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Enzymatic Digestion Pattern of Varicella Zoster Virus ORF38 and ORF54 in Chickenpox Patients Using RFLP Technique

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ABSTRACT

***Background*:** Varicella zoster virus (VZV) causes chickenpox in children and zoster (zona) in the elderly. Using RFLP-PCR method for detection of VZV specific SNPs ORF38, 54 and 62 could distinguish the profile of VZV isolates. The aim of this study was to investigate enzymatic digestion pattern of VZV ORF38 and ORF54 in chickenpox patients using RFLP technique.

***Methods*:** Thirty-eight chickenpox patients, who referred to the hospitals of Iran University of Medical Sciences in Tehran from May 2010 to June 2015 were enrolled in this cross sectional study. After the DNA extraction, PCR amplification of 38 VZV isolates performed by specific primers of ORFs 38 and 54, then RFLP assay and digestion carried out by *Pst*I (for ORF38) and *Bgl*I (for ORF54) restriction enzymes.

***Results*:** Of 38 positive VZV DNA, the mean age (yr)±SD was 34.4±23.3 (range: 7-89). 22 (57.9%) were female and 16 (42.1%) were male. The predominant VZV profile of *Bgl*I+*Pst*I+ were 89.5% (34/38) followed by 10.5% (4/38) *Pst*I+*Bgl*I^{-}. Statistical analysis showed that there was no significant relationship between genotype, age, sex, and year of infection variables (*P* value> 0.05). The common VZV genotype among Iranian patients with chickenpox and zona infection is genotype *Bgl*I+*Pst*I+ followed by *Pst*I+*Bgl*I^{-}.

Conclusion: There are different VZV circulating genotypes that call for for more research on this field by widely population and other methods such as nucleotide sequencing to justify the accurate VZV genotype prevalence in Iran.

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Introduction

Varicella zoster virus (VZV) is a herpes virus that causes chickenpox (varicella) and shingles (zoster) (1, 2). The primary infection in children causes varicella or chickenpox that is one of the six exanthemas of childhood. Elderly infection results in painful complications with some skin lesions, called zona or shingles (3, 4). Chickenpox is one of the major childhood diseases that is prevalent in temperate zones with significant mortality (1, 5). Children may be hospitalized

for chickenpox by 2-4%, with a mortality rate of 0 to 0.05 per 100,000 cases. Neuralgia pain and sometimes morbidity and mortality of Herpes zoster in the elderly (>50 yr ages) is due to reactivation of latent VZV infection in dorsal root of ganglia (1, 3, 4, 6).

A live attenuated vaccine, called Oka, is created by a Japanese scientist, Takahashi in 1970s, povides a long-lasting protection in the introduced countries and decreases the spread of VZV infection dramatically (7-9). In order to use the live attenuated vaccine, surveillance of circulating strains is important (7, 10). Phylogenetic tree of VZV strains by sequence analysis demonstrates different genotypes that categorized in distinct clades globally, although the virulence genetic markers of circulating strains are unknown (11). Furthermore, homologous recombination effects on VZV evolution. Analyzing one or a few loci could distinguish the VZV genotypes (11). There are various methods to differentiate wild type (WT) and vaccine VZV strains, including single stranded conformational polymorphism analysis, long PCR, PCR-RFLP analysis, and Light Cycler Real-Time PCR; although Restriction Fragment Length Polymorphism (RFLP) is the original method and PCR-RFLP widely used (6, 12, 13).

There are three salient single nucleotide polymorphisms (SNPs) in VZV vaccine surveillance and epidemiological studies that could be distinguished by the RFLP analysis, like open reading frames (ORFs) 38, 54, and 62, each of which had a specific silent base mutation at position 943 (A to G), 744 (C to T), and 2872 (C to T) alleles; these were used to determine the digestion pattern profiles by PstI (for ORF 38), BglI (for ORF 54) and SmaI (for ORF 62) restriction enzymes, respectively (10, 14, 15).

PstI positive strains (BglI/SmaI negative) were dominant in the Europe and the North America in that wild type strains, PstI and BglI positive strains (SmaI negative) contain the Oka like strains and African or Asian strains (10). The specific restriction site of the varicella vaccine strains (vOka) was SmaI that located on ORF 62. Thus, RFLP could be used to distinguish strains from WT, vaccine, and or their geographical dominance (10, 16).

The aim of the prospective study was to distinguish wild type or vaccine strains of VZV by enzymatic digestion pattern of ORF38 and ORF54 in chickenpox patients using RFLP technique which prepared for the first time in Iran.

Materials and Methods

Study population

From May 2010 to June 2015, 38 patients (16 males, 22 females) referred to the hospitals of Iran University of Medical Sciences in Tehran City (the capital of Iran) and practitioners detected them by clinical manifestations enrolled in this cross sectional study. All of the patients suffered from the chickenpox or herpes zoster by further molecular diagnosis for VZV-DNA using PCR. Samples were collected from cerebrospinal fluid (CSF), skin lesions, and vesicle fluids in viral transport media and were stored at -70 ^{0}C for later detection.

