

ABO blood grouping from hard and soft tissues of teeth by modified absorption-elution technique

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Abstract

Background: Teeth have always been known as stable tissue that can be preserved both physically and chemically for long periods of time. Blood group substances have been known to be present in both the hard and soft tissues of the teeth. **Objectives:** This study aimed at detection of ABO blood group substances from soft and hard tissues of teeth and also to evaluate the reliability of teeth stored for a relatively long period as a source of blood group substances by absorption–elution technique with some modifications. **Results:** Blood group obtained from the teeth was compared with those obtained from the blood sample. Pulp showed a very large correlation in both fresh and long-standing teeth though it decreased slightly in the latter. Hard tissue showed a large correlation in both the groups indicating that hard tissue is quite reliable to detect blood group and that there is no much difference in the reliability in both the groups. However, combining pulp and hard tissue, correlation is moderate. Correlation of blood grouping with the age, sex, and jaw distribution was carried out. **Conclusion:** Blood group identification from hard and soft tissues of teeth aids in the identification of an individual.

Key words: ABO, absorption–elution, blood grouping, dentin, forensic odontology, pulp, teeth

Introduction

Blood grouping has been one of the corner stones of identification of biological material. The term “Blood group” is applied to inherited antigens detected on the red cell surface by specific antibodies.^[1] The ABO blood group system was first described by Karl Landsteiner in 1900 and remains the bulwark of forensic blood group investigation. The reasons for this are manifold. It is the primary, most common, conspicuous, and easily detectable

groups.^[2] Though advances have been made in terms of Deoxyribonucleic acid (DNA) analysis, fingerprinting, etc., blood grouping still has a major role in forensic science in the field of person identification, paternity dispute, and other scenarios. This is attributed to the fact that genetic and antigenic constituents of an individual are not affected by environmental conditions.^[3] Lattes has aptly said “*The fact that belonging to a definite blood group is a fixed character of every human being and can be altered neither by the lapse of time nor by intercurrent disease.*” Blood group like fingerprint is an unalterable primary character.^[4]

Teeth being the hardest of all tissues can be preserved intact for long periods of time after death. They are stable chemically and they retain their characteristic even in the most adverse condition.^[5] The presence of blood group substances and other genetic markers such as enzymes in soft and hard dental tissues makes it possible to assist in the identification of highly decomposed bodies.^[6]

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Pulpal tissue being contained within dental hard tissues. Post-mortem changes are seen very late. Since tooth pulp is highly vascular, blood group antigens are most certainly bound to be present. In dentin, it is presumed that these substances are located in the dentinal tubules. The possible distribution of ABO substances from the pulpal cavity wall to the dentin edge and to the enamel gradually decreases because of fewer possibilities of diffusion of antigens from both blood and saliva.^[7]

Absorption-elution (AE) technique was devised by Siracusa^[8] in 1923 and has been refined by Kind.^[9] Various modifications have taken place since then to improve its sensitivity. This technique has been used extensively to determine blood group from dried stains, tissues, secretions, and teeth in various forensic laboratories. This method is very sensitive, highly specific, and least interfered with the nature of the substrata. It has also been found that the material once used is available for re-use with practically no loss in its antigenic property.^[10] Recent tooth specimen could be expected to provide good sources for determination of blood groups. However, the effect of autolysis, dehydration, loss of pulp antigens, or high number of errors due to foreign antigen borne by bacteria in carious teeth may lead to variation in the study. Therefore, it was thought justifiable to conduct this study to determine ABO blood group substances from both soft and hard tissues of not only relatively fresh teeth but also from teeth kept standing for a relatively long period of 6-10 months by the AE technique with certain modifications.

Materials and Methods

Sixty clinically sound human permanent teeth were collected randomly along with the respective blood samples from patients who reported for extraction due to orthodontic and therapeutic purposes. Carious teeth, grossly attrited, and abraded teeth were excluded from the study. The study was divided into two groups: Group I (fresh teeth) comprised of 30 teeth which were analyzed within a period of 6 weeks. Group II (teeth kept for long period of time) comprised of 30 teeth that were collected and kept for a period of 6-10 months and then analyzed.

At the time of extraction of teeth, subsequent to a written consent, 1 ml of venous blood was drawn under aseptic condition and stored in labeled Ethylene Diamine Tetra Acetic Acid (EDTA) bottles. This blood sample was then subjected to blood grouping using the forward grouping slide method at room temperature. The blood groups thus determined were tabulated and used as the gold standard. The extracted teeth were washed under running water to remove blood, saliva, and debris attached to it. The teeth were dried with gauze and placed in labeled plastic recipients until used in dry state.

