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CORRESPONDENCE

Ancient DNA (aDNA) Studies of Man and Microbes: General Similarities, Specific Differences

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ABSTRACT The study of ancient DNA (aDNA) using molecular methods is an increasingly valuable tactic in bioarchaeology. While this method must be carefully undertaken to ensure that the molecules detected are representative of the ancient sample and not modern contaminates, there is a danger that a 'one size fits all' approach to validation will lead to misinterpretation and/or missed opportunities of valuable findings. When comparing human and pathogen aDNA, there are many shared technical means that can ensure best practice. However, there are a number of assumptions that should not be used for both scenarios. We discuss these aspects in reference to a recent article published by this journal and highlight some of the latest advances in molecular detection of ancient pathogen DNA that can further improve this endeavour. Copyright © 2009 John Wiley & Sons, Ltd.

Key words: ancient DNA; PCR; validation

Introduction

Roberts and Ingham writing in this journal (2008) have recently reviewed a number of publications employing ancient DNA analyses to diagnose disease in human remains, against suggested validation criteria. Many of the original measures (Cooper & Poinar, 2000) were appropriate and have stood the test of time. For example (a) the use of multiple extraction and template blanks, (b) reproducibility, (c) appropriate molecular behaviour, (d) confirmation of product identity with sequencing and (e) replication of key findings at a separate centre. However, the discussion on avoiding contamination is incomplete and we cannot agree with the assertion that all the criteria apply equally to pathogenic DNA. This may explain why some workers have not shown a greater adoption rate of particular recommendations over time. Furthermore, there is no discussion about potential reasons for false negative results other than that the sample may have degraded. We are concerned that this paper will be used by individuals less familiar with the field (editors, reviewers, etc.) and feel that some clarification within the overall framework of the critique is necessary.

Mitochondrial DNA (mtDNA)

The authors suggest that 'mitochondrial DNA results should be obtainable if pathogen DNA is

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detected'. Mitochondrial DNA (mtDNA) is multi-copy and although it may sound intuitive to suggest that this should be detectable when pathogen DNA persists our experience gained over the last 15 years would suggest otherwise. Human cellular DNA is far less likely to persist in old cases even when pathogen DNA, such as from *Mycobacterium tuberculosis* complex or leprosy, is detectable. Consequently, suggesting that mtDNA be detected to support any pathogen aDNA finding is incorrect and would inevitably complicate the analyses. Polymerase chain reaction (PCR) methods for detecting mtDNA sequences can be extremely difficult to maintain contamination-free due to the ubiquity of human DNA sequences. Sourcing of PCR reagents is also very important in this respect. The idea of setting up a genetics database for recovered pathogen and human sequences is certainly feasible but it is important to remember that 'taking of DNA records for all personnel' to identify sources of exogenous DNA carries with it the need to seek approval from the relevant local ethical committee. Moreover, this strategy is only useful if the ancient and modern target sequences differ.

For the above reasons, we would suggest that amplification of human DNA from ancient samples should be left to those with expertise in this area or, if it is intended to study both pathogen and human samples, through collaborative studies.

'Modern' contamination

Contamination of ancient samples with modern pathogen DNA is often quoted as one of the main concerns of aDNA studies. This is a major error in thinking as modern pathogen DNA can be excluded from the laboratory relatively easily; the key is that this must be monitored with suitable control assays. This is fundamental to all molecular biology work using PCR.

In practice, contamination from robust positive ancient specimens poses a far greater risk than that from modern DNA. It is easy to overlook that some samples may be strongly positive for pathogen DNA and cross-contamination to other cases or extraction controls is possible if handled together. This can occur regardless of personnel wearing suitable protective clothing and gloves and is not helped by using dedicated laboratories. Sporadic contamination from such cases may also occur during PCR cycling, hence the need to monitor experiments with multiple blanks.

Sequencing

The authors state that sequencing after cloning must be undertaken, 'cloning being an improvement on direct sequencing'. This statement is simply wrong as the two approaches address different aspects: direct sequencing for rapidly confirming the identity of the amplicon and cloning for seeking evidence of additional templates. Cloning is arguably less useful for pathogens like tuberculosis, where it is now possible to design highly specific PCR methods based on species-specific deletions in the genome (Brosch *et al*., 2002). It is useful for confirming VNTR analysis (Taylor *et al*., 2006) but it must be remembered that when sequencing clones, polymorphisms may also be present due to errors introduced by Taq polymerase and replication errors (especially when looking at repetitive loci, Hauge, 1993), in addition to genuine template heterogeneity. Finally, cloning ideally requires an additional facility as the clones that are generated provide the perfect source of contamination since each bacterium contains numerous copies of the molecule of interest.

Problems due to false negatives

The authors make no mention of potential reasons why a false negative result may occur. Careful storage of a sample will favour continued survival of the nucleic acid once it has been sampled and there are many ways to do this. The optimum extraction procedure is essential, both to produce a representative quality of extract and to remove inhibitors. Even then, PCR inhibition remains a leading potential source of false negative results and may interfere with quantitative studies. It is relatively easy to overcome this through a range of measures such as sample dilution, addition of extra Taq polymerase or inclusion of bovine serum albumin (Al-Soud &

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Rådström, 2001). If negative results are to be published (and we agree that they should be), it is vital that false negatives be avoided.