The study was approved by the Ethics Committee of the university and patients were consent to participate the study.

PCR:

VZV DNA was extracted from 200 µl of the collected specimens using DNA extraction kit (Qiagen GmbH, Hilden, Germany) according to manufacturer's instructions. After the process, Nanodrop spectrophotometer (Thermo Scientific, Wilmington, USA) was used for evaluating the quality and quantity of the extracted DNA and the resultant DNA stored at -20 °C for further use.

Primers of ORF 54 were prepared from Muir et al. study (15): forward primer

5'-CGTAATGCATAACAGGCCAACAC-3' (95005–95027) and reverse primer 5'-GAAACCTGGCGTCAAACATTACA-3' (95501–95479), product size was 497 bp; ORF 38 from Sauerbrei et al. study (17): forward primer 5'-AAGTTTCAGCCAACGTGCCAATAAA-3' (69060–69084) and reverse primer 5'-AGACGCGCTTAACGGAAGTAACG-3' (69706–69684) product size was 647 bp. Calculation of physical and chemical features of the primers, specificity and secondary structures performed by Genamics Expression software version 1.100©2000 and BLAST.

The conventional PCR method carried out for ORF54 (497bp) and ORF38 (647bp) with the above mentioned primers. Twenty-five µl reaction mixture used for amplification reaction by the following protocol: template DNA or controls corresponding to 0.2-0.5 µM concentration, dNTPs mix 40 mM (10 mM each dATP, dTTP, dGTP, dCTP) corresponding to 0.5 mM concentration, each forward and reverse primers of ORF 54, 38 corresponding to 0.5 µM concentration, MgCl2 solution 50 mM corresponding to 1.5 µM concentration, 10X reaction buffer corresponding to 1X concentration, Taq DNA polymerase (Fermentas GmbH, Germany) 5 units/µl concentration, and sterilized D.W. added to justify the total volume.

Thermocycler program for the PCR was: One cycle at 95 ^{0}C, 5 min for initial denaturation; 35 cycle at 95 ^{0}C, 30 second (denaturation); hybridization of ORFs 54 and 38 at 62 ^{0}C and 65 ^{0}C, 30 second according to melting temperatures; and extension were at 72 ^{0}C, 40 second for ORF 54 and 60 second for ORF 38, according to the length of their products; one cycle at 72 ^{0}C 5 minute for final extension. The visualization prepared by 2% Agarose gel electrophoresis, then gel duck Carestream Gel Logic 212 Pro (Carestream Inc., New Haven, CT 06511 USA) were used for imaging the products band.

Restriction fragment length polymorphism (RFLP)

PCR-RFLP method was used to detect Single Nucleotide Polymorphism (SNP) of ORF 38 and 54. The PstI and BglI restriction enzymes (Thermo Fisher Scientific Inc, Waltham, MA USA) was used for digestion reactions with the following protocol: 18 µl Nuclease-free water, 10 µl of PCR product, 2 µl of 10X endonuclease buffer 0, and 1 µl digestion enzymes PstI or BglI. Heating prepared at 37 ^{0}C for 15 h. Visualization performed by 2% Agarose gel electrophoresis.

Statistical Analysis

After the calculation of arithmetic means and SD of the mean, fisher-exact test and t test were administered to compare genotypes frequency among various groups of SPSS version 20 software (SPSS Inc., Chicago, IL, USA). $P < 0.05$ was significant.

Results

Patients

The mean age (yr)±SD of 38 positive VZV DNA isolates was 34.4±23.3 (Range: 7-89 yr), 42.1% were male and 57.9% were female. Table 1 indicates the demographic characteristics and laboratory parameters.

Table 1
Demographic characteristics, laboratory parameters of positive VZV DNA isolates

Year	2010	2011	2012	2013	2014	2015	Total
Total No.	2	6	2	6	12	10	38
Male/Female	2/0	4/2	2/0	0/6	6/6	2/8	16/22
Age(y), Mean±SD	39.3±36.8	25.8±24.5	38.6±19.3	20.5±17.6	46.3±21.1	23.0±14.9	34.4±23.3

Fig. 1
Some of the confirmed PCR products of ORF 38 for RFLP. (39-44: samples, 46: control positive, 47: control negative, Ladder: size marker 100-1000bp)

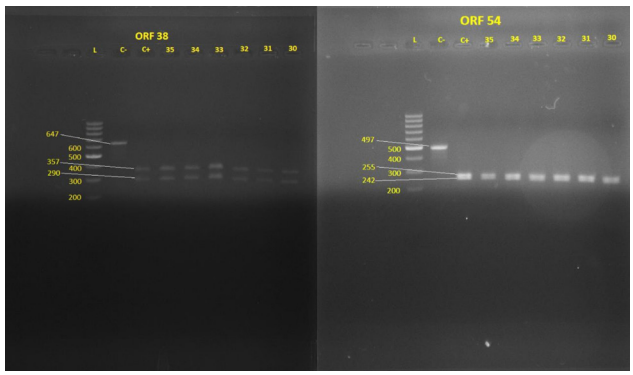

Fig. 2
Digestion of some PCR products of ORF 38 and 54 by PstI and BglI restriction enzymes. (30-35: samples, C+: control positive, C-: control negative, L: Ladder)

