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Effect of Argentine Ant Contamination on ABO Blood Typing of Human Saliva Samples

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ABSTRACT Ants are among the many small invertebrates that readily come into contact with materials later collected as evidence at the scene of a crime. Ants perhaps might contaminate these materials and produce spurious results resembling human ABO blood types in subsequent laboratory analyses. In one such case, we collected Argentine ants, *Iridomyrmex humilis* (Mayr), from the site of a rape and murder. Dried saliva found on the victim's body and subjected to laboratory analysis was reported to have produced antigenic reactions characteristic of blood type B. This blood type did not agree with that of the victim, known associates, or the individuals accused of the crime. However, ants had been noted on the body at the time of its discovery. Our absorption/inhibition and absorption/elution assays of Argentine ant body parts and of materials fed upon or walked upon by ants demonstrate that crushed heads and/or thoraxes of *I. humilis* produce a false positive type A response. This does not appear to be associated directly with digestive tract contents although clover honey which they were fed in the laboratory produced a similar false response. None of the materials walked upon by Argentine ants, including slides and filter papers coated with the saliva of an AB nonsecretor, gave any antigenic response, although our test for trail-following behavior demonstrated that *I. humilis* deposited trail-marking compounds.

EVIDENCE CONCERNING insects is most frequently introduced in criminal court proceedings in order to establish time of death on the basis of the development of dipterous larvae (maggots) (see reviews by Meek et al. [1984] and Keh [1985]). We report here results from a rather unusual forensic study to investigate the possibility that ants contaminated criminal evidence in one of the rape-murders in Los Angeles, Calif., attributed to the "Hillside Strangler."

Body secretion evidence such as saliva and semen are frequently associated with violent crimes involving rape. Two complementary immunological assays, absorption/inhibition (A/I) and absorption/elution (A/E), are employed with such secretions to detect antigenic activity indicative of the blood type of the biological donor. Results of these assays may aid in the identification of criminal suspects and be introduced as evidence in criminal court proceedings.

The legal aspects of this study are discussed elsewhere (Grunbaum et al. 1984, 1986). Briefly, we wanted to determine whether ants collected from the site of a rape-murder could have interfered with an antigen assay of dried saliva (indicated by a positive starch iodine test for amylase) detected on the body of the victim. At trial (*State of Calif. v. Buono* [1983]), a crime laboratory analyst reported that this material had exhibited antigenic activity indicative of ABO blood type B. However, the victim was reported to have ABO blood type A, and both men accused of the crime were ABO type AB nonsecretors. The blood type of a nonsecretor usually cannot be detected in body secretions. Thus, attorneys for the prosecution were placed in the unusual position of attempting to discount the meaning of this physical evidence and the analysis of it by their own police crime laboratory. In a true role reversal, the defense attorneys wished to sustain the validity of this forensic analysis, since it raised the possibility that an unknown person having blood type B may have been involved in the crime.

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Ants were clearly present in photographs of the body taken at the time of its discovery. Testimony in an earlier trial by a prosecution expert witness (*State of Calif. v. Shively and Crane* [1981]) suggested that antigen-grouping assays of homogenized ant bodies (species unknown) might produce a spurious reaction resembling type B antigenic activity. Such false positive ABO antigenic reactions have been reported from various substances such as wood, house dust, and bacteria (Kind & Lang 1976). The possibility was raised by the prosecution that materials deposited on the body by foraging ants might account for the type B activity.

At the request of the defense attorneys, we collected Argentine ants, *Iridomyrmex humilis* (Mayr), from several colonies in the immediate vicinity of the crime scene on a hillside in the Eaglerock section of Los Angeles, Calif. We were unable to collect any other species of ants in this area, although the 6-year interval between the murder and our collections leaves open the possibility that other species may have been present at that time. Indeed, in later rebuttal testimony for the prosecution a colleague emphasized the likelihood of this possibility. Unfortunately, no insect specimens were collected at the time of the crime and the results of the immunological assays performed at that time did not become an issue until the trial 6 years later.

Although the specific legal question initiating our study was whether materials deposited by ants could mimic antigenic activity indicative of ABO blood type B, we chose to address the more general problem of whether ants or materials deposited by ants could exhibit antigenic responses associated with any human blood types. We extended our investigation to include the ants themselves, their food materials, and the soil in which they tunneled.

Every effort was made to minimize contamination during collection and maintenance of the ant colonies and preparation of samples for immunological analyses. All materials employed were newly purchased and, if not purchased in sterile condition, were rinsed with steam distilled water and 95% ethanol before use. Disposable gloves were worn at all times.

At the crime scene, soil containing the ants was placed in plastic containers (22 by 22 by 15.2 cm) with tightly fitting lids. The containers were transported to the laboratory in Berkeley. Each container was provided with a food source of clover honey (0.5 ml, pure U.S. fancy white,

Empress) in a small plastic petri dish lid (35 by 10 mm) placed on the soil surface. Lids and honey were changed every 3–4 days and colonies were maintained in the laboratory for ca. 1 month before experimentation.

