Isolation of Sulfur Reducing and Oxidizing Bacteria found in Drywall.

D.G. Hooper[1] M.D., Ph.D., J. Shane, Ph.D. [2],David C. Straus, Ph.D.,[3], V. Bolton, M.D.,[1], K.H. Kilburn, M.D., [4] F.T. Guilford, M.D., [5]

[1] RealTime Laboratories, Dallas, TX, [2] ProLab, Weston, FL, [3] Texas Tech University, Lubbock, TX., [4] Neuro-Test, Inc., Pasadena, CA., [5]Your Energy Systems, LLC, Palo Alto, CA.

ABSTRACT

Dry wall from China has been reported to release sulfur producing products which are corrosive to metals in the home, result in noxious odors, and represent a significant health risk. It has been reported that these emissions produce medical symptoms such as respiratory or asthma type problems, sinusitis, gastrointestinal disorders, and vision problems in home owners and their household pets. We are reporting a method of identifying a causative agent for the emissions by sampling affected gypsum wall board and subjecting that extraction to Real Time Polymerase Chain Reaction (RT-PCR) studies. Specific DNA probes and primers have been designed and patented that detect a specific iron and sulfur reducing bacteria (e.g. *Thiobacillus ferrooxidans*). One hundred percent of affected dry wall samples obtained from homes located in the southeastern United States were found to test positive for the presence of *T. ferrooxidans*. All negative controls consisting of reported unaffected wall board and internal controls were found to test negative within our limits of expectation. Because the problem appears in these cases to be associated with a bacterium, a solution to the problem of dry wall contamination using a patented zeolite suspension is also presented. This compound has the potential to act as an “antibiotic” and mitigate the problem in the contaminated drywall.

INTRODUCTION

Corrosive imported drywall refers to drywall imported into the United States from China from 2004 to 2007. "The U.S. Consumer Product Safety Commission in recent months has received more than 550 reports from people in 19 states and the District of Columbia involving odors, health symptoms and corrosion problems they blame on imported Chinese drywall. The complaints involve "rotten egg" smells and corrosion of wiring and other metals in the homes." It is reported that the imported drywall emits gases that produce a sulfurous odor. These gases are also reported to cause significant property damage in heating, ventilation, and air conditioning (HVAC) systems, electrical wiring, copper plumbing and appliances as well as causing significant health problems. Some believe that the real source and cause of the property damage has not been identified. There are, however, many who conjecture that the gases arise from tainted drywall manufactured in China which is both the source and cause of both the environmental and health problems reported. One theory is that the tainted drywall was manufactured with gypsum mined in China and that the gypsum is then mixed with fly ash, a waste material that is a byproduct from power plants using coal. Samples of Chinese drywall that were tested by United Engineering, however, found that the wallboard consisted of 5-15% organic material, which would contradict the theory that Chinese drywall was made using waste from coal fired power plants. It is now theorized that the tainted drywall from China comes from mined gypsum, not synthetic gypsum made from coal ash. Since this wallboard contains such a high concentration of organic material verse wallboard made with synthetic gypsum, a current theory in the industry is that Chinese drywall contains an organism that is degrading iron and sulfur compounds to produce sulfur odors. This theory has been supported by culture results from some university studies conducted in the U.S. in late 2009 (private communication). The organisms identified were iron and sulfur oxidizing bacterium.

The literature identifies over one hundred species of sulfur and iron-oxidizing organisms. In this study six organisms were studied for presence in contaminated drywall. The iron-oxidizing bacterium *Thiobacillus ferrooxidans* is an important bacterium for the leaching of sulfide ores.*T. ferrooxidans* can oxidize both Fe^{2+} and reduced sulfur compounds. It has been reported by Sugio e.t. al. that *T. ferrooxidans* has the ability not only to oxidize metal ions but also to reduce them. Two enzymes have been isolated from *T. ferrooxidans* which can use Fe^{3} as an electron acceptor for the oxidation of sulfide and sulfite ions. These enzymes, hydrogen sulfide: ferric ion oxidoreductase (SFORase) and sulfite: ferric ion oxidoreductase, have been purified from *T. ferrooxidans. A n*ew route for sulfur oxidation in *T. ferrooxidans* was reported by Sugio et al in 1990. They reported that the substrate for SFORase in solid elemental sulfur oxidation by *T. ferrooxidans* was found to be hydrogen sulfide. This system involves a hydrogen sulfide-binding protein (SBP), which reversibly binds hydrogen sulfide and supplies SFORase with hydrogen sulfide as a substrate. Thus, the existence of SBP demonstrated that hydrogen sulfide is synthesized in T. *ferrooxidans* cells. This work concluded that hydrogen sulfide plays a role as an energy reserve in T. ferroxoidans. Sugio et. al. in 1992 and in 2000 substantiated the existence of a hydrogen sulfide:ferric ion oxidoreductase in iron-oxidizing bacteria such as *T. ferrooxidans*.

