International Journal of Psychosocial Rehabilitation, Vol. 24, Issue 05, 2020
ISSN: 1475-7192

Molecular Detection and Genotypic Study of Leptotrichia SPP by 16SrRNA Gene that Isolated from Bacterial Vaginosis Miscarriage and Non-miscarriage Women in AL-hillah City

Ilham A. Bunyan, Alaa K. Hameed and Asmaa K. Gatea

Abstract--- *This study was aimed to determine Leptotrichia spp by culture independent method from both bacterial vaginosis women without miscarriage and vaginosis women with miscarriage. Also other aim, sequencing of the 16SrRNA gene was conducted for phylogenetic tree study of local isolates of Leptotrichia spp in comparison to world Leptotrichia spp isolates in NCBI Gen bank and lastly deposition of the current isolates in Gen bank. One hundred fifty (150) high vaginal swabs were collected from women with vaginosis (Seventy five samples were taken from married vaginosis women without miscarriage and Seventy five samples from vaginosis women with miscarriage) from Babylon city hospital and private clinics. The age of patient (15– 45) years. The sample was collected by disposable swabs, 16s rRNA gene detection by polymerase chain reaction technique. Results revealed that 63(84.00%) and 42(56.00%) of Leptotrichia spp out of 150 swabs obtained by PCR from miscarriage and non-miscarriage vaginosis women respectively . phylogenetic study of the 16S rRNA gene indicated that local Leptotrichia spp (NO.1 and NO.2) isolates shared higher homology with other Leptotrichia spp isolates available in the GenBank. The homology of the nucleotides was between (99.06%) for both isolates.*

Keywords--- *Leptotrichia Spp, 16SrRNA Gene, Bacterial Vaginosis, DNA Sequencing, Molecular Identification, Miscarriage.*

I. INTRODUCTION

Bacterial vaginosis (BV) is a state of vaginal normal flora disturbance, in which the typically plentiful hydrogen peroxide producing lactobacillus are scarce and increased the growth of other anaerobe bacteria such as Gardnerella vaginalis and Atopobium vaginae, Bacteroides spp., Mobiluncus spp. and Prevotella spp [1].

Bacterial vaginosis is a main causes of vaginal complaints in women of childbirth age (pregnant and non-pregnant) [2]. BV is correlated with adverse pregnancy outcomes like preterm labour , miscarriage and increasing the risk for infections that transmitted sexually such as human immunodeficiency virus (HIV) [3].

Leptotrichia spp(Sneathia) bacteria are long, Gram-negative, non-motile rods, non-sporulating and belong to Fusobacteria [4].

Significant levels of G. vaginalis , Leptotrichia/ Sneathia species, Megasphaera phylotype 1-like species, Mycoplasma hominus and Mobiluncus spp and Atopobium spp. during second trimester of pregnancy have been

Ilham A. Bunyan, Department of Microbiology, College of Medicine, University of Babylon, Iraq. E-mail: Ilhamalsaedi2008@gmail.com
Alaa K. Hameed, Department of Microbiology, College of Medicine, University of Babylon, Iraq.
Asmaa K. Gatea, Department of Obstetrics and Gynaecology, College of Medicine, University of Babylon, Iraq.

DOI: 10.37200/IJPR/V24I5/PR201790
Received: 13 Feb 2020 | Revised: 07 Mar 2020 | Accepted: 23 Mar 2020

related with an increased risk of spontaneous preterm birth (SPTB). Few studies have analyzed the role of them BV associated bacteria and the risk of miscarriage [5][6].

Sneathia amnii is considerably fastidious, which can elucidate why it has not been identified by conventional culture depended microbiological methods . so in vaginal samples from women with and without BV. S. amnii was observed through the use of molecular techniques in 40% of high vaginal samples out of 736 women involved in the human microbiome study, these patients admitted to the urban outpatient clinics for reasons such as pregnancy, annual examination, vaginal discharge, screening for sexually transmitted infection (STI), and, etc. [7].

