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Comment on “DNA from Pre-Clovis Human Coprolites in Oregon, North America”

Hendrik Poinar,¹ Stuart Fiedel,² Christine E. King,¹ Alison M. Devault,¹ Kirsti Bos,¹ Melanie Kuch,¹ Regis Debruyne¹

Gilbert *et al.* (Reports, 9 May 2008, p. 786) analyzed DNA from radiocarbon-dated paleofecal remains from Paisley Cave, Oregon, which ostensibly demonstrate a human presence in North America predating the well-established Clovis complex. We question the authenticity of their DNA results and argue that in the absence of intact stratigraphy and diagnostic artifacts, and in view of carbon isotope anomalies, the radiocarbon dates of the oldest specimens are unreliable.

Gilbert *et al.* (1) interpreted data retrieved from three paleofecal samples from Paisley Cave, Oregon, dating from more than 12,000 years before the present (yr B.P.) [14,000 calendar (cal) yr B.P.], as proof of

¹McMaster Ancient DNA Centre, Department of Anthropology, McMaster University, 1280 Main Street West, Hamilton, ON L8S 4L9 Canada. ²Louis Berger Group, 801 East Main Street, Suite 500, Richmond, VA 23219, USA.

*To whom correspondence should be addressed: E-mail: poinarh@mcmaster.ca

a pre-Clovis occupation of North America. The logic of their argument rests on six main points: (i) each coprolite sample contains only one of the five known Native American mitochondrial DNA (mtDNA) haplogroups, (ii) leaching of DNA from overlying sediments has been excluded, (iii) canid DNA in the feces does not preclude the authenticity of Native American human sequences, (iv) none of the team members working with the samples possess Native American mtDNA haplotypes, (v) the presence of human

antigens in the feces supports their human origin, and (vi) obsidian hydration dates for “associated” artifacts and radiocarbon dating of the feces support a pre-Clovis age. Here, we successively address the relevance of these points.

Gilbert *et al.*'s first experiment, a multiplex single-nucleotide polymorphism (SNP) assay, was crucial to their study, as all downstream experiments were based on its results (Fig. 1). This assay targeted one conserved SNP from each of the five known Native American haplogroups (A, B, C, D, and X) (2) and three non-Native American haplogroups (M, N, and R). A central tenet of their hypothesis requires the presence of only one Native American sequence in each fecal sample, because any additional ones would open the possibility of leaching from younger strata. By pooling all eight primer pairs into a single reaction, and neglecting to provide sensitivity or optimization data for the assay, Gilbert *et al.* appear to have ignored any amplification biases introduced through competing amplicon sizes [71 to 105 base pairs (bp)] and sequence compositions, which can result in decreased sensitivity and efficiency of each individual amplification (3, 4). Although the detection of the haplogroup A SNP or the nondiagnostic 9-bp deletion was confirmed in at least one independent laboratory (Uppsala or Leipzig), inconsistencies in the purported semiquantitative

