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Short Communication

Antibacterial activity of reduced iron clay against pathogenic bacteria associated with wound infections

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ABSTRACT

Clay is a substance historically utilized by indigenous cultures for the treatment of superficial wound infections. This study evaluated the effects of a recently identified clay – OMT Blue Clay – against staphylococci, streptococci, *Enterobacteriaceae* and non-fermenting Gram-negative bacilli. The clay and its aqueous leachate were evaluated against the bacteria in biofilm and planktonic states. Time-kill studies were used to assess planktonic activity. Biofilms on medical-grade Teflon discs were treated with a hydrated clay suspension or leachate. For the planktonic studies, clay and leachate exhibited bactericidal activity against all strains tested, with the exception of leachate against *Staphylococcus aureus* IDRL-6169 and USA300. All strains treated with clay suspension and leachate resulted in statistically significant biofilm population reductions compared with controls, except *S. aureus* IDRL-6169 and USA300 ($P \leq 0.05$). OMT Blue Clay and its aqueous leachate exhibited bactericidal activity against a range of human pathogens in the planktonic and biofilm states.

1. Introduction

Humans have used clay for medicinal purposes since prehistoric times, and this practice is now being considered for adoption into the biomedical compendium. A recent report described the application of a French green clay to Buruli ulcers, resulting in apparent activity against *Mycobacterium ulcerans* and wound re-epithelialization [1,2]. This example and others like it have generated interest in the pharmacologic potential of certain clays for treatment of infected wounds.

Clay is a size classification of natural minerals referring to particulate diameters of < 2 µm [3]. The mineralogical and chemical compositions of individual clay deposits vary by geologic environment. Clays identified as potentially antibacterial share mineralogical and chemical compositions that provide buffering capacity to fluids in contact with them, which include reduced transition metals (most commonly Fe^{2+}) and whose immense surface areas (hundreds of m^2/g) control the water chemistry, which is key to sustained mineral viability [1,4]. Many underlying mechanisms for the antibacterial activity of various clays have previously been investigated [5-8].

A variety of clays were previously evaluated against planktonic liquid cultures incubated with equal parts of a clay suspension followed by quantitation over 24 hours [4,9]. In these studies, one Fe^{2+}-bearing clay from a deposit in Oregon (mined by Oregon Mineral Technologies, OMT) exhibited superior activity compared with controls when evaluated against: *Escherichia coli* ATCC 25922; extended-spectrum β-lactamase-producing *E. coli* ATCC 51446; *Pseudomonas aeruginosa* ATCC 27853; *Salmonella enterica* subspecies *enterica* serovar Typhimurium ATCC 14028; *Staphylococcus aureus* ATCC 29213; *S. aureus* USA300; *Staphylococcus epidermidis* ATCC 14990; methicillin-resistant *S. epidermidis* (MRSE) ATCC 35984; and a methicillin-resistant *S. aureus* (MRSA) isolate from Sonora Quest Laboratories, Tempe, AZ [6,9,10]. This natural clay is dominated by illite-smectite (a group of clay minerals containing an expandable interlayer structure) pyrite, Ca-plagioclase, and quartz [1,9]. The smectite interlayer region acts as a reservoir from which metals, which may have antibacterial effects, are gradually released via cation exchange [10,11]. When clay containing reduced transition metals is taken from its natural environment and mixed with oxygenated water, soluble metals from the minerals likely provide aqueous reactants that drive an antibacterial process [9].

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Table 1
Bacterial species studied, including strain, source, and susceptibility profile.