Trail following behavior is well documented in ants (Parry & Morgan 1979) and *I. humilis* is no exception (Wilson and Pavan 1959, Van Vorhis Key et al. 1981). However, legal proceedings place great weight upon direct evidence, and it was necessary from a legal point of view that we demonstrate the capability of the ants we collected to deposit materials on the substrate as they walk. This demonstration was also necessary in order to infer a direct relationship between test substrates exposed to Argentine ants in the laboratory and substrates walked over by ants in the field.

To collect trail pheromone, a foraging arena was established within an ant-rearing container by securing a petri dish containing honey to the top of a glass rod inserted into the soil. Thus, the ants had to walk up the glass rod to reach the honey. A Whatman No. 1 filter paper wrapped tightly around the glass rod collected any materials deposited by the ants as they moved to and from their food source. After 72 h, this filter paper was removed and extracted in 1 ml dichloromethane for 48 h. A clean filter paper was similarly extracted as a control. Twenty behavioral assays were then performed with the treatment solution and 20 with the control solution.

In each behavioral assay, a single ant was placed in a glass petri dish (150 by 20 mm) containing a piece of tracing paper upon which a straight 10-cm artificial trail had been drawn with a microliter syringe containing 10 μl of one of the two test solutions. A positive response was recorded if, during a 20-min period, the ant followed at least half of the 10-cm line and paused upon reaching a terminus. Each ant and each artificial trail was tested only once to eliminate the possibility of trail reinforcement. The results clearly establish that compounds eliciting trail-following are indeed deposited by foraging ants: 17 (85%) of the 20 ants exposed to artificial trails of the treatment extract responded in a positive manner, while none of the 20 ants exposed to the control extract responded in any manner.

An experiment was conducted to rule out the possibility that the substance removed from the victim's body and identified as saliva could have come from an animal other than a human. Detection of amylase by a starch/iodine test is the most extensively used procedure for the identification of salivary stains. Iodine solutions cause starch to turn a deep blue. Absence of blue signals the presence of the starch-hydrolyzing amylase enzyme, which is found in large quantities in human saliva.

Dog saliva, cat saliva, snail track substance, slug track substance, and human saliva controls were placed into wells made in a thin layer of starch gel. The gel was incubated for 24 h at 37°C to permit any amylase to interact with the starch. Subsequently, the gel was flooded with an iodine solution. As expected, the human saliva, which is known to contain a high level of amylase, produced large circular areas free of blue iodine. The dog saliva produced a very small area free of blue. Neither the cat saliva nor the slug or snail track substances produced any reactions. This experiment strengthened the likelihood that the substance removed from the victim's body was human saliva.

To collect materials for immunological testing, three substrates were exposed separately to foraging ants within the rearing containers: Whatman No. 1 filter papers, filter papers coated around the perimeter with a 0.6-ml sputum sample from the defendant (an AB nonsecretor), and glass slides coated with 0.1-ml of the same sputum sample. Each of the test substrates was placed, along with a small (diam, 35 mm) petri dish lid containing 0.5-ml of honey, in an upturned plastic petri dish lid (150 by 15 mm) on the soil surface within the rearing containers. Foraging ants could only reach the honey by traversing the test substrates. Ants were observed traversing all substrates throughout the exposure period. Uncoated filter papers were removed after 72 h of exposure, and the sputum-coated papers and slides after 24 h. These specimens were refrigerated for 2–5 days (4°C) before antigen-grouping analysis.

In addition, 100 ant workers were removed from the rearing containers and separated at their abdominal constriction (petiole) into a head-and-thorax section and an abdomen (gaster) section. Ants had been fed on honey and many (75 ants) had been exposed to AB nonsecretor sputum samples. The 100 heads-and-thoraxes and 100 abdomens were separately macerated in sterile physiological saline and refrigerated (4°C) for 48 h.

Specimens were subjected to both A/I and A/E assays. These assays are indirect immunological methods that are used when the test samples do not contain intact red blood cells. The A/I test is relatively insensitive. The A/E test, although ca. 100-fold more sensitive, is more likely to give false or misleading results. The procedures used are described in detail by the Metropolitan Police Forensic Science Laboratory (1978). Any modifications have been noted.

Saliva specimens of known ABO type were used as controls. In addition to macerated sections of ant bodies, test specimens included filter paper and sputum-covered papers and slides traversed by ants, honey on which the ants had fed for 72 h, soil from the collection site, clean filter paper, clean fresh honey, and saliva from a group AB nonsecretor (the defendant). Each experiment required nine samples from each of these specimens: three samples of the neat extract, three samples diluted to one-half, and three samples diluted 5-fold.

In both the A/I and A/E assays, the test samples were exposed to specific antisera during which time antigen/antibody complex would have formed in the presence of corresponding antigen and antibody (absorption).

