Original Article

Genomic identification of human vaccinia virus keratoconjunctivitis and its importance as a laboratory-acquired infection

Zahra Movahedi Motlagh, Azam Mokhtari, Mohammadreza Mahzounieh

Context: Vaccinia virus (VACV) is a member of orthopoxvirus genus of the family Poxviridae. VACVs are enveloped, double-stranded DNA viruses. Several species of this family, for example, molluscum contagiosum, smallpox, deerpox, horsepox, rabbitpox, and VACVs may cause conjunctivitis. **Aims:** Given the high incidence of keratoconjunctivitis in Iran (approximately 3.6%–53.9%) and insufficient clinical diagnostic measures, laboratory tests for detection of its causes and determination of accurate keratoconjunctivitis/conjunctivitis prevalence due to different pathogens are essential. **Settings and Design:** In this research, conjunctival samples collected from 100 patients with keratoconjunctivitis signs were referred to an eye hospital of Iran. **Subjects and Methods:** After DNA extraction, polymerase chain reaction (PCR) was carried out for detection of VACV. PCR-positive products were further subjected to DNA sequencing. **Statistical Analysis Used:** The results were analyzed using Chi-square test. **Results:** In this study, 28% of the samples were positive and a statistically significant relationship obtained between working in medical or research laboratories and VACV prevalence ($P < 0.05$). **Conclusions:** This study showed a high rate of VACV keratoconjunctivitis, and therefore, further studies for its prevention and control are necessary.

Key words: Keratoconjunctivitis, polymerase chain reaction, vaccinia

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Vaccinia virus (VACV) is an orthopoxvirus from Poxviridae family that used in smallpox vaccine. VACV keratitis (VACVK) begins with a finely granular opacification of the cornea that may progress to ulceration, endothelial keratitis, and diffuse interstitial keratitis.[1]

Vaccinia keratitis has been divided into four clinical subtypes, each differing in course and severity. The most form is benign and is a simple punctuate epithelial keratitis. However, this form may progress to one of the most severe forms. The second form is marginal keratitis, which typically affects the lower cornea. In this form, the spread of virus from the lower lid or conjunctival lesions may occur. Marginal keratitis usually heals within 2–3 weeks but can cause significant scarring and vascularization. The scarring may spare the visual axis but can still cause significant astigmatism.[2,3]

Central disciform keratitis is the third form. It lasts longer than its marginal counterpart, with an active period of weeks. Due to its location in the visual axis, severe scarring and vascularization may result, causing overrunning visual loss.[4]

Pustular keratitis is the fourth form. Pustules begin as phlyctenule-like lesions that progress to stromal destruction, perforation, and leukoma formation. Bacterial superinfection and panophthalmitis may occur in this keratitis form. The end result in severe cases, as in variolar pustular keratitis, can be phthisis and blindness. In summary, keratoconjunctivitis caused by poxviruses often begins in the eyelid area and then passes the cornea and the choroid. Patients have lid wounds that are small, round, waxy, white, nodules on the eyelids and may be one or more. Infected eyes are red and with secretion.[5]

VACVK was estimated to occur in up to 30% of all ocular vaccinia cases.[1] The recent compensation of vaccination of military personnel, hospital staff, and first responders has led to a reevaluation of the vaccines for adverse reactions.[5]

Recently, for investigation of orthopoxvirus biology and as a tool in molecular biology and immunology, VACV is increasingly used in research laboratories.[6-9] Vaccinia can cause mild-to-moderate infection in healthy hosts and can be transmitted to their contacts.[8,10-12] Although routine smallpox vaccination has been stopped, vaccination is still recommended for healthcare and laboratory workers who handle nonattenuated orthopoxviruses.[10] Ocular vaccinia is a common side effect of smallpox vaccination too. In primary vaccinees, ocular vaccinia occurred, and symptoms included conjunctival disease, iritis, and keratitis.[1-4]

