

Survival of Methicillin-Resistant Coagulase-Negative Staphylococci from Floor Dust

Plykaeow Chaibenjawong^{1*}, Kannipa Tasanapak¹, Sutthirat Sitthisak¹,
Thaniya Wongwan², Suttipalin Suwannakul³

1. Department of Microbiology and Parasitology, Faculty of Medical Science, Naresuan University, Phitsanulok 65000, Thailand.
2. Department of Community Medicine, Faculty of Medicine, Naresuan University, Phitsanulok 65000, Thailand.
3. Department of Preventive Dentistry, Faculty of Dentistry, Naresuan University, Phitsanulok 65000, Thailand.

Abstract

MRCoNS (Methicillin-Resistant Coagulase-Negative Staphylococci) are a group of opportunistic pathogens that possess abilities to survive desiccated environments and resist many antimicrobial drugs. From floor dust collected within community and hospital buildings, 78 penicillin-resistant staphylococci were isolated and confirmed to be coagulase negative. Their antibiotic resistances were investigated according to CLSI guidelines and the presence of the *mecA* gene. We found 72 isolates (92.31%) classified as MDR (multidrug resistance), of which 9 isolates (11.54%) indicated resistance against 7 classes of antibiotics. Fifty-nine isolates (75.64%) were specified as MRCoNS; 8 isolates (10.26%) from the community, and 51 isolates (65.38%) from the hospital. All isolates survived a week of desiccation with only 2 log¹⁰CFU decrease, and were identified as *S. warneri*, *S. arlettae*, *S. equorum*, *S. haemolyticus*, *S. saprophyticus*, *S. capitis*, and *S. cohnii*. Among them, *S. capitis* was found only from the hospital, while *S. cohnii* was the most prevalent species recovered from both places.

Nearly all of them possess characteristics of MDR or MRCoNS. With ability-to-survive-desiccation on floor dust, they could become a hidden threat as a crucial source of antibiotic-resistant genes, which is needed to be monitored to prevent the emergence of multidrug resistant staphylococci with high virulence potential.

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Introduction

CoNS (Coagulase – Negative Staphylococci) had previously been thought to be harmless. Their definition was coined to differentiate them from a virulent coagulase-positive species, *Staphylococcus aureus*. Presently, they are perceived as typical opportunistic pathogens, causing an important nosocomial infection in, especially, immunocompromised patients¹. While MRSA (Methicillin-Resistant *S. aureus*) had evolved with capability to resist major groups of antibiotics, MRCoNS (Methicillin-Resistant Coagulase-Negative Staphylococci) have also developed in

parallel and become the center of interest amongst the use of indwelling intravascular devices in modern medicine^{2,3}. Generally, MRCoNS can be found on human bodies⁴, animal farms⁵, foods⁶, or even in the air in parks and metro stations⁷. The high potential survival from desiccation of their relative *Staphylococcus aureus* suggests them a good candidate as a source of antibiotic resistant genes⁸. Through horizontal gene transfer, these genes could be transmitted into virulent staphylococci, upgrading them to be a multidrug resistant superbug, which jeopardizes the antibiotics treatment situation of seriously ill patients⁹⁻¹¹. A previous *in vitro* study has proved the feasibility of *mecA* gene transfer from *S. epidermidis* to *mecA*-negative *S. pseudintermedius*; thus, encouraging a new MRCoNS character¹².

The high prevalence of MRCoNS isolated from high contact fomites, such as computer mice and keyboards, tables, books, banknotes, etc.¹³, also supports the hypothesis of desiccation tolerance of MRCoNS.

