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Research paper

DETERMINING THE FREQUENCY OF COLTON BLOOD GROUP ANTIGENS *Co*^{a} AND *Co*^{b} IN THE MALTESE POPULATION

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Abstract. This study aimed at identifying the frequency of two blood group antigens of interest within the Colton (Co) blood group system, *Co*^{a} and *Co*^{b}, the higher and lower prevalence antigens respectively. The antigenic frequency was determined using the polymerase chain reaction-allele specific primer extension (PCR-ASPE) technique on a sample size of 68 adult Maltese blood donor samples and 351 neonatal cord blood samples. The aforementioned methodology enabled the molecular typing of the missense variant in the *aquaporin 1* (*AQP1*) gene (*NM_198098:c.134C>T; rs28362692*), responsible for the single amino acid change of Ala45Val (alanine for *Co*^{a} and valine for *Co*^{b} at position 45) (*NP_932766:p.Ala45Val*) within the AQP1 protein chain. As a result of the missense variant, the Colton phenotypes Co(a+b-), Co(a+b+) and Co(a-b+) could be identified through the stipulated PCR-ASPE technique, and the rare phenotype, Co(a-b+), if detected, would also be subjected to DNA sequencing. Analysis of raw data unveiled that the Co(a+b-) phenotype was the most prevalent in both the donor pool (88.23%) and the cord blood pool (86.04%), whilst the Co(a+b+) phenotype revealed a lower occurrence in the donor pool (5.88%) and the cord blood pool (6.98%). The Co(a-b+) was not encountered in the donor and cord blood samples tested, negating the need for DNA sequencing. The novelty of this study can be seen in the unprecedented determination of the frequency of the *Co*^{a} and *Co*^{b} antigens within the Maltese population. Outlining the Colton blood group antigen frequencies is a significant step to understand any susceptibilities to the development of the pertinent antibodies, thus aiding in the reduction of Haemolytic Transfusion Reactions (HTRs) and Haemolytic Disease of the Foetus and Newborn (HDFN). Moreover, research of this calibre would expand the local database of molecular typing of blood group antigens, improving transfusion.

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1 INTRODUCTION

Red cell antigens play a vital role in transfusion medicine, with their attributable polymorphic characteristics that render them alloantigenic, indicating the presence of blood group activity. Such a feature has the capacity to stimulate alloantibody production in individuals who lack the pertinent antigens. Transfusion medicine studies value the generation of alloantibodies due to the potentially detailed output apropos of the red cell surface architecture (Daniels 2007). The clinical significance of particular alloantibodies is further outlined in reported cases of Haemolytic Transfusion Reactions (HTR) and Haemolytic Disease of the Fetus and Newborn (HDFN), which indicate that the alloantibodies in question were causative of such adverse reactions of varying severity (Halverson & Peyrard 2010).

A total of 35 blood groups exist, which are categorised into protein-based groups, such as the Colton (Co) blood group system, or carbohydrate-based groups, such as the ABO blood group system (Daniels 2007). The types of blood groups present in an individual, and by extension, the type of alleles that influence antigen production of said blood groups are inherited from maternal and paternal origins (Dean 2005).

With respect to the Colton blood antigens, Preston et al. (1994) maintain that studies on the protein aquaporin 1 (AQP1), a water-channel protein located on the red cell membrane, have resulted in its correlation to such antigens. The Co antigens are in fact produced by the alleles of the same gene that produces AQP1 protein. The first antigen to be discovered was *Co*^{a} (Heisto et al. 1967), followed by its antithetical antigen *Co*^{b} (Giles et al. 1970). Ancillary antigens are *Co*^{3} and *Co*^{4} (Preston et al. 1994), which for the purpose of the study were not included. The antibodies corresponding to the Co antigens often belong to the immunoglobulin G (IgG) class (Daniels 2002; Issitt & Anstee 1998), with some anti-Co^a, – Co^b and – Co^3 binding complement (Covin et al. 2001).

In fact, anti-*Co*^{a} has been reported as a causative factor to significant delayed or acute transfusion reactions or

HDFN (Simpson 1973; Kurtz et al. 1982) and anti-Co^3 as causative factor to HDFN of great severity, necessitating neonatal transfusion (Savona-Ventura, Grech & Zieba 1989). Conversely, anti-Co^b was rarely encountered, and if so, in the presence of other alloantibodies in patient's sera, with reports indicating that no significant HDFN was observed (Daniels 2002; Issitt et al. 1998).