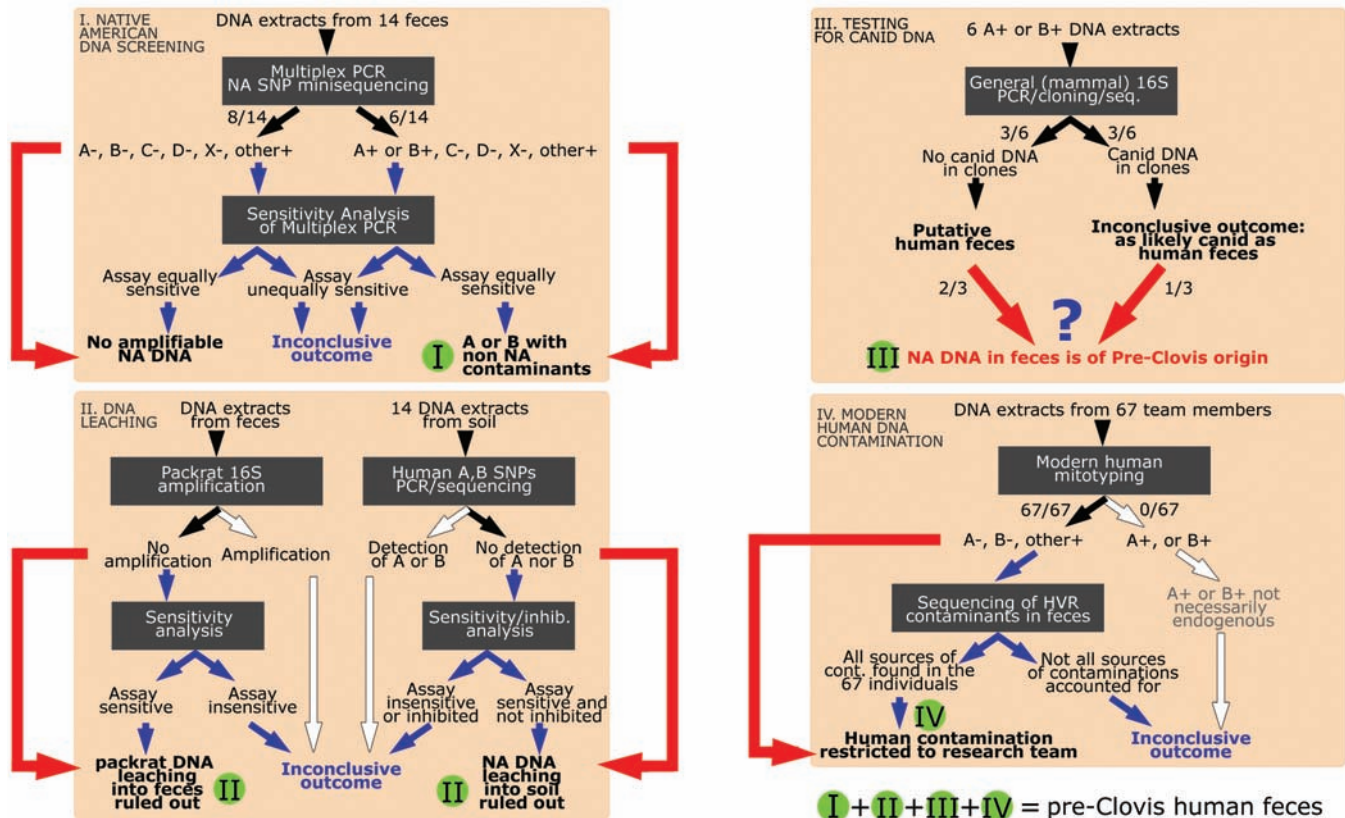


Fig. 1. Overview of the four main DNA experiments in Gilbert *et al.* Blue arrows, experiments we deem necessary; red arrows, actual experiments performed; black arrows, common pathways between both; white arrows, possible but unobserved outcomes.

data derived in each lab undermine their authenticity. Sequence authenticity is better supported by direct quantitation of DNA in ancient extracts (5–7). Gilbert *et al.* did not provide quantitative data but rather the ratio of alleles present in their polymerase chain reaction (PCR) end products (Table 1). These ratios indicate a mixture of putative endogenous (13% to 98%) and exogenous DNA, present in all six fecal samples, and do not give any indication of the absolute number of starting molecules present in the PCR. In addition, Gilbert *et al.* provided sequences of cloned PCR products, which again show a mixture of sequences containing low amounts of Native American mtDNA (Table 1). In only a single case does the ratio favor a putative endogenous DNA source; however, this is the second youngest sample (6640 yr B.P.) tested, and thus it is irrelevant to the pre-Clovis claims.

To exclude the possibility that the Native American mtDNA within the coprolites derived from leaching of younger strata, Gilbert *et al.* performed two tests (Fig. 1). In the first test, packrat DNA was targeted using a 16S ribosomal DNA assay on the fecal DNA extracts. No amplification products were observed, from which Gilbert *et al.* inferred that leaching had not occurred. However, without specific controls demonstrating the sensitivity of this assay in the presence of fecal extracts, the results are not probative. In the second test, 14 randomly chosen sediment samples were targeted for human mtDNA overlapping a haplogroup A SNP, and a 9-bp deletion found in Native American haplogroup B. Only 13 of the 28 PCR attempts yielded amplification products, a surprisingly low result because contaminating human DNA has been reported in every other PCR performed. Sequences of 247 clones from these 13 products

show the presence of both human mtDNA (non-A and non-B mtDNA) and nuclear mitochondrial pseudogenes. The absence of the Native American A SNP and a 9-bp deletion is used to disprove leaching, but the lack of specific data on the sensitivity of this assay in the presence of the contaminating (likely better preserved) human DNA and inhibitors in the extracts renders the results inconclusive.

The authors' assertion that "substantial amounts of liquid water are required to move free DNA molecules between strata" is based on the work of Haile *et al.* (8). However, that study showed that sheep DNA had leached through sediment representing >1500 years' accumulation in "dry caves"; the leaching was attributed to "copious" yet ultimately unknown amounts of urine. Thus, the possibility of DNA leaching in environments of limited circulating water and across substantial time spans cannot be excluded, nor has this process been adequately studied by any group working in this field (9).

