INTRODUCTION

Polycythaemia and erythrocytosis are frequently used synonymously. Traditionally, the term polycythaemia refers to a cluster of disorders, typically characterised by a persistent elevation in haematocrit (HCT) due to an increase in erythrocytes in the blood circulation [1]. It is suspected when the individual presented with a Hb level above the normal reference range (i.e. Hb level is above 18.5 g/dL or the packed cell volume (PCV)/ HCT is greater than 0.52 in male; and 16.5 g/dL or 0.48 in female respectively) [2].

Erythrocytosis encompasses a number of disorders characterised by increased circulating red blood cells (RBCs) which can be classified into relative, idiopathic and absolute erythrocytosis [3]. Relative erythrocytosis refers to a condition in which the HCT is elevated, normal red cell mass (RCM), while the plasma volume may be reduced. It is associated with various events such as acute hypoxia, cigarette smoking, excessive alcohol intake and use of diuretics [4]. Meanwhile, idiopathic erythrocytosis is defined as an increased in RCM without an identified cause, i.e. the underlying causes for an acceleration in erythropoiesis is unknown. It is diagnosed based on the exclusion of various primary or secondary polycythaemias (congenital or acquired) [5].

Absolute erythrocytosis can be further divided into primary and secondary forms. Primary erythrocytosis is characterised by expansion of erythropoietic compartment independent of erythropoietin (EPO) and/or abnormally increased in the response of the haematopoietic precursors to EPO due to hereditary or acquired somatic mutations. On
the other hand, in secondary erythrocytosis, the erythroid precursors show normal response towards erythropoietin despite the increased level of circulating factors driving erythropoiesis (most commonly erythropoietin, insulin growth factor I, angiotensin II/angiotensin receptor axis aberrations and cobalt) [6]. Secondary erythrocytosis may arise from several causes including inappropriate erythropoietin production, renal tumours and other kidney diseases [7].

Erythrocytosis in blood donors may indicate an underlying disease state, thus thorough investigations must be carried out to exclude them. In some previous studies, such values have been investigated to exclude or confirm the presence of secondary causes of erythrocytosis such as smoking and respiratory problems, as well as primary myeloproliferative neoplasm (MPN), such as polycythaemia vera (PV) [8]. A previous study also showed that JAK2 V617F mutation has been found in the majority of patients with PV and also, in other MPN. A point mutation in the JAK2 V617F gene was identified most frequently in PV (65-97%), essential thrombocythaemia (23-57%) and myelofibrosis (35-57%). It is also occasionally present in myelodysplastic syndrome, chronic myelomonocytic leukaemia and other atypical myeloid disorders [9].

Clonal, acquired point mutation of JAK2 V617F gene, causing constitutive activation of the tyrosine kinase is believed to confer erythropoietin-independent hypersensitivity and erythropoietin-independent survival to the myeloid-stem cell. Thus, mutation in the JAK2 V617F caused this kinase to remain active even without growth factors stimulation, resulting in continuous proliferation of mature cells [9].

Detection of JAK2 V617F mutation at very low level in healthy individuals who have perfectly normal blood count, has also been reported in another study. The conclusion made, that this type of mutation is probably unrelated to the progression of the disease but its presence may indicate that a very early molecular event has occurred prior to the manifestation of haematological disorders, especially MPN phenotype. This mutation itself may not be adequate enough to provoke MPN [10]. However, the diagnosis and clinical assessment in blood donors may be difficult and complicated by frequent, regular blood donations that can mask an underlying pathological disease such as MPN, particularly PV, due to steady reduction of blood mass caused by the blood donations [8].

A study on a group of patients with underlying MPN has found that 18.1% of the patients were or had been blood donors. Interestingly, there was no other single feature except that blood donation was common in the history of those patients. Because of that, the importance of paying due attention to the blood cell count of the donors was highlighted [11]. In view of their finding, we strongly suggest that blood donors with high Hb level should be investigated thoroughly in order to exclude any significant pathological conditions that are associated with erythrocytosis. In this study, we investigate the occurrence of the JAK2 V617F gene mutation in a cohort of blood donors with erythrocytosis.

METHODS

This pilot cross-sectional study was conducted at Hospital Sultanah Aminah, Johor Bahru (HSAJB) and Hospital Universiti Sains Malaysia (HUSM), Kubang Kerian. The protocol for this study was approved by the Medical Research and Ethics Committee (MREC) and School of Medicine Science Research and Ethical Committee. Written consent was obtained from all subjects of this study.