*Corresponding author:

Plykaeow Chaibenjawong
Department of Microbiology and Parasitology,
Faculty of Medical Science, Naresuan University,
Phitsanulok 65000, Thailand.
E-mail: plykaeowc@nu.ac.th

MRSA was found to survive for 318 days in screw-top bottles with dust¹⁴. In the presence of airborne dust, aerosolized *S. aureus* could survive with high viability, inflammogenicity and biofilm forming capacity¹⁵. To date, few reports have focused on the survival of MRCoNS from dust particles. Whether MRCoNS could survive from floor dust of hospital environments and go on infecting immunocompromised patients is unknown. This research explored the survival of MRCoNS from dust collected from community and hospital building floors, investigated their abilities to survive desiccation, identified their species, and determined their antibiotic resistant patterns. These basic data would support the monitoring systems to prevent the emergence of multidrug resistant pathogens and the distribution of antibiotic resistant genes in the hospitals and communities.

Materials and methods

Materials

Culture media and rabbit's plasma EDTA were purchased from HiMedia (India). Antibiotics, chemicals and reagents were from Sigma-Aldrich (Singapore). Primers and DNA sequencing were ordered or sent to Macrogen (Korea).

Dust sampling and basic screening for drug-resistant staphylococci.

Floor-dust samples were collected in the area of Naresuan university, Phitsanulok province, Thailand. As representatives of community and hospital environments, 10 dust samples were taken from floors of each place; educational buildings (faculty of Medical Science) and hospital buildings (Naresuan University Hospital). All 20 dust samples were naturally desiccation-tolerance screened by keeping at room temperature (~30 °C) for 7 days before penicillin-resistance screened by plating on Mannitol Salt Agar (MSA) supplemented with penicillin G (0.3 µg/ml). Grown colonies of presumptive staphylococci were confirmed using Gram's strain, catalase test, and coagulation of rabbit's plasma EDTA¹⁶, as well as the PCR method amplified by 16S rRNA specific primers (Table 1)¹⁷.

Analysis of antibiotic resistant patterns

Antibiotic resistant patterns were carried out using standard disk diffusion and Minimum Inhibitory Concentration (MIC) assays according

to CLSI guidelines¹⁸. Ten types of antibiotic discs, i.e., cefoxitin (surrogate test for oxacillin) (30 µg), gentamicin (10 µg), erythromycin (15 µg), tetracycline (30 µg), ciprofloxacin (5 µg), clindamycin (2 µg), trimethoprim-sulfamethoxazole (1.25/23.75 µg), chloramphenicol (30 µg), rifampin (5 µg) and linezolid (30 µg) were used as representatives to evaluate staphylococci resistance for each of drug class. Penicillin disc (10 units) was also used to confirm penicillin-resistant cultures isolated. Any *Staphylococcus* that has the ability to resist 3 or more drugs (except penicillin) will be classified as MDR¹⁹.

Primer	Sequence (5' → 3')	DNA fragment (bp)
16S 387F	CGA AAG CCT GAC GGA GCA AC	16S rRNA (528) ¹⁷
16S 914R	AAC CTT GCG GTC GTA CTC CC	
<i>mecA</i> -F	TGG CTA TCG TGT CAC AAT CG	<i>mecA</i> (310) ²⁰
<i>mecA</i> -R	CTG GAA CTT GTT GAG CAG AG	
SA-(F)	GCC AAA AGA GAC TAT TAT GA	<i>dnaJ</i> (920) ²¹
SA-(R)	ATT GYT TAC CYG TTT GTG TAC C	

Table 1. Primers used in this study.

Confirmation for MRSA and MRCoNS

Isolation of presumptive MRSA and MRCoNS were conducted by plating penicillin-resistant staphylococci on MSA supplemented with oxacillin (4 µg/ml). Confirmation of their methicillin resistance were analyzed by 2 steps; 1) the presence of the *mecA* gene using PCR with *mecA* specific primers (Table 1)²⁰, and 2) Their antibiotic resistant patterns, which indicate oxacillin resistance with the MIC of ≥ 0.5 µg/ml for MRCoNS (except *S. lugdunensis*), and ≥ 4 µg/ml for MRSA and *S. lugdunensis*¹⁸.