Although the source has not yet been identified, analytical testing of contaminated Chinese drywall samples has revealed strontium sulfide, hydrogen sulfide, and sulfur dioxide (SO_2) (Public Health Statement, May 7, 2009). Emissions from the drywall have been blamed for the reported corrosion of plumbing and electrical systems and homeowners have blamed the gases for their health ailments (numerous local and national newspaper articles, Florida Department of Health releases). In a report released from the Florida Department of Health on October 15, 2009 many unanswered questions remained. Although sulfur gases were found, there is no evidence of high emissions in any of the samples evaluated which do not explain the difficulties in human ailments due to the exposure. Dose response relationships for hydrogen sulfide in human subjects cannot be developed directly because of known adverse effects on function of the brain, eye, lung and skin. The best data has been assembled from observed episodes rather than from experimental exposures. These data showed impairment of balance, color discrimination, and reaction time, verbal memory and other functions at average daily exposures to H2S of 1 ppm to 5 ppm. (Kilburn K.H., 1999, and 2008). There is also evidence of serious eye and CNS impairment from brief spikes of H2S for 1 to 3 breaths at 50 ppm to 200 ppm and coma at average levels of 100 ppm and higher concentrations. Also, levels that corrode metal cause corneal ulcers and asthma in human subjects.

The organisms used in this study were: *Thiobacillus (Acidithiobacillus) ferrooxidans, Sulfobacillus thermosulfidooxidans, Leptosprillium ferroxidans, Thiobacillus (Acidithiobacillus) caldus, Actinobacillus thiooxidans, and Desulfotomaculum ruminis.* All work was conducted on either ATCC cultures or DNA obtained directly from ATCC. RealTime polymerase chain reaction studies (rt-pcr) were conducted on the six organisms. A provisional patent has been filed on the method and composition of the DNA work reported here. The data indicate a very strong correlation between the presence of the bacterium T. ferrooxidans and the presence of the associated myriad problems with the contaminated crywall.

MATERIALS AND METHODS

SAMPLES: Samples were obtained from 25 homes in the southeastern United States. These samples were submitted from Pro-Lab, Florida, and were subjected to multiple toxicology, mycology and microbiology examination. All studies were non-contributory to causation of the production of hydrogen sulfide and sulfur dioxide. A two square inch piece of dry wall was submitted under chain of custody to RealTime Laboratories. Each specimen was placed in a clean, sterile crucible and crushed into dry powder. One gram of powdered dry wall was then subjected to extraction.

CONTROLS: Samples of US manufactured dry wall, not exuding H_2S and SO_2 were purchased from a local building supply store and sampled in the same matter as the contaminated dry wall sections. Positive controls were non-contaminated dry wall that was spiked with various concentrations of six different bacterial organisms to study. (Table 1). Internal controls consisted of a known fungal spore, *Geotricum sp.*, (GEO Control) which is present to insure that that extraction system is working.

EXTRACTION OF SAMPLES: Positive and negative controls and all samples were extracted using a commercially available extraction system that was optimized for the extraction of bacterial genomic DNA. Powdered samples were extracted by pretreatment of Proteinase K and lysis solution in the presence of silica beads for bead beating. A set volume of internal control spores was introduced to the all samples, including the negative control. All samples were incubated at 55º C and 72º C and then further subjected to a second lysis solution. After ethanol addition, the samples were placed into spin columns containing a silica filter. DNA in the mixture was bound to the silica filter, washed several times to remove contaminants and eluted from the spin column filter utilizing a low salt solution. Samples were stored at -20º C until tested.