Conjunctivitis is one of the most common and highly contagious eye diseases in Iran. It is also one of the most challenging diseases that expected physicians to do a proper diagnosis and etiological treatment for it. Conjunctivitis frequently causes recourse to the hospital and repeated absence in work and school in Iran. Due to the overlap of conjunctivitis

Department of Pathobiology, Faculty of Veterinary Medicine, University of Shahrekord, Shahrekord, Iran

Correspondence to: Dr. Azam Mokhtari, Department of Pathobiology, Faculty of Veterinary Medicine, University of Shahrekord, Saman-Shahrekord Highway, Shahrekord, Iran. E-mail: a.mokhtari@alumni.ut.ac.ir

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symptoms, it seems very difficult to diagnose it precisely and definitely based on clinical symptoms.[13]

In Iran, little research has been done in this field; because ocular viral diseases are given treatment based on clinical appearance. These few studies showed infective conjunctivitis causes chiefly by adenoviruses and herpesviruses which have worldwide distribution.[14]

Conjunctivitis diagnosis in Iran is based on history taking and physical examination. However, there are no key signs for the etiologic diagnosis of conjunctivitis. Early symptoms of the disease are not specific, and accurate distinction between viral and bacterial causes is almost impossible.[15]

Although with numerous use of antibiotics for the treatment of bacterial conjunctivitis, the risk of adverse complications is reduced, this strategy leads to drug resistance, drug toxicity, and economic costs to patients.[13]

In the previous studies reported from Iran, the prevalence of the various bacterial and viral conjunctivitis and keratoconjunctivitis causes has been reported (the prevalence varies from 3.6% to 53.9%).[13-16] However, about the possible importance of VACV to cause these complications, any study from Iran is not available.

The mass vaccination program led to the complete eradication of smallpox in Iran in 1978. From 1978 to the last decade, no reports of smallpox have been reported from Iran.[17] Consequently, now, there is no immunization program against any of human pox viruses in Iran. Hence, in the first place, it seems poxviruses may not be notable causes of conjunctivitis in this country.

Although there are published reports from Iran that reported a variety of possible causes of conjunctivitis, in some of the reports, despite the presence of symptoms, none of the likely and common causes of conjunctivitis has been isolated.[13-17] It may be due to this reason that those keratoconjunctivitis cases may not be infectious or may be due to less common conjunctivitis causes such as VACV.

In recent years, some studies on camelpox virus strains isolated in Iran showed them to be members of the variola/vaccinia subgroup of poxviruses. In addition, some reports showed the emergence of vaccinia infections in areas where smallpox was eradicated.[18,19] Considering the increasing use of VACV in research experiments and its use in medical laboratories in Iran as well as other countries and the possibility of ocular vaccinia as a vaccine complication in recipients and injectors, this study was designed. The vaccinia prevalence was calculated primarily and secondarily any document indicated that vaccinia keratoconjunctivitis maybe a laboratory-acquired infection was investigated.

Subjects and Methods

Blood sampling and DNA/RNA extraction

This study was a cross-sectional survey and the target group was all patients who referred to an ocular disease hospital and have shown general keratoconjunctivitis symptoms. The conjunctival swab samples were obtained during 2013–2014 and were analyzed to detect vaccinia gene. All the procedures followed in this study were in accordance with the ethical standards and with the Helsinki Declaration of 1975 as revised in 2000. None of the patients had been vaccinated against smallpox. A complete history of work experience in the laboratory or health centers was collected. The patients' eyes were washed with sterile saline, and a sterile cotton swab was rubbed onto the conjunctiva. Then, it immersed in Eagle's minimal essential medium supplemented with penicillin, amikacin, amphotericin B, and 1% fatal bovine serum. DNA was extracted according to phenol-chloroform DNA extraction protocol.

