Assessing the effectiveness of antimicrobial agents infused in polyurethane composite rubber flooring products in the control of bacterial pathogens

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ABSTRACT

The viability of nosocomial and community-acquired bacterial pathogens was assessed on polyurethane composite rubber flooring products containing three different antimicrobial agents. Samples containing three concentrations of zinc pyrithione, N-butyl-1,2-benzisothiazolin-3-one, or inorganic silver-glass zeolite were tested in three separate assays using gram (+) and gram (-) bacterial species. A direct contact assay (JISZ 2801) was optimized to mimic real-world conditions and a time-course study revealed that zinc pyrithione incorporated into the polyurethane moisture cure adhesive used as a binder for the rubber flooring composition was effective at inhibiting bacterial growth, including community- and hospital-acquired methicillin resistant *Staphylococcus aureus* (MRSA) strains. MRSA strains isolated from rubber flooring at a local exercise facility were found to exhibit differential sensitivity to antimicrobial agents, demonstrating that levels of inhibition can vary substantially depending on the strain used. Other factors affecting sensitivity to the antimicrobial agents include metabolites in the incubation solution, incubation temperature, and age of rubber sample or the extent they have been washed. Results suggest that the inclusion of zinc pyrithione based antimicrobial agents in polyurethane composite rubber flooring products can effectively inhibit the growth of pathogenic bacteria commonly encountered in gymnasiums or other public environments that utilize resilient rubber flooring products. Approaches to maximize the level of control will be discussed, as well as areas of future research.

INTRODUCTION

RB Rubber Products produces a wide variety of flooring and surfacing products based on molded recycled rubber-polyurethane composites. These products include resilient underlayment for tile and other hard surface flooring products, decorative rubber tiles and roll flooring products for health clubs and gymnasiums, safety surface tiles for fall impact attenuation under playground equipment, and agricultural flooring products.

Reports of Methicillin-Resistant *Staphylococcus aureus* (MRSA) infections obtained from hospital and community environments have increasingly appeared in the news and scientific literature over the past several years. One notable report estimated the total number of deaths caused by MRSA infection in 2005 in the U.S. to be nearly 19,000, a rate much higher than previously believed (1). According to this study, approximately 94,000 patients developed an MRSA infection and nearly one in five died. A common form of transmission of MRSA strains occurs via contact with inanimate objects, which includes polyurethane composite rubber flooring products that are used in hospitals, gymnasiums, locker rooms, playgrounds and childcare facilities. In one well-publicized case, members of the St. Louis Rams professional football team were found to spread an MRSA strain to teammates and to opposing players (2). Taken together with reports of nosocomial (hospital-acquired) and other community acquired bacterial infections, the scope of problem appears to be worsening and studies that aim to reduce the spread of bacterial infections like MRSA are becoming increasingly important.

RB has historically incorporated biocide in selected polyurethane composite rubber products to inhibit the growth of fungi (mold and mildew). In order to evaluate whether this technology could be modified or optimized to be effective in inhibiting the growth of microbial growth on urethane composite rubber products, RB entered into a research project with Saint Louis University Department of Biology to study the prevalence of microbial contaminants on public use surfaces and the effectiveness of various biocidal additives.

Here, we describe the results of an unbiased survey of microbial contaminants detected on recycled rubber-polyurethane composites located in a variety of community environments. Repeat sampling at sites within three distinct locations where rubber products are widely used revealed the presence of a broad range of microbial organisms, including several human bacterial pathogens. Using a selection of the bacterial isolates, together with standard strains, the effectiveness of antimicrobial agents infused into polyurethane composites at controlling the growth of the bacterial pathogens was assessed. An assay was developed to mimic real world conditions and demonstrated that composites containing zinc pyrithione were highly effective at inhibiting bacterial growth, whereas two other compounds tested showed only a modest level of control.

EXPERIMENTAL PROCEDURES

Sampling of rubber products

Samples were taken from rubber flooring and other rubber products at three different locations; 1) an exercise facility, 2) indoor children's play areas, and 3) outdoor playgrounds (**Fig. 1**). Approximately 8-12 sites were sampled at each location and sites that were heavily trafficked or areas that would likely come in contact with bare skin were emphasized. The materials sampled included: rubber pads, interlocking rubber flooring tiles, drain-through rubber modular tiles, and rubber grips. In an effort to record differences that may occur throughout the year due to changes in environmental conditions, multiple samplings were carried out at each facility over a period of 10 months and the temperature and humidity levels at each location were recorded. For example, the outdoor play area was sampled during a period when temperature and humidity were high, shortly after a heavy rain shower and 24 hours after a hard frost. In addition, the time of last human use was noted at the sites, if known, to assess the longevity of the microbial contaminants. For example, periodic sampling in the spinner room at the exercise facility that is locked after each spinner class made it possible to assess the relative amount of viable bacteria that remained at specific time points.

An 11cm x 11cm area was sampled using a sterile cotton swab and three swabs were taken from each sampling site. Two of the swabs were streaked onto Brain Heart Infusion (BHI) agar plates and incubated at 25°C and 37°C for bacterial growth. One swab was streaked onto Saboraud Dextrose Agar or Cooke Rose Bengal Agar and incubated at 30°C for fungal growth. The total number of colony forming units (CFU) was estimated on each plate and pure cultures of unique colony types were obtained and saved for further analysis. Bacteria were classified by the size and shape of bacterial cells and their response to gram staining. Further bacterial identification was carried out by differential staining methods and growth on differential media (**Fig. 2**). Methicillin resistance of *Staphylococcus aureus* strains was determined by zone of inhibition assays, and polymerase chain reactions were used for confirmation (3). Fungal identification was made by observing colony morphologies and differential stains & media.

Figure 1: Sampling locations; exercise facility, indoor play area and outdoor playground (left to right).