From each tooth, pulp was extirpated and the remaining tooth structure was pulverized in the following way. The tooth is split horizontally at the cervical margin with a carborundum disc using a micromotor. A drop of saline was put into the pulp chamber and root canal to wet the pulp, which was extirpated using a barbed broach. The remaining tooth is pulverized with straight fissure bur starting from the pulpal end. The extirpated pulp and powder are stored in sterile labeled recipients. In a blind study, pulp and pulverized tooth powder were then subjected to AE test. Tooth pulp was divided into two equal parts and put into sterile labeled test tubes. Similarly, 10 mg of powder each was placed in labeled test tubes. Two drops of anti-A or anti-B sera were added, respectively, to each test tube containing pulp samples. Similarly, antisera was added to the powder sample also and mixed. Confirming the test samples being sufficiently soaked with antiserum, the test tubes were plugged with cotton and were placed at 4°C overnight to allow absorption.

After removing antisera, each sample was washed 6 times with 10 ml ice cold saline solution in the following way. After addition of saline, the test samples were agitated and then centrifuged for 5 min at 4,000 rpm. The supernatant was decanted and sucked away with a Pasteur pipette. Any excess saline was removed. Then two drops of 10% bovine serum albumin (BSA) in saline was added to the test samples and the test tubes were placed in a pre-heated water bath at 56°C for 10 min to elute the antibodies. The powder test samples in BSA were centrifuged at 1,500-2,000 rpm for 1-2 min. the supernatant was pipetted and used for agglutination reaction. A drop of 0.5% A and B red blood cell (RBC) suspensions is immediately put into the respective test tubes; the samples were gently shaken and incubated at 37°C for 30 min to enhance agglutination. Later, one drop of the solution was placed on a microscopic slide, then cover slip was placed, and finally agglutination was read microscopically at a magnification of ×10 and ×40 [Figures 1-3].

Results

Sixty teeth were collected from patients in the age group of 10-60 years. The age, sex, and jaw distribution of the samples in both the groups are as shown in Table 1. When blood grouping from the pulp and hard tissue correlated with blood group of person which was identified from the reference, the test result was recorded as "positive" and if they did not match, then it was recorded as "negative" and tabulated.

The comparison of blood groups between reference, pulp, and hard tissue were done using online Java-Script tests. Two groups were considered to be statistically similar if $P > 0.05$.

In Group I ($n = 30$), when pulp was compared with the reference, 28 teeth showed positive results, while two were negative. The sensitivity of pulp in comparison to blood was found to be 93%. Hard tissue when compared with blood showed 24 positive samples and six negative results, with

80% positivity. Teeth (pulp + hard tissue) correlated only in 22 samples with a sensitivity of 73.3%. In Group II ($n = 30$), for pulp, 25 samples showed positive results and five showed negative result with a sensitivity of 83.3%. Comparison of hard tissue with blood showed 23 samples correlating with seven negative results with a sensitivity of 76.6%. Complete correlation for teeth was seen in only 20 samples with 10 negative results showing a sensitivity of 66.6% [Table 2].



