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Animal DNA in PCR reagents plagues ancient DNA research

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Abstract

Molecular archaeology brings the tools of molecular biology to bear on fundamental questions in archaeology, anthropology, evolution, and ecology. Ancient DNA research is becoming widespread as evolutionary biologists and archaeologists discover the power of the polymerase chain reaction (PCR) to amplify DNA from ancient plant and animal remains. However, the extraordinary susceptibility of PCR to contamination by extraneous DNA is not widely appreciated. We report the independent observation of DNA from domestic animals in PCR reagents and ancient samples in four separate laboratories. Since PCR conditions used in ancient DNA analyses are extremely sensitive, very low concentrations of contaminating DNA can cause false positives. Previously unidentified animal DNA in reagents can confound ancient DNA research on certain domestic animals, especially cows, pigs, and chickens.

Keywords: *Sus scrofa*; *Bos taurus*; *Gallus gallus*; Deoxynucleoside triphosphates

1. Introduction

Ancient DNA analyses are becoming more successful and widespread. These studies depend upon the polymerase chain reaction (PCR) to amplify minute quantities of heavily damaged template. To achieve the sensitivity necessary to detect ancient DNA, extreme measures are taken. High-cycle PCR can detect as few as 10 copies of modern template DNA (Shanks et al., 2005), and possibly even single molecules (Hofreiter et al., 2001). To amplify highly degraded and damaged DNA, amplicons are kept short, increasing the probability that an ancient sample will contain the targeted region. Many studies use universal primers that can amplify a variety of animal species. These factors increase the likelihood that PCR will amplify traces of highly fragmented template, including ancient DNA and extraneous modern DNA.

Problems with modern human DNA contamination are widely appreciated in ancient DNA research (Abbott, 2003; Kolman and Turross, 2000), although they are sometimes still ignored. However, species closely associated with humans in everyday life or biotechnological processes also contribute a detectable amount of DNA into the environment and thus potentially confound ancient DNA results (Shanks et al., 2005). Here we present evidence that DNA from cow (*Bos taurus*), pig (*Sus scrofa*), and chicken (*Gallus gallus*) contaminates PCR reagents and possibly ancient samples.

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2. Methods

2.1. Assessing previous studies

First, a survey of anomalous sequences observed in past studies from a wide variety of non-domestic animal species in four independent ancient DNA laboratories [Oregon State University (OSU), Smithsonian Institution (SI), Max Planck Institute for Evolutionary Anthropology (MPI), and the University of California, Los Angeles (UCLA)] was conducted. Data from studies targeting several families of birds, several families of mammals, and a reptile were included. These projects were entirely independent, used different extraction methods, different primer sets, different brands and batches of reagents, and were performed by different people in different laboratories at different times. Sequences generated from both PCR no-template controls and from PCR positives of non-target animal species were included.

2.2. Estimating levels of animal contamination

The prevalence of contamination in ancient DNA research was assessed through separate analyses of large numbers of no-template PCR controls and PCR reactions involving DNA from one species and species-specific primers for another species, in three independent laboratories (OSU, Smithsonian Institution, and MPI, Table 1). All three of these ancient DNA facilities have dedicated equipment and are spatially isolated from main laboratories where PCR products and modern DNA are handled. They are further protected by strict rules prohibiting people, reagents and other laboratory materials from moving from areas where high quality DNA is present to ancient DNA facilities.

To screen for levels of extraneous DNA in reagents, 779 no-template PCR reactions were performed at OSU. A variety of mtDNA primer sets that targeted fragments ranging in size from 116 to 292 base pairs (bp) were used (Shanks et al., 2004, 2005). All reactions that yielded product were sequenced, and the species of origin was identified by BLAST search (http://www.ncbi.nlm.nih.gov/BLAST/).

Experiments designed to estimate the level of pig contamination in PCR and extraction reagents or on samples were conducted at the Smithsonian Institution. Forty-eight PCR assays with DNA extracted from several species of birds, equids, canids, and squirrels (each replicated three times) were performed with pig-specific primers that amplify a 182-bp fragment of the mitochondrial control region (PIG3F 5′-GTACATCGCACA TATCATGTC and PIG3R 5′-GAACCAGATGCCTGTTA). Additionally, 175 no-template PCR control reactions were performed with the same primers. A subset of the reactions that yielded product was sequenced, and the species of origin was identified by BLAST search.

