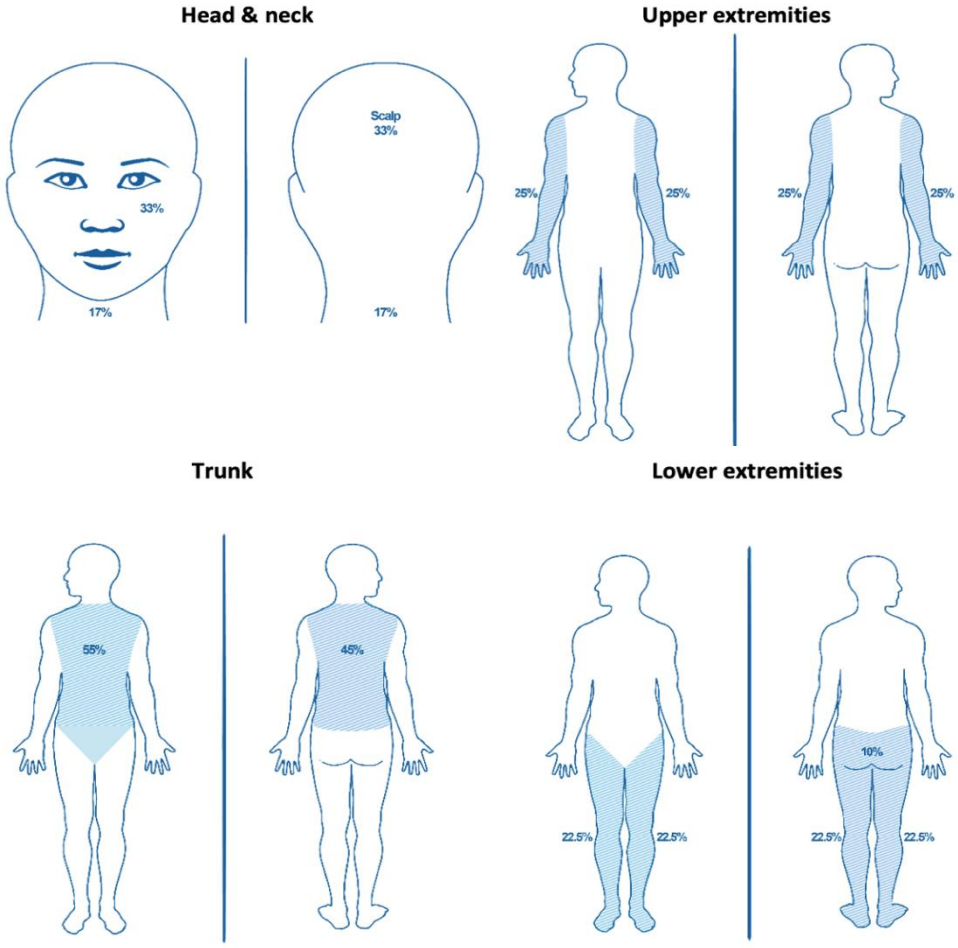
Agarose gel (1.5 g) was suspended and dissolved by heating in 100 ml of 0.5X TBE buffer.

APPENDIX C

DATA COLLECTION AND CASE RECORD FORM (CRF)

1. Eczema Area and Severity Index (34)

1.1 Extent of eczema per body region (4 body regions)



% involvement	0	1-9%	10 - 29%	30 - 49%	50 - 69%	70 - 89%	90 - 100%
Region score	0	1	2	3	4	5	6

Figure 1. Extent of eczema per body region (4 body regions).

1.2 lesion severity atlas (34)

