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TECHNICAL NOTE

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Use of Solid-Phase Double-Antibody Radioimmunoassay to Identify Species from Small Skeletal Fragments

ABSTRACT: Protein radioimmunoassay (pRIA) offers the potential to identify species in small skeletal fragments submitted as forensic evidence. The technique consists of protein extraction followed by a solid-phase double-antibody radioimmunoassay using controls of antisera (raised in rabbits) and radioactive (iodine-125) antibody of rabbit gamma globulin (produced in donkeys). Species determination results from evaluation of radioactivity uptake. To demonstrate the potential of this technique, six known bone samples (three human and three nonhuman, including one from a deer [*Odocoileus virginianus*]) were submitted for blind analysis. pRIA correctly distinguished the human from the nonhuman samples. Using 200 mg or less of each sample, species of the deer specimen was identified correctly, given the choices of cow, deer, dog, goat, and pig.

KEYWORDS: forensic science, radioimmunoassay, bone fragments, species identification

Species determination of submitted skeletal remains constitutes an important component of forensic anthropological analysis. Usually, such determinations can be made easily from morphological indicators when specimens are relatively complete and well preserved (e.g., 1–3). In many cases, species analysis may be needed only to distinguish human from nonhuman remains. Occasionally, it may be necessary to identify the nonhuman species, a problem also usually easily resolved through consultation with comparative collections and/or zoological specialists if the remains are well preserved.

When remains are fragmentary, poorly preserved, or otherwise morphologically compromised, determinations can prove problematic. These problems frequently arise in the submission of fragmentary evidence for DNA analysis. Fragments of bone and tooth can resemble other types of small particles recovered from crime scenes. In such cases, SEM/EDS can help distinguish the former from the latter (4), but offers little help in separating human from other vertebrate species.

When separation can not be made on morphological evidence, researchers frequently turn to histological approaches. Microscopic analysis of bone cross-sections may reveal a plexiform type of bone or an osteon banding pattern diagnostic of nonhuman origin (5–9). However, it is more problematic to use histological evidence to determine human status because the human pattern can be shared with some other vertebrate species. The need clearly exists for methodology to determine species in such cases.

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Protein Studies

In 1980, Lowenstein (10) noted that proteins such as collagen and albumin are present in all metazoans "from sponges to man" and can be preserved even in ancient materials. He noted however, that due to the small quantities preserved in fossil specimens and species similarity of collagen structure, analysis of constituent amino acids can be difficult. Lowenstein introduced a solid-phase radioimmunoassay technique (pRIA) that yields species specific information from fossils as old as 1.9 million years. The technique involved extraction of collagen or albumin from the unknown, exposure of the extract to rabbit serum with species specific antibodies, followed by exposure of the product to radioactively labeled goat antibody to rabbit gamma globulin. The radioactivity of goat antibody allowed the extent of binding to be measured and thus to determine the most logical species represented. The technique was successfully (with logical results) applied to a variety of fossil material of considerable antiquity. Subsequently, the technique has been applied to examine evolutionary relationships of extinct animals (11) as well as to examine the species of bloodstains on ancient stone weapons and fossilized urine from archeological deposits (12).

More recently, a highly specific enzyme-linked immunosorbent assay involving monoclonal antibodies has been used to detect human proteins in blood stains on buried cloth (13) as well as in ancient bone (14,15). This method also has detected human albumin in cremated bone as ancient as 3000 years (16). Additional research on human and bovine samples indicates that bone integrity represents a factor in protein survival (17).

In an innovative 1999 study, Cattaneo et al. (18) examined human/nonhuman differences in 35 documented burned bone samples using quantitative and standard light microscopy, mitochondrial DNA amplification, as well as the biomolecular analysis outlined above aimed at identifying human albumin. The quantitative

histology approach proved useful and complemented the other methods. Human albumin was identified in five of the 15 human samples, using the ELISA assay approach.