To further quantify the level of pig contamination in PCR reagents, and to explore the possibility of a carrier effect when a DNA extract is present in a PCR, a third experiment was done at the MPI. Duplicate PCRs were performed for 143 DNA extracts from bonobo (*Pan paniscus*) feces, 50 extraction controls and 42 no-template PCR controls. Pig-specific mitochondrial 12S primers that amplify a 137-bp region were used (primer 1: 5′-AACTCTTGCCAATTCAGCC and primer 2: 5′-TGTAGCCCATTTCTTTCCAA) in a 60 cycle PCR using AmpliTaqGold. All products were sequenced, and species of origin were identified by BLAST search.

2.3. Origin of PCR contamination

Four parallel no-template PCR experiments were performed at OSU to investigate deoxynucleoside triphosphates (dNTPs) as a possible source of human contamination. The experiments were identical except that a different lot of dNTPs was used each time. Thirty-six PCR reactions targeting a 250-bp fragment of the human mtDNA d-loop region were performed. Each 50 µl reaction contained 10 mM Tris, pH 8.5, 50 mM KCl, 200 µM (each) dATP, dCTP, and dGTP,

Table 1
Details of representative polymerase chain reactions

Lab	PCR assay					Animal(s)
	Primers	Gene	Target (bp)	Specificity	Reference	
OSU	L15684, H15760	Cytb	116	Mammals excluding humans	Irwin et al. (1991)	Cow, pig
OSU	12S01, 12S03	12S	137	Phasianinae subfamily	Shanks et al. (2005)	Chicken
MPI	Primer 1, Primer 2	12S	137	*Sus scrofa* (pig)	This report	Pig
MPI	12S a′, 12S o	12S	~150[a]	General mammalian, also many bird species	Höss et al. (1996), Poinar et al. (1998)	Pig, cow, chicken, mouse, goat
SI	Pig3F, Pig3R	CR	182	*Sus scrofa* (pig)	This report	Pig
SI	Cytb2rc, Cytb-wow	Cytb	268	Birds, mammals	Dumbacher et al. (2003)	Chicken, cow
SI	Cytb2, CytbS2h	Cytb	121	Birds	Dumbacher et al. (2003)	Chicken
SI	t-lys, A6MNH	ATP	234	Birds	Slikas et al. (2000)	Chicken
SI	L5758, H5791	ND2	232	Birds	Fleischer et al. (2006)	Chicken
UCLA	12SA, 12SL	12S	176	Mammals excluding humans	Unpublished	Cow
UCLA	cCB51, cCB52	Cytb	225	Mammals excluding humans	Leonard et al. (2000)	Pig

Primer sets described in the literature or here and information about what region of the mitochondria, what size fragment they target and their specificity and which non-human animals they have amplified are listed. Laboratories are listed in the first column (OSU for Oregon State University, MPI for Max Planck Institute, SI for Smithsonian Institution and UCLA for the University of California, Los Angeles).

[a] Variable between species.

400 μM dUTP (Lot 1: Pharmacia; Lot II: Pharmacia; Lot III: Pharmacia; and Lot IV: TaKaRa), 1 μM of each human-specific HPLC-purified primer (L16159 5′-TACTTGACC ACCTGTAGTAC and H16364 5′-GAATTCTGAGGGGGGT CATCCATGGG), 2.5 U *Taq* DNA polymerase, and 2.5 mM $MgCl_2$. Thermal cycle conditions were 92, 52, and 72 °C (1 min each) for 40 cycles. To eliminate the 10× PCR buffer, $MgCl_2$, and *Taq* DNA polymerase as a source of human contamination, each reagent was prepared at OSU under ultra clean conditions in a hood supplied with HEPA-filtered air. All PCR reagent preparations, manipulations, and experiments were performed by the same researcher who has a rare polymorphism (T-to-C transition at 16,288) in the target d-loop mtDNA region making it possible to discriminate between human contamination introduced from the laboratory environment and PCR reagents. PCR products were cloned, sequenced, and human d-loop haplotypes were identified by BLAST. Sequences were aligned with ClustalW (Thompson et al., 1994), and a neighbor-joining phylogeny was constructed with PAUP version 4.0b10 (Swofford, 2002). Statistical support for internal nodes was estimated with 1000 bootstrap replicates also in PAUP version 4.0b10.