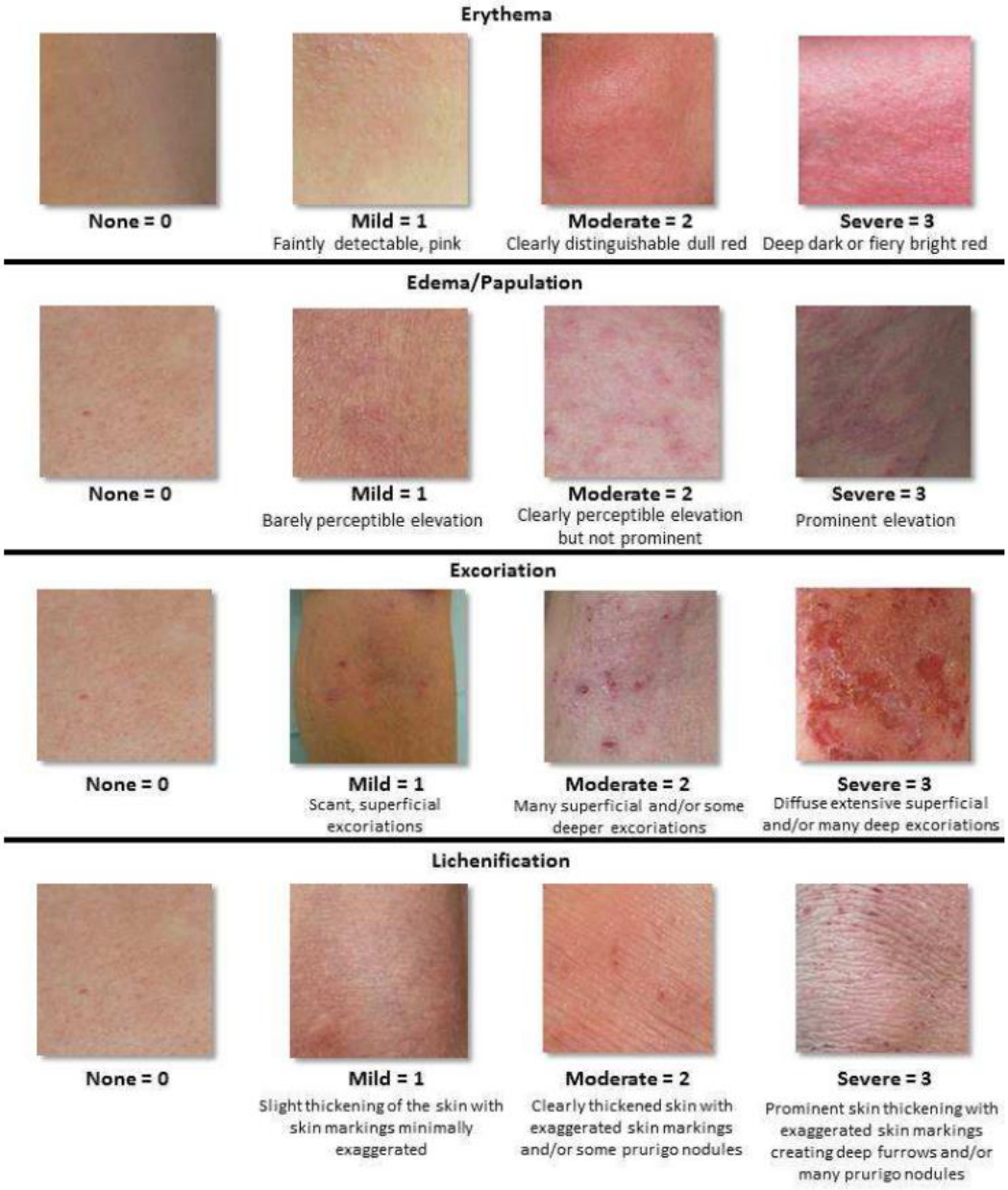


Figure 2. Lesion severity atlas

1.3 Eczema Area and Severity Index (EASI) case report form (34)

Table 1. Eczema Area and Severity Index (EASI) case report form.

☐ **Age < 8 yrs**: Eczema Area and Severity Index (EASI) case report form

Body region	Erythema	Ederma/Papulation	Excoriation	Lichenification	Region score	Multiplier	score
1. Head/neck	(+	+	+	)	x	x 0.1	
2. Trunk	(+	+	+	)	x	x 0.3	
3. Upper extremities	(+	+	+	)	x	x 0.2	
4. Lower extremities	(+	+	+	)	x	x 0.4	
						The final EASI score (total score = 0-72)	

☐ **Age ≥ 8 yrs**: Eczema Area and Severity Index (EASI) case report form

Body region	Erythema	Ederma/Papulation	Excoriation	Lichenification	Region score	Multiplier	score
1. Head/neck	(+	+	+	)	x	x 0.2	
2. Trunk	(+	+	+	)	x	x 0.3	
3. Upper extremities	(+	+	+	)	x	x 0.2	
4. Lower extremities	(+	+	+	)	x	x 0.4	
						The final EASI score (total score = 0-72)	