Species	Strain	Source	Antimicrobial susceptibility (if known)
Staphylococcus aureus	IDRL-6169	Hip (prosthetic joint)	Resistant to methicillin and mupirocin (high level of resistance)
S. aureus	USA300	Unknown	Resistant to methicillin
Staphylococcus epidermidis	ATCC 35984	Catheter sepsis	Resistant to methicillin
Streptococcus pyogenes	IDRL-7467	Knee (prosthetic joint)	Unknown
Streptococcus dysgalactiae	IDRL-10052	Knee (prosthetic joint)	Susceptible to penicillin, ceftriaxone, erythromycin, and vancomycin
Pseudomonas aeruginosa	IDRL-11465	Urine	Resistant to cefepime, ceftazidime, ceftazidime/avibactam, meropenem, and aztreonam
P. aeruginosa	IDRL-10628	Unknown	$bla_{\mathrm{VIM-2}}$; resistant to ceftazidime and ceftazidime/avibactam
Enterobacter cloacae	IDRL-10306	Knee (prosthetic joint)	Resistant to ampicillin and cefazolin
E. cloacae	IDRL-10375	Unknown	bla_{KPC}; resistant to ceftolozane/tazobactam, imipenem, meropenem, ertapenem, ceftriaxone, and cefepime
Acinetobacter baumannii	ARLG-1268	Hip	Resistant to amikacin, ampicillin, cefepime, ceftazidime, ciprofloxacin, and tobramycin
Klebsiella pneumoniae	IDRL-10377	Unknown	bla_{KPC}; resistant to ceftolozane/tazobactam, imipenem, meropenem, ertapenem, ceftriaxone, and cefepime
Escherichia coli	IDRL-10366	Unknown	bla_{KPC}; resistant to ceftolozane/tazobactam, imipenem, meropenem, ertapenem, ceftriaxone, and cefepime
E. coli	ATCC 25922	Clinical isolate	Pan-susceptible

The proposed general mechanism for the antibacterial activity of OMT Blue Clay is that hydration of the clay results in dissolution of reduced Fe^{2+} and Al^{3+} from the minerals, which together damage the bacterial membranes, allowing excess Fe^{2+} to cause intracellular protein damage by oxidation [9]. The soluble metals assumed to be involved in the antibacterial action are protected from rapid oxidation by adsorption into the expandable clay interlayer, potentially conferring to the clay a more sustained effect than metals in the leachate solution alone [1,9].

The current study examined the effect of the OMT Blue Clay and its aqueous leachate on monomicrobial pathogenic bacteria in planktonic and biofilm states.

2. Materials and methods

2.1. OMT Blue Clay and leachate preparation

Vials containing clay were autoclaved, followed by adding sterile water (Barnstead™ Nanopure™, Thermo Fisher Scientific™ Marietta, OH) for a clay concentration of 200 mg/mL; the mixture was homogenized on a stir plate overnight. A leachate was extracted from a portion of the equilibrated suspension by ultracentrifugation at 9000 rpm for 1 hour at room temperature; the supernatant constituted the leachate and was collected and stored at 4°C. The leachate remained at pH < 4 and Eh $\approx$ 400–600 mV [9] for the duration of experimentation.

2.2. Microorganisms

Twelve bacterial species (Table 1) representing common pathogens in superficial non-healing wounds were tested. Each organism was subcultured from Microbank™ vials (Pro-Lab Diagnostics, Round Rock, TX), which had been stored at –80°C, onto trypticase soy agar containing 5% sheep blood and incubated at 37°C in 5% CO_2 for 24 hours.

2.3. Planktonic experiments

Three to five isolated colonies were placed into 4 mL 20% Luria broth (LB) and grown to 0.5 McFarland visual turbidity standard [1.5×10^8 colony forming units (cfu)/mL]. They were then diluted to a 10^7 cfu/mL concentration in 20% LB. A 1:1 ratio of diluted bacteria and sterile water comprised the positive control. A 1:1 ratio of inert clay suspension or leachate with 20% LB, without bacteria, was used as a negative control. Treatment groups were prepared in a 1:1 ratio of diluted bacteria to either OMT Blue Clay or leachate. Culture tubes were placed on their sides to maintain the clay or leachate in suspension and incubated at 37°C in air on an orbital shaker at 180 rpm. At 4, 8, 12, and 24 hours, clay-treated and leachate-treated bacteria, and positive controls, were quantitatively cultured. The contents of the treatment tubes were subsequently transferred into tubes of 10 mL tryptic soy broth (TSB), incubated for 24 hours, and examined for growth.