Detection of vaccinia virus by polymerase chain reaction assay

The presence of VACV was detected using the primers designed by Beacon Designer software (PREMIER Biosoft, USA). The sequences of the forward and reverse primers were 5′-accgtcatcattctttgctttcg-3′ and 5′-actgtaatcccgtatttcgtgagg-3′, respectively. Each polymerase chain reaction (PCR) reaction was performed in a final volume of 25 μl containing 11 μl of deionized sterile water, 10 μl of Taq DNA Polymerase 2x Mix Red-Mgcl2 2 mM (GeneAll, Cat. No. A180301), 1 pmol of each primer, and 2 μl of DNA template.

The thermal cycling conditions for the amplification were 1 cycle for 4 min at 94°C, 35 cycles of 30 s at 94°C, 45 s at 55°C, and 45 s at 72°C, with a final extension step of 5 min at 72°C. Positive and negative controls (from Veterinary Laboratories Agency, UK) were included in each analysis. 6 μl of the amplified products was loaded on a 1.3% agarose gel and visualized by staining with ethidium bromide and compared to DNA markers (50 base pair ladder, Fermentas).

Sequencing

Two PCR-positive samples in a volume of 50 ml were sent to Bioneer Company for sequencing.

Results

Infection and statistics

One hundred conjunctival swab samples were obtained from an eye hospital. Of these, 28 samples (28%) were positive for VACWR002 nucleotide fragment. Twenty of the cases had a history of working in the research or medical laboratories or health centers, and 18 cases of them were VACV-positive. In this study, there was a statistically significant association between VACV and lab working status using Chi-square test ($P < 0.05$).

VACV prevalence results by considering other factors such as age, sex, and history of working in a medical or research laboratory include the time between exposure and VACV positivity and are listed in Table 1.

There were no statistically significant associations between VACV status and sex or age using Chi-square test ($P < 0.05$).

Polymerase chain reaction

VACV was detected using PCR test specific for the VACWR002 nucleotide fragment. The VACV-specific band with the size of 163 bp was detected in DNA-positive control. The positive PCR products were in the same size as those from the positive control [Fig. 1].

Sequencing

After sequencing, alignment of expected amplicon with the read sequences confirmed the presence of the VACWR002 fragment in the positive samples [Figs. 2-4].

Table 1: The prevalence of vaccinia virus among different groups

VACV condition	Sex		Age group				Working at lab		Lab exposure condition	
	Female	Male	<15	15-30	30-45	>45	Yes	No	Previous	Current
VACV-positive	13	15	1	12	10	5	18	10	2	16
VACV-negative	37	35	24	13	15	20	2	70	0	0
Total	50	50	25	25	25	25	20	80	2	16

VACV: Vaccinia virus

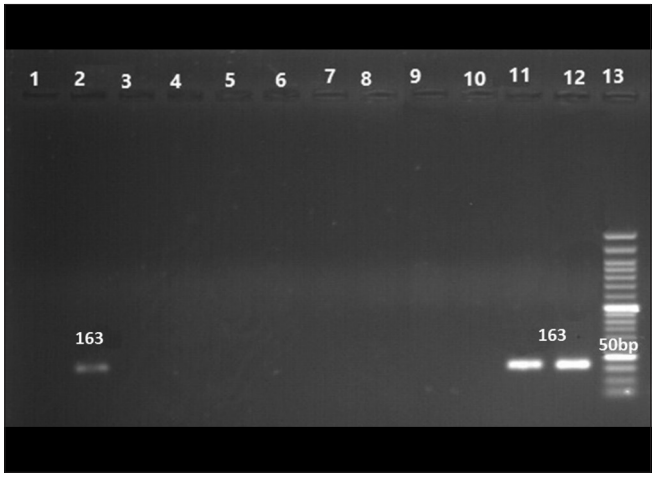

Figure 1: VACWR002 polymerase chain reaction amplification products following electrophoresis. 13:50 bp DNA ladder, 12: Positive control, 11 and 2 positive samples, 1: Negative control