Determination of desiccation tolerance

To evaluate ability-to-survive-desiccation of all isolated staphylococci, the standard desiccation assay⁸ was adapted. Briefly, isolated CoNS were grown overnight in brain heart infusion (BHI) broth. Cells were washed and resuspended in phosphate buffered saline (PBS). The cell suspension was diluted to 0.05 (OD₆₀₀) before placing (20 µl) onto a plastic support, followed by air-drying at 30°C for 7 days. The CFU count was performed by resuspending desiccated cells in 1 ml of PBS and then plating onto BHI agar. Difference in desiccation survival of CoNS was evaluated by subtraction of the number of microbial survivals (in log₁₀ CFU) between day 0 (before desiccation) and day 7 (7th day of desiccation). Factors expected to

encourage their survival from desiccation, i.e., sample-collecting place and methicillin-resistant character, were analyzed and calculated as average reductions in log₁₀CFU for each group. The calculated data were then statistically compared using the Student *t* test with 95% confidence (*P*=0.05).

Species identification of CoNS

Identification of all isolated staphylococci at species level was performed according to *dnaJ* gene sequence-based assay, which indicated higher discrimination than the commonly used 16S rRNA sequence-based assay²¹. Species of CoNS identified with similarities of *dnaJ* gene sequence > 97% compared to sequence databases of the National Center for Biotechnology Information (NCBI) were reported. All relevant primers are listed in Table 1.

Results

The screening steps were used as a presumptive method to isolate naturally desiccation-tolerant and antibiotic-resistant staphylococci from highly contaminated dust samples. Initially, we had expected to find some penicillin-resistant *S. aureus* strains, which might be later leading to the discovery of desiccation-tolerant MRSA. However, not a single penicillin-resistant *S. aureus* was found from all dust-samples collected. All 78 presumptive staphylococci were isolated and confirmed to be only CoNS; 13 isolates (16.67%) came from floors within the faculty of Medical Science, and 65 isolates (83.33%) from hospital floors.

Analysis of antibiotic resistant patterns

Analysis of all 78 isolated penicillin-resistant CoNS for their antimicrobial resistance against 10 classes of antibiotics revealed the patterns of multidrug resistance for each isolate (Figure 1). From floors of community buildings, 11 of 13 isolates were grouped as MDR (84.62%), of which 10 isolates were resistant to 3-4 drug classes and 1 isolate indicated the ability to resist 7 drug classes. Sixty-one of 65 isolates from floors of hospital origin were analyzed as MDR (93.85%); 34 isolates showed the resistance against 3-4 drug classes, 19 isolates resisted 5-6 drug classes, and up to 8 isolates possessed the ability to resist 7 drug classes. Considering MDR-CoNS with the ability to resist 5 or more classes

of antibiotics as potential MDR, we found a significantly high number of 27 from 65 isolates (41.54%) recovered from floors of hospital setting, compared to 1 of 13 isolates (7.69%) found on floors of community buildings. Altogether, 72 of penicillin-resistant CoNS isolated from both places were classified as MDR (92.31%), of which 9 isolates (11.54%) exhibited resistance against up to 7 drug classes. The first 4 drug classes that indicates the high number of isolated CoNS with drug resistance are macrolide (erythromycin; 76 isolates), lincosamide (clindamycin; 65 isolates), penicillinase-stable penicillin (cefoxitin; 59 isolates) and folate pathway antagonist (trimethoprim-sulfamethoxazole; 41 isolates). The numbers of CoNS isolated from both places indicate the similar order of resistance against these 4 representative drug classes (Table 2).

Antibiotics	Cefoxitin	Gentamicin	Erythromycin	Tetracycline	Ciprofloxacin	Clindamycin	Trimethoprim-sulfamethoxazole	Chloramphenicol	Rifampin	Linezolid
Community building	8	1	13	4	1	11	7	3	0	0
Hospital building	51	33	63	14	22	54	34	3	9	2
Total	59	34	76	18	23	65	41	6	9	2

Table 2. The number of penicillin-resistant CoNS isolated from dust collected on community building floors (faculty of Medical Science) and hospital floors (Naresuan University Hospital) that exhibit resistance against each class of antibiotics.

