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UDC: 616.14-005.6-02:575
DOI: https://doi.org/10.2298/VSP190402086T

# Association of prothrombin, FV Leiden and MTHFR gene polymorphisms in the Montenegrin patients with venous thromboembolism

## Povezanost polimorfizama za protrombin, FV Leiden i MTHFR gen sa venskim tromboembolizmom kod bolesnika u Crnoj Gori

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**Abstract**

**Background/Aim.** Polymorphisms of the factor V Leiden (FV G1691A), prothrombin (FII G20210A), and methylenetetrahydrofolate reductase (MTHFR C677T) genes are the most commonly investigated inherited risk factors for developing venous thromboembolism (VTE). Despite this fact, there is insufficient data regarding their clinical burden and distribution in the Montenegrin population. The aim of the study was to determine the frequency of these polymorphisms in Montenegrin patients with VTE. **Methods.** This case-control study was conducted on 160 Caucasian subjects. The study group was composed of 80 patients (35 men and 45 women) with VTE. The control group consisted of 80 healthy individuals (32 men and 48 women) without previous thromboembolic episodes. Genotyping of the FV G1691A, FII G20210A, and MTHFR C677T polymorphisms was performed by allele-specific polymerase chain reaction (PCR). **Results.** The frequency of heterozygotes (HET) for FII G20210A and FV G1691A was significantly higher in the VTE group compared to the healthy control group ($\chi^2$ = 11.7; $p$ = 0.001 and $\chi^2$ = 17.69; $p$ < 0.001, respectively). The association of FII G20210A and FV G1691A polymorphisms with an increased risk of VTE [odds ratio (OR) 10.5; 95% confidence interval (CI) = 2.34 to 47.27, and OR 14.8; 95% CI = 3.34 to 65.43; $p$ < 0.001, respectively] was confirmed. Recessive homozygotes (RH) for FII G20210A and FV G1691A were not found in any of the investigated groups. Regarding MTHFR C677T, the difference between the frequency of HET and RH in the control and VTE group was not significant. **Conclusion.** Our study showed that FII G20210A and FV G1691A polymorphisms are significantly associated with VTE. Detection of the above-mentioned polymorphisms prior to VTE development can contribute to the prevention of further VTE occurrence, especially among patients' relatives who are carriers of these polymorphisms.

**Key words:**
**factor v; genes; mutation; polymorphism, genetic; prothrombin; thromboembolism.**

**Apstrakt**

**Uvod/Cilj.** Polimorfizmi u genima koji kodiraju faktor V Leiden (FV G1691A), protrombin (FII G20210A) i metilentetrahidrofolat reduktazu (MTHFR C677T) su najčešće ispitivani nasledni faktori rizika od nastanka venskog tromboembolizma (VTE). Uprkos tome, ne postoji dovoljno podataka o kliničkom značaju i distribuciji tih polimorfizama u crnogorskoj populaciji. Cilj rada bio je da se utvrdi frekvencija tih polimorfizama kod bolesnika sa VTE u Crnoj Gori. **Metode.** Istraživanje je sprovedeno kao studija tipa slučaj-kontrola na 160 ispitanika kavkaskog porekla. Studijsku grupu sačinjavalo je 80 bolesnika (35 muškaraca i 45 žena) sa VTE, a kontrolna grupa se sastojala od 80 zdravih ispitanika (32 muškarca i 48 žena), koji nisu imali tromboembolijske epizode bolesti. Genotipizacija polimorfizama za FV G1691A, FII G20210A i MTHFR C677T izvršena je alel specifičnom, lančanom reakcijom polimeraze (PCR). **Rezultati.** Učestalost heterozigota (HET) za FII G20210A i FV G1691A bila je značajno viša u VTE grupi u poređenju sa kontrolnom grupom ($\chi^2$ = 11,7; $p$ = 0,001 i $\chi^2$ = 17,69; $p$ < 0,001). Potvrđena je povezanost polimorfizama za FII G20210A i FV G1691A sa povećanim rizikom od VTE [*odds ratio* (OR) 10,5; 95% *confidence interval* (CI) = 2,34 do 47,27; $p$ = 0,001 i OR 14,8; 95% CI = 3,34 do 65,43; $p$ < 0,001]. Recesivni homozigoti (RH) za FII G20210A i FV G1691A nisu pronađeni ni u jednoj

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od ispitivanih grupa. Za polimorfizam MTHFR C677T nije pronađena značajna razlika u učestalosti HET i RH između VTE grupe i kontrolne grupe. **Zaključak.** Naša studija je pokazala da su polimorfizmi za FII G20210A i FV G1691A značajno povezani sa VTE i njihovo pravovremeno otkrivanje može doprineti prevenciji VTE, posebno kod srodnika bolesnika koji su nosioci tih polimorfizama.