From a total of 2238 blood donors who had donated blood during the 9-month study period, 175 donors with erythrocytosis (Men; Hb > 16.5g/ and Women; Hb> 13.8g/dl) were included. However, only blood from 45 donors with TWC > 12.0 x 10⁹/l, platelet > 450 x10⁹/ l and Hb > 17 g/dL (male), >16 g/dL (female) were subjected to allele-specific polymerase chain reaction (PCR) because of budget constraint. This molecular-based method qualitatively detects JAK2 V6127F mutation for any possibilities of MPN in blood donors with erythrocytosis.

Whole blood of approximately 4 ml in volume, was collected in EDTA from each donor. Genomic DNA was extracted using QiAmp® DNA Blood Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer’s instructions. The
extracted DNA was used as a template and was kept at 4°C until further use.

In order to detect clonal JAK2 V617F mutation, the portion of the JAK2 V617F gene that acquired the mutation was amplified by Allele Specific Oligonucleotide (ASO) Polymerase Chain Reaction (PCR). Briefly, the PCR was performed using a master mix containing Gene Amp 10x PCR Buffer II, Gene Amp MgCl2 25 mM, GeneAmp dNTP blend 10 mM, 5 µM forward control primer (FC), 5 µM forward specific primer (FS), 5 µM reverse primer ®, AmpliTaq Gold 5 U/µL and PCR water, as well as 20-200 ng of template DNA. A list of primers and their nucleotide sequences is shown in Table 1. The amplification conditions include, initial denaturation at 95°C for 10 min, followed by 14 cycles of denaturation (94°C for 20 sec), annealing (65°C, 60 sec) and extension (72°C for 60 sec). These cycles were followed by a final extension step at 72°C for 5 min. Subsequently, the PCR products were visualised by agarose gel electrophoresis, using a 100 bp DNA ladder (Promega, USA) as the molecular weight marker after staining with ethidium bromide.

Table 1 Primers used in the amplification of JAK2 V617F genes

<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer sequences (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward control</td>
<td>ATC TAT AGT CAT GCT GAA AGT AGA AAG</td>
</tr>
<tr>
<td>Forward specific</td>
<td>AGC ATT TGG TTT TAA ATT ATG GAG TAT ATT</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>CTG AAT AGT CCT ACA GTG TTT TCA GTT TCA</td>
</tr>
</tbody>
</table>

Each sample was run in duplicates along with no template control (NTC; blank), OLIGO, positive and negative controls. The positive and negative controls were samples which have been previously validated. A PCR product with only a distinct band of 364-bp size was considered negative and reported as no mutation in the JAK2 V617F gene, and a sample which gave two distinct bands of 364 bp and 203 bp, was considered positive and reported as presence of mutation in JAK2 V617F gene.

In the event where one of the duplicates was positive (mutation detected) while the other duplicate showed the absence of any bands, the result is considered negative. However, if one of the duplicate samples was positive (mutation detected) while the other was negative (mutation is not detected), the test should be repeated. In addition, if the duplicate sample did not show any band, the test was repeated.

RESULTS

Among 2238 blood donors, a total of 175 (7.8%) blood donors were noted to be erythrocytosis, based on the inclusion criteria. A majority (53%) of the blood donors with erythrocytosis in this study were males and their age ranged from 20-29 years old. Most of them were Malays, followed by Chinese, Indians and other ethnics (Figure 1). More than half of male donors were active smokers whereas all of the female donors were non-smokers. The majority of the blood donors donated their blood once to 20 times during the study period (Table 2).

![Figure 1](image_url) The number of donors with erythrocytosis according to age group

Table 2 Demographic data of blood donors with erythrocytosis

<table>
<thead>
<tr>
<th>Variable</th>
<th>Frequency (n=175)</th>
<th>%</th>
<th>Mean</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td></td>
<td>25.66</td>
<td>17-55</td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>92</td>
<td>53</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>83</td>
<td>47</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Smoking (Male only)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Smoker</td>
<td>49</td>
<td>53.26</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-smoker</td>
<td>43</td>
<td>46.74</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of donation</td>
<td></td>
<td>4.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 – 20</td>
<td></td>
<td>167</td>
<td></td>
<td></td>
</tr>
<tr>
<td>21 – 40</td>
<td></td>
<td>5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>41 – 60</td>
<td></td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>61 – 80</td>
<td></td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;80</td>
<td></td>
<td>1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
The descriptive data on haematological parameters in the blood donors with erythrocytosis is shown in Table 3. Mean Hb level for male and female (averaged) were 15.99 (g/dL). None of the 45 donors’ samples examined was positive for a 203-bp product indicative of JAK2 V617F mutation, but all of them were positive for the internal control (representative gel is shown in Figure 2). Thus, the JAK2 V617F mutation was absent in all of our donors.

