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# Turbidity: The Answer to Inconclusive "Ring Test" For Forensic Samples

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## Introduction

Blood is one of the most important and prevailing biological evidence commonly encountered by investigators at the scene of a crime. It is present in several forms such as liquid blood stains, adulterated with other biological and/or physical pieces of evidence, and blood patterns. Species identification of bloodstain found at the crime scene is of paramount importance in forensic investigation since it helps in solving the crime of violence, and accidental cases and it provides the link between the victim, suspect, and the place of commission. Human and animal blood are indistinguishable to the naked eye, however, divergent at the biochemical and molecular levels. Though omnipotent, DNA typing has some inherent limitations in forensic cases as it is unable to establish the nature of the biological material which may or may not be important in a particular case. The importance of identification versus typing tests with an evidence item must be considered in the context of the case. The identification and species-determination aspects of a forensic examination can sometimes be more important for a suspected hit-and-run case. The suspect may be absolved of suspicion by finding that bloodstains on his vehicle were of nonhuman origin. The earliest precipitin test method was the "ring test," [1], where an aqueous solution of antigen is layered over the denser antiserum solution in a tube. The formation of precipitate at the interface between the layers indicates a positive test. The precipitin test can also be done using the antisera, raised against the hemoglobin of the species, which detects the origin of species as well as confirms the presence of blood. Unfortunately, the blood stain often found in the crime scene is dried and is not a true mixture of blood cells and serum. Depending on the clotting time (before drying or after drying) the ratio of cell/serum often varies, which prevents the formation of a proper "Ring' in the "Ring Test" [2]. In addition, multiple factors, such as ionic strength, temperature, incubation time, and pH limit the efficacy and sensitivity of the test and have a direct influence on the formation of the precipitin band. A recent publication concluded that human blood was sensitive to the precipitin test but only for a specific period (up to 32$^{nd}$ day) [3]. The surface of the object and the presence or absence of antigen in the blood did affect the 'ring formation' for the anti-human serum [4]. Therefore, this method was largely abandoned when gel-based methods, such as Double Diffusion' were developed, which was also based on the same principle and takes approximately. 24-48 hours to detect [5]. Although the immune-diffusion assay is highly sensitive, this technique requires high concentrations of both antigen and antibody and is relatively insensitive to antibodies with low affinities [6].

Following the precipitin test, identified by Paul Uhlenhuth in 1990 [1], presently multiple advanced techniques have been employed including microscopic examination, immunological method, chemical examination, spectrophotometric analysis, etc [7-10]. For the qualitative and semi-quantitative determination of blood, the crossed-over electrophoresis technique is also used. In this technique, under the influence of an electric field, the antigen and the antibody migrate toward each other, and precipitin is formed at the point of their interaction. This method is comparatively much more rapid and gives a higher electrophoretic resolution and better quantitative information than classical immune electrophoresis, however, this method requires a lot of other buffers, chemicals, and a gel appararus to perform that [11].

The advancement continues and some of the highly specific anti-hemoglobin precipitin sera have been used for the identification of human bloodstain in a single procedure. Other methods that include an iso-enzymes method and rapid immunoassay are also used. Immunoassay test for human blood involves the reaction of antigens in the extract with monoclonal

antibodies within the test strip, resulting in the extract antigen-antibody complex which reacts with dye particles to create suitable visible reactions.

The rapid Immunoassay methods is considered a highly specific and sensitive assay that confirms the existence of human blood in stain extracts. These assays are easily performed, applicable to various types of samples, and produce results in a timely manner, usually within minutes of application to the test strips [12]. This last feature allows for confirmation of blood in supernatants of samples which will be subjected to DNA analysis prior to testing, without consumption of stain portions necessary for obtaining DNA. Such rapid assays, like ABACard, Hematrace, and Hemdirect Hemoglobin method also prevent extensive delays in genetic marker testing. However, the disadvantage of this method was the 'high dosage' effect, which may yield false negative results and usually cross-react with the protein homologs in higher primates.

Presently, the traditional precipitin tests have been largely supplemented by lateral flow immunochromatographic assays targeting either hemoglobin or glycophorin A, a red cell membrane Since, the. The species specificity of the hemoglobin test is not absolute; in addition to the expected cross-reactions with primate hemoglobin, false positive results have been observed with hemoglobin from ferrets and several other non-primate species. However, no cross-reactions have been reported for the glycophorin A test. Spectrophotometric identification of hemoglobin also confirms the species origin of the blood stain. Some spectroscopic techniques, including Infrared Raman spectroscopy, and spectrophotometry are employed to analyze multiple dry blood samples for species identification. The various forms of hemoglobin (oxyHb, deoxy-Hb, metHB, etc.) have characteristic absorption spectra in the visible range; a strong absorption peak (the Soret band) in the 400-425nm range, and one or two weaker absorption peaks (α and β bands) in the 500-650nm range. The presence of hemoglobin is characterized by the strong single peak of the Soret band with its narrow spectral range. Since the porphyrin compounds and their derivatives from animal or plant sources may share similar spectral characteristics with hematin, hemochromogen, or hemoglobin, modernization has taken place by utilization of modern analytical methods based on the combination of Raman Spectroscopy and advanced statistics to analyze the composition of blood traces from several species [13-15]. Despite the proven sensitivity and accuracy the routine day-to-day utility of the latest methods is largely limited by the unaffordable expenses of the kits. Furthermore, these kits are applicable only for human origin.

It is our view, that 'Turbidity measurement' not the 'Ring formation' is the answer in the "Precipitin" test for forensic samples, where most of the parameters are not ideal. One hundred (100) samples of dried/aged forensic blood stains were taken, suspended in 0.9% saline solution, centrifuged and the supernatant has been used as an antigen for further analysis. Around 80 samples were found to be of human origin by double diffusion. Those samples were subjected to the 'Ring Test'. Within 10 minutes, a visibly clear turbidity could be observed, at room temperature, instead of proper 'ring formation', which was measurable against respective negative control, at the visible range (403nm) by using a normal spectrophotometer. Within 45 minutes the turbidity increased almost 1.7-1.9-fold, and remained static afterwards, for various forensic samples. This method can be useful in all forensic analysis, especially in 'open & shut 'cases. Moreover, it is equally applicable to other biological fluids, such as semen & saliva. Therefore, turbidity measurement of the antigen-antibody complex may be the more effective tool for species determination of the forensic aged samples as it takes less time and requires minimum instruments to get a conclusive result.

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