Microorganisms that actually cannot be cultivated, including A. vaginae, Megasphaera, Leptotrichia, and BV-associated bacterium 1 (BVAB1), BVAB2, and BVAB3 have been described by molecular techniques like the 16SrRNA Polymerase Chain Reaction (PCR) [8]

Recently, techniques of 16S rRNA gene amplicon sequencing have permitted a more detailed understanding of the bacterial composition of communities that residing in the vagina and allowed identify of several clusters, called community state types (CSTs), that differ according to bacterial taxa composition and relative abundance. The CSTs are dominated by different species of Lactobacillus, or by a small number of Lactobacillus spp. The last CSTs are composed of a different anaerobic bacteria including Gardnerella vaginalis and Sneathia spp [9]. The low-Lactobacillus CSTs is collectively called molecular-BV, since they represent identical low-Lactobacillus states that can be captured by the criteria of Nugent and Amsel's [10].

II. MATERIALS AND METHODS

Sample Collection

The total number of samples were collected (150) high vaginal swabs samples diagnosed as bacterial vaginosis by the physician (seventy five vaginosis women with miscarriage and seventy five vaginosis women without miscarriage) were recovered All samples or individual were attended to Maternity and Pediatrics Hospital and outpatient clinics of Gynecology in Al-Hillah city/ Iraq, during the duration from (November 2018 to June 2019). After taking the permission from the patients for examination and sampling, three cotton swab of high vaginal discharge obtained from each woman by brushing a swab across the vaginal wall.

DNA Extraction

G-SpinTM Total DNA extraction kit (iNtRON/ Korea) was using for extracting DNA from all frozen high vaginal swabs according to manufacture instructions. The concentration and purity of DNA that extracted from high vaginal swab was checked by using Nano drop spectrophotometer, that checked and measurement by reading the absorbance in at (260 /280 nm).

Primer Design and Uniplex PCR

Molecular detection was conducted by uniplex PCR with a primer as illustrated by[8] and imported from Macrogen Company as shown in the table (1). The reaction of PCR for detection 16SrRNA gene was done in a volume 20 µl, which included : Maxime PCR Pre mix kit (Bioneer, Korea), 5 µl (20 ng/µL) of sampling DNA, 1 µL

DOI: 10.37200/IJPR/V24I5/PR201790
Received: 13 Feb 2020 | Revised: 07 Mar 2020 | Accepted: 23 Mar 2020

(10pm/µl) of each forward and reverse primers and Nuclease-free water was used to complete the volume of the mixture .

PCR thermocycler program were: 95°C for 5 minutes then 38 cycles of 95°C for 30 second, 55°C for 30 seconds, and 72°C for 1 minutes followed by 5-minute extension at 72°C. After that PCR amplicon was confirmed by agarose gel electrophoresis on a 1.5% agarose gels by visualization against UV light.

Table 1: Primer for Amplification of 16SrRNA Gene of Leptotrichia spp[8].

Bacterium	**Sequence of Primer (5' -------- 3')**		**PCR amplicon size**	**GenBank code**
Leptotrichia spp	F	CAATTCTGTGTGTGTGAAGAAG	230	AY724742
	R	ACAGTTTTGTAGGCAAGCCTAT		

DNA Sequencing

DNA sequencing of the amplicon was carried out by Macrogen Company in Korea by using the AB DNA sequencing system. phylogenetic tree analysis was conducted dependent on the identify alignment on the NCBI-Blast and neighbor distance phylogenetic tree analysis and Multiple sequence alignment analysis based ClustalW alignment analysis.

Statistical Analysis

The result was analyzed by using the statistical software package SPSS 23. Pearson Chi-square test and odds ratio with (95%) confidence was used to determine the statistical difference between groups.

III. RESULTS AND DISCUSSION

Bacterial vaginosis is a vaginal cavity dysbiotic disease with deleterious consequences during pregnant [11].

A variety of microorganisms are responsible for bacterial vaginosis, One of which is anaerobes that considered as vaginal flora and there presents in great numbers due to reduce in the growth of Lactobacillus lead to causing vaginal infections, which are considered as a common cause of miscarriage [12].

The current results showed that the number of Leptotrichia spp was (70.00%), the distribution of this microorganism in vaginosis women with miscarriage was (84.00%). While there distribution in vaginosis women without miscarriage was (56.00%) as showed in table (2) and figure (1). Statically a significant difference observed with a P value ($p \leq 0.005$).

The present study agree with study conducted by [13] who used 16SrRNA genes to investigate the relationship between bacterial morphotypes and bacterial vaginosis in infected women. The 16SrRNA genes used in PCR targeting the variable region of the gene with species-level detection used to investigate the relation between the existence of individual species of bacteria and clinical diagnostic characteristics of BV [14].

