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thesis entitled

POLYMERASE RESISTANCE TO PCR INHIBITORS IN BONE

presented by

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has been accepted towards fulfillment
of the requirements for the

M.S. degree in Criminal Justice

Major Professor's Signature

7/9/07

Date

POLYMERASE RESISTANCE TO PCR INHIBITORS IN BONE

By

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A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTERS OF SCIENCE

School of Criminal Justice

2007

ABSTRACT

POLYMERASE RESISTANCE TO PCR INHIBITORS IN BONE

By

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The molecularly based forensic identification of aged and degraded human skeletal remains requires optimization of the polymerase chain reaction (PCR). Several substances have been reported to disrupt the PCR process, leading to a decrease in amplification efficiency. PCR inhibitors are often encountered during the analysis of skeletal remains; however, methods conventionally employed to separate inhibitors from DNA may be less than ideal, as purification techniques tend to reduce the amount of sample DNA. Forensic analyses of skeletal material are most commonly conducted with variants of *Taq* DNA polymerase such as AmpliTaq™ Gold, which are derived from the thermophilic bacterium *Thermus aquaticus*. While *Taq* is suitable for routine PCR, it is susceptible to PCR inhibitors that can co-extract with skeletal DNA. Researchers have demonstrated that DNA polymerases derived from other thermophilic species may exhibit improved resistance to particular PCR inhibitors, though skeletally-derived inhibitors have not been investigated. In this study, ten polymerases were analyzed for inhibitor resistance to ancient skeletal DNA extracts, type I collagen, calcium ions, and humic acid. Replicates were performed with and without the addition of bovine serum albumin (BSA) to characterize mechanisms of relief with each inhibitor source. *Ex Taq*™ HS and *Tth* DNA polymerases were identified as superior to AmpliTaq™ Gold for tolerating bone-derived inhibitors, and the addition of BSA was shown to alleviate skeletally-derived PCR inhibition for many assayed polymerases.

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INTRODUCTION

The amplification of nucleic acid through the polymerase chain reaction (PCR) has become a powerful tool and staple technique in forensic science. The ability to amplify DNA from trace biological material allows for genetic identification when other forms of identification are impossible. PCR is very sensitive, capable of reliably generating sufficient copies of targeted genetic material for downstream analysis from only a few intact cells. As such, the technique is employed in several applications that require analysis from a limiting amount of DNA, including forensic identification, phylogenetic reconstruction, and pathogen detection in medicine and food science.

It is possible to amplify DNA from post-mortem samples if it is sufficiently intact. Over time, DNA degrades via hydrolytic deamination, or by single-stranded breaks in its phosphate backbone as a result of hydrolysis or base loss (reviewed by Mitchell et al. 2005), with the rate of degradation being influenced by environmental factors such as temperature, humidity, pH, and enzymatic attack (Lindahl 1993, Burger et al. 1999, Smith et al. 2003). Generally, the efficiency of PCR depends on the quantity and quality of DNA added to the reaction, with DNA degradation reducing that efficiency. Modern forensic analyses amplify short tandem repeats (STR)—stretches of nuclear DNA that are 100–500 nucleotides in length (Wallin et al. 1998, Cotton et al. 2000, Krenke et al. 2002). If the targeted DNA is extensively degraded, STR analysis will fail (Whitaker et al. 1995, Chung et al. 2004). Mitochondrial DNA analysis often proves more successful than STR analysis when DNA samples are heavily degraded, which may be due to a number of reasons, including higher copy number per cell (Robin and Wong 1988) and superior preservation due to cellular location (Foran 2006).

The Polymerase Chain Reaction

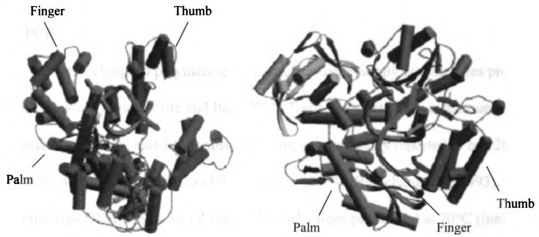
The PCR amplification process is facilitated by a polymerase, an enzyme that catalyzes template-directed synthesis of nucleic acids from nucleoside triphosphates. The DNA polymerase synthesizes a new DNA strand (amplicon) from a targeted template, under conditions favorable for enzymatic activity *in vitro*, including optimized pH and levels of PCR components. Template DNA is targeted with the inclusion of short, complementary stretches of DNA known as primers or oligonucleotides, which anneal to single-stranded DNA. The polymerase binds to the template-primer complex and provides a catalytic center for the incorporation of deoxynucleoside triphosphates.

Traditional PCR is conducted by manipulating the temperature of the reaction in a cyclical fashion; each cycle includes a denaturation stage, an annealing stage, and an extension stage. DNA is first made single-stranded by heating the reaction to near-boiling (92–94°C), exposing annealing sites on the template for complementary primers. The temperature is then lowered for primer annealing. The temperature and duration of this stage, generally 45°–68°C for 30–60 seconds, are optimized around the primers to promote annealing specificity, and are dependent on the primer length and base composition. Finally, the temperature is raised to an optimal level for DNA extension, which is dependent on the polymerase, PCR buffer, and nature of the template (Innis et al. 1995, Su et al. 1996), but is typically set to 68–72°C. The length of the extension stage must be sufficient to allow complete replication of the targeted amplicon, and depends on polymerase extension rate and processivity (the number of nucleotides added per association/disassociation with template DNA).

The DNA Polymerase

Organisms from all Kingdoms utilize some type of polymerase for DNA (or RNA) synthesis (Ishino et al. 1998, Bell and Dutta 2002, Lehman 2003) and most employ several DNA polymerases for efficient and accurate replication of their genome. For example, a typical eukaryotic cell contains at least 15 types of DNA polymerases, which are distinguished by structure, enzymatic function, and abundance (Hubscher et al. 2002). All DNA polymerases discovered to date can be classified into six families: A, B, C, D, X, and Y (reviewed by Filée et al. 2002), and contain a shared architecture, presumably owing to the conserved nature of DNA. Three major subunits exist, which structurally resemble a clenching right hand (Figure 1) and are traditionally identified as "palm", "finger" and "thumb" domains (Kohlstaedt et al. 1992). The palm domain interacts with two divalent metal cations (Mg^{++}) to serve as the catalytic center for phosphoryl transfer, while the finger domain stabilizes interactions between incoming nucleotides and the template. The thumb domain functions as an additional stabilizing and processivity factor (reviewed by Steitz 1998). The catalytic (palm) domain of DNA polymerases is most highly conserved through polymerase families; however, the thumb and finger domains differ structurally among families and appear to contribute to the enzymes' variable properties (Steitz 1999).

Figure 1: Structure of DNA Polymerase



Polymerases for which crystal structures have been developed share a common phenotype resembling a right hand that includes "palm" (red), "finger" (purple) and "thumb" (khaki) domains. Examples include *Taq* (left), an A family polymerase, and *Tok* (right), an archaeal B family polymerase. Image from Pavlov et al. (2004).

PCR-Utilized Polymerases

The most extensively utilized replicative enzyme in PCR is *Taq* DNA polymerase, derived from the thermophilic bacterium *Thermus aquaticus,* which was isolated from the hot springs of Yellowstone National Park. Kary Mullis is credited with proposing the use of *Taq* in a PCR setting (Saiki et al. 1988), though the ability to synthesize nucleic acids *in vitro* with replicative enzymes and temperature manipulation was described prior to Mullis' proposal (e.g., Kleppe et al. 1971). Before the isolation of *Taq,* the primary polymerase utilized for DNA amplification was the Klenow fragment of *E. coli* Pol I (reviewed by Uhlmann et al. 1998). The Klenow fragment is denatured at high temperatures however, requiring the repeated addition of polymerase with every

amplification cycle. *Taq* is inherently heat-resistant and requires addition to PCR only once, making DNA amplification more cost-effective while allowing for the automation of PCR.

Taq belongs to polymerase family A, the same family that includes prokaryotic Pol I enzymes (Braithwaite and Ito, 1993). *Taq's* thermostability is measured as a function of activity half-life at sustained temperature, and is reported to be 120 minutes at 92.5°C, 40 minutes at 95°C, and 9 minutes at 97.5°C (Blanchard et al. 1993). Native *Taq* synthesizes DNA at a maximal rate of 75 nucleotides per second at 70°C (Innis et al. 1988, Abramson et al. 1990) and incorporates approximately 60 nucleotides per binding event (Abramson et al. 1990, King et al. 1993, Takagi et al. 1997). Fidelity measurements vary among authors (Tindall and Kunkel 1988, Keohavang and Thilly 1989, Tse and Forget 1990, Ling et al. 1991, Lundberg et al. 1991, Cariello et al. 1991, Tindall and Kunkel 1992, Brail et al. 1993, Lawyer et al. 1993, Cline et al. 1996, Takagi et al. 1997, Patel et al. 2001) but is estimated to be 2.1×10^{-4}–8.2×10^{-6} errors per nucleotide polymerized. The enzyme exhibits an inherent 5'–3' exonuclease activity (Longley et al. 1990) but no "proofreading" 3'–5' exonuclease activity (Tindal and Kunkel 1988), meaning that it can degrade DNA in a forward direction during replication, but cannot correct its own base pair misinsertions. *Taq* also exhibits marginal reverse transcriptase activity (the ability to produce DNA from an RNA template; Jones 1989); however, the enzyme is not utilized *in vitro* for this purpose.

Several modifications have been applied to *Taq* in attempts to increase one or more aspects of enzyme performance. One of the earlier variants of *Taq* was the Stoffel fragment, an N-terminal deletion derivative that lacks the 5'–3' exonuclease domain.

The Stoffel fragment was reported to be more thermostable than the full enzyme and to exhibit optimal polymerase activity under a larger range of Mg^{++} concentrations (Lawyer et al. 1993). This derivative was traditionally preferred over *Taq* for amplification of GC-rich templates where a higher denaturation temperature was required (Newton 1994). KlenTaq (or *Taq* large fragment) is a similar N-terminal deletion mutant lacking the 5'–3' exonuclease domain that reportedly exhibits a two-fold higher fidelity rate than the whole enzyme (Barnes 1992).

Many variants of *Taq* have been modified for "hot-start" functionality in order to increase specificity to template DNA. In hot-start PCR, the polymerase is rendered inert at low temperatures where non-specific annealing of primers to template may occur. As the temperature is increased, primer annealing becomes more specific, and at the same time the polymerase is activated. This is accomplished via a number of methods, including paraffin-imbedded reagents that are released when heated (Herbert et al. 1993) or polymerase-binding ligands that sterically hinder protein-DNA interactions until released by heat (Birch et al. 1996). AmpliTaq™ Gold (Applied Biosystems) is simply *Taq* bound to an inhibitory chemical ligand, which requires several minutes of high PCR temperatures before the molecule is dissociated and the enzyme is activated. *Ex Taq*™ HS (Takara) is another *Taq* variant that utilizes a polymerase-specific antibody to block DNA interactions until sufficiently heated. HotMaster™ *Taq* utilizes a proprietary chemical ligand that competitively binds the polymerase at low temperatures and is released at high temperatures with each cycle of PCR, thereby increasing template specificity (Eppendorf commercial literature).

Though *Taq* has proven a reliable enzyme for mainstream DNA amplification, it has its limitations and may not necessarily be optimal for situation-specific PCR. Many thermostable polymerases have been isolated and found suitable for PCR, which are derived from myriad thermophilic bacteria and archaea and made commercially available in natural or recombinant forms. A list of enzymatic properties for several thermostable polymerases is provided in Table 1. Most of these belong to families A (prokaryotic repair polymerases) or B (archaeal and eukaryotic replicative polymerases) (Pavlov et al. 2004), though recently there has been considerable effort to isolate thermostable Y-family polymerases for specialized PCR applications, namely the improved synthesis of UV-damaged DNA (McDonald et al. 2006). The enzymatic properties of polymerases (e.g., nuclease activity, fidelity, processivity, or extension rates) vary among families and species of origin, and commercial products are touted according to one or more intrinsic properties that surpass *Taq*'s capabilities. For example, *Pfu* DNA polymerase, which is derived from the hyperthermophilic archaebacterium *Pyrococcus fusorious*, reportedly exhibits a 12-fold higher fidelity rate than *Taq* (Lundberg et al. 1991). *Tth* DNA polymerase from *Thermus thermophilus* functions as both a polymerase and as a reverse transcriptase (Myers and Gelfand 1991), and may be utilized for one-tube reverse transcriptase-PCR (RT-PCR).

One of the most notable variations among polymerases is an increased fidelity due to 3'–5' exonuclease activity (i.e., proofreading). B family polymerases exhibit proofreading activity and tend to exhibit higher fidelity rates than other polymerases (Cline et al. 1996). However, in practice this advantage comes at a cost, as proofreading enzymes can degrade primers and template when not actively participating in extension

(Haff and Smirnov 1997), resulting in reduced DNA yield. This is particularly problematic for forensic or anthropological samples that are already low in template copy number. Template and primer degradation can be minimized by conducting PCR with nucleotide concentrations of 200–400 μM for each dNTP. Lower concentrations may result in depletion of the dNTP pool during DNA synthesis, which promotes exonucleolytic activity as the dominant function of the enzyme (Roche commercial literature, New England Biolabs commercial literature). Additionally, it is advised to add the polymerase immediately prior to PCR cycling, so as to minimize potential template degradation (reviewed by Rolfs et al. 1992). Primers can also be made nuclease-resistant with the use of phosphorothioate-modified nucleotides during synthesis, which is recommended when using proofreading polymerases (reviewed by Liao et al. 2005).