Discussion

Vaccination against smallpox is associated with a high risk of harmful reactions, such as mild fever and muscle pain or systemic infection, encephalitis, myocarditis, and death.[4,20-23] One common adverse reaction to smallpox vaccination is ocular vaccinia, resulting from accidental transmission of VACV from the inoculation site to the eye. Accidental transfer of virus to another part of the body is not uncommon and the most usual sites being the face, genitalia, and anus.[2] Accidental transfer of the virus to the eye occurred in ~1–4 recipients per 40,000 primary vaccinations during the smallpox eradication attempt.[2,4] The estimated rates of corneal involvement (keratitis) ranged from 6% to 30% of ocular vaccinia cases, depending on reporting conditions.[1,3]

After the complete eradication of smallpox, no reports of smallpox have been reported from Iran. Furthermore, no vaccination program against any of human pox viruses has been performed up to now. Therefore, poxviruses are not considered as important causes of conjunctivitis in Iran.[24] However, unpredictably, in this study, 28% of the samples was positive and a statistically significant relationship obtained between working in medical or research laboratories and VACV prevalence ($P < 0.05$).

In fact, 64.28% of VACV-positive patients had a history of working in a research or medical laboratory. Ninety percent of these people worked directly with the virus in the laboratory, or there was a unit in their workplace where the recombinant VACV was manipulated for research purposes. Of these people, 88.88% when tested for VACV were currently carrying out their research on vaccinia or have been commuting in the research units of the laboratory where recombinant vaccinia was handled. We would believe that mere working in the laboratory may not lead to this condition; however, the exposure/handling of the VACV would put the individual with a risk of developing this condition. Since none of the patients had not been vaccinated against smallpox, the results mentioned in the above confirmed authors' hypothesis that the VACV may be important as a laboratory-acquired infection.

Although the exact mechanism of infection could not be specified, the location of the principal lesion at the inner canthus suggests either unintentional inoculation from hand to eye or transmission through aerosolization of the virus.[10] Regardless, both mechanisms indicate that existing biosafety precautions in the laboratory were presumably inadequate. Biosafety level 2 (BSL-2) precautions are recommended for laboratories and persons who manipulate strains of VACV.[25] This recommendation supposes that all such persons will be sufficiently vaccinated against the virus. However, this report and others of laboratory-acquired vaccinia infections showed that vaccination is being forgone in certain institutions.[6-8,26-28] No usual instructions exist for the level of precautions to be used by unvaccinated personnel. Vaccination may probably prevent or attenuate these patients' infection, and it should continue to be recommended for laboratory workers who handle vaccinia. However, given that vaccination has side effects of its own that might reduce its use (including a rate of ocular complications of 10–20/1 million immunizations),[10,11,24,29] biosafety recommendations for unvaccinated laboratory workers should be exactly mentioned.

The Centers for Disease Control has previously recommended increased biosafety precautions for laboratories with unvaccinated personnel who manipulate monkeypox virus.[30] Implementing certain BSL-3 precautions in this case, for example, performing all manipulations of virus in the biosafety cabinet or other enclosed equipment, frequent glove changing squired by handwashing, and always wearing goggles or face shields at the time of manipulation virus outside of a primary abnegation device, might have prevented this infection or would have minimized the potential human mistakes. Use of eye protection should be specially assured as critical eye infections can occur even in previously vaccinated persons.[31]

In this study, the prevalence of ocular vaccinia among employees of laboratories and health centers was considerably high (90%). These results emphasize on the issue that protecting of laboratory and health care personnel from the complications of VACV must be considered. The results of our study are