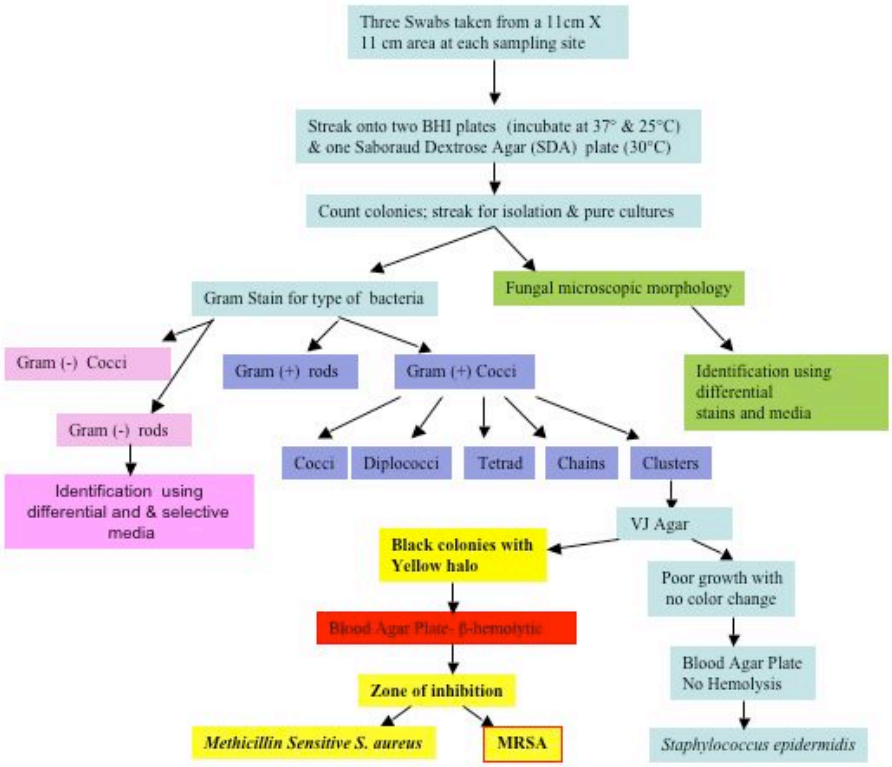

Figure 2: Procedures used to characterize microbial contaminants isolated from sampling sites.

Assessing the effectiveness of antimicrobial compounds infused in composite polyurethane rubber products

The effectiveness of zinc pyrithione (ZP), N-butyl-1, 2-benzisothiazolin-3-one (NB) and inorganic silver-glass zeolite (SZ) infused in polyurethane composites in inhibiting the growth of bacteria was assessed. Three amounts of antimicrobial compounds were analyzed: for zinc pyrithione, 0.05% (active by weight; ZP-0.05), 0.1% (ZP-0.1), and 0.15% (ZP-0.15); for N-butyl-1, 2-benzisothiazolin-3-one, 0.05% (NB-0.05), 0.1% (NB-0.1), and 0.2% (NB-0.2); and for inorganic silver-glass zeolite, 0.25% (SZ-0.25), 0.5% (SZ-0.5), and 0.75% (SZ-0.75). Samples containing no antimicrobial agent were included with all tests.

Two assays were used to assess the effectiveness of the compounds in inhibiting bacterial growth: 1) a "zone of inhibition" assay to test diffusible bacteriostatic activity, and 2) a "direct contact" assay to test bactericidal activity on suspensions of known density. In the zone of inhibition assay**,** plates having Mueller Hinton agar were inoculated with *Staphylococcus aureus*, a community-acquired methicillin resistant *Staphylococcus aureus* subsp. *aureus* ATCC® BAA-1556 ™ (USA300), a hospital-acquired methicillin resistant *Staphylococcus aureus* subsp. *aureus* ATCC® BAA-700699 ™ (Mu50), and two MRSA strains isolated by our survey of a local exercise facility (MRSA1 and MRSA2). In addition to these gram positive organisms, three gram negative species, *Escherichia coli* B ATCC® 11303™, *Klebsiella pneumoniae* subsp. *pneumoniae* ATCC® 132™, and an *Enterobacter agglomerans* strain isolated from the survey were also tested. Each of the bacterial samples was streaked onto the Mueller Hinton agar plate using a sterile cotton swab so that a lawn of bacterial growth could be obtained. A 10mm X 10mm rubber sample was placed in the center of the plate so that it firmly adheres to the agar surface. The plates were incubated at 37°C and the zone of inhibition (i.e. the diameter of the area showing little or no bacterial growth) was measured after 18 to 22 hours of incubation (**Table 3**).

The direct contact assay followed the Japanese standard protocol (JIS Z 2801:2000) for testing bactericidal activity (4). A set volume (200 µl) of bacterial suspension containing $2x10^4 – 4x10^5$ cfu/ml was placed on a 25 x 25 mm rubber sample. Initially, bacterial suspensions were made with de-ionized water as specified by the standard

protocol. However, most experiments used artificial perspiration solution (Pickering Laboratories, Inc., Mountain View, CA) as the suspension media as it more closely mimics real world conditions (**Figure 3**). The bacterial suspension was covered with a glass cover slip and incubated at 37°C for 0, 1, 2, 4, & 24 hours. Additional studies were carried out at 25˚C but are not reported here. After each time point, the cover slip and rubber sample were washed thoroughly using 3 mls of a sterile phosphate buffered saline solution. The wash was collected, mixed thoroughly, and 100 µl of the wash solution was plated onto agar plates having Brain Heart Infusion (BHI) media. Plates were incubated at 37°C for 18-22 hours and the number of colony forming units was determined. To calculate the bacteriocidal activity, CFUs resulting from each treatment condition were compared to number of colonies that formed on plates that had been plated with wash solution derived from rubber samples that had been collected immediately after contact. Tests were conducted for five *S. aureus* strains described above, as well as *Enterobacter agglomerans* (a gram negative) species detected in our survey. The percent viability was calculated for each assay and is presented as a log reduction value in the histograms along with the standard deviations. A log reduction value of greater than two is considered significant.

