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ORIGINAL ARTICLE

Optimization and application of a high-resolution melting protocol in the characterization of avian infectious laryngotracheitis virus

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Abstract A previous sequence analysis of a *US5* gene fragment of infectious laryngotracheitis virus (ILTV) performed in an Argentinian epidemiological study allowed to differentiate between wild and vaccine strains. This analysis also defined five ILTV haplotypes with specific variations at positions 461, 484, 832, 878 and 894 of the *US5* gene. This characterization of viral strains may also be accomplished using the High-Resolution Melting Analysis (HRMA), which has been described as an effective, fast and sensitive method to detect mutations in PCR products. In the present study, an HRM protocol was developed with the aim of characterizing the circulating ILTV strains in Argentina. The specificity of this tool was confirmed in different DNA diluents, without interference from heterologous DNA or other cellular metabolites. Additionally, the salt concentration in the elution buffer used for DNA extraction did not alter the curve profiles. Higher concentrations of DNA (Ct ≅ 26.0) displayed well-defined curve profiles, whereas lower concentrations (Ct ≅ 32.5) exhibited more heterogeneous curves. The HRMA showed 97.49% concordance with the reference technique, *i.e.*, sequencing. The HRM protocol has the capability to perform DNA amplification prior to its characterization. Thus, eventually this technique may be used simultaneously as a diagnostic tool. This advantage implies a significant reduction in the time and effort involved in sample processing.

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PALABRAS CLAVE
Análisis de la disociación en alta resolución;
Secuenciación;
Caracterización;
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Optimización y aplicación de un protocolo de desnaturalización de alta resolución en la caracterización del virus de la laringotraqueítis infecciosa aviar

Resumen En un estudio epidemiológico realizado previamente en Argentina, se analizó la secuencia de un fragmento del gen *US5* del virus de la laringotraqueítis infecciosa (ILTV), lo que permitió diferenciar las cepas de campo de las vacunales. También esto permitió definir cinco haplotipos del ILTV, con variaciones específicas en las posiciones 461, 484, 832, 878 y 894 del gen *US5*. La caracterización de las cepas virales también puede lograrse mediante el análisis de la disociación de alta resolución o *high-resolution melting analysis* (HRMA), descripto como un método efectivo, rápido y sensible para detectar mutaciones en productos de PCR. En el presente estudio se desarrolló un protocolo de disociación de alta resolución con el objetivo de caracterizar cepas del ILTV circulantes en Argentina. Para ello,se confirmó la especificidad de esta herramienta en diferentes diluyentes del ADN de las muestras, sin observarse interferencias en presencia de ADN heterólogo u otros metabolitos celulares. Asimismo, la concentración de sales en el *buffer* de elución utilizado durante la extracción de ADN no alteró los perfiles de las curvas. Se obtuvieron perfiles bien definidos con concentraciones de ADN más elevadas (Ct ≅ 26.0), mientras que concentraciones más bajas presentaron curvas heterogéneas (Ct ≅ 32.5). El HRMA mostró una concordancia del 97.49% con la técnica de referencia, la secuenciación. El protocolo de disociación de alta resolución amplifica el ADN antes de su caracterización, por lo que esta técnica podría ser eventualmente utilizada para confirmar la presencia del ILTV y, al mismo tiempo, distinguir haplotipos, optimizando su valor como herramienta de diagnóstico. Esta característica implica una reducción significativa en el tiempo dedicado al procesamiento de muestras.

Introduction

Infectious laryngotracheitis (ILT) is a worldwide occurring respiratory chicken disease, generally observed in areas of highly intensive poultry production[14].

The causal agent of this disease is the infectious laryngotracheitis virus (ILTV) or *Gallid Herpesvirus-1* (GaHV-1), a member of the genus *Iltovirus* of the family *Herpesviridae*[9], whose genome consists of a linear double stranded DNA of 153 to 155 kbp[12,18].

ILTV infection is characteristically localized in the ocular conjunctiva and respiratory tract (larynx and trachea) and affects chickens from 14 days of age[11]. The clinical signs include different grades of conjunctivitis, nasal discharge, coughing and depression. On occasions, more virulent ILTV strains can lead to a more severe form of the disease, with high respiratory distress as a consequence of tracheal obstruction with fibrino-hemorrhagic exudates, causing asphyxia and death[14].

