21/02/2023

Dear Bernard,

As discussed with you last week telephonically, we have had success in extracting useable proteins from Homo naledi teeth. As you are aware, in the first round of permits issued we extracted material successfully by thin sectioning the teeth. However, during the course of this work, and after examining closely the Australopithecus sediba teeth to be sampled, and with the desire to minimize the use of invasive methods, a new method was invented to extract proteins practically non-invasively by an acid etching technique. I am pleased to let you know that this minimally invasive technique has worked both independently and comparatively with the sectioned teeth. Interestingly, all of the naledi individuals have returned results indicating they are female. This is a surprising result, and after detailed analyses of the results we are confident that they are correct. As you know, we have sampled at least one of the most robust naledi specimens which has been identified morphologically as a male, it has also returned a result indicating it is female with high confidence. The odds that the first five specimens to be selected for sampling are all female is very low, and this intriguing result requires further investigation prior to publication. In addition, the new method of acid etching is so minimally invasive as to effectively leave no evidence of the technique being applied to the specimen other than a slight dulling of the enamel surface. I am sure you agree that the development of a minimally invasive technique to sample is an equally important result of this experiment as the acquisition of ancient proteins.

As background to this work, the recovery of ancient proteins from hominid dental enamel has previously been achieved starting from specimens up to ~2 million years old retrieved in temperate and sub-tropical environments [1,2]. The dental enamel proteome includes amelogenin, a protein that, in humans, is expressed in two isoforms encoded in the non-recombinant regions of the heterochromosomes, namely amelogenin X (AMELX) on the X chromosome and amelogenin Y (AMELY) on the Y chromosome. The detection of AMELY-specific peptides allows the straightforward assignment of the analysed dental specimen to a male individual. On the other hand, the exclusive detection of AMELX-specific peptides is ambiguous, because it can either be interpreted as deriving (i) from a female individual, or alternatively from (ii) a male individual whose signal for the AMELY-specific peptides is too low to be detected, due to diagenetic degradation. These two cases can now be discriminated with a semi-quantitative mass spectrometric approach based on the determination of the threshold intensity of AMELX-specific peptides below which the signal for AMELY-specific peptides is lost. This method however requires the identification of AMELY-specific peptides in *at least one* dental specimen.

As a review of the experiment so far, we analysed five *H. naledi* dental specimens: UW101-020, UW101-809, UW101-886, UW102b-511 and U.W. 102. We are confident these all represent different individuals. In a first round of analysis performed on a set of four specimens (UW101-020, UW101-809, UW101-886, UW102b-511), we compared the results obtained destructively sampling an enamel fragment cut from the tooth versus micro-destructive acid etching. We found that enamel etching performed similarly to cutting in terms of ancient protein recovery. All the four specimens yielded very good protein recovery and high AMELX sequence coverage, however no AMELY-specific peptide was detected. To clarify whether (i) this result could represent the identification of four teeth originating from female individuals, or whether (ii) any of these teeth originated from a male individual whose AMELY-specific peptide signal was lost due to diagenetic degradation, we then analysed, as a possible positive control, a fifth specimen (U.W. 102, left M^3 - "Neo") previously identified as a male, based on morphological evidence. Ancient proteins from Neo's left M^3 were exclusively retrieved by acid etching, without altering its morphology and minimally altering its appearance, Fig. 1. Unexpectedly, despite the recovery of dental enamel proteins was generally good and the intensity of the AMELX peptide signal was quite high, it has not been possible to observe any

AMELY-specific peptide, as instead expected from a male individual. As a conclusion, we still cannot determine whether the absence of AMELY-specific peptide is due to: (i) methodological, e.g. detection limits, (ii) biological, e.g. no expression of AMELY in *H. naledi*, or (iii) cultural, i.e. exclusive presence of female *H. naledi* individuals, reasons. In modern humans, a small percentage of males do not express AMELY, AMELY null. Depending on the population and sample size the deletion of AMELY ranges from 0.02 to 4.5 % [3–8]. Is *H. naledi*, or at least the *H. naledi* male population from Rising Star Cave, AMELY null? Is there an 'over representation' of female individuals within the *H. naledi* population at the Rising Star Cave? To put our results into perspective, 5 female assignments from 5 specimens represents a 3.125% probability under the assumption that males and females are represented in the population in equal numbers. A ~3% probability of finding 5 out of 5 females is not beyond the realms of expectation, although it is sufficiently low to merit further investigation especially when minimally destructive sampling, Fig. 1, is possible. For this reason, it would be advisable to screen a larger, highly heterogeneous, sample set of *H. naledi* dental specimens, to further attempt the detection of AMELY-specific peptides in *H. naledi* using exclusively acid etching. The negligible alteration of the enamel of each dental specimen analysed, Fig. 1, minimises the impact of the approach suggested.