Our result similar to study conducted in Iraq by[15] who detected Leptotrichia/Sneathia from bacterial vaginosis women. The extremely anaerobe Leptotrichia/Sneathia species tend to be an infectious agent in this disease , since relatively recent PCR studies have been shown the concentration of these species are significantly higher in samples demonstrating bacterial vaginosis in contrast with normal samples [16] [17].

DOI: 10.37200/IJPR/V24I5/PR201790
Received: 13 Feb 2020 | Revised: 07 Mar 2020 | Accepted: 23 Mar 2020

Table 2: PCR Detection Percentage of Leptotrichia spp

Types of samples	PCR Number	
	Positive (%)	Negative (%)
Vaginal swab from miscarriage women	63(84.00%)	12(16.00%)
Vaginal swab from non-miscarriage women	42(56.00%)	33(44.00%)
Total	105(70.00%)	45(30.00%)
P –value	0.042	

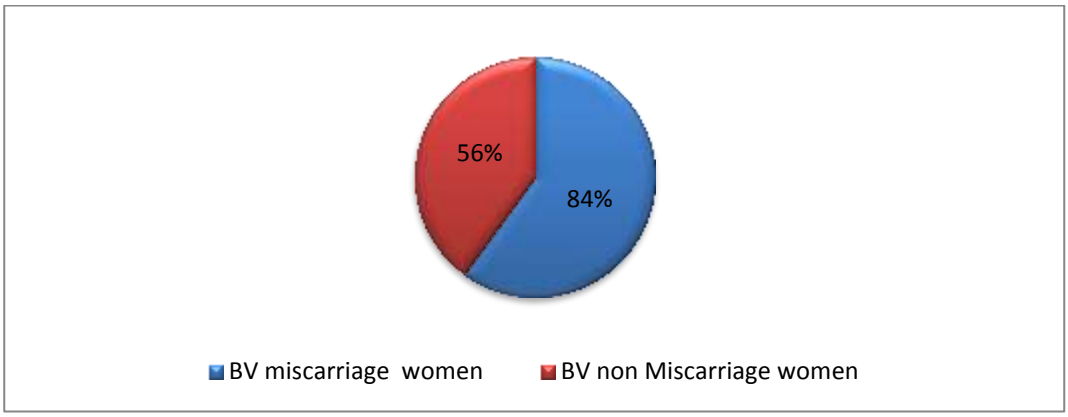

Figure 1: Relative Distribution Of Leptotrichia spp From Vaginosis Miscarriage and Non-Miscarriage Women

Amplification of 16SrRNA gene of Leptotrichia spp by PCR to validate the existence of 16S rRNA gene that appeared in molecular weight 230 bp as proposed by [18] figure(2) was exclusively used to proceed for the sequencing analysis. Five species of bacteria including : A. vaginae, G. vaginalis, Eggerthella-like, Megasphera ph. 1, and Leptotrichia/Sneathia, were identified in majority of subjects with BV and can therefore be regarded as bacterial indicators of this disease(Janulaitiene et al .,2017).

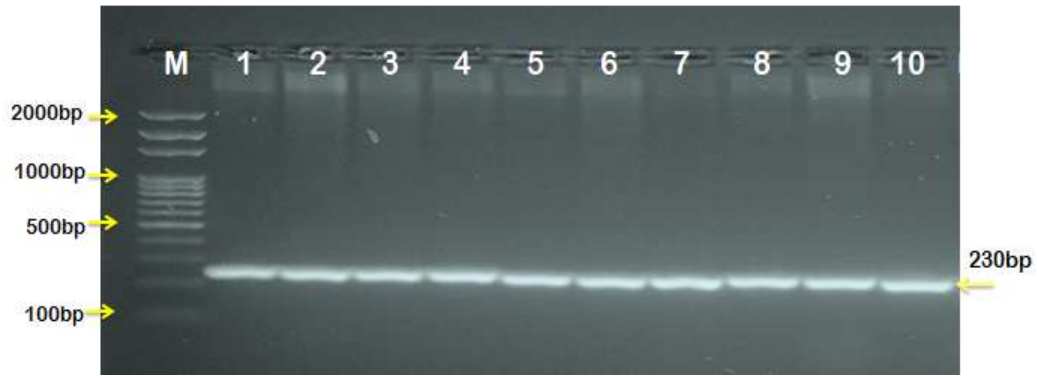

Figure 2: PCR Amplicon Analysis For 16SrRNA Gene In Leptotrichia spp by Agarose Gel Electrophoresis. M (Marker Ladder 2000-100bp). Positive Leptotrichia spp. Sample Shown at Lane (1-10) with 230bp Product Size.[21]