**Ključne reči:**
**faktor v; geni, mutacija; polimorfizam, genetički; protrombin; tromboembolija.**

## Introduction

Venous thromboembolism (VTE) is a multifactorial disease that results from the interaction between acquired and genetic risk factors with an incidence of 1–2 per 1,000 persons annually. The most common clinical manifestations of VTE are deep venous thrombosis (DVT) and pulmonary thromboembolism (PTE). Below the age of 25, thrombosis is rarely diagnosed, but after the age of 40, it shows an increasing tendency, and it is often repeated and additionally complicated by pulmonary embolism [1–3].

Thrombophilia represents a group of inherited and acquired coagulation abnormalities associated with thrombosis. Although thrombophilia itself is not a disease, it increases the risk of developing VTE in response to the provocation/perturbation by environmental factors [4, 5]. Numerous acquired and hereditary risk factors are known to be responsible for the occurrence of VTE [5, 6]. Genes encode proteins of the hemostasis system, affecting their synthesis and activity, and the acquired factors stimulate the tendency of hypercoagulability. Many studies suggested a multifactorial etiology of VTE. Therefore, the hereditary predisposition only represents an increased risk and does not determine whether the disease will necessarily manifest in the genetically affected population [7–11].

The most common single nucleotide polymorphisms (SNPs) tested in the genes associated with VTE are genes for factor II (prothrombin, FII G20210A), factor V Leiden (FV G1691A), and methylenetetrahydrofolate reductase (MTHFR C677T) [11]. Many previous studies have shown that the frequency of polymorphisms [recessive homozygotes (RH) and heterozygotes (HET)] of these genes was significantly higher in patients with VTE compared to the healthy population [11–14]. Nevertheless, there are studies with controversial results, especially on the role of MTHFR gene polymorphisms in VTE [15–16]. Besides, the prevalence of genetic polymorphisms in the healthy population can vary regarding different geographical regions and differ among ethnic groups [17, 18].

According to the literature, FV G1691A polymorphisms are the most important genetic risk factors for the manifestation of VTE. The gene for FV consists of 25 exons, and it is located on chromosome 1q23. FV G16961A mutation leads to the replacement of arginine for glutamine at position 506 in the protein resulting in reduced sensitivity of FV to the inhibitory effect of activated protein C (APC) and the balance of the hemostatic system moves to a state of hypercoagulability [19].

Coagulation factor V (FV) is an important protein (cofactor), with a double role in maintaining hemostatic balance due to the same influence in both procoagulation and the anticoagulation mechanism of blood clotting [19]. The relative risk of thrombosis in HET FV G16961A carriers is 3 to 7 times, and for the RH of FV A16961A carriers, 50 to 80 times higher, compared to noncarriers of these polymorphisms [20]. The prevalence of FV G16961A increases from West to East Europe and from North to South Europe. In a healthy European population, HET for FV G16961A is present with a frequency of 5%–7%, and 15%–50% in VTE patients [18, 21]. This mutation is rarely found in the populations of Africa, Australia, and South Asia [22, 23]. A large epidemiologic study conducted in the USA presented a 5.27% incidence of HET for FVG1691A in European individuals, 2.21% in Latinos, 1.23% in Afro-Americans, 1.25% in American Indians, and only 0.45% in Asians [22].