RESULTS & DISCUSSION

Survey of Microbial Contaminants Found on Rubber Products

Bacterial and fungal contaminants were detected at all sites sampled at each of the three locations surveyed. Although the sampling methods used were not designed to accurately determine the amount of microbial contaminants at each site, general inferences can be drawn from the data due to the reproducibility of the results and by comparison with controls (i.e. rubber products that were not handled or exposed to patrons at the facilities sampled). As indicated in **Table 1**, the overall level of bacterial contamination was high at all three locations, while the amount of fungal organisms recovered was slightly more variable.

Table 1: Microbial contamination detected on rubber products located in three different locations

Sampling site & date	Relative level of contamination[1]		Morphological features of bacterial isolates (number of unique colony forming units)[2]					
	Bacteria	Fungi	G(+) rods	G(+) cocci in clusters	G(+) cocci in chains	G(+) cocci	G(+) tetrad	G(-) rods
Exercise facility								
5-16-07	+	+	5	1	1	1	0	1
6-7-07	+	+	6	3	0	0	2	1
8-27-07	+++	+	7	8	0	0	0	1
10-22-07	+++	+++	6	8	0	2	2	3
11-12-07	+++	n. t.	2	5	0	0	1	3
2-22-08	+++	+++	13	9	0	3	1	1
Indoor play area								
6-26-07	+++	++	11	6	0	4	0	0
7-11-07	+++	++	7	6	0	0	1	1
7-24-07	+++	++	4	10	0	3	2	1
10-4-07	+++	+++	5	13	0	1	0	3
Outdoor playground								
7-19-07	++	++	4	4	0	1	0	0
8-14-07[3]	+++	+++	20	4	0	1	3	3
9-18-07	+++	+++	12	5	0	0	0	6
11-08-07[4]	+++	+++	11	0	0	0	0	1

[1] + = low level, ++ = moderate level, +++ = high level, based on total number of bacterial or fungal colonies isolated from all sites examined; n.t. = not tested.
[2] G(+) = gram positive, G(-) = gram negative.
[3] Sampling occurred 30 hours after a heavy and sustained rainfall.
[4] Sampling occurred 24-30 hours after a hard frost.

For indoor facilities (i.e. exercise facility and indoor child's play area), the overall level of contamination correlated with the relative amount of human activity at the sites. That is, higher levels of contamination were found at peak periods of use of the facilities. Interestingly, the level of both bacterial and fungal contamination at the outdoor playground showed little variation and was high even after a hard frost. However, the diversity of the organisms recovered at the outdoor playground was lower than that found at the indoor facilities and, as described below, substantially fewer potential pathogens were recovered.

Table 1 indicates the diversity of bacterial organisms recovered by recording the number of unique colony types identified within each morphological class. Species that have the same morphological features [i.e. gram (+) rods] commonly have different colony morphologies (i.e. size, color, shape, margin etc…), thus number of unique colony types can serve as a good indicator of diversity. When further characterized, the most common bacteria recovered at all sites were *Bacillus subtilus* and *Bacillus cereus* species which are gram (+) rods that inhabit the soil. It is likely that the patrons track these organisms into the facilities on their shoes. Soil dwelling organisms are generally tolerant of extreme environmental conditions, which could also explain why they are overrepresented and why high levels were detected at the outdoor playground after a hard frost.

TABLE 2: List of pathogenic and/or opportunistic bacteria detected on rubber products at three locations surveyed

Species	EF[1]	IP[2]	OP[3]	Characteristics
Acinetobacter hemolytica		X		Gram (-) rod; Common soil dwelling bacteria that contribute to the mineralization of aromatic compounds in the soil. They are also known to cause infection in immunocompromised patients.
Actinomyces spp.	X	X		Gram(-) rod; Colonizes skin, wounds, respiratory and gastrointestinal tracts in humans. Of clinical importance because of the emergence of strains that are resistant to commercially available antibiotics. Survives desiccation, which promotes the transmission through fomites.
Citrobacter spp.	X		X	Gram(-) rod; Commonly found in soil, water and human intestines. Most of the species are non-pathogenic although they have been known to cause urinary tract infections, infant meningitis and sepsis.
Enterobacter agglomerans	X	X	X	Gram(-) rod; Found on plant surfaces, seeds, fruits and human or animal feces. It is known to be an opportunistic pathogen that causes wound, blood and urinary tract infections in immunocompromised.
Kingella spp.	X			Gram(-) rod; Found in human respiratory tract and can cause skeletal infections, endocarditis, bacteremia, and rarely pneumonia, and meningitis.
Listeria spp.	X			Gram(+) rod; Rarely cause disease in healthy adults, yet it can be lethal to fetuses when it crosses the placenta from the infected mothers. It also causes bacteremia and meningitis in immunocompromised patients.
Proteus mirabilis			X	Gram(-) rod: Causes urinary tract infections in humans, particularly with the catheters. *Proteus* is also resistant to many antimicrobial drugs.
Shigella spp.	X			Gram(-) rod; The genus *Shigella* has four well defined species that are all pathogenic to humans. All the four species cause severe dysentery called Shigellosis, which is characterized by abdominal cramps, fever, diarrhea, and bloody stools.
Staphylococcus aureus	X	X	X	Gram(+) cocci; An opportunistic pathogen and causes wide range of infections like boils, impetigo, epidermal necrosis, osteomyelitis, meningitis, endocarditis, pneumonia, mastitis, bacteremia, enterocolitis, food poisoning & toxic shock syndrome.
MRSA	X			Gram(+) cocci; Methicillin resistant strain of *S. aureus*
Staphylococcus epidermidis	X			Gram(+) cocci; A normal inhabitant of the skin, skin glands, and mucous membranes of humans. It is an opportunistic pathogen that can cause urinary tract infections, wound infections, endocarditis, and septicemia.

