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The Use of Isoelectric Focusing to Identify Rhinoceros Keratins

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ABSTRACT: Keratins represent the principal structural proteins of hair. They are also found in horn, nail, claw, hoof, and feather. Hair and nail samples from human and canine sources and hair samples from mule deer, white tail deer, cat, moose, elk, antelope, caribou, raccoon, and goat were studied. Parrot and goose feathers were also analyzed. Keratins are polymorphic, and species differences are known to exist. Proteinaceous extracts of deer and antelope antlers and bovine and rhinoceros horn were prepared by solubilizing 10 mg of horn sample in 200 μL of a solution containing 12*M* urea, 74m*M* Trizma base, and 78m*M* dithiothreitol (DTT). Extraction took place over a 48-h period. A 25-μL aliquot of extract was removed and incubated with 5 μL of 0.1*M* DTT for 10 min at 25°C. Keratins were then separated by isoelectric focusing (IEF) on 5.2% polyacrylamide gels for 3 h and visualized using silver staining. At least 20 bands could be observed for each species studied. However, band patterns differed in the position of each band, in the number of bands, and in band coloration resulting from the silver staining process. Horn from two species of rhinoceros was examined. For both specimens, most bands occurred in the pH range of 4 to 5. Although similar patterns for both species were observed, they differed sufficiently to differentiate one from the other. As might be expected, the closer two species are related phylogenetically, the greater the similarity in the IEF pattern produced from their solubilized keratin. Ten samples were removed from each species horn under study and every sample was extracted and run on an IEF gel. Approximately 50 keratin extracts from each species were analyzed by IEF.

KEYWORDS: criminalistics, keratins, species identification, serology, isoelectric focusing

Five species of rhinoceros survive in the world today, three in Asia and two in Africa. The Great Indian Rhinoceros (*Rhinoceros unicornis*) is the largest of the Asian species and the most numerous. The Great Indian Rhino and the Javan Rhino (*Rhinoceros*

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...ntis) have one horn, whereas the Sumatran Rhino (*Didermocerus sumatrensis*) has ... The Javan Rhino is probably the rarest large animal in the world. The other two ...cies, located in Africa, are the Black Rhino (*Diceros bicornis*) and the White Rhino (*...ceros simum*).

The ingestion of powdered rhinoceros horn has not proven efficacious as a stimulant ...male potency; however, some still believe that in ointment form, it may be of some ...p to impotent men [1]. The horn is also believed to help relieve pain during childbirth ...o prolong life. Thus, high prices paid by Asian druggists for rhinoceros horn make ...aching very financially attractive. While rhinos are strictly legally protected in India, ...y can still be shot in Nepal with special permission from the King. The rhinoceros has ...most become extinct, and all five species are officially listed by the U.S. Fish and ...ildlife Service [2] and by international agreement at the Convention on International ...de in Endangered Species of Wild Fauna and Flora [3]. There are increasing amounts ...rhinoceros horn entering the United States illicitly. The development of a simple, ...id reliable species identification technique is an important step which will lead to ...e frequent and successful prosecution of poachers and associated rhino horn dealers.

Basis of Investigation

The microscopic examination of the morphological properties of hair has been routinely ...ed in forensic science for species identification. The value of this method for individualization, however, is somewhat limited because an individual can produce a variety ...hair types with different cortical and medullary characteristics.

The principal structural proteins of hair are the keratins. They are also found in antler, ...n, nail, claw, hoof, and feather. Keratins are a group of tissue proteins of ectodermal ...gin which are resistant to digestion by pepsin or trypsin and are insoluble in water, ...ganic solvents, weak acids, and bases [4].

Most land mammals produce at least two forms of hard keratin: (1) hair keratin and (2) either claw, nail, or hoof keratin. Sometimes a third form, either horn or quill, may be produced. In all cases the keratins appear to be structurally similar, consisting of filaments (microfibrils) with a diameter of about 7.0 nm, often aligned, and embedded in a nonfilamentous matrix [5]. The microfibrils in hard mammalian keratin are low-sulfur proteins. Microfibrils can be thought of as one-dimensional (1-D) crystals of low-sulfur proteins. The alpha-helical sections of these proteins are almost straight, but may be distorted into supercoils or related structures. The matrix is believed to contain high-sulfur proteins and possibly high-glycine-tyrosine proteins [5]. Keratin is not a single substance but a complex mixture of proteins and the sulfur containing diamino acid cystine. This distinguishes it from collagen, elastin, and myofibrillar proteins.