Table 1: Properties of Various PCR-Suitable DNA Polymerases

DNA Polymerase	Fidelity (errors/base /duplex)	Processivity (nt)	Ext. Rate (nt/sec)	Exonuclease Activity 5'–3'	Exonuclease Activity 3'–5'	RT Activity	Special Properties	References
Taq	$2.1x10^{-4}$ – $8.2x10^{-6}$	42	60–150	yes	no	yes†		Tindall and Kunkel 1988, Keohavang and Thilly 1989, Tse and Forget 1990, Ling et al. 1991, Lundberg et al. 1991, Cariello et al. 1991, Tindall and Kunkel 1992, Brail et al. 1993, Lawyer et al. 1993, Cline et al. 1996, Takagi et al. 1997, Patel et al. 2001
Stoffel Fragment	ND	20-40	>50	no	no	no	Broad Mg^{++} Range‡	Rolfs et al. 1992, Pavlov et al. 2002
Tth HB8	$3.0x10^{-5}$	ND	25	yes	no	yes	RT-PCR	Carballeira et al. 1990, Sellmann et al. 1992, Frey and Suppmann 1995
Tfi	ND	ND	ND	yes	no	no		Shandilya et al. 2004
Tli (Vent)	$1.6x10^{-5}$ – $2.8x10^{-6}$	7	17	no	yes	yes†		Brail et al. 1993, Kong et al. 1993, Cline et al. 1996
Tfl (Replinase)	$4.0x10^{-6}$	30-40	33-67	no	no	yes†		Mattila et al. 1991, Rolfs et al. 1992, Brail et al. 1993, Pavlov et al. 2004 Epicentre commercial literature
Pfu	(2.5–3.7) $x10^{-5}$	2.1-20	25	no	yes	no		Brail et al. 1993, Cariello and Skopek 1993, Takagi et al. 1997, Pavlov et al. 2002

Table 1: Properties of Various PCR-Suitable DNA Polymerases (cont'd.)

DNA Polymerase	Fidelity (errors/base /duplex)	Processivity (nt)	Ext. Rate (nt/sec)	Exonuclease Activity 5'–3'	Exonuclease Activity 3'–5'	RT Activity	Special Properties	References
Tgo	4.9×10^{-7}	ND	ND	no	yes	no		Roche commercial literature
KOD1 (Pfx)	ND	>300	106-138	no	yes	no		Takagi et al. 1997
GB-D (DeepVent)	2.7×10^{-6}	<20	23	no	yes	no		Cline et al. 1996, Takagi et al. 1997
Pwo	3.2×10^{-6}	20-30	ND	no	yes	no		Frey and Suppman 1995, Roche Diagnostics 1999

Thermostable polymerases listed by name, as well as enzyme characteristics including fidelity, processivity, extension rate, and presence or absence of 5'–3' exonuclease activity, 3'–5' exonuclease activity, or reverse-transcriptase (RT) activity. Other notable enzyme properties are also listed if applicable.
RT-PCR: reverse-transcriptase-PCR
Ext.: extension
† Exhibits negligible but detectable RT activity.
‡ Exhibits optimal activity in the presence of 2–10 mM Mg^{++}.

In an effort to overcome the limitations of individual polymerases, multiple enzymes can be simultaneously employed in one PCR reaction (Barnes 1994). Polymerase blends are typically comprised of a primary enzyme (for DNA amplification) mixed with a secondary polymerase, in order to improve some aspect of amplification efficiency (e.g., inclusion of proofreading to reduce nucleotide misincorporation). A list of commercially available polymerase blends is found in Table 2.

Table 2: Commercial DNA Polymerase Blends.

Polymerase Blends	Supplier	Major Polymerase	Supporting Polymerase
Advantage®-HF	Clontech/Takara	KlenTaq	Proprietary Proofreader
EXL® DNA Polymerase	Stratagene	*Pfu*	*Taq*
Expand™ High Fidelity PCR System	Roche	*Taq*	*Tgo*
LA Taq™ Polymerase	Takara	*Taq*	DeepVent
Platinum® *Taq* High Fidelity	Invitrogen	*Taq*	DeepVent
TaqPlus® Precision PCR System	Stratagene	*Taq*	*Pfu*

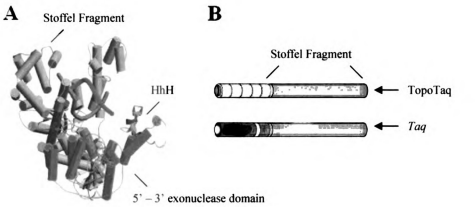
The manufacturer of available commercial polymerase blends as well as the primary and supporting enzymes utilized.

A large body of research has focused on reengineering thermostable polymerases to enhance fidelity and processivity or to confer additional functionality to the native enzyme. These modifications either take the form of point mutations or genetic recombination. Random mutagenesis screens of *Taq* have resulted in mutant cold-sensitive variants for hot-start PCR applications (e.g., Kermekchiev et al. 2003), while site-directed mutagenesis has been utilized to create variants with lowered fidelity for

mutagenic PCR (e.g., Patel et al. 2001) as well as mutants that more effectively incorporate ddNTPs for sequencing applications (Tabor and Richardson 1995, Li et al. 1999).

Several DNA polymerase chimeras have also been developed (e.g., Villbrandt et al. 2000, Pavlov et al. 2002, Davidson et al. 2003, McDonald et al. 2006), which are genetically engineered enzymes that incorporate functional domains and structural motifs from native polymerases and other proteins into a "best of all worlds" construct. This is made possible because of the modular and independently-folding nature of polymerase domains, which allows for cooperative domain behavior despite different species origins (Pavlov et al. 2004). Most attempts to engineer chimeras have focused on improving commonly utilized PCR polymerases in order to enhance their natural capabilities. For instance, *Taq* was modified for increased processivity and fidelity through its fusion with the thioredoxin binding domain from T3 bacteriophage DNA polymerase (Davidson et al. 2003). The processivity of both *Taq* and *Pfu* were greatly enhanced with the fusion of the double-stranded DNA binding protein Sso7d from the hyperthermophilic archaeabacteria *Sulfolobus solfataricus* (Wang et al. 2004). Similarly, polymerase processivity, thermostability, and resistance to salt inhibition were increased with the fusion of sequence-nonspecific DNA binding helix-hairpin-helix motifs to *Taq* and *Pfu* (Pavlov et al. 2002). This technique has also been applied to the Stoffel fragment and is commercially marketed as TopoTaq™ (Figure 2). A list of commercially available chimeric polymerases can be found in Table 3.

Figure 2: *Taq* and TopoTaq™ Domain Organization



(A) Schematic representation of *Taq* DNA Polymerase, highlighting the Stoffel fragment of *Taq* (gray), 5'–3' exonuclease domain (green), and single helix-hairpin-helix (HhH) motif in the 5'–3' exonuclease domain (yellow). (B) Cartoon of TopoTaq™ and *Taq* domain organization. The domains of the TopoTaq™ chimera are modeled to resemble native *Taq,* where DNA-binding HhH repeats (yellow) are positioned N-terminal to the Stoffel fragment. TopoTaq™ combines multiple HhH repeats from DNA Topoisomerase V with the Stoffel fragment to create an enzyme with increased processivity over *Taq.* Image from Pavlov et al. (2002).

Table 3: Commercial DNA Polymerase Chimeras

Polymerase Chimeras	Supplier	Structural Composition	Chimeric Advantage
TopoTaq™	Fidelity Systems	*Taq* DNA polymerase + Topo V HhH motifs	Increased processivity, thermostability, and salt resistance
Pfx50™	Invitrogen	*Thermococcus zilligi* DNA polymerase + ssDNA-binding domain	Increased processivity
PfuUltra™ II Fusion HS	Stratagene	*Pfu* + dsDNA-binding domain	Increased processivity
Herculase® II Fusion	Stratagene	*Pfu* + dsDNA-binding domain	Increased processivity
Phusion™	Finnzymes / New England Biolabs	GB-D DNA polymerase + dsDNA-binding domain	Increased processivity

Name and manufacturer of commercially available polymerase chimeras. Original polymerases are listed along with domain modifications, as well as conferred advantage relative to the original enzyme.

PCR Inhibition

PCR is very sensitive when conducted under conditions that allow for optimal protein-nucleic acid interactions. Suboptimal reaction conditions include improper parameters for reagents (e.g., incorrect annealing temperature for primer pairs) or an inefficient ratio of PCR components leading to reduced amplification (e.g., insufficient Mg^{++} or dNTPs). Further, several substances have been reported to inhibit PCR. The degree of inhibition can be partial or whole, resulting in less efficient amplification, which reduces detection sensitivity, or a complete disruption of amplification.

The major mechanisms of PCR inhibition can be classified into three categories (Wilson 1997). The first class of inhibition involves a failure of cell lysis, leaving target DNA physically isolated from the PCR components. The second category includes materials that directly degrade or capture template DNA or primers, including competing DNA-DNA interactions. For example, primers and intact template DNA may anneal to sheared DNA fragments, creating multiple substrates for polymerase binding and resulting in excessive non-targeted amplification (Lienert and Fowler 1992). The third category of inhibition involves molecules that interact with the polymerase directly, leading to reduced or failed amplification. The specific mechanisms of this interaction vary widely among inhibitors. Some molecules bind the polymerase directly and prevent DNA interactions, such as heme, a component of blood (Akane et al. 1994), and humic substances from soil (Tsai and Olsen 1992). Other molecules (e.g., phenol, detergents) can denature the enzyme (Rossen et al. 1992, Katcher and Schwartz 1994), and yet others can enzymatically degrade the enzyme, such as proteinases present in food samples (Powell et al. 1994).

Polymerases can also be inhibited indirectly if required cofactors are unavailable. Divalent cations such as Mn^{++} and Ca^{++} are thought to competitively bind the enzyme's active site where Mg^{++} is required for catalytic activity (Bickley et al. 1996). Further, metal chelators like EDTA inhibit PCR by sequestering Mg^{++} in solution (Rolfs et al. 1992).

PCR Inhibitors Encountered in the Forensic Analyses of Biological Samples

PCR inhibition is regularly observed in forensic evidence submitted for DNA analysis. Blood contains multiple PCR inhibitors, including heparin (Beutler et al. 1990), heme (Akane et al. 1994), and immunoglobulin G (Abu Al-Soud et al. 2000). Other common sources of PCR inhibition in forensic samples include melanin in skin and hair (Eckhart et al. 2000), urea from urine (Mahoney et al. 1998), bacteria from vaginal secretions (Lienert and Fowler 1992), and bile acids, bilirubin and polysaccharides in feces (Olive 1989, Monteiro et al. 1997, Lantz et al. 1997). Other inhibitors are derived from secondary sources rather than the biological material itself. Serological fluids must sometimes be extracted from unorthodox substrates (e.g., carpet material, flooring, fabric) that prove difficult to extract biological evidence from or contain substances that co-extract with DNA and inhibit PCR. For example, DNA extracted from leather can be inhibited in PCR due to co-extracted tanning agents (Bright and Petricevic 2004). Another common example is indigo, a dye that imparts pigmentation to denim and other fabrics, which strongly inhibits PCR (Shutler et al. 1999).

Combating PCR Inhibition

Several techniques have been developed to overcome the effects of inhibitors present in DNA extractions. The most straightforward method is simple dilution of the extract. If DNA quantities are high enough, they can be diluted to a point where inhibitory effects are relieved, permitting amplification. However, the success of this

method is ultimately sample-dependent, as excessive dilution of template DNA can reduce amplification efficiency. Some traditional DNA purification methods allow for the co-isolation of PCR-inhibitory substances. For example, phenol-chloroform extractions may fail to remove water-soluble inhibitors like urea or humic acid (Moreira 1998), Fe^{++} (Kreader 1996), and components of feces (Wilde et al. 1990), while Chelex™ chelating resin has proven ineffective at removing inhibitors from blood samples (Jung et al. 1991, Greenspoon et al. 1998). As such, alternative DNA purification methods have been utilized to increase DNA yield or overcome inhibition from biological mixtures. For example, Rossen et al. (1992) developed a hot-NaOH DNA extraction method for use on dairy-related food samples to alleviate inhibitory effects encountered during pathogen detection assays. Likewise, an alkaline lysis procedure for use with hair shafts was developed by Graffy and Foran (2005) that reduced PCR inhibition compared to conventional grinding methods, and was particularly effective at improving amplification success from chemically treated hair.

Additionally, extracted DNA may be re-purified prior to PCR. Methods include selective precipitation (e.g., Wallace 1987), silica membrane spin column filtration (e.g., Greenspan et al. 1998), Microcon® filtration (e.g., Makristathis et al. 1998), gel filtration (e.g., Gur'ev et al. 1998), DNA-binding silica beads (e.g., Zink et al. 2005), and immunomagnetic DNA separation (e.g., Ye et al. 2004). All can be effective at separating PCR inhibitors from the DNA; however, purification usually results in a loss of sample DNA (reviewed by Miller et al. 1999), so caution is advised with low DNA copy number samples.

The addition of chemical adjuvants to PCR can also lead to improved amplification, either by optimizing PCR component interactions or combating inhibitors directly. Dimethyl sulfoxide (DMSO) has been shown to improve PCR and reverse transcriptase-PCR yield, presumably by stabilizing nucleic acid complexes and improving primer annealing efficiency (Shen and Hohn 1992, Sidhu et al. 1996). Kreader (1996) demonstrated that the addition of bovine serum albumin (BSA) or T4 gene 32 protein could relieve PCR inhibition in the presence of 10–1000 times higher Fe^{++}, hemin, and humic substance levels than without, although neither improved inhibition from bilirubin, EDTA, NaCl, bile salts, or detergents.