[1] EF = exercise facility
[2] IP = indoor child's play area
[3] OP = outdoor playground

A primary goal of the survey was to assess whether potential human pathogens could be recovered from rubber products. The initial procedures to characterize the microbial contaminants aimed to recover all bacterial and fungal organisms, while additional tests focused on the identification of potential human pathogens, particularly *Staphylococcus aureus*. **Table 2** shows a list of potential human pathogens recovered at the three different facilities. *S. aureus* was found at all three locations; eleven isolates at the exercise facility, eight at the indoor child's play area and one at the outdoor playground. Notably, two isolates of MRSA were also recovered from the exercise facility. As *S. aureus* is normal inhabitant on skin carried by an estimated 30% of the population, it is not surprising that it was recovered from locations that have a high level of human activity, such as a gymnasium or play area. Several other potential pathogens were recovered, many of which are also common inhabitants of human skin, as well as bacteria that inhabit mucosal tissues or the intestines.

The indoor facilities were kept at fairly constant temperatures and no obvious correlations between the relative amount of microbes at different environmental temperatures or humidity levels were detected. Interestingly, *Staphylococcal* strains were readily recovered on rubber flooring material beneath a spinner bike 40 hours after last use, indicating that they can persist for long periods of time. Numerous fungal species with distinct colony morphologies were also isolated. Although the precise identity has yet to be determined, *Saccharomyces spp.* and *Penicillium spp.* appear to be the most commonly occurring fungal isolates found at all the sites.

Assessing the effectiveness of antimicrobial compounds infused in composite polyurethane rubber products

The zone of inhibition studies were carried out with all rubber samples using five strains of *S. aureus* and three gram negative species (**Table 3**). The strains used include a methicillin sensitive *S. aureus*, known hospital-acquired (Mu50) and community-acquired (USA300) methicillin resistant strains and the two MRSA isolates recovered from the survey. Zone of inhibition studies provide information about the inhibitory effect of the antimicrobial agents, yet are also a measure of the solubility of the agents. Thus, the size of the cleared area around the rubber sample in which the bacteria fail to grow is not only an indication of the bacteriostatic activity of the compound, but it is also a reflection the solubility of the agent and/or the ability of the compound to be released from the samples.

Control samples having no antimicrobial agent were not inhibitory against any of the bacteria tested. This suggests that composite rubber products either do not exhibit antimicrobial activity or this activity is not readily diffusible. Of the three antimicrobial agents tested, zinc pyrithione was the most effective at inhibiting bacteria tested. Notably, it was the only antimicrobial agent effective against hospital- and community-acquired MRSA strains. However, N-butyl-1, 2-benzisothiazolin-3-one was more effective than zinc pyrithione in inhibiting *S. aureus*, MRSA1, and MRSA2 strains, which may reflect greater solubility of N-butyl-1, 2-benzisothiazolin-3-one. Interestingly, N-butyl-1, 2-benzisothiazolin-3-one was not effective in inhibiting any of the gram negative bacteria tested. The inorganic silver-glass zeolite was not effective in inhibiting any of the bacteria tested although the highest concentration of the antimicrobial agent (0.75%) was slightly effective against *Klebsiella pneumoniae.*

Table 3: Zone of inhibition[1] studies with five Staphylococcus strains and three gram (-) species

Sample	*S. aureus*	MRSA1	MRSA2	MRSA USA300	MRSA MU50	*E. coli*	*K. pneumoniae*	*E. agglomerans*
Control	—	—	—	—	—	—	—	—
Zinc pyrithione								
ZP-0.05	—	—	—	+	+++	+	—	++
ZP-0.10	+++	—	+	+++	+++	++	—	+++
ZP-0.15	+++	++	+	+++	++++	+++	++	+++
N-butyl-1, 2-benzisothiazolin-3-one								
NB-0.05	—	+++	—	—	—	—	—	—
NB-0.10	+++	+++	—	—	—	—	—	—
NB-0.20	++++	++++	+++	+	+	—	—	—
Inorganic silver-glass zeolite								
SZ-0.25	—	—	—	—	—	—	—	—
SZ-0.50	—	—	—	—	—	—	—	—
SZ-0.75	—	—	—	—	—	—	+	—

[1] The diameter of the zones of inhibition are represented by: — = no zone detected; + = ≤ 10mm; ++ = 11mm - ≤14mm; +++ = 15mm - 20mm; ++++ = >20mm.

DIRECT CONTACT ASSAYS

Direct contact assays involve placing a known concentration of bacteria in a liquid suspension on top of a rubber sample for a specified length of time and measuring the reduction in viability. These assays were initially performed using sterile dH_2O for the bacterial suspension placed on the rubber samples. Since bacteria are likely introduced to the rubber products in association with human secretions (e.g. sweat), a solution of artificial perspiration was used as the suspension solution to more closely mimic the real world conditions. **Figure 3** compares the relative inhibitory effect of the antimicrobial containing rubber samples using water versus artificial perspiration. The reduction in the number of viable bacteria recovered after contact with the rubber samples as compared to the control is recorded as log reduction values, with each number reflecting a 10 fold reduction in the number of bacteria. Log reduction values greater than 2.0 (100 fold reduction) are considered significant.

Using dH_2O, samples having zinc pyrithione (ZP) were effective at inhibiting *S. aureus* after one-hour incubation. In contrast, when artificial perspiration was used, only the rubber sample with highest concentration of antimicrobial agent showed a significant effect after 2 hours of incubation, suggesting that the bacteria have a higher survival rate when artificial perspiration is used as a suspension media.

