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Original Article

Impact of Age and Microbial Growth on the Accuracy of Preliminary Tests and Blood Grouping of Dried Blood Stains in Forensic Investigations

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ABSTRACT

The purpose of this research was to determine the impact of age and microbial growth on the benzidine and phenolphthalein tests along with blood grouping. The paper compared the efficacy of the absorption elution method to that of the ammonia extraction method for blood grouping. In this study, 50 bloodstained samples spanning a decade in age were tested. The accuracy of the benzidine test was 90% and that of the phenolphthalein test was 86%. It was found that age played no role in both preliminary tests nor in blood grouping. Ammonia extraction proved to be more effective than absorption elution with an accuracy of 69% versus 61%. Owing to the absence of impact of age on the samples, microbial growth was studied in detail. On *in-vitro* culturing, samples showed the presence of various cultures including bacteria, fungi such as *Aspergillus* and *Penicillium* and an abundance of yeasts. Based on the experimental findings, it is evident that even on samples with no visible contamination, extensive microbial colonisation can occur. Such growth can also effect the efficiency of performed tests producing false positives and negatives. This highlights the issue of necessary reformations to current practices to improve quality of investigations.

Keywords: Blood, Benzidine test, Phenolphthalein test, Absorption elution, Ammonia extraction, Microbial contamination, Forensic serology.

INTRODUCTION

The study aimed at analysing the impact that age and microbial contamination could have on-dried bloodstains. This study also discusses the efficacy of the benzidine and phenolphthalein tests on old bloodstains. Since any microbial colony present would release metabolites as part of their growth process[1], the research conducted determined if these products would affect the samples in any way that would potentially affect future investigations. During the course of the research, the accuracy of the absorption elution method and ammonia extraction method was also compared.

In 1908, DelCarde and Benoit[2] performed the phenolphthalein test on old and putrefied blood, obtaining positive results on a control bloodstain which was 26 years old. Robert P. Spalding, during the course of his work with the FBI, found that the phenolphthalein and benzidine tests gave positive results on bloodstains up to 56 years old. Kind[3] had detailed the absorption elution process for dried blood crusts, with Outteridge[4] and Ueno[2] proposing later modifications for his work. Studies by Kind and Cleevely[5] and later Kind and Land[27] examined the action of the ammonia extraction method on bloodstains. Yada[7] and Benciolini and Cortivo[27] stressed the possibility of contamination affecting blood grouping results. Gerbal and Ropars[8] as well as Yip *et al.*[9] have detailed the occurrence of the acquired B phenotype, which is a direct effect of bacterial contamination. Studies conducted by Pfaller *et al.*[10] and Osman *et al.*[11] highlighted various organisms that were found colonising biological samples, which included fungi, bacteria and yeasts.

METHODOLOGY

Sampling

The samples used in the course of this study were obtained with permission from the Department of Forensic Medicine, St. John's Medical College, Bengaluru. A total of 50 bloodstained cloth samples were collected in separate sealed and labelled airtight pouches, using sterile cutting implements. A total of 50 control cloth samples were collected and stored in a similar manner. The sampling process was done using random sampling and all details of the persons to whom the clothing belonged were kept confidential.

Preliminary tests – Reagent preparation

Two preliminary tests were performed: benzidine and phenolphthalein. For the benzidine test 0.25 g of benzidine was mixed with 25 ml of glacial acetic acid. The stock solution for the phenolphthalein test was prepared by boiling 2 g of phenolphthalein, 20 g of potassium hydroxide and 20 g of zinc dust in 100 ml distilled water till the formation of a clear and colourless solution. On cooling, the above solution was decanted and stored at 4 °C. The working solution was prepared by mixing 2 ml of stock solution with 2 ml of ethanol and 10 ml distilled water. In both tests, 3% hydrogen peroxide was used to facilitate the reaction.