Special Considerations for DNA Extraction and PCR from Skeletal Material

DNA may be recovered from skeletal material if soft tissue or serological fluid is not analyzable. Post mortem, bone tends to degrade more slowly than softer tissue—a property that proves useful in anthropological and forensic DNA analyses. The recovery of DNA from skeletal material is traditionally accomplished by drilling or pulverizing bone into powder to maximize surface area (e.g., Höss and Pääbo 1993, Edson et al. 2004), followed by cell lysis and DNA extraction. Several extraction methods have been described in the literature (e.g., Meijer et al. 1992, Höss and Pääbo 1993, Baron et al. 1996, Yang et al. 1998), but the standard strategies involve sample digestion with an EDTA/detergent buffer (to dissolve the inorganic components of bone and lyse cells) followed with either phenol-chloroform or silica-based techniques to selectively extract

DNA from cellular debris (reviewed by Hummel et al. 2003, Rohland and Hofreiter 2007).

The amplification of DNA from aged or damaged skeletal samples can be especially challenging due to the combination of degraded template and co-extracted PCR inhibitors. Inhibition has been reported with bones exposed to soil (e.g., Hagelberg et al. 1991, Yang et al. 1998, Sutlovic et al. 2005) and water (e.g., Kalmár et al. 2000, Sørensen et al. 2003). The major inhibitors cited in these scenarios are humic substances (e.g., humic and fulvic acids), which are polyphenolic compounds originating from decomposed plant and animal matter and characterized by their structural heterogeneity (Li et al. 2003). Humic substances can accumulate in skeletal samples via prolonged exposure to water or soil (Tsai and Olsen 1992), and are thought to disrupt PCR by denaturing or covalently binding to the polymerase, preventing DNA replication (Tebbe and Vahjen 1993, Young et al. 1993).

Bone is composed of approximately 70% hydroxyapatite [$Ca_5(PO_4)_3(OH)$], which forms the calcium phosphate lattice that imparts rigidity to it. Standard DNA extraction methods first utilize EDTA to decalcify skeletal hydroxyapatite by binding Ca^{++}. It is not known how much Ca^{++} persists through skeletal DNA extraction and purification procedures; however, Ca^{++} has been shown to inhibit DNA amplification in non-skeletal studies. For instance, Abu Al-Soud and Rådström (1998) demonstrated that concentrations of 1 mM Ca^{++} inhibited PCR when using *Taq* and AmpliTaq™ Gold DNA polymerases, presumably due to Mg^{++}/Ca^{++} competitive binding at the polymerase active site.

The remaining 30% of bone is organic material, with approximately 27% being collagen (Scholz et al. 1998), which is also a proven PCR inhibitor (Kim et al. 2000). Collagen is an extracellular protein found in various forms of connective tissue, and is classified into several distinct types based on amino acid sequence. Mature collagens exist as right-handed triple helixes composed of three polyproline II-like α-chains (Brodsky and Ramshaw 1997). The primary constituent of bone is type I collagen, a heterotrimer 300 nm in length consisting of two α1(I) and one α2(I) chains (Dalgleish 1997, Von Der Mark 1999). Intact type I collagen is insoluble in water and should be removed from skeletal digests following organic extraction (reviewed by Hummel et al. 2003); however, Scholz et al. (1998) identified degraded type I collagen (using UV-Vis spectrophometry, SDS-PAGE, and collagenase treatments) as a potent PCR inhibitor in ancient skeletal DNA samples that had undergone phenol-chloroform extraction. While both intact and degraded type I collagen are inhibitory (Scholz et al. 1998, Kim et al. 2000), the mechanism by which either affects PCR is not clear. Kim et al. (2000) found that inhibition by type I collagen could be partially reversed with the addition of extra Mg^{++}, suggesting that collagen may sequester available Mg^{++} in PCR.

Differential Polymerase Resistance to PCR Inhibitors

Multiple researchers have observed that polymerases derived from different thermophilic species exhibit varying degrees of resistance to particular inhibitors. Abu Al-Soud and Rådström (1998) contrasted the ability of nine DNA polymerases to amplify bacterial DNA in the presence of complex biological mixtures, including blood, meat,

cheese, and feces. Additionally, polymerases were spiked with high levels of Mg^{++}, Ca^{++}, K^{+}, and Na^{+} to simulate ionic concentrations that might be present in food samples and inhibit PCR pathogen detection assays. The authors demonstrated that AmpliTaq™ Gold and *Taq* (Boehringer Mannheim) were more sensitive to Ca^{++} and inhibitors from blood than were *Pwo, Tub, Tfl,* and *Tli.* Few studies exist that include such a comprehensive variety of polymerases; however, other researchers have reported differential inhibitor resistance among a small number of enzymes. For instance, *Tth* was found to retain polymerase activity in 5% phenol, whereas *Taq* was inhibited by a trace amount (Katcher and Schwartz 1994). *Tth* and *Tfl* have been shown to be more resistant to inhibition than *Taq* in the presence of vitreous eye fluid (Wiedbrauk et al. 1995). In a food pathogen detection assay, *Pwo* was more sensitive to inhibition than *Taq* in the presence of collagen (Kim et al. 2000). The apparent relative inhibitor resistance of some polymerases suggests that the selection of polymerase may be a useful tool in overcoming PCR inhibition encountered with skeletal DNA amplification.

Goals of This Project

Many forensic DNA laboratories utilize only one polymerase for the analysis of biological samples, based on their existing standard operating procedures. A prime example is the Armed Forces DNA Identification Laboratory (AFDIL), which is one of the leading laboratories in the world for the processing of degraded skeletal remains. AFDIL utilizes AmpliTaq™ Gold as the exclusive enzyme for all skeletal DNA amplification (Edson et al. 2004). Further, the enzyme is integrated into several

commercial kits extensively utilized by the forensic community (e.g., Applied Biosystems AmpF*l*STR® Identifiler PCR Amplification Kit, Applied Biosystems AmpF*l*STR® Yfiler PCR Amplification Kit, and Promega PowerPlex® 16 System). However, AmpliTaq™ Gold has been shown in previous studies (see above) to more be sensitive to PCR inhibitors compared to some other enzymes.

The purpose of this study was to investigate polymerase inhibitor resistance as a means towards improving the amplification of DNA from skeletal material. To the author's knowledge, an assay of polymerase-specific resistance to skeletally-derived inhibitors has never been conducted. It was hypothesized that commercially available DNA polymerases isolated from various thermophilic species would behave differently in the presence of PCR-inhibitory substances derived from bone, and some polymerases might exhibit an increased inhibitor resistance relative to conventionally utilized standards such as AmpliTaq™ Gold.

Towards this goal, a series of assays was designed to analyze ten commercially available thermostable polymerases for resistance to skeletally-derived PCR inhibitors. Five *Taq* enzymes or variants (including AmpliTaq™ Gold), and five polymerases derived from thermophiles other than *T. aquaticus,* were evaluated for sensitivity to inhibitors from skeletal DNA extracts as well as type I collagen, calcium ions, and humic acid. PCR reactions were conducted with and without the addition of BSA in order to examine the adjuvant's alleviating effects on PCR inhibition. The overall goal of the study was to identify inhibitor-resistant polymerases that would most effectively facilitate the genetic identification of aged and degraded skeletal remains, as well as to better characterize PCR inhibition mechanisms encountered with the analysis of bone.

MATERIALS AND METHODS

Human Bones Selected as Sources of PCR Inhibition

Ancient human bone was chosen to serve as a genuine source of skeletally-derived inhibitors for the polymerase-specific resistance assay. Michigan State University graduate students collected sets of skeletal samples from burial sites in Butrint and Diaporit, Albania. Sample sets included whole or fragmented remains of skulls, long bones, vertebrae, ribs, pelvic bones, metacarpals, and metatarsals (Murray 2006). Bone fragments from burials designated 5010 and 213 exhibited high incidences of PCR inhibition during previous genetic analyses (M. Mutolo, personal communication), thus one bone was chosen from both sets. Burial 5010 is an adult female, excavated from Butrint and dated to the 5^{th}–7^{th} Century A.D. Burial 213 is an adult male, excavated from Diaporit and dated to the 5^{th}–7^{th} Century A.D. A tibia fragment was selected from burial 5010, and a thoracic vertebra fragment was selected from burial 213. Positive control DNA was isolated from a fresh porcine ox coxae fragment (pork loin chop).

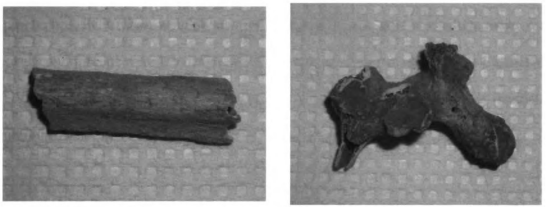
Persistence of Color-Causing Substances in Bone Extractions

Mutolo (2006) reported unusual coloring of bone powder and subsequent DNA extractions generated from Albanian excavation skeletal samples during previous analyses. The discoloration varied widely among skeletal sets, ranging from yellow to dark brown. The author noted that the color could not be eradicated with Microcon®

purification. Discoloration of skeletal extractions has traditionally been attributed to soil-derived humic substances or Fe^{++} that persist through various purification techniques and co-extract with DNA (Hagelberg and Clegg 1991, Cooper 1992, Tuross 1994).

The two fragments selected from burials 5010 and 213 are depicted in Figure 3 to show native bone discoloration. The tibia fragment from 5010 was brown in color, while vertebra fragment 213 was red in color. During phenol-chloroform extraction, the aqueous layer from 5010 retained a light brown discoloration, while the extract from 213 had a red discoloration consistent with the original bone. Discoloration was retained following Microcon® purification. With subsequent dilution, the coloring disappeared, and no discoloration was visible in the 1:10 and 1:20 dilutions of either extract.

Figure 3: Tibia Fragment 5010 and Thoracic Vertebra Fragment 213



Discoloration of skeletal samples 5010 (left) and 213 (right) prior to powder collection. Tibia fragment 5010 is a yellowish-brown color, while vertebra fragment 213 is a dull red color. Color-attributing substances persisted through DNA phenol-chloroform extraction and Microcon® purification.

Reagent and Tool Preparation for DNA Extraction

Personal protective equipment, including latex gloves, surgical mask, and long-sleeve laboratory coat, was utilized to minimize DNA contamination at all points of preparation, extraction, and DNA amplification of skeletal samples. Handling of skeletal material and DNA extraction were performed in a Labconco Purifier PCR Enclosure, which was cleaned with 10% bleach and 70% ethanol prior to use. A "bone wash" reduced-EDTA digestion buffer (10 mM EDTA, 0.5% SDS, 20 mM Tris pH 8.0) was prepared according to Mutolo (2006). Bone wash, standard digestion buffer (50 mM EDTA, 0.5% SDS, 20 mM Tris pH 8.0) and water were purified with a .22-micron filter. A Dremel tool was cleaned with 10% bleach and 70% ethanol. Dremel collets, rings, and $1/16^{th}$ inch drill bits were soaked in bleach for 15 minutes, rinsed with sterile water and allowed to air dry. Bone wash, digestion buffer, water, Dremel tool, drill bits, pipettes, pipette tips, PCR tubes, weigh boats, weigh paper, 1.5 mL microcentrifuge tubes, cotton swabs and forceps were UV-irradiated for 10 minutes and then placed in a Cleanspot UV/PCR Workstation (cleaned with 10% bleach and 70% ethanol), and UV-irradiated until utilized.

Skeletal Material Processing

Skeletal samples were placed into weigh boats. The Dremel tool was fitted with a $1/16^{th}$ inch drill bit, cleaned again with 70% ethanol, and utilized to remove a small portion of bone surface material approximately 1 mm deep. The bone fragment was

transferred to a piece of weigh paper, and the surface powder was discarded. The exposed bone surface was swabbed with bone wash twice and allowed to air dry. The Dremel tool was disassembled, fitted with a new drill bit, and cleaned with 70% ethanol. A hole was drilled into the exposed surface of bone to generate powder. One hundred milligrams of powder was collected into two 1.5 mL microcentrifuge tubes for each ancient human bone fragment, and 50 mg of powder was collected into two tubes for the porcine bone. Three hundred microliters of digestion buffer and 5 µL of 20 mg/mL proteinase K were added to each tube. A reagent blank containing 300 µL digestion buffer and 5 µL of proteinase K was also prepared. Samples and reagent blanks were vortexed for 15 seconds and incubated at 55°C for 48 hours.

Skeletal DNA Extraction

DNA was extracted using a phenol-chloroform method. Three hundred microliters of phenol was added to each sample tube, then vortexed for 30 seconds and centrifuged at 14,000 rpm for 5 minutes. The aqueous layer was transferred to a new tube. Three hundred microliters of chloroform was added to each tube, then vortexed for 30 seconds and centrifuged at 14,000 rpm for 5 minutes. The aqueous layer was transferred to a Microcon® YM-100 centrifugal filter device and centrifuged at 500 x g for 12 minutes. Three hundred microliters of water was added to each tube, then centrifuged at 500 x g for 15 minutes. DNA from human extractions was eluted in 50 µL of sterile water, and porcine DNA was eluted in 100 µL of sterile water. Human and porcine samples were mixed with respective duplicate extractions to ensure consistent

concentrations of DNA and inhibitory elements. To prevent DNA degradation as a result of multiple freeze-thaw cycles, the porcine DNA extraction was aliquoted into 10 volumes of 20 µL. A portion of the DNA extraction from samples 5010 and 213 was aliquoted and diluted with sterile water at 1:5, 1:10, and 1:20 of the original concentration. All extractions were stored at -20°C.