The molecular point of interest in *Co* is the alanine amino acid at position 45 and a cytosine (C) nucleotide at base pair (bp) position 134 in the Co^a, and the valine amino acid at position 45 and a thymine (T) nucleotide at bp position 134 in the Co^b. The Colton blood group displays a type of missense variant, the single nucleotide polymorphism (SNP) also known as Co^a/Co^b SNP, which arises from a single amino acid substitution of alanine to valine at position 45 (NP_932766:p.Ala45Val) caused by the nucleotide change of C>T at position 134 in exon 1 of AQP1 gene (NM_198098:c.134C>T; rs28362692). The AQP1 protein architecture is also indicative of the presence of the SNP – in fact, this multi-pass protein, containing a number of extracellular and intracellular loops, has the Co^a/Co^b SNP on its extracellular loop (extracellular loop A). Apart from this, Colton blood group activity within the AQP1 protein structure is correlated to the presence of consensus motifs of amino acid sequences asparagine-proline-alanine (Asn-Pro-Ala). Furthermore, the motif instigates water channel formation to an electrostatic field, which is attributed to the AQP1 protein function (Halverson & Peyrard 2010).

The phenotypic expression of the Co blood group system was highlighted in a study conducted by Lewis et al. (1977). In this study, Co blood group phenotypes and allele frequencies were derived from a sample size of 726 random Canadian Caucasian adults, with an inheritance in 57 unrelated families, with at least one Co^b-positive parent. It was reported that the homozygous wildtype phenotype Co(a+b-) had an observed occurrence of 89.81% (n=652); the homozygous alternative phenotype Co(a-b+) had an observed occurrence of 0.41% (n=3); and the heterozygous phenotype Co(a+b+) had an observed occurrence of 9.78% (n=71). The constituent codominant alleles, Co^a and Co^b which respectively give rise to the Co^a and Co^b antigens present on the red cell surface, are manifested in the abovementioned phenotypes (Halverson & Peyrard 2010) An analysis by Rogers, Stiles & Wright (1974) reported the presence of the null phenotype Co(a-b-), which could be attributable to varied possibilities of molecular alteration, such as a large deletion affecting most of exon 1 or a 113C>Tpolymorphism on the cDNA (complementary DNA), amongst others (Preston et al. 1994).

In light of the phenotypic occurrence defined by the previous studies, the incumbent study aimed at determining the frequency of the Co^a and Co^b antigens and the prevalent phenotypes in the Maltese population. This was conducted by testing for the characteristic missense variant Co^a/Co^b SNP in 351 neonatal cord blood samples and 68 Maltese blood donor samples by polymerase chain reaction-allele specific primer extension (PCR-ASPE).

The clinical significance in determining the frequency of Co^a and Co^b antigens is bolstered by the production of alloantibodies, which has reportedly led to adverse reactions HTR and HDFN (Halverson & Peyrard 2010). This unprecedented research on the local scenario of the Colton blood group system would therefore contribute to safer transfusion practices and better transfusion medicine.

2 METHODOLOGY

2.1 *E*thical Approval & Sample Selection

A total of 351 neonatal cord blood samples, obtained from the Malta Biobank at the Laboratory of Molecular Genetics at the University of Malta, and 68 Maltese blood donor samples were obtained from the Hospital Blood Bank at Mater Dei Hospital. It was permissible for the latter samples to be used for laboratory tests, without a permission form from the donors, due to a binding agreement in the National Blood Transfusion Service (NBTS) questionnaire. Permission was granted to utilise the cord blood samples (reference number: 48/2002) and the donor blood samples (reference number: 17/2016) by the University of Malta Research Ethics Committee.

2.2 DNA Extraction

DNA extraction of a volume of 1 mL donor whole blood was the first step taken in the methodology in order to achieve downstream application of molecular methods. The salting out technique (Miller, Dykes & Polesky 1988) was employed to obtain purified DNA for further testing. The underlying principle involves the disruption and digestion of the preliminary cell membrane, the exposure to a highly saline environment, the separation of protein precipitate from DNA-laden supernatant by centrifugation, and DNA collection through precipitation- inducing solutions such as ethanol (Diego & Lyn 2014).