Confirmation for MRCoNS

According to CLSI criteria, 59 isolates (75.64%) appeared to be MRCoNS with the inhibition zone of cefoxitin (30µg) ≤ 24 mm. All MRCoNS were subsequently confirmed with the presence of *mecA* gene and with MIC of > 0.5 µg/ml, by which 3, 7, 3 and 1 isolates of MRCoNS or 23.73% showed the MIC of oxacillin at 4, 8, 16 and 32 µg/ml, respectively. The remaining 45 isolates (76.27%) indicated the MIC of > 32 µg/ml (Figure 2). From this study, 8 MRCoNS identified (10.26%) were traced back to floors of community buildings, while 51 (65.38%) were from floors of hospital buildings. The ratios of MRCoNS to CoNS recovered between the community (8/13) and the hospital (51/65) were not significantly different.

Determination of desiccation tolerance

All isolated penicillin-resistant staphylococci had abilities to survive a week of desiccation on plastic supports with average reduction of $1.91 \pm 0.72 \log_{10}\text{CFU}$. Due to the different strategies of routine cleaning between community and hospital building floors, ability-to-survive-desiccation of cultures isolated from both places were compared and found not significant difference, with average reduction of 1.76 ± 0.52 (community) and 1.94 ± 0.75 (hospital) in $\log_{10}\text{CFU}$ (Figure 3a). Another factor that might affect their survival ability. i.e., methicillin-resistant character was also analyzed. We found that the cultures identified as MRCoNS exhibited the similar levels ($P > 0.05$) of desiccation survival, compared to cultures identified as not MRCoNS, with average reductions of 1.87 ± 0.73 and 2.06 ± 0.66 in $\log_{10}\text{CFU}$, respectively (Figure 3b).

settings, while all *S. capitis* were determined as MDR (Figure 1). With specific characters of MRCoNS previously confirmed, 3 species from 5 MRCoNS isolated from community buildings were identified as *S. arlettae* (1 isolate), *S. saprophyticus* (2 isolates) and *S. cohnii* (2 isolates), whereas 6 species from 41 isolates, i.e., *S. arlettae* (1 isolate), *S. equorum* (2 isolates), *S. haemolyticus* (2 isolates), *S. saprophyticus* (6 isolates), *S. capitis* (9 isolates), and *S. cohnii* (21 isolates) came from hospital origin. *S. cohnii* appears to be the main species of MRCoNS identified from the hospital.

Discussion

We could not detect MRSA or even a single penicillin-resistant *S. aureus* from dust collected from community and hospital building floors. All survivors from floor dust and routine floor cleaning were identified as CoNS and MRCoNS. Previous studies also showed poor survival of MRSA on public inanimate objects. Only one MRSA was isolated from 740 handles of shopping baskets²², while attempts to isolate MRSA from the university environment resulted in the isolation of solely MRCoNS¹³.

In our study, all MRCoNS survived a 7-day period of screening and showed the ability of desiccation tolerance with just $2 \log_{10}\text{CFU}$ decrease. This factor possibly helps MRCoNS survive in the air of metro stations, hospitals, and parks, as well as from university and farm environments^{5,7,13}. Most MRCoNS identified from university environments were found to possess *qacA/B* genes, the gene encoding various antiseptic and antibiotic resistances¹³. This could signify that using common disinfectants for routine cleaning on floors of hospital and community buildings was sufficient to reduce contamination from MRSA but not MRCoNS to below the detection level. Although some MRSA might survive sanitizing agents used, they were unable to be resuscitated following the 7 days of desiccation period applied in the screening step compared to MRCoNS. In HIV-infected patients, MRCoNS carriers were reported as less likely to have MRSA colonization²³, demonstrating the better adaptation *in vivo* of MRCoNS over that of MRSA; though, the factors behind this may be different from *in vitro* experiments.