Thermostable Polymerases Selected for Assays

Five *Taq* polymerases from different commercial sources, as well as five non-*Taq* polymerases, were selected for the inhibitor resistance assays. The polymerase names, bacteria of origin, and commercial suppliers are listed in Table 4. *Tfl, Tfi,* and *Tth* belong to the A family of polymerases that includes *Taq*, while *Tgo* and Pfx50™ are archaeal B family polymerases that exhibit proofreading ability.

Table 4: Assayed Polymerases

Name	Thermophile	Supplier
Taq	*Thermus aquaticus*	New England Biolabs
Taq	*Thermus aquaticus*	Promega
AmpliTaq™ Gold	*Thermus aquaticus*	Applied Biosystems
HotMaster™ *Taq*	*Thermus aquaticus*	Eppendorf
Ex Taq™ HS	*Thermus aquaticus*	Takara
Tfl	*Thermus flavus*	Promega
Tfi	*Thermus filiformis*	Invitrogen
Tgo	*Thermococcus gorgonarius*	Roche
Tth	*Thermus thermophilus*	Roche
Pfx50™	*Thermococcus zilligii*	Invitrogen

The different assayed polymerase products listed by name, thermophilic bacterium of origin, and commercial supplier.

Composition of PCR Buffers

Commercial polymerase products come supplied with a PCR buffer solution optimized for enzymatic efficiency. PCR buffer composition varies among commercial sources and the formula is often proprietary. Buffers contain a pre-optimized concentration of Mg^{++}, or alternatively, an external Mg^{++} source is provided for manual optimization. The buffer compositions of the polymerases in this study are listed in Table 5.

Table 5: PCR Buffer Composition

Polymerase	Buffer Composition
Taq (New England Biolabs)	10X ThermoPol Reaction Buffer: 200 mM Tris-HCl, 100 mM $(NH_4)_2SO_4$, 100 mM KCl, 20 mM $MgSO_4$, 1% Triton X-100 (pH 8.8 @ 25°C)
Taq (Promega)	10X Reaction Buffer: 100 mM Tris-HCl, 500 mM KCl, 1% Triton X-100 (pH 9.0 @ 25°C) Separate vial of 25 mM $MgCl_2$
AmpliTaq™ Gold (Applied Biosystems)	10X GeneAmp PCR Buffer II: 100 mM Tris-HCl, 500 mM KCl, (pH 8.3 @ room temperature) Separate vial of 25 mM $MgCl_2$
HotMaster™ *Taq* (Eppendorf)	Proprietary (10X buffer contains 25 mM $MgCl_2$)
Ex Taq™ HS (Takara)	Proprietary (10X buffer contains 20 mM $MgCl_2$)
Tfl (Promega)	10X Reaction Buffer: 200 mM Tris-acetate, 100 mM $(NH_4)_2SO_4$ (pH 8.9 @ 25°C) Separate vial of 25 mM $MgCl_2$
Tfi (Invitrogen)	5X Reaction Buffer: 250 mM Tris-HCl, 75 mM $(NH_4)_2SO_4$, 25 mM KCl, 1 mM DTT, proprietary stabilizers (pH 8.4) Separate vial of 50 mM $MgCl_2$
Tth (Roche)	10X Reaction Buffer: 100 mM Tris-HCl, 15 mM $MgCl_2$, 1 M KCl, 500 µg/mL BSA, 0.5% Tween 20 (v/v) (pH 8.9 @ 25°C)
Tgo (Roche)	5X Reaction Buffer: 50 mM Tris-HCl, 87.5 mM $(NH_4)_2SO_4$, 6.25 mM $MgCl_2$, 2.5% Tween 20 (v/v), 7.5% DMSO (v/v) (pH 8.5 @ 25°C)
Pfx50™ (Invitrogen)	Proprietary (10X buffer contains 12 mM $MgSO_4$, undisclosed concentration of BSA)

Lists the PCR buffers for each polymerase product. Prepackaged buffers are optimized for standard PCR. Note that suppliers often provide several buffer solutions for use; the listed buffer is the variant utilized in inhibitor assays.

PCR Conditions for Bone Inhibition Assay

Primer sequences and PCR cycling parameters are outlined in Table 6. A master mix was prepared for 10 μL PCR reactions and included the following: 1X PCR reaction buffer, 1 μL of 20 μM forward primer Pig-F, 1 μL of 20 μM reverse primer Pig-R, 1 μL of deoxynucleoside 5'–triphosphates (200 μM each dNTP), 1 U of polymerase, 1 μL of 60 ng/μL porcine DNA, 1 μL of human bone DNA extraction (at varied concentrations; see below) and water to a final volume of 10 μL. If required, Mg^{++} was added to the master mix at a reaction concentration of 2.5 mM. PCR reactions were spiked with DNA extractions from bone samples 5010 and 213 using 1 μL of the original extraction or 1 μL of a 1:5, 1:10, or 1:20 dilution of the extraction. Primers Pig-F or Pig-R were designed according to Lahiff et al. (2000) and are specific for a 212 bp porcine mtDNA amplicon. Positive and negative controls were conducted with every reaction set, and all reactions were performed in duplicate. A single replicate including nonacetylated bovine serum albumin (FisherBiotech) was also conducted for the 213 extraction, which substituted 1 μL of 4 μg/μL BSA for 1 μL of water in each reaction. After cycling, 5 μL of each PCR product was loaded onto a 2% agarose gel and electrophoresed. PCR products were visualized with ethidium bromide staining and UV illumination, and analyzed for successful target amplification.

Table 6: Primer Sequences and PCR Parameters

Bone Extraction Assays

Target	Primer Name	Sequence (5'-3')	Amplicon Size	PCR Parameters	
ATPase subunit 8	Pig-F	gcctaaatctcccctcaatggta	212		94°C 2 min
(porcine mtDNA)	Pig-R	atgaaagaggcaaatagattttcg		35 cycles	94°C 30 sec
					53°C 1 min
					72°C 30 sec
					72°C 7 min

Individual Inhibitor Assays

Target	Primer Name	Sequence (5'-3')	Amplicon Size	PCR Parameters	
HVI	F15989	cccaaagctaagattctaat	421		94°C 2 min
(human mtDNA)	R16410	gaggatggtggtcaagggac		35 cycles	94°C 30 sec
					55°C 1 min
					72°C 1 min
					72°C 7 min

Includes primer sequences and PCR cycling parameters for the bone inhibition assay and individual inhibitor assays. Porcine primers were developed by Lahiff et al. (2000). Hypervariable Region I (HVI) primers are utilized by the Armed Forces DNA Identification Laboratory (AFDIL) for human identification (Edson et al. 2004).

PCR Conditions for Individual Inhibitor Assays

Assayed polymerases were also analyzed for their ability to amplify DNA in the presence of inhibitors thought to co-extract with DNA during skeletal sample extraction, including collagen, Ca^{++}, and humic acid. PCR reactions were prepared in 10 µL volumes using the parameters described above with the following substitutions: 1 µL of

20 μM forward primer 15989F, 1 μL of 20 μM reverse primer 16410R, 1 μL of 2 ng/μL human genomic DNA, 1 μL of 4 μg/μL BSA, 1 μL of inhibitor (see below), and water to a final reaction volume of 10 μL. The concentrations of inhibitors added to PCR reactions are summarized in Table 7. PCR reactions were cycled according to parameters listed in Table 6 and visualized as previously described. All reaction sets were performed in duplicate. PCR reactions were conducted with and without BSA. Type I collagen from calf skin (Sigma-Aldrich, product number C9791) was dissolved in a NaH_2PO_4 buffer (adjusted to pH = 3) to remove protein crosslinks, and neutralized with 5 N NaOH to pH 7.0. Collagen was diluted with water into the following stock concentrations: 1 μg/μL, 5 μg/μL, 10 μg/μL, 15 μg/μL, and 20 μg/μL. A blank of neutralized NaH_2PO_4 buffer was prepared for inclusion in positive controls to demonstrate that any material included in the PCR reactions due to the necessary acidification/neutralization step did not inhibit DNA amplification. This solution was substituted for 1 μL of water in the positive controls for each collagen-spiked PCR reaction set. Granular calcium chloride dihydrate (J. T. Baker) was dissolved in water to the following stock concentrations: 10 mM, 25 mM, 50 mM, 75 mM, and 100 mM. Powdered humic acid (Alfa Aesar) was dissolved in water to the following stock concentrations: 1 ng/μL, 10 ng/μL, 50 ng/μL, 100 ng/μL, and 1000 ng/μL.

Table 7: Concentrations of Inhibitors

Inhibitor Source	Supplier	Final Concentration in PCR				
		1	2	3	4	5
Collagen (Type I, calf skin)	Sigma	0.1 µg/µL	0.5 µg/µL	1 µg/µL	1.5 µg/µL	2 µg/µL
Calcium ($CaCl_2 \cdot 2\ H_2O$)	J. T. Baker	1 mM	2.5 mM	5 mM	7.5 mM	10 mM
Humic Acid	Alfa Aesar	0.1 ng/µL	1 ng/µL	5 ng/µL	10 ng/µL	100 ng/µL

Source and final concentration of collagen, calcium, and humic acid added to PCR reactions. Reactions were performed in duplicate, both with and without BSA.

Optimizing Polymerases for Bone Inhibition and Individual Inhibitor Assays

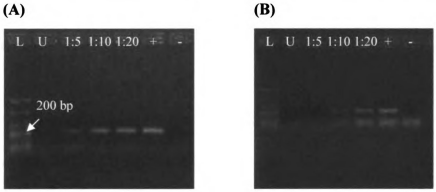
When possible, polymerases were assayed using manufacturer's recommended protocols, except that all PCR reactions were scaled to 10 µL volumes. All assayed polymerases except *Tfl* were able to amplify the 212 bp porcine amplicon. When attempting to amplify the 421 bp amplicon, *Tgo* and Pfx50™ required further optimization to generate a target band in positive controls. Pfx50™ activity was restored when dNTP concentrations were increased to 400 µM. Attempts to vary dNTPs, Mg^{++}, or polymerase concentrations in *Tgo* reactions did not yield positive amplification, nor did adding template DNA. *Tgo* activity was restored in reactions that contained 20 ng DNA, 300 µM dNTPs, 3.75 mM Mg^{++}, and 0.1 units polymerase, therefore these parameters were adopted for subsequent PCR.

RESULTS

Polymerase-Specific Inhibitor Resistance Assays—Ancient Bone Extractions

Polymerase amplification failure correlated with an increase in inhibitor concentration in reactions that were spiked with ancient bone extracts. Because PCR reactions were conducted using constant PCR cycling parameters and reagent concentrations (with exceptions; see *Materials and Methods*), some additional qualitative discrimination could be made between PCR products of differing intensities when visualized with ethidium bromide/UV illumination. Inhibition was witnessed as a gradual decline in band intensity or the absence of a band as the concentration of inhibitor source increased (Figure 4).

Figure 4: Relief of Inhibition with Dilution of Skeletal Inhibitory Elements



Typical results for PCR reactions when spiked with skeletal DNA extracts. (A) represents dilutions of extract 5010 amplified using AmpliTaq™ Gold. (B) represents dilutions of extract 213 amplified using HotMaster™ *Taq*. Lane "L" contains 1 µL of a New England Biolabs Low Molecular Weight Marker. Target amplicon is 212 bp. Lane "U" contains 5 µL of reactions spiked with undiluted bone extract, and lanes "1:5", "1:10", and "1:20" contain 5 µL of reactions that were spiked with respective extract dilutions. Lanes "+" and "-" contain 5 µL of positive and negative controls. Note the gradual decrease in band intensity as inhibitor concentration increased.

DNA Amplification in the Presence of Tibia Fragment 5010

The results of the polymerase-specific inhibitor resistance assay utilizing the 5010 extract are displayed in Table 8. The two unmodified *Taq* variants exhibited different levels of inhibition susceptibility, as the Promega-supplied product amplified DNA in the presence of a 1:5 inhibitor dilution, while the New England Biolabs *Taq* failed to amplify DNA even in the 1:20 dilution. The three hot-start *Taq* variants consistently amplified DNA in all dilutions, but not at full strength. *Tgo* polymerase was inhibited at the 1:10 dilution, while *Tth* and Pfx50™ amplified DNA in the undiluted extraction. Polymerases *Tfl* and *Tfi* failed to amplify in the presence of any dilution of the 5010 extract, and surprisingly also failed when no inhibitor was added to the porcine bone DNA. Positive

control PCR was conducted substituting human non-skeletal DNA for the porcine bone-derived DNA (with human HVI primers; see Table 6), whereupon successful amplification was observed for both enzymes.

