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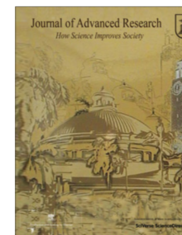

ORIGINAL ARTICLE

Dexamethasone abrogates the antimicrobial and antibiofilm activities of different drugs against clinical isolates of *Staphylococcus aureus* and *Pseudomonas aeruginosa*

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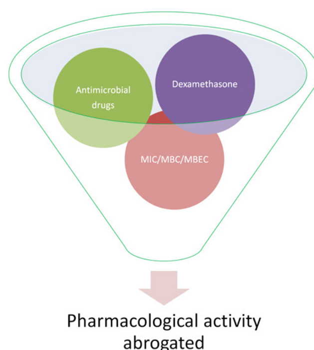
GRAPHICAL ABSTRACT



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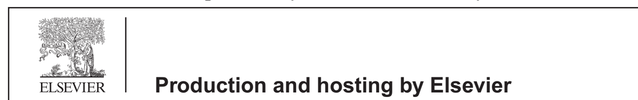

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ABSTRACT

Staphylococcus aureus and *Pseudomonas aeruginosa* are part of the human microbiota and are also important bacterial pathogens, for which therapeutic options are lacking nowadays. The combined administration of corticosteroids and antimicrobials is commonly used in the treatment of infectious diseases to control inflammatory processes and to minimize potential toxicity of antimicrobials, avoiding sequelae. Although different pharmaceutical dosage forms of antimicrobials combined to corticosteroids are available, studies on the interference of corticosteroids on the pharmacological activity of antimicrobials are scarce and controversial. Here, we provide evidence of the interference of dexamethasone on the pharmacological activity of clinically important antimicrobial drugs against biofilms and planktonic cells of *S. aureus* and *P. aeruginosa*. Broth microdilution assays of minimal inhibitory concentration (MIC), minimum bactericidal concentration (MBC), and minimum biofilm eradication concentration (MBEC) of gentamicin, chloramphenicol, oxacillin, ceftriaxone and meropenem were conducted with and without the addition of dexamethasone. The effect of all drugs was abrogated by dexamethasone in their MIC, MBC, and MBEC, except gentamicin and meropenem, for which the MBC was not affected in some strains. The present study opens doors for more investigations on *in vitro* and *in vivo* effects and safety of the combination of antimicrobials and glucocorticoids.

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Introduction

The treatment of bacterial infections presently faces major challenges due to the constant emergence of antimicrobial resistant strains. The rate of disease occurrence and mortality is increasing worldwide, as clinical treatments are steadily failing [1]. This microbial resistance picture has become a serious threat to public health, especially in developing countries, where health policies often do not include antimicrobial stewardship programs [2]. In addition, the number of new approved antimicrobial drugs has been decreasing since the 1950s. Because of the lack of novel antimicrobial drugs in the pharmaceutical market, scientists are worried on the possibility of the post-antibiotic era, in which scarce pharmacological options will be available for the treatment of even minor infectious diseases [1,2].

Several drug-resistant species have been detected in community and hospital outbreaks of infections. *Staphylococcus aureus* is a Gram-positive species which is part of the human microbiota, and is also an important opportunistic pathogen, which colonizes around 20% of the population [3]. Different diseases can be caused by *S. aureus* infections, including osteomyelitis, endocarditis, and otitis, and drug resistance among strains of this species is steadily growing worldwide [4,5]. *Pseudomonas aeruginosa* is an ubiquitous Gram-negative aerobic species, frequently isolated from aquatic and terrestrial environments and of the human microbiota [6,7]. Pathogenic strains of this species are commonly associated with chronic lung infections, and are capable of causing a wide range of opportunistic infections. High levels of phenotypic diversity of pathogenic strains have been described, and a clinically relevant consequence of this diversity is a poor antimicrobial susceptibility profile, making chronic *P. aeruginosa* infections very difficult to eradicate [1,8].