2.4. Biofilm experiments

Three to five colonies of viable bacteria were inoculated into 2–4 mL TSB, and incubated at 37°C in air until a visual turbidity equivalent of a 0.5 McFarland standard was achieved. Broth cultures were diluted 1:100 in TSB and 2 mL added to each of 12 wells in a 24-well plate. Medical grade, autoclaved, Teflon discs (12.5×1 mm) were aseptically transferred to each of the wells and incubated at 37°C air for 24 hours on an orbital shaker (120 rpm) to allow biofilm formation on the discs. Discs were rinsed in sterile saline and placed in 1.5 mL of the following: a 1:1 solution of sterile water and 20% LB (positive control); or solution of 1:1 20% LB and OMT Blue Clay or OMT leachate as treatments, respectively. Control and treated discs were incubated at 37°C in air for 24 hours.

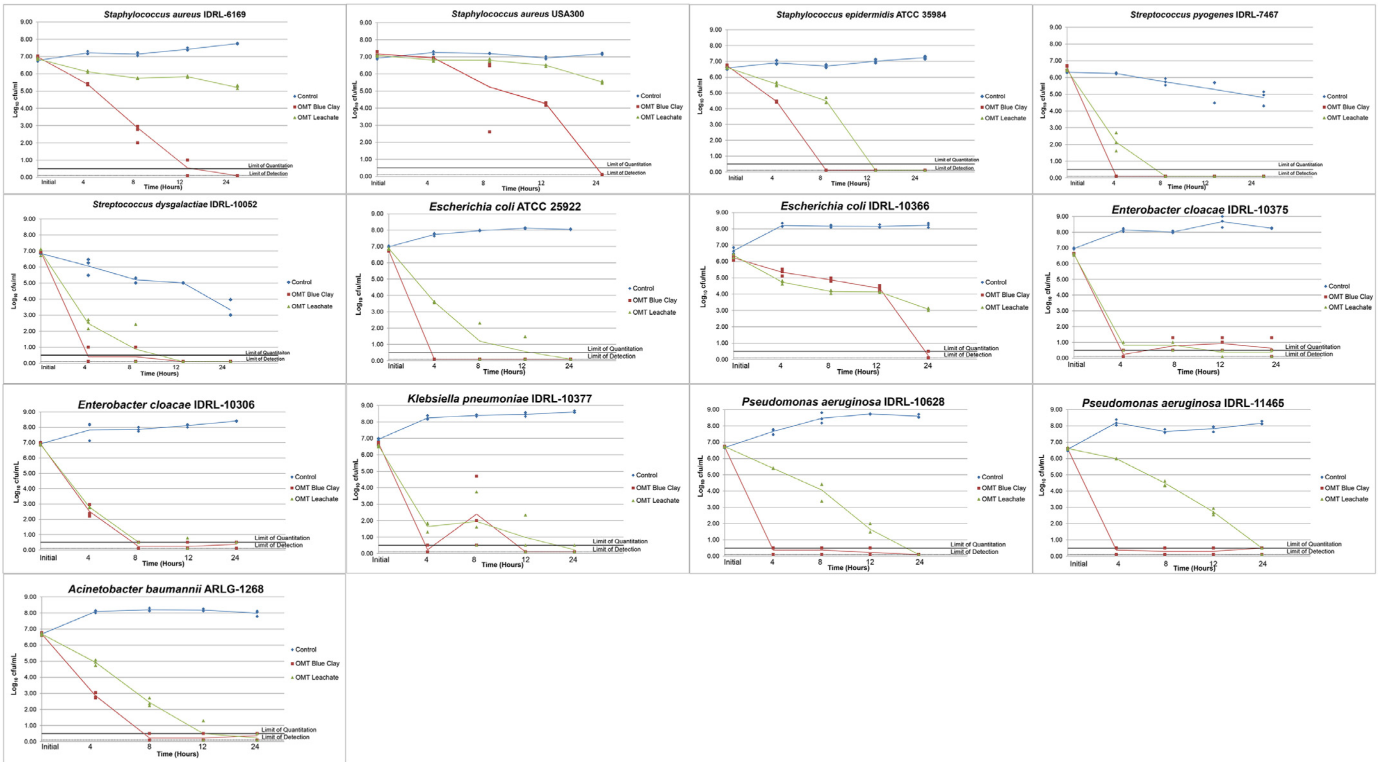

Fig. 1. Planktonic bacterial population densities following treatment with OMT Blue Clay or OMT leachate after 4, 8, 12, or 24 hours.