Preliminary tests – Execution

For the benzidine test, a small piece of the bloodstained sample was placed against a white background and one drop of the prepared benzidine reagent was added, followed by one drop of 3% hydrogen peroxide. A positive result was recorded whenever immediate blue colouration was observed. The same procedure was adopted for the control samples. In the phenolphthalein test, one drop of the working solution followed by one drop of 3% hydrogen peroxide was added to a small piece of the bloodstained sample, placed on a white background. In this case, the positive result was recorded when an immediate intense pink colour was observed[12].

Absorption Elution and Ammonia Extraction Methods

Indicator cell suspensions of blood groups A and B were prepared in 0.85% saline, by centrifuging thrice at 4,000 rpm for 3 min to prepare a stock solution. The resultant stock solution was suspended in 50 drops of 0.85% saline to prepare a working solution.

To perform the ammonia extraction procedure, 5 x 5 mm pieces of the fabric samples were cut and placed in labelled wells of a porcelain cavity plate. The blood was extracted using four to five drops of 5% ammonia solution. The extracts were transferred to a separate cavity plate, heat-fixed on a water bath at 56°C, treated with a single drop of corresponding anti-serum and placed in a room temperature moisture box for 5 min.[13] Excess anti-serum was blotted away using Whatmann's filter paper and placed in a normal saline container at 4°C for 10 min. The plates were removed, blotted dry and a drop of corresponding indicator cell suspension was added to the wells. Finally the plates were placed in a moisture chamber at 37°C for 15 min before their subsequent removal and transfer to a room temperature moisture chamber. The plates were rotated and the results were examined.

The absorption elution method was conducted by soaking 2 × 2 mm pieces of bloodstained samples overnight in anti-sera in Eppendorf tubes at 4°C. Ice-cold saline was used for washing the samples and a single drop of fresh saline was added to the samples before they were placed in a hot water bath at 56°C for 15 min. On removal of the tubes, a single drop of indicator cell suspension was added and the tubes were placed at 4°C for half an hour before they were centrifuged at 2,000 rpm for 1 min.[13]

Microbial Growth

Nutrient Agar (pH 7) and Potato Dextrose Agar (pH 5.6) were prepared and sterilised using an autoclave, along with the requisite glassware, cotton swabs and distilled water. The melted media was poured in labelled petri plates and allowed to solidify. Cotton swabs were dipped in distilled water, swabbed over the samples and then swabbed over the surface of the media in a zig-zag manner. A 2 × 2 mm piece of the bloodstained region of fabric was placed at the centre of each corresponding plate. The Nutrient Agar plates were incubated at 37°C for two days and the Potato Dextrose Agar plates at 24°C for a week. Observed bacterial colonies were stained using Gram's stain whereas fungal colonies were stained using Lactophenol Cotton Blue.

RESULTS

The results of the conducted blood grouping tests, microbial analysis and preliminary tests are represented in Tables 1, 2 and 3 respectively.

No growth was observed in the Nutrient Agar plates. However, with the help of both microscopic and macroscopic analysis, the above was observed in the Potato Dextrose Agar plates. Although bacterial colonies were observed (both spherical and rod shaped), it was not possible to identify the colonies (Table 2).

The results of the conducted preliminary tests are as shown below in Table 3.

DISCUSSION

Discrepancies that were observed between experimental results and reference data were explained on several bases. It was postulated that there might have been potential manual error owing to release of dye from the fabric and incomplete blotting with the Whatmann's filter paper. Dried blood in the wells closely resembled agglutination until it was teased with a pair of needles. Cross-contamination was avoided at all stages during experimental testing; however, improper storage conditions could have contributed to initial contamination and degradation. Bacterial contaminants could sometimes resemble antigenic determinants which could have produced a false positive for certain blood groups[14].

The age and possible treatments[15] provided to the owners of the clothing might have impacted testing as well. In the case of absorption elution, slight temperature fluctuations were unavoidable due to a heat wave. Finally, pH of the anti-sera used could have played a role in the errors noted[9].