A common virulence factor involved in drug resistance by *S. aureus*, *P. aeruginosa* and several other microbial species are biofilms. Biofilms can be defined as microbial communities that grow attached to biological tissues or to abiotic surfaces, set in a matrix of extracellular polymeric substances (EPS) [9,10]. The EPS matrix is generally composed of polysaccharides, lipids, proteins and extracellular DNA, and has a protective and adhesive role in biofilm formation [11]. When planktonic bacteria start the transition to biofilms, varied biochemical-genetic regulatory pathways are activated to allow microbial attachment to surfaces, followed by microbial growth and EPS matrix production [12]. As microbial growth reaches a critical level for biofilm stability, the *quorum* sensing mechanism, an intracellular population-based communication system, is triggered, and micro-organisms are then detached from the biofilm [13]. The detached micro-organisms may attach to any near surfaces and form new biofilms, starting a new cycle of hard-to-treat infections [12].

Biofilm formation is associated with most of the known infectious diseases, and less than 0.1% of the known microorganisms live as planktonic (free) forms in the environment [10,13]. Biofilm-embedded strains have been described as more than 1000 times resistant to antimicrobial drugs than their planktonic counterparts due to the protective effect of the EPS, which may adsorb or react with antimicrobial drugs [14]. As a consequence, the entrance of active drugs into the biofilm is reduced, and it is possible that the adsorbed drugs, even at sub-inhibitory concentrations, can trigger transcription of genes associated with several resistance mechanisms [15].

In hospital settings, the treatment of infectious diseases in which a strong and extensive inflammatory process is noticed, the combined use of antimicrobials and corticosteroids is commonly adopted by prescribers [16,17]. Dexamethasone (1-dehydro-16α-methyl-9α-fluorohydrocortisone - DEXA) is a synthetic glucocorticoid widely used in such combinations on the treatment of infectious diseases, in order to modulate the immune responses triggered by microbial extracellular DNA, lipopolysaccharide and varied toxins [16,17]. Beyond its strong immunosuppressive properties, DEXA has the ability to penetrate the central nervous system, being used on the treatment

of bacterial meningitis and encephalitis [17]. Common associations available at the pharmaceutical market in Brazil and elsewhere include the following: DEXA, neomycin and polymyxin B Sulfate (ophthalmic suspension); DEXA and tobramycin (ophthalmic suspension); DEXA and ciprofloxacin (otologic suspension), and DEXA, framycetin and gramicidin D (ophthalmic suspension). Interestingly, despite the use of such combinations being a common practice evidenced in guidelines and standardized protocols, few evidences of safety and effectiveness are available [17].

This work presents the discovery that DEXA abrogates the activity of different antimicrobial drugs when combined *in vitro* against microbial biofilms of *S. aureus* and *P. aeruginosa*, and provides evidence for the first time of possible risks of the combined used of DEXA and the tested drugs, for instance, in drip devices for intravenous drug administration. Moreover, the data presented here open doors for investigations on the effect of these combinations *in vivo* for the treatment of infectious diseases caused by pathogens of these species.

Material and methods

Bacterial isolates

All samples used in this study were from the clinical isolates collection of the Microbiology Research Laboratory, at University Vale do Rio Doce (Governador Valadares, Brazil). *P. aeruginosa* strains consisted of pathogenic tracheal isolates, and *S. aureus* isolates were isolated from catheter tips. Isolates of both species were obtained of adult patients, and a total of 10 strains of each species were used in this study. All isolates were cultured overnight in brain hear infusion (BHI) broth (Difco, Becton Dickinson, USA) at 35 ± 2 °C for activation, and tested with Gram-positive and Gram-negative bacteria identification cards for VITEK 2 system (bioMérieux, Marcy l'Etoile, France) for identity confirmation up to species level. Each card was inoculated with a bacterial suspension prepared in saline solution from VITEK 2 kit and analyzed according to the manufacturer's instructions.

Antimicrobial drugs

Stock solutions of 4 mg/mL of Gentamicin (Mantecorp, São Paulo, Brazil), Chloramphenicol (Pfizer, New York, USA), Oxacillin (Bristol Myers Squibb, New York, USA), Ceftriaxone (Roche, Basel, Switzerland) and Meropenem (AstraZeneca, Cambridge, UK) were prepared in warm DMSO, and serially diluted in PBS for the antimicrobial assays in order to reach final concentrations with the bacterial inoculum ranging from 1000 μg/mL to 1.95 μg/mL.