2.4.1. Baseline biofilm growth

After incubation, three discs were separately placed into 1 mL sterile saline following rinsing, and vortexed for 30 seconds, sonicated for 5 minutes, and vortexed for another 30 seconds. The resulting sonicate fluid was quantitatively cultured and incubated in room at 37°C in air for 24 hours, with results recorded as cfu/cm^2.

2.4.2. Treated biofilms

Control and treatment discs were vigorously rinsed and separately placed in 1 mL TSB. Sonicate fluid was obtained as above and incubated at 37°C in air for 24 hours, with results recorded as cfu/cm^2. After quantitation, an additional 4 mL TSB was added to each disc and incubated in air at 37°C for 24 hours for broth-only cultures.

2.5. Statistical analyses

Experiments were performed in triplicate as technical replicates. Statistical analyses were completed using the Wilcoxon rank-sum test. No adjustments for multiple comparisons were made due to the small sample size. All tests were two sided and *P*-values $\leq$ 0.05 were considered statistically significant. Analyses were performed using SAS v.9.4 software (SAS Inc., Cary, NC). For planktonic studies, the OMT Blue Clay and leachate were considered bactericidal if there was a $\geq$ 3 $\log_{10}$ reduction in bacterial counts compared with controls [12]. Limits of quantitation and detection were numerically designated as 0.5 and 0.1 log cfu/mL, respectively, if agar plates yielded no colonies but the incubated sonicate fluid gained turbidity and yielded viable growth on subculture, or if neither plates nor sonicate fluid indicated growth.

3. Results

3.1. Planktonic experiments

The OMT Blue Clay and leachate were bactericidal against all Gram-positive bacteria tested over 24 hours, with the exception of OMT leachate-treated *S. aureus* IDRL-6169 and USA300 (Fig. 1). Growth of all Gram-positive organisms was reduced to the limit of detection over 24 hours following OMT Blue Clay and leachate treatments except leachate-treated *S. aureus* IDRL-6169 and USA300. The OMT Blue Clay and leachate were also bactericidal against all Gram-negative bacteria tested over 24 hours, reducing all populations to the level of detection, with the exception of the leachate-treated *E. coli* IDRL-10366.

3.2. Biofilm experiments

Compared with controls, the size of the disc-associated bacterial population for all organisms was substantially reduced when exposed to both clay and leachate, except leachate-treated *S. aureus* IDRL-6169 and USA300.

With the exception of *S. aureus* USA300, all Gram-positive bacterial populations exposed to the clay were at the threshold of detection, meaning that no bacterial colonies were observed through the quantitative culture process (Fig. 2a). Additionally, all species exhibited population reductions when treated with the leachate, barring *S. aureus* IDRL-6169 and USA300. Except where noted, all clay-associated and leachate-associated bacterial reductions were statistically significant compared with controls ($P \leq 0.05$).

Gram-negative bacteria similarly exhibited attenuation following OMT Blue Clay or leachate treatment (Fig. 2b). While the application of clay did not result in total elimination of viable bacteria, with the exception of *Acinetobacter baumannii* ARLG-1268,