With the exception of immunoglobulins, keratins appear to be more heterogeneous than any other type of protein. Although several explanations for this variability have been proposed, it appears that multiple gene loci may be responsible for the great variation [6,7]. Evidence for this hypothesis is provided by sequence studies which show that groups of keratins with closely related amino acid sequences are present in a single individual [6,7]. The observed heterogeneity may be related to the fact that the keratinized epidermis is the boundary between the animal and its physical surroundings. A pool of genes coding for closely related proteins may enable more rapid adjustment to a changing environment.

Because the synthesis of keratins is under genetic control and because keratins are known to be polymorphic, the analysis of these proteins can be used for species identification. There may also be considerable differences between subspecies, varieties, breeds, and perhaps even individuals within a single species.

Materials and Methods

Acrylamide, bis-acrylamide, and Pharmalyte ampholytes were purchased from Sigma Chemical Co., St. Louis, Missouri. The silver staining solutions were purchased from Bio-Rad Laboratories Inc., Rockville Center, New York.

Test Samples

Hair and nail samples were obtained from human and canine sources. Hair samples from mule deer, white tail deer, cat, moose, elk, antelope, caribou, raccoon, and goat were provided by Robert Adamo, Department of Laboratories and Research, Valhalla, New York. Feather samples were obtained from parrot and goose. Shavings of elephant tusk ivory, bovine and rhinoceros horn, and antelope antlers were obtained from the U.S. Fish and Wildlife Service, Lawrence, New York. Deer antlers were purchased locally.

Extraction of Keratins

Approximately 10 mg of each sample is treated with an extraction solution to obtain solubilized keratins. The technique is adapted from Carracedo et al. [8]. The extraction solution consists of 3.6-g urea, 0.045-g Tris(hydroxymethyl)aminomethane ("Trizma base"), 60 mg of dithiothreitol (DTT), and 4.8 mL of distilled water. Each sample is extracted in 200 μL of this solution for 48 h at 25°C. Ten minutes before focusing, 5 μL of 0.1*M* DTT is added to 25 μL of each extract. The samples are now ready for isoelectric focusing (IEF).

Isoelectric Focusing Procedure

Gel Preparation– The gel is prepared according to the method of Gill and Sutton [9] with minor modifications as follows: 7 mL of 29.1% acrylamide, 7 mL of 0.9% bis-acrylamide, 5 g of sucrose, 1.0 mL of Pharmalyte (pH range 2.5 to 5), and 1 mL of pharmalyte (pH range 5 to 8) are added to 24 mL of distilled water. The mixture is degassed and 0.3 mL of riboflavin (10 mg/dL) is added. The gel is allowed to polymerize for 90 min while exposed to light produced by a fluorescent lamp and is stored overnight at 5°C to promote hardening before use. Gels are 20 cm by 15 cm by 0.5 mm with a gel concentration of $T = 5.2\%$ (T = acrylamide + bis/100) and cross-linking of $C = 3.0\%$ (C = bis/acrylamide + bis). Generally, ten samples are run on each gel.

Isoelectric Focusing—Paper applicators are impregnated with the DTT-treated sample (see Extraction of Keratins subsections) and then placed on the gel. Samples are applied at a distance of 2 cm from the cathodal wick. The cathodal solution is 1.0*M* sodium hydroxide and the anodal solution is 0.9*M* phosphoric acid. IEF is conducted at 5 W, constant power. A maximum voltage of 2500 V with unlimited current is used. The gel is seated on a cooling platform maintained at 4°C. Electrofocusing is carried out for 60 min, and then the sample applicators are removed. Focusing continues for another 120 min to complete the run [9].

Staining Procedure—The staining procedure follows the Bio-Rad Laboratories protocol for silver staining. The technique of silver staining isoelectric focused keratins has been previously described by Carracedo et al. [10]. After focusing, the gel is fixed in 200 mL of a mixture of 30% methanol/10% trichloroacetic acid/3.5% sulfosalicylic acid for 1 h, followed by a bath in several volumes of 30% methanol/12% trichloroacetic acid (TCA) for at least 2 h to insure removal of all ampholytes. The gel is then soaked in 400 mL of 10% ethanol/5% acetic acid (v/v) for 30 min. All reagents are prepared in deionized water. The gel is immersed for 3 min in an oxidizing solution that contains potassium bromate and nitric acid. A rotating shaker is used to agitate the gel during each bath. It is then placed into the Bio-Rad silver nitrate staining reagent for 15 min and bathed in sodium carbonate/paraformaldehyde. The gel is soaked in the latter solution until dark brown bands appear against a pale background. Band development is terminated by placing the gel in a 200 mL bath of 5% acetic acid for 5 min. The gel is then soaked in deionized, distilled water and subsequently immersed in the latter for storage.