On comparing the results obtained for absorption elution and ammonia extraction, an agreement of 70% was found between the two methods. It was postulated that if samples wherein the data from the hospital were not available could be cross-referenced, then the accuracy percentages would increase[16]. Based on a study conducted by Yip *et al*.[9], absence of the A antigen which was observed in some samples could be explained. In their study it was postulated that when the A sites were engaged due to activity of bacterial enzymes, such as

Table 1: Results of the conducted blood grouping tests

Sample No.	Year	Hospital data	Ammonia extraction	Absorption elution
1.	2011	NA	O	O
2.	2011	NA	B	B
3.	2012	O	O	O
4.	2012	NA	A	A
5.	2011	NA	O	O
6.	2011	B	B	O
7.	2011	B	O	B
8.	2011	NA	O	O
9.	2011	B	B	AB
10.	2011	B	B	O
11.	2012	NA	O	B
12.	2012	O	O	O
13.	2012	O	O	O
14.	2012	B	A	A
15.	2012	O	O	O
16.	2011	A	A	O
17.	2011	NA	B	B
18.	2011	O	AB	A
19.	2011	NA	B	B
20.	2011	AB	AB	A
21.	2012	B	B	B
22.	2012	A	A	A
23.	2006	NA	AB	AB
24.	2012	B	AB	B
25.	2012	O	O	O
26.	2011	B	B	B
27.	2011	NA	O	AB
28.	2011	O	O	O
29.	2012	NA	O	O
30.	2012	B	AB	O
31.	2012	NA	O	O
32.	2011	NA	A	A
33.	2012	A	A	A
34.	2011	O	O	O
35.	2011	O	O	O
36.	2012	NA	A	A
37.	2012	NA	B	B
38.	2011	NA	B	B

Table 1 cont.....

Sample No.	Year	Hospital data	Ammonia extraction	Absorption elution
39.	2011	NA	B	B
40.	2012	A	A	A
41.	2013	NA	A	O
42.	2012	NA	A	A
43.	2012	NA	B	B
44.	2012	A	AB	B
45.	2011	O	B	O
46.	2011	NA	A	A
47.	2011	B	AB	B
48.	2014	NA	B	B
49.	–	NA	O	O
50.	–	NA	O	O

deacetylase, they would no longer be free to react with the A antigen and hence would test negative for the same.

In both of the cases, it did not appear, though the age of the samples was affecting the accuracy of the blood grouping tests. Instead, microbial growth and storage conditions appeared to have a greater impact. This is in accordance with studies conducted by Yada[7] and Benciolini and Cortivo[27]. Yada showed that irrespective of the age of the samples, the blood group could be determined and both studies independently proved that microbial contamination was responsible for discrepancies in the results.

Although no growth was observed on the Nutrient Agar plates, extensive growth was observed on the Potato Dextrose Agar plates, possibly due to the fact that the former is an extremely basic medium.

The predominant organism that was found growing on the plates was yeast, followed by the reported fungi. Most of these organisms can be found growing on blood and yeast is a common part of the human micro-flora[17, 18]. In the study, yeast grew most rapidly and occupied the majority of the plate area, often showing antagonistic

Table 3: Results of conducted preliminary tests

Sample	Age	Benzidine test	Phenolphthalein test
1	NA	+	+
2	2011	+	+
3	2012	+	+
4	2012	+	+
5	2011	–	–
6	2011	–	–
7	2011	+	+
8	2011	+	+
9	2011	+	+
10	2011	–	–
11	2012	+	+
12	2012	+	+
13	2012	+	+
14	2012	+	+
15	2012	+	+
16	2011	+	+
17	2011	+	+
18	2011	+	+
19	2011	+	+
20	2011	–	–
21	2012	+	+
22	2012	+	–
23	2006	+	+
24	2012	+	+
25	2012	+	+
26	2011	+	-
27	2011	+	+
28	2011	+	+
29	2012	+	+
30	2012	+	+
31	2012	+	+
32	2011	+	+
33	2012	+	+
34	2011	+	+

Table 2: Results of the microbial growth analysis

Name	Aspergillus	Penicillium	Mucor	Cladosporium	Trichoderma	Yeast
Number	12	12	17	12	16	44

Table 3 cont.....