Phylogenetic Analysis

DNA sequencing was carried out in order to phylogenetic confirmative of Leptotrichia spp based on 16S rRNA gene detection . Two isolates(one from vaginosis miscarriage women and one from vaginosis non miscarriage women), were sent for sequencing after that submission in NCBI-GenBank database to get accession number codes

DOI: 10.37200/IJPR/V24I5/PR201790
Received: 13 Feb 2020 | Revised: 07 Mar 2020 | Accepted: 23 Mar 2020

(MN165524 and MN165525) frequently.

phylogenic study of 16SrRNA gene of Leptotrichia spp isolated from vaginosis women with miscarriage and vaginosis women without miscarriage illustrated that the local Leptotrichia spp isolates (No.1) and (No.2) were genetically related to NCBI Blast Uncultured Leptotrichia sp. clone MZH0805-11 isolate (EF120364.1) at sequence homology identity (99.06%) for both isolates whereas other NCBI-Blast uncultured Leptotrichia sp. showed differences out of the tree in total genetic alteration (0.001-0.004%) as illustrated in figure (3,4) and table (3) .

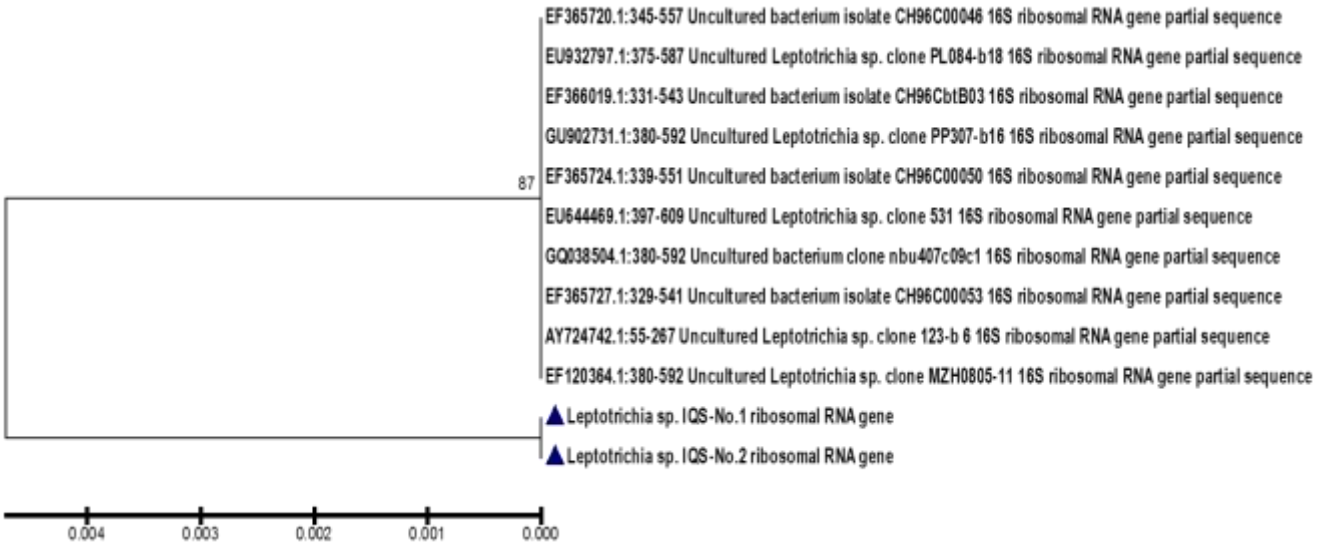

Figure 3: Phylogenetic Tree Analysis Depend on Partial Sequence of 16SrRNA Gene of Local Leptotrichia spp. (IQB.L.No.1 and IQB.L.No.2). Phylogenetic Tree was Conducted Using (MEGA 6.0 Version). In a Total Genetic Alteration (0.001-0.004%)