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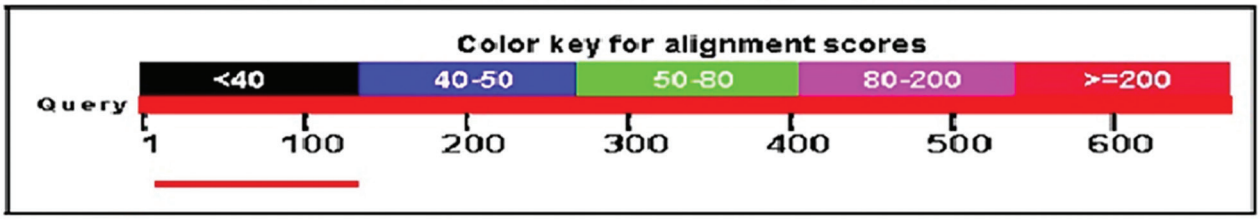
Nucleotide Sequence (672 letters)

RID UTC1EVSE114 (Expires on 07-21 16:24 pm)

Query ID	lcl|Query_57465	**Subject ID**	lcl|Query_57467
Description	None	**Description**	None
			See details
Molecule type	nucleic acid	**Molecule type**	nucleic acid
Query Length	672	**Subject Length**	163
		Program	BLASTN 2.2.32+

Distribution of 1 Blast Hits on the Query Sequence



Descriptions

Sequences producing significant alignments:

Description	Max score	Total score	Query cover	E value	Ident	Accession
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Alignments

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Range 1: 42 to 163

Score	Expect	Identities	Gaps	Strand	Frame
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Features:

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Sbjct  42   AATTTT-TGTTATTGCATT-TAATCTCGAAATTCAGAGTGCACACCTTTCTCCTGTAAAG  99

Query  71   AAACCTGAAGTTGCTACCTTATTAAGGACGGAGAAGTATTCCTCACGAAATACGGGATTA  130
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Sbjct  100  AAACCTGAAGTTGCTACCTTATTAAGGACGGAGAAGTATTCCTCACGAAATACGGGATTA  159

Query  131  CAGT  134
            ||||
Sbjct  160  CAGT  163
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Figure 2: Alignment of reading sequence of polymerase chain reaction products with the expected amplicon. There is 99% identity between polymerase chain reaction product sequences and expected amplicon

consistent with results of Lewis *et al*. (2004) that documented ocular vaccinia infection in an unvaccinated laboratory worker and described the associated laboratory infection.[31] These findings were found by Silva *et al*.

On May 1, 2015, Hsu *et al*. reported laboratory-acquired VACV infection in a recently immunized person too that shows that handling of virus even by immunized people is also needed to employ special measures.[27,28]