I am requesting you apply, as Curator of Collections, for an addition to this permit, including enough specimens to sample above the %100 statistical probability of there being no male in the sample. Discovering a male within the naledi sample is, as you might imagine, critical for the experiment. If there is no male in the sample, or we are seeing an anomalous result based upon a unique hereditary condition in the *H. naledi* population, the result is of considerable importance to the study of this ancient population.

We propose to eventually sample the following specimens and would like them included in the permit:

1. U.W. 101-1463 right maxillary M1. (DH1)
2. U.W. 101-006 right maxillary M3. (DH3)
3. U.W. 110-2 maxillary right P4 crown. (Letimela)
4. U.W. 101-344 right maxillary M1. (Hill Antechamber)
5. U.W. 101-1686 right mandibular deciduous M2 (1400 mandible)
6. U.W. 101-1396 right maxillary M1
7. U.W. 101-525 right maxillary M1.
8. U.W. 101-583 right maxillary M1.
9. U.W. 101-2175 left mandibular P4 (Feature 1 mandible. Undescribed)
10. U.W. 101-1846 right maxillary M1 (Hill. Undescribed)
11. U.W. 101-1915 right maxillary M1 (Hill. Undescribed)
12. U.W. 101-2021 right maxillary M1 (Hill. Undescribed)
13. U.W. 101-1571 left mandibular deciduous canine (early juvenile 4)
14. U.W. 101-824 left mandibular deciduous canine (early juvenile 3)
15. U.W. 101-593 right maxillary M2 (early juvenile 2)
16. U.W. 101-516 left mandibular M3 (antimere of 001 mandible)
17. U.W. 102c-589 Lesedi mandible
18. U.W. 101-010 mandible fragment
19. U.W. 101-652 cusp of developing tooth germ
20. Hill antechamber feature (3 or possibly 4)

The etching procedure used for the analysis of UW101-020, UW101-809, UW101-886, UW102b-511 and U.W. 102 represents a development of a methodology previously used on modern teeth samples as described by Stewart *et al* (2016) [9]. The entire procedure is performed in a Class 2

Biosafety Cabinet inside an ancient biomolecule extraction facility, Fig. 2. A graphical representation of the workflow is shown in Fig. 3.

Briefly, the enamel surface to be acid etched is first cleaned gently with molecular biology grade water and dried with absorbent paper. A total of 125 µL of 10% v/v trifluoroacetic acid (TFA) are then added to the inner lid surface of a 0.5 mL microcentrifuge tube. The enamel surface to be acid etched is brought into contact with the 10% TFA droplet for 1 minute. This initial 1 minute serves as a further cleaning step and the acid is discarded. Another 125 µL of 10% v/v TFA is then added to the inner lid surface of a new 0.5 mL microcentrifuge tube and the enamel surface to be acid etched is brought into contact with the 10% TFA droplet for 5 minutes to perform the extraction. After 5 minutes the etched surface is inspected and the extraction etching may be repeated for a maximum of 2 more times (the 10% TFA in the inner lid surface may need to be topped up due to evaporation). The maximum exposure to 10% TFA is therefore 15 minutes for extraction and an additional 1 minute for the cleaning step.

Once extraction has been completed the acid is removed from the inner lid surface and placed in a 1.5 mL microcentrifuge tube. The inner lid surface is then washed with 75 µL of 10% TFA to maximise recovery. This wash is combined with the extraction volume in the same 1.5 mL tube. The combined extraction volume is then processed according to the digestion free palaeoproteomics workflow [1,2], i.e. peptide cleanup using C18 StageTips and nano-liquid chromatography tandem mass spectrometry.

Fig. 1. U.W. 102 Neo M[3] post etching

Fig. 2. Ancient biomolecules extraction facility and setup.

Fig. 3. Graphical overview of the acid etching procedure and downstream palaeoproteomic analysis (from Stewart *et al.* 2016, modified)[9].

I am personally confident that the method is so minimally invasive as to be safe continuing the experiment and will have little or no effect on future studies of the specimens being sampled. I am

proposing the permitting of the following individual specimens to be sampled by this method. We are confident that all of these teeth come from discrete individuals. With the exception of the teeth in the Hill Antechamber (which remain encased in their plaster jacket), they are all described in the upcoming publication on the naledi dentition. We are not intending to sample all of these teeth at once, but likely in batches of five to seven so that the experiment can be adapted and improved as we continue.

My request is to permit this sample of teeth at once, and the experiment will then continue over the next 12 to 24 months. I am proposing that I carry the first sample of these teeth in mid-April to Copenhagen, where they will be sampled in the laboratory of Enrico Cappellini. This work will form part of the PhD of Palesa Madupe as well, increasing the impact of outcomes.

As usual, I will personally hand carry the specimens on the airplane, delivering them in person to Enrico in his lab, where they will be held under the strictest security.

I appreciate your attention to this request. If you have any further questions about this proposed experiment, please feel free to contact me at any time.

Sincerely,

Lee

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