Figure 1: Photomicrograph (x10) showing agglutination of RBCs

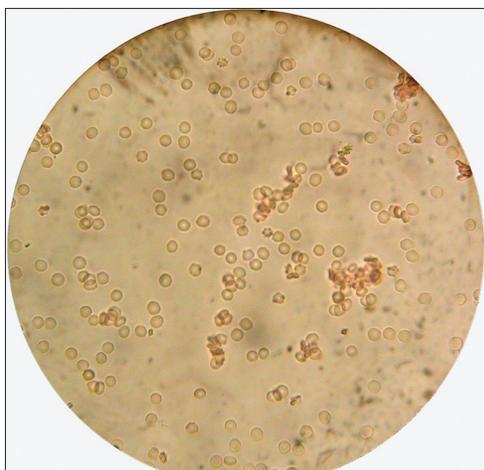


Figure 2: Photomicrograph (x40) showing agglutination of RBCs

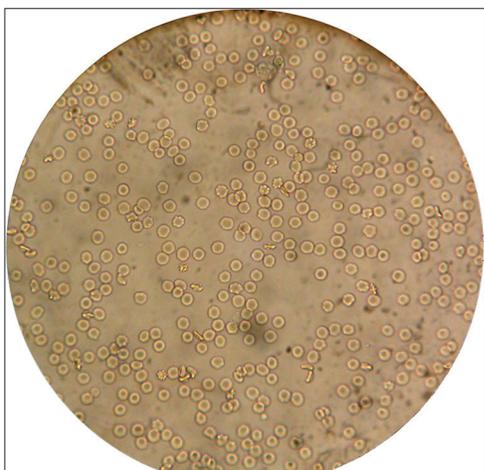


Figure 3: Photomicrograph (x40) showing no agglutination of RBCs

Analyzing pulp in both the groups in relation to the age of the sample showed that in teeth from older subjects, there was a decrease in the sensitivity [Table 3]. When pulp and hard tissue were analyzed with regard to maxillary and mandibular teeth, no difference is found in Group I. However, in Group II, maxillary teeth showed a better performance compared to mandibular teeth [Table 4]. When the performance of pulp and hard tissue was analyzed with regard to both the sexes, it was found that samples from males showed better results [Table 5].

Analysis of pulp for individual blood groups against the reference samples in Group I showed complete correlation for "B" group (6 of 6) with a P value of 0.5. Two samples each were grouped wrongly for "A" and "O" blood groups. In Group II, complete correlation was seen for "A" group (13 of 13) and "AB" blood group (1 of 1). Two samples each showed negative result for "B" and "O" groups [Table 6]. When hard tissue was analyzed for individual blood groups against reference sample, Group I showed complete correlation in "O" group (17 of 17). Four samples each in "A" and "B" blood groups showed negative result. However, in Group II, complete correlation was not found for any of the blood groups. "A" group shows 12 of 13 positive, and "B" showed 7 of 10 test result [Table 7].

Goodman-Kruskal's gamma co-efficient was used to find the correlation between pulp, hard tissue, and teeth with reference. In the analysis of pulp against the reference samples, a very large correlation was seen in Group I and a large correlation for Group II indicating that pulp is very reliable for the purpose of blood grouping in both the samples though it decreases slightly in Group II. In the analysis of hard tissue against reference sample, a large correlation is seen in both the groups indicating that there is no much difference in the reliability and that hard tissue is quite good to detect blood group. However, combining pulp and hard tissue, correlation is moderate [Table 8].

Discussion

Over the past three-quarters of a century, information from studies on blood groups have been applied to medico-legal application. The use of blood group substances in medico-legal examination is based on the fact that once a blood group is established in an individual, it remains unchanged throughout his life.^[1] Recent developments in

Table 1: Age, sex, and jaw distribution of two groups

	Age in years (n=30)					Sex distribution (n=30)		Jaw distribution (n=30)	
	12-20	21-30	31-40	41-50	51-60	Males	Females	Maxillary	Mandibular
Group I	10	6	-	12	2	10	20	15	15
Group II	14	6	1	7	2	11	19	16	14

Table 2: Performance of pulp, hard tissue, and teeth in relation to reference sample

Correlation with control	Group I (n=30)		Group II (n=30)	
	Positive	Pick-up rate (%)	Positive	Pick-up rate (%)
Pulp	28	93.3	25	83.3
Hard tissue	24	80.0	23	76.7
Teeth (pulp+hard tissue)	22	73.3	20	66.6

Table 3: Performance of pulp in various age distributions of two groups

Age in years	Group I		Group II	
	Total	Number positive (%)	Total	Number positive (%)
12-20	10	10 (100.0)	14	13 (92.9)
21-30	-	-	6	5 (83.3)
31-40	6	6 (100.0)	1	1 (100.0)
41-50	12	10 (83.3)	7	6 (85.7)
51-60	2	2 (100.0)	2	0

Table 4: Performance of pulp, hard tissue of maxillary and mandibular teeth

Teeth	Group I			Group II		
	No. of teeth (n=30)	No. correlated		No. of teeth (n=30)	No. correlated	
		Pulp (%)	Hard tissue (%)		Pulp (%)	Hard tissue (%)
Maxillary	15	14 (93.3)	12 (80)	16	15 (93.7)	14 (87.5)
Mandibular	15	14 (93.3)	12 (80)	14	11 (78.5)	10 (71.4)