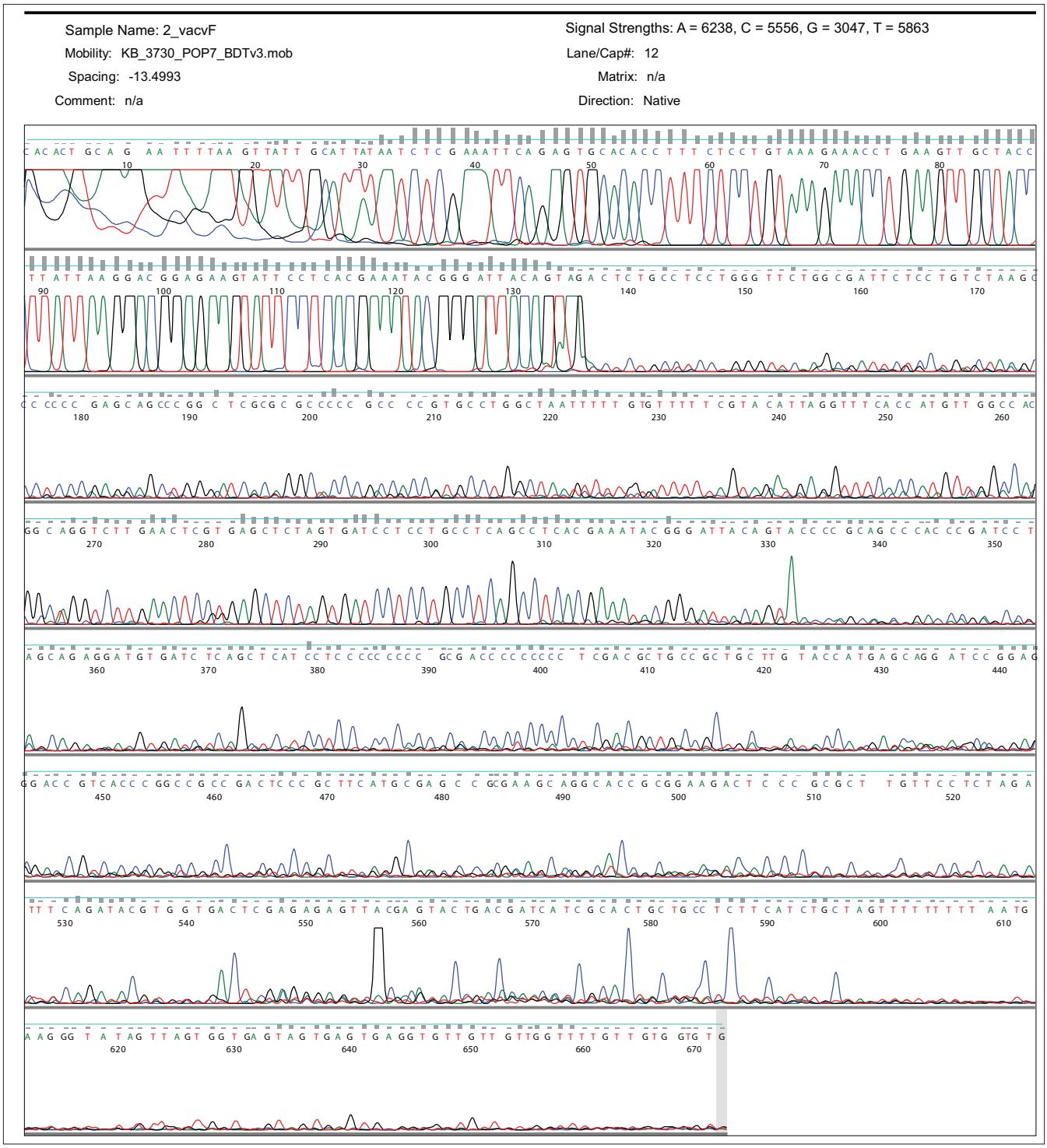

Figure 3: Reading sequence electrophoretogram of vaccinia virus polymerase chain reaction products

However, no principled monitoring of vaccinia infection in laboratory workers currently exists; hence, the full condition of the problem is unclear. Further investigation of laboratory practices involving vaccinia is guaranteed. Currently, vaccination is the best way to prevent or mitigate accidental infection[9] and should continue to be recommended for personnel handling nonattenuated strains of virus. If vaccination is impossible, workers should implement more stringent biosafety practices.[2]

Overall, the present study showed a high rate of VACV keratoconjunctivitis in Iran, and a statistically significant relationship was obtained between working in medical or research laboratories and VACV prevalence ($P < 0.05$).