This was carried out by: (i) thawing the whole blood stored at – 20°C, at room temperature for approximately one hour on a rotator machine (ii) incubating an aliquot of 1mL blood in 3mL of 1x Erythrocyte lysing buffer (ELB) (84.5g/L NH4Cl; 10.0g/L $KHCO_3$; 0.3722g/L Na_2EDTA) for 15 minutes, and centrifuging at 2500g for 10 minutes at 5°C; (iii) discarding the supernatant and washing the remaining white cell pellet with 1mL of 1x ELB and centrifuging as before (this step was repeated to procure a clean pellet); (iv) re-suspending the pellet with 1mL 1x Sodium Ethylenediaminetetraacetic acid (EDTA) (SE) buffer (43.9g/L NaCl; 93.1g/L Na_2EDTA; 1% Sodium Dodecyl Sulfate; 1μL proteinase K); (v) incubating the tubes overnight in a water bath at 37°C; (vi) adding 1mL of 1x SE buffer, 750μL of 6M NaCl and 3.75mL of chloroform to the tube, rotating the tubes and intermittently vortexing them; (vii) centrifuging the mixture for 10 minutes at 2000g and aspirating the top aqueous DNA layer into another tube; (viii) mixing the DNA layer with double the volume of 100% ethanol to precipitate the DNA strand; (ix) purifying the resultant DNA strand with repeated washes of 1mL of cold 75% ethanol and centrifugation at 11,000g for 4 minutes; (x) decanting the mixture and allowing the extracted DNA

to air dry; and (xi) resuspending the DNA pellet in 500 μL Tris-acetate EDTA (Tris-hydrochloric acid; 0.5M EDTA; pH 8.0), leaving the sealed DNA tubes to rotate overnight, subsequently being refrigerated at 4°C.

The quality of the samples was noted during the gel electrophoresis process through the visualisation of gels under an imaging system. Extracted DNA was also quantified and checked for its purity with the Nanodrop 2000c UV-VIS (ultraviolet-visible) Spectrophotometer (Thermo Scientific™, USA)

2.3 DNA Quality Checks and Quantification

DNA quality checks were carried out by means of gel electrophoresis, and revealed that 37 out of the original 105 donor samples and 63 out of the original 414 cord blood samples were of poor integrity. The nature of their integrity would have been detrimental to the interpretation of the results and thus had to be removed from the final sample size, accounting for the 68 donor samples and 351 cord blood samples used for testing. Moreover, quantification of DNA showed that the DNA samples were highly concentrated (indicating the need for the dilution of samples) and the majority exhibited high purity due to their approximate value of 1.8 in the 260/280 DNA purity ratio (Matlock 2015).

2.4 PCR-ASPE

PCR-ASPE enables the identification of SNPs through an allele-specific single reaction under standard PCR conditions. Key components of the PCR-ASPE are its primers; the common reverse primer, two forward allele – specific primers with different tails and the primer that serves as an internal control. The main stages of this technique involve: the denaturation of the double stranded DNA, the annealing of allele-specific different-tailed primers to their complementary DNA sequence and their amplification into allele-specific PCR products of varied lengths. The products are subsequently separated by gel electrophoresis for an enhanced visualisation of the results (Gaudet et al. 2009).

The following primers were used for the purpose of the study: Co^a forward primer (5'-GAA CAA CCA GAC GGC – 3'), Co^b forward primer (5'-GGG AAC AAC CAG ACG GT-3'), a common reverse primer (5'-CTG AGA GGA TGG CGG TGG-3') (Jungbauer 2012), and as an internal control, a human growth hormone (HGH) forward primer (5'-GCC TTC CCA ACC ATT CCC TTA-3') and a *HGH* reverse primer 5'-TCA CGG ATT TCT GTT GTG TTT C-3') in order to ascertain that the PCR run was carried out in the correct manner (Xuereb, Debono & Borg, 2015). The primers were prepared and reconstituted prior to the master mixes preparations for the detection of Co^a and Co^b. The constituents and their pertinent volume both can be found in the Table 1.

Table 1: *Co^a and Co^b master mixes (volume in μL)*

Reagents	Co^a Master mix	Co^b Master mix
5x FIREPol® Master Mix Ready to Load	4	4
Sterile water	14.8	14.8
Forward *HGH* primer	0.1	0.1
Forward Co^a primer	0.1	N/A
Forward Co^b primer	N/A	0.1
Reverse *HGH* primer	0.1	0.1
Reverse *Co* primer	0.1	0.1

For the purpose of this study, 96-well PCR plates were used, with the first 48 wells being used for the detection of Co^a and the second set of 48 wells being used for Co^b detection using the same samples. The same 48 samples of cord blood or donor samples were used for the detection of both antigens.