Table 5: Performance of pulp, hard tissue of teeth from males and females

Sex	Group I			Group II		
	No. of teeth (n=30)	No. correlated		No. of teeth (n=30)	No. correlated	
		Pulp (%)	Hard tissue (%)		Pulp (%)	Hard tissue (%)
Males	10	10 (100)	9 (90)	11	10 (90.9)	9 (81.8)
Females	20	18 (90)	15 (75)	19	15 (78.9)	14 (73.6)

Table 6: Comparison of individual blood groups between reference group and pulp of both groups

Blood groups	Group I (n=30)			Group II (n=30)		
	Reference group (%)	Pulp (%)	P value	Reference group (%)	Pulp (%)	P value
A	7 (23.3)	5 (16.7)	0.285	13 (43.3)	13 (43.3)	0.500
B	6 (20.0)	6 (20.0)	0.500	10 (33.3)	8 (26.7)	0.322
AB	-	-	-	1 (3.3)	1 (3.3)	0.500
O	17 (56.7)	19 (63.3)	0.372	6 (20.0)	8 (26.7)	0.297

Table 7: Comparison of individual blood groups between reference group and hard tissue of both groups

Blood groups	Group I (n=30)			Group II (n=30)		
	Reference group (%)	Hard tissue (%)	P value	Controls (%)	Reference group (%)	P value
A	7 (23.3)	3 (10.0)	0.103	13 (43.3)	12 (40.0)	0.422
B	6 (20.0)	10 (33.3)	0.159	10 (33.3)	7 (23.3)	0.235
AB	-	-	-	1 (3.3)	2 (6.7)	0.258
O	17 (56.7)	17 (56.7)	0.500	6 (20.0)	9 (30.0)	0.224

Table 8: Performance of pulp and hard tissue in relation to reference

Tests	No. correlated	No. not correlated	Goodman-Kruskal's gamma co-efficient
Group I			
Pulp vs. reference	28	2	0.87
Hard tissue vs. reference	24	6	0.60
Teeth vs. reference	22	8	0.47
Pulp vs. hard tissue	22	8	0.47
Group II			
Pulp vs. reference	25	5	0.67
Hard tissue vs. reference	23	7	0.53
Teeth vs. reference	20	10	0.33
Pulp vs. hard tissue	22	8	0.47

DNA profiling technique allow highly efficient personal identification using minute amount of forensic specimens such as blood stains, salivary stains, and other tissues. ABO blood grouping still is informative and has a niche in for forensic identification. In Japan, ABO blood grouping is examined in suspects of criminal cases before DNA profiling.^[11] Mukherjee and Chattopadhyay^[12] have reported a case, wherein precise identification of the diseased was done by blood grouping of teeth by AE technique. Kuo^[13] has reported a homicide case in which a conviction of first-degree murder was achieved and the identity of the missing person was possible by determining the genetic inheritance from blood grouping.

Various sero-genetic markers such as ABO, Phosphoglucomutase-1 (PGM₁), Adenylate kinase (AK), 6-phosphogluconate dehydrogenase (6-PGD), Adenosine deaminase (ADA), Glucose-6-phosphate dehydrogenase (G-6-PD) are present in the teeth.^[6,14] Blood grouping from teeth is not a straight forward technique. For several decades, forensic scientists have been in search of a

reliable method for blood grouping of teeth. Conventional absorption inhibition method was employed by Shimura^[15] and Suzuki.^[16] However, this method required complicated pre-treatment procedures such as decalcification and results were less than ideal.^[17] Using mixed agglutination technique preceded by decalcification, Kramer^[18] concluded that it was not possible to demonstrate A and B antigens in human dentin.

The current choice for typing of dried blood stains is AE technique.^[9,14,19] Various studies^[6,7,12,17,20-24] have obtained positive results for blood grouping on teeth using AE technique. However, low concentration of blood group antigens in tooth material as well as potential for oral and environmental bacterial contamination might affect the blood grouping results. Blood group like antigens are present ubiquitously in plants, animals, bacteria, fungi, and the possible presence of such contaminants may lead to blood group mistyping due to acquired antigen activity. Studies have shown contamination of dental tissue with aerobic gram-negative bacteria which possess ABO blood group like antigens simulating a B-type blood group. A large number of aerobic gram-negative bacteria are present in saliva or on dental tissues. It has to be kept in mind that together with yeasts, the aerobic gram-negative bacteria tend to grow exclusively in contaminated stored specimens or putrefying material and they tend to overgrow other species. It may be an important source of blood typing error and occasional mistyping of blood groups from oral material (e.g., teeth and alveolar bone) may be caused by the aerobic gram-negative oral flora.^[25]