Minimal inhibitory concentration (MIC) assay

MIC assays were conducted in untreated sterile 96-well polystyrene microtiter plates (Kartell, Italy) as described by the Clinical and Laboratory Standards Institute (CLSI) [18]. Bacterial cultures were prepared in Mueller Hinton broth (Difco) in 1 McFarland scale by adjusting the optical density to 1 at 600 nm wavelength in a microplate reader (Biorad, USA), and 100 μL was dispensed in the wells. Sequentially, the wells received each of the antimicrobials serially diluted in final concentrations ranging from 1 mg/mL to 7.8 μg/mL, creating a final concentration of the bacterial inoculum equal to 0.5 McFarland scale ($\sim 1.5 \times 10^8$ CFU/mL). Plates were then incubated at 37 °C overnight. A 0.1 g/L resazurin (Sigma, St Louis, USA) solution was used for staining procedures [42]. MIC was established as the lowest concentration in which resazurin staining had negative result (no color modification from blue to pink) in all strains. Drugs were used as a negative control for resazurin staining. This assay was performed in triplicate.

Minimum bactericidal concentration (MBC) assay

MBC assays were conducted using the CLSI method [18] for each tested drug. Aliquots of 100 μL of each well in which resazurin staining result was negative (indicating no bacterial growth) were dispensed in Mueller–Hinton agar (Difco) plates and inoculated through spread plate technique. Drugs were used as a negative control. All plates were incubated overnight at 37 °C and bacterial growth was observed. MBC was established as the lowest concentration that yielded no bacterial growth of all isolates in agar plates.

Minimum biofilm eradication concentration (MBEC) assay

MBEC assays were conducted as previously described [10]. Biofilms were formed overnight at 37 °C in non-treated 96 wells polystyrene plates. Cultures were prepared in 0.5 McFarland scale turbidity in BHI broth (Difco), as described previously [10]. Following, biofilms were washed three times and exposed to 200 μL of the aforementioned antimicrobial drugs, diluted in fresh Mueller Hinton broth (Difco) in concentrations ranging from 1000 to 3.9 μg/mL. Plates were incubated overnight at 37 °C. Resazurin staining (0.1 g/L) was used to assess the antibiofilm activity of the drugs after overnight incubation at 37 °C. A total of 20 μL of the solution was dispensed in each well, and plates were incubated for 10 min at 37 °C. Metabolically active bacteria converted resazurin (blue) in resofurin (pink). The lower concentration in which resazurin was not converted in resofurin was considered the MBEC. Fresh Mueller Hinton broth (Difco) aliquots with and without drugs in the higher concentration (1000 μg/mL) were used as controls. This experiment was performed in triplicate.

DEXA interference experiments

To investigate the possible interference of DEXA on the antibiofilm potential of the antimicrobial drugs, biofilms were formed as described in the previous section. The antimicrobial drugs were diluted in fresh Mueller Hinton broth to reach the MBEC, and 200 μL was dispensed in each biofilm. A stock solution of DEXA (Merck Sharp Dohme, USA) was prepared in DMSO (Vetec, Brazil) and diluted in sterile saline to reach the concentration of 1000 μg/mL. Following, 50 μL of DEXA at 1000 μg/mL was added to each well. Plates were than incubated overnight at 37 °C, and resazurin staining (0.1 g/L) was used as described in the previous section to assess the interference of DEXA on the antimicrobial drugs. As a control, the concentrations used in antibiofilm experiments were used to assess possible antimicrobial and antibiofilm effects of DEXA alone. These experiments were performed in triplicate. Moreover, to exclude the possibility of the interference being barely

a dilution effect, experiments were repeated using only the same volume of DMSO free of DEXA and the results were assessed as described.

Statistical analysis

Differences in antimicrobial activity results were analyzed using ANOVA and post hoc Tukey test. Significance level was set as $P < 0.05$. Analyses were conducted in Biostat 5.0 for Windows.

Results

MIC, MBC and MBEC determination

Standard methods were used to determine the MIC and MBC parameters of gentamicin, chloramphenicol, oxacillin, ceftriaxone and meropenem against the clinical bacterial isolates (Table 1). Most of the tested drugs presented low values of MIC and MBC, and in some cases, such values were equal, indicating the bactericidal effect of the drug. In others, MIC was lower than MBC, and it was possible to infer a bacteriostatic effect [19].