TARGET DESIGN: Primers and probes specific for each of 6 bacterial targets plus an internal control assay were designed by RealTime Labs, LLC and synthesized by Sigma-Aldrich (St. Louis, MO). All probes used for the assays are hydrolysis probes with the reporter FAM attached to the 5’ end and the quencher BHQ (Black Hole Quencher) attached to the 3’ end. Primers and probes were received lyophilized and re-suspended and mixed into working stocks containing probe and primer at required concentrations. The enzyme for the reaction was purchased as a lyophilized bead from Cepheid and manufactured specifically for the SmartCycler® system. Each 25.0 uL SmartCycler® reaction tube contained reaction mix in the formula: 0.5 bead, 16.5 µl of PCR grade water, 3.5 µl primer/probe working stock, and 5.0 µl of extracted DNA. The internal control was run for each extracted sample and a negative control was added as an independent assay to insure a quality control step for the extraction. All 6 bacterial assays and the internal control had a cycling profile containing an initial hot start followed by 45 denature and anneal cycles. All assays were designed and operated as qualitative detection assays. The capacity of a single SmartCycler® instrument varies from 16 to 96 reactions depending on the configuration. Multiple bacterial assays can be processed on a single SmartCycler® run with each assay containing samples, a negative control and a positive control. Data generated from the assays were analyzed utilizing the SmartCycler® Software and positives were defined as any target that crosses the Cepheid recommended threshold of 20 on the fluorescence scale during processing on the instrument.

ASSAY DESIGN: All assays were designed using sequence from the strains listed in Table 1 with the assays being designed and utilizing commercially available assay design software (GenBank). Specificity was checked between the organisms using an assembly program. The probe and primer sequence specificity was checked using the Blast tool available at the NCBI website.

ASSAY OPTIMIZATION:

A: Rehydration of Lyophilized Stocks: All lyophilized stocks were rehydrated to 100 µM concentration. Master Mix Stocks were made for each assay by using the following formulation for 700.0 µl of Master Mix Stock:

Primer 1 15.0 µl
Primer 2 15.0 µL
Probe 10.0 µL
Water 660.0 µL
TOTAL 700.0 µL

B: Standard Curves:
Assays for organisms were optimized utilizing target DNA in 10 fold serial dilutions formulating a curve. Data were collected and evaluated. The assays were determined to be optimized to the specification of RealTime Laboratories.

RESULTS

A. CONTROLS FOR RT-PCR BACTERIA

Positive Control – Targets were bacterial extractions of the organisms listed in Table 1 which were obtained from ATCC. A positive control for each target of interest (Primer/Probe sets) was processed along with each drywall sample in each real-time PCR run. The positive control shows that the primer/probe set for each target is not being inhibited and shows that a negative result is a true negative (**Figure 1).**
Negative Control – A negative control for each target of interest (Primer/Probe sets) was processed along with each drywall clinical sample in each real-time PCR run. This negative control can be extracted from tissue or water but must be lot checked prior to use. The negative control shows that the primer/probe set, water and extraction reagents for each target is not contaminated with the target and shows that a positive result is a true positive **(Figure 1).**
Internal Control – Each drywall sample examined was inoculated with fungal spores from the internal control target *Geometrica sp.* to show that a negative target result is a true negative result and not related to the extraction of the sample. The samples were processed through the extraction protocol and amplified and detected utilizing primer and probes specific for *Geometrica sp.* (**Figure 2**).

Figure 1. Representative Control Curves for Bacteria Tested

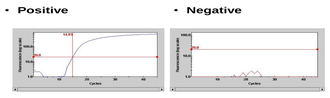
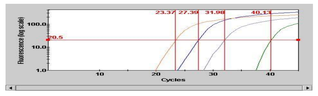
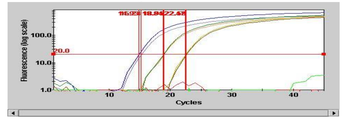


Figure 2. Representative GEO control for all tests conducted to represent internal control. Different concentrations of GEO were added to the same amount of drywall material.