The volume of Co^a and Co^b master mixes were multiplied by 55, to account for the volume required for the 48 wells used to detect Co^a *and* Co^b, as well as an extra 7μL to overcome pipetting errors. Therefore, the total volume of 5x FIREPol® Master Mix Ready to Load was 220 μL, sterile water 814 μL, forward *HGH* primer 5.5 μL, forward Co^a primer 5.5 μL, forward Co^b primer 5.5 μL, reverse *HGH* primer 5.5 μL and reverse *Co* primer 5.5 μL.

Wells D12 and H12 were used as negative controls, whereby they solely contained the master mix. PCR optimisation was an essential step to ascertain proper interpretation of the bands on the agarose gel, thus avoiding the presence of hindering non-specific secondary bands. This was tackled by optimising the annealing temperature by means of gradient PCR, which sought to test the effect of varying annealing temperatures ranging from 55–65°C within the same run. By means of the Mastercycler® thermal cycler (Eppendorf s.r.l, Germany), a finalised profile was established through a system of trial and error, as seen in Table 2.

Table 2: *Thermal cycler profile for the PCR-ASPE detection of Colton antigens*

Number of cycles	Temperature (°C)	Duration	Stage
1 cycle	95	5 minutes	Initial hot-start
28 cycles	95	50 seconds	Denaturation
	62	30 seconds	Annealing
	72	45 seconds	Extension
1 cycle	72	10 minutes	Final extension
1 cycle	4	hold	Incubation

*2.5 A*garose Gel Electrophoresis

The PCR products generated by the PCR-ASPE process were then separated by agarose gel electrophoresis. Such a method separates DNA by size, depending on their rate of migration, through the gel across the electric field, facilitating the movement of negatively-charged molecules towards the anode electrode (Yilmaz, Ozic & Gok 2012).

A 1.5% agarose gel was prepared (Yilmaz et al. 2012) and an aliquot of 7μL of PCR product was subsequently pipetted into each well, with the final well being used to load 7μL of 100bp DNA ladder. Each gel would contain 48 loaded samples to be tested for the presence of either Co^a or Co^b. The gel electrophoresis run was set at 130V for 20 minutes with variable current. The gel was subsequently visualised by means of UV-transilluminator Bio – Doc-It®2 imaging system (Analytic Jena, Germany).

Should Co antigens be present, the Co^a would manifest itself as a band of 221bp, whilst the Co^b would be depicted by a band of 223bp (Jungbauer 2012). As established in previous studies the internal control *HGH* would be seen as a band of 434bp. Since the Colton products are much smaller than the HGH products, the bands of the former would migrate at a faster rate than the latter (Yilmaz et al. 2012).

3 DATA ANALYSIS

The IBM SPSS® Statistics 20 package was predominantly used on the compiled dataset to generate frequency tables and bar charts, and perform Pearson Chi-Square tests. The frequency tables and bar charts were required to determine the genotypic frequencies of the donor and cord blood populations in relation to each other, as well as within each respective population, separated by phenotype as well as by gender. The data extracted from the aforementioned tests would be extended to the manual calculation of the allelic frequencies. The Pearson Chi-Square tests were used to deduce a relationship between the following variables: the homozygote wildtype Co(a+b-) and the heterozygote Co(a+b+) phenotype within the donor and cord blood populations; and the phenotypic differences between males and females within the cord blood (or donor) populations. Moreover, the Online Encyclopaedia for Genetic Epidemiology Studies was used to access the Hardy-Weinberg Equilibrium calculator (Rodriguez, Gaunt & Day 2009), in order to determine whether genetic and allelic frequencies both the cord and donor populations are constant, reaching Hardy-Weinberg equilibrium.