Another important genetic risk factor for VTE is FII G20210A (prothrombin). Prothrombin is the precursor of thrombin and has an important role in the formation of fibrin in the coagulation process. Substitution of guanine (G) for adenine (A) in the FII gene at position 20210 is associated with an increase in prothrombin plasma concentrations. Gene for FII is located on chromosome 11p11, in a 3'-untranslated region [24]. The prevalence of polymorphism of *FII* G20210A in the European population is 2%–4% [25]. The prevalence of FII G20210A in Northern Europe is 1.7%, and in the Mediterranean region is twice as high. In patients with VTE, HET for FII G20210A is present in 6%–18% of the cases [25]. This polymorphism was found to be very rare or even absent in African and Asian populations, as well as in American Indians and Australian Aborigines [25]. RH variant for FII G20210A is very rare. The risk becomes 50-fold higher among individuals with two copies of the 20210A allele [26].

MTHFR is the key enzyme in regulating the metabolism of folate and homocysteine levels, which catalyzes the reaction of 5, 10-methylenetetrahydrofolate to 5-methylenetetrahydrofolate, which functions as a methyl donor in the conversion of homocysteine to methionine. Increased level of homocysteine in the blood has a toxic effect on the vascular structure [27]. RH and HET of MTHFR C677T result in the reduction of synthesis of 5-methylenetetrahydrofolate, leading to an increased concentration of homocysteine in the plasma, which increases the risk of arterial and venous thrombosis [27]. The gene for MTHFR is located on chromosome 1 at position 1p36.3 [28]. The HET prevalence of the MTHFR C677T gene in a healthy European population is high (30–50%), and it is not associated with an increased risk of thromboembolism. The prevalence of MTHFR C677T in Northern Europe is significantly lower than in Southern Europe, so the MTHFR C677T polymorphism in Norway accounts for about 28%, and in Italy about 44%. The prevalence of RH in the healthy European population is 5–15% [29, 30].

The SNPs in FV G16961A, FII G20210A, and MTHFR C677T genes have been broadly investigated worldwide. Although the majority of studies confirmed the importance of these polymorphisms as the most common inherited risk factors for

the development of VTE, there are still some inconsistencies, especially regarding the different ethnic populations [17, 18, 23]. To our knowledge, there is no published data on these polymorphisms in patients with VTE and the general population in Montenegro, although similar investigations of polymorphisms have been conducted on the population of pregnant women with adverse pregnancy outcomes and pregnant women with successful procreation [31, 32].

The aim of this case-control study was to determine the frequency of FV G16961A, FII G20210A, and MTHFR C677T gene polymorphisms in both patients with VTE and healthy subjects in Montenegro and to assess their association with VTE development. This is the first investigation of SNPs in genes for thrombophilia susceptibility (FII, FV, and MTHFR) in Montenegrin patients with VTE, and we believe that it could serve as knowledgeable data for comparison with different populations from the region or broader.

## Methods

This case-control study consisted of 160 Caucasian subjects. The study group was composed of 80 patients (35 men and 45 women) after experiencing at least one clinically confirmed episode of VTE (DVT and/or PTE). The evidence of VTE had been documented in their medical records with appropriate diagnostic methods and specialists' expertise.

The main criterion for inclusion of patients in the study group was one or more recurrent episodes of VTE without any of well-known comorbidity (cancer, diabetes mellitus). The control group (CG) consisted of 80 healthy persons (32 men and 48 women) who had not experienced the thromboembolic episode by the time they accepted the participation in the study. For the CG, we randomly selected 80 voluntary blood donors (32 men and 48 women) with no history of the thromboembolic episode, who were as similar as possible to the VTE group regarding age and gender.

The Ethics Committee of the Clinical Center of Montenegro approved this case-control, retrospective study (Number: 03/01-5005/1). All participants provided their informed consent to take part in the research, and the study was performed in accordance with the Declaration of Helsinki. The research was conducted at the Center for Medical Genetics and Immunology, Clinical Center of Montenegro, in the period from January 2015 to July 2017.