2. Severity Scoring of Atopic Dermatitis (SCORAD) (33)

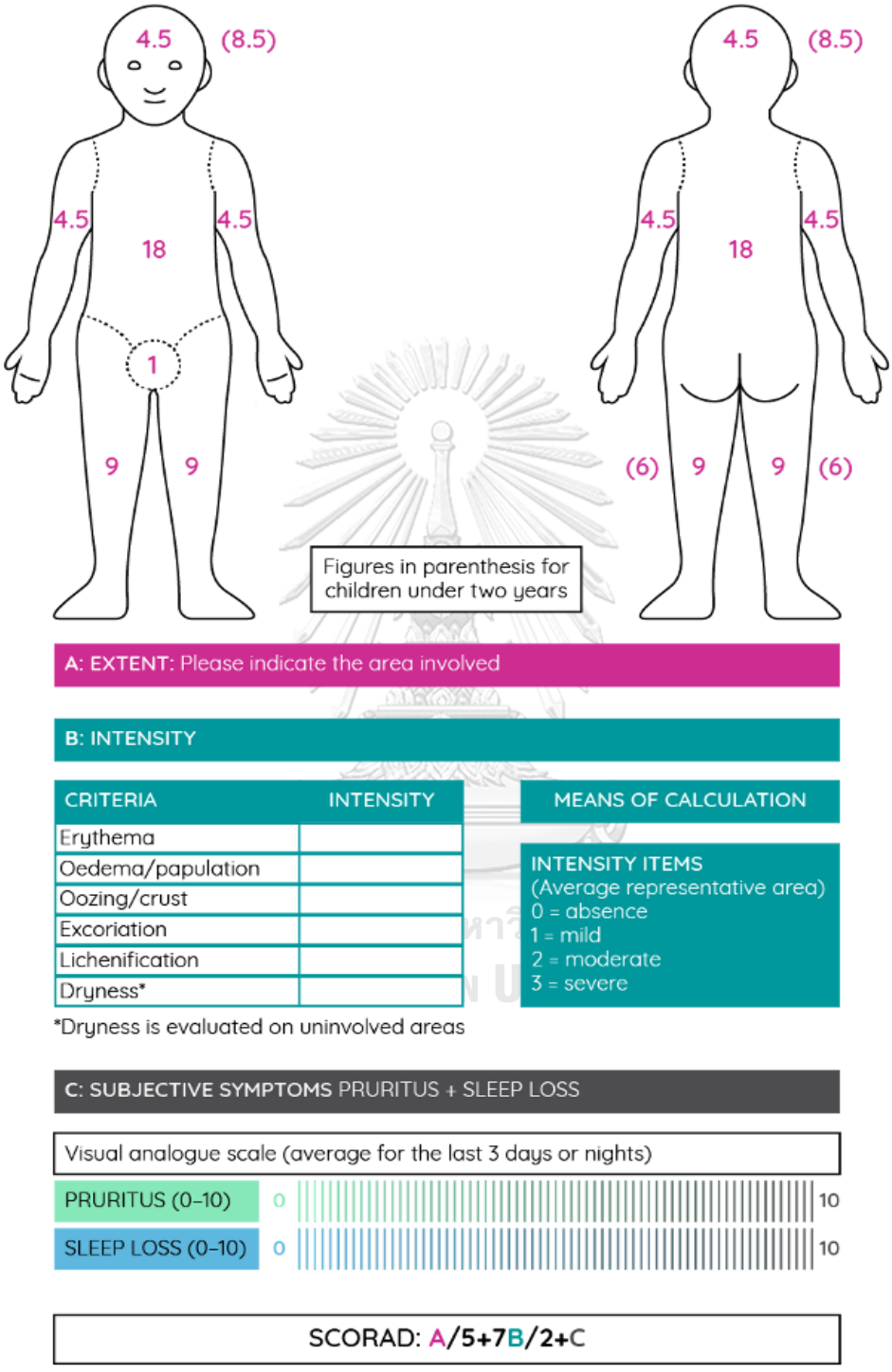


Figure 3. Severity Scoring of AD (SCORAD)

