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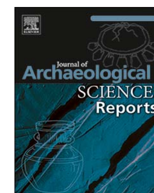

Finding the unexpected: A critical view on molecular diagnosis of infectious diseases in archaeological samples

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ABSTRACT

In the 1980s the polymerase chain reaction (PCR) technique was developed and opened the possibility to analyze ancient DNA (aDNA) in archaeological remains and identify the etiological agent of several infectious diseases. The main concern in analyzing aDNA is to successfully verify its authenticity and the information that it contains. Primers are often designed to target the desired DNA sequences, however they can target unknown sequences amplifying different organisms. With the development of next-generation sequencing platforms, the production of sequence data is increasing. There is a universe of unknown sequences that are to be discovered and deposited in the database. Could we then state in the future that we have species-specific primers? We analyzed bones of human and ground sloth remains for *Trypanosoma cruzi* by PCR. Although the samples analyzed did not amplify this parasite some bacteria were amplified. Normally this would be interpreted as a contamination and would not be reported. Yet, in this article we ask researchers to rethink about these findings, especially due to the age of these samples and consider the archaeological context for the data interpretation.

1. Introduction

In the 1980s the polymerase chain reaction (PCR) technique was developed opening the possibility to analyze ancient DNA in archaeological remains and to identify the etiological agent of several infectious diseases, establishing what is called molecular Paleoparasitology and Paleomicrobiology. While DNA from ancient organic remains has been extracted in a number of cases, there are still complications with analyses. The main concern in analyzing aDNA (ancient DNA) is to successfully verify its authenticity and the information that it contains (Fulton, 2012). However, not only there are issues with contamination and conservation of aDNA, but also with primers design. Primers are often designed to target the desired DNA sequences, still in some situations primers target unknown sequences amplifying different organisms than the one specific to the primers (Rose et al., 1998).

With the development of next-generation sequencing platforms, the production of sequence data is increasing vastly (Kodama et al., 2012). In 2016, the National Institutes of Health (NIH) Sequence Read Archive (SRA) contains ~3 petabases of sequence information (Solomon and Kingsford, 2016). In April 2016, a total of 193,739,511 sequences, and 211,423,912,047 bases were deposited in the Genbank by direct submission, collaboration/agreement, and/or internal NCBI/NLM curation (National Center for Biotechnology Information et al., n.d.; NCBI Resource Coordinators, 2016).

Herein, we will discuss results obtained by using the primers TC/TC1 and TC2 described by Souto et al. (1996) and explore the possibility of updating primers as new sequences are deposited in the database.

2. Methodology

We analyzed bones of human remains from Brazil: Justino site, Sergipe ($n = 7$), dated from 4380 to 3200 BP (Before Present); and São Jorge Church, Rio de Janeiro ($n = 7$) dated from the end of the 18th

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century. Some ($n = 5$) bone fragments were also analyzed from an individual extinct giant ground sloth of the genus *Eremotherium* spp., from Lagoa dos Porcos site, Piauí, Brazil dated from 30.000 BCE (Before Christ). Samples were provided by the Collection of Coprolites and Paleoparasitological Materials Luiz Fernando Ferreira – Fiocruz, and also by the National Historic and Artistic Heritage Institute – IPHAN (01500.002205/2013-84).

In this study, we used primers that amplify a part of the intergenic region of *Trypanosoma cruzi* mini-exon genes: 5′-GTGTCCGCCACCTCCTTCGGGCC (TC1 – group 1-specific); 5′-CCTGCAGGCACACGTGTGTGTG (TC2 – group 2-specific); 5′-CCCCCCTCCCAGGCCACACTG (TC, common to both groups). These DNA markers define two phylogenetic lineages of *Trypanosonoma cruzi* (TC1 and TC2), which show a high phylogenetic divergence and were used as primers for *T. cruzi* identification using archaeological remains subsequently with successful results (Lima et al., 2008; Fernandes et al., 2008). Samples were analyzed and sequenced at the Laboratory of Parasitology at the Fluminense Federal University.

Further information about sample preparation, DNA extraction and products amplification can be accessed at Pucu et al. (submitted).

3. Results

All samples tested with TC2 primer were negative. For the TC1 primer 3 samples had amplifications matching 350 base pairs that corresponds with the TC1 primer amplification base pairs for *T. cruzi*. The one sample from Justino Site had 99% of similarity with the bacteria *Propionibacterium acnes* strain A1–14 (accession number Genbank: CP013693.1), and one from São Jorge Church had 71% of similarity solely with the bacteria *Agrobacterium tumefaciens* strain Ach5 (aCP011247.1), for the ground sloth remains one sample had 89% of similarity with a bacteria *Pseudomonas putida* strain AA7 (CP018846.1). The sequence alignment is at the Data in Brief.

4. Discussion

All DNA sequences amplified were deposited after the TC1 primer was designed, i.e. in 1996, showing a probable congruence among the sequences. Some primers specificity might be put to a test due to the universe of new information and the daily sequencing of new organisms. Everyday new DNA sequences are being deposited in different databases; besides, it is very common for primers' to amplify unknown sequences even in studies with modern samples. However, some of these sequences are unknown and are not reported in the studies, therefore not deposited in the Genbank database.