The literature discussed above indicates that protein detection and analysis represents a promising approach to distinguish human from nonhuman tissues in forensic contexts. The specific techniques introduced by Lowenstein and colleagues are especially useful because they: 1. Utilize very small samples; 2. Not only separate humans from nonhumans but also identify nonhuman species; 3. Offer the degree of scientific certainty required of forensic analysis; and 4. Have proven useful even with samples of extreme antiquity. The research reported here demonstrates how current applications of this technique can be utilized for species identification from forensic samples.

Materials and Methods

Samples were collected by the first author from skeletal remains of three humans and three nonhuman animals. Although the species of each individual was known, the samples themselves were too small and fragmentary to allow species identification. Each sample clearly represented bone, but morphological markers were not present to distinguish human from nonhuman animal or to recognize nonhuman animal species. Details of the individual samples are presented in Fig. 1.

All samples were labeled only as A through F and sent to the third author for specimen preparation. Analysis by pRIA was directed to first determine which of the specimens were human and which were nonhuman. Once the nonhuman specimens were identified as such, the second and third authors were instructed to determine the species of "F", given the choices of cow, deer, dog, goat and pig. These species were selected to include the correct species (deer) as well as others commonly found in forensic contexts that present morphological similarity to deer.

Upon receipt of the samples, the third author used a clean, 1 mm stainless steel Dremel drill bit to remove and discard the outer 1 to 2 mm surfaces of each sample. The core bone matter then was reduced to small particles and powder using a separate, clean drill bit. This material was considered to be free of significant external protein contamination and was utilized in the analysis.

To isolate the protein, the particles and powder of each sample were placed in a 10 mL vaccutainer, which was then capped. A solution of 1M EDTA was added using a syringe inserted through the septa of the cap. The same syringe then was utilized to produce a partial vacuum within the container. The bone matter was then dissolved in the EDTA solution by gently shaking and rotating the container for two to five days.

Once the bone matter was dissolved, 25 mL of the EDTA extract were placed in each of the wells of a 98-well polystyrene microtiter plate. The extract was allowed to sit within the wells for 2 h at room temperature. By the end of the 2 h period, some of the protein in the

FIG. 1—*Submitted samples A through F: (a) Adult modern human, 1.3 g from the sternal end of a left lower rib; (b) nonhuman large ungulate, 1.1 g of cortical bone; (c) nonhuman apparent dog, 1.8 g of cortical bone; (d) adult human, 1.5 g of cortical bone from the distal diaphysis of a left femur originating from a prehistoric skeleton from Ecuador; (e) adult modern human, 1.7 g from the diaphysis of a left fibula; and (f) nonhuman deer, 1.1 g from the horizontal ramus of a left mandible (Scales in centimeters).*

TABLE 1—*pRIA analysis of standards and six submitted samples.*

Applied Anti-sera Standard		Known Origin of Anti-sera Used								
		Human	Bison	Bear	Rat	Elephant	Elk	Goat	Pig	Dog
					% uptake I–125					
human		**22**	3	3	6	1	0			
bison		2	**16**	1	0	1	1			
bear		8	3	**23**	3	5	2			
rat		9	4	6	**22**	3				
elephant		8	3	8	1	**20**				
elk		6	6	8			**20**			
goat			10				8	**39**	9	13
pig			7				8	20	**33**	23
dog			8				3	12	4	**35**
					Test 1: Animal vs. Human.					
Lab No.	Submitter No.									
MA-1506	A	**10**	4	1	0	0				
MA-1507	B	3	**10**	1	0	0	4	7	2	2
MA-1508	C	11	3	**17**	2	2				
MA-1509	D	**3**	2	0	0	0	2			
MA-1510	E	**23**	4	7	5	7				
MA-1511	F	5	6	6	0	6	**17**	13	6	10

solution was bound to the plastic plate, creating the "solid phase" of the procedure. The liquid content of each well was then washed out with a soy protein solution which coated the plate to prevent any further protein binding.

Following this procedure, 25 mL of antisera raised in rabbits were added to each well and left at room temperature overnight. This antisera originated from rabbits that had been exposed to albumins or sera of different species of animals, including human. Known species-specific rabbit antisera was thus added to each of wells. During the overnight period, the species-specific antibody bound to the antigen already present on the plastic plate. The greatest binding occurred with the antibody that was most specific to the antigen.