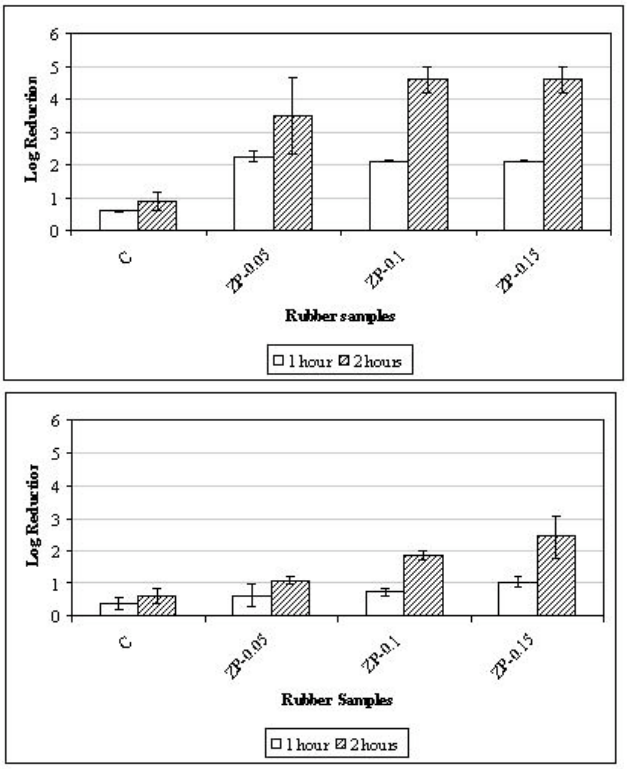

Figure 3: Direct Contact Assay with *S. aureus* using dH_2O (top) and artificial perspiration (bottom) as suspension solutions. Concentrations of zinc pyrithione in samples were 0.05%, 0.1% and 0.15%.

The use of artificial perspiration resulted in a clear dose-dependent relationship between the amount of biocide in the samples and log reduction levels and was found to be more reproducible, so remaining studies were carried out using artificial perspiration rather than deionized water as a suspension medium. **Figures 4-9** indicate the relative effectiveness of the antimicrobial agents against 5 strains of *S. aureus* and *Enterobacter agglomerans*.

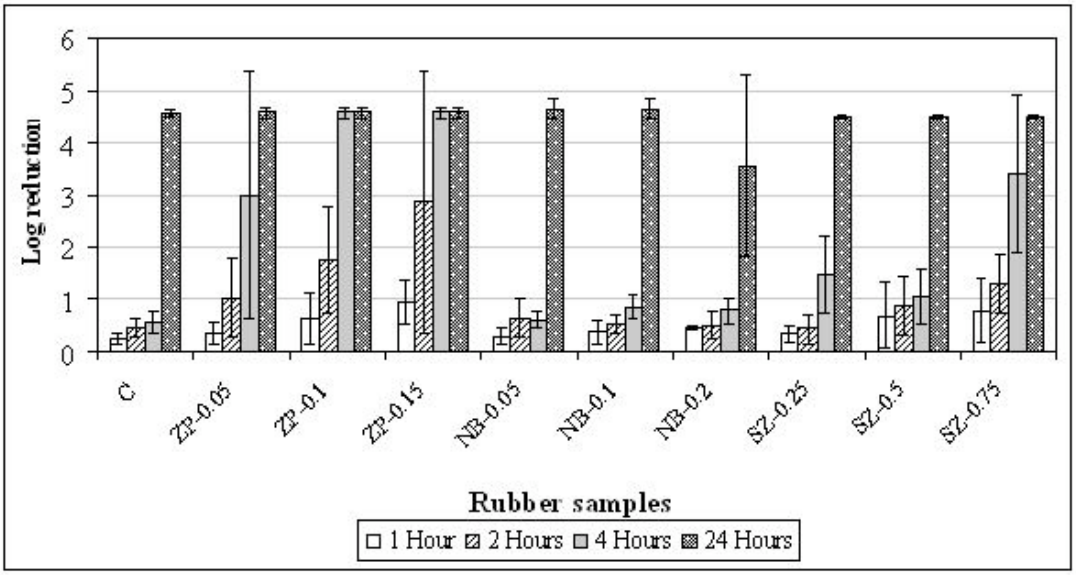

Figure 4: Direct contact assay performed with *S. aureus* suspended in artificial perspiration. Concentrations of antimicrobial agents are: zinc pyrithione (ZP) 0.05%, 0.1% and 0.15%; N-butyl-1, 2-benzisothiazolin-3-one (NB), 0.05% 0.1%, 0.15%; inorganic silver-glass zeolite (SZ) 0.25%, 0.5%, 0.75%. Contols (C) are rubber samples that do not contain antimicrobial agents.

- Zinc pyrithione samples were more effective at inhibiting *S. aureus*, followed by N-butyl-1, 2-benzisothiazolin-3-one samples. All the rubber samples including the control sample without any antimicrobial were effective after 24 hours incubation.

