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Technical Note

Case study: Loss of Kastle-Meyer test specificity on jeans

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ABSTRACT

A pair of jeans produced false positive results upon testing for the presence of blood using the Kastle-Meyer (KM) test. Positive reactions were obtained from all unstained areas of the fabric tested. The peroxidase used in the manufacture of some jeans may be the causative agent for the observed false positive reactions; however, it was not possible to confirm this theory.

1. Introduction

The Kastle-Meyer (KM) test is a presumptive test for blood that is widely used in forensic science. Peroxidases are a class of enzymes that catalyze the oxidation of compounds in the presence of a peroxide. Hemoglobin has a peroxidase activity, which forms the basis of the KM test, by catalyzing the oxidation of phenolphthalin in the presence of hydrogen peroxide, producing a pink colour [1]. Chemical oxidants produce a colour change before the addition of hydrogen peroxide, and can therefore be eliminated as a source of false positive results. A variety of studies examining common stain sources including other bodily fluids, fruits, vegetables and various chemicals demonstrated that, in contrast to benzidine, the KM reaction is highly specific [1–5]. Rare examples of false positive KM reactions have been observed from crushed root nodules from legume plants (e.g. pea, bean, clover, alfalfa) as they contain leghemoglobin [6]. Stains from these substances are not, however, blood-like in appearance. Consequently, the Centre of Forensic Sciences considers a positive KM test specific for blood when conducted as a three-stage test on staining with a blood-like appearance.

2. Casework example

A pair of dark blue jeans worn by an individual involved in an alleged blood-letting incident was received by the laboratory. Blood-like staining was observed along the front middle section of the left leg, with a few additional light to moderate red-brown stains on the right knee, inside the front pockets, and one on the back left buttock (Fig. 1). KM rub testing was performed on representative areas by sequentially applying a drop each of methanol (Acros Organics), phenolphthalin solution (prepared in-house following SOP; 0.5% *w/v* phenolphthalein from EMD Chemicals, 10% w/v sodium hydroxide from Fisher Scientific, 2% w/v zinc from Sigma-Aldrich, boiled under reflux until clear), and 6% hydrogen peroxide (EMD Millipore) to a tiny amount of a suspected bloodstain rubbed onto a filter paper. These stains all tested positive using the KM test. A number of yellowish stains that did not appear obviously blood-like also tested KM positive. This prompted the testing of multiple areas on the jeans (not all shown in Fig. 1) that appeared unstained under a 4× magnification light, all of which produced positive results.

KM reagents are tested at the beginning of each workday using a known bloodstain as positive control and a clean filter paper as negative control. Given the unusual results obtained on the exhibit jeans, reagents were further tested using a negative control denim sample from a separate pair of jeans and were confirmed to be working appropriately. A second set of KM reagents and a new box of filter papers were also used to test unstained areas on the exhibit jeans, with reproducible KM positive results.

It is not uncommon to obtain a KM positive result from an unstained area on an item when abundant bloodstaining is present in other areas, due to transfer of small amounts of dried blood when the item is folded. However, it is unexpected to obtain a positive reaction from numerous tests, and in this case, all tests performed on the item where blood did not appear to be present, given that the item was not excessively bloodstained. Approximately 50 KM tests were done on representative unstained areas on the outside front and back, inside front and back pockets, and inside surfaces, all with positive results. A pink colour only appeared after addition of the third chemical, and its intensity

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Fig. 1. Inside front pocket (top photo) and outside back pocket (bottom photo) of exhibit jeans showing blood-like staining (B) and unstained areas (C) tested by KM, ABAcard® HemaTrace®, and DNA analysis.

increased as is observed with an enzymatic reaction. The reactions were indistinguishable from those obtained from bloodstains (i.e. observed within 10s), and were stronger (immediate) as a greater amount of indigo dye transferred to the filter paper from the denim material.

As it appeared that the KM test could not be relied upon to indicate blood on this particular item, an alternate test was employed. The ABAcard® HemaTrace® test is an immunochromatographic test for human blood based on an antibody complex formed with hemoglobin [7]. Positive ABAcard® HemaTrace® results were obtained from staining with a blood-like appearance and negative results were obtained from unstained areas tested away from the blood-like staining (Fig. 1). Given that the ABAcard® HemaTrace® test is more sensitive than the KM rub test [7,8], it is unlikely that trace blood was present in the unstained tested areas. DNA analysis showed the tested blood-like stains (1256–5128 pg/μL) had on average 67 times more DNA than their unstained controls (31–67 pg/μL). Blood was ultimately reported as being present on the jeans considering the results of the ABAcard® HemaTrace® test together with the appearance of the staining and the amount of DNA obtained.