Densitometry—The stained gels were photographed and banding patterns recorded directly from the gel using an LKB Bromma 2202 Ultroscan Laser Densitometer linked to a Hitachi D-2000 Chromato-Integrator printer. The gel was scanned at 20 mm/min using a wavelength of 633 nm. The densitometric tracings were used to produce a schematic diagram illustrating the banding patterns which were obtained from the analysis of keratin samples from various animal species.

Results

The keratin patterns of different species obtained via IEF analysis in the region from pH 2.5 to pH 8 are illustrated in Figs. 1 through 6. The patterns were phenotyped from at least 20 samples from each of the 25 animal species studied. Multiple determinations showed good replication. Figures 1 to 6 illustrate the wide scope of the samples examined. The particular species used in this study were selected based upon their comparative values and also because these animal species are often illegally trapped or destroyed by poachers who market materials obtained from the animal, or both. Because deer and antelope antler and bovine horn are often used as substitutes for rhinoceros horn by individuals seeking profits from uninformed buyers, these specimens were also included in this study. Although antler and horn originate from different biological areas (antlers are an extension of the skull, whereas horn grows from underlying layers of the skin), both contain keratin detectable by the IEF method.

The majority of the diagnostic bands relative to species specificity are observed on the gels in the pH region between approximately 2.5 and 5.0. The remaining portion of the gel (pH 5.0 to 8.0) is less informative for determining the animal species based upon those samples used in this study.

Figure 1 illustrates keratin banding patterns resulting from IEF analysis of samples obtained from several different species. Although the banding patterns from different species show a certain degree of similarity, nonetheless, differences between species can

FIG. 1—*IEF gel, pH 2.5 to 8.0, of keratins extracted from various sources:* (1) *raccoon hair,* (2) *cat hair,* (3, 4) *bovine horn,* (5) *dog hair,* (6) *goose feather,* (7) *human fingernail,* (8) *deer hair, and* (9) *goat hair. Protein concentration of each sample is approximately 1 mg/10* μL.

be quite clearly discerned, with regard to the numbers and positions of the individu bands as well as with regard to the intensity of color produced via the silver stainin procedure. There is broad variation found among raccoon, cat, dog, deer, and goat hai bovine horn, goose feather, and human fingernail samples. The keratin pattern obtain with goose feather is strikingly different than the others due to the predominance bands having isoelectric points in the range of 3.0 to 6.0.

A very important, albeit predictable, observation resulting from these studies is th the closer species are phylogenetically related, the greater the similarity in their kerat banding patterns. This is illustrated in Fig. 2 for various species of deer. Almost n difference can be discerned in the various banding patterns. The patterns obtained fr samples of moose, elk, antelope, and deer hair are illustrated. A sample of bovine ho was also run on this gel. Apparently the yield of keratin from the extraction procedur was lower than expected (approximately 1-mg protein/10 μL), and therefore the band appear less intense than the corresponding bands seen in Fig. 1. Similarly, goat and raccoon hair samples are also present in Fig. 2 and appear more intense than the sample from these species seen in Fig. 1 as a result of greater concentrations of keratin in th former.

The IEF banding patterns obtained from samples of rhinoceros and bovine horn ar shown in Figs. 3 and 4. These samples were run simultaneously on the same gel due t the importance of being able to distinguish these two species based upon differences in their keratin IEF banding patterns. In powdered form, it is impossible to distinguish samples from these species by visual comparison. This fact is often exploited by dealer who claim to be providing buyers with authentic rhinoceros horn, but actually the horn is of bovine origin. The main differences observed in the banding patterns obtained from the above species is found in the pH region between 2.5 and 3.0. Samples obtained from the anterior and posterior horns of the black rhinoceros were run side by side to determin if any intra-individual sample variation exists in the same animal. No significant differ ences were observed in this particular case.

Also shown in Fig. 4 are the keratin banding patterns obtained from the horns of th black and white rhinoceros as well as bovine horn. Samples of black rhinoceros horn illustrated in Fig. 4 were obtained from the anterior and posterior horns, respectively. The color differences observed on the original photographs shown in Figs. 3 and 4 may be due to insufficient removal of ampholytes before silver staining. It is also evident from inspection of the bands in the pH region between 2.5 and 3.0 that bovine horn can be readily distinguished from horn obtained from both rhinoceros species. In contrast to the

