Determining Laboratory Reference Values of TREC and KREC in Different Age Groups of Iranian Healthy Individuals

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ABSTRACT

Assessment of the number of T-cell receptor excision circles (TREC) and kappa-deleting recombination excision circles (KREC) copies has been recently described as biomarkers of newly formed T and B cells respectively. In this study, we aimed to explore the effects of demographic variables including age, gender, weight, height and ethnicity on these two episomal DNA molecules. Second, for the first time in our country, we determined the reference values of TREC and KREC copy numbers in different age groups of Iranian healthy individuals as a threshold for identifying T cell and B cell lymphopenia.

The TREC and KREC copy numbers were evaluated in 251 dried blood spot (DBS) samples from healthy volunteers (age range: 0-60 years). Six primary immunodeficiency (PID) patients were included as disease controls.

TREC and KREC copies were markedly reduced with increasing age. Although the levels of TREC and KREC were higher in females than males, this difference did not reach statistical significance.

These findings suggest that demographic variables including age should be considered for interpretation results of the TREC/KREC assay.

Keywords: Immunodeficiency; Kappa-deleting recombination excision circles; Reference value; T-cell receptor excision circles

INTRODUCTION

T-cell receptor excision circles (TREC) are circular DNA fragments produced during T cell receptor (TCR) gene rearrangement in thymocytes. TRECs are then exported from the nucleus to the cytoplasm of recent thymic emigrants (RTEs) as episomal DNA which not being replicated during mitosis. Therefore, the TREC concentration shows the thymic generation of naïve T lymphocytes.¹,³ In 2005, TREC was applied by Chan and Puck for the screening of newborns for severe combined immunodeficiency (SCID) and other T cell immunodeficiencies.⁵ It has also been shown that the
TREC assay can be used in evaluating T cell generation as well as the history of T cell replication in selected clinical conditions, including human immunodeficiency virus (HIV) infection, the aging process, autoimmune diseases, hematopoietic stem cell transplantation (HSCT), and the outcome of chemotherapy. Kappa-deleting recombination excision circles (KREC) are the circular by-product of B cell receptor (BCR) gene rearrangements and are present in naïve B cells. Therefore, measuring KREC copy numbers is a powerful tool to quantify the generation of naïve B cells and their subsequent replication history, especially in B cell lymphopoenia disorders like X-linked agammaglobulinemia (XLA). More recently, a multiplex quantitative real time PCR method has been developed to identify the T and B cell lymphopoenias through simultaneous measurement of TREC and KREC copy numbers. Some patients with a family history of immunodeficiency can be identified by prenatal diagnosis of the disease while other patients may be recognized following the onset of severe infections. However, TREC/KREC assay can be useful for early diagnosis of these patients in the newborn period, which reduce treatment costs and increase the survival. This assay has other advantages including high sensitivity, high throughput, low cost, and ability to apply DNA extracted from a minimal volume of blood samples collected on Guthrie cards and could detect other conditions involving T and/or B cell lymphopoenia.

Accordingly, it would be necessary to determine the reference values of TREC and KREC based on age, gender, ethnicity and other related factors in the Iranian population for precise interpretation of each parameter in different pathophysiologial situations. In this study, we aimed to determine the normal values of TREC and KREC in Iranian healthy individuals. Due to the noticeable effects of demographic conditions on the interpretation of KREC and TREC numbers, we also analyzed the results based on age, gender, ethnicity as well as the weight and length of newborns at birth.

**MATERIALS AND METHODS**

**Sample Collection**
A total of 251 blood samples of healthy individuals (99 males and 152 females; age range 0-60 years) without any clinical manifestation of primary immunodeficiency disorders were dropped onto GE 903 Guthrie cards (GE Healthcare Life Sciences Corp, Marlborough, Massachusetts, USA). Blood samples from six primary immunodeficiency (PID) patients with a definitive diagnosis of SCID (2 males), XLA (2 males) and common variable immunodeficiency (CVID, 1 male, and 1 female) were used as disease controls. The Guthrie card samples were dried at room temperature and stored at -20 for a maximum of two months, until being tested as previously described.

Finger, for adults, and heel, for newborns, stick blood samples were obtained at the Immunology, Asthma, and Allergy Research Institute (IAARI) and the Valiasr Hospital (both under the supervision of the Tehran University of Medical Sciences) from 4 May till 4 July 2017.