3. Hanifin and Rajka Diagnostic Criteria 1980 for Atopic dermatitis (30-32)

Table 2. Hanifin and Rajka Diagnostic Criteria 1980 for Atopic dermatitis
Major criteria: Must have three or more of: 1. Pruritus 2. Typical morphology and distribution ☐ Flexural lichenification or linearity in adults ☐ Facial and extensor involvement in infants and children 3. Chronic or chronically relapsing dermatitis 4. Personal or family history of atopy (asthma, allergic rhinitis, atopic dermatitis)
Minor criteria: Should have three or more of: 1. Xerosis 2. Ichthyosis, palmar hyper linearity, or keratosis pilaris 3. Immediate (type 1) skin-test reactivity 4. Raised serum IgE 5. Early age of onset 6. Tendency toward cutaneous infections (especially *S. aureus* and herpes simplex) or impaired cell-mediated immunity 7. Tendency toward non-specific hand or foot dermatitis 8. Nipple eczema 9. Cheilitis 10. Recurrent conjunctivitis 11. Dennie-Morgan infraorbital fold 12. Keratoconus 13. Anterior subcapsular cataracts 14. Orbital darkening 15. Facial pallor or facial erythema 16. Pityriasis alba 17. Anterior neck folds

APPENDIX D

THE RESULTS OF ALL TESTS IN THIS STUDY

1. Gram staining

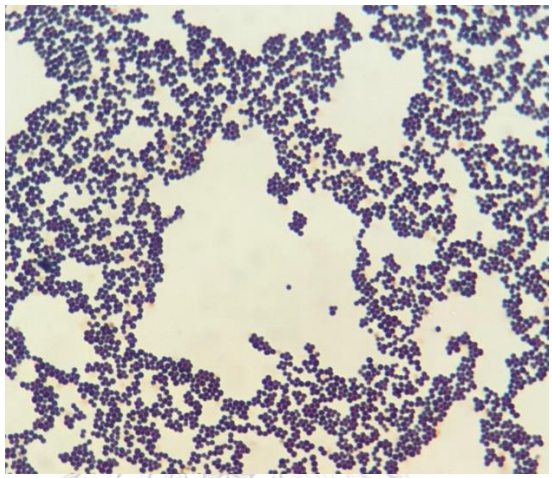


Figure 4. Gram staining of *Staphylococcus aureus* isolates

2. Biochemical tests

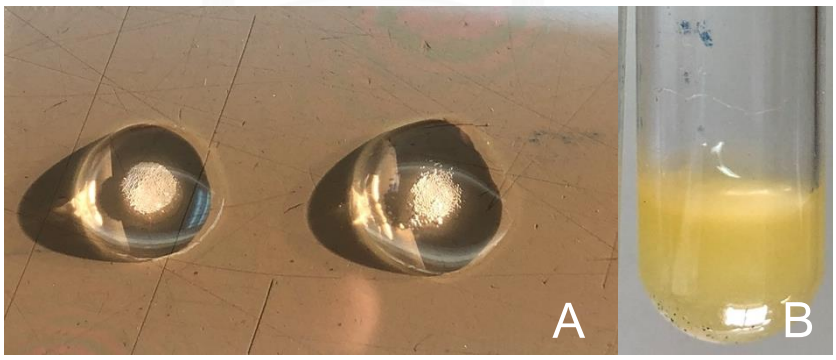


Figure 5. Biochemical tests

3. Biochemical test for confirm all species of *Staphylococcus*

Table 3. Biochemical test of identification *Staphylococcus* spp.

Species	Characteristic										
	Expression of		Novo	Sugar fermentation from:							
	Alk	Ure		Tre	Man	Mann	A	Mal	Lac	Suc	VP
S. aureus	+	d	-	+	+	+	-	+	+	+	+
S. epidermidis	+[m]	+	-	-	-	+	-	+	d	+	+
S. haemolyticus	-	-	-	+	d	-	-	+	d	+	+
S. hominis	-	+	-	d	-	-	-	+	d	+	+
S. xylosus	d	+	+	+	+	+	d	+	d	+	+

d; 11-89% of strain positive., Alk: Alkaline phosphatase, Ure: Urease, Novo: Novobiocin resistance, Tre: Trehalose, Man: Mannitol, Mann: Mannose, A: Arabinose, Mal: Maltose, Lac: Lactose, Suc: Sucrose and VP: Voges–Proskauer.

4. The susceptibility test of *S. aureus* with different antibiotics using disk diffusion method.

Table 4. Zone diameter of antimicrobial agents of *S. aureus* (N=28) in AD patients.

