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Insect artifacts are officially categorized as bloodstains produced as a result of insect activity [1]. This definition encompasses any insect that interacts with a corpse or associated exuded blood, although flies, ants and cockroaches are the most common culprits [2]. The problem with fly artifacts in particular is that they can be virtually indistinguishable from certain types of human bloodstains. Insect stains produced by fly regurgitation or fecal elimination are morphologically very similar to impact (i.e., forward, back, and mist-like spatter), projected, sneezed, and expired bloodstains [3-4], and cannot be reliably distinguished using presumptive or confirmatory tests for identification of human blood [5-7]. The use of molecular methods, namely DNA typing for person's identification, does not overcome these limitations since viable human DNA can be extracted from fly stains [7-9]. Insect transfer patterns that result in translocation stains or tarsal tracks are presumed to be chemically indistinguishable from the source (i.e., stain, fluid, or food) that the fly made direct contact with [10]. However, empirical testing of such artifacts has not occurred.

The inability to consistently and reliably distinguish insect artifacts from human body fluid stains represents a serious deficiency with respect to entomological contaminants at crime scenes [10]. There has been modest success with a few methods designed for visual, contextual, or chemical analysis of fly artifacts [11], but none are satisfactory based on several limitations. The deficiencies include a lack of reliability, the fact that no single technique is suitable for all fly species, none make a distinction from other forms of body fluids that may also be present at crime scenes, all are presumptive not confirmatory tests, and assessment of artifact morphology is dependent on a very small pool of forensic experts. In addition, very few forensically important species known worldwide have been assessed by the reported methods for discerning

fly artifacts from human bloodstains or other bodily fluids. This makes it very difficult to come to any consensus on the typical classification of fly artifacts or accurate methods of detection. Some investigators have suggested that use of two methods in conjunction with one another (i.e., presumptive blood testing coupled with visual analysis) might improve the precision in distinguishing fly artifacts from human bloodstains. While in theory this idea makes sense, the reality is that the techniques alone or in combination still should be viewed as inconsistent and non-quantifiable, especially in terms of the visual analysis component. As a consequence, identification of insect artifacts at a crime scene relies on qualitative interpretation to distinguish fly evidence from bloodstains.

One avenue that has not been explored with respect to insect artifacts is the development of confirmatory tests based on the chemical composition of insect-derived stains. Regurgitate stains deposited by *Protophormia terraenovae* Robineau-Desvoidy (Diptera: Calliphoridae) have been shown to possess at least three digestive enzymes (trypsin-like, chymotrypsin-like, and pepsin-like) that were also found in the crop of the adult fly, independent of the food source [12]. The pepsin-like enzyme appears to be a cathepsin D-like proteinase adapted for functionality in the strongly acidic midgut environment of larvae and adult flies [13]. Sequence analyses of digestive cathepsin D-like proteinase from the common house fly *Musca domestica* L. (Diptera: Muscidae) show that the enzyme lacks the proline loop (of motif DxPxPx(G/A)P)) typical of other insects as well as vertebrates, yielding a functional protein similar to vertebrate pepsin [14]. Thus, the cathepsin D-like proteinase in *M. domestica* is functionally similar to vertebrate pepsin but is structurally a cathepsin D aspartic proteinase [15]. This proteinaceous enzyme has been shown to be localized in the crop of adult *P. terraenovae*, and to be deposited in regurgitate after

ingesting bovine or human blood. Cathepsin D-like proteinase is predicted to be present in gut secretions of all cyclorrhaphous Diptera that digest bacteria.

During this grant period, we proposed a program of research aimed at developing a confirmatory diagnostic test based on antibody detection that allows for discrimination between fly artifacts from bloodstains. Our approach 1) focused on polyclonal antibodies generated toward M. dom-3, a synthetic peptide demonstrated in our preliminary research to have the highest reactivity with the synthetic peptide and in detection of enzymes in fly regurgitate during preliminary testing; 2) the resulting antibodies were used to develop western and dot blot detection assays to distinguish fly artifacts from human bloodstains; 3) we characterized the specificity of M. dom-3 antibodies in recognizing fly artifacts (both regurgitate and feces) from several species of flies common to crime scenes in the United States; 4) we examined the potential for polyclonal antibodies to distinguish fly artifacts from other human body fluids, including saliva, semen and urine, and to assess the length of time after stain deposition that the antibodies are useful in discriminating artifacts from human body fluid stains; and 5) evaluated the utility of the polyclonal antibodies in detection of fly artifacts from a range of household materials, including different floor covering (carpet types, ceramic and vinyl tiles, wood floors), wall materials, and furniture fabrics. The experiments were designed to test our hypotheses that fly antibodies generated against antigenic sites associated with the enzyme cathepsin D-like proteinase can be used to discriminate fly artifacts produced by multiple species of flies from human bloodstains and other bodily fluids.