To differentiate between potential human and canid origins, the six coprolite samples were screened using a general mammalian 16S PCR assay. Sequences of coyote, dog/wolf, or fox were reported for three samples, although the original sequence data were not presented. Because morphology and protein analyses are inadequate for unambiguous attribution of paleofeces to humans, and these samples contain both human and canid DNA in unspecified relative quantities, the samples are equally as likely to be canid as human, leaving open the strong likelihood of leaching (Fig. 1).

Gilbert *et al.* (1) claimed that because the cave sediments were undisturbed, the only possible sources of contamination were people at the archaeological site or in the genetics lab-

oratories. To demonstrate this, one must link all putative contaminating sequences found from the fecal and sediment extracts with the 67 individuals said to have participated in the research. To do this, Gilbert *et al.* sequenced the mtDNA hypervariable region (HVR) and typed the A haplogroup SNP and the 9-bp deletion from all 67 individuals. However, comparable HVR sequences were never presented from the coprolite or sediment samples, and because no other diagnostic positions of the human mtDNA were sequenced, there is no way to link the 67 investigators with the contaminating sequences present in the coprolites or sediment (Fig. 1). Thus, these data alone are inadequate for pinpointing the source(s) of contamination. The possibility remains that DNA sources from outside this group of researchers have not been accounted for, which could, of course, include Native American haplogroups. This line of evidence does not prove that the Native American mtDNA sequences present within the feces are endogenous.

In support of their identification of endogenous human DNA, Gilbert *et al.* cite the results of cross-immunoelectrophoresis (CIEP) testing. CIEP remains highly controversial in the field, is notably insensitive, has little experimental support in the literature (10–13), and has yielded some failures (14). Gilbert *et al.* identified human protein in three of the six samples containing little endogenous DNA as judged from the ratio in the clones. Surprisingly, the two youngest samples, one of which had the highest percentage of putatively "endogenous" human DNA (Table 1), did not yield any detectable antigens by CIEP.

Aside from the ambiguous DNA evidence, there is no artifactual evidence of a pre-Clovis,

Table 1. All data presented from the 14 paleofecal samples screened for Native American DNA in Gilbert *et al.* (1). NP, not presented; NA, not attempted; Non, non-A/non-B; +, detection of canid DNA in clones of 16S rDNA PCR product; ?, unknown (i.e., unsure if other sequences

were present in clones of this assay); √, samples containing human antigens; –, no human antigens detected. Samples supporting putative pre-Clovis humans in North America, based on radiocarbon dates, are in bold.

Sample	¹⁴ C	Presence or % SNP detected in PCR or pyrosequencing				16S rDNA mammal canid/other	Clones sequenced total (Endo:Exo)	Protein
		Miniseq A, B, other	% Hg A, % Hg B 663, 8281	% A2 8027, 12007	% B2 4977, 11177			
1294-PC-5/7D-4	1,308	B, other	<2, 50-98	<2, <2	34, 27	+/?	NP	–
1374-PC-1/2A-28	6,640	B, other	<2, 48-98	<2, <2	97, 100	?	27 (25:2)*	–
1294-PC-5/6B-40	10,050	B, other	<2, 38-97	<2, <2	100, 89	+/?	5 (1:4)*‡	√
1294-PC-5/6B-50	12,260	A, other	13-41, <2	12, 16	<2, <2	+/?	NA	√
1294-PC-5/7C-31	12,290	B, other	<2, 98	<2, <2	21, 19	?	22 (3:19)†	√
1374-PC-5/5D-31	12,400	B, other	NP	NA	NA	?	24 (3:21)*	–
1294-PC-2/3C-19	NP	Non	NA	NA	NA	?	NA	NA
1294-PC-5/10D-8	NP	Non	NA	NA	NA	?	NA	NA
1294-PC-2/3C-16	NP	Non	NA	NA	NA	?	NA	NA
1294-PC-1/4C-23	NP	Non	NA	NA	NA	?	NA	NA
1294-PC-5/7D-12	NP	Non	NA	NA	NA	?	NA	NA
1374-PC-2/1A-12	NP	Non	NA	NA	NA	?	NA	NA
1374-PC-1/1A-13	NP	Non	NA	NA	NA	?	NA	NA
1374-PC-5/5B-25	NP	Non	NA	NA	NA	?	NA	NA

*Clones sequenced in Copenhagen. †Clones sequenced in Leipzig. ‡Informative sequences of clones presented in the supporting online material in (1) for sample 1294-PC-5/6B-40 appear to be missing; thus, the total number of clones is not exactly known.