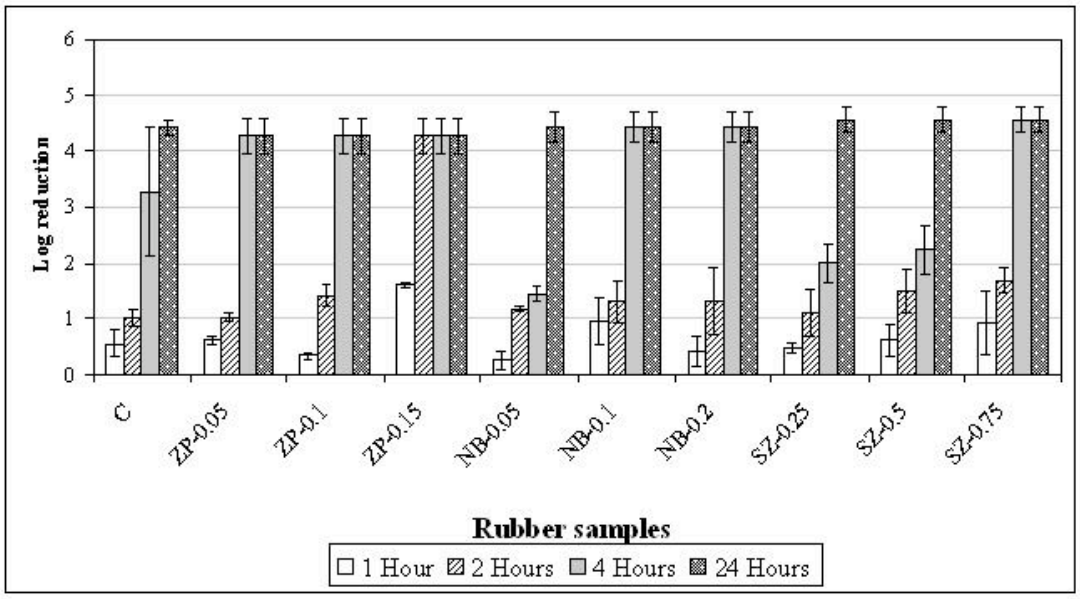

Figure 5: Direct contact assay conducted with MRSA isolate 1. Concentrations of antimicrobial agents are: zinc pyrithione (ZP) 0.05%, 0.1% and 0.15%; N-butyl-1, 2-benzisothiazolin-3-one (NB), 0.05% 0.1%, 0.15%; inorganic silver-glass zeolite (SZ) 0.25%, 0.5%, 0.75%.

- Zinc pyrithione samples were the most effective in inhibiting MRSA isolate 1 at 2 and 4 hours of incubation followed by N-butyl-1, 2-benzisothiazolin-3-one. Inorganic silver-glass zeolite was the least effective in inhibiting MRSA1 under the concentrations tested.

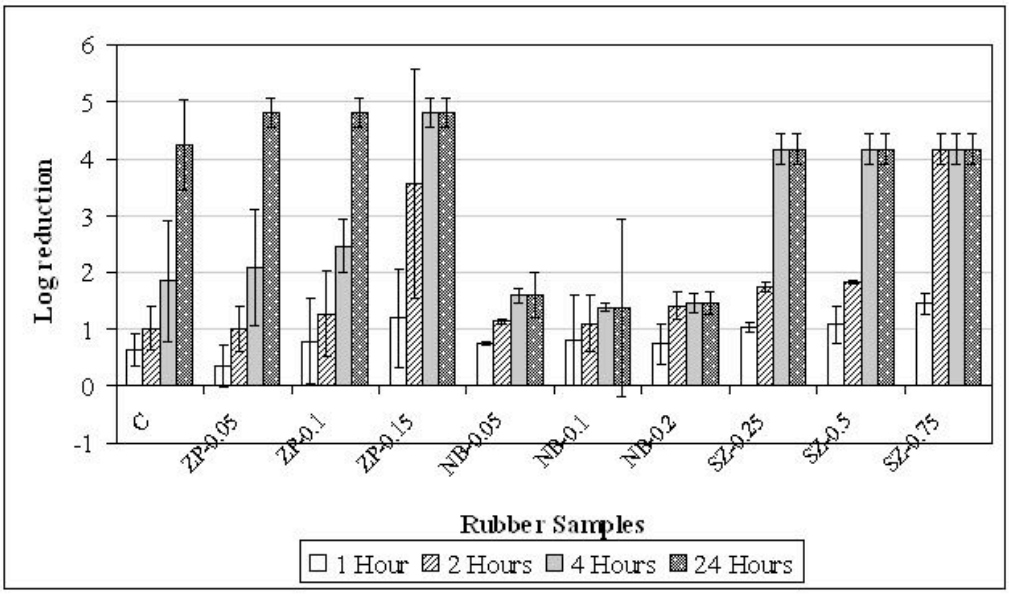

Figure 6: Direct contact assay conducted with MRSA isolate 2. Concentrations of antimicrobial agents are: zinc pyrithione (ZP) 0.05%, 0.1% and 0.15%; N-butyl-1, 2-benzisothiazolin-3-one (NB), 0.05% 0.1%, 0.15%; inorganic silver-glass zeolite (SZ) 0.25%, 0.5%, 0.75%.

- Inorganic silver-glass zeolite rubber samples were the most effective in inhibiting MRSA isolate 2 followed by zinc pyrithione samples.