In our study, the AE technique was used with certain modifications for blood grouping from hard and soft tissues of teeth which includes storing, separation of tissue, pulverization of hard tissue, absorption, elution, and agglutination reaction. In our study, pulp was extirpated using a barbed broach after splitting the tooth at the cervical region. This technique is simpler and increases the chances of extirpation of pulp in one piece compared to the procedure used by Smeets *et al.*^[7] The principle of AE test lies in the absorption of blood group specific agglutinin on the surface of a substance having blood group agglutinogens, elution of the antibody so absorbed under a high temperature, and the agglutination of the blood cells possessing corresponding antigens.^[20] Consequently, the surface area of the material used becomes important. Hence, the hard tissue of teeth is pulverized to fine powder to increase the surface area for reaction. In our study, absorption of agglutinins was facilitated by placing the test samples in antisera overnight at 4°C.^[21,26] Placing the samples at 4°C and later washing the samples at the same temperature increases agglutination intensities.^[19]

Ten percent BSA in saline was used in this study instead of physiological saline as a medium to carry out elution and

agglutination. BSA of 5-10% increases the sensitivity of the reagents by two to eight times. This phenomenon is likely due to the action of albumin. Several seed agglutinins of small molecular size induce hemagglutination not in saline but in BSA solution. BSA may neutralize ionic repulsion between erythrocytes and enhance specific linkage between corresponding antigen and antibodies. They are also found to block non-specific binding of antibodies on to the specimen.^[11]

After elution of the antibodies, prior to the addition of the red cell suspension, the test samples containing hard tissue powder are centrifuged at 1,200-1,500 rpm for 1-2 min. The supernatant is then decanted and sucked and to this, the red cell suspension is added. This step is included as it is noted that tooth powder interferes with reading of the agglutination results [Figures 2 and 4].

Carious teeth are excluded from the study as it has been found that bacteria in caries interfere with the blood group antigens.^[6,22,27] Dental tissues from pathologically affected or damaged teeth are often invaded by certain bacteria which impart their adventitious antigenic activities to them and cause false-positive reactions. Bacteria may also cause false-negative results because of the loss of blood group activity or occasionally hemolysis of the cells.^[6]

In Group I, pulp showed a sensitivity of 93% for blood grouping. Studies by Ballal and David,^[28] Garg and Garg,^[29] Lele,^[30] Sharma and Chattopadhyay,^[6] and Smeets *et al.*^[7] have shown a sensitivity of 90%, 92%, 86%, 100%, and 80%, respectively. In Group II, pulp showed a sensitivity of 83.3%. Similar studies by Sharma and Chattopadhyay^[6] and Smeets *et al.*^[7] show 100% and 86% sensitivity, respectively. The negative results of pulp for blood grouping in this study can be attributed to insufficient quantity of pulp, reduction in fibrosed tissue in the pulp with increasing age, and increased calcification of the canal.^[3] It was found in our

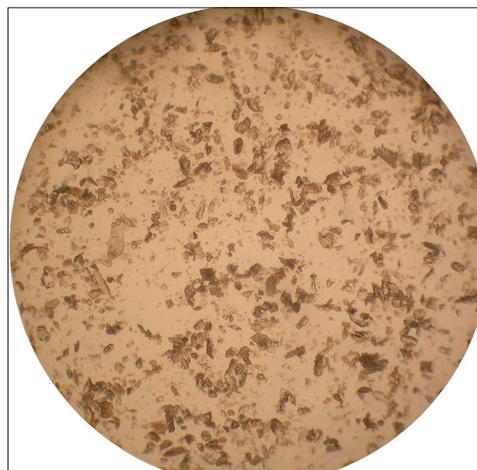


Figure 4: Photomicrograph showing no agglutination in hard tissue with interference of tooth powder

study that a majority of teeth that showed negative results in pulp were teeth in the age group of 40 years and above. The negative results may be due to failure of technique, contamination of the sample, and lysis of the cell.

Hard tissue when compared with blood showed 86% positivity in Group I and 76.6% positivity in Group II. This study cannot be compared with previous studies as previous studies have taken the sensitivity of each component of hard tissue separately. The negative results in the study can be attributed to the low amount of antigens in the hard tissue,^[17] inaccessibility of blood group substances in the dentin because of high degree of calcification,^[18] contamination of sample, or failure of technique.