3. Results

3.1. Assessing previous studies

The survey of anomalous PCR results from past ancient DNA projects on non-domestic species yielded a very strong pattern across laboratories. Cow, pig, and chicken were identified in independent experiments in all four laboratories. Laboratory animals were also identified, including mouse (*Mus musculus*), goat (*Capra hircus*), rabbit (*Oryctolagus cuniculus*) and guinea pig (*Cavia porcellus*), but at lower frequencies. Mouse was identified in two independent laboratories (MPI and SI), and goat, rabbit and guinea pig were each identified in a single lab (MPI, SI and OSU, respectively).

3.2. Estimating levels of animal contamination

Of the 779 no-template PCR control reactions done at OSU, 763 (98%) did not yield any product. All contaminant PCR products were sequenced and identified by BLAST. The most common contaminant identified was cow (14/16, 87.5%), followed by pig and chicken (each 1/16, 6.25%).

Three percent (7 of 223) of the control reactions using pig-specific primers at the Smithsonian Institution yielded PCR products. All spurious amplifications were in no-template PCR control reactions. Two of these products were sequenced and identified by BLAST. Both sequences were identical to pig mtDNA.

Experiments to estimate pig DNA prevalence undertaken at the Max Planck Institute yielded similar results, with 5% of the reactions yielding PCR products (19 of 378). Unlike the results from the Smithsonian, all of the positive reactions were from PCRs containing DNA extracts from another species (bonobo feces, $n = 286$). All 50 extraction controls and 42 no-template PCR controls did not yield products. Although each extract was used as template in two separate PCR tests, pig DNA was never amplified in duplicate reactions from the same DNA extract.

3.3. Origin of PCR contamination

Human mtDNA (d-loop) was amplified from four lots of dNTPs at OSU. Eight of 36 (22%) no-template PCRs yielded products. Amplicons were cloned and sequenced from PCR reactions containing dNTPs from each of the four lots. Forty clones were sequenced. Twenty-one human mitochondrial haplotypes were identified (GenBank accession numbers DQ325279–DQ325299). Efforts to prevent human contamination from the laboratory environment were successful because none of the human sequences matched the haplotype of the OSU researcher. Six haplotypes (Pharmacia lot 1, haplotypes 1, 2, 6 and 8; Pharmacia lot 3, haplotype 1, and TaKaRa lot 1, haplotype 3) matched published sequences (Adcock et al., 2001; Lewis et al., 2005; Trejaut et al., 2005; Wen et al., 2004, Fig. 1). Two haplotypes were identical to DNA sequences previously assigned to Amerindian populations (Pharmacia 3-1 = haplogroup C; GenBank accession number DQ144532) (Lewis et al., 2005) and an ancient human skeleton (haplotype LM15; GenBank accession number AF328747) (Adcock et al., 2001). The human d-loop sequences amplified in no-template PCR reactions were correlated with source of dNTPs (Fig. 1).

4. Discussion

Contamination of experiments with extraneous DNA has a long history of confounding ancient DNA analyses (Cano et al., 1993; Golenberg et al., 1990; Pääbo, 1989; Poinar et al., 1993; Soltis et al., 1992; Woodward et al., 1994). While the existence of DNA contamination is now widely accepted in ancient DNA analyses of human remains (Hofreiter et al., 2001; Kolman and Turross, 2000; Malmström et al., 2005; Serre et al., 2004; Wandeler et al., 2003), analyses of other ancient animal remains are seen as much less problematic. Some ancient DNA studies now use domestic animals as a proxy to study ancient human populations, because they are perceived to be much less prone to contamination.