Analysis of associated faunal remains and soil samples

The authors state that analysis of faunal remains and soil samples should form part of the study. In practice, biomolecular scientists are usually asked to examine human material only after a lengthy period of time has elapsed. In some instances, this may take place years after the excavation, making analysis of soil from the relevant contexts impossible. The authors suggest that soil be sampled to identify any contaminating pathogenic DNA. This is a sensible suggestion if the DNA of interest is likely to be present in the soil. Most pathogens studied, such as tuberculosis and leprosy, are not free-living in the environment, being obligate parasites. Even if shed from an infected animal, the survival of naked mycobacterial DNA in soil is limited to a few days whereas whole cells may persist for a period of time measured in months (Young *et al.*, 2005). This is an ongoing area of research (Taylor *et al.*, 2003), but the chance of free DNA or even non-viable pathogenic mycobacteria persisting within soil for hundreds or thousands of years and contaminating human remains is exceedingly remote. PCR methods exploiting large sequence polymorphisms (LSPs) in the genomes of pathogens of interest can be readily used to avoid false-positives due to environmental contamination.

The study of animal remains is certainly an area in which more work is required, particularly when zoonotic diseases are detected in human skeletons. However, the study of these poses well recognised problems in their own right. Identification and study of infectious lesions in animal bones from archaeological sites is complicated by the fragmented and disarticulated nature of these remains and the frequent lack of an adequate baseline of known cases for diagnosing disease. In addition, unlike most human remains, the depositional history of faunal remains is often complex and they have generally been subject to cooking and scavenging.

Excavation protocols

The authors remind us that handling bones and teeth contaminates them with modern DNA, and suggest sterile sampling procedures at excavation in order to circumvent this. Contamination in this manner is largely a problem for human DNA studies, rather than ancient pathogen DNA which seems to be the topic of Roberts and Ingham's (2008) contribution. However, even for ancient human DNA work, such procedures are highly impractical. It is rarely known at excavation whether ancient human DNA work will be carried out at all, let alone which particular skeletons in a cemetery will be sampled. One could, of course, suggest that archaeological excavation and post-excavation study of skeletal material be routinely carried out under sterile conditions to facilitate future DNA work. However, it seems impractical and unreasonable to impose these sorts of conditions in perpetuity on the off-chance that some future workers might wish to carry out DNA work on remains (DNA work is conducted on only a tiny minority of cemetery populations). A more practical solution would seem to be the use of an appropriate sampling strategy to avoid handled surfaces and adoption of protocols that have been shown to be useful for detecting and removing contamination with modern DNA. For example, recording of appropriate molecular behaviours (Malmström *et al.*, 2007) and the further use and development of techniques which have been demonstrated to be of value in decontamination (Dissing *et al.*, 2008), particularly as they are already necessary for DNA work on existing museum specimens.

Case reports versus population studies

The authors advocate the greater use of population studies as opposed to individual case studies which they see as 'haphazard analysis of individual skeletons and mummies that have been the target in the past'. Individual cases have often been studied because they have lesions characteristic of, or are early examples of, a particular disease. When DNA has been demonstrated there has been a natural tendency to study

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the case in some detail to recover as much genetic information as possible. The presence of pathognomonic lesions made these logical starting points for early investigators looking for feasibility of aDNA recovery. In some instances, they have been studied on more than one occasion as techniques improve or additional informative loci are recognised (Fletcher *et al*., 2003a,b). Population studies are certainly commendable undertakings but pose greater challenges when it comes to obtaining multiple data points from a larger sample size and require clear research questions.

Quantitation

One criteria mentioned briefly in the paper was quantitation, although there appeared to be some confusion as to the definition of quantitation in the context of aDNA analysis. Under this heading came the need to assess 'sporadic instances of contamination' as well as 'the copy number of the DNA target'. The authors also state that 'investigators should be wary that low copy numbers of the starting DNA template could lead to sporadic instances of contamination and must therefore be noted in any results published'. This does not follow as template copy numbers are commonly low in aDNA extracts. We deduce that what was meant was that low template numbers could be confused with sporadic contamination (presumably with mtDNA). We would regard any assays with evidence of sporadic pathogen DNA contamination as unacceptable, requiring repeat analysis.

We suggest that the use of a real-time PCR platform should be the method of choice as these instruments have many advantages and are becoming routine in molecular laboratories. A real-time platform facilitates the monitoring of multiple blanks, highlights inhibited samples and allows melt analysis of putative products. Any product formation during early cycles (low cycle threshold, (Ct) values) can be suggestive of amplicon contamination. Inhibition in ancient samples may be assessed by their ability to 'right-shift' Ct values of standardised inhibition assays (Nolan *et al*., 2006). The sample data are conveniently stored and available for inspection, forming part of the laboratory record of all isolates studied in the laboratory. If used to quantitate the aDNA template as suggested by Roberts and Ingham (2008), modern DNA standards must eventually be included (although this should be done only after completion of all other amplifications and subject to the laboratory strategy separating pre and post PCR stages).

It is worth remembering that the use of PCR in routine clinical diagnostic and forensic laboratories depends on the ability of the operator to process patients' samples alongside DNA standards ranging from a single genome equivalent to several million, and this is regularly achieved with good working practice. If an operator is unable to add modern DNA to one reaction without contaminating the ancient DNA reaction then they surely cannot be trusted to work with two ancient DNA samples without risking cross contamination. Amplification of aDNA should be regarded as another method in which low copy number detection is required, with the appropriate controls, and not a mysterious process which need be limited to a few dedicated centres.

We applaud attempts to enhance laboratory protocols in ancient DNA but observe that successful formulation of protocols for such work are likely to arise through collaboration with those actively involved in the practical aspect of such work. Guidelines should be suggestive and not replace thought, as different sub areas of aDNA study may require different approaches or provide additional opportunities for validation. Finally, it is inevitable that this field will begin to see contributions from other specialities and emerging technologies such as DNA repair, (Ballantyne *et al*., 2007), microarray analysis and various nanotechnologies (Park *et al*., 2002). These will provide alternative means of detecting disease biomarkers and new opportunities for data validation.

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