Table 8: DNA Amplification Results with Tibia Fragment 5010

	PCR reactions containing 1 µL of skeletal inhibitors:			
DNA Polymerase	Undiluted	1:5 Dil.	1:10 Dil.	1:20 Dil.
Taq (New England Biolabs)	–/–	–/–	–/–	–/–
Taq (Promega)	–/–	–/+	+/+	+/+
AmpliTaq™ Gold (ABI)	–/–	+/+	+/+	+/+
HotMaster™ *Taq* (Eppendorf)	–/–	+/+	+/+	+/+
Ex Taq™ HS (Takara)	–/–	+/+	+/+	+/+
Tfl† (Promega)	–/–	–/–	–/–	–/–
Tfi† (Invitrogen)	–/–	–/–	–/–	–/–
Tth (Roche)	+/+	+/+	+/+	+/+
Tgo (Roche)	–/–	–/–	–/–	+/+
Pfx50™ (Invitrogen)	+/+	+/+	+/+	+/+

Inhibitory effects of tibia fragment 5010 on the DNA amplification efficiency of assayed polymerases. PCR reactions were spiked with 1 µL of undiluted 5010 extract or a 1:5, 1:10, or 1:20 dilution of the extract. "+" denotes visible targeted amplification after 35 cycles, while "–" denotes no visible targeted amplification after 35 cycles. All results are from two independent PCR reactions.
† Enzyme failed to amplify skeletally-derived DNA when no inhibitor was added, but amplified human, non-skeletal DNA when no inhibitor was added.

DNA Amplification in the Presence of Vertebra Fragment 213

Results of the polymerase-specific inhibitor resistance assay utilizing the 213 extract are displayed in Table 9. The extract appeared to be more inhibitory than the

5010 extract, requiring additional dilution before polymerase activity was restored. All enzymes except *Tth* were inhibited in the presence of the undiluted extraction, and all except *Tth* and *Ex Taq*™ HS were inhibited in the presence of the 1:5 dilution. AmpliTaq™ Gold and HotMaster™ *Taq* amplified DNA in the presence of the 1:10 dilution, while *Tgo* and *Tfi* were inhibited at the 1:10 dilution. Unmodified *Taq* variants and Pfx50™ were inhibited in all concentrations of the 213 extract. *Tfl* was unable to amplify skeletally-derived DNA in the presence or absence of the exogenous inhibitor.

Table 9: DNA Amplification Results with Vertebra Fragment 213

	PCR reactions containing 1 µL of skeletal inhibitors:			
DNA Polymerase	Undiluted	1:5 Dil.	1:10 Dil.	1:20 Dil.
Taq (New England Biolabs)	–/–	–/–	–/+	–/–
Taq (Promega)	–/–	–/–	–/–	–/–
AmpliTaq™ Gold (ABI)	–/–	–/–	+/+	+/+
HotMaster™ *Taq* (Eppendorf)	–/–	–/–	–/+	+/+
Ex Taq™ HS (Takara)	–/–	+/+	+/+	+/+
Tfl† (Promega)	–/–	–/–	–/–	–/–
Tfi (Invitrogen)	–/–	–/–	–/–	+/+
Tth (Roche)	–/+	+/+	+/+	+/+
Tgo (Roche)	–/–	–/–	–/–	–/+
Pfx50™ (Invitrogen)	–/–	–/–	–/–	–/–

Inhibitory effects of vertebra fragment 213 on the DNA amplification efficiency of assayed polymerases. PCR reactions were spiked with 1 µL of undiluted 213 extract or a 1:5, 1:10, or 1:20 dilution of 231 extract. "+" denotes visible targeted amplification after 35 cycles, while "–" denotes no visible targeted amplification after 35 cycles. All results are from two independent PCR reactions.
† Enzyme failed to amplify skeletally-derived DNA when no inhibitor was added, but amplified human, non-skeletal DNA when no inhibitor was added.

DNA Amplification in the Presence of Vertebra Fragment 213—BSA Included

When BSA was added to PCR reactions, most polymerases exhibited an improved amplification ability in the presence of higher inhibitor concentrations from the 213 extract (Table 10). All enzymes except *Taq* (New England Biolabs), *Tfl*, and Pfx50™ generated a visible target amplicon in the presence of undiluted 213 extract. BSA did not restore *Tfl* amplification in the presence or absence of the 213 extract.

Table 10: DNA Amplification Results with Vertebra Fragment 213—BSA Included

	PCR reactions containing 1 µL of skeletal inhibitors:			
DNA Polymerase	Undiluted	1:5 Dil.	1:10 Dil.	1:20 Dil.
Taq (New England Biolabs)	–	+	+	+
Taq (Promega)	+	+	+	+
AmpliTaq™ Gold (ABI)	+	+	+	+
HotMaster™ *Taq* (Eppendorf)	+	+	+	+
Ex Taq™ HS (Takara)	+	+	+	+
Tfl† (Promega)	–	–	–	–
Tfi (Invitrogen)	+	+	+	+
Tth (Roche)	+	+	+	+
Tgo (Roche)	+	+	+	+
Pfx50™ (Invitrogen)	–	+	+	+

Inhibitory effects of vertebra fragment 213 on the DNA amplification efficiency of assayed polymerases when 4 µg of BSA was added to each 10 µL reaction. PCR reactions were spiked with 1 µL of undiluted 213 extract or a 1:5, 1:10, or 1:20 dilution of 213 extract. "+" denotes visible targeted amplification after 35 cycles, while "–" denotes no visible targeted amplification after 35 cycles.
† Enzyme failed to amplify skeletally-derived DNA when no inhibitor was added, but amplified human, non-skeletal DNA when no inhibitor was added.

Polymerase Resistance to Collagen

The results of the inhibitor resistance assay that utilized type I collagen as an agent of PCR inhibition are displayed in Table 11. All polymerases successfully amplified DNA in the presence of 1 and 5 μg collagen in a 10 μL PCR volume. *Taq* (New England Biolabs) failed to amplify DNA in the presence of 10 μg collagen, while *Taq* (Promega), AmpliTaq™ Gold, *Tfl*, and *Tfi* were inhibited at 10 μg collagen in one replicate. *Tth* and *Tfl* were inhibited in one replicate in the presence of 15 μg collagen, and HotMaster™ *Taq* consistently amplified DNA at 15 μg collagen. All polymerases except *Tgo* were completely inhibited in the presence of 20 μg collagen. *Tgo* did not amplify 2 ng DNA, even when no inhibitor was added. When 20 ng DNA was added to PCR, *Tgo* amplified DNA in the presence of 20 μg collagen. The base-neutralized phosphate buffer used to solubilize collagen was incorporated into positive controls and did not inhibit any of the assayed polymerases.

Table 11: DNA Amplification Results with Type I Collagen

	PCR reactions containing collagen at:				
DNA Polymerase	**1 µg**	**5 µg**	**10 µg**	**15 µg**	**20 µg**
Taq (New England Biolabs)	+/+	+/+	–/–	–/–	–/–
Taq (Promega)	+/+	+/+	+/–	–/–	–/–
AmpliTaq™ Gold (ABI)	+/+	+/+	+/–	–/–	–/–
HotMaster™ *Taq* (Eppendorf)	+/+	+/+	+/+	+/+	–/–
Ex Taq™ HS (Takara)	+/+	+/+	+/+	–/–	–/–
Tfl (Promega)	+/+	+/+	+/–	+/–	–/–
Tfi (Invitrogen)	+/+	+/+	+/–	–/–	–/–
Tth (Roche)	+/+	+/+	+/+	+/–	–/–
Tgo† (Roche)	+/+	+/+	+/+	+/+	+/+
Pfx50™ (Invitrogen)	+/–	+/+	+/+	–/–	–/–

Inhibitory effects of type I collagen on the DNA amplification efficiency of assayed polymerases. PCR reactions of 10 µL volumes were spiked with 1, 5, 10, 15, or 20 µg collagen. "+" denotes visible targeted amplification after 35 cycles, while "–" denotes no visible targeted amplification after 35 cycles. All results are from two independent PCR reactions.
† PCR reactions contained 20 ng DNA instead of 2 ng DNA.

Polymerase Resistance to Collagen—BSA Included

The results of the BSA-facilitated inhibitor resistance assay that utilized type I collagen as an agent of PCR inhibition are displayed in Table 12. When BSA was added, polymerases consistently amplified in the same or higher concentrations of collagen versus respective reactions that lacked the adjuvant. All assayed polymerases successfully amplified in the presence of 1 and 5 µg collagen. *Taq* (New England Biolabs) was inhibited with 10 µg collagen. *Taq* (Promega) and AmpliTaq™ Gold were inhibited with 15 µg collagen. HotMaster™ *Taq*, *Ex Taq*™ HS, *Tth*, and Pfx50™ were

inhibited with 20 μg collagen. *Tfl* and *Tfi* successfully amplified DNA in the presence of 20 μg collagen. *Tgo* did not amplify 2 ng DNA, even when no inhibitor was added. When 20 ng DNA was added to PCR, *Tgo* amplified DNA in the presence of 20 μg collagen.

Table 12: DNA Amplification Results with Type I Collagen—BSA Included

	PCR reactions containing collagen at:				
DNA Polymerase	**1 μg**	**5 μg**	**10 μg**	**15 μg**	**20 μg**
Taq (New England Biolabs)	+/+	+/+	–/–	–/–	–/–
Taq (Promega)	+/+	+/+	+/–	–/–	–/–
AmpliTaq™ Gold (ABI)	+/+	+/+	+/–	–/–	–/–
HotMaster™ *Taq* (Eppendorf)	+/+	+/+	+/+	+/+	–/–
Ex Taq™ HS (Takara)	+/+	+/+	+/+	+/+	–/–
Tfl (Promega)	+/+	+/+	+/+	+/–	+/–
Tfi (Invitrogen)	+/+	+/+	+/+	+/+	+/–
Tth (Roche)	+/+	+/+	+/+	+/+	–/–
Tgo† (Roche)	+/–	+/–	+/+	+/+	+/–
Pfx50™ (Invitrogen)	+/–	+/+	+/+	+/–	–/–

Inhibitory effects of type I collagen on the DNA amplification efficiency of assayed polymerases when 4 μg of BSA was added. PCR reactions of 10 μL volumes were spiked with 1, 5, 10, 15, or 20 μg collagen. "+" denotes visible targeted amplification after 35 cycles, while "–" denotes no visible targeted amplification after 35 cycles. All results are from two independent PCR reactions.
† PCR reactions contained 20 ng DNA instead of 2 ng DNA.

Polymerase Resistance to Calcium

The results of the inhibitor resistance assay that utilized calcium as an agent of PCR inhibition are listed in Table 13. *Tth* was inhibited at 2.5 mM Ca^{++}, while *Taq* (New

England Biolabs and Promega), AmpliTaq™ Gold, *Tfl, Tfi,* and Pfx50™ were inhibited at 5 mM Ca^{++}. HotMaster™ *Taq* and *Ex Taq*™ HS were inhibited at 7.5 mM Ca^{++}. *Tgo* did not amplify 2 ng DNA, even when no inhibitor was added. When 20 ng DNA was added to PCR, *Tgo* amplified DNA in 7.5 mM Ca^{++}. All assayed polymerases were completely inhibited in 10 mM Ca^{++}.

Table 13: DNA Amplification Results with Calcium

	PCR reactions containing Ca^{++} at a reaction concentration of:				
DNA Polymerase	**1 mM**	**2.5 mM**	**5 mM**	**7.5 mM**	**10 mM**
Taq (New England Biolabs)	+/+	+/+	–/–	–/–	–/–
Taq (Promega)	+/+	+/+	–/–	–/–	–/–
AmpliTaq™ Gold (ABI)	+/+	+/+	–/–	–/–	–/–
HotMaster™ *Taq* (Eppendorf)	+/+	+/+	+/+	–/–	–/–
Ex Taq™ HS (Takara)	+/+	+/+	+/–	–/–	–/–
Tfl (Promega)	+/+	+/+	–/–	–/–	–/–
Tfi (Invitrogen)	+/+	+/+	–/–	–/–	–/–
Tth (Roche)	+/+	–/–	–/–	–/–	–/–
Tgo† (Roche)	+/+	+/+	+/+	+/+	–/–
Pfx50™ (Invitrogen)	+/+	+/–	–/–	–/–	–/–

Inhibitory effects of calcium on the DNA amplification efficiency of assayed polymerases. PCR reactions of 10 µL volumes were spiked with 1 µL of a 10, 25, 50, 75, or 100 mM Ca^{++} solution. "+" denotes visible targeted amplification after 35 cycles, while "–" denotes no visible targeted amplification after 35 cycles. All results are from two independent PCR reactions.
† PCR reactions contained 20 ng DNA instead of 2 ng DNA.

Polymerase Resistance to Calcium—BSA Included

The results of the BSA-facilitated inhibitor resistance assay that utilized calcium as an agent of PCR inhibition are listed in Table 14. When BSA was added, some polymerases amplified in higher concentrations of calcium versus respective reactions that lacked the adjuvant. HotMaster™ *Taq* showed the greatest improvement and generated a visible amplicon in 10 mM Ca^{++}. BSA also improved DNA amplification with *Tfl*, *Tfi*, and *Tth*, as the enzymes amplified DNA in 5 mM, 7.5 mM, and 2.5 mM Ca^{++}, respectively. *Taq* variants other than HotMaster™ *Taq*, as well as *Tgo* and Pfx50™, did not exhibit improved amplification when BSA was added.