We have found, in four independent laboratories, that extraneous DNA from non-human animal species is routinely amplified under a wide variety of PCR conditions. Using different protocols and primers, we amplified extraneous sequences from both no-template controls and DNA extracts of other species. The most pervasive sources of non-human animal contamination were cow, pig and chicken. These domestic species are of great interest for ancient DNA analyses due to the profound influence livestock has had on the development of human societies (Alves et al., 2003; Edwards et al., 2003; Götherström et al., 2005; Larson et al., 2005; Vilà et al., 2001; Watanobe et al., 2001, 2002, 2004). Our results indicate that DNA of certain animal species, particularly those closely associated with everyday human life, may be more

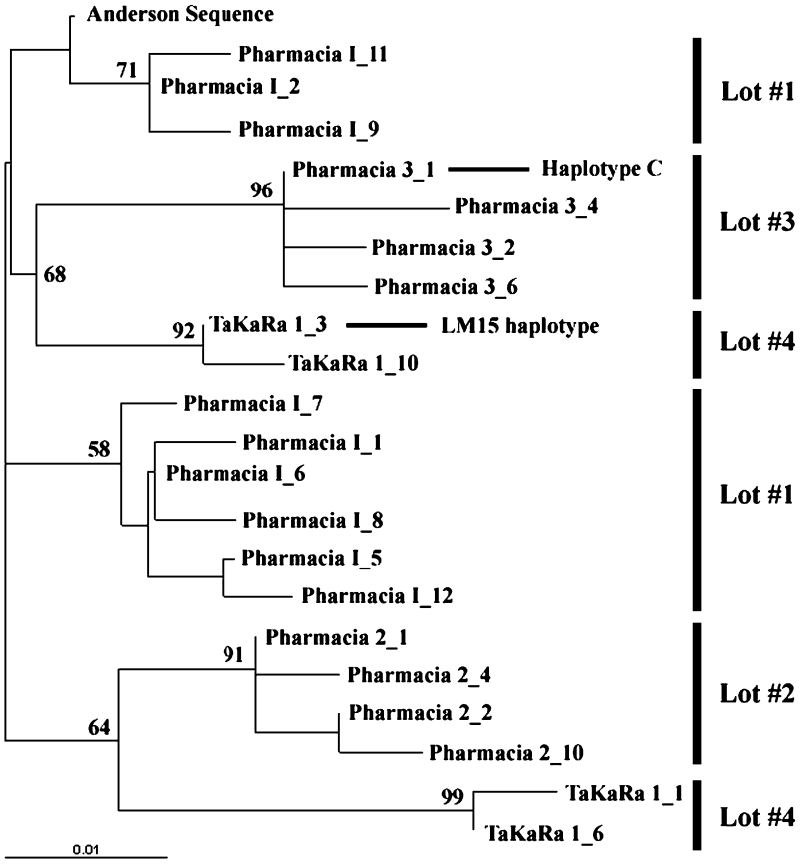

Fig. 1. Neighbor-joining phylogeny of 21 human mtDNA d-loop haplotypes amplified from no-template PCR reactions at OSU. The Anderson reference sequence is labeled "Anderson sequence" (Anderson et al., 1981). Bootstrap values >50% are indicated on branches, based on 1000 steps. Sequences clustered into groups that mirror dNTP lots.

frequent in both PCR reagents and the environment than originally anticipated. Further, our results strongly indicate that contamination of PCRs targeting domestic animals may occur on at least two levels, as contamination in the reagents used and on the specimens investigated.

Contamination of PCR reagents with human DNA was expected because they are handled by humans, both during manufacture and set-up of PCR experiments. However, contamination of PCR reagents with DNA from domestic animals is more difficult to explain. The minimal PCR mixture consists of water, buffer, $MgCl_2$, *Taq* DNA polymerase, primers, and dNTPs. Primers are chemically synthesized and purified by HPLC. Although PCR primers are used at comparatively low concentrations, primers are a possible source of extraneous DNA. Water, $MgCl_2$, and buffers are inorganic reagents unlikely to contain animal DNA. *Taq* DNA polymerase is derived from bacteria, but it is potentially exposed to cow or pig DNA during enzyme purification, where it is common practice to stabilize the enzyme in either bovine serum albumin or gelatin. However, researchers from OSU prepared *Taq* DNA polymerase excluding animal-derived chemicals and still identified extraneous cow and pig DNA in no-template PCR control reactions.