The MBEC of the tested drugs (Table 2), as expected, was considerably higher than MIC and MBC values. *S. aureus* biofilms were more sensible to oxacillin than the other tested drugs ($P < 0.05$). Biofilms of *P. aeruginosa* strains, on the other hand, were equally susceptible to the tested drugs ($P > 0.05$), except for chloramphenicol. Factors such as the reduced metabolism compared to planktonic cells, the protective effect of the EPS and the compact nature of biofilms that hampers the entrance of molecules, antimicrobial compounds are often unable to eradicate biofilms. Sessile bacteria can be 10–1000 times less sensitive to antimicrobial drugs than planktonic bacteria [15].

The effect of DEXA on the pharmacological activity of the antimicrobials

For the first time, the *in vitro* effects of DEXA and antimicrobial drugs against clinical isolates of *S. aureus* and *P. aeruginosa* are described. As expected, DEXA presented no antimicrobial or antibiofilm potential in the tested concentrations (data not shown). The combined use of DEXA abrogated the antimicrobial and antibiofilm effects of the tested drugs in their MIC (Table 3), MBC (Table 3) and MBEC (Table 4), for most of the strains of both species. Furthermore, the possibility of the interference being only a dilution effect was excluded, once the experiments with DMSO free of DEXA have not altered the antimicrobial effect of the tested drugs.

Table 1 Antimicrobial susceptibility of *S. aureus* and *P. aeruginosa* isolates.

Parameter	Antimicrobials (μg/mL)			
	Genta	Chloram	Oxa	Merop
(*S. aureus*)				
MIC	12.5	6.25	6.25	6.25
MBC	100	12.5	6.25	6.25
Parameter	**Antimicrobials (μg/mL)**			
	Genta	Chloram	Ceft	Merop
(*P. aeruginosa*)				
MIC	12.5	6.25	25	12.5
MBC	25	25	100	12.5

MIC: Minimal inhibitory concentration. MBC: Minimum bactericidal concentration. Genta: Gentamicin; Chloram: Chloramphenicol; Ceft: Ceftriaxone; Oxa: Oxacillin; Merop: Meropenem. Data are referent to the lowest concentrations observed to all isolates.

Discussion

In this study, *S. aureus* isolates obtained from catheter were investigated regarding their susceptibility to different antimicrobials. As described, the bacterial inoculum was prepared in 1 McFarland Scale in order to create a final concentration equal to 0.5 McFarland scale, as a dilution was expected by the addition of the drugs in aqueous solution [18]. All the tested drugs are used in Brazil and several other countries for the treatment of infectious diseases in hospital settings. Although Meropenem is of hospital use only, Gentamicin and Chloramphenicol are widely available for purchasing in drugstores in different dosage forms such as tablets and topical ointments/creams, as well as Ceftriaxone. Considerably low values of MIC were observed in most of the tests, although a high MBC value was observed for gentamicin, which was eight times higher than its MIC. Similarly, *S. aureus* strains isolated from suppurative lesions presented poor susceptibility to gentamicin, chloramphenicol, ciprofloxacin, erythromycin, methicillin, tetracycline and cotrimoxazole [21]. Resistance to oxacillin has been described in European *S. aureus* strains [5], and poor susceptibility of *S. aureus* strains isolated from samples such as blood and urine, and body sites such as eyes, ears, throat, skin, and also from catheter tips, was detected for cotrimoxazole, tetracycline, penicillin and amoxicillin [20].

The susceptibility of *P. aeruginosa* strains was also investigated in this study. In general, the MIC values were low for the tested drugs. On the other hand, the MBC of ceftriaxone and chloramphenicol was four times higher than the MIC, suggesting a bacteriostatic effect [19]. Interestingly, although chloramphenicol is actually a bacteriostatic drug, ceftriaxone is a bactericidal β-lactam. An investigation on possible production of β-lactamases would be of interest for further studies. Resistance of *P. aeruginosa* to ceftazidime, cefepime, imipenem, meropenem, gentamicin, amikacin, and ciprofloxacin was observed in 36% of a collection of strains isolated from varied hospital departments in Malaysia [22]. Moreover, Swedish clinical samples were also resistant to meropenem [23]. *P. aeruginosa* strains associated with nosocomial-acquired pneumonia were described to be resistant to ciprofloxacin, levofloxacin, ceftazidime, piperacillin, imipenem, tazobactam, tobramycin, gentamicin, cefepime, amikacin and meropenem [24].