Figure 4: Partial Sequence Alignment Analysis of 16SrRNA Gene of Local Leptotrichia spp. (IQB.L.No.1 and IQB.L.No.2) With Gene Bank Leptotrichia spp. Isolates 16SrRNA. Partial Sequence Alignment Analysis was Conducted by Clustalw Alignment Tool In (MEGA 6.0 Version). Which Showed The sequence Alignment Identity As (*) With Different Leptotrichia spp

DOI: 10.37200/IJPR/V24I5/PR201790
Received: 13 Feb 2020 | Revised: 07 Mar 2020 | Accepted: 23 Mar 2020

Table 3: The NCBI-BLAST Sequence Homology (%) between Local Isolates of Leptotrichia spp.(IQB.L.No.1) and (IQB.L.No.2) 16S rRNA Gene and Gen Bank Deposited Leptotrichia spp. Isolates

Local isolates		**NCBI-BLAST Homology Sequence identity**		
	Identity Isolates on NCBI BLAST	**Accession Number**	**Country**	**Homology (%)**
Isolate of *Leptotrichia spp.* No. 1	Uncultured *Leptotrichia sp*. clone MZH0805-11	EF120364.1	USA	99.06%
***Isolate* of *Leptotrichia spp.* No.2**	Uncultured *Leptotrichia sp*. clone MZH0805-11	EF120364.1	USA	99.06%

The Nucleotide variations substitution analysis between local Leptotrichia sp isolates 16SrRNA gene and NCBI BLAST Leptotrichia sp isolates were showed no transitional substitutions (0)% from total nucleotides. Whereas highly nucleotide variations Substitution at transversionsal substitutions were showed at (16.67%) between (A) nucleotide that substituted by (T) and (C) nucleotide. As showed in table (4).

Table 4: Nucleotide Variations Substitution Analysis between Local Leptotrichia spp Isolates 16SrRNA Gene and NCBI Leptotrichia spp Isolates

	A	**T**	**C**	**G**
A	-	*11.03*	*8.92*	**0**
T	*16.67*	-	**0**	*13.38*
C	*16.67*	**0**	-	*13.38*
G	**0**	*11.03*	*8.92*	-

IV. CONCLUSIONS

The detection of unculturable , fastidious bacteria associated with bacterial vaginosis would support the early diagnosis in pregnancy and promote early curative to decrease the complication of pregnancy like miscarriage and preterm delivery.

REFERENCES

[1] Yzeiraj-Kalemaj, L., Shpata, V., Vyshka, G., and Manaj, A. (2013). Bacterial vaginosis, educational level of pregnant women, and preterm birth: a case-control study. *ISRN Infectious Diseases*, 2013.

[2] Tuddenham, S., Ghanem, K. G., Caulfield, L. E., Rovner, A. J., Robinson, C., Shivakoti, R., ... and Brotman, R. M. (2019). Associations between dietary micronutrient intake and molecular-Bacterial Vaginosis. *Reproductive health*, *16*(1), 151.

[3] Fujisaki, M. (2020). Bacterial Vaginosis. *In Preterm Labor and Delivery (pp. 175-180). Springer, Singapore*.

[4] Eribe, E., R., K.; Bruce J. Paster, B., J.; Caugant, D., A.; Dewhirst, F., E.; Stromberg, V., K.; Lacy, G., H.; and Olsen, I. (2004). Genetic diversity of Leptotrichia and description of Leptotrichia goodfellowii sp. nov., Leptotrichia hofstadii sp. nov., Leptotrichia shahii sp. nov. and Leptotrichia wadei sp. nov. *International Journal of Systematic and Evolutionary Microbiology.* 54: 583-592.

[5] Nelson, D. B., Hanlon, A., Nachamkin, I., Haggerty, C., Mastrogiannis, D. S., Liu, C., and Fredricks, D. N. (2014). Early pregnancy changes in bacterial vaginosis-associated bacteria and preterm delivery. *Paediatric and perinatal epidemiology*, 28(2), 88-96.

[6] Menard, J. P., Mazouni, C., Salem-Cherif, I., Fenollar, F., Raoult, D., Boubli, L., ... and Bretelle, F. (2010). High vaginal concentrations of Atopobium vaginae and Gardnerella vaginalis in women undergoing preterm labor. *Obstetrics and Gynecology*, 115(1), 134-140.