Table 14: DNA Amplification Results with Calcium—BSA Included

DNA Polymerase	PCR reactions containing Ca^{++} at a reaction concentration of:				
	1 mM	**2.5 mM**	**5 mM**	**7.5 mM**	**10 mM**
Taq (New England Biolabs)	+/+	+/+	–/–	–/–	–/–
Taq (Promega)	+/+	+/+	–/–	–/–	–/–
AmpliTaq™ Gold (ABI)	+/+	+/–	–/–	–/–	–/–
HotMaster™ *Taq* (Eppendorf)	+/+	+/+	+/+	+/+	+/–
Ex Taq™ HS (Takara)	+/+	+/+	–/–	–/–	–/–
Tfl (Promega)	+/+	+/+	+/–	–/–	–/–
Tfi (Invitrogen)	+/+	+/+	+/+	+/–	–/–
Tth (Roche)	+/+	+/–	–/–	–/–	–/–
Tgo† (Roche)	+/–	+/+	+/+	+/–	–/–
Pfx50™ (Invitrogen)	+/–	+/–	–/–	–/–	–/–

Inhibitory effects of calcium on the DNA amplification efficiency of assayed polymerases when 4 µg of BSA was added. PCR reactions of 10 µL volumes were spiked with 1 µL of a 10, 25, 50, 75, or 100 mM Ca^{++} solution. "+" denotes visible targeted amplification after 35 cycles, while "–" denotes no visible targeted amplification after 35 cycles. All results are from two independent PCR reactions.
† PCR reactions contained 20 ng DNA instead of 2 ng DNA.

Polymerase Resistance to Humic Acid

The results of the inhibitor resistance assay that utilized humic acid as an agent of PCR inhibition are listed in Table 15. All assayed polymerases amplified DNA in the presence of 1 ng humic acid in a 10 µL PCR volume. *Taq* (Promega), HotMaster™ *Taq*, and *Tfl* and were inhibited at 10 ng humic acid. *Taq* (New England Biolabs), AmpliTaq™ Gold, *Ex Taq*™ HS, and *Tfi* were inhibited at 50 ng humic acid. *Tth* became inhibited at 100 ng humic acid, and Pfx50™ became inhibited at 1000 ng humic

acid. *Tgo* did not amplify 2 ng DNA, even when no inhibitor was added. When 20 ng DNA was added to PCR, *Tgo* was inhibited at 50 ng humic acid.

Table 15: DNA Amplification Results with Humic Acid

	PCR reactions containing humic acid at:				
DNA Polymerase	**1 ng**	**10 ng**	**50 ng**	**100 ng**	**1000 ng**
Taq (New England Biolabs)	+/+	+/–	–/–	–/–	–/–
Taq (Promega)	+/+	–/–	–/–	–/–	–/–
AmpliTaq™ Gold (ABI)	+/+	+/–	–/–	–/–	–/–
HotMaster™ *Taq* (Eppendorf)	+/+	–/–	–/–	–/–	–/–
Ex Taq™ HS (Takara)	+/+	+/+	–/–	–/–	–/–
Tfl (Promega)	+/+	–/–	–/–	–/–	–/–
Tfi (Invitrogen)	+/+	+/+	–/–	–/–	–/–
Tth (Roche)	+/+	+/+	+/+	–/–	–/–
Tgo† (Roche)	+/+	+/+	–/–	–/–	–/–
Pfx50™ (Invitrogen)	+/+	+/+	+/+	+/+	–/–

Inhibitory effects of humic acid on the DNA amplification efficiency of assayed polymerases. PCR reactions of 10 µL volumes were spiked with 1, 10, 50, 100, or 1000 ng humic acid. "+" denotes visible targeted amplification after 35 cycles, while "–"denotes no visible targeted amplification after 35 cycles. All results are from two independent PCR reactions.
† PCR reactions contained 20 ng DNA instead of 2 ng DNA.

Polymerase Resistance to Humic Acid—BSA Included

The results of the BSA-facilitated inhibitor resistance assay that utilized humic acid as an agent of PCR inhibition are listed in Table 16. Inhibition was consistently relieved with the addition of BSA. All polymerases amplified DNA in the presence of 100 ng humic acid, though *Tgo* required 20 ng DNA before amplification was observed.

All polymerases were inhibited in the presence of 1000 ng humic acid. For most of the polymerases tested, the addition of BSA permitted successful amplification in 10 to 100-fold more humic acid than without.

Table 16: DNA Amplification Results with Humic Acid—BSA Included

	PCR reactions containing humic acid at:				
DNA Polymerase	**1 ng**	**10 ng**	**50 ng**	**100 ng**	**1000 ng**
Taq (New England Biolabs)	+/+	+/+	+/+	+/+	–/–
Taq (Promega)	+/+	+/+	+/+	+/–	–/–
AmpliTaq™ Gold (ABI)	+/+	+/+	+/+	+/+	–/–
HotMaster™ *Taq* (Eppendorf)	+/+	+/+	+/+	+/+	–/–
Ex Taq™ HS (Takara)	+/+	+/+	+/+	+/+	–/–
Tfl (Promega)	+/+	+/+	+/+	+/+	–/–
Tfi (Invitrogen)	+/+	+/+	+/+	+/+	–/–
Tth (Roche)	+/+	+/+	+/+	+/+	–/–
Tgo† (Roche)	+/+	+/+	+/+	+/+	–/–
Pfx50™ (Invitrogen)	+/+	+/+	+/+	+/+	–/–

Inhibitory effects of humic acid on the DNA amplification efficiency of assayed polymerases when 4 µg of BSA was added. PCR reactions of 10 µL volumes were spiked with 1, 10, 50, 100, or 1000 ng humic acid. A "+" denotes visible targeted amplification after 35 cycles, while "–" denotes no visible targeted amplification after 35 cycles. All results are from two independent PCR reactions.
† PCR reactions contained 20 ng DNA instead of 2 ng DNA.

DISCUSSION

Variable Polymerase Resistance to Inhibitors in Bone Extracts

The purpose of this study was to investigate thermostable DNA polymerases for their ability to amplify DNA from skeletal remains. An ideal polymerase should generate high DNA yields from low copy number samples while exhibiting superior resistance to inhibitors likely to be present in skeletal DNA extracts. The most direct way to examine resistance among polymerases was to subject each to skeletal samples that have previously proven inhibitory to PCR. Human tibia fragment 5010 and vertebra fragment 213 represented some of the most challenging ancient skeletal samples to which our laboratory had access, as previous attempts to amplify DNA from them were unsuccessful due to apparent PCR inhibition, even after extensive organic extraction and Microcon® filtration (Mutolo 2006). These bones provided an excellent opportunity to examine the inhibitor sensitivity of assayed polymerases.

The assays that utilized bone as an inhibitor source were designed such that polymerase-specific inhibition (reviewed by Wilson 1997) would be the only explanation for failed DNA amplification. To accomplish this, it was necessary to rule out PCR failure simply resulting from a lack of template DNA; thus the inhibited human skeletal material was not used as the DNA source. Instead, template DNA was obtained from fresh porcine bone, and all PCR reactions contained the same quantity of target material, estimated at 60 ng by UV spectrophotometry. The use of porcine-specific PCR primers

meant that human DNA, either from the bones or from the investigator, would not be a source of contamination.

DNA amplification success among polymerases varied widely in the presence of bone extracts (before BSA was added), though there were some general trends in polymerase performance between both bones. Hot-start *Taq* variants and *Tth* fared better in high concentrations of the 5010 and 213 inhibitors (Tables 8 and 9), while *Taq* and *Tgo* had activity only at more dilute concentrations. Among the hot-start polymerases, *Ex Taq*™ HS was the least susceptible to the 213 extract, followed by AmpliTaq™ Gold and HotMaster™ *Taq*, respectively. *Tfl* and *Tfi* performed relatively poorly, amplifying DNA only in dilute concentrations of the 213 extract, and both enzymes failed to amplify DNA with any added 5010 extract. Additionally, *Tfl* and *Tfi* failed to amplify skeletally-derived porcine DNA, even in the absence of exogenous inhibitors. The reason for the latter finding is unclear; however, amplification failure might be due to the enzymes' susceptibility to even the small amount of (apparent) skeletal inhibitors present in the porcine extract. *Tfi* did amplify DNA in the presence of the most dilute 213 extract, but band intensity was extremely weak (data not shown), even in positive (porcine) controls, suggesting that inhibition was still occurring as a result of the porcine extract. Interestingly, BSA relieved inhibition due to the 213 extract for *Tfi* but not *Tfl*. When PCR assays were conducted using non-skeletal DNA as template, amplification was successful with both enzymes. Additionally, neither enzyme exhibited substantial susceptibility relative to the other polymerases in collagen, Ca^{++}, or humic acid-spiked reactions, and BSA relieved inhibition for both in the presence of individual inhibitor sources (discussed in detail below), indicating that collagen and Ca^{++} were not present in

the porcine extract in sufficient quantities for inhibition. Further, it is extremely unlikely that humic acid was present in the extract, as the porcine bone was not in contact with soil. Therefore, the specific inhibitor that led to DNA amplification failure with *Tfl* and *Tfi* remains unidentified. Nonetheless, the results are grounds to omit either enzyme from PCR reactions that would benefit from low susceptibility to skeletally-derived inhibitors.

Most of the assayed enzymes exhibited a decrease in amplification efficiency in the presence of the 213 extract relative to the 5010 extract. The cause of this variation is unknown; however, the two skeletal fragments are from different excavations and displayed different discoloration, so it is possible that they contained different inhibitors (or proportions of specific inhibitors) from the bone and/or soil. The brown discoloration of tibia fragment 5010 and its corresponding DNA extract is indicative of soil-derived humic acids (Stevenson 1982), which are thought to bind the polymerase directly (discussed in detail below). Vertebra fragment 213 and its extract had a reddish tint. The distinctive color of red soil is characteristic of iron content, primarily in the form of hematite (Fe_2O_3), magnetite (Fe_3O_4), or goethite (FeOOH) (Wang et al. 1993, Pai et al. 2003). Iron concentrations of 10 mM and higher have been shown to inhibit *Taq* (Kreader 1996, Abu Al-Soud and Rådström 2000), presumably by interfering with polymerase-Mg^{++} interactions. If humic substances and iron were both present in the 213 extract, then it could have inhibited PCR by multiple mechanisms, helping to explain the high inhibition observed with assayed polymerases. BSA relieves the effects of both inhibitors (Kreader 1996), which probably accounts for the low inhibitor susceptibility observed with extract-spiked *Tth* reactions (whose buffer contained BSA) versus polymerases that did not benefit from the adjuvant. The high inhibitor susceptibility of

Pfx50™ (which also had BSA in its buffer) to the 213 extract is puzzling, especially considering that no inhibition was observed with the 5010 extract, though this phenomenon further implies that inhibitors were affecting polymerase activity by multiple mechanisms.

PCR reactions with Pfx50™ differed from the other reactions in two major respects: amplification was conducted with the lowest amount of Mg^{++} relative to other polymerases (1.2 mM in the 1X commercial buffer), and Pfx50™, unlike the other assayed enzymes, is a chimeric construct. Low Mg^{++} may have contributed to the inhibition observed with the 213 extract (especially if some component were binding Mg^{++} or competing for the polymerase active site), a possibility that could be addressed in future studies by manipulating Mg^{++} levels. However, lack of available Mg^{++} did not appear to be the primary cause of inhibition, as PCR reactions utilizing *Tth* contained only 1.5 mM Mg^{++}, yet the enzyme was highly resistant to inhibitors in the 213 extract. It is also possible that the structural nature of the chimera contributed to inhibitor susceptibility. U.S. patent records indicate that Pfx50™ consists of *T. zilligi* DNA polymerase fused to a single-stranded DNA binding protein derived from *S. solfataricus*, though details such as domain organization remain proprietary (Lee et al. 2007). Enzyme processivity data for Pfx50™ are not available; however, archaeal family B polymerases are characteristically nonprocessive (reviewed by Perler et al. 1996). If an inhibitor inactivated the accessory protein, the enzyme would likely experience a decrease in processivity (and DNA amplification efficiency). This may explain why Pfx50™ suffered additional inhibition not experienced by polymerases that did not require the

support of a non-polymerase domain to amplify DNA at efficiencies necessary for typical PCR.

Variable Polymerase Resistance to Collagen, Calcium, and Humic Acid

To the author's knowledge, only one study (Scholz et al. 1998) has been published on the identification and quantification of collagenous material from skeletal DNA extractions, yet many cite the study as authoritative evidence that collagen is a primary PCR inhibitor in bone (e.g., Kalmár et al. 2000, Chelomina 2004, Chung et al. 2004, Kemp et al. 2006). In the study, 21 ancient (400–700 A.D.) skeletal samples were digested and extracted using the phenol-chloroform method. Eighteen of the twenty-one extractions contained a negatively-charged substance that fluoresced blue under UV illumination following agarose gel electrophoresis. Further, this fluorescence persisted after spin column filtration (30 kDa cutoff). DNA amplification failed for all samples containing the fluorescent substance, and PCR using contemporary DNA failed when spiked with the ancient DNA extracts. Amplification was restored for 15 of 21 samples when collagenase (a protease specific for collagen, see below) was substituted for proteinase K in the bone digestion step prior to DNA extraction. The fluorescent substance disappeared after 30 hours of collagenase treatment. The authors demonstrated that contemporary type I collagen also fluoresced blue under UV light, and inhibited PCR. Because of this, they surmised that degraded collagen was co-extracting with the skeletal DNA and inhibiting PCR.

The effects of collagenase observed by Scholz et al. (1998) raise some questions regarding the possible mechanisms of collagen inhibition in skeletal remains. Collagenase is a highly specific protease that cleaves at a single bond on each peptide chain of the collagen triple helix to initiate protein degradation *in vivo* (reviewed by Chung et al. 2004). The apparent relief from collagen inhibition with collagenase (Scholz et al. 1998) initially suggests that the inhibitory effects of collagen are reduced by cleaving the triple helix (or denatured peptide chains) into large pieces. Unfortunately, the authors failed to mention the commercial source of collagenase used in the study, which makes interpretation of the results difficult, as many "crude" collagenase products are actually mixtures of several proteases that work together to degrade collagen into small peptides (Sigma-Aldrich commercial material). Collagen may have been degraded sufficiently to be removed by spin column filtration, but this was not investigated. Further, the authors did not attempt to relieve PCR inhibition by applying collagenase to the ancient DNA extracts themselves, so it is not certain that collagen remnants were in fact co-extracting with DNA. However, the DNA extracts contained inhibitors that were only neutralized when the bone samples were treated with collagenase, so it is possible that collagen is capable of persisting through phenol-chloroform DNA extractions.