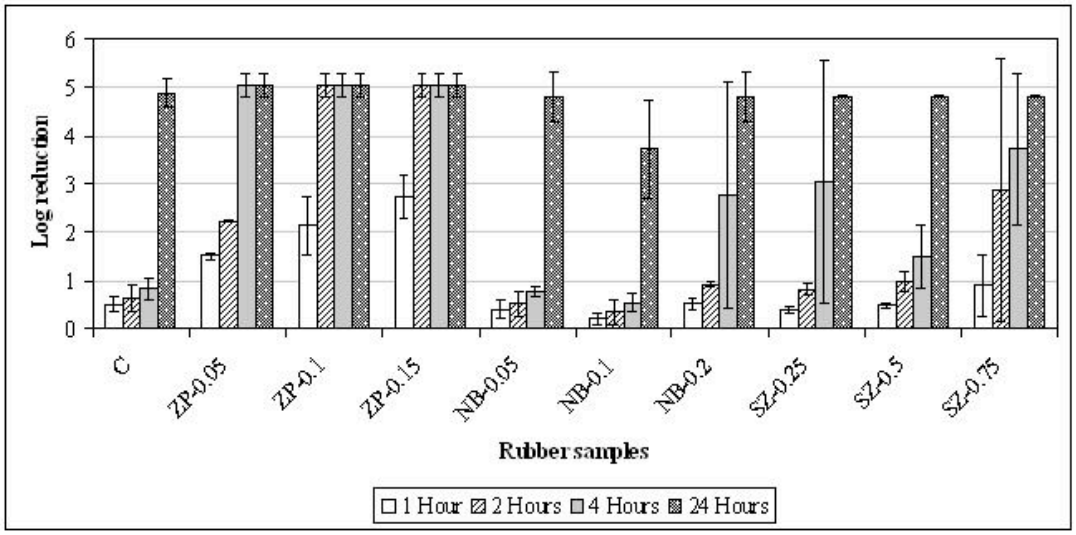

Figure 7: Direct contact assay conducted with MRSA USA300. Concentrations of antimicrobial agents are: zinc pyrithione ZP) 0.05%, 0.1% and 0.15%; N-butyl-1, 2-benzisothiazolin-3-one (NB), 0.05% 0.1%, 0.15%; inorganic silver-glass zeolite (SZ) 0.25%, 0.5%, 0.75%.

- Zinc pyrithione samples were very effective in inhibiting community acquired MRSA strain (USA300), even after two hour in direct contact with the samples.

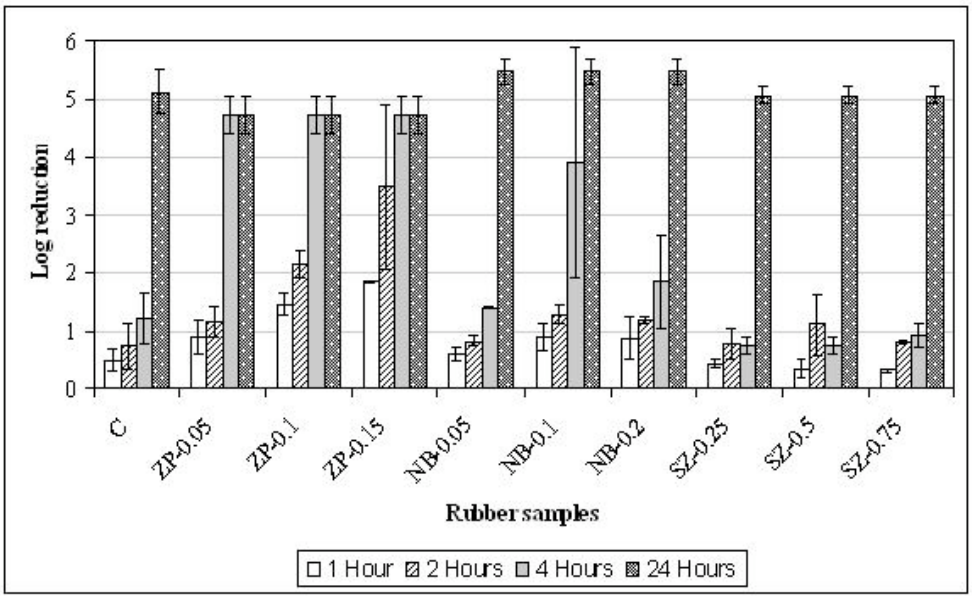

Figure 8: Direct contact assay conducted with MRSA strain MU50. Concentrations of antimicrobial agents are: zinc pyrithione (ZP) 0.05%, 0.1% and 0.15%; N-butyl-1, 2-benzisothiazolin-3-one (NB), 0.05% 0.1%, 0.15%; inorganic silver-glass zeolite (SZ) 0.25%, 0.5%, 0.75%.

- Zinc pyrithione -containing samples were effective in inhibiting hospital-acquired MRSA strain Mu50.

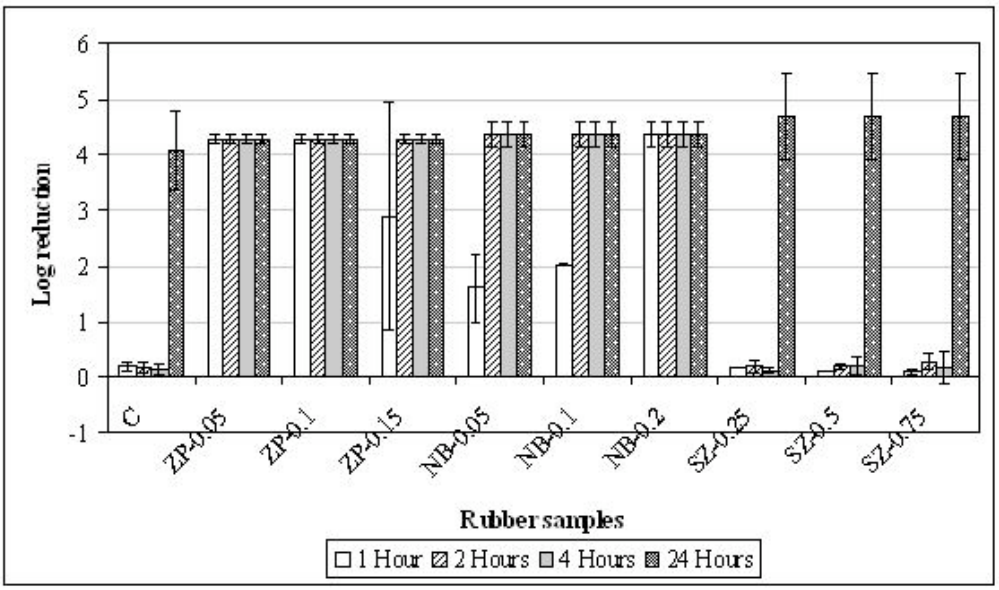

Figure 9: Direct Contact Assay with the gram negative species, *E. agglomerans.* Concentrations of antimicrobial agents are: zinc pyrithione (ZP) 0.05%, 0.1% and 0.15%; N-butyl-1, 2-benzisothiazolin-3-one (NB), 0.05% 0.1%, 0.15%; inorganic silver-glass zeolite (SZ) 0.25%, 0.5%, 0.75%.