To identify factors that influence desiccation survival of CoNS, our results

Species (Number of isolates)	<i>S. warneri</i>	<i>S. arlettae</i>	<i>S. equorum</i>	<i>S. haemolyticus</i>	<i>S. saprophyticus</i>	<i>S. capitis</i>	<i>S. cohnii</i>	Total isolates identified
Penicillin-resistant CoNS	2	3	4	8	9	11	27	64
Community building	1	2	-	-	2	-	5	10
Hospital building	1	1	4	8	7	11	22	54
MRCoNS	-	2	2	2	8	9	23	46
Community building	-	1	-	-	2	-	2	5
Hospital building	-	1	2	2	6	9	21	41

(Naresuan University Hospital).

Table 3. Distribution of penicillin-resistant CoNS and MRCoNS species isolated from dust collected on community building floors (faculty of Medical Science) and hospital floors.

Species identification of CoNS

Based on the *dnaJ* sequencing method²¹, 71 isolates of CoNS had been examined at species level, however, we lost the remaining 7 isolates due to some technical reasons. The BLAST results showed 7 staphylococci species identified from 64 isolates as *S. warneri* (2 isolates), *S. arlettae* (3 isolates), *S. equorum* (4 isolates), *S. haemolyticus* (8 isolates), *S. saprophyticus* (9 isolates), *S. capitis* (11 isolates), *S. cohnii* (27 isolates), and unidentified (7 isolates). Table 3 illustrates the frequency of species isolated from each place, with *S. cohnii* as the most prevalent species identified. Three species, i.e., *S. equorum*, *S. haemolyticus* and *S. capitis*, were found only on floors of hospital

indicated low possibilities for 2 factors, i.e., ability-to-resist-methicillin (MRCoNS versus penicillin-resistant CoNS but not MRCoNS), and places (with different floor cleaning strategies between community and hospital settings). The similar low effects of methicillin resistance had appeared on desiccation survival of MRSA and other *S. aureus*^{24,25}. The second factor also shows the same desiccation survival of CoNS isolated between both places. Although previous study reported the lab strain *S. aureus* SH1000 survived desiccation better than clinical strains⁸, CoNS isolated from hospital floors in current study were not clinical strains or had been clinical strains but would have adapted to survive desiccation and disinfectants after contaminating floor, dust, air, or other inanimate surfaces in hospital environments.

Analysis of antibiotic resistant patterns reveals high prevalence of MDR-CoNS (92.31%) as well as MRCoNS (75.64%) distributed on floors of community and hospital buildings. The ratios of MRCoNS to CoNS isolated from both places were not significantly different, similar to the case of MDR-CoNS. However, the number of potential MDR-CoNS (5 or more classes of antibiotic resistance) was found much higher on floors of hospital settings. This situation is actually common around the world due to antibiotic treatment for hospitalized patients. MDR-CoNS resistant to 6-8 antibiotics also found in hospital air in China, while those isolated from air in metro stations and parks indicated resistance against the lower number of antibiotics. Furthermore, both MDR-CoNS from metro stations and hospitals exhibited the orders of antibiotic resistance for the first 3 drugs resembling our study⁷. Similar results are also found on MRCoNS isolated from high contact surfaces¹³. Contrary, multidrug resistance in CoNS recovered from 117 non-hospitalized volunteers in Germany showed a different pattern, in which tetracycline, fosfomycin and erythromycin are the first 3 drug resistance²⁶. The effect of different habitats or regions seems to affect the pattern of their drug resistances.