Table 3 Descriptive data on haematological parameters of blood donors with erythrocytosis

<table>
<thead>
<tr>
<th>Variables</th>
<th>Mean</th>
<th>SD</th>
<th>Range</th>
<th>Normal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hb (g/dL)</td>
<td>15.9</td>
<td>1.52</td>
<td>13.9–19.1</td>
<td>12.0–16.5 (male)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>9.8–13.8 (female)</td>
</tr>
<tr>
<td>RBC (x10^12/L)</td>
<td>5.48</td>
<td>0.56</td>
<td>4.3–7.5</td>
<td>3.8–5.2</td>
</tr>
<tr>
<td>HCT (%)</td>
<td>47.64</td>
<td>4.20</td>
<td>37.2–55.4</td>
<td>34.9–46.9</td>
</tr>
<tr>
<td>MCV (fL)</td>
<td>87.32</td>
<td>4.13</td>
<td>63.4–97.3</td>
<td>80.8–100.0</td>
</tr>
<tr>
<td>MCH (pg)</td>
<td>29.39</td>
<td>2.26</td>
<td>20.9–33.7</td>
<td>26.4–34.0</td>
</tr>
<tr>
<td>WBC (x10^9/L)</td>
<td>8.20</td>
<td>2.36</td>
<td>3.2–20.1</td>
<td>4.5–11.0</td>
</tr>
<tr>
<td>Platelet (x10^12/L)</td>
<td>292.5</td>
<td>71.25</td>
<td>159–468</td>
<td>150–450</td>
</tr>
</tbody>
</table>

*The normal range for Hb level in accordance to a study by Roshan et al., 2009.

Discusssion

Measurement of Hb levels before each blood donation is mandatory in every blood centre in the world. Any abnormally low or high level of Hb detected must be taken seriously and warrants further investigations. Some studies carried out in the 80s until now, show that such donors are found to have hidden pathological disorders as serious as renal artery stenosis, as well as MPN [12, 13].

In the present study, we found the prevalence of erythrocytosis among our cohort of blood donors was 7.8%. However, this finding is much higher compared to a previous local study where the prevalence of deferred blood donors due to high Hb was 1.7% [14]. In the Turkish population, 2.8% of blood donors were deferred due to high Hb [15]. It was reported that 2.4% of Iranian donors were also deferred due to the same reason.

In this study, we found that all of our donors with erythrocytosis did not have any mutation in their JAK2 V617F gene. Scott et al. (2007) [16] reported the possible causes which might be underdiagnosed in these donors, were other types of PV with negative JAK2 V617F mutation or PV with exon 12 mutations [16]. In addition, other investigations conducted in the United Kingdom and Vienna showed around 5% of PV patient were negative for JAK2 V617F mutation [17, 18].

Detection of JAK2 V617F mutation at very low level in healthy individuals with perfectly normal blood count had also been reported in a few other studies. These studies also concluded that the mutation is probably unrelated with the progression of the disease but its presence may indicate that a very early molecular event has occurred prior to the manifestation of haematological disorders, especially MPN phenotype. This mutational event itself may not be adequate to provoke MPN. This study data was supported by the finding of low annual incidence of MPN reported compared to the frequency of individuals harbouring JAK2 V617F mutation [19].

Interestingly, the prevalence and frequency of JAK2 V617F mutation was also found to be higher in smokers compared to non-smokers, possibly contributed by the acceleration of erythropoiesis which causes the haematopoietic cells susceptible to JAK2 V617F mutation. However, there is no correlation that can be concluded between the prevalence of the JAK2 V617F mutation or the frequency of the mutation with RBC, HCT or WBC count [20]. Acceleration of erythropoiesis, myelopoesis and thrombopoiesis in smokers was partly due to continuous activation of the JAK/STAT signalling pathway [16].