The currently available ILT vaccines (live attenuated and vectored) are capable of controlling the disease. However, it was proved that they were not equally efficient against the ILTV challenge[29]. Usually, those differences can also be observed under field conditions and they might depend not only on the type of vaccine but also on the route of administration and virulence of the field strain[7]. Thus, despite the extensive use of attenuated and vectored viral vaccines, outbreaks are still common. This situation has motivated several studies aiming at differentiating vaccine strains from field isolates. Diverse genomic regions as well as molecular methodologies have been applied to study circulating variants in several countries. The implemented techniques have evolved from restriction fragment length polymorphism (RFLP) analysis of the entire viral genome to sequence analysis of some ILTV genes[1,3–5,8,16,19,20,23].

Recently, our group has demonstrated that the sequence analysis of a fragment of the *US5* gene (glycoprotein J or gJ) allowed to characterize Argentinian field strains into five groups (haplotypes), two of them belonging to vaccine strains[8]. Through this analysis, we detected specific variations at five critical positions over a 1279-bp region of the *US5* gene, and therefore stated that five haplotypes circulate in this country.

Mutation scanning may also be accomplished using the High-Resolution Melting Analysis (HRMA). This technique is effective, fast and highly sensitive, and allows to detect punctual mutations along the sequence of PCR products[10,22,25,26]. In this methodology, the fragment of interest is amplified in the presence of a fluorescent dye at saturating concentrations; subsequently, the PCR product is denatured by gradual temperature increments and, finally, the characterization is achieved by comparing variations in fluorescence as a function of melting point temperatures[10,17]. Since the melting temperature (Tm) of a product is dependent on the GC content, length, and sequence of the sample, PCR products can be distinguished by their melting curves[27]. The amplification and subsequent mutation scanning performed in an HRM protocol occur in the same tube, which makes it less laborious and eliminates contamination concerns[21,26].

The purpose of the present study was to design and implement an HRM protocol in order to characterize ILTV strains circulating in Argentina and to compare the results with the reference sequencing technique.

Materials and methods

Viral samples

ILTV samples were obtained from the tracheas of animals infected during field outbreaks in different locations of Argentina, between 2006 and 2012 (Table 1).

Two vaccines were also included in the study, CEO attenuated by several passages in chicken embryo (Laringo-Vac™ Solvay Animal Health) and TCO attenuated by tissue culture passages (LT-IVAX™ Schering Plough).

DNA extraction

Viral DNA was extracted from tracheal swabs or tracheal homogenates in PBS medium supplemented with antibiotics as described by Craig et al[8]. Briefly, tracheal mucosa samples were scraped and homogenized with sterile sand and PBS supplemented with penicillin 10 000 IU/ml, streptomycin 5000 μg/ml, gentamicin sulfate 1000 μg/ml, kanamycin sulfate 700 μg/ml, and amphotericin B 10 μg/ml (Sigma Chemical Co™, St. Louis, MO, USA). Subsequently, the homogenates were centrifuged for 1 min at 5000 *g* to eliminate cell debris and sand. Tracheal swabs were obtained by a strong rubbing of the mucosal zone and suspended in 2 ml of sterile PBS supplemented with antibiotics. Finally, DNA was extracted from the supernatants using the QIAamp DNA Mini Kit (Qiagen Inc., Valencia, CA, USA), according to the manufacturer's instructions.

Primers

Two sets of primers (Suppl. Table 3) were designed using ''Primer Quest Tool'' software (https://www.idtdna.com/site) on sequences previously published in GenBank. These primers were selected avoiding secondary structures (because this may result in unusual melting profiles) and with an annealing temperature of 60 °C. These primers amplify two regions within the *US5* gene: zone 1 (Z1 of 137 bp) and zone 2 (Z2 of 136 bp). These two regions together include the five varying nucleotide positions that define the five haplotypes. Three concentrations of the primers were tested (0.1 μM, 0.3 μM and 0.5 μM) to optimize the protocol.