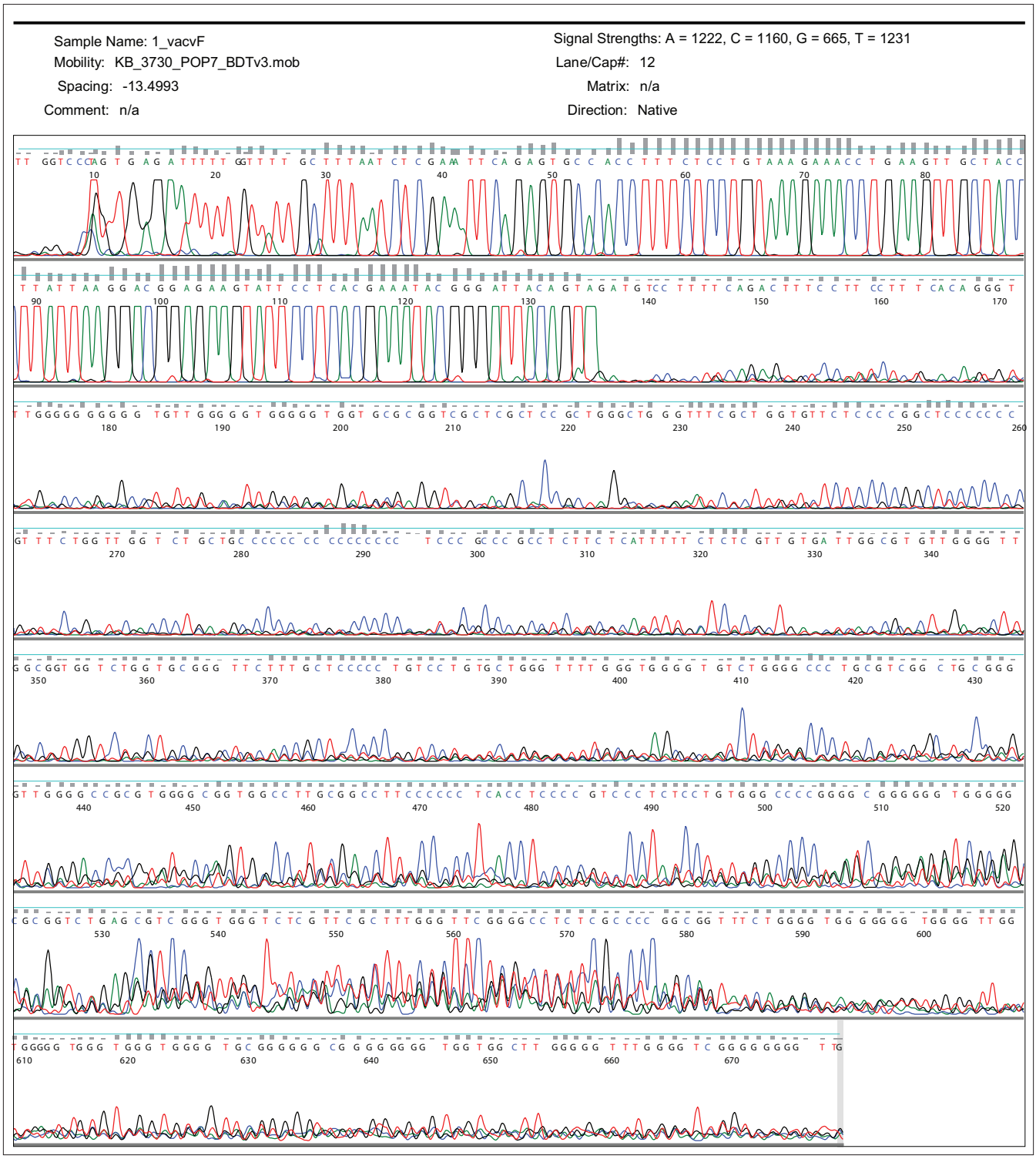

Figure 4: Reading sequence electrophoretogram of vaccinia virus polymerase chain reaction products (2)

Undoubtedly, further studies for determining its prevalence at a larger scale and, consequently, methods of its prevention should be done in Iran.

Conclusion

However, the results of this report and other similar studies can't definitively confirm that vaccinia is a laboratory-acquired infection, but the high prevalence of the virus among individuals tested in this study and the significant VACV prevalence among the laboratory personnel provide valuable data and the hypothesis that vaccinia may be a laboratory-acquired infection should be further studied.

This study suggests to prevent vaccinia complications in people who may be infected with the virus, particularly who

are working in the health care and research laboratories; the preventive measures should be implemented.

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Conflicts of interest

There are no conflicts of interest.

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