DNA extraction from 3.2 mm punched DBS samples (~3 microliters of whole blood) and multiplex quantitative real-time PCR were performed as previously described with minor technical modifications. Following DNA extraction, TREC-KREC-ACTB triplex real time PCR was performed on a final volume of 30 microliters according to the manufacturer’s instructions (ImmunoIVD, Stockholm, Sweden). Finally, TREC and KREC levels were calculated per the punch of the Guthrie cards. The β-actin (ACTB) copy numbers were used to confirm the quality of the DNA extraction from the DBS punches and also the real time PCR process. Samples with ACTBs higher than 1000 copies/3.2 mm punched DBS were regarded as having a high quality. The standard curves were generated using serial dilutions of the standard plasmids containing 10-100,000 copies of TREC and KREC and 100-1,000,000 copies of ACTB provided in the kits (SCREEN-ID Neonatal Screening Kits, ImmunoIVD AB, Stockholm, Sweden). TREC/KREC assay was performed at the ImmunoIVD AB Company, Stockholm, Sweden.

**Statistical Analysis**
Data analysis was performed in Excel 2013 (Microsoft Office, USA) and IBM SPSS Statistics, version 21 (IBM Corp., Armonk, N.Y., USA). Quantitative variables were analyzed by Kolmogorov-Smirnov test for normal distribution and then expressed as mean±standard deviation and median as well as lower (Q1) and upper (Q3) quartile. Mann-Whitney and Kruskal-Wallis tests were used for mean comparisons between two and multiple groups for non-parametric data, respectively. Independent-samples t-test was also
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applied to compare the means of two groups for parametric data. Pearson and Spearman correlation coefficients were performed for parametric and non-parametric data, respectively. A p-value of <0.05 was considered statistically significant.

Ethics
This study was approved by the ethics committee of Tehran University of Medical Sciences (N.: IR.TUMS.IAARI.REC.1395.382) and samples were collected after signing the informed consent forms by participants or their guardians.

RESULTS

Gender and Ethnicity-Related Differences for TREC and KREC Copy Numbers
Higher levels of both TREC and KREC copies were detected in females than males at all ages, although the differences were not statistically significant (Table 1).

To explore the effect of gender and age simultaneously on TREC and KREC levels, we compared two groups of males and females at different age ranges. We only observed a significant dissimilarity in TREC values between females and males in the age range of 5-15 y (p=0.005) (Figure 1A and B).

In the present study, we also evaluated the impact of ethnicity on TREC and KREC copies of thirty-three healthy individuals (age range 0-35 y) divided into four groups (Kord, Tork, Lor and Fars, the four main ethnicities in Iran). Despite small changes, we could not find any significant variations (data not shown).

Comparing Weight and Height at Birth with TREC and KREC levels
Pearson correlation was calculated between TREC and KREC levels, weight and height at birth in 36 newborns (0-7 days after birth).

![Figure 1. Comparison of T-cell receptor excision circles (TREC) (A) and kappa-deleting recombination excision circles (KREC) (B) copy numbers between females and males among healthy individuals (group 0-1 m F=18 M=18, 1 m-5 y F=17 M=14, 5-15 y F=26 M=22, 15-35 y F=62 M=21 , 35 y<F=29 M=21). Data as shown means ± SD. Mann-Whitney for non-parametric data and independent-samples t-tests for data showed normal distribution. (**p-value<0.01). m: month; y: year; F: female; M: male; DBS: dried blood spot.]

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Table 1. The results of comparison of T-cell receptor excision circles (TREC) and kappa-deleting recombination excision circles (KREC) copy numbers between healthy male and female individuals aged 0-60 years

<table>
<thead>
<tr>
<th></th>
<th>Female</th>
<th>Male</th>
<th>p-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>TREC</td>
<td>125 ± 67</td>
<td>117 ± 69</td>
<td>0.28</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KREC</td>
<td>70 ± 42</td>
<td>64 ± 43</td>
<td>0.30</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

DBS: Dried blood spot. * Mann-Whitney test was done.