[7] Harwich, M. D., Serrano, M. G., Fettweis, J. M., Alves, J. M., Reimers, M. A., Buck, G. A., and Jefferson, K. K. (2012). Genomic sequence analysis and characterization of Sneathia amnii sp. nov. BMC genomics, 13(8), S4.

DOI: 10.37200/IJPR/V24I5/PR201790
Received: 13 Feb 2020 | Revised: 07 Mar 2020 | Accepted: 23 Mar 2020

[8] Fredricks, D. N., Fiedler, T. L., Thomas, K. K., Oakley, B. B., and Marrazzo, J. M. (2007). Targeted PCR for detection of vaginal bacteria associated with bacterial vaginosis. *Journal of clinical microbiology*, *45*(10), 3270-3276.

[9] Ravel, J., Gajer, P., Abdo, Z., Schneider, G. M., Koenig, S. S., McCulle, S. L., ... and Brotman, R. M. (2011). Vaginal microbiome of reproductive-age women. *Proceedings of the National Academy of Sciences*, *108*(Supplement 1), 4680-4687.

[10] McKinnon, L. R., Achilles, S. L., Bradshaw, C. S., Burgener, A., Crucitti, T., Fredricks, D. N., ... and Kwon, D. S. (2019). The evolving facets of bacterial vaginosis: implications for HIV transmission. *AIDS research and human retroviruses*, *35*(3), 219-228.

[11] Grine, G., Drouet, H., Fenollar, F., Bretelle, F., Raoult, D., and Drancourt, M. (2019). Detection of Methanobrevibacter smithii in vaginal samples collected from women diagnosed with bacterial vaginosis. *European Journal of Clinical Microbiology and Infectious Diseases*, 1-7.

[12] Bunyan, I. A., Umran, B. J., and Salman, Z. K. (2017). Analyzing the correlation of TLR-4 Asp299Gly andThr399Ile polymorphism in women with Bacterial Vaginosis in Hillah city. *Research Journal of Pharmacy and Technology*, *10*(12), 4178-4182.

[13] Srinivasan, S., Morgan, M. T., Liu, C., Matsen, F. A., Hoffman, N. G., Fiedler, T. L., ... and Fredricks, D. N. (2013). More than meets the eye: associations of vaginal bacteria with gram stain morphotypes using molecular phylogenetic analysis. *PloS one*, 8(10), e78633.

[14] Srinivasan, S., Hoffman, N. G., Morgan, M. T., Matsen, F. A., Fiedler, T. L., Hall, R. W., ... and Fredricks, D. N. (2012). Bacterial communities in women with bacterial vaginosis: high resolution phylogenetic analyses reveal relationships of microbiota to clinical criteria. *PloS one*, 7(6), e37818.

[15] Abed, K. A., and Kandala, N. J. (2016). Molecular and Bacteriological Detection of Some Bacterial Vaginosis Associate Bacteria in Women. *Iraqi Journal of Science*, *57*(3B), 1926-1936.

[16] Fredricks, D. N., Fiedler, T. L., Thomas, K. K., Mitchell, C. M., and Marrazzo, J. M. (2009). Changes in vaginal bacterial concentrations with intravaginal metronidazole therapy for bacterial vaginosis as assessed by quantitative PCR. *Journal of clinical microbiology*, 47(3), 721-726.

[17] Zozaya-Hinchliffe, M., Lillis, R., Martin, D. H., and Ferris, M. J. (2010). Quantitative PCR assessments of bacterial species in women with and without bacterial vaginosis. *Journal of clinical microbiology*, 48(5), 1812-1819.

[18] Bunyan, I. A., Gatea. A.K., Hameed .A.K.(2019).Molecular detection of Bacterial Vaginosis and its association with miscarriage in AL-Hillah city. *Annals of Tropical Medicine and Public Health*.

[19] Janulaitiene, M., Paliulyte, V., Grinceviciene, S., Zakareviciene, J., Vladisauskiene, A., Marcinkute, A., and Pleckaityte, M. (2017). Prevalence and distribution of Gardnerella vaginalis subgroups in women with and without bacterial vaginosis. *BMC infectious diseases*, *17*(1), 394.

DOI: 10.37200/IJPR/V24I5/PR201790
Received: 13 Feb 2020 | Revised: 07 Mar 2020 | Accepted: 23 Mar 2020