HRMA sensitivity assay

A sensitivity assay was performed with a clone containing a 512-bp DNA fragment Z1-Z2 from the TCO vaccine by transforming *E. coli* DH5α with a ''pGEM®-T easy vector'' (Promega Co., Fitchburg, WI, USA), following the protocol described by Sambrook[28]. Differences between results obtained in the presence of heterologous DNA were evaluated by performing serial ten-fold dilutions of the clone (final concentrations of 10^{10} copies/μl to 1 copy/μl). The dilutions were prepared in two different diluents (a) nuclease-free water and (b) DNA solution extracted from a negative sample for ILTV (called matrix). Finally, a real time PCR of each dilution was performed in a StepOnePlus™ System (Applied Biosystems™, Foster City, CA, USA). The cycling program includes a final denaturation step performed from 60 °C to 95 °C for 15 s, with a temperature rising slope of 0.3%, which corresponds to a variation of about 0.1 °C/s in the instrument (Suppl. Table 4). Reaction mixes were prepared in a final volume of 20 μl, using ''MeltDoctor™ HRM Master Mix'' (Applied Biosystems™) and 0.3 μM of each set of primers (gJZ1-Fw and gJZ1-Rv, and gJZ2-Fw and gJZ2-Rv).

The values of slope and intercept of each obtained standard curve were compared applying Multiple Linear Regression ($p < 0.05$).

HRMA haplotype test

Five clones were used as controls for this trial: one for each ILTV haplotype circulating in Argentina. For this purpose, the amplified Z1-Z2 fragment was cloned from the TCO vaccine (haplotype 1), the CEO vaccine (haplotype 2) and field samples BA12_223 (haplotype 3), ER09_02 (haplotype 4) and ER13_122 (haplotype 5). These control clones are henceforth called H1, H2, H3, H4 and H5, depending on the haplotype to which they belong. Then, serial ten-fold dilutions were prepared for each clone separately using (a) nuclease-free water and (b) the provided elution buffer (QIAamp DNA Mini Kit, Qiagen Inc.) to determine if the presence of salts in the buffer could interfere with resultant profiles. Dilutions with 10^4 and 10^2 DNA copies/μl in both media were selected (Ct = 32.5 and 26.0, respectively, based on the results of the *sensitivity assay*) and analyzed in triplicate by HRM. PCR conditions were similar to those of the *sensitivity assay*, except for the last denaturation step, when the temperature increase was achieved on a continuous mode with the addition of a final step (Suppl. Table 4).

The results were analyzed with High-Resolution Melt Software v3.0.1. (Thermo Fisher Scientific, Waltham, MA, USA). One replica of each clone (H1 to H5) was assigned as ''control'' for each expected HRMA profile, whereas the other two replicas were kept as ''unknown sample''. Additionally, pre-melting and post-melting limits were set around the melting peak to minimize the noise in the reading along the program (Fig. 1).

HRMA repeatability assay

The same previously described controls for haplotypes 1 to 5 (with 10^4 DNA copies/μl in nuclease-free water) were used following the procedure described for the HRMA haplotype test. PCR conditions are detailed in Suppl. Table 4.

Intra-plate and inter-plate variability were analyzed in this assay. For intra-plate variability, each haplotype was seeded in triplicate in a 96-well plate and this scheme was repeated varying positions in the same plate. Inter-plate variability was assessed by performing and repeating this latter scheme for three consecutive days. Each day was considered a replica.

Table 1 Viral strains characterized by sequencing and HRMA.