The bacterial biofilms investigated in this study were poorly susceptible to the tested antimicrobials. Studies on biofilm susceptibility to antimicrobial drugs remain scarce. Biofilms of *S. aureus* isolated from central venous catheters, endotracheal tubes and wound drainage tubes showed high MBEC values for vancomycin, gentamicin and rifampin [25]. Rifampicin alone or combined with vancomycin was ineffective against biofilm-producer methicillin resistant *S. aureus* (MRSA) strains [26]. Biofilms of MRSA strains isolated from bloodstream infections were resistant to vancomycin [27].

Table 2 Biofilm susceptibility to antimicrobial drugs.

S. aureus	Antimicrobials (μg/mL)			
	Genta	Chloram	Oxa	Merop
MBEC	1000	750	125[*]	500
P. aeruginosa	Antimicrobials (μg/mL)[**]			
	Genta	Chloram	Ceft	Merop
MBEC	500	1000	500	500

MBEC: Minimal biofilm eradication concentration. Genta: Gentamicin; Chloram: Chloramphenicol; Oxa: Oxacillin; Ceft: Ceftriaxone; Merop: Meropenem. Data are referent to the lowest concentrations observed to all isolates.
[*] $P = 0.041$ – statistically significant difference.
[**] $P = 0.093$ – no statistically significant difference.

Table 3 Percentage of isolates in which the addition of DEXA abrogated the antimicrobial activity of the tested drugs.

Parameter	Antimicrobials + DEXA			
	Genta (%)	Chloram (%)	Oxa (%)	Merop (%)
(*S. aureus*)				
MIC interference	100	100	100	100
MBC interference	40	100	100	50
Parameter	Antimicrobials + DEXA			
	Genta (%)	Chloram (%)	Ceft (%)	Merop (%)
(*P. aeruginosa*)				
MIC interference	100	100	100	100
MBC interference	60	100	100	50

MIC: Drugs tested in their minimal inhibitory concentration. MBC: Drugs tested in their minimum bactericidal concentration. Genta: Gentamicin; Chloram: Chloramphenicol; Ceft: Ceftriaxone; Oxa: Oxacillin; Merop: Meropenem.

Table 4 Percentage of isolates in which the addition of DEXA abrogated the antibiofilm effect of the tested drugs.

S. aureus	Antimicrobials + DEXA			
	Genta	Chloran	Oxa	Merop
	100%	100%	100%	100%
P. aeruginosa	Antimicrobials + DEXA			
	Genta	Chloran	Ceft	Merop
	100%	100%	100%	100%

Genta: Gentamicin; Chloram: Chloramphenicol; Oxa: Oxacillin; Ceft: Ceftriaxone; Merop: Meropenem. Drugs were tested in their MBEC value.

Poor susceptibility of biofilms of *P. aeruginosa* clinical isolates was observed for colistin, meropenem, tobramycin, ticarcillin-clavulanate, ciprofloxacin, cefepime, ceftazidime [28], and tobramycin and ceftazidime [29]. Biofilm-producing strains of *P. aeruginosa* isolated from the wastewater of a burn care center were resistant to gentamicin, imipenem, tobramycin and piperacillin [30]. Resistance to levofloxacin, moxifloxacin, ertapenem, and ceftriaxone was described for a *P. aeruginosa* biofilm obtained from a urinary tract infection patient [31].