Even though some primers in the literature are used many times, with positive results, there are some surprises concerning their specificity in new studies. Hänsch et al. (2015) showed evidence that the *pla* gene, thought to be specific to *Yersinia pestis* and widely used in aDNA studies, also occurs in strains of other bacteria species, such as *Escherichia coli*. The authors suggest a combination of at least two different molecular targets for proper identification of *Y. pestis*.

Propionibacterium acnes is an anaerobic Gram-positive bacillus that can be found in the human skin and maintains the inflammatory phase of acne. The complete DNA sequence amplified was deposited in 23 December 2015. It is considered a commensal organism that can occur in the conjunctiva, mouth, and intestine; and it is a common contaminant of cultures (Zylberberg et al., 1996; Levy et al., 2008). *P. acnes* has also been identified in sites with bone infection causing osteomyelitis, and associated with the presence of foreign bodies, immunosuppression, preceding surgery, trauma, diabetes, obstruction of sinuses or ducts, and has been associated with the development of prostate cancer (Funke et al., 1997; Levy et al., 2008; Bae et al., 2014).

Barnes and Thomas (2006) published a study that tested tuberculosis and syphilis on archaeological remains. One of their samples, positive for tuberculosis morphologically, matched portions of the 16SrRNA of *P. acnes* with 99% of identity. Although *P. acnes* is a human skin commensal there have been cases of infection, indicating that there is a probability of the individual we analyzed to be immunosuppressed. Even though *P. acnes* was identified in this previous study it was considered a contamination, however, perhaps there is a link between bone lesions caused by tuberculosis and other pathogens with the presence of *P. acnes*. Tuberculosis can cause immunosuppression in the host, which can benefit the occurrence of *P. acnes*. Hence, further studies are needed to understand the pathogenicity of this bacterium, and its co-occurrence with other pathogens. Many times, an unexpected result might indicate another previous disease that it was in fact the cause of the dissemination of an etiological agent, which in an individual immunocompetent would not cause any harm.

Agrobacterium tumefaciensis (= *Rhizobium radiobacter*) an aerobic gram-negative soil pathogen that can cause crown gall tumors on infected dicotyledonous plants (De Groot et al., 1998). The complete sequence of the strain Ach5 chromosome linear was deposited in 23 June 2015. The presence of this bacterium might indicate that it was in the soil in direct contact with the bones we analyzed. However, in this case it is difficult to establish that this contamination occurred at the time the remains were buried or later, when the bones were excavated.

Pseudomonas putida is an aerobic, gram-negative soil bacterium that occurs in the soil and can bio-catalyze some chemical products, i.e. polystyrene foam (Nicoletti et al. 2015). The genome sequence was deposited in 13 March 2017. The presence of *P. putida* shows that the contamination of archaeological samples with these bacteria might be high, since this species is also found in the soil. In some archaeological context, the soil will be in direct contact with bones until they are excavated, and due to the bone's porosity not only minerals but also the soil might replace and impregnate the bone matrix. Even with the removal of the outer layer of the bone the soil will not be cleaned off, regardless of the piece of bone used (we used the bone marrow). This can be aggravated when scientific collections wrap the samples with polystyrene for transportation. Therefore, we advise archaeologists to seek for a different material to prevent impact to prevent contamination.

Generally, primers are used and designed for "pure cultures" and/or isolated organisms. However, the archaeological material can be considered an environmental sample that translates not only the host's information and its parasites, but also the environment of where it lived, where it was deposited until excavated, and even what happens to the material during its transportation, storage, and laboratory experiments. When we apply these primers primarily designed for modern samples in archaeological ones, they can reflect unique and unpredictable contexts and results.

Although checking primers is a premise in any research, not always this strategy will have the expected efficiency. Several organisms deposited recently did not exist when many primers were designed years ago, and even today the hybridization area of primers might not be in the sequence database. Besides, there is a universe of unknown sequences that are to be discovered and deposited in the database. Could we then state in the future that we have species-specific primers? How many researchers have found in analyses almost "perfect sequences" that when compared to the database they have zero similarity with any organism? However, there is a tendency to "discard" these results because they did not match what was looked for, and we believe that this is a mistake. Unexpected results, especially ones from archaeological materials, should be reported not only because it is a rare material, but also due to the fact that we can be facing new discoveries. With unexpected results, methodologies can be improved and new findings reported and analyzed even though it is a challenge to discover whether results from aDNA are authentic or a contamination.

5. Conclusion

In Brazil, there is a saying: *"aimed at what I saw but hit what I did not*

foresee," that agrees with research in the paleoparasitological field that aims at what you might expect to find. Nonetheless, in this letter we call attention to what we did not expect we would find.

Even though the possibility of contamination is very high, since *P. acnes* normally occurs on human skin; *A. tumefaciens* in the soil; and *P. putida* in soil and polystyrene, we advise researchers to report unexpected results as discussed above. These organisms could also be present at the time the individuals were living, since negative controls of extraction and PCR did not amplify these organisms. Due to the importance of the materials we stress that results should not be discarded; owing to the archaeological context that might indicate that it was in fact an endogenous and ancient DNA.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jasrep.2017.04.019.

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