Sample	Age	Benzidine test	Phenolphthalein test
35	2011	+	+
36	2012	+	+
37	2012	+	+
38	2011	+	+
39	2011	+	+
40	2012	+	+
41	2013	–	–
42	2012	+	+
43	2012	+	+
44	2012	+	+
45	2011	+	+
46	2011	+	+
47	2011	+	+
48	2014	+	+
49	NA	+	+
50	NA	+	+

Note: In all three tables, the abbreviation 'NA' refers to 'Not Available', wherein the data were not immediately available for inclusion in the study.

action towards the other fungi. On prolonged incubation, it was assumed that this antagonism would intensify.

Acidic pH produced by decomposing bodies is ideal for fungi to proliferate[19]. Several fungi that affect humans or grow on blood are slow growing and take several weeks to grow[20], which was seen in the case of *Cladosporium*. Sporulation was not seen in some cases because it often takes a nutritionally deficient medium to facilitate the formation of spores[21].

Given the conditions of storage[22] and lack of a viable food source for bacteria on the clothing, it was not expected that there would be bacteria growing on the media. The Nutrient Agar medium showed no growth and the pH of the Potato Dextrose Agar was set to 5.6 to enable better growth of fungi[23]. However, there are some explanations, such as a mutation to use a component of blood, such as the heme group[24], which might account for the pink colouration of the bacterial colonies.

This ability to use the products obtained on break down and degradation would also explain the presence of the bacteria on an acidic medium, when most bacteria prefer a neutral pH[25]. The bacteria continued to grow even in the presence of fungi, which was interesting as normally, many fungi such as *Penicillium* would have produced substances which would have inhibited the growth of bacteria[26]. The presence of the cocci and the bacilli is of importance because *Staphylococci* and gram negative rods are two of the species of bacteria known to affect blood grouping results[15].

The benzidine test showed accuracies of 90% and 86% of the samples which were positive for the phenolphthalein test. In the phenolphthalein test, sample 11 gave a positive result even for the control. The oldest sample (2006) gave a positive result for both the preliminary tests. Two out of the 50 samples were negative for both the preliminary tests.

The reasons for the negative results on the samples could be due to improper preservation of the samples as they were bundled up and sealed in plastic bags. Cross contamination due to this could have been the reason for the control sample giving a positive result in the preliminary test. The bacterial and fungal growth found on some samples could also be the reason for obtaining negative results in the preliminary tests. Degradation of haemoglobin in the bloodstains due to factors such as exposure to sunlight, chemical reagents or other undetermined factors could have affected the test results. Robert P. Spalding, more recently, has determined that the age of the sample does not have an impact on either the benzidine or phenolphthalein tests.

From the experimental results obtained, it can be concluded that age of the samples does not influence either preliminary tests or blood group determination. This is concurrent with Robert P. Spalding's previous findings. However, the observed microbial growth may have affected the results and this same growth appeared to have also impacted the results of the blood grouping tests, which has previously been noted by both Yada[7] as well as Benciolini and Cortivo[27].

Future testing practices could involve the use of an anti-sera which is at a lower pH, as suggested by Yip *et al.*[9], as this had been revealed to reduce the error percentages for blood grouping. Further sub-culturing of the cultures, usage of more extensive media and biochemical tests

would be useful to get a clearer picture of the organisms and produced metabolites.

The knowledge that the age of the bloodstain does not affect the aforementioned tests will ensure more effective dispensing of justice in investigations.

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