Original article

Direct and synergistic hemolytic reactions triggered by indoor airborne mold

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Objective Adverse human health effects from indoor fungi probably result from exposure to spores, fungal fragments, and their metabolites. Some fungi take advantage of hemolytic factors production for acquisition of the iron required for growth and survival in the host environment. To examine common types of airborne molds and their hemolytic reactions, air samples were collected from buildings in each of three seasons (hot, cool, and rainy) during the period January 2008 to October 2009.

Methods Samples were obtained using a modified air collector and the settle plate method. Isolated molds were tested for their ability to produce hemolytic factors directly on complete solid media supplemented with either human or sheep blood. Along with direct hemolytic activity, *Aspergillus* or *Penicillium* isolates were simultaneously tested for their cooperative hemolytic (CAMP-like) reactions with four common bacteria found in the respiratory tract.

Results *Cladosporium* was the predominant mold throughout the year, but it was found at higher concentrations in the cool season. It was followed in abundance by *Aspergillus*, but was found in higher concentrations in the hot and the rainy seasons. One third of the common mold-tested isolates, including *Aspergillus*, *Cladosporium*, *Fusarium*, *Penicillium*, and non-sporulating mold, could lyse human blood better than sheep blood at 28ºC and/or 37ºC with the exception that all *Cladosporium* and some *Penicillium* tested could not grow at 37ºC. Most of the tested mold isolates had synergistic CAMP-like reactions with *Staphylococcus aureus* or *Streptococcus pneumoniae* on sheep blood agar, but reactions with *Streptococcus pneumoniae* were varied.

Conclusions Common indoor airborne mold found throughout the year were *Cladosporium*, *Aspergillus*, *Penicillium*, *Fusarium*, and non-sporulating mold. One third of the common mold-tested isolates had hemolytic activities on human blood better than sheep blood. Most of the *Aspergillus* and *Penicillium* isolates had synergistic hemolytic reactions on sheep blood with *Staphylococcus aureus*. **Chiang Mai Medical Journal 2018;57(3):143-50.**

Keywords: hemolytic, synergistic, *Aspergillus*, *Penicillium*, *Staphylococcus aureus*

Introduction

Microfungi are commonly found in the environment. Many species are able to disperse their spores and hyphal fragments into the air because of their small size. Mold spores are ubiquitous in the outdoor air but can also be found indoors as they enter the indoor envi-

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ronment from the outdoor air. In the home or workplace where there is moisture or moldy material, the level of exposure is higher than in other indoor areas. In many reports of building-related illnesses, fungi were suggested as a useful indicator of indoor air quality (1). In an indoor environment, the toxic effects and irritations probably are the result of a chronic or high level of exposure to airborne spores, hyphal fragments, and their metabolites. Many species have been reported to produce metabolites into indoor air including volatile organic compounds, mycotoxins, and hemolysins. Hemolysin generally acts by creating pores in cell membranes and is produced by many indoor molds (2-4). One advantage for microorganisms of producing hemolytic factors is the acquisition of iron which is required for their growth and survival in the host environment. Vesper and colleague (4) demonstrated that stachylysin, which is produced by the toxigenic fungus *Stachybotrys chartarum*, caused pores in sheep red blood cell membranes. In addition, when injected into *Lumbricus terrestis*, the erythrocruorin hemoglobin was released resulting in a lethal effect (5). Hemolytic activities have also been reported in various pathogenic fungi and some endemic fungi (2,6) such as *Histoplasma capsulatum*, *Cryptococcus neoformans*, *Trichophyton rubrum*, *Trichophyton mentagrophytes*, *Candida albicans* (7), and *Malassezia* sp. (8). Along with the hemolysis resulting directly from hemolytic factors, Schaufuss and colleagues (9) demonstrated the enhancement of hemolysis indirectly via cooperation between some dermatophytes and skin bacterial flora. A potential cooperative interaction between mold and bacteria in the upper human airway may be an alternative avenue for lysing red blood cells and other lining cells. In this study, we examined the types of airborne mold found during three different seasons in academic buildings in Chiang Mai Province in northern Thailand. We determined on solid media whether those common molds could secrete factors capable of lysing red blood cells directly to acquire iron and also whether they could cooperate with some common bacteria found in the respiratory tract, e.g., *Staphylococcus aureus*, to indirectly lyse red blood cells.