### *Gene analysis*

Deoxyribonucleic acid (DNA) was isolated from peripheral blood collected in 4.5 mL tubes with Na-citrate (9 N coagulation sodium citrate 3.2%) and extracted with a commercial test QIA amp DNA Blood Mini Kit (Qiagen, Germany). Extracted DNA was dissolved in 200 μl buffer AE and stored at -20°C. SNPs for FV G1691A, FII G20210A, and MTHFR C677T were detected by allele-specific polymerase chain reaction (PCR). DNA amplification was performed by Attomol Quick type, factor II 20210 G > A, factor V G1691A and MTHFR 677C > T. HotStarTaq DNA polymerase was provided by Qiagen. Heterozygous control template DNA for FII G20210A, FV G1691A, and MTHFR C677T were used as positive PCR controls. PCR was performed in a thermocycler (Mastercycler gradient Eppendorf) using the temperature regime: initial activation (15 min 95°C), 5 cycles (1 min, 94°C; 1 min, 63°C; 1min, 72°C), 30 cycles (30 s, 94°C; 30 s, 63°C, 30 s, 72°C), final elongation synthesis (2 min, 72°C). Amplified DNA samples were analyzed after electrophoresis (2.5% agarose gel, stained with ethidium bromide) and visualized on Ultra Violet (UV) transilluminator.

### *Statistical analyses*

Statistical analyses were performed using the IBM SPSS software program (version 21.0). Descriptive statistics were used for the demographic characteristics. The significance of the differences in the distribution of HET and RH polymorphisms between the VTE group and the control group was investigated by the $\chi^2$ test. Moreover, the odds ratio (OR) with their corresponding 95% confidence interval (CI) was used to represent the association between SNPs and VTE risk. The *p* values less than 0.05 were considered statistically significant.

## Results

The baseline demographic and clinical characteristics of participants are presented in Table 1. The VTE group includes 51 (63.75%) patients with DVT, 17 (21.25%) patients with PTE, and 12 (15%) patients with both DVT and PTE. In the VTE group, the youngest patient who had DVT was 10 years old. The majority of patients from the VTE group (69%) had the first episode of DVT and/or PTE before the age of 50.

**Table 1**

**Demographic characteristics of the patients in the venous thromboembolism (VTE) subgroups and the control group**

| Parameter | Groups | | | | |
|---|---|---|---|---|---|
| | DVT | PTE | DVT + PTE | Total | Control |
| Patients, n (%) | 51 (63.75) | 17 (21.25) | 12 (15) | 80 (100) | 80 (100) |
| Sex, n (%) | | | | | |
| male | 22 (43.14) | 8 (47.06) | 5 (41.67) | 35 (43.75) | 32 (40) |
| female | 29 (56.86) | 9 (52.94) | 7 (58.33) | 45 (56.25) | 48 (60) |
| Age (years) | | | | | |
| mean ± SD | 42.88 ± 14.34 | 46.35 ± 14.22 | 47.08 ± 18.29 | 44.25 ± 14.91 | 44.61 ± 6.87 |
| median (range) | 43 (10–73) | 51 (27–64) | 42.5 (16–75) | 43 (10–75) | 43 (30–77) |
| Age (years) of the first episode of VTE, n (%) | | | | | |
| before 50 | | | | 55 (69) | |
| after 50 | | | | 25 (31) | |

**DVT – deep vein thrombosis; PTE – pulmonary thromboembolisms; SD – standard deviation.**

The results of the allele and genotype frequencies for SNPs in FV G1691A, FII G20210A, and MTHFR C677T within the examined groups are presented in Table 2. The results obtained by comparing the VTE group with the CG showed that the frequency of HET for FII G20210A and FV G1691A were higher in the VTE group ($\chi^2$ = 16.26; $p$ = 0.001 and $\chi^2$ = 17.69; $p$ < 0.001). RH for FII G20210A and FV G1691A were not found in any investigated group. Compared to the CG, the incidence of RH alleles A for FII G20210A and FV G1691A was higher in the VTE group. Risk estimate analyses showed that the risk for VTE was significantly higher in the presence of FII G20210A (OR = 10.5; 95% CI = 2.34–47.27; $p$ = 0.001) and FV G1691A (OR = 14.8; 95% CI = 3.34–65.43; $p$ < 0.001). Regarding MTHFR C677T, a difference in the frequency of HET, RH, and wild type (WT) between the VTE group and the CG group was not significant ($p$ = 0.603). Recessive allele T for the MTHFR gene was approximately equally distributed in both examined groups.

Statistically significant difference was not found when comparing the distribution of examined genotypes between genders within each group (results were not presented in the tables).

The distribution of individual and multiple polymorphisms of the investigated genes within the study groups demonstrated that only 18.75% of patients in the VTE group did not have any HET and RH for investigated polymorphisms as opposed to 36.25% in the CG group (Table 3). Furthermore, the presence of two or more investigated polymorphisms were detected in a larger percentage of subjects in the VTE group comparing with the CG (FV G1691A with MTHFR C677T: 16.25% and 1.25%, respectively; FII G20210A with MTHFR C677T: 8.75% and 2.5%, respectively).