4 RESULTS

*4.1 P*CR Products on Agarose Gel

The electrophoresis gel in Figure 1 indicates the presence of *HGH* (434bp) and Co^a (221bp) positive bands, which were visible in the majority of lanes on the gel, and were measured against the 100bp DNA ladders found in lanes 25 and 50 and the negative control present in lane 47. The electrophoresis gel in Figure 2 demonstrates the presence of *HGH* (434bp) and the predominant absence of strong Co^b results. In fact, a rarer strong positive Co^b result can be seen in lane 28, measured against the 100bp DNA ladder (lanes 25 and 44). The negative control in lane 42 served as a vital measure in order to evade the misinterpretation of Co^b bands. Both Figures 1 and 2, demonstrated the presence of primer dimers, which are the result of secondary reactions between the primers themselves, depicted as the third (and last) band. Primer dimers stand out in all lanes of the first gel, whilst in the second gel, the bands, albeit fainter, are also seen in all lanes. Moreover, negative results were also observed, indicating a problem with the viability of the particular samples tested. For instance, lane 44 in Figure 1 and lanes 12, 16 and 17 in Figure 2 are prime examples of negative results due to poor sample viability.

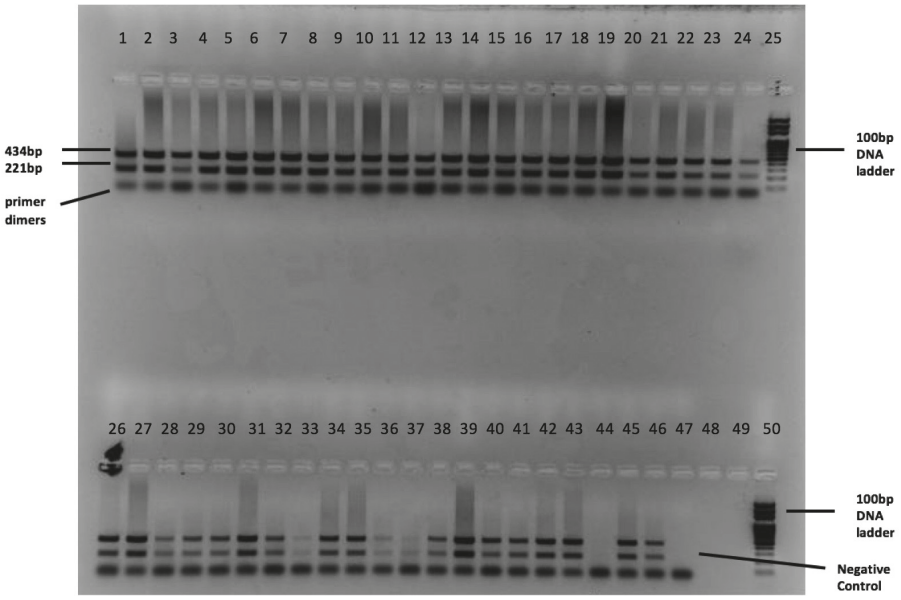

Figure 1: Gel electrophoresis depicting *Co*[a] positive results. Top row lanes 1–24 indicate the consistent presence of the *HGH* internal control at 434 bp and the *Co*[a] bands at 221 bp, measured against a 100bp DNA ladder in lane 25. Bottom row lanes 26–43 indicate a similar consistency, with a few lanes depicting weaker results, such as lanes 33,36 and 37. Lane 44 indicates a negative result due to an unviable sample, lane 47 represents the negative control, lanes 48–49 are empty, and lane 50 represents the 100bp DNA ladder. This image also denotes the presence of primer dimers (secondary reaction) in all lanes.