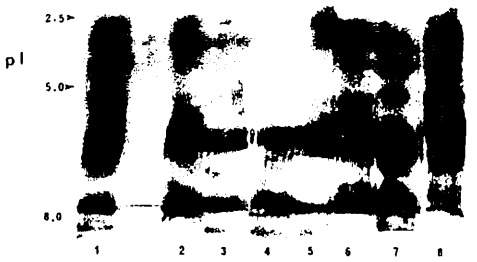

FIG. 2—*IEF gel illustrating keratin patterns of samples obtained from* (1) *moose hair,* (2) *elk hair,* (3) *antelope hair,* (4, 5) *bovine horn,* (6) *deer hair,* (7) *goat hair, and* (8) *raccoon hair.*

FIG. 3—*IEF gel illustrating keratin patterns of samples obtained from* (1) *bovine horn,* (2) *black rhino horn (anterior),* (3) *black rhino horn (posterior), and* (4) *white rhinoceros horn.*

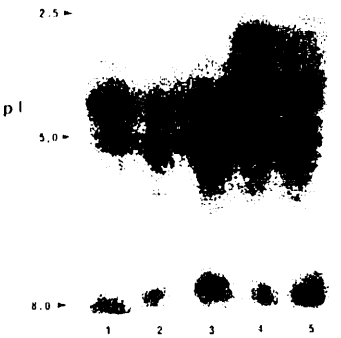

FIG. 4—*IEF polyacrylamide gel illustrating banding patterns of keratins obtained from* (1) *black rhino horn (posterior),* (2) *black rhino horn (anterior),* (3) *white rhinoceros horn, and* (4, 5) *bovine horn.*

position of keratin bands obtained from rhinoceros horn, bands from bovine horn are present in the more acidic region of the gel.

In Fig. 5, the IEF banding patterns of antlers from several species of antelope, as well as black rhinoceros horn (anterior), sheep wool, and bovine hair and horn are shown. The slight curvature of the bands is probably due to excess buffer flooding at the electrode strip, resulting in curved equipotential surfaces produced during the focusing run. In this case also (compare with Fig. 4), the bovine bands are seen in the more acidic region (pH between 2.5 and 3.5), whereas bands from rhinoceros horn are found in the pH region between 3.0 and 5.0.

FIG. 5—*IEF polyacrylamide gel illustrating banding patterns of keratins obtained from* (1–3) *various antelope species (lechwe, roan, and African),* (4) *white rhinoceros,* (5) *Dorset sheep wool,* (6) *human hair, and* (7) *bovine horn.*

The bovine hair and horn banding patterns show a high degree of similarity. Presumably, this reflects the fact that the keratins are derived from similar structures within the same species. Low power light microscopy clearly reveals the compacted, hair-like nature of rhinoceros horn. The antler keratin bands are formed in the same region as the bands from the rhinoceros horn keratins; their distribution is quite different, however, and they are readily distinguished.

In Fig. 6, the IEF banding patterns generated from gels shown in Figs. 1 to 5, as well as others, are depicted schematically. The gels were laser scanned and the resulting densitometric tracings used to construct the schematic patterns. The bands shown are those which most clearly allow differentiation among the species considered in this study.

Discussion

IEF has been successfully used to distinguish between the 25 animal species studied in this report. Although some patterns show a certain degree of similarity, differences between species are clear both in regard to the number, position, and color of the bands. Nevertheless, it was observed that the closer the phylogenetic proximity of the species, the smaller the differences in the patterns. There are often considerable differences between breeds and even occasionally slight individual differences which in no way limit the identification of a particular species as such.

Since this method can be applied to even a single hair, it can be used for species identification in forensic science laboratories. However, in most laboratories trace microscopical examination by an experienced microscopist would probably be more efficient and is nondestructive. An extension of the approach reported in this study could result in better methods for individualization of human hair and should be further investigated in this regard.

Because it is sometimes difficult visually to analyze bands that are closely positioned, it is thought that use of a scanning laser densitometer should be incorporated into the analysis to improve resolution of the banding pattern. By comparing densitometric tracings obtained from multiple keratin banding patterns, similarities and differences can easily be discerned and precise band positions can be denoted. Densitometry also allows

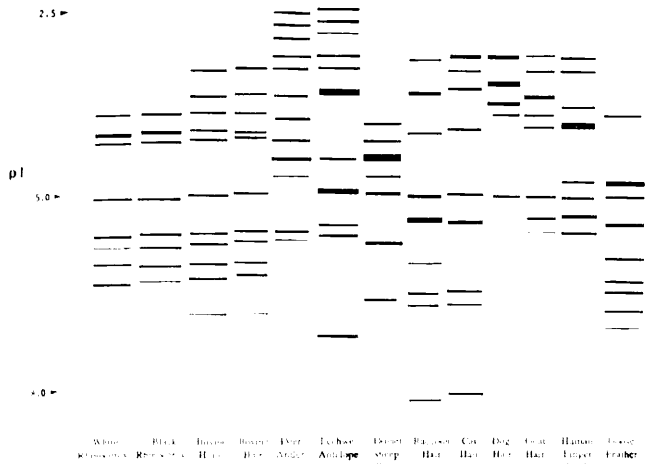

FIG. 6—*Schematic diagram representing keratin banding patterns of many of the samples examined.*

the calculation of relative concentrations of each molecular species of keratin by integration of the various specific peaks seen on each tracing.