Details of commercial dNTP preparation are proprietary. However, one supplier revealed that deoxynucleoside monophosphates are obtained by hydrolysis of animal DNA and then phosphorylated chemically to produce triphosphates. Thus, dNTPs are the only PCR reagent with a known connection to animal DNA. dNTPs are therefore the most likely source of extraneous animal DNA in PCR reagents. Human mtDNA haplotypes detected in no-template PCR control reactions formed distinct clusters of sequence similarity based on manufacturer and reagent lot (Fig. 1), suggesting that dNTPs also contain measurable amounts of human DNA. Some ancient DNA researchers irradiate PCR reagent cocktails (excluding *Taq* DNA polymerase and template DNA) with UV light in an attempt to eliminate DNA contamination (Kaestle and Smith, 2001). However, this strategy is ineffective because dNTPs absorb UV light, thereby shielding deliberately added modern DNA templates from damage (Tilley, Shanks, Hodges, and Ream, unpublished data).

Three independent laboratories quantified the prevalence of PCR reagent contamination by performing hundreds of no-template PCR controls with primers that amplify both pig and cow templates. Each of the independent surveys detected contamination in 2–5% of reactions. In the experiment with the DNA extracts from bonobo feces, pig sequences were detected in 7% of PCRs containing DNA extract. The observation that amplification of pig DNA in particular extracts could not be replicated suggests that stochastic effects underlie

these results. Considering the large number of reactions involved in ancient DNA projects and the use of high-cycle PCR tests designed to detect trace quantities of short, highly degraded DNA, even small amounts of extraneous DNA could lead to spurious sequences in projects targeting cow, pig, chicken, and human.

The amplification of contaminating DNA in 5% of polymerase chain reactions may not sound like a lot – a 5% rate of error is often considered acceptable. However, in many ancient DNA studies based on archeological remains, the percentage of reactions yielding products is less than 10%. The 5% amplification of contamination detected represents 5% of all reactions, not just of the ones that yield product. In the case of having 10% of reactions yielding products, half of these positives could thus be expected to be contamination. In other words, 50 percent of the results would represent false positives, a level far greater than any commonly accepted level.

This problem can be magnified in ancient DNA studies if a carrier effect increases the rate at which false positives are generated through amplification of extraneous DNA in PCR reagents. We found evidence for a possible carrier effect in the experiments with bonobo feces, where 92 control reactions failed to amplify pig contaminant DNA, whereas 19 of 378 bonobo DNA extracts tested positive for pig DNA. Although the 3% false positive rate in 233 reactions detected at the Smithsonian Institution was not significantly different from the 0% false positive rate in 92 reactions at the Max Plank Institute ($p = 0.06$), false positive rates between the no-template control reactions and PCR reactions containing fecal extracts were significantly different ($p = 0.0013$). The large increase in frequency of amplification of pig contaminants in reactions with DNA extracts from bonobo feces compared to no-template reactions (0%) indicates that a carrier effect (Handt et al., 1994) increased the rate of false positives in reactions containing DNA extract. However, it should be noted that a recent study on human contamination in ancient DNA studies found no evidence for a carrier effect influencing detection of contaminating DNA (Malmström et al., 2005). A carrier effect may be sensitive to other unknown factors.

Another possible explanation for these results is contamination of the samples due to handling. Cow, pig and chicken contamination was observed in all four laboratories, and these animals are common food items in many parts of the world. DNA from these three species may be transferred to samples during handling, resulting in sample contamination (Gilbert et al., 2005). However, this does not explain the occurrence of pig DNA in no-template controls performed at OSU and Smithsonian Institution. A more likely explanation is that contamination originated from manufacturing practices used to isolate the building blocks of dNTPs.

5. Conclusions

Our results suggest that attempts to study ancient DNA from cow, pig, and chicken remains can suffer from problems of contamination similar to those that plague ancient DNA research on humans (Abbott, 2003). DNA from domestic and laboratory animals in commercially prepared dNTPs, or modern DNA contaminating samples, make ancient DNA studies involving these species highly questionable. These problems will be exacerbated by samples of marginal quality because low rates of success are expected and highly sensitive PCR conditions are used. To control one source of contamination, we urge companies to disclose species from which their reagents are derived. However, a heavy burden of proof lies on researchers presenting results of ancient DNA studies on domestic animals such as cows, pigs, and chicken, comparable to that for ancient DNA studies on humans.

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