According to CLSI guidelines, MIC criteria for MRCoNS except *S. lugdunensis* is ≥ 0.5 $\mu\text{g/ml}$. In this study, we found a high number of MRCoNS (76.27%) indicating the much higher level of MIC (> 32 $\mu\text{g/ml}$) than those specified in the standard. This could be a risk if these microorganisms have a chance to colonize on

any indwelling devices attached to long-stay patients. Many of them were identified as *S. capitis* and *S. cohnii*, which can cause catheter-related blood stream and foreign body-related infections¹. All *S. capitis* in the current study were found only from the hospital. They exhibit not only a high level of MIC for oxacillin, but also a high number of drug resistance (between 4-7 classes). Their multidrug resistant character was previously reported on *S. capitis* associated with neonatal sepsis or sterile sites such as body fluids, subcutaneous wounds and abscesses^{27,28}.

In spite of being found on floors of both places, most isolates of *S. cohnii* from hospital dust were identified as MRCoNS with high levels of MIC for oxacillin. *S. cohnii* with the character of MRCoNS was reported to be the predominant species isolated from outdoor surfaces of university environments¹³. Their multidrug resistances were common on *S. cohnii* isolated from German dairy farms²⁹. Another important species in our results with similar MDR character and isolated places to *S. cohnii* but with diminished numbers are *S. saprophyticus*. *S. saprophyticus* is a major cause of urinary tract infection that has epidemiologic and genomic evidence related to the meat-production chain³⁰. With MDR or MRCoNS characters identified from environmental samples, they were found in relatively low proportion in other studies^{7,13}.

Noticeably, *S. haemolyticus* were found only on hospital floors, and most of them were classified as MDR but not MRCoNS. In contrast to the high proportion of their MRCoNS character that had been isolated from the university canteen¹³. Other 3 species of CoNS identified from floor dust with low numbers are *S. warneri*, *S. arlettae*, and *S. equorum*, in which some isolates of the latter 2 are also MRCoNS. Noteworthy, we could not isolate a single *S. epidermidis* out of floor dust taken from both places. *S. epidermidis* is a major CoNS usually presented in the body and environment, and appears in most of the previous studies^{6,7,13,26,27}. This will be interesting to find out for our future investigation.

Conclusions

Most MRCoNS and MDR-CoNS from floor dust were found as a great collection of multidrug-resistant genes. Armed with the ability to survive desiccation on floor dust, they can

become a hidden threat as opportunistic pathogens. This work highlights the ability of MRCoNS or MDR-CoNS to survive desiccation on floor dust apart from their antibiotic-resistant abilities. With the feasibility of *mecA* gene transfer between staphylococci, a new virulent MRSA or MRCoNS could emerge anytime. However, the possibilities of these staphylococci to infect immunocompromised patients and behave as a reservoir of antibiotic resistant genes in the real world are still needed to be proved.

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Declaration of Interest

The authors report no conflict of interest.