The inhibitory potential of skeletally-derived collagen may depend on its state of degradation. Each peptide chain of the type I collagen triple helix consists primarily of non-polar amino acids that follow a regular Gly-X-Y tripeptide pattern, with X and Y often occurring as proline and hydroxyproline, respectively (Dalgleish 1997). Intact type I collagen is water-insoluble and should be removed during phenol-chloroform

extraction. However, less frequent, highly polar tripeptide sequences also exist in type I collagen, (e.g., Gly-Glu-Hyp, Gly-Ala-Arg; Dölz and Heidemann 1986), which could be sufficiently soluble in the aqueous phase of phenol-chloroform extractions to co-extract with DNA if collagen in skeletal material were degraded to smaller units. In this case, collagen fragments might bind polymerases at key amino acid residues, preventing the molecular interactions required for DNA synthesis.

Peptide binding need not take place within the polymerase active site to inhibit PCR, but could occur at finger or thumb domains, leading to decreased processivity as template-polymerase contacts are disrupted. Melanin, a recognized PCR inhibitor, appears to interfere with DNA amplification via a similar mechanism. Eckhart et al. (2000) demonstrated that PCR reactions of larger amplicons were more susceptible than smaller amplicons to inhibition by melanin, given identical PCR parameters, suggesting that the inhibitor reduced enzyme extension rate or processivity. Further, the addition of BSA to melanin-spiked samples restored DNA amplification, indicating that the adjuvant was providing an alternative binding substrate and relieving polymerase-specific inhibition. As polymerase species vary in regards to domain structure, some enzymes might be more vulnerable to inhibition by highly charged peptides. It would be interesting to test this idea by spiking PCR with short, synthesized peptides that resemble the collagen sequence. If variable inhibitor resistances were observed among polymerases, the experiment would help to identify those best suited for amplifying DNA from aged skeletal samples where collagen degradation is more likely.

In the current study, *Taq* variants exhibited different susceptibilities to type I collagen, but all amplified DNA in at least five times the previously reported minimum

inhibitory concentration for *Taq* (0.08 μg/μL collagen; Kim et al. 2000). *Taq* from New England Biolabs was the most susceptible to inhibition (Table 11), as no amplification was observed in the presence of 1 μg/μL collagen, while *Taq* (Promega), AmpliTaq™ Gold, and *Ex Taq*™ HS became inhibited at 1.5 μg/μL collagen. HotMaster™ *Taq* exhibited the lowest collagen inhibition susceptibility of the *Taq* variants, not being seen at concentrations less than 2 μg/μL. HotMaster™ *Taq* might owe its comparatively higher resistance to structural modifications engineered into the enzyme; it is advertised as an altered form of native *Taq* (Eppendorf commercial literature). Unfortunately, any details of these modifications are proprietary. Attempts to contact the manufacturer yielded no additional information; the product rights were sold from Eppendorf to 5Prime in April of 2007, and technical documentation is not yet available for existing technical staff (D. Reed, 5Prime technical support, personal communication). It is noteworthy that HotMaster™ *Taq* was inhibited by whole bone extracts (Tables 8 and 9), indicating that collagen was not a major source of inhibition in those samples.

Most non-*Taq* polymerases exhibited collagen susceptibility similar to hot-start *Taq* variants. *Tgo* was an exception, and was not inhibited at the most concentrated level of collagen (2 μg/μL) when attempting to amplify 20 ng of template DNA (Table 11). However, the enzyme failed to amplify 2 ng of DNA (the assay standard) in all instances, including positive controls. To investigate whether increased template DNA resulted in *Tgo*'s low inhibitor susceptibility, a collagen-stressed PCR replicate was conducted with *Ex Taq*™ HS using 20 ng DNA instead of 2 ng. Interestingly, no additional inhibition relief was observed (data not shown), suggesting that *Tgo* is indeed resistant to the effects of collagen, given that sufficient template is available for amplification. This is likely

due, at least in part, to the high concentration of Mg^{++} in *Tgo* reactions that resulted from PCR optimization (3.75 mM versus 2.5 mM for most polymerases; see *Materials and Methods*). Kim et al. (2000) observed that PCR inhibition by collagen could be partially reversed with the addition of Mg^{++}. Taken together, these observations suggest that collagen molecules sequester Mg^{++} or inhibit the polymerase by some other mechanism that can be reversed by increasing enzyme access to Mg^{++}. Given this, it is possible that other polymerases would have amplified DNA in higher collagen concentrations with the addition of more Mg^{++}, though this was not explored in the current research. Increasing Mg^{++} in PCR might help to offset the effects of an inhibitory substance, but it is not itself an ideal solution to overcoming PCR inhibition, as excessive Mg^{++} can reduce PCR sensitivity, resulting in non-specific amplification (e.g., Kim et al. 2000), inhibiting the reaction at sufficiently high concentrations (>5 mM, depending on polymerase; Abu al-Soud and Rådström 1998).

The next substance examined, Ca^{++}, has been reported in food science research (e.g., Bickley et al. 1996, Abu al-Soud and Rådström 1998) and clinical studies (e.g., Thomas et al. 1999) as a PCR inhibitor. While calcium has not been definitively reported as a PCR inhibitor in skeletal DNA extractions, it is a major component of hydroxyapatite, and Opel (2006) speculated that it could persist through DNA extraction and inhibit PCR. DNA has been shown to bind Ca^{++} *in vitro* at an average of 1 ion for every 40 nucleotides (Dobi and Agoston 1998), providing a theoretical mechanism for Ca^{++} presence in skeletal DNA extractions, even following spin column filtration. Dobi and Agoston (1998) reported that EDTA washings only partially disrupted these interactions, supporting the possibility of Ca^{++} remaining in skeletal DNA extractions.

Bickley et al. (1996) demonstrated that PCR reactions utilizing *Taq* and 1.5 mM Mg^{++} were inhibited when detecting *L. monocytogenes* in the presence of 3 mM Ca^{++}, and suggested that the different ions were competitively binding the polymerase active site. Their theory is consistent with the results in the current study, as the range of $[Mg^{++}]$ in Ca^{++}-stressed PCR reactions was 1.25–3.75 mM, and most instances of PCR inhibition were observed with Ca^{++} concentrations higher than 2.5 mM (Table 13). *Tgo* amplified DNA at higher Ca^{++} concentrations than did other polymerases, possibly because at least 50% more Mg^{++} was present as a result of PCR optimization. *Tth* and Pfx50™ PCR buffers contained relatively low concentrations of Mg^{++} (1.5 and 1.2 mM, respectively), and both proved to be the most susceptible to Ca^{++} inhibition. The mechanism of Ca^{++} inhibition proposed by Bickley et al. (1996), as well as the data presented here, indicate that DNA amplification is largely influenced by the Mg^{++}/Ca^{++} ratio in solution. Other factors that contribute to low inhibitor susceptibility might include polymerase affinity for Mg^{++} versus Ca^{++}, as well as PCR buffer components that are capable of absorbing Ca^{++}. The latter could explain why HotMaster™ *Taq* was less susceptible than other *Taq* variants to Ca^{++} (despite equivalent amounts of Mg^{++}), as the HotMaster™ buffer contains a proprietary molecule that acts as a weak chelator for excess Mg^{++}, purportedly "self-adjusting" Mg^{++} concentrations (Halley and Prezioso 2007). The molecule may bind Ca^{++} as well, which would account for low susceptibility to Ca^{++} inhibition, especially if the chelator exhibited a preferential affinity for Ca^{++}. When BSA was added, Ca^{++} inhibition was further relieved, which is consistent with the chelator theory, as additional Ca^{++} neutralizers in solution would assist in restoring DNA amplification. *Tfi*'s notable improvement with BSA may be due to a similar additive, as the buffer

includes "proprietary stabilizers". It would be interesting to apply the HotMaster™ and *Tfi* buffers to Ca^{++}-inhibited PCR using *Taq* from a relatively inhibitor-susceptible commercial source. If the alleviatory effects were buffer-related, one would expect the new polymerase to become more inhibitor-resistant. Identified buffers could then be used whenever Ca^{++} inhibition was suspected.

PCR inhibition by phenolic compounds such as humic acid is well documented (e.g., Tsai 1992, Tebbe and Vahjen 1993, Tuross 1994, Kreader 1996, Cipollaro et al. 1999, Kolman and Tuross 2000). Humic substances denature biological molecules by bonding to N-substituted amides, and oxidized humic acids form quinones that covalently bond to enzymes (Young et al. 1993). It is thought that humic acids from soil (Tuross 1994, Cipollaro et al.1999) or water (Kreader 1996) persist through DNA extraction and purification, resulting in PCR inhibition. The molecular structures of humic substances have proven difficult to characterize, but are understood to be 600–10,000 Da heterogeneous molecules that associate via weak dispersive forces (Yamada et al. 2000, Piccolo and Spiteller 2003) to form macromolecular complexes 100 kDa and larger (Yamada et al. 2000). This means that individual humic molecules are small enough to pass through standard Microcon® filters (30 or 100 kDa); therefore, they either remain associated in large complexes or bind to DNA itself in order to persist through sample purification via spin column filtration. Humic substances can present in skeletal DNA extracts as a result of soil that adheres to the surface of bones after excavation, which must be removed before DNA isolation. Bone also appears to absorb humic substances (or other soil-derived inhibitors), as PCR inhibition can occur even if the outer bone surface is discarded and only underlying material is used for DNA extraction (e.g.,

Mutolo 2006). It is unclear to what degree humic substances accumulate in bone over time, but given their ubiquity in soil, they remain the most likely candidates for PCR inhibitors in ancient skeletal samples.

PCR was highly inhibited by humic acid in all reactions without BSA, indicating that humic acid affects all polymerases by some general mechanism. *Tth* and Pfx50™ include BSA in their commercial buffers, and exhibited the lowest susceptibilities to humic acid (Table 15). When BSA was included in reactions with the other polymerases, humic acid inhibition was similarly relieved (Table 16). The pattern of polymerase behavior in the presence of humic acids is quite similar to that observed with the 213 skeletal extraction (Tables 9 and 10), in that inhibition occurred with most polymerases (the exceptions being *Tth* and Pfx50™), and BSA was nearly always effective at neutralizing the inhibitors. This suggests that the primary inhibitors in the bone fragments were indeed humic substances. Soil-derived phenolic compounds other than humic acid, such as fulvic and tannic acids, were not investigated in this study; however, it is likely that they inhibit PCR by similar mechanisms (i.e., covalently bonding the polymerase), as the inclusion of BSA allows at least 100-fold more humic, fulvic, or tannic acids to be accommodated in PCR (Kreader 1996).

Possible Mechanisms of Inhibition Relief with BSA

When BSA was added to PCR reactions containing the inhibitors from the 213 extract, DNA amplification improved for most polymerases (Table 10); all enzymes except *Taq* (New England Biolabs), *Tfl,* and Pfx50™ amplified DNA in the presence of

the original, undiluted extract. BSA has long been utilized as a PCR adjuvant (e.g., Höss et al. 1992), enhancing DNA amplification by relieving inhibitory effects of various substrates, and appears quite effective against certain skeletally-derived PCR inhibitors (particularly humic substances). However, BSA's mechanisms for attenuating the effects of various inhibitors are poorly understood (discussed in detail below).

Replicate amplification reactions were performed with and without BSA in an attempt to elucidate mechanisms of inhibition by skeletally-derived substances. BSA markedly improved DNA amplification for all polymerases in humic acid-spiked PCR; however, in collagen or Ca^{++}-spiked reactions, BSA only improved DNA amplification with certain polymerases (see below). Kim et al. (2000) reported that BSA did not relieve collagen inhibition when using *Taq*. This is consistent with the results of the current study, as BSA did not improve DNA amplification in collagen-spiked reactions for either of the unmodified *Taq*s, nor did it assist two of the three hot-start variants (Tables 11 and 12). *Ex Taq*™ HS, *Tfl, Tfi*, and Pfx50™ were less inhibited by collagen when BSA was added, while HotMaster™ *Taq*, *Tfl*, *Tfi*, and *Tth* were less inhibited by Ca^{++} when BSA was present (Tables 13 and 14). Initial inhibitor susceptibility might be attributed to some critical buffer component that was compromised with the addition of collagen or Ca^{++} and only relieved when BSA was added. As an example, *Ex Taq*™ HS and HotMaster™ *Taq* were the only *Taq* variants wherein amplification improved with the addition of BSA when in the presence of collagen and Ca^{++}, respectively. PCR buffer formulations suspected of contributing to inhibitor susceptibility could be confirmed (and subsequently avoided) by conducting similar experiments utilizing the buffer(s) in question and *Taq* from another, relatively inhibitor-resistant product.