As the first step toward developing a chemical test to recognize fly artifacts, polyclonal antisera were generated in rats against three distinct antigenic sequences of fly cathepsin D-like proteinase, an enzyme that is structurally distinct in cyclorrhaphous Diptera from other animals.

The resulting rat antisera bound to artifacts produced by *Protophormia terraenovae* and synthetic peptides used to generate the polyclonal antisera, but not with any type of mammalian blood tested in immunoassays. Among the three antisera, anti-md3 serum displayed the highest reactivity for fly stains, demonstrated cross-reactivity for all synthetic peptides representing antigenic sequences of the mature fly enzyme, and bound artifacts originating from the fly digestive tract.

The serum was then used to test the hypothesis that digestive artifacts produced by an array of necrophagous flies associated with human decomposition could be detected with the immunoassay. Anti-md3 serum was able to bind artifacts from twenty-seven species of flies representing nine families. The antiserum reacted with both regurgitate and defecatory stains, but not transfer patterns. Stains from four fly species displayed no reactivity with anti-serum in dot blot assays. Anti-md3 serum did not bind to either human or bovine blood stains on filter paper. However, when both types of blood were spiked with synthetic md3 peptide the antiserum was able to bind. Dot blot assays displayed positive reactions with stains produced from larvae and teneral adults of *Sarcophaga bullata*, and with artifacts as old as 7-years after deposition. These observations indicate that the immunoassay permits distinction of artifacts from a wide range of species from human bloodstains, from multiple development stages, and from artifacts that remain at crime scenes for many months to years after deposition. Further work is needed to determine whether the detection of fly artifacts using the antiserum is suitable for non-laboratory conditions.

The confirmatory immunoassay was then used to determine if artifacts produced by four species of necrophagous flies (*Protophormia terraenovae*, *Calliphora vicina*, *Cynomya cadaverina*, and *Sarcophaga bullata*) could be distinguished from a range of human body fluids

(e.g., blood, semen, urine, saliva, and feces). Adult flies were fed *ad libitum* human blood, semen, urine, feces or saliva for 24 h at 25°C and permitted to deposit artifacts on a range of household materials: ceramic tile, carpet (plush), t-shirt (cotton), wood block, and unfinished drywall. A lift technique was developed that permitted transfer of fly artifacts from the test materials to filter paper (Whatman #4 110 mm Ø) for dot blot analyses. Artifact transfers were confirmed visually and with ALS using a 450 nm emission filter and an orange contrast filter. All species readily deposited artifacts on all test household materials regardless of diet consumed. Despite differences in texture and porosity of the household materials, artifacts of all species transferred to saturated filter paper (Dulbecco's PBS) with apparent equal efficiency based on visual inspection. With all fly species, anti-md3 sera bound to artifacts produced after feeding on semen, blood, feces, urine and saliva. Binding appeared proportional to the size of the artifact transferred during the lifts. By contrast, none of the human fluids tested positive in the immunoassays, nor did lifts from household materials not exposed to flies. There was no evidence of false positives with any of the fly species tested, regardless of diet consumed. Similarly, there was also no indication of false negatives with any of the dot blot assays. However, flies did deposit artifacts not derived from the digestive tract on the test materials that, as expected, did not yield positive reactions with the immunoassay. Such artifacts generally cannot be visually distinguished from regurgitate and defecatory stains and thus can yield results perceived as false negatives. These observations suggest that immunoassays using anti-md3 sera coupled with a simple lift technique can be used effectively as a confirmatory assay to distinguish fly regurgitate and fecal stains from human body fluid stains.

Implications for Criminal Justice Policy and Practice in the United States

Current methods of visual, contextual, and chemical analysis of fly artifacts do not permit reliable or quantifiable discrimination between the fly artifacts and human body fluid stains [11]. The results from this basic research will lead to the development of a detection assay that will overcome the deficiencies of current methods. The antibody detection assay developed by this research will not only lead to a confirmatory test for fly artifacts at crime scenes but is anticipated to have similar ease of use as presumptive blood tests, will be highly specific, repeatable, quantifiable, and will not require extensive training to use. Thus, any crime scene responders will be able to perform the tests, permitting widespread adoption across the U.S. This in turn will lead to independent and unbiased forensic analysis.

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