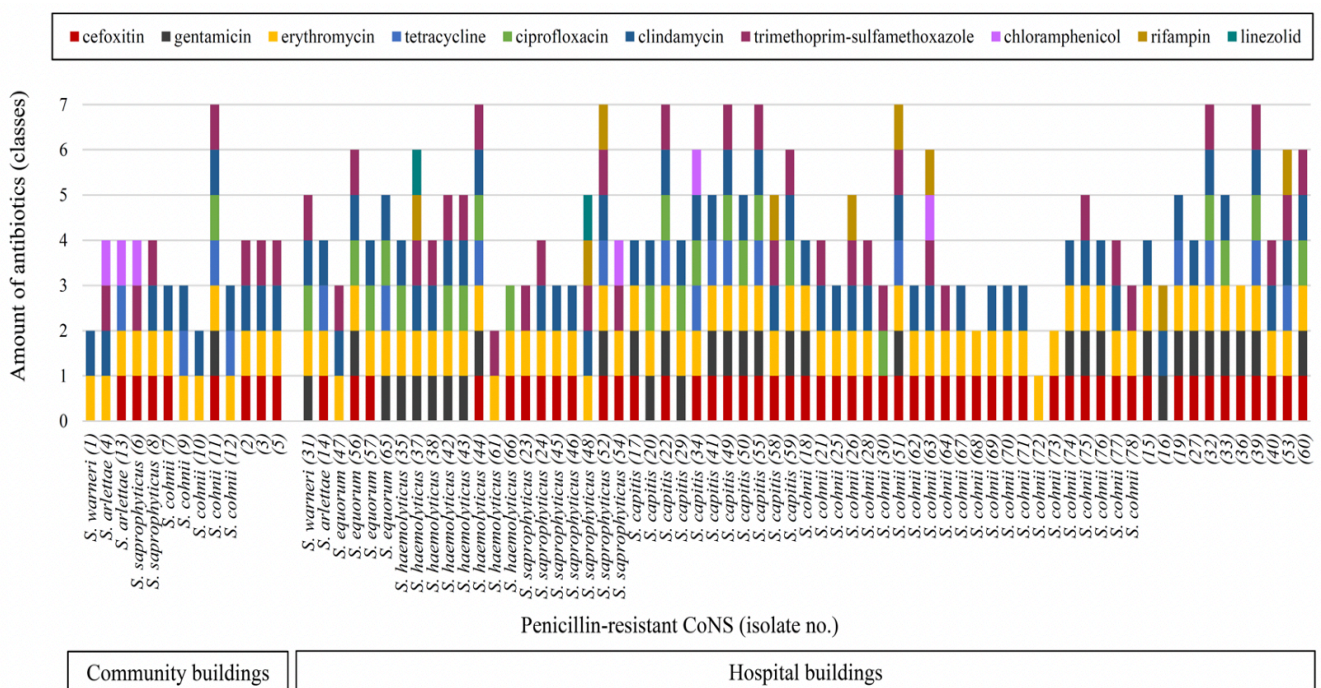


Figure 1. Multidrug resistant patterns of all isolated CoNS and their abilities to resist diverse antibiotics. Various color squares represent each class of antibiotics. Identifiable species are labelled and grouped along sample collected places; community (faculty of Medical Science) and hospital. Unidentifiable species are shown only isolate numbers in parentheses.