Although the ABAcard® HemaTrace® test was useful in this circumstance, it is not a test that is used to screen items for the presence of blood. When compared to the KM test, the ABAcard® HemaTrace® test is more costly (a kit is utilized versus a few drops of solutions), and also more time consuming to perform (up to 15 min per test versus several seconds). Given that numerous tests may be performed to examine an item for the presence of blood, it is not feasible to use the ABAcard® HemaTrace® test routinely.

3. Follow-up testing

The implications of a false positive KM test on an item that may be routinely encountered in casework prompted an investigation to account for these observations, to determine whether this may be a more widespread phenomenon.

It was discovered that peroxidases may be used in the manufacture of jeans. For example, Novozymes, a company that supplies enzymes to the textile industry, introduced a new product in 2014 called DeniLite® Cold. This uses peroxidase to bleach denim to create different colour tones, improving upon traditional enzymatic bleaching technology that uses laccases [9]. Additionally, cellulases have been shown to re-adsorb on the fabric and bind to indigo causing back-staining during industrial denim washing [10–12]. It was hypothesized that peroxidase added during the manufacturing process could bind to indigo dye and remain immobilized on the denim substrate; this source of peroxidase could oxidize the phenolphthalin with the addition of KM hydrogen peroxide [13], rather than hemoglobin, causing a positive KM test from unstained areas of the jeans.

An attempt was made to obtain a new pair of jeans of the same brand and style as that of the exhibit described above. However, a representative from the company indicated that this 2012 style was no longer available. Furthermore, the jeans were produced by a factory in Cambodia that closed in 2015, and the company representative could not determine whether peroxidase had been used during its industrial wash, nor was there an opportunity to obtain a sample of denim from this factory.

A pair of jeans of the same brand, processed using peroxidase in a Mexican factory was acquired. The denim tone was much lighter blue than that of the exhibit jeans, and KM testing of the outside and inside surfaces produced negative results. Further KM testing was conducted on a variety of jean styles of the same brand, and various jean brands being sold in retail stores, all with negative results.

A sample of DeniLite® Cold (30–50% sodium percarbonate, 5–10% violuric acid, < 5% peroxidase) was obtained from Novozymes (Tianjin, China). A 0.3% solution of these granules dissolved in water (a concentration within the range of manufacturing conditions) gave a positive KM reaction after the addition of phenolphthalin (since sodium percarbonate, a component of DeniLite® Cold, is an adduct of sodium carbonate and hydrogen peroxide). New denim swatches soaked in the solution for 45 min did not test positive, nor did the solution itself after 45 min had elapsed. A swatch that was soaked for 2 min only in a new solution tested KM positive when first removed, but no longer tested positive 45 min later (although still wet), nor when dried.

No evidence was found to confirm that peroxidase used in denim manufacturing caused the false positive KM test results observed in casework. One cannot rule out other compounds to explain the false positive results, from complex chemistry that continues to evolve in denim manufacturing, or from other sources to which this pair of jeans may have been exposed. To that end, one method used in the past to dye cotton yarns involved adding indigo to a mixture of ferrous sulphate with lime [14]. In this process, a large quantity of indigo is lost due to formation of an insoluble complex between dye and iron [15]. The decomposition of hydrogen peroxide by ferrous iron produces a highly oxidizing hydroxyl radical in what is known as the Fenton reaction [16]. Venneman et al. [17] described false positive results when adding KM reagents to a 10% solution of ferrous sulphate, although it was seen as a pink ring around an orange-brown centre. We rule this out as a cause of our observed false positives, as an orange-brown colour change is expected prior to addition of hydrogen peroxide. No such colour changes were observed when testing the exhibit jeans described in this case study.

Ultimately, it is important to remain aware that even with tests that are routinely used in the laboratory, and have proven to be robust over time, conditions may exist that produce false positive results, as was observed here. Recognizing such circumstances and performing

requisite control testing are key to ensuring the validity of any reported result.

Conflict of interest

The authors declare no conflicts of interest or disclaimers.
This work has not been presented previously.

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