It is probable that enzyme variability contributed to the differential relief observed after BSA was added. For example, *Taq* differs from *Tfl, Tth*, and Pfx50™ in that it is faster at synthesizing DNA, extending at a rate of up to 150 nucleotides per second, while *Tfl*, *Tth*, and Pfx50™ extend DNA at maximal rates of 25, 67, and 67 nucleotides per second, respectively (Table 1, Invitrogen commercial literature). If collagen or Ca^{++} disrupt PCR component interactions (making successful interactions less frequent), and PCR parameters are kept constant (e.g., a 45 second extension step), then "slow" polymerases are less likely than "fast" polymerases (in this case, *Taq*) to achieve complete synthesis of a target amplicon. Adding BSA would relieve inhibition, promoting full amplicon extension with the slower polymerases. Indeed, *Tfl, Tth,* and Pfx50™ were less inhibited by collagen or Ca^{++} after BSA was added, while DNA amplification with the faster *Taq* variants generally did not improve.

Extension rate variability alone does not explain why BSA failed to improve DNA amplification with *Taq* at high inhibitor concentrations. However, multiple performance characteristics (e.g., processivity, inhibitor affinity) likely contributed to the variable improvement among polymerases. The roles of extension rate and processivity on enzyme inhibitor susceptibility could be explored with a PCR inhibitor assay that includes PCR stage duration as a variable. If the rate of DNA synthesis influences the degree of inhibition, one would expect DNA amplification to be restored with a longer extension step. It also follows that a polymerase with high extension rate and processivity, such as KOD1 (Table 1), would be especially inhibitor-resistant. If KOD1 was equally or more inhibitor-susceptible than slower polymerases, then low extension rate and processivity could be excluded as primary contributors of inhibition.

To the author's knowledge, there has been no evidence presented to suggest that BSA interacts with polymerases directly, so the adjuvant most likely interacts with inhibitors, promotes PCR component interactions (e.g., via macromolecular crowding, discussed below), or both. Therefore, it is logical that BSA should confer its alleviatory effects on inhibited PCR independent of the polymerase species. In the current study, there was only a single instance (*Ex Taq*™ HS, Ca^{++}; Table 14) where a PCR band was generated at a higher inhibitor concentration when BSA was omitted than when it was present. Considering this, it is likely that BSA was conferring its effects with all polymerases, as was apparent with humic acid-spiked reactions (Tables 15 and 16). While improvements in PCR success were not obvious with all collagen and Ca^{++}-inhibited reactions, it is possible that BSA was less effective at relieving inhibition caused by the substances (relative to humic acid) and the assay design was insufficiently sensitive to demonstrate subtle improvements. If BSA improved DNA amplification only slightly, then the effect would not be observed unless a particular polymerase was already nearly capable of generating a visible target band (under UV illumination) at a particular inhibitor concentration. To confirm whether BSA was improving DNA amplification with all polymerases, a similar PCR inhibition assay could be designed using quantitative PCR, which estimates DNA copy number with much higher resolution than simple PCR band intensity measurements.

Considering that BSA alleviates inhibition caused by many different substances (e.g., ions, proteins, dyes, polymeric acids), it seems apparent that the adjuvant enhances PCR via multiple mechanisms. Kreader (1996) first suggested that BSA provides an alternative binding substrate for polymerase-targeting inhibitors. This would explain the

DNA amplification improvement observed in reactions spiked with humic acid, as BSA provided new binding sites for the inhibitor, decreasing the occurrence of polymerase-inhibitor interactions. *In vivo*, the major function of serum albumin is to bind and transport fatty acids in plasma circulation (Spector 1975, Carter and Ho 1994, Peters 1995); however, it has the capacity to bind some ions as well. For example, BSA can bind free Ca^{++} *in vitro* at a maximum of eight ions per albumin molecule at pH 7.4 (Carr 1953). PCR inhibition by Fe^{++} is alleviated with the addition of BSA (Kreader 1996), presumably via a similar mechanism. It is interesting that the addition of the adjuvant does not appear to bind Mg^{++} at levels that result in the loss of polymerase activity. Human serum albumin shares approximately 76% sequence identity with BSA (Carter and Ho 1994) and has been shown to exhibit a stronger affinity for Ca^{++} than Mg^{++} at pH 8.0 (Irons and Perkins 1962); therefore, BSA may be preferentially binding Ca^{++} in spiked PCR reactions.

It is also possible that BSA increases PCR success indirectly via macromolecular crowding. Zimmerman and Harrison (1987) demonstrated that saturation of PCR reactions with large, relatively inert molecules such as polyethylene glycols increased the amplification efficiency of *E. coli* Pol I and T7 DNA polymerase in the presence of inhibitory K^{+} concentrations. This was attributed to increased DNA-polymerase interactions, which in turn increased the rate of DNA polymerization. *In vitro* macromolecular crowding conditions also enhance ligase activity (e.g., Zimmerman and Pheiffer 1983). BSA has been utilized as a crowding agent to study protein folding and molecular interactions in an environment that approaches *in vivo* conditions (reviewed by Chebotareva et al. 2004). BSA may then improve inhibited PCR reactions (where some

required component of PCR is sequestered or inactivated) by reducing the free space in the reaction, raising the effective concentration of various PCR components. Improvements in DNA amplification among polymerases would vary based on an enzyme's capability to synthesize DNA at the adjusted component concentrations.

Kreader (1996) found that the effects of BSA on inhibited PCR were concentration-limited. In her study, 400 ng/μL was determined to be the optimal concentration of BSA to relieve PCR inhibition from humic acid, as more BSA did not additively relieve inhibition with increased inhibitor. Experiments to vary BSA concentration were not conducted in the current study, but 400 ng/μL consistently improved DNA amplification in the presence of inhibitory bone extracts as well as humic acid. The possibility of excessive BSA inhibiting PCR has not been investigated, though Kreader (1996) reported no PCR inhibition by BSA using concentrations as high as 3 μg/μL. Two of the PCR buffers used in the current study contained BSA; however, the concentration of BSA in the 1X *Tth* buffer was only 50 ng/μL (the BSA concentration for Pfx50™ being proprietary), while PCR amplification efficiency was consistently improved for both enzymes when extra BSA was added. It is probable that BSA was originally included in these buffers for the purpose of relieving PCR inhibition; however, the concentration could be raised considerably for maximum effectiveness.

Special Properties of Polymerases and Their Relationship to Overcoming PCR Inhibition

Polymerase inhibitor susceptibility may be intrinsically tied to variability in the different enzymes' amino acid sequence. While domain structure is highly conserved

among polymerase families (Filée et al. 2002), single amino acid residues can play large roles in catalytic activity and substrate specificity (Loh and Loeb 2005). Researchers have altered key residues within *Taq* to develop mutants for specific applications (e.g., sequencing or high-mutation PCR; reviewed by Loh and Loeb 2005). For example, Tabor and Richardson (1995) found that mutating a single amino acid residue in *Taq* resulted in a variant that incorporated ddNTPs up to 8000 times more efficiently than native *Taq,* and the substitution is now included in polymerases designed for sequencing applications, such as Thermo Sequenase™ from USB and AmpliTaq™ FS from Applied Biosystems (Parker et al. 1996, Vander Horn et al. 1997). Attempts to engineer inhibition resistance into thermostable polymerases via point mutations or other modifications that alter substrate specificity have not been conducted; however, it is an enticing concept that could be revisited as our understanding of polymerase-specific inhibition mechanisms grows, and the notion of inhibitor-resistant "designer" polymerases seems feasible. Existing polymerases could be mutated to minimize or eliminate inhibitor susceptibility, and modification strategies would depend on the mechanism of inhibitor attack. For example, if an inhibitor interacted with charged amino acids, then the course of action might be to introduce neutral amino acid substitutions. If the inhibitor target was specific and critical for function, (e.g., the active site), it would be desirable to increase the stringency of the target for appropriate PCR interactions, possibly through targeted point mutations or domain swapping (see *Introduction*). One might expect a trade-off between replicative efficiency and any inhibitor resistance gained by mutation, although a less efficient, highly inhibitor-

resistant polymerase would still be preferable to an efficient, inhibitor-susceptible version when amplifying DNA from challenging skeletal samples.

The results of this study suggest that the various polymerases were primarily responsible for differential resistances to skeletally-derived inhibitors, much like *Tth*'s resistance to phenol (Katcher and Schwartz 1994). However, is also apparent that PCR buffers can play as great a role (or greater) in PCR inhibitor resistance. In a recent study (Rohland and Hofreiter 2007), eleven commercially available polymerase products (containing either *Taq* or *Taq* plus a proofreading enzyme) were compared for relative PCR success when attempting to amplify DNA from an ancient cave bear, which was diluted to the point that permitted AmpliTaq™ Gold to amplify DNA in approximately 50% of the attempts. PCR success ranged from 0–72% among polymerases, even though the enzymes all originated from *T. aquaticus*. AmpliTaq™ Gold and HotMaster™ *Taq* were enzymes common to that study and the current research. In that study, HotMaster™ *Taq* performed poorly compared to AmpliTaq™ Gold (28% versus 67% success rate, respectively), whereas in the current study, both enzymes were similarly inhibited by the 213 and 5010 skeletal extracts and humic acid, while HotMaster™ *Taq* was less susceptible to collagen and Ca^{++} inhibition. It is unclear why the relative inhibition susceptibilities of the two polymerases appear inconsistent between studies, though the inhibitory nature of the samples may have been quite different, given their environmental histories. Continued testing of different commercial polymerase products on challenging skeletal samples, as carried out by Rohland and Hofreiter (2007) and in this study, may prove to be the most practical method of identifying which mixtures reliably overcome PCR inhibition.

Future Research in Characterizing and Overcoming PCR Inhibition

The most common methods to overcome PCR inhibition involve the removal of inhibitors from target DNA. Sample purification is effective when DNA copy number is sufficiently high, but the loss of target DNA that can occur with each cleanup step poses a fundamental problem with low copy samples. Utilizing polymerases that are resistant to PCR inhibitors is a promising alternative to sample purification that does not involve the sacrifice of DNA, but will require a more detailed analysis of PCR inhibitor mechanisms. To investigate these, it would be helpful to determine which molecules are the most prevalent (or potent) inhibitors. Chemical analysis of skeletal DNA extracts (such as mass spectrometry or elemental analysis) would help explain the variable inhibition observed with skeletal samples of differing age, states of degradation, and taphonomic histories. For example, PCR inhibition has been reported with buried (e.g., Tuross 1994), non-buried (e.g., Scholz et al. 1998), and sea-soaked bones (e.g., Sørensen et al. 2003), yet it is not certain what substances were responsible for failed DNA amplification. Their identification might prove useful in future analyses that examine polymerases for their capacity to amplify DNA from inhibitor-rich samples.

It is likely that differences in soil type influence the degree of PCR inhibition. The amount and composition of humic substances in soil differ geographically as a result of native fauna and flora (Kononova 1966, Schnitzer 1982, Stevenson 1982, Swift 1996), suggesting that some soils are more inhibitory than others. Further, it is doubtful that humic substances are the only soil-derived molecules affecting PCR success. Chemical

analysis could be used to identify other soil-derived inhibitors. Such research would also show which DNA extraction methods most effectively remove inhibitors from DNA.

It would be interesting to investigate the degree to which PCR inhibition affects the analysis of DNA from different bone types. The current perception of skeletal DNA degradation is that highly compact bones (e.g., femora) are more reliable sources of DNA than spongy bones (e.g., ribs and vertebrae), presumably because DNA in compact bone is better protected from environmental weathering. This is particularly evident when contrasting PCR success among different bone types from individual burials (e.g., Misner 2004, Mutolo 2006), wherein femora were better sources of DNA than corresponding ribs, pelves, or crania. However, the range of PCR failure observed among different bone types may be a function of variable inhibitor accumulation (likewise due to bone density) rather than insufficient template. This indicates that DNA analysis is less successful with spongy bone relative to compact bone because *Taq* is more susceptible to the accumulated inhibitors. If this is the case, amplification should be possible with the spongy bone DNA extracts after inhibitors are removed. However, one might expect purification methods (which also remove DNA) to be ineffective at restoring amplification for the inhibitor-rich samples if they are already low in DNA copy number (a likely scenario for aged skeletal remains), and the application of an inhibitor-resistant polymerase may be a better way to neutralize the effects of PCR inhibitors in such instances.

CONCLUSIONS

Thermostable polymerases selected for DNA amplification should be chosen on the basis of enzyme characteristics that best suit the desired application. PCR inhibitor resistance is an important property of polymerases, as successful DNA amplification from biological samples (particularly forensic) is often impossible without minimizing the effects of PCR inhibitors (Akane et al. 1994, Kontanis and Reed 2006). High PCR inhibitor resistance is especially desirable when amplifying DNA from degraded samples where DNA copy number is too low for extensive sample purification.

The results of this study indicate that BSA should be included in all PCR amplifications of skeletally derived-DNA. Polymerase products that are prepackaged with BSA in the PCR buffers are more suitable for overcoming skeletally-derived inhibitors relative to products that lack the adjuvant. However, further addition of BSA (at least 400 ng/μL) should be considered for optimal relief from inhibitors.

To minimize PCR inhibition due to components from skeletal extracts, the use of an especially inhibitor-resistant polymerase is advised. *Tfl, Tfi, Tgo,* and Pfx50™ were found to be susceptible to skeletal inhibitors and are not good candidates for skeletal DNA analyses. Hot-start *Taq* variants are recommended over unmodified *Taq,* and the results of this study indicate that *Ex Taq*™ HS exhibits higher inhibitor resistance than AmpliTaq™ Gold and HotMaster™ *Taq*. *Tth* consistently outperformed all other polymerases in high concentrations of PCR inhibitors from skeletal extracts. Therefore, of the enzymes considered, *Ex Taq*™ HS and *Tth* are recommended for the amplification of DNA from skeletal material, especially if PCR inhibitors are likely to be present.

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