According to the A/I method, titered antiserum (anti-A, anti-B, or anti-H lectin) was added to each of the test samples. For example, anti-A was added to one neat extract and two dilutions, anti-B was added to a similar series, and anti-H was added to the remaining three samples. The titer of the antisera had been adjusted so that most of the antibodies would be bound (absorbed) if the corresponding antigen were present in the sample. The specific antiserum would therefore no longer be available for agglutination of indicator cells. Group A, B, or O indicator cells (human red blood cells of known ABO type) were added to the samples that had received the corresponding antisera. Agglutination was read after centrifugation. Inhibition (lack of agglutination) indicated the presence of specific antigens in the control saliva samples. In our experiment, inhibition in any of the test samples was regarded as a pseudoantigenic reaction.

With the A/E method, an excess specific antiserum was added to each test sample at 4°C. If antigens were present in the sample, the homologous antibody would be specifically absorbed. Any unabsorbed antiserum from the specimens was removed by washing. Indicator cells were then added. Dissociation of the antigen and antibody was brought about by subjecting the samples to heating at 56°C (elution). The test samples were then subjected to slow rotation for 30 min at room temperature and the samples were examined under a microscope for agglu-

tination. Agglutination in a series of dilutions is customarily interpreted as an indication of the presence of a specific antigen in the specimen. In our experiment agglutination in the test samples was interpreted as a pseudoantigenic reaction.

Results are shown in Table 1. With the highly sensitive A/E assay, only the macerated ant heads and thoraxes, the fresh honey, and the honey upon which ants had fed exhibited unexpected agglutination. In all three cases, the reactions resembled ABO type A antigenic activity and were not affected by dilution of the samples. The A/I test gave complete "group A" inhibition at all dilutions of the specimen made from ant heads and thoraxes; there was no inhibition with the samples derived from honey. None of the filter papers or slides traversed by the ants exhibited any pseudoantigenic activity, and no reactions resembling type B antigenic activity were observed in any samples except the standard type B control.

We conducted a second antigen grouping assay in order to determine whether the false positive type A activity associated with the ant heads and thoraxes was attributable solely to honey ingestion in the rearing containers. Another group of 100 ants was collected from the crime scene, starved for 24 h and separated into head-and-thorax and abdomen (gaster) sections. Results of antigen grouping assays with these ants, which to our knowledge had never been exposed to honey, were compared with those obtained with a second group of 100 ants which had been fed honey in the laboratory. In the A/I and A/E assays, the heads and thoraxes of both groups of ants exhibited reactions characteristic of type A antigenic activity. The abdomens of ants fed with honey and the starved ants gave no inhibition with the A/I assay and incomplete agglutination with the A/E assay. These results indicate that false positive type A activity was not an experimental artifact resulting from honey consumption in the laboratory, but do not completely exclude contamination of appendages, mouthparts or esophagus by dietary components (e.g., honeydew) consumed in the field. However, ant grooming behavior and the 24-h starvation period before dissection would tend to negate such contamination.

Factors present in the head and/or thorax of *I. humilis* mimic the antigenic activity associated with human blood type A. Our results, however, do not support secretion of these factors by Argentine ants trailing over a substrate. Thus, so long as no ant body parts contaminate forensic samples, Argentine ant trails would appear to have no effect on the ABO grouping of physiological materials of human origin. We found no evidence to support the hypothesis that a type B blood antigen response could result from the presence of *I. humilis* body parts or trails, although the possibility exists that other ant species may produce such a response.

Whether other ant species, other ubiquitous invertebrates or miscellaneous environmental variables have an effect on ABO blood typing remains to be discovered. Certainly, forensic scientists should be aware of the extrinsic factors that could impinge upon their results. The scientific and legal ambiguity of forensic analyses can only be dispelled by a thorough record of the environment at the crime scene and by collection and analysis of any necessary control samples in addition to the primary evidential sample.

Table 1. Apparent ABO phenotype as indicated by antigenic activity

Sample	A/I	A/E
Saline blank	NR	NR
Saliva controls:		
A_2 secretor	AO	AO
A_1 nonsecretor	NR	A
B secretor	B	B
O secretor	O	O
O nonsecretor	NR	NR
AB nonsecretor (defendant's saliva)	NR	NR
Defendant's saliva (AB nonsecretor) from glass slide over which ants tracked for 24 h	ND	NR
Defendant's saliva (AB nonsecretor) on filter paper over which ants tracked for 24 h	NR	NR
Filter papers:		
Not exposed to ants	NR	NR
Exposed to ants for 72 h	NR	NR
Soil from collection site	NR	NR
Honey on which ants fed for 72 h	NR	A
Honey control	NR	A
Iridomyrmex humilis ($N = 100$):		
Heads and thoraxes	A	A
Abdomens	NR	NR

A/E reactions are ca. 100-fold more sensitive than A/I. NR, no reaction; ND, not done.

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