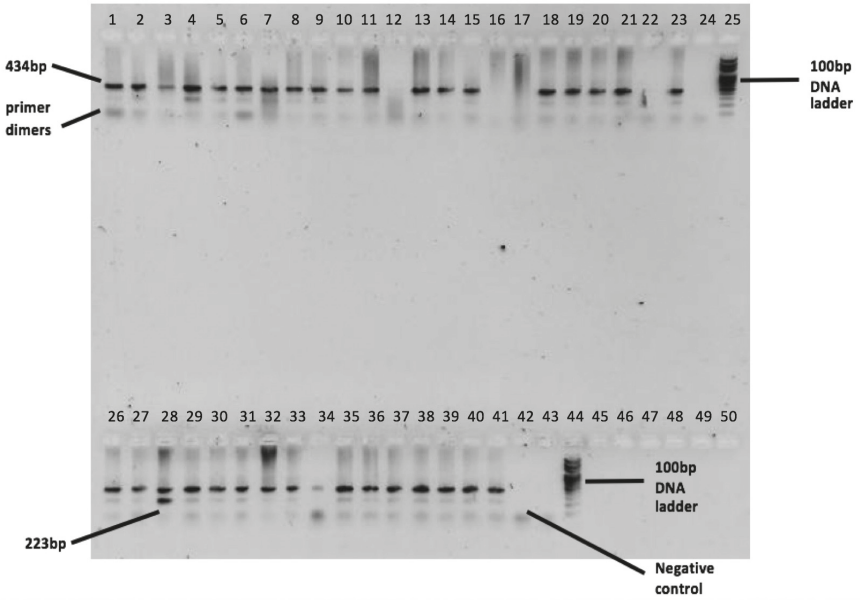

Figure 2: Gel electrophoresis depicting *Co*[b] positive results. Top row lanes 1–24 display an array of very faint, equivocal *Co*[b] results, and the *HGH* internal control at 434bp, as seen in lanes 1–11, 13–15, 18–21 and 23, measured against the 100bp DNA ladder in lane 25. Failed samples due to sample unviability can be seen in lanes 12, 16–17, 22 and 24. Bottom row lanes 26–44 indicate a strong positive *Co*[b] result at 223bp in lane 28, faint equivocal *Co*[b] results in lanes 26–27 and 29–41, the *HGH* internal control at 434bp, a negative control in lane 42, an empty well in lane 43 and a 100bp DNA ladder in well 44. This image also denotes the presence of primer dimers (secondary reaction) in all wells.

5 Statistical findings

5.1 Genotypic and Allelic Frequencies

SPSS frequency tables were generated in order to calculate the genotype count and percentage occurrence for both sample populations and their respective gender segments of the homozygous wildtype Co(a+b-), heterozygote Co(a+b+). The homozygous alternative Co(a-b+) phenotype was not encountered in both populations (0% genotype occurrence), and was therefore omitted from the calculations found in Table 3. Table 3 also demonstrates the allele frequencies, which were calculated by taking into account the co-dominant alleles Co^a and Co^b, and Co^{ab} for both the donor and cord blood populations.

Table 3: *Genotypic and allelic frequencies of the donor and cord blood study populations.*

Phenotypes	Sample	Genotype Count	Genotype Occurrence (%)
Co(a+b-)	**Donor population**	**60**	**88.2**
	Males	33	86.8
	Females	27	90.0
Co(a+b+)	**Donor population**	**8**	**11.8**
	Males	5	13.2
	Females	3	10.0
Co(a+b-)	**Cord blood population**	**302**	**86.0**
	Males	157	86.3
	Females	145	85.8
Co(a+b+)	**Cord blood population**	**49**	**14.0**
	Males	25	13.7
	Females	24	14.2
Allele Number*	**Sample**	**Allele frequency****	**Allele occurrence (%)*****
Co^a *120*	Donor population	0.94	94
Co^{ab} *8*			
Co^b *0*	0.06	6	
Co^a *604*	Cord blood population	0.93	93
Co^{ab} *49*			
Co^b *0*	0.07	7	

5.2 Chi-Square test & Hardy-Weinberg equilibrium (HWE)

The SPSS statistical package was also used to conduct the Pearson Chi-Square (χ_2) test – a test used to deduce whether there was a relationship between the following sets of categorical variables: (i) occurrence of Co blood group phenotypes in cord blood population and occurrence of Co blood group phenotypes in donor population; (ii) occurrence of Co blood group phenotypes in males and occurrence of Co blood group phenotypes in females within the cord blood population; (iii) occurrence of Co blood group phenotypes in males and occurrence of Co blood group phenotypes in females within the donor population. The phenotypes used for statistical analysis were Co(a+b-) and Co(a+b+) since Co(a-b+) was not encountered. Table 4 represents the statistical output of three χ_2 tests on the abovementioned variables.

Table 4: A cross-tabulation of the $\chi 2$ analysis performed on the test variables.

Variables	Value	df	Asymptotic significance (2 sided)
p.Ala45Vall*Population2	.234a	1	.629
p.Ala45Val*Gender3	.016b	1	.900
p.Ala45Val*Gender4	0.161c	1	.688

df = degrees of freedom

* = The relationship between the two tested variables

1 = The protein name p.Ala45Val was used as the umbrella term for the Co(a+b-) and Co(a+b+) phenotypes

2 = Population refers to the donor and cord blood population

3 = Gender of the cord blood population

4 = Gender of the donor population

a = 0 cells (.0%) have expected count less than 5. The minimum expected count is 9.25

b = 0 cells (0.0%) have expected count less than 5. The minimum expected count is 23.59

c = 2 cells (50.0%) have expected count less than 5. The minimum expected count is 3.53

With reference to the primary χ_2 analysis between the two Co blood group phenotypes within cord blood and donor population, the value of the test statistic is 0.234, the p-value is 0.629 and the degrees of freedom is 1 with a level of significance of 0.05. Since the p-value is greater than the level of significance, there is no statistical difference between phenotypes within the two population samples, thus indicating similar phenotypic frequencies in the donor and cord blood samples. Furthermore, the cross-tabulations of the Co blood group phenotypes and the gender reveal that the p-value in the case of the cord blood population and the donor population is greater than the 0.05 level of significance (0.900 in cord blood sample and 0.688 in donor samples).