We only found a significant negative correlation between birth height and TREC copies (R: -0.408; p=0.035). While no significant association was detected between the copies of KREC and height (R: -0.069; p=0.701 and R:-0.239; p=0.181, respectively) (Figure 2A, B) as well as TREC and KREC levels with weight at birth (R:-0.009; p=0.999) (Figure 2C, D).

Age-Related Differences for TREC and KREC Copy Numbers

To investigate copy numbers of TREC and KREC for different age ranges, we analyzed the levels of TREC and KREC in DBS samples from healthy individuals by triplex quantitative real time PCR. As shown in Figure 3, both TREC and KREC levels decreased significantly with increasing age (p<0.001). However, no significant changes were found between the 1 m-5 y group and newborns (0-1 m).

We also calculated the reference ranges of TREC, KREC, and ACTB, categorized into five different age groups (0-1 m, 1 m-5 y, 5-15 y, 15-35 y, >35 y) and observed significant age-related alterations in the levels of both TREC and KREC as shown in Table 2. The median copy numbers of TREC, KREC, and ACTB were also evaluated in these five groups (Table 2). By using these reference ranges, TREC copies in samples from two patients with XLA (P3 and P4) and one patient with CVID (P5) fell within the normal range (Table 3). As expected, two patients with autosomal recessive SCID as well as one with CVID (P6) showed low or undetectable levels of TREC. All six patients showed zero copies of KREC.

Table 2. Reference ranges of T-cell receptor excision circles (TREC) and kappa-deleting recombination excision circles (KREC) in DBS samples from Iranian healthy individuals at different groups of ages

<table>
<thead>
<tr>
<th>Age at time of sampling</th>
<th>Sex (female/male)</th>
<th>The reference range of TREC (copies/3.2mm DBS)</th>
<th>The reference range of KREC (copies/3.2mm DBS)</th>
<th>The reference range of ACTB (copies/3.2mm DBS)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n, n)</td>
<td>Median (Q1-Q3) of TREC (copies/3.2mm DBS)</td>
<td>Median (Q1-Q3) of KREC (copies/3.2mm DBS)</td>
<td>Median (Q1-Q3) of ACTB (copies/3.2mm DBS)</td>
</tr>
<tr>
<td>0-1 m</td>
<td>(18, 18)</td>
<td>270 ±135</td>
<td>90 ±50</td>
<td>9722 ±3921</td>
</tr>
<tr>
<td></td>
<td></td>
<td>246 (188-312)</td>
<td>53 (53-125)</td>
<td>8215 (7150-11952)</td>
</tr>
<tr>
<td>1 m-5 y</td>
<td>(17, 14)</td>
<td>219 ±126</td>
<td>137 ±101</td>
<td>8503 ±3460</td>
</tr>
<tr>
<td></td>
<td></td>
<td>215 (103-293)</td>
<td>94 (63-220)</td>
<td>8235 (5548-10249)</td>
</tr>
<tr>
<td>5-15 y</td>
<td>(26, 22)</td>
<td>83 ±48</td>
<td>53 ±28</td>
<td>8510 ±2651</td>
</tr>
<tr>
<td></td>
<td></td>
<td>72 (43-119)</td>
<td>47 (34-70)</td>
<td>8248 (6669-9677)</td>
</tr>
<tr>
<td>15-35 y</td>
<td>(62, 21)</td>
<td>29 ±23</td>
<td>24 ±16</td>
<td>9168 ±3404</td>
</tr>
<tr>
<td></td>
<td></td>
<td>23 (15-40)</td>
<td>20 (12-34)</td>
<td>8330 (7299-11132)</td>
</tr>
<tr>
<td>35 y &lt;</td>
<td>(29, 21)</td>
<td>0-35</td>
<td>29 ±22</td>
<td>24 (13.42-36)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6 (0.21-12)</td>
<td>24 (13.42-36)</td>
<td>7043 ±1751</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>6870 (6037-7863)</td>
</tr>
</tbody>
</table>

DBS: Dried blood sample; Y: year; m: month
Figure 2. Correlation analysis of T-cell receptor excision circles (TREC) and kappa-deleting recombination excision circles (KREC) levels with demographic variables. TREC and KREC copy numbers and weight, as well as height at birth, were depicted as scatter diagrams with a linear regression line for newborns (age: 0-7 days after birth, n=36). A significant negative correlation was found between the birth height and TREC \( (R=-0.408; \ p\text{-value}=0.035) \). DBS: dried blood spot; cm: centimeter; g: gram.
Figure 3. Comparison of T-cell receptor excision circles (TREC) (A) and kappa-deleting recombination excision circles (KREC) (B) levels at different ranges of age in healthy individuals (group 0-1 m n=36, 1 m-5 y n=31, 5-15 y n=48, 15-35 y n=83, 35 y < n=50). Data as shown means±SD. Kruskal-Wallis test was done (**p-value<0.01, ***p-value<0.001, related to TREC and KREC level at birth (0-1 m)). DBS: dried blood spot; y: year; m: month.