- Zinc pyrithione and N-butyl-1, 2-benzisothiazolin-3-one samples were effective in inhibiting *E. agglomerans* even after 1 hour incubation. Inorganic silver-glass zeolite samples were not effective in inhibiting even after 4 hours of incubation, although after 24 hours incubation all the rubber samples including the control samples were effective.

CONCLUSIONS FROM DIRECT CONTACT ASSAYS

The direct contact assays were effective at measuring the bactericidal activity of rubber samples having antimicrobial agents. The control samples (i.e. those without any antimicrobial compounds) showed inhibitory activity after 24 hours of incubation, whereas samples containing antimicrobial agents demonstrated a dose-dependent relationship and, depending on the agent and microbe, were often effective after one hour of incubation. The studies indicate that the survival rate of bacteria is greater when artificial perspiration is used as the suspension media instead of deionized water. The use of artificial perspiration led to more reproducible results and a more consistent dose-dependent relationship between the amount of biocide and log reduction values. Even though the use of artificial perspiration increased the viability level of the bacteria, it falls short of duplicating the actual environmental conditions. In the direct contact assays, log reduction values were high after 24 hours, even with the control samples, yet surveys taken from the spinner room more than 40 hours after human use demonstrated that viable bacteria could still be recovered. In nature, bacteria exist in communities rather than in isolation and are often co-dependent on neighboring organisms. Thus, while the controlled studies with individual strains provide valuable empirical information, the actual effectiveness of the antimicrobial agents is best evaluated under authentic conditions.

The assays revealed that zinc pyrithione was the most effective of the three antimicrobial agents in inhibiting the bacteria tested, with the exception of the MRSA2 strain. In this case, inorganic silver-glass zeolite was more effective. In most assays, log reduction values of two or greater were only observed at incubation times of 4 hrs or greater. This is not to say that the antimicrobial agents were not effective at shorter time points, rather, under the conditions tested (i.e. specific bacterial concentrations, with artificial perspiration at 37˚C) a significant level of reduction was not evident until 4 or more hours of incubation. Additional studies would be needed to assess the relative impact of the antimicrobial agents using lower bacterial concentrations or under different incubation conditions.

It is noteworthy that the MRSA strains tested exhibited differential sensitivity to antimicrobial agents. This suggests that subtle genetic differences in the strains may result in significant differences in the response to biocidal compounds. With the apparent increase in spread of MRSA in hospital and community environments, coupled with the notorious ability of bacteria to develop resistance to control agents, our studies demonstrate that approaches to control of MRSA may be even more challenging than currently perceived. At the least, it illustrates the importance of testing multiple variants, rather relying on a few standard strains. Interestingly, the effects of the antimicrobial agents on the survival of gram negative bacteria were also variable and revealed a large differences between the biocidal activity of the antimicrobial compounds. Differences in the level of growth inhibition were observed between the incubation temperatures of 25˚ C and 37˚ C with the three gram negative organisms tested. In one case, the level of control was not dose-dependent and, surprisingly, there were instances in which the bacterial numbers actually increased (data not shown).

FUTURE DIRECTIONS

In studies not shown, the inhibitory effect of rubber products *per se* (i.e. without containing antimicrobial compounds) appeared to diminish after the samples had been washed extensively. This suggests that there are antimicrobial activities associated with polyurethane-recycled rubber composites, most probably the rubber curatives used in the vulcanizing process, and that these activities are removed and/or affected by washing or other treatments. Additional studies are needed to determine whether the antimicrobial agents added to the composites are depleted following extensive washes, yet the observation that bacteria have a higher survival rate on older rubber products indicates the inclusion of compounds like zinc pythrione could play a role in controlling microbial contaminants in rubber composites as they age under field conditions. Studies that assess the effectiveness of the washing agents and their ability to maintain the longevity of the biocidal activity are planned.

As mentioned, significant differences in log reduction values were observed between studies that were carried out at different incubation temperatures. In most cases, log reduction values were greater at 37˚C rather than 25˚C. This could reflect the rate of release of the antimicrobial compounds from the polyurethane composites or an enhanced effect on bacteria at higher temperatures. Other explanations are also possible. While incubation at 25˚C (77˚F) more closely reflects the ambient temperature bacteria are likely to experience in gymnasiums or other places that have rubber products, most published reports use direct contact assays that are carried out incubations at 37˚ C (98.6˚C). Additional studies are needed to better understand the influence of incubation temperature and other variables that influence the effectiveness of antimicrobial agents, especially on the gram negative bacteria.

Our studies also reveal differences in the response of MRSA isolates to the antimicrobial agents. Tests to genetically characterize the two isolates recovered from the exercise facility are underway. In addition, future

studies are planned to install and monitor polyurethane-rubber composite surfaces under actual field conditions to assess their effectiveness against MRSA and other pathogens.

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BIOGRAPHIES

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Dr. John C. Kennell earned a M.S. degree in Molecular, Cellular & Developmental Biology and Botany from Iowa State University in 1984 and Ph.D. in Plant Pathology from the University of Florida in 1987. Following postdoctoral studies at Ohio State University, his first academic appointment was as the James Trustee Assistant Professor of Biology at Southern Methodist University. He joined the faculty in the Department of Biology at Saint Louis University in 2001 and is currently an Associate Professor and serves as Associate Chair of the Department. His primary research interests involve the study of mitochondria of filamentous fungi and his lab is funded by a grant from the National Institute of Health.

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