**Table 2**

**Allele and genotypes frequencies in patients with VTE and healthy controls**

| Genotype/allele, n (%) | | VTE group | Control group | OR (95%CI)* | $\chi^2$ |
|---|---|---|---|---|---|
| | | | | $p$ value | |
| *FV* G1691A | WT (G/G) | 58 (72.5%) | 78 (97.5%) | 14.8 (3.34–65.43) | 17.69 |
| | HET(G/A) | 22 (27.5%) | 2 (2.5%) | **< 0.001** | **< 0.001** |
| | RH(A/A) | 0 | 0 | | |
| | allele G | 138 (86.3%) | 158 (98.8) | 12.6 (2.90–54.52) | 16.26 |
| | allele A | 22 (13.8%) | 2 (1.2%) | **< 0.001** | **< 0.001** |
| *FII* G20210A | WT (G/G) | 63 (78.8%) | 78 (97.5%) | 10.5 (2.34–47.27) | 11.70 |
| | HET(G/A) | 17 (21.3%) | 2 (2.5%) | **0.001** | **0.001** |
| | RH(A/A) | 0 | 0 | | |
| | allele G | 143 (89.4%) | 158 (98.8) | 9.4 (2.13–41.36) | 10.96 |
| | allele A | 17 (10.6%) | 2 (1.2) | **0.001** | **0.001** |
| *MTHFR* C677T | WT(C/C) | 22 (27.5%) | 25 (31.3%) | 1.2 (0.60–2.36) | 0.27 |
| | HET(C/T) | 43 (53.8%) | 43 (53.8%) | 0.603 | 0.603 |
| | RH(T/T) | 15 (18.8%) | 12 (15.0%) | | |
| | allele C | 87 (54.4%) | 93 (58.1%) | 0.86 (0.55–1.34) | 0.317 |
| | allele T | 73 (45.6%) | 67 (41.9%) | 0.573 | 0.573 |

***Wild – type vs. heterozygous + homozygous; WT – wild type; HET – heterozygous; RH – recessive homozygous; OR – Odds Ratio; 95% CI – 95% Confidence Interval.**

**Table 3**

**Representation of individual and multiple polymorphisms in genes FV G1691A, FII G20210A and MTHFR C677T within the study groups**

| Polymorphisms | VTE group (n = 80) | Control group (n = 80) |
|---|---|---|
| | n (%) | n (%) |
| FV G1691A | 4 (5) | 1 (1.25) |
| FII G20210A | 5 (6.25) | 0 |
| MTHFR 677* | 31 (38.75) | 47 (58.75) |
| FV G1691A<br>FII G20210A | 3 (3.75) | 0 |
| FV G1691A<br>MTHFR 677 * | 13 (16.25) | 1 (1.25) |
| FII G20210A<br>MTHFR 677* | 7 (8.75) | 2 (2.5) |
| FV G1691Aw<br>FIIG20210A<br>MTHFR 677* | 2 (2.5) | 0 |
| None† | 15 (18.75) | 29 (36.25) |

***MTHFR C677T and MTHFR T677T; †There is not one of heterozygous and recessive homozygous; VTE – venous thromboembolism.**

## Discussion

The occurrence of the thromboembolic disease is in line with the simultaneous presence of gene polymorphisms and environmental risk factors which can partly explain the inconsistent results of similar studies conducted in different geographic regions [10, 11, 33]. The results of our study showed that the presence of polymorphisms FII G20210A and FV G1691A was significantly higher in the VTE group compared to the CG. RH for these two coagulation factors are absent both in the VTE group and the CG. A recent meta-analysis performed on a large sample, including 11,000 cases and 21,000 controls, has shown a significant association of HET for FV G1691A and FII G20210A with VTE [11], and it is in concordance to findings of our study. Literature data have shown that the combined effect of more than one genetic polymorphism for thrombophilia susceptibility can double or triple the risk for VTE [9, 29, 33]. The combination of the most significant genetic risk factor, FV G1691A and FII G20210A, with HET and RH in the MTHFR gene, has been frequently found in patients with VTE [11, 29]. We also found a higher presence of two or more polymorphisms in the VTE group compared to the CG. In two patients, polymorphisms for all three examined genes were observed simultaneously.