Williams DM et al. (2007) [21] identified two unrecognized JAK2 V617F mutations and three unrecognized MPL mutations in JAK2 V617F-negative PV, erythrocytosis, and idiopathic myelofibrosis patients. Furthermore, they also
identified JAK2 exon 12 lesions in 30% of JAK2 V617F-negative PV patients. They further reported that either JAK2 V617F or JAK2 exon 12 lesions were present in 9% of erythrocytosis patients [21]. Most of these patients acquire the somatic gain-of-function mutation of JAK2 gene, JAK2 V617F, or another functionally similar to JAK2 V617F mutation, such as JAK2 exon 12 mutations that result in the proliferation of all three haematopoietic cell lines (erythroid, myeloid and megakaryocytes) causing panmyelosis [22]. More recently, another type of mutations in exon 12 of JAK2 was found in individuals with PV who lacked the V617F mutation but still showing a predominantly erythroid phenotype [23]. A rising number of these mutations has also been described and revealed that even though the mutated clone is small but their presence demonstrates a clonal disease [24].

Previously, investigators in Milan showed that around 8% of regular volunteer blood donors had high Hb and HCT level. They also found that some donors with an upper limit of Hb and HCT level have underlying acquired pathological conditions such as early stage of PV; a type of MPN, as well as respiratory failure. Thus, the study concluded that some donors with upper limit Hb and HCT levels may have hidden medical problems [25].

Geeta et al. (2010) [26] reported a case of a consistent rise in haemoglobin and haematocrit with negative JAK2 V617F mutation analysis. A diagnosis of PV was made on the basis of the British Committee for Standards in Haematology (BCSH) guidelines [26].

An extensive list of acquired secondary causes of erythrocytosis also needs to be considered since a number of patients do not have an identifiable cause of erythrocytosis. Erythrocytosis can be classified into relative, absolute and idiopathic erythrocytosis [27].

The most common cause of relative erythrocytosis is a contraction of plasma volume possible due to dehydration, contributed by increased water and salt loss via sweating due to hot weather, inadequate liquid intake, excessive coffee or tea intake as well as smoking. In fact, smoking can further aggravate the effect of dehydration by increasing the plasma viscosity [28]. We observed that more than half of male donors were smokers. A study done on the smoking habit of the Danish population revealed that cigarette smoking has an escalating effect on Hb level which is proportional to the number of cigarettes consumed [29].

Besides, most of our blood donation drives were conducted during daytime where the temperature is usually at its peak. As a fact, Malaysia is a Southeast Asia country, which is situated in a tropical region and known to have a hot and humid climate with heavy tropical rains throughout the year [30].

In absolute erythrocytosis, the Hb, RBC and HCT were all high, exceeding the upper limit of the normal reference range. The levels of Hb, HCT and RBC count were significantly higher in a smoker compared to a non-smoker [13, 29, 31]. Acceleration of erythropoiesis, myelopoiesis and thrombopoiesis in smokers may partly be due to continuous activation of the JAK/STAT signalling pathway [32].

High Hb in blood donors may be a normal variation or classify as an idiopathic erythrocytosis. However, idiopathic erythrocytosis is a diagnosis of exclusion, thus thorough investigations must be proceeded to exclude all other causes of primary or secondary erythrocytosis before this diagnosis is being made.

We observed that donors with higher number of blood donations have higher Hb level. Our finding is similar to a recent local study who demonstrated a significant correlation between Hb and the number of donations [33]. The postulated mechanism behind this is thought to be due to prolonged and continuous stimulation of myeloid cells by phlebotomies, can induce inhibition of the feedback control of cells committed for the erythropoiesis [11]. Other postulated mechanism suggested that repeated increase in Hb level in healthy blood donors may indicates that the person have acquired JAK2 mutation [8].

**CONCLUSION**

Erythrocytosis in blood donors may indicate an underlying disease state, thus thorough investigations and follow-up must be done to exclude them. Findings of this study suggest the need for future studies with additional tests, such as specific primers for JAK2 exon 12 to improve the diagnosis of JAK2 617F negative blood donors with persistent erythrocytosis.
Conflict of Interest
Authors declare none.

Acknowledgement
This work was supported by short-term grant 304/PPSP/61313044. We thanked the members of the Molecular Laboratory (Department of Haematology), Puan Narishah, Puan Salwana, Puan Mira and Puan Salamah for the helpful discussions and technical support.

REFERENCES