The plate was then again washed out with the soy protein and radioactive (iodine-125 labeled) antibody (raised in donkeys) against rabbit gamma globulin was applied to the plate wells and left overnight at room temperature. The greatest binding of this antibody occurred in the wells with the greatest quantity of rabbit gamma globulin that was already bound to the original antigen in the wells of the plate. Subsequently, excess donkey antibody was washed out with sterile water and the radioactivity of the wells was quantified using a scintillation counter. In this method, the highest counts reflect the greatest binding of the specific antisera to specific antigens, allowing the species to be determined. The counts were measured as the percentage uptake of the radioactive material in the binding process. Each sample was analyzed twice.

Results

Measurement of the uptake of the radioactive material correctly identified samples A, D, and E as human and samples B, C, and F as nonhuman. Although D (specimen from prehistoric Ecuador) was identified as human, it was noted that the measurement was close to the limit for identification. Elemental analysis of this specimen revealed a low phosphorous peak indicating taphonomic alteration consistent with its considerable antiquity.

Details of this analysis are revealed in Table 1. To determine human/nonhuman status, each of the samples was tested using antisera standards of human, bison, bear, rat, elephant, elk, goat, pig, and dog. Table 1 indicates that the highest values were obtained for human in samples A, D, and E.

Once sample F was correctly identified as being nonhuman, it was further examined to determine which of the suggested animal species was represented (cow, deer, dog, goat, or pig). This analysis correctly identified the species as deer. Goat, pig, and dog were directly represented in the initial analysis (Table 1). Of the other two possible species, cow was closely related taxonomically to bison and deer was closely related to elk. Because the highest value in the test was for elk (17), deer was the most likely animal represented of the five suggested possibilities.

Analysis of sample D (human from prehistoric Ecuadorian sample) documents the usefulness of this technique in applications to forensic samples and/or those from archeological or ancient contexts. The pRIA analysis indicated a human origin for this specimen but the species difference in radioactive uptake was minimal compared to the other samples (Table 1). As shown in Fig. 2, the elemental analysis of sample D (MA-1509) revealed very low concentrations of phosphorus in comparison with MA-1510 and other normal bone. Figure 3 further reveals that the overall average amino acid concentration was depleted in sample D in comparison with the other samples and normal standards. The specific combined values, as well as the individual amino acid values are presented in Table 2.

Discussion

pRIA with associated elemental analysis and testing for amino acids represents a valuable approach to identify species in fragmentary bone cases from forensic contexts. Because such small quantities are required, pRIA can be used to test for species prior to DNA analysis. The test clearly can differentiate human from nonhuman species usually, even in ancient material. As indicated in the analysis of sample D, antiquity may lead to low amino acid levels and protein preservation problems, depending on the

TABLE 2—*Amino acid analysis of standards and six submitted samples.*

Sample Designation			Racemization Results		Concentration Ratios			Specific Concentration Results					
			1st Test: Is D/L Asp < 0.1? 2nd Test: Is D/L Ala < D/L Asp?		Sample values should be close to the Expected and modern values. The closer the values for the samples are to the Expected values, the better the specimen.			Values represent the percent (%) amino acid in the sample relative to a modern specimen (+/− 20%). Values ~100% or greater mean identical to modern bone. Lower values for the COMBINED AVERAGE indicate less preservation and a lower quality specimen.					
Lab No.	Submitter Number	Material	D/L Asp	D/L Ala	Asp/Glu	Ser/Glu	Ala/Glu	Asp	Glu	Ser	Gly	Ala	Combined Average
Mdn	Expected value for a modern specimen		<0.05	<0.009	0.68	0.47	1.51	100%	100%	100%	100%	100%	100%
Mdn	Reference standard run with the samples		0.013	0.006	0.72	0.49	1.66	104%	103%	103%	88*	106%	101%
MA-1506	A	bone powder	0.044	0.022	0.71	0.48	1.57	104%	106%	104%	94%	103%	102%
MA-1507	B	bone powder	0.030	0.004	0.72	0.48	1.66	104%	105%	104%	103%	108%	105%
MA-1508	C	bone powder	0.016	0.005	0.72	0.49	1.58	99%	99%	99%	101%	97	99%
MA-1509	D	bone powder	0.136	0.030	0.82	0.41	1.54	6%	5%	4%	4%	5%	5%
MA-1510	E	bone powder	0.037	0.003	0.70	0.49	1.72	106%	109%	109%	96%	116%	107%
MA-1511	F	bone powder	0.022	0.003	0.72	0.48	1.65	90%	91%	89%	77%	93%	88%