A result of this nature ascertains that there is no statistical difference in phenotypic occurrence in males and females of both the cord blood and donor population. Donor and cord blood samples were also tested for HWE (Hardy-Weinberg Equilibrium) by means of the online HWE calculator (Online Encyclopaedia for Genetic Epidemiology 2008). The resultant outcome, expressed as a p-value, would in fact verify or negate a statistical difference between expected and observed values of the genotypic frequencies, thus determining whether they are in equilibrium or not (Rodriguez et al. 2009). The statistical test was performed, taking into consideration that there were 68 samples in the donor population (60 homozygous wildtype phenotypes and 8 heterozygous phenotypes), and 351 cord blood samples (302 homozygous wildtype phenotypes and 49 heterozygous phenotypes), with the results depicted in Table 5.

Table 5: *HWE statistical calculation for donor and cord blood population*

Sample	Genotype	Expected frequency		Observed frequency	
		n*	%	n	%
Donor population	Co(a+b-)	60.24	88.58	60	88.24
	Co(a+b+)	7.53	11.07	8	11.76
	Co(a-b+)	0.24	0.35	0	0
Cord blood population	Co(a+b-)	303.71	86.53	302	86.04
	Co(a+b+)	45.58	12.99	49	13.96
	Co(a-b+)	1.71	0.49	0	0
Sample	**p allele frequency****	**q allele frequency*****		**p-value ($\chi 2$)**	
Donor population	0.94	0.06		0.27	
Cord blood population	0.93	0.07		1.98	

*n = count

**p allele frequency = dominant allele frequency (Co^a)

***q allele frequency = recessive allele frequency (Co^b)

As depicted in Table 5, the expected and observed values of the phenotypes of the donor and cord blood samples respective are approximately the same. Furthermore, the p-value of the donor population (0.27) and that of the cord blood population (1.98) are greater than the 0.05 level of significance, thus outlining that there is no statistical difference between the two observed and expected values, implying that the populations are within HWE.

*5.3 P*henotypic occurrence of the Colton blood group in the Maltese population

Complementing the above data, the occurrence of the three Colton blood group phenotypes in question within the donor pool or cord blood pool was calculated, as seen in Table 6.

Table 6: *Percentage phenotypic occurrence within the two study populations.*

Phenotype	Donor population* (%)	Cord blood population* (%)
Co(a+b-)	88.23	86.04
Co(a+b+)	5.88	6.98
Co(a-b+)	0	0

Calculations for phenotypic occurrence explained:

*The allele number was divided by the total number of alleles in one study population (calculated by doubling the total genotypic number of that population), and then multiplied by 100 to convert it to a percentage. For instance, Co(a+b-) = 120/(68*2)*100 = 88.23%

6 DISCUSSION

A comparative analysis of the literature bolstering percentage occurrences of the Co blood group phenotype within test populations was sought to denote any correlations with the compiled statistics within the Maltese population. Two noteworthy studies were utilised for the scope of this preliminary analysis: the previously mentioned study of Lewis et al. (1977) that exhibited an observed occurrence of Co(a+b-) at 89.81%, Co(a+b+) at 9.78% and Co(a-b+) at 0.41%; and an ancillary study by Race and Sanger (1975) who outlined the percentage occurrence of Co(a+b-) at 91.4%, Co(a+b+) at 8.4% and Co(a-b+) at 0.2% within their test population.

The data procured from the Maltese samples revealed how similar the Co blood group antigen frequency patterns are, with an 88.23% and 86.04% occurrence of Co(a+b-) in the donor and cord blood populations respectively. The 5.88% and 6.98% occurrence of Co(a+b+) in the donor and cord blood populations respectively was also quite a similar result, albeit slightly lower in comparison. The phenotype Co(a-b+) was not encountered in the study, which was still a reflective result in comparison to the low percentage occurrences found in the other studies. It was also interesting to note that this study, which employed a genotyping method, yielded comparable results to those published by Lewis et al. (1977), who tested samples serologically, using anti-Co^a and anti-Co^b by means of the capillary papain testing.