Assays for all organisms were optimized utilizing target DNA in 10-fold serial dilutions formulating multiple curves. **(Figure 3).** Data were collected and evaluated and the assays were determined to be optimized to the specification of RealTime Laboratories. **(Table 2).**

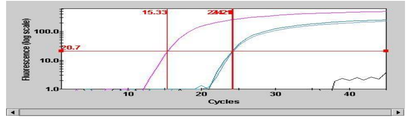
Figure 3. Standard Curves for *Thiobacillus ferroxidans*



1:10 dilutions made of *Thiobacillus ferroxidans* in water to demonstrate sensitivity of system. Positive results cross the 20.0 fluorsecence (log scale). Negatives do not cross.

C. TESTING OF SPIKED DRYWALL: Assays for all organisms were conducted by spiking known ATCC organisms into normal drywall that was purchased from a local building supply store. This dry wall was not emitting any sulfur compounds. Drywall was tested using each of the six DNA probes. A positive GEO control and negative drywall sample was also tested. In all cases of the spiking test, all six organisms were detected. (**Figure 4)**.

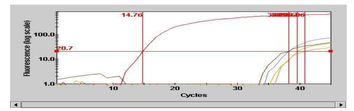
Figure 4. Testing of Spiked Drywall



Drywall preparations were spiked with various concentrations of bacteria. Positive controls crossed the 20.0 line first with dilutions crossing later. Negative controls never crossed the 20.0 line.

TESTING OF DRYWALL UNKNOWNS:
Assays using probes for all organisms in this study were conducted using all six organism noted in Table 1. Results are shown in **Figure 5** and further summarized in **Table 2**. *A. ferrooxidans* was detected in all twenty-five samples tested. A possible weak positive result with *T. caldus* was detected in one of twenty-five drywalls tested. Further testing of the six organisms in all twenty-five boards is continuing in order to optimize recovery.

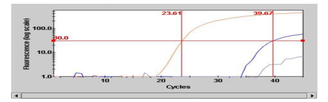
Figure 5. Testing of Drywall Unknowns



Examples of dry wall from contaminated homes. Control *T. ferroxidans* crosses at 14.76. The drywalls cross at 38-41. Negatives did not cross.

•**TESTING OF CONCENTRATED DRYWALL UNKNOWNS:**
Assays for *T. caldus* and *A. ferrooxidans* were run with concentrated versions of three samples to determine if increased sample could be used to optimize recovery. Results obtained demonstrated that more than 1 gm of the sample overloaded the filter in the collection tube and no DNA was recovered (**Figure 6**).

Figure 6. Testing of Concentrated Drywall unknowns.



Drywall extractions were concentrated by putting more extraction material over the silica filters. Here three curves show positive control (23.61), standard preparation (39.67), and concentrated amount (does not cross line).

Table 2. Drywall test results for validation studies of sulfur and iron reducing and oxidizing bacteria.

Bacteria	Standard Curves Drawn	Spiked Drywall Showing No Inhibition of Curves # spiked / # Positive	Drywall Unknowns Positive/Total (% Positive)	Concentrated Drywall Unknowns + /Total
Thiobacillus (Acidithiobacillus) caldus	Yes	6/6	1 weak/25 0 %	0/3
Sulfobacillus thermosulfidooxidans	Yes	6/6	0/25 0%	0/3
Leptospirillum ferrooxidans	Yes	6/6	0/25 0%	0/3
Actinobacillus thioxidans	Yes	6/6	0/25 0%	0/3
Thiobacillus (Acidithiobacillus) ferrooxidans	Yes	6/6	25/25 100%	0/3
Desulfotomaculum ruminis	Yes	6/6	0/25 0%	0/3

SUMMARY AND CONCLUSIONS

Contaminated drywall can be defined as drywall that emits hydrogen sulfide and sulfur dioxide gases. The control group in this case consists of drywall that does not emit the same types of gases. The hypothesis in this study tested the following:
Null Hypothesis: There is not a significant difference in the presence of sulfur and iron reducing/oxidizing bacteria between the groups of contaminated and non-contaminated drywall.
Alternative Hypothesis: There is a significant difference in the presence of sulfur and iron reducing/oxidizing bacteria between the groups of contaminated and non-contaminated drywall.
Results: Reject the null hypothesis; there is sufficient evidence to indicate that there are bacteria that can reduce/oxidize sulfur and iron in contaminated drywall and these bacteria are not present in the non-contaminated dry wall.