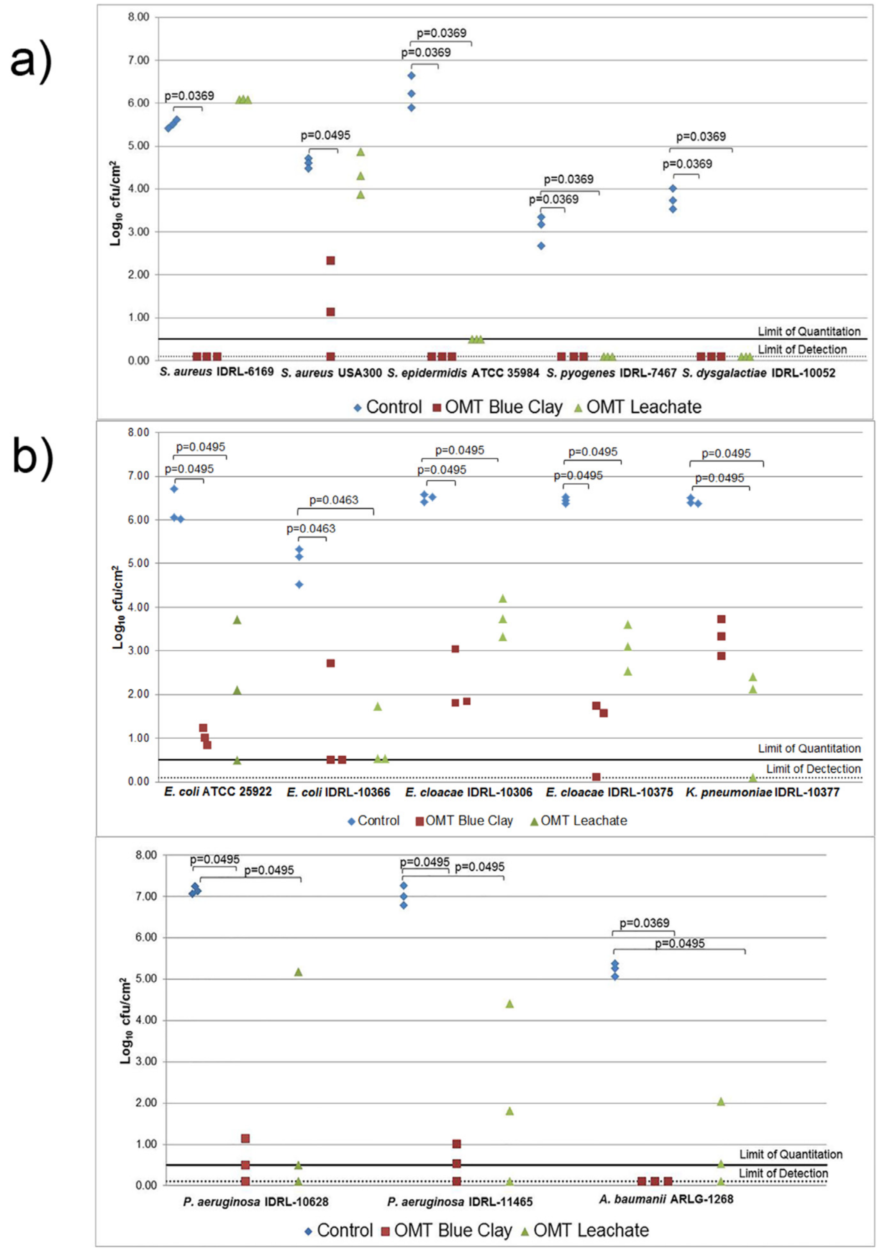

Fig. 2. Disc-associated biofilm population densities for a) Gram-positive and b) Gram-negative species 24 hours after treatment with OMT Blue Clay or OMT leachate compared with controls.

all species demonstrated statistically significant population reductions compared with controls. The same was also observed in all leachate-treated Gram-negative biofilms compared with controls.

Five species exhibited statistically significant population reductions when treated with OMT Blue Clay vs. leachate: *S. aureus* IDRL-6169 ($P = 0.037$), *S. aureus* USA300 ($P = 0.049$), *S. epidermidis* ATCC 35984 ($P = 0.025$), *Enterobacter cloacae* IDRL-10306 ($P = 0.049$), and *E. cloacae* IDRL-10375 ($P = 0.049$) (Fig. 2). In the case of *S. aureus* IDRL-6169, leachate-treated biofilms exhibited greater population density than the control ($P = 0.049$). Also, leachate-treated biofilms of *Klebsiella pneumoniae* IDRL-10377 were diminished in cfu/cm^2 vs. clay-treated counterparts ($P = 0.049$) (Fig. 2).

4. Discussion

While clays have previously been used in a variety of medical applications, the antibacterial activity of clays is just beginning to be scientifically evaluated [1,2,7,9,13,14]. This study investigated the activity of a specific clay against pathogenic bacteria common to wound infections in both the planktonic and biofilm states. The OMT Blue Clay that was tested has been mineralogically and chemically defined in detail [1].