Table 3. The results of T-cell receptor excision circles (TREC) and kappa-deleting recombination excision circles (KREC) in DBS samples from Iranian primary immunodeficiency (PID) patients

<table>
<thead>
<tr>
<th>Patients</th>
<th>Age of sampling</th>
<th>Sex</th>
<th>TREC (copies/3.2mm DBS)</th>
<th>KREC (copies/3.2mm DBS)</th>
<th>Mutated gene</th>
<th>Diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>1 m</td>
<td>Male</td>
<td>0</td>
<td>0</td>
<td>RAG2</td>
<td>SCID</td>
</tr>
<tr>
<td>P2</td>
<td>2 m</td>
<td>Male</td>
<td>1</td>
<td>0</td>
<td>ADA</td>
<td>SCID</td>
</tr>
<tr>
<td>P3</td>
<td>14 y</td>
<td>Male</td>
<td>129</td>
<td>0</td>
<td>BTK</td>
<td>XLA</td>
</tr>
<tr>
<td>P4</td>
<td>18 y</td>
<td>Male</td>
<td>30</td>
<td>0</td>
<td>BTK</td>
<td>XLA</td>
</tr>
<tr>
<td>P5</td>
<td>24 y</td>
<td>Male</td>
<td>16</td>
<td>0</td>
<td>ND</td>
<td>CVID</td>
</tr>
<tr>
<td>P6</td>
<td>17 y</td>
<td>Female</td>
<td>0</td>
<td>0</td>
<td>ND</td>
<td>CVID</td>
</tr>
</tbody>
</table>

DISCUSSION

To our knowledge, this is the first study investigating the effects of aging, gender, ethnicity, birth weight and height on TREC and KREC copy numbers in Iranian healthy individuals. Our findings showed no significant changes between females and males, regardless of age, which is in accordance with previous studies.15,16 In contrast, selected previous studies have suggested a higher level of TREC numbers among females than males1,17 which was only seen in the 5-15 year age group. In addition, another study concluded on neonates reported higher levels of TREC in females than males.18 Although other evidence indicated that TREC copy numbers in adults (age range from 24 to 60 years) are dependent on gender, KREC
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copies were not influenced by this factor.\textsuperscript{19,20} Due to the substantial immunomodulatory role of sex hormones on immune responses, these sex-related alterations may be explained as a result of hormonal variations.\textsuperscript{21-23}

Some previous studies have reported ethnic-related changes in TREC and KREC values suggesting that the changes observed in this study might be related to genetic variations,\textsuperscript{24-26} and/or more exposure to various environmental factors such as infectious agents among different ethnicities.\textsuperscript{27} However, in the current study, no significant differences were seen but insufficient sample size was a limitation of our study that may affect the results.

A correlation between weight and TREC value has been analyzed in several studies.\textsuperscript{7,17,28} A recent report on DBS samples of pre-term neonates showed a positive association between copy numbers of TREC and KREC with birth weight.\textsuperscript{7} Another study showed a positive relationship between birth weight which was divided to three groups (very low, low and normal birth weight of pre-term newborns, <28 weeks gestation) and TREC copies but there was no association between full term and late pre-term (32 to lower than 37 weeks gestation) neonates.\textsuperscript{18} While a negative association between TREC numbers with the obesity indicators in adult atomic-bomb survivors have been described in another study concluding that obesity can increase the apoptosis of thymocytes.\textsuperscript{17} Some previous studies have tested the correlation between immune function and body height of healthy adults.\textsuperscript{29,30} We also investigated the correlation between both TREC and KREC copies with birth height. In the current study, no significant correlation was observed between both marker and weight while there was a significant negative correlation between the length of full-term neonates and TREC copies.