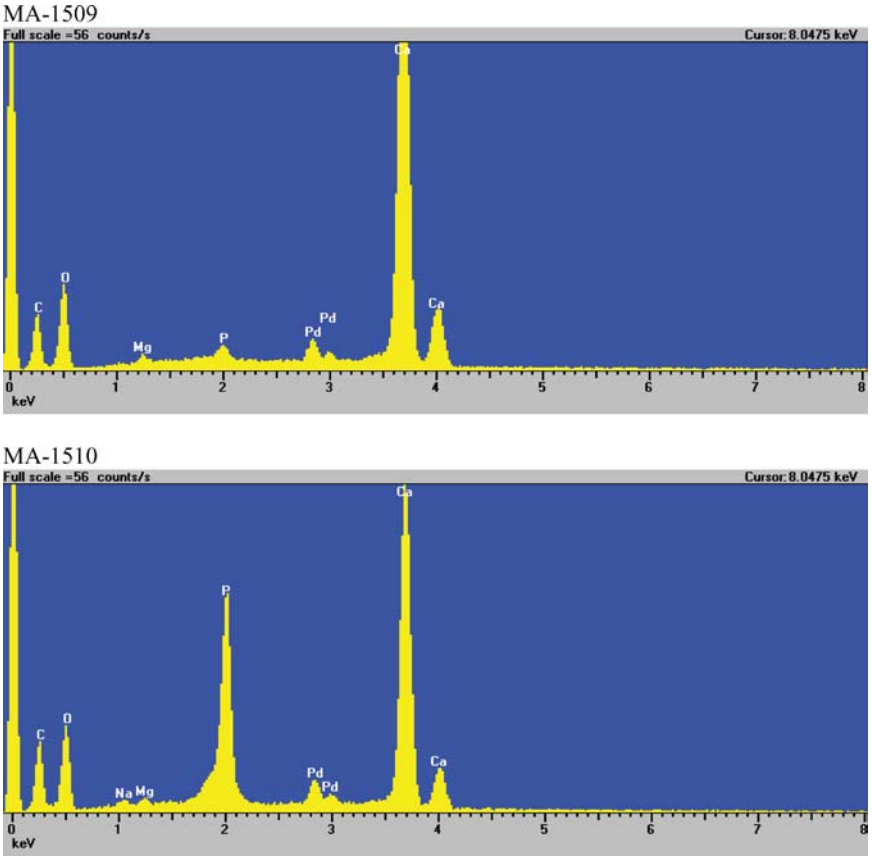

FIG. 2—*Elemental analysis of submitted samples MA-1509 (D) and MA-1510 (E).*

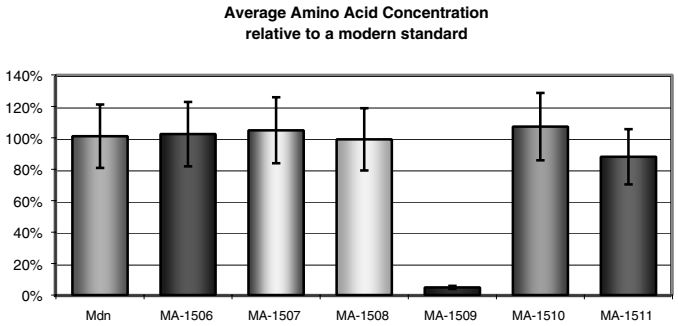

FIG. 3—*Average amino acid concentration in submitted samples relative to a modern standard.*

particular sample and taphonomic situation. pRIA also can determine nonhuman animal species in cases where it is important to do so.

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