For the first time, we describe here that DEXA hampers the pharmacological activity of different antimicrobial drugs, abrogating their effect in the MIC, MBC and MBEC. Moreover, in recent investigations, DEXA has decreased the post-antibiotic effect of the same drugs used in this study [32]. DEXA crosses cellular membranes and binds to cytoplasmic receptors of glucocorticoid, which bind to glucocorticoid response elements. This system binds to DNA regions resulting in increased transcription of lipocortins, proteic inhibitors of phospholipase A_2, the primary enzyme involved in inflammatory mediators synthesis pathway, resulting in control or suppression of the inflammatory processes [41]. The pharmacological properties of DEXA and the results of this study do not support inferences on competitions for pharmacological targets in bacterial cells between DEXA and the antimicrobial drugs, given that corticosteroids have no molecular target in bacteria.

The results reported herein can be partially explained when we consider that it is possible that chemical interactions may have inactivated the antimicrobial drugs before binding to their molecular targets. Curiously, the activity of gentamicin and meropenem in their MBC was not affected by DEXA in some strains. Gentamicin binds to specific 30S ribosome subunit proteins, what interferes with the synthesis of essential

proteins, and chloramphenicol binds to the 50S ribosome subunit, inhibiting protein synthesis as well [41]. The β-lactams oxacillin and meropenem inhibit cell wall synthesis by binding to penicillin-binding proteins on bacterial membranes [41]. Concerning the aforementioned drugs, we believe that they have reached their target on the bacterial cells before DEXA would impair its pharmacological action.

The effects of the combined use of corticosteroids and antimicrobial drugs *in vivo* have been described, and are very controversial, given that the bacterial strains and the clinical contexts vary widely among the studies. The combined use of DEXA and cloxacillin was more effective than cloxacillin alone on the treatment of bacterial arthritis caused by *S. aureus* in Swiss mice [33]. DEXA also did not interfere on the effectiveness of fluconazole in a murine model of cryptococcosis [34]. The combined use of hydrocortisone and mupirocin was equally effective to control the colonization of the skin of patients with eczema and atopic dermatitis by *S. aureus*, suggesting that antimicrobial drugs can be avoided in later stages of the diseases or in mild conditions, in order to prevent the development of bacterial resistance [35]. More recently, the combined use of methylprednisolone and imipenem in children with severe pneumonia was described to be more effective than the drug alone when considering clinical outcomes such as fever, leucocytes counts, complications due to the course of the disease, and the need of invasive interventions [36].

Negative evidences in this context have also been provided in the latest years, what contributes to the current controversial picture that is the combined use of corticosteroids and antimicrobials. As corticosteroids can induce extensive immunosuppression, infectious diseases caused by pathogens (mainly opportunistic species) from the microbiota of the patient or from clinical settings such as hospitals or laboratories are likely. In this context, the combined use of DEXA and ceftriaxone resulted in therapeutic failure in the treatment of bacterial meningitis by *Streptococcus pneumoniae* in a rabbit model [37]. Similar observations were also reported for the combined use of DEXA and vancomycin in a rabbit model of pneumococcal meningitis, although the use of rifampicin and DEXA was suggested to be safe [38]. More recently, it was observed that patients treated with corticosteroids and antimicrobials during cancer chemotherapy or after graft-versus-host disease presented subclinical bacteremia, although they presented no classical symptoms such as fever and chills [39].

Despite the relevance of this data, the present study is not without limitations. Although clinical isolates were used in this study, our sample is limited to 10 strains of each species; thus, researches with larger samples are important to confirm our observations [40]. Despite the antimicrobials used in this study are highly relevant in clinical treatments of infectious diseases, it would be of interest to conduct the assays with a larger number of drugs from different pharmacological groups, in order to explore the combined used of DEXA with drugs of distinct mechanisms of action. Further, time-kill experiments are important to compare the kinetics of bacterial death exposed to antimicrobials combined or not to DEXA.

Conclusions

The results presented here provide evidence for the existence of negative interference risks involving the joint administration of antimicrobial drugs and dexamethasone. However, more studies should be conducted, including *in vivo* experiments, given that it is not possible to infer that the impairment of the antimicrobial activity caused by DEXA observed *in vitro* will be also detected in living systems, due to interferences of metabolism and of complex events involved in drug distribution. Nevertheless, this has no implication on the measuring of the potential effects of combining antimicrobials and glucocorticoids.

Conflict of Interest

The authors have declared no conflict of interest.

Compliance with Ethics Requirements

This article does not contain any studies with human or animal subjects.

Acknowledgments

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jare.2016.12.001.

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