The existence of blood group substances in both the hard and soft tissues and their nature (water soluble or alcohol soluble) has been a subject of debate for a long time.^[17] Since tooth pulp contains a lot of blood vessels, blood group antigens are almost certainly bound to be present in tooth pulp.^[7,17] Hence, determination of blood group from pulp should be relatively straightforward. This has been confirmed in many studies.^[6,7,17,21,22,27] The major contemporary theory for the origin of blood antigens in dental hard tissue is based on infusion-sedimentation phenomenon combined with inherently present antigen. The infusion-sedimentation theory describes the infusion of water soluble antigens from saliva into the tooth surface. It is also presumed that blood group antigens are present in dentinal tubules.^[7] Studies have shown that the odontoblasts including the dentinal process are one of the positive sources of blood group antigenicity. It has been shown that blood group activity of the tooth fragments is markedly decreased when the odontoblastic zone is scrapped off.^[31] Imai *et al.*^[32] observed that the outer part of dentin has smaller amounts of blood group substances than the inner, whereas predentin shows maximum concentration. This has been explained on the basis of organic content of these layers. Predentin is uncalcified and the inner dentin layer adjacent to it has high organic content. Calcification begins in the outer dentin layer which have lower organic content and hence lower amount of blood group substance.^[6]

Teeth as a whole showed a sensitivity of 73.3% and 66.6% for blood group determination in Groups I and II, respectively. This is much higher when compared with study by Smeets *et al.*^[7] This is probably due to the use of 10% BSA instead of saline as an elution and agglutination medium.^[11]

When pulp was analyzed for individual blood groups, in Group I, "B" group showed complete correlation and in Group II, "A" and "AB" groups showed complete correlation. Negative results have been observed in other groups. Similarly for hard tissue, "O" group showed complete correlation in Group I, whereas no blood group is completely and correctly matched with the reference group

in Group II. There are no studies available in the literature on analysis for individual blood group. False results may be due to bacterial contamination and presence of acquired antigen^[23] or failure of the technique. False "O" group may be due to progressive breakdown of antigenic substances.^[22] Studies have shown that N-acetyl-d-galactosamine, the determinant of blood group "A" changes to "B" specific D-galactose under the influence of moisture and heat.^[33]

As age increases, it was found in the study that the performance of pulp decreases. This is consistent with other studies by Lele *et al.*^[30] and Parekh.^[21] This can be attributed to the reduced amount of pulpal tissue available for the study. As age progresses, volume of pulp chamber decreases due to increased fibrosis or increased calcification of the canal.^[30] However, Garg and Garg^[29] in their study did not show any age variation.

Pulp and hard tissue of maxillary teeth show better results in both Groups I and II. Though no exact explanation is available, it could be due to morphological difference in the size of tooth and larger amount of pulp available. Similar findings were noted by Lele *et al.*^[30] In this study, pulp and hard tissue from the teeth of males show higher percentage of positive results than females. This may be attributed to difference in the morphological size of the tooth between both sexes and the availability of pulp for identifying blood groups. However, studies by Garg and Garg,^[29] Lele *et al.*,^[30] and Parekh^[21] show no sex difference in their result.

Thus, in this study, it was found that pulp showed a very large correlation in Group I and a large correlation for Group II indicating that the pulp is very reliable for blood grouping in both fresh and long-standing teeth though it decreases slightly in Group II. In the analysis of hard tissue against reference sample, a large correlation was seen in both the groups indicating that there is no much difference in the reliability and that hard tissue is quite reliable to detect blood group. However, combining pulp and hard tissue, correlation was moderate.

Conclusion

The blood typing from both hard and soft tissues of teeth by AE method can be used for identification. Though blood grouping does not give a positive identity but only a positive non-identity, it has been used to positively identify individuals. In view of particularly significant positive results of ABO blood groups obtained from dental pulp in this study, it can be concluded that dental pulp can be used to establish identity, where teeth happen to be the only remnants available for personal identification. Though blood group substances have been detected from dentin, it may not be as reliable a source as shown in the study. Blood group determination from teeth warrants further research as it is inexpensive, does

not require sophisticated equipment and skill. A need for sophisticated technique or modification of current technique is recommended especially in cases of hard dental tissue, where the amount of antigen may be low. Further research is also needed with a larger sample size and more time duration in conditions simulating the natural environment from where the problem arises. This study is thus a quantum of what has been learned and how much more needs to be learned in the challenging branch of forensic odontology.

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