or even Clovis-era, occupation at Paisley Caves. Although the obsidian hydration ages [table S8 in (I)] might appear suggestive of an early human presence, such dates are notoriously unreliable (15). Notably, the obsidian artifact dated to 16,910 yr B.P. is some 2500 years older than the reported age of the dung from the same stratigraphic context. Typologically, none of the stone tools recovered from the site are Paleoindian. The oldest reliably dated artifacts from the cave are the string fragments that are ^{14}C -dated to 10,550 and 10,290 yr B.P. (post-Clovis ages). The Clovis or post-Clovis-aged fecal specimen 1294-PC-5/6B-40 [no. 2 in figure 1B in (I)] was derived from much deeper than two of the ~12,300 yr B.P. feces [nos. 5 and 6 in figure 1B in (I)], which highlights the obvious problem of stratigraphic disturbance in the cave. A coprolite dated to 4130 ± 40 yr B.P. (~4700 cal yr B.P.) was transported below a zone with Mt. Mazama ash dated ~7600 cal yr B.P.; it lay 10 cm above bones of extinct fauna (earlier than ~12,900 cal yr B.P.) and some 40 cm above fecal sample 1294-PC-5/7C-31, dated to ~14,300 cal yr B.P. [no. 4 in figure 1B in (I)]. The acknowledged penetration of later human material, via rodent burrowing, into the lower strata is obviously relevant to the issues raised earlier of possible contamination of samples by urine leaching and intermixing.

Lacking typologically distinctive artifacts in stratigraphic order, the case for pre-Clovis human presence in the cave is reduced to several equivocal broken animal bones and three coprolites reported to contain Native American mtDNA. Apart from the DNA issues raised above, are the three pre-Clovis dates (14,300 cal yr B.P.) accurate? Although the concordance of dates for three split samples initially seems convincing, the ^{14}C dates for another split coprolite (1294-PC-5/6B-40) present a problem: Beta-Analytic dated one piece to $10,050 \pm 50$ bp, with $\delta^{13}\text{C}$ stated as -14.7 per mil, whereas Oxford dated the other piece to $10,965 \pm 50$ bp, with $\delta^{13}\text{C}$ of

-23 per mil. These large discrepancies of both age and $\delta^{13}\text{C}$ suggest either field or laboratory errors in processing or, alternatively, the presence within the coprolite of organic materials of differing inherent age.

Closer inspection of the $\delta^{13}\text{C}$ values of the ostensibly older coprolite samples reveals inconsistencies that raise questions about both their dates and their human origin. Beta and Oxford reported substantially different $\delta^{13}\text{C}$ values for specimen 1374-PC-5/5D-31-2, -18.4 per mil and -16.6 per mil, respectively. Although not as extreme as the discrepancy noted in 1294-PC-5/6B-40, this difference (1.8 per mil) appears to be too great for what should be the same dated material, and both values differ sharply from those reported for the other coprolite samples of the same age (~ -25 per mil). A possible explanation of the ^{13}C variability is the presence of remnants of ingested organisms of differing inherent age. Plants, fish, or waterfowl derived from the waters of a radiocarbon-depleted lake potentially could cause a “freshwater reservoir effect,” making dates too old by ~1000 years (16). Several studies have clarified the quantitative relationship of $\delta^{13}\text{C}$ in plants, the herbivores that consume them, and carnivores (17–19). Herbivores’ feces are generally enriched in $\delta^{13}\text{C}$ by -0.9 per mil relative to the plants in their diet. The local terrestrial plants near Paisley Cave ~12,500 cal yr B.P. evidently had a $\delta^{13}\text{C}$ of -25 per mil (grass) or -24 per mil (sagebrush); at ~14,300 cal yr B.P., plant $\delta^{13}\text{C}$ may have been about -22 per mil. Apart from sample 1374-PC-5/5D-31-2, discussed above, the $\delta^{13}\text{C}$ of the supposedly pre-Clovis human dung is reported as -25.4 – -24.5 per mil and -25 – -25.6 per mil. If derived solely from terrestrial sources, these values appear to imply a strictly herbivorous diet of local grasses, which is improbable for normally omnivorous humans.

These issues might be addressed in the future by the separate dating of identifiable plant and animal tissue fragments in the coprolites.

Until that is done, given the aberrant dating of one split sample, the radiocarbon dates for the rest cannot be regarded as secure. This uncertainty, the inconclusive identification of Native American mtDNA in these fecal samples as a result of inadequate experimental controls, inconsistency of results, unsubstantiated claims to have identified all sources of contamination and to have precluded leaching between strata, as well as the acknowledged presence of canid DNA in three samples, together render the inference of widespread pre-Clovis occupation tenuous.

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