Several studies have examined the impact of aging on thymopoiesis by measuring the age-related changes in TREC copy numbers.\textsuperscript{31-33} In a study published by Douek et al the authors found that blood samples of older children and adults had 10 and 100 fold lower numbers of TREC than infants, respectively. These findings reflected a reduced production of newly formed T cells with increasing age.\textsuperscript{34} Another study on combined variable immunodeficiency (CVID) patients and healthy controls indicated decreased levels of TREC with advancing the age in both patients and controls, despite stable levels of KREC,\textsuperscript{35} while a striking decrease of these two was reported in samples of healthy children (age range from 2 months to 16 years).\textsuperscript{36} Indeed, a state of immunodeficiency appears during the aging process, possibly as a result of thymic involution since the first year of life.\textsuperscript{36,37} As both TREC and KREC persist in the cell and are not replicated during mitosis, they are diluted after cell division.\textsuperscript{20} In the present study, we observed decreased levels of TREC and KREC during aging, starting at about 6 years of age. Both TREC and/or KREC were detectable in elderly individuals in our study as well as some previous evidence,\textsuperscript{1,38,39} indicating that the generation of T- and B- cells could remain as a result of the thymus and bone marrow activity even in old age, respectively.\textsuperscript{3,38} Other studies showed that TREC levels were reduced in healthy individuals and HIV infected patients by about 95\% between almost twenty and sixty-five years of age.\textsuperscript{39,40} Others demonstrated a strong decrease of both TREC and KREC since the age of three years,\textsuperscript{16} or only TREC from birth to about four years of age.\textsuperscript{19} Some previous studies have determined the reference ranges of TREC and/or KREC in healthy individuals in different age groups.\textsuperscript{15,41} Because the level of these two molecules gradually reduced during aging, this assay has proven for newborn screening although some studies used it to measure T- and B- cell neogenesis in children and adults with PIDs, autoimmune diseases and for the monitoring of the immune recovery after therapies including hematopoietic stem cell transplantation (HSCT), enzyme replacement as well as antiretroviral treatment for HIV infected patients.\textsuperscript{5,19,38} Before the current study, we determined cut-offs of both TREC and KREC for detecting newborns with PID (Manuscript was submitted for publication). For investigating the levels of both molecule in PID patients aged higher than 1 month, which are be exploring by us in another study, it is necessary to calculate referenced values of these two markers in healthy individuals for different age groups. Thus, in this study, we took the population group of 0-60 years due to the most PID patients referred to IAARI placed at this age range. For this choice, we also considered selected age ranges in some previous studies.\textsuperscript{15,19,42} As disease controls, selected PID patients were evaluated for their TREC/KREC copy numbers. Our results indicated low levels of both TREC and KREC values in DBS samples of two autosomal recessive SCID patients (P1 and P2) which agrees with previous reports.\textsuperscript{4,7,9} However, both XLA patients (P3 and P4) showed normal TREC levels.
but zero copies of KREC which were associated with low to zero numbers of B cells in these patients, which was in accordance with previous reports.²⁹,³⁰ Two CVID patients (P5 and P6) had undetectable levels of KREC. TREC copies in P5 were in normal range while P6 showed zero copy numbers of TREC. Our findings were in accordance with a study by Serana et al.³⁵ that demonstrated decreased levels of both two parameters in CVID adult patients. On the contrary, some other studies revealed normal copies of these two molecules in newborns with CVID.³⁶,³⁷ As shown in the results, the concentration of both two target molecules were reduced gradually in healthy individuals during aging.¹,¹¹ Therefore, age should be considered at the time of sampling.

The difficult conditions for transferring these kits to Iran and the inability to access to some data of included people were the limitations of our study. Despite these limitations, the findings of our study able us to interpret and compare the results of this assay in samples of adults with PID to reference values of these two molecules in related age groups, so this could reduce false positive results in our examinations.

Our findings show that the age should be considered as the most important confounder for measurement of TREC/KREC copy numbers than factors like ethnicity, weight, and height. Therefore, we calculated the reference ranges of these two parameters for different age groups in order to facilitate the interpretation of the results for newborn screening as well as monitoring of T and B cell immune alterations following HSCT and antiretroviral therapy.

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REFERENCES


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