In our study, the frequency of FV G1691A in healthy subjects was 2.5%, which is in correlation with the published data on the white European population [11]. We found that the overall frequencies of FV G1691A polymorphisms were significantly higher in patients with VTE increasing the risk for VT in FV G1691A carriers for almost 15 times (OR = 14.8; 95% CI = 3.34–65.43; $p < 0.001$). This finding is consistent with the reported prevalence of FV G1691A for other countries in the region (Italy 15.3%, Croatia 16%, Macedonia 21.1%, Serbia 29.3%, Bulgaria 25%, Bosnia and Herzegovina 18%, and Greece 31.9%) [11, 22]. Our study showed that allele A was present in 13.8% of subjects with VTE vs. 1.2% in healthy subjects.

Polymorphism FII G20210A leads to an increased prothrombin production, which may result in 30% to 70% higher levels of prothrombin in HET and RH compared to the production in the absence of this condition [25]. In our study, 17 out of 80 patients (21.3%) with VTE were HET for FII G20210A polymorphism. In the CG, FII G20210A polymorphism was presented in 2.5% of participants. Risk for development of VTE in FII G20210A carriers was 10.5 times higher (OR = 10.5; 95% CI = 2.34–47.27; $p = 0.001$) than in wild-type carriers. The results of our research are in correlation with literature data, in which it is reported that HET of FII 20210 is present in 6–18% of patients with VTE and 2–4% in the healthy European population [24]. Our results similarly correlate with the results in Serbia (11.6% of VTE patients) [13]. Other studies from our region report different results in which HET for FII 20210 were presented in patients with VTE with lower frequency (Croatia 4% and Bosnia and Herzegovina 2.7%) [30, 33].

The role of MTHFR polymorphisms in the development of VTE is controversial: some authors have shown an association between MTHFR C677T polymorphism with VTE [32, 33], while others have proven the contrary [11, 34, 35]. Our results showed that HET and RH for MTHFR C677T were present in a large and approximately equal percentage in both examined groups (53.8% and 18.8% vs. 53.8% and 15%, respectively). We also reported previously that the frequency of HET and RH for MTHFR C677T was similar in the population of healthy pregnant women and women with adverse pregnancy outcomes [31, 32]. These results confirm previously published data of similar studies [5, 11, 13, 16]. We did not observe the difference in the frequencies of FV G1691A, FII G20210A, and MTHFR C677T polymorphisms in investigated genes between genders in the patients' group or in the CG. Determining the distribution of these polymorphisms according to gender is especially important in women because they undergo hemostatic changes during pregnancy [32].

It is estimated that 40–50% of thrombosis cases result from thrombophilia. Therefore, the genetic testing of this condition is significant in the prevention and treatment of thrombosis, with an important role in determining the duration of secondary anticoagulant prophylaxis, especially in the case of increased risk of thromboses such as surgery, pregnancy, immobilization, and trauma. Knowledge about the presence of these risk factors can support the prevention of these diseases, especially among patients' relatives who are carriers of these polymorphisms.

Our study showed a significantly higher presence of FV G16961A and FII G20210A polymorphisms in patients with VTE, compared to healthy controls with no history of VTE. In contrast, we did not detect any significant associations between the homozygous MTHFR 677TT and HET MTHFR C677T genotype and VTE. This study included a small sample of patients, and thus, conclusions should be confirmed by future research in the field.

The multifactorial etiology of VTE implies a necessity of further research of more genes related to thrombophilia and their interplay with environmental risk factors, which should lead to a more comprehensive and reliable genetic counseling and prevention of VTE.

## Conclusion

Our study showed that FII G20210A and FV G1691A polymorphisms are significantly associated with VTE. Detection of the above-mentioned polymorphisms prior to VTE development can contribute to the prevention of further VTE occurrence, especially among patients' relatives who are carriers of these polymorphisms.

## Conflict of interest

The authors declare that they have no competing interests.

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Received on April 2, 2019.
Revised on July 24, 2019.
Accepted July 26, 2019.
Online First September, 2019.