The current results support the hypothesis that treatment of monomicrobial biofilms with OMT Blue Clay generates population reductions of sufficient magnitude to warrant *in vivo* evaluation. Both clay and leachate demonstrated bactericidal activity towards all organisms tested in the planktonic state, with the exception of leachate-treated *S. aureus* IDRL-6169 and USA300 (Fig. 1). The *S. aureus* strain studied here was different from those examined in previous work, potentially pointing to strain and/or methodological differences [6,9,10]. Furthermore, oxidation of metals in solution may occur when clays are not present to buffer the aqueous conditions where Fe^{2+} is soluble, abrogating the leachate's antibacterial effect [1].

Both clay and leachate showed statistically significant growth attenuation of established biofilm communities, with the exception of leachate-treated *S. aureus* IDRL-6169 and USA300. Five organisms demonstrated greater biofilm population reduction when treated with clay *vs*. leachate (Fig. 2). It was hypothesized that the antibacterial action on biofilms is similar to that of planktonic bacteria [1,5,9,14-18]. It has previously been observed that the clay's mineralogy provides an enhanced antibacterial effect over metals in solution alone [9], which is consistent with the present results.

Limitations of this study were that only single concentrations of clay and leachate were evaluated, rather than ranges of concentrations. While the concentrations were selected based on activity of the same OMT clay previously achieved by the current group, the referenced study had applied this concentration to planktonic bacteria only, with some divergence in strains tested [5,6,14]. A second limitation was the monomicrobial design of planktonic and biofilm experiments, which lack the ecologic complexity of a real, infected wound bed. Without accounting for the diversity of human-associated microbiomes, the current results obtained may only provide a limited prediction of treatment response in the target location.

As clay is a mineralogically heterogeneous substance, thorough understanding of the material is necessary before medical use. Toxic metals (e.g. As, Hg) harbored within certain clays may become absorbed through the integumentary barrier. Many clays do not reduce bacterial populations; they may either be innocuous or actually enhance bacterial growth [2]. While recent studies [4,8,9,13–15] have ascribed antibacterial activity to particular chemistries, the preferred advancement would be to synthesize a similar mineral composition with quality control of chemical makeup.

5. Conclusions

In summary, this study demonstrated that the application of OMT Blue Clay and its aqueous leachate to monomicrobial planktonic communities and biofilms results in significant reductions in population size. These results are provocative because they demonstrate susceptibility to clay-based treatment among both Gram-positive and Gram-negative bacteria, including strains resistant to traditional antibiotics. While this line of antibacterial therapeutics requires further development, the current results generate support for future *in vivo* testing and demonstrate promise for new antibacterial designs.

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Competing interests

Dr. Patel reports grants from CD Diagnostics, BioFire, Curetis, Merck, Hutchison Biofilm Medical Solutions, Accelerate Diagnostics, Allergan, and The Medicines Company. Dr. Patel is or has been a consultant to Curetis, Qvella, St. Jude, Beckman Coulter, Morgan Stanley, Heraeus Medical GmbH, CORMATRIX, Specific Technologies, Diaxonit, Selux Dx, GenMark Diagnostics, LBT Innovations Ltd, PathoQuest and Genentech; monies are paid to Mayo Clinic. In addition, Dr. Patel has a patent on *Bordetella pertussis/parapertussis* PCR issued, a patent on a device/method for sonication with royalties paid by Samsung to Mayo Clinic, and a patent on an anti-biofilm substance issued. Dr. Patel has served on an Actelion data monitoring board. She receives travel reimbursement from ASM and IDSA and an editor's stipend from ASM and IDSA, and honoraria from the NBME, Up-to-Date and the Infectious Diseases Board Review Course. Dr. Williams holds an international patent with Arizona State University and the U.S. Geological Survey on synthetic antibacterial clay compositions and methods.

All other authors: none to declare.

All authors have read and approved the article in its current form.

Ethical Approval

Ethical approval was not required for the execution of this research.

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