Isolates no. (N=28)	Zone diameter (mm.) of antimicrobial agents							
	Cefoxitin (30 µg.)	Bactrim (5 µg.)	Fusidic acid (10 µg.)	Mupirocin (200 µg.)	Tetracycline (30 µg.)	Ciprofloxacin (5 µg.)	Clindamycin (2 µg.)	Erythromycin (15 µg.)
CA001	S (26)	S (31)	S (28)	R (10)	S (25)	S (24)	S (26)	S (26)
CD004	S (26)	S (30)	R (20)	S (33)	S (27)	S (23)	S (24)	S (26)
CD007	S (27)	S (30)	R (20)	S (32)	S (27)	S (24)	S (25)	S (26)
CA003	S (28)	S (30)	R (20)	S (33)	R (8)	S (27)	S (25)	S (26)
CA004	S (27)	S (28)	S (30)	S (32)	R (9)	S (27)	S (24)	R (9)
CD001	S (28)	S (26)	S (28)	S (33)	S (26)	S (26)	R (0)	R (0)
CD017	S (27)	S (30)	S (31)	S (33)	R (9)	S (25)	R (0)	R (12)
CD018	S (26)	S (30)	S (29)	S (32)	S (27)	S (28)	R (18)	R (0)
CA002	S (28)	S (29)	S (34)	S (33)	S (27)	S (30)	S (27)	S (27)
CD008	S (30)	S (28)	S (28)	S (38)	S (23)	S (34)	S (27)	S (27)
CD014	S (27)	S (29)	S (29)	S (34)	S (27)	S (26)	S (25)	S (28)

Isolates no. (N=28)	Zone diameter (mm.) of antimicrobial agents							
	Cefoxitin (30 µg.)	Bactrim (5 µg.)	Fusidic acid (10 µg.)	Mupirocin (200 µg.)	Tetracycline (30 µg.)	Ciprofloxacin (5 µg.)	Clindamycin (2 µg.)	Erythromycin (15 µg.)
CD021	S (30)	S (27)	R (0)	S (31)	S (29)	S (26)	S (25)	S (28)
CD022	S (27)	S (32)	S (30)	S (34)	S (28)	S (31)	S (29)	S (27)
CD024	S (28)	S (30)	S (31)	S (35)	R (8)	S (26)	S (25)	S (29)
CD025	S (28)	S (33)	S (32)	S (37)	S (30)	R (6)	S (24)	R (6)
CD027	S (30)	S (31)	S (31)	S (35)	R (9)	S (24)	S (26)	S (29)
CD028	S (27)	S (30)	S (27)	S (31)	R (9)	S (27)	S (25)	S (27)
CD029	S (28)	S (29)	S (29)	S (34)	S (26)	S (27)	S (28)	S (26)
CD030	S (29)	S (29)	S (30)	S (31)	S (25)	S (24)	S (28)	S (27)
CD031	S (31)	S (30)	S (30)	S (35)	S (27)	S (29)	S (27)	S (26)
A003	S (26)	S (30)	S (28)	S (31)	R (10)	S (25)	S (24)	S (26)
A008	S (29)	S (30)	S (30)	S (35)	R (9)	S (28)	S (25)	S (27)
A013	S (27)	S (30)	S (28)	S (32)	S (25)	S (26)	S (22)	S (27)
A014	S (28)	S (30)	S (29)	S (33)	S (28)	S (26)	S (26)	S (26)
A020	S (27)	S (29)	S (29)	S (32)	S (27)	S (27)	S (26)	S (25)

Isolates no. (N=28)	Zone diameter (mm.) of antimicrobial agents							
	Cefoxitin (30 µg.)	Bactrim (5 µg.)	Fusidic acid (10 µg.)	Mupirocin (200 µg.)	Tetracycline (30 µg.)	Ciprofloxacin (5 µg.)	Clindamycin (2 µg.)	Erythromycin (15 µg.)
A021	S (26)	S (28)	S (26)	S (31)	S (23)	S (27)	S (23)	S (25)
A022	S (28)	S (33)	S (30)	S (35)	S (27)	S (29)	S (27)	S (28)
A023	S (30)	S (31)	S (28)	S (31)	S (28)	S (26)	S (26)	S (27)

Note: S; Susceptibility, R: Resistant

APPENDIX E

DNA CODON

One- and Three-Letter symbols for the amino acid

A	Ala	Alanine
B	Asx	Asparagine or aspartic acid
C	Cys	Cysteine
D	Asp	Aspartic acid
E	Glu	Glutamic acid
F	Phe	Phenylalanine
G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
K	Lys	Lysine
L	Leu	Leucine
M	Met	Methionine
N	Asn	Asparagine
P	Pro	Proline
Q	Gin	Glutamine
R	Arg	Arginine
S	Ser	Serine

T	Thr	Threonine
V	Val	Valine
W	Trp	Trytophan
Y	Tyr	Tyrosine
Z	Gix	Gin or Glu

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