Name	Year of isolation	Location	HRMA	Sequencing
TCO Vaccine (LT-IVAX™)			H1	H1
BA10_149.647	2010	Pergamino, BA	H1	H1
BA10_149.672	2010	Pergamino, BA	H1	H1
ER11_162	2011	Villa Elisa, ER	H1	H1
CEO Vaccine (Laringo-Vac™)			H2-3	H2
ER06_01	2006	Herrera, ER	H2-3	H2
ER06_02	2006	Herrera, ER	H2-3	H2
ER08_04	2008	Crespo, ER	H2-3	H2
ER08_11	2008	Crespo, ER	H2-3	H2
BA09_068	2009	BA	H2-3	H2
NQ09_103	2009	Neuquén, Nq	H2-3	H2
RN09_109	2009	Cipolletti, RN	H2-3	H2
RN10_33	2010	Cipolletti, RN	H2-3	H2
BA10_141	2010	Chivilcoy, BA	H2-3	H2
BA10_152	2010	BA	H2-3	H2
BA10_154.740	2010	Pergamino, BA	H2-3	H2
BA10_154.741	2010	Pergamino, BA	H2-3	H2
BA11_176	2011	Pergamino, BA	H2-3	H2
BA11_195.300	2011	San Andrés de Giles, BA	H2-3	H2
BA11_195.349	2011	Gral. Conesa, BA	H2-3	H2
Mza12_203	2012	Guaymallén, Mza	H2-3	H2
BA12_210	2012	Gral. Conesa, BA	H2-3	H2
BA12_224	2012	BA	H2-3	H2
Cba12_245	2012	Río Cuarto, Cba	H2-3	H2
BA08_051	2008	Lobos, BA	H2-3	H3
BA08_057	2008	Carmen de Areco, BA	H2-3	H3
ER09_01[a]	2009	Mansilla, ER	Var.	H3
ER09_08	2009	Mansilla, ER	H2-3	H3
BA09_071	2009	BA	H2-3	H3
BA09_092	2009	BA	H2-3	H3
BA09_096	2009	BA	H2-3	H3
BA09_097	2009	BA	H2-3	H3
BA09_099.397	2009	Cañuelas, BA	H2-3	H3
ER10_01	2010	Rocamora, ER	H2-3	H3
BA10_111	2010	Roque Pérez, BA	H2-3	H3
BA10_149.675	2010	Virrey del Pino, BA	H2-3	H3
BA11_156	2011	Pergamino, BA	H2-3	H3
BA11_163	2011	BA	H2-3	H3
BA11_180	2011	San Nicolás, BA	H2-3	H3
BA11_195.304	2011	San Andrés de Giles, BA	H2-3	H3
BA11_196	2011	BA	H2-3	H3
BA12_223	2012	BA	H2-3	H3
ER08_03	2008	Crespo, ER	H4	H4
ER08_05	2008	San Cipriano, ER	H4	H4
ER09_02	2009	Caseros, ER	H4	H4
ER09_03	2009	San Justo, ER	H4	H4
ER09_086	2009	ER	H4	H4
ER12_07	2012	Villa Elisa, ER	H4	H4
ER12_14	2012	Villa Elisa, ER	H4	H4
ER12_77	2012	1° de Mayo, ER	H4	H4
ER12_81	2012	Basavilbaso, ER	H4	H4
ER07_01	2007	ER	H5	H5
ER07_02	2007	ER	H5	H5
ER08_01	2008	San José, ER	H5	H5
ER08_06	2008	1° de Mayo, ER	H5	H5
ER08_07	2008	3 de Febrero, ER	H5	H5

Table 1 (*Continued*)

Name	Year of isolation	Location	HRMA	Sequencing
ER08_27	2008	1° de Mayo, ER	H5	H5
ER08_30	2008	Villa Elisa, ER	H5	H5
ER09_09	2009	Pronunciamiento, ER	H5	H5
ER10_25	2010	Villa Elisa, ER	H5	H5
ER12_63	2012	Villa Elisa, ER	H5	H5
ER12_66	2012	Villa Elisa, ER	H5	H5
ER12_67	2012	Villa Elisa, ER	H5	H5
ER12_75	2012	Villa Elisa, ER	H5	H5
ER12_84	2012	Villa Elisa, ER	H5	H5
ER12_85	2012	Cnia. Las Pepas, ER	H5	H5
Field strains[b]				
ER13_14	2013	Basavilbaso, ER	H2-3	H3
ER13_21	2013	3 de Febrero, ER	H5	H5
ER13_22	2013	Villa Elisa, ER	H5	H5
ER15_37	2015	Herrera, ER	H5	H5
ER15_38	2015	Herrera, ER	H5	H5

[a] Sequence without concordance between sequencing and HRMA.
[b] Field samples used to check HRMA accuracy in unknown sequence samples.
TCO: Tissue culture origin, CEO: Chicken embryo origin. BA: Buenos Aires, ER: Entre Ríos, Nq: Neuquén, RN: Río Negro, Mza: Mendoza, Cba: Córdoba.