PCR-RFLP

The PCR products of 38 VZV-DNA positive was confirmed by appropriate markers and VZV positive controls (Fig. 1). RFLP analyses of ORF54 by specific restriction enzymes showed that all 34 isolates were cleaved by BglI enzyme into 255, 242 bp fragments (89.5% BglI+) and 4 were negative (10.5% $BglI^{-}$). Digestion of ORF 38 by PstI restriction enzyme illustrated that all isolates were cleaved into 357, 290 bp fragments (100% PstI+) as visualized by 2% Agarose gel electrophoresis (Fig. 2). There were no $PstI^{-}$ isolates.

Statistical analysis revealed that there were no significant correlations between the genotype, age, and sex variables (P value> 0.05).

Discussion

Actually, childhood exanthema and elderly neuralgia with significant skin lesions is caused by the varicella-zoster virus infection (18, 19). Live attenuated Oka vaccine declined the occurrence of chickenpox in communities (20).

Reactivation of VZV in order to zona presentation could be decreased by use of vaccination (9, 20). Vaccination of individuals could spread the VZV vOka strain to regions that already had other predominant strains (20). Furthermore, the VZV dominant genotypes monitoring and molecular surveillance of circulating strains is important (13, 20) even for areas without VZV vaccine or in processing program countries such as Iran.

Generally, genotyping of VZV strains was discussed by different strategies (21). One of them is RFLP-PCR genotyping which carried out by digestion profile of VZV ORF 38, 54, and 62 with PstI, BglI and SmaI restriction enzymes. The RFLP-PCR method could distinguish wild type strains from vaccine derived strains (6, 10, 20, 22).

Digestion pattern of VZV strains represents their genotype. Digestion pattern of Oka vaccine strains $PstI^{-}$BglI+SmaI+, Japanese Oka like wild type strains $PstI^{-}$/PstI+BglI+$SmaI^{-}$, African and Asian wild type strains BglI+, North America and Europe PstI+$BglI^{-}$; $PstI^{-}BglI^{-}$ has not been identified so far (Table2) (15, 16, 20-24).

In comparison with our results which 34 isolates digestion profile PstI+BglI+ similar to other African/Asian or Oka like wild type strains (pOka), 2 isolates were PstI+$BglI^{-}$ like American/European strains and there were no isolates containing Oka vaccine strains ($PstI^{-}$ BglI+).

In the present study, the PCR-RFLP method was used to analyze the varicella-zoster virus

genotype by investigation of digestion profile for the first time in Iran. There are some studies on antibody prevalence in different populations in Iran which report VZV specific antibodies are about 70% to 90%, so, there has not been any data from Iranian circulating VZV genotypes, respectively (5, 18, 19).

According to different genotyping strategies, scientists have been introduced a different nomenclature of genotypes (20). A method was described by Loparev et al. used Single Nucleotide Polymorphism of ORFs 38 (PstI) and 54 (BglI) along with sequencing the short region of ORF 22 (447 bp) (25). In this method three genotypes and some subgenotypes were distinctive. The major and minor genotypes are J, E (E1, E2) and M (M1, M2, M3 and M4) (25, 26). Every genotype has a particular geographical distribution, although some regions may contain a mixture of them. Moreover, every genotype has a certain profile of digestion by PstI and BglI restriction enzymes (25).

According to genotyping strategy of Loparev et al., the M genotype strains have PstI+BglI+ digestion profile (21) and is assumed to be reproduced by combination of J and E genotypes (13, 16, 21); The J genotype strains (Japanese Oka like wild type strains) have PstI$^-$/PstI+BglI+ digestion profile. (13, 16, 21). In this paper, we detected the predominant 89.5% (34/38) PstI+BglI+ that could be the M or J genotype. The M genotype is dominant in tropical and subtropical zone like Africa (Chad, DRC and Morocco), South America, Western Australia and Asia (Nepal, India, China and Bangladesh) (21, 22); and the J genotype is dominant in Japan and other Eastern and Asian countries and Australia. Asian immigrant of Western North America and Australia were exposed to the imported strains (16, 21, 25).

Our results showed that we have 10.5% (4/38) PstI+BglI$^-$ strains which the E genotype strains uniformly bear the PstI+BglI$^-$ marker based on Loparev et al. studies (13, 16, 21). The E genotype strains are dominant genotype in Europe, Eastern Australia, Asian Russia, Kazakhestan, Canada, and the United States (16, 21, 25). This could show another circulating genotype of our country and could be due to the high level of migration to Europe and/or other countries that E genotype is dominant.

Conclusion

These data are indicative of the presence of some diversity in VZV genotypes of our population, moreover, nucleotide sequencing could be combined with RFLP-PCR for better detection.

Results suggest that we need to increase the basic concept of our VZV genotypes and more research stidues are required for this topic. Monitoring of circulating VZV genotypes is vital, particularly for initiation of vaccination program and these data call for investigation in a broader number of specimens for confirmation.

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