Methods

Air sampling and mold identification

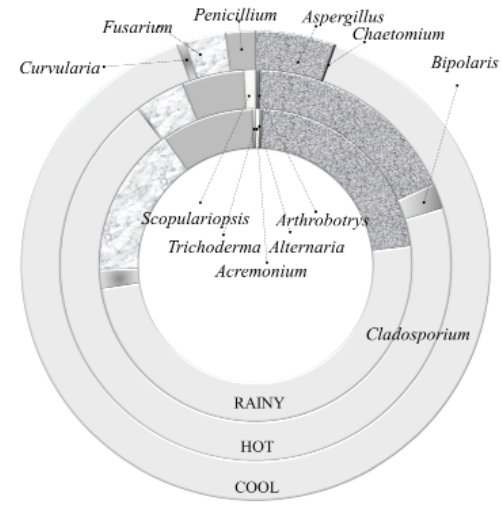
To determine the quantities and types of airborne mold found in different seasons, indoor air samples were collected using the settle plate method and a modified air collector. The air collector used in this study was modified from a small electric sweeper in which the suction speed was adjusted equal that of a modified Anderson sampler (10). The aerosol fungal fragments and spores contained in an air volume of 0.025 m^3 were transferred directly via suction onto the surface of a Sabouraud dextrose agar (SDA) plate over a period of 30 seconds. The air samples were obtained from locations approximately three meters apart in a closed atmosphere. Simultaneously, SDA plates were placed at the same spot as the modified air collector and left there for 15 minutes. Air sampling was performed in January 2008, April 2008, and October 2009, representing the cool, hot, and rainy seasons, respectively. Three complete air sample collections were done in each of the three seasons from three different rooms which had a possibility of moisture or visible mold including two lecture halls (B and C) and a microbiology preparation room (D). Two additional air samples were obtained in cool and the hot seasons from two additional workplaces in academic buildings (A and E). All sampling sites were located in buildings of the Faculty of Medicine and the Faculty of Associated Medical Science, Chiang Mai University. The relative humidity (RH) and temperature (ºC) in each room were measured simultaneously once during air sampling. Molds were counted, isolated, and identified by genera based on the characteristics of their colonies and microscopic morphologies.

Hemolytic activity assay

To determine whether certain airborne molds could lyse red blood cells on solid media, mold isolates were inoculated on SDA or Tryptic soy agar (TSA) plates supplemented with 5% human blood (Blood Bank of Maharaj Hospital, Chiang Mai University) or sheep blood [(defibrinated with citrate phosphate dextrose adenine (CPDA-1)]. The plates were incubated at one of three different temperatures, 28ºC, 37ºC, or 28ºC for three days and were then continuously grown at 37ºC (28ºC was increased to 37ºC), and observed daily for ten or more days. Hemolytic activity was demonstrated by clear zones of hemolysis surrounding or beneath the colony.

Cooperative hemolytic (CAMP-like) reaction assay

To determine whether cooperative (CAMP-like) hemolytic reactions can be triggered by *Aspergillus* or *Penicillium* with some common bacteria found in the respiratory tract, CAMP-test, modified from Schaufuss et al(9), was performed using strains of airborne *Aspergillus* or *Penicillium*, four clinical bacterial isolates (*Staphylococcus aureus* ATCC25923, *Streptococcus pneumoniae* ATCC49619, *Streptococcus pyogenes* GA3 and *Staphylococcus epidermidis* SP350), and TSA supplemented with red cells from either human or sheep blood. After the tested mold was inoculated and incubated at 28°C for three days, each type of bacterium was streaked in a straight line across the plate approximately 15-25 mm distant from the mold colony and was then incubated for an additional seven days. As a known CAMP-like positive reaction with *S. aureus*, a *Rhodococcus equi* (soil isolate) was streaked in a straight line two to three cm in length at a right angle of bacterial line and incubated for an additional 24 hours. Cooperative hemolytic reaction was demonstrated by a hemolysis zone between the mold colony and the bacterial colony.



	COOL	HOT	RAINY
°C	23.4-26.3 (24.5)	30.9-33.8 (32.6)	28.2-31.1 (29.2)
%RH	43.5-56.2 (51.1)	45.5-49.3 (47.1)	56.3-60.4 (58.0)
CFU/m³	512-2892 (1423±1285)	360-1888 (919±843)	280-724 (605±284)

Figure 1. Average seasonal indoor mold ratios observed in three rooms (B, C, and D) in the cool, hot, and rainy seasons

Results

Amounts and types of mold in academic rooms and workplaces

The average colony-forming units (CFU)/m^3 (mean and standard deviation) of the total culturable indoor mold colonies calculated from the three rooms (B, C and D) with air samples collected in the cool, hot and rainy seasons using a modified air collector were calculated to be approximately 1423±1285 CFU/m^3 (cool season), 919±843 CFU/m^3 (hot season), and 605±284 CFU/m^3 (rainy season). Molds collected included *Alternaria*, *Aspergillus*, *Bipolaris*, *Chaetomium*, *Cladosporium*, *Curvularia*, *Fusarium*, *Penicillium*, *Trichoderma*, and non-sporulating mold. *Cladosporium* was the predominant genus, followed by *Aspergillus*, *Fusarium*, and *Penicillium* species (Figure 1). To isolate pure mold colonies for hemolytic activity assay, simultaneous air sampling with a settle plate was performed. Using the settle plate method, not only pure colonies but some additional types of mold which could not be isolated using the modified air collector were able to be isolated including *Acremonium*, *Arthrobotrys*, and *Scopulariopsis*.

The most common types of mold found in the indoor atmosphere included *Cladosporium*, *Aspergillus*, *Penicillium*, and *Fusarium*, with variations in the amounts and types among the rooms and in different seasons. *Cladosporium* predominated in all seasons but was highest in the cool season. *Aspergillus* was most prevalent in the hot and rainy seasons, as were *Penicillium*, *Fusarium*, and non-sporulating molds.

Hemolytic activity of indoor mold on human and sheep red blood cells

Approximately one-third of each species of common indoor mold isolates tested, including *Aspergillus*, *Cladosporium*, *Curvularia*, *Fusarium*, *Penicillium* as well as non-sporulating molds, were identified as hemolytic activity positive with human blood (Figure 2a). Characteristics of HA positive strains incubated for ten days varied in terms of genera and isolates (Figure 2c). Although *Cladosporium* was the predominant genus and had hemolytic activity on human blood agar at both 28ºC and 28ºC increased to 37ºC, they were all heat intolerant when initially germinated at 37ºC. In contrast, although there were fewer *Aspergillus*

Table 1. Characteristics of hemolytic activity of common molds during growth on human blood agar at different temperature of 28°C and/or 37°C

		28°C			37°C			28°C to 37°C		
		3d	6d	≥9d	3d	6d	≥9d	3d	6d	≥9d
Aspergillus		-	-	-	-	~	+	-	~	n
Cladosporium	1	-	-	+	x	x	x	-	~	~
	2	~	-	+	x	x	x	-	~	~
	3	-	~	+	x	x	x	-	~	+
	4	-	~	~	x	x	x	-	~	~
	5	-	~	~	x	x	x	-	~	+
	6	-	-	-	x	x	x	-	~	+
	7	-	-	-	x	x	x	-	~	+
Fusarium	1	-	-	-	-	-	~	-	-	+
	2	-	-	-	-	-	-	-	-	-
Penicillium	1	-	-	~	-	~	+	-	~	+
	2	-	-	+	x	x	x	-	+	+
	3	~	~	~	~	+	+	-	+	+
	4	-	+	+	x	x	x	~	+	+

*;spore germinated initially at 28°C for 3 days and continuously grown at 37°C , -; no activity, ~; incomplete hemolysis, +; complete hemolysis, -; no growth, n-not done

and *Penicillium* than *Cladosporium*, they could lyse red blood cells at 28ºC, 37ºC, or 28ºC increased to 37ºC, with the exception of some *Penicillium* isolates (Table 1).

Synergistic hemolytic reactions of indoor mold with *S. aureus* **or** *S. pneumoniae*

Synergistic cooperative hemolytic reactions of *Aspergillus* or *Penicillium* with *S. aureus* on SBA were shown as a half-moon-shaped hemolysis within the α-hemolytic zone of bacteria, e.g., *Aspergillus* (Figure 2d-ii) and *Penicillium* (Figure 2d-vii). A synergistic zone of an *Aspergillus* sp. also occurred on HBA, but with quite much less reaction (Figure 2d-i). The synergistic hemolytic zones of *Aspergillus* with *S. pneumoniae* were shown as a slightly half-moon shaped clear zone below the bacterial colonies (Figure 2d-iii). No CAMP-like reaction could be detected between *Aspergillus* or *Penicillium* and *S. epidermidis* or *S. pyogenes*.

In Figure 2b, a CAMP-like test with sheep blood, synergistic hemolytic reactions were identified in most isolates of *Aspergillus* or *Penicillium* with *S. aureus*, but the results were varied when tested with *S. pneumoniae*, especially in *Penicillium*. In HA positive strains, all tested *Aspergillus* and two thirds of *Penicillium* had CAMP-like reactions. Interestingly, most HA negative strains of *Aspergillus* and *Penicillium* also had synergistic hemolytic reactions with *S. aureus*, but the reactions were weak. Only some of the isolates of *Aspergillus*, but none of *Penicillium*, had reactions with *S. pneumoniae*. No CAMP-like reaction could be detected with human blood in any of the tested fungal strains with the exception of a weak reaction with one strain (Figure 2d-i).

Discussion

The airborne mold count varied normally with the geographic location and seasonal period. The average culturable indoor mold counts found in this study were approximately 1423±1285, 919±843 and 605±284 CFU/m^3 collected in the cool, hot, and rainy seasons, respectively. A large variety of fungal species can be found in indoor air. The most common genera are *Alternaria*, *Aspergillus*, *Cladosporium* and *Penicillium*. A study in Chiang Mai Province, Northern Thailand by Tankaew et al (11) reported that *Aspergillus*, *Cladosporium*, *Mucor*, *Rhizopus*, *Penicillium*, *Scopulariopsis*, *Fusarium*, and non-sporulating mold were isolated from hospital operating rooms and classrooms before cleaning. In this report, *Acre-*

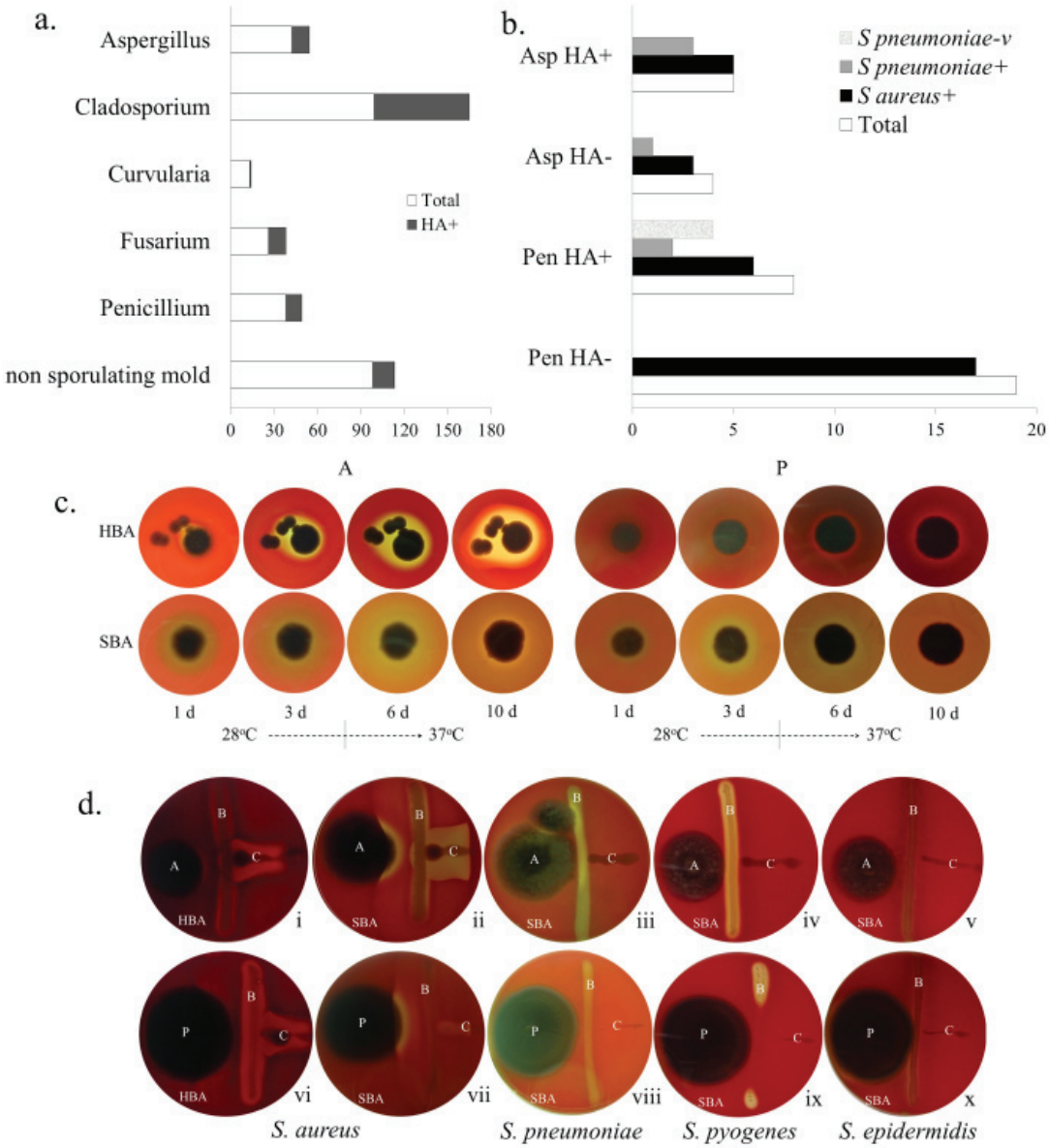


Figure 2. Hemolytic reactions of common indoor mold on complete solid media supplemented with human (HBA) and/or sheep (SBA) blood cells. a; ratio between total tested mold and HA positive incubated at 28°C to 37°C. b; ratio between HA pos. and HA neg. of *Aspergillus* and *Penicillium*, and synergistic hemolytic reactions with *Staphylococcus aureus* and *Streptococcus pneumoniae* at 37°C. In an *Aspergillus* sp. (A) and a *Penicillium* sp. (P), c; characteristics of HA in 10 days. d; cooperative hemolytic reactions between mold (A or P) and four distinct bacteria (B) on SBA (ii-v and vii-x) and/or HBA (i and vi) showed synergistically clear zones only with *S. aureus* on SBA, *Rhodococcus equi* (C) was used as a control.

monium, *Alternaria*, *Arthrobotrys*, *Aspergillus*, *Bipolaris*, *Chaetomium*, *Cladosporium*, *Curvularia*, *Fusarium*, *Penicillium*, *Scopulariopsis*, *Trichoderma*, and non-sporulating mold were found in indoor air throughout the year. In the rainy season, although the higher relative humidity may be suitable for mold growth, some spore and fungal fragments have been shown

to be less aerosolized than in other seasons.

Mold spores and small hyphal fragments can produce allergens, volatile organic compounds, and metabolites including mycotoxin and hemolytic factors. Their fragments and metabolites can cause diseases in atopic and immunocompromised individuals and may cause toxic effects and irritation in healthy individuals. Although indoor mold may cause adverse health effects, the severity of the impact depends upon the type and amount of mold present as well as the susceptibility and sensitivity of the individual to exposure to mold. The effect of the level of mold exposure on health was reviewed by Eduard (12) that respiratory symptoms and airway inflammation in highly exposed working populations began to appear at exposure levels of 10^5 spores/m^3. Thus, at mold exposure levels of diverse mold species less than 10^5 spores/m^3, such as found in this study, might not affect fairly on health in non-sensitized populations.