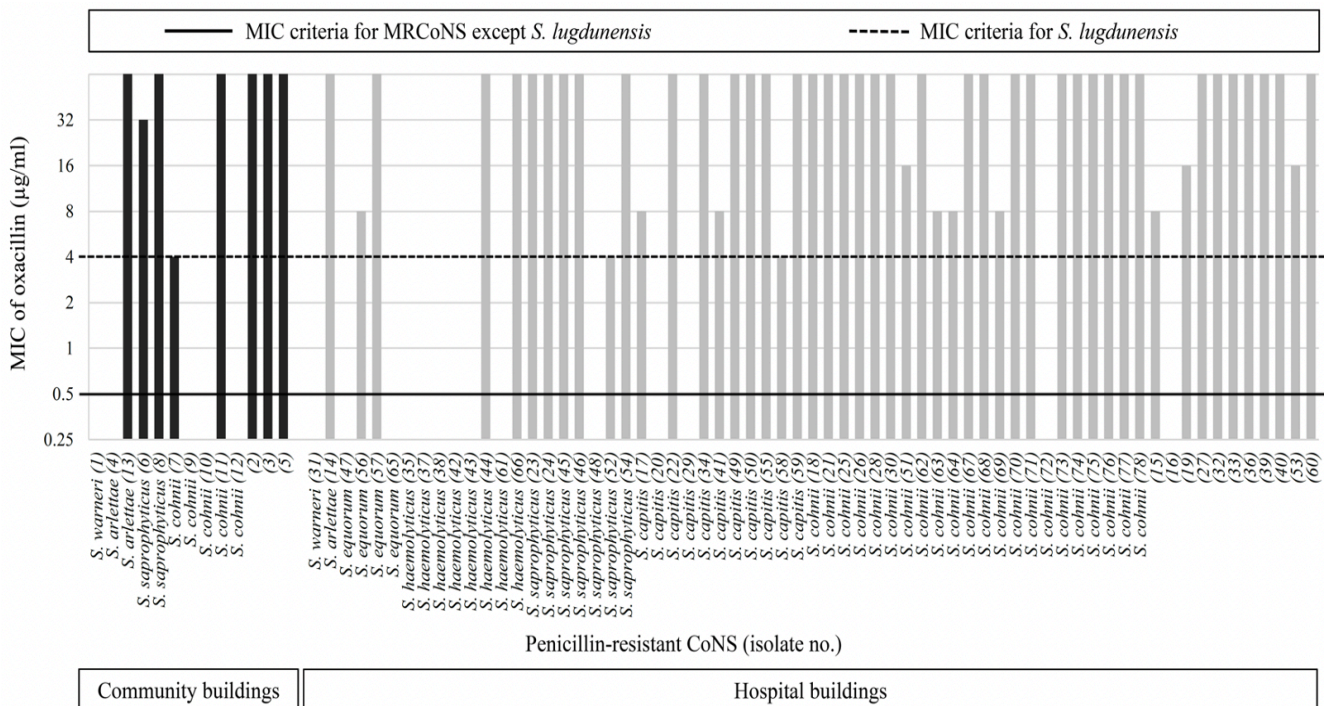


Figure 2. MIC (Minimum Inhibitory Concentration) of all isolated CoNS that showed resistance to the oxacillin surrogate disc (30 µg cefoxitin). Horizontal black solid and dash lines represent MIC criteria for MRCoNS. All concentrations labelled higher than 32 µg/ml are not shown. Identifiable species are labelled and grouped along sample collected places; community (faculty of Medical Science) and hospital. Unidentifiable species are shown only isolate numbers in parentheses.

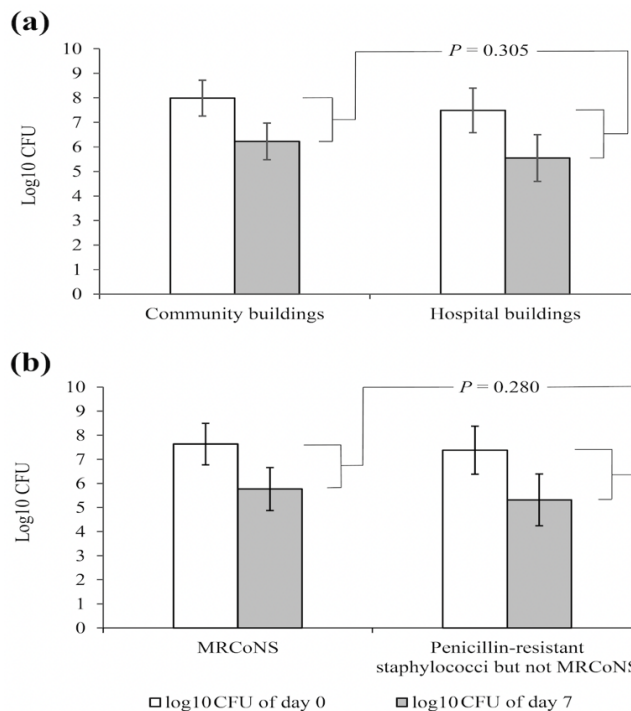


Figure 3. The effect of sample-collecting place (a) and methicillin-resistant character (b) on survival of CoNS isolated from dust samples collected from floors of faculty of Medical Science (community buildings) and hospital buildings. Mean survival with error bars (SD) are shown. White and gray squares represent the number of colonies in log₁₀CFU of the day 0 (before desiccation) and day 7 (7th day of desiccation). Calculated data were statistically compared with 95% confidence ($P=0.05$).

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