A closer look at the data obtained from the Maltese population samples would indicate that there is no significant difference between Co(a+b-) and Co(a+b+) in the donor pool and cord blood pool and no statistical difference between the phenotypic occurrence in males and females in either study populations. Furthermore, both the donor populations and the cord blood populations are in HWE, indicating that the expected and observed occurrence of each phenotype in both population groups are similar.

In order to achieve such results, the serological analysis methodology was initially weighed against the Colton antigen genotyping methodology. The typical technique for serological analysis is haemagglutination, often referred to as the "Gold Standard" method, whereby red cell antigens are detected through antigen-antibody interactions (Reid 2009). Studies conducted by both Reid (2009) and Jungbauer (2012) outlined the limitations to such a technique for the detection of Co blood group antigens due to potentially costly testing and feasibility.

In fact, the monoclonal and polyclonal anti-Co^a and anti-Co^3 are exclusively reserved for reference laboratories in small quantities, rendering it difficult to choose the haemagglutination method. In light of such limitations, it was essential to favour the genotyping methods in order to enable the typing of Co^a and Co^b alleles through the 134C>T SNP detection, and thus facilitate the identification of the Colton phenotypes in question (Halverson & Peyrard, 2010). In fact, the utilisation of PCR-ASPE as a means of detecting SNPs served as a comprehensive way of amplifying genes that express blood group antigens. Moreover, it was a solid basis for genotypic and phenotypic classification of blood groups (De Mattos 2011). With regards to the specific detection of Colton antigens, this method was beneficial since it used allele-specific primers, which were previously tested in the study by Jungbauer et al. (2011), thus ascertaining that they were specific to the alleles in question. In addition to this, the previously tested primers shortened the time required to optimise the PCR run.

It is worth noting that a number of limitations were encountered throughout the study, and were mainly the DNA quality and viability, the quantity of samples available and aspects of the chosen methodology. The DNA pool included a number of old samples that deteriorated in quality and integrity due to their exposure to repeated freeze-thaw cycles. Furthermore, there was the possibility that some donor samples were deteriorated during their interim storage

period at the National Blood Transfusion. To overcome said problems, a number of test repeats were conducted by gel electrophoresis on certain samples that gave dubious or false negative results. Samples that showed negative results, particularly at the HGH band, were discarded. Such a situation resulted in a decreased quantity of samples that could be successfully tested, which lead to the utilisation of a smaller sample size, particularly in the case of the donor pool.

The second limitation was the tedious nature of utilising PCR-ASPE, since the Coa and Cob primers were not used simultaneously in the same PCR run. In fact, the same DNA sample was used twice to test the separate allele mixes, therefore indicating that more time and resources were used. Another obstacle encountered was the formation of false positive bands on the electrophoresis gel, due to non-specific amplification of the alleles. This was overcome by repeating PCR cycles and increasing the annealing temperature up to 62ºC, reflecting the finalised annealing temperature.

The mode of detection of the Co blood group itself posed its limitations due to the lack of serological data and limited access of anti-sera (Halverson & Peyrard 2010), which would have given a clear picture of gene expression and interaction. Despite this issue, alternative methodologies were employed to achieve the results regardless.

When taking into consideration such limitations, future studies related to the identification of genotypic and allelic frequencies of blood group antigens should consider and employ alternative techniques such as Sanger sequencing, high-throughput sequencing, microarray and multiplex technologies instead of PCR-ASPE in order to improve the efficiency and quality of results for blood group genotyping.

7 CONCLUSION

A study of this nature has enabled the identification of Coa and Cob blood group antigens, the results of which were derived from testing a small collection of donor and cord blood samples, that were representative of a larger collection within the Maltese population. Statistical analysis reveals the similarity in frequencies between the binary gender segments, the donor and cord blood populations, between the Maltese population and other test populations in the aforementioned ancillary studies.

The importance of studying the Co blood group has already been highlighted due to the association of HTR and HDFN occurrence. Apart from mitigating adverse transfusion reactions, conducting such testing lays the groundwork for increased interdependence between transfusion and genetics, ultimately enriching the molecular research database.

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10 CONFLICT OF INTEREST

Authors report no conflict of interest.

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