In this study, the spores of isolated indoor molds were germinated initially at room temperature for three days and then grown for a further seven days at 37°C to observe their hemolytic activity. We found one half the common airborne indoor molds presented greater hemolytic activities when cultured on solid media supplemented with human blood than with sheep blood. Of these, *Cladosporium* spp. were hemolytically active, but they could not survive and grow at 37°C, whereas most species of *Aspergillus* and *Penicillium* could grow and hemolyze well at this human body temperature as reported previously (3,13,14). Some common airborne *Aspergillus* and *Penicillium* species have been reported to produce hemolytic factors (2) including *A. fumigatus*, *A. niger*, *A. terreus*, *A. flavus*, *A. clavatus*, *A. nidulans*, *A. oryzae*, and *P. chrysogenum*. Well characterized Asp-hemolysin produced by *A. fumigatus*, which has often been suggested as being involved in fungal virulence, was found to be a major secreted protein during growth in minimal medium despite no hemolysis, cytotoxic and attenuation in virulence in the mutant strain (15). However, hemolysins produced by *A. niger* and *P. chrysogenum* have been found to have adverse effects such as toxicity in mouse neuron cells (14) and increased production of macrophage inflammatory protein-2 (MIP-2) (13), respectively. Sago-contaminated mold, particularly *Penicillium citrinum*, based on the frequency of isolation from sago starch and their hemolytic activities on sheep and human blood agar, has been identified as a possible candidate in the etiology of sago hemolytic disease (SHD) which affects people in rural Papua New Guinea (16).

In addition to the direct production of hemolytic factor, the ability to lyse red blood cells through CAMP-like reactions might be an alternative for molds to acquire iron. In this study, although most of the *Aspergillus* and *Penicillium* tested could lyse human blood better than sheep blood, cooperative hemolytic reactions of many *Aspergillus* and *Penicillium* with bacteria could be generated well on SBA. Most *Aspergillus* and *Penicillium*, either hemolytic activity-negative or -positive, could generate synergistic CAMP-like reactions with *S. aureus*, but the reactions with *S. pneumoniae* were varied. Synergistic hemolytic reactions between dermatophytes and *S. aureus*, *Staphylococcus intermedius*, or *Listeria ivanovii* have been previously reported (6); however, this study is the first report of synergistic hemolytic reaction between *Aspergillus* or *Penicillium* and *S. pneumoniae*. Further study on CAMP-like reactions between more strains of indoor or pathogenic molds and *S. pneumoniae* are needed to confirm this interaction.

In conclusion, *Cladosporium* was predominant throughout the year, with higher levels in the cool season, while *Aspergillus* as well as *Penicillium*, *Fusarium*, and non-sporulating mold were found to be more abundant in the hot and rainy seasons. Hemolytic activity could be detected on human blood agar better than sheep blood in many types of common airborne molds, predominantly *Cladosporium*. Most of the *Aspergillus* and *Penicillium* isolates tested not only had hemolytic activity but also had synergistic hemolytic reactions on sheep blood with some common bacteria, particularly *S. aureus*. The information obtained in this study may be helpful in understanding

indoor airborne mold exposure for public health monitoring. Further studies on purification and characterization of hemolytic factors and CAMP-like factors produced by certain airborne *Aspergillus* and *Penicillium* may lead to a better understanding of the involvement of these fungi in terms of cytolytic activities in red cells and other cells in the respiratory tract.

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ปฏิกิริยาการสลายเม็ดเลือดแดงโดยตรงและเสริมกันที่เกิดจากราในอาคาร

มาลี เมฆาประทีป, ศิริพร จองแก และ นงนุช วณิตย์ธนาคม
ภาควิชาจุลชีววิทยา คณะแพทยศาสตร์ มหาวิทยาลัยเชียงใหม่

วัตถุประสงค์ ผลกระทบต่อสุขภาพจากราภายในอาคารอาจเกิดจากผู้ที่อาศัยอยู่สัมผัสกับเชื้อรารวมทั้งสารเมทาบอไลท์ที่ราสร้าง ราบางชนิดอาจสร้างสารช่วยสลายเม็ดเลือดแดงซึ่งเป็นข้อได้เปรียบที่ช่วยให้ราได้รับธาตุเหล็กที่จำเป็นต่อการอยู่รอดและเจริญเติบโตในคน ได้ทำการเก็บตัวอย่างราจากอากาศภายในอาคารในสามฤดูระหว่างเดือนมกราคม พ.ศ. 2551 ถึงเดือนตุลาคม พ.ศ. 2552 เพื่อศึกษาชนิดของราในอากาศที่พบบ่อยและทดสอบปฏิกิริยาหรือฤทธิ์ในการสลายเม็ดเลือดแดงของราเหล่านี้

วิธีการศึกษา เก็บตัวอย่างโดยใช้วิธีการดูดอากาศลงบนจานอาหารด้วยเครื่องดูดฝุ่นประยุกต์และโดยการเปิดจานอาหารโดยตรง ทดสอบฤทธิ์สลายเม็ดเลือดแดงของราที่แยกได้ โดยการเพาะเลี้ยงรา และ/หรือเพาะเลี้ยงราที่เจริญใกล้กันกับเชื้อแบคทีเรียที่พบได้บ่อยในทางเดินหายใจ บนอาหารวุ้นแข็งที่ผสมเม็ดเลือดคนหรือเม็ดเลือดแดงแกะที่อุณหภูมิ 28 องศาเซลเซียส และ/หรือ 37 องศาเซลเซียส

ผลการศึกษา คลาโดสปอเรียมปนเปื้อนในอากาศปริมาณมากกว่าราชนิดอื่นตลอดปีโดยเฉพาะในฤดูหนาว ส่วนแอสเปอร์จิลลัสพบปริมาณน้อยกว่าแต่ค่อนข้างมากในฤดูร้อนและฤดูฝน หนึ่งในสามของชนิดราที่เพาะแยกได้มีฤทธิ์สลายเม็ดเลือดแดงได้โดยตรง โดยสลายเม็ดเลือดแดงของคนได้ดีกว่าของแกะ ราเหล่านี้ได้แก่ แอสเปอร์จิลลัส คลาโดสปอเรียม ฟิวซาเรียม เพนนิซิเลียม และรากลุ่มที่ไม่สร้างสปอร์ โดยเกือบทั้งหมดมีฤทธิ์สลายเม็ดเลือดแดงได้ที่อุณหภูมิ 28 องศาเซลเซียส และ/หรือ 37 องศาเซลเซียส ยกเว้นคลาโดสปอเรียมทุกสายพันธุ์และเพนนิซิเลียมบางสายพันธุ์ไม่พบการเจริญเติบโตที่ 37 องศาเซลเซียส นอกจากนี้การทดสอบเพิ่มเติมในแอสเปอร์จิลลัสหรือเพนนิซิเลียม ยังพบว่าขณะราเจริญเติบโตใกล้กันกับเชื้อแบคทีเรีย มีฤทธิ์สลายเม็ดเลือดแดงแกะแบบเสริมกัน โดยเฉพาะระหว่างรากับสแตปฟิโลคอคคัส ออเรียส ส่วนปฏิกิริยาเสริมกันกับสเตรปโตคอคคัส นิวโมเนียอี พบได้แต่ยังคงมีความแปรปรวนในการทดสอบ

สรุป ราที่พบบ่อยในอากาศในอาคารตลอดปี ได้แก่ คลาโดสปอเรียม แอสเปอร์จิลลัส ฟิวซาเรียม เพนนิซิเลียม และรากลุ่มที่ไม่สร้างสปอร์ หนึ่งในสามของชนิดราที่เพาะแยกได้มีฤทธิ์สลายเม็ดเลือดแดงได้โดยตรง โดยสลายเม็ดเลือดแดงของคนได้ดีกว่าของแกะ และพบว่าขณะราแอสเปอร์จิลลัสหรือเพนนิซิเลียม เมื่อเจริญเติบโตใกล้กันกับสแตปฟิโลคอคคัส ออเรียส มีฤทธิ์สลายเม็ดเลือดแดงแกะแบบเสริมกัน **เชียงใหม่เวชสาร 2561;57(3):143-50.**

คำสำคัญ: ฤทธิ์สลายเม็ดเลือดแดง ฤทธิ์เสริมกัน แอสเปอร์จิลลัส เพนนิซิเลียม สแตปฟิโลคอคคัส ออเรียส