The fact that the rhinoceros horn can be distinguished from other types of horn and hair is an important one. All five species of rhinoceros are on the endangered species list. This IEF method will help the forensic scientist to distinguish rhinoceros horn from other similar materials, thus providing a deterrent to illegal game hunting. Hopefully, the rhinoceros will be spared from the threat of extinction at the hand of man.

The usefulness of the method described in this report is still somewhat limited since not all of the samples studied had been obtained in a homogeneous or in a pure form. Unfortunately, in many instances, rhinoceros horn represents only a small fraction of a complex mixture of biological materials, many of which contain keratin, which are packaged and illegally imported for "medicinal purposes." Analysis of these samples after keratin extraction often reveals a confusing pattern which is difficult to phenotype and results are usually inconclusive. Better extraction methods must therefore be developed before the IEF method can be put into routine use for species identification. We have found that despite our attempts to replicate the sample handling and extraction procedure to ensure that the final concentration of keratin placed on the gel is always the same, occasionally banding patterns will appear either more or less intense than expected. Further studies are needed to develop procedures that will result in consistent band intensities and that will enhance the sensitivity of the technique so that even small samples, in the microgram range, can still be successfully analyzed.

In this day and age when many species of animal are near extinction (because of the commercial value placed on a much-wanted body part which they possess) we still see the frivolous and carefree attitudes of man; instead of preserving our natural wildlife, he has become the major factor in bringing many types of wildlife to the brink of

extinction, solely for financial gain. Further studies should be performed on additional species so that a database can be established which would facilitate species identification by IEF analysis of keratins. This technique will not help save the particular rhinoceros whose horn is being subjected to analysis; however, it will serve as a tool for wildlife agencies to use in investigating wildlife product evidence and to facilitate the punishment of perpetrators of illegal game hunting. It is hoped that this method may contribute in a significant way to giving the existing rhinoceroses a chance for survival.

Acknowledgments

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The Cocaine Body-Packer Syndrome: Evaluation of a Method of Contrast Study of the Bowel

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ABSTRACT: The questionable reliability of the conventional procedures for detection of ingested drug packages triggered us to evaluate the accuracy of a method of contrast study of the bowel in 23 nonsurgically managed cocaine body packers. A single dose (60 mL) of a water soluble contrast compound (amidotrizoate + meglumine) was given orally after initial clinical examination and drug detection in urine. Thereafter, roentgenograms were performed daily after spontaneous passage until obtaining two packet-free stools and negative views. Roentgenograms showed packages when performed at least 3 h after the ingestion of the contrast compound. Sensitivity and specificity of the method with respect to the detection of residual packets in the body, assessed by subsequent examination of stools, was good and did not diminish as the number of packages decreased during the time spent in ward. No side effects were observed. We conclude that oral administration of a water soluble contrast compound is an easily performed, efficient, and safe method for the nonsurgical management of cocaine body-packers.

KEYWORDS: criminalistics, cocaine, X-ray analysis, body-packing, contrast study of the bowel, water-soluble compound, drug smuggling

The smuggling of illicit drugs is increasing throughout Western countries [*1,2*]. Drug determination in urine is a fairly reliable test for detection of body-packers, but positive results are not diagnostic per se [*3*]. In all cases, confirmation of body-packing by subsequent radiological investigations is required [*4–9*]. Ordinary plain films of the abdomen are commonly used, but the percentage of false negatives is substantial [*5,6,9*]. A contrast study of the bowel after oral barium administration has been used in a few cases, but the procedure was judged as being too cumbersome [*5,9*]. In rare reports, a contrast method using a water-soluble compound has been said to be helpful when radiolucent foreign bodies could not be identified otherwise [*8,10*]. Furthermore, during the course of elimination of cocaine packages, spontaneous elimination is now considered to be a safe and simple alternative to surgical treatment of the body-packers [*6,8*].

However, the medical management requires that the physician observe the patient for two main complications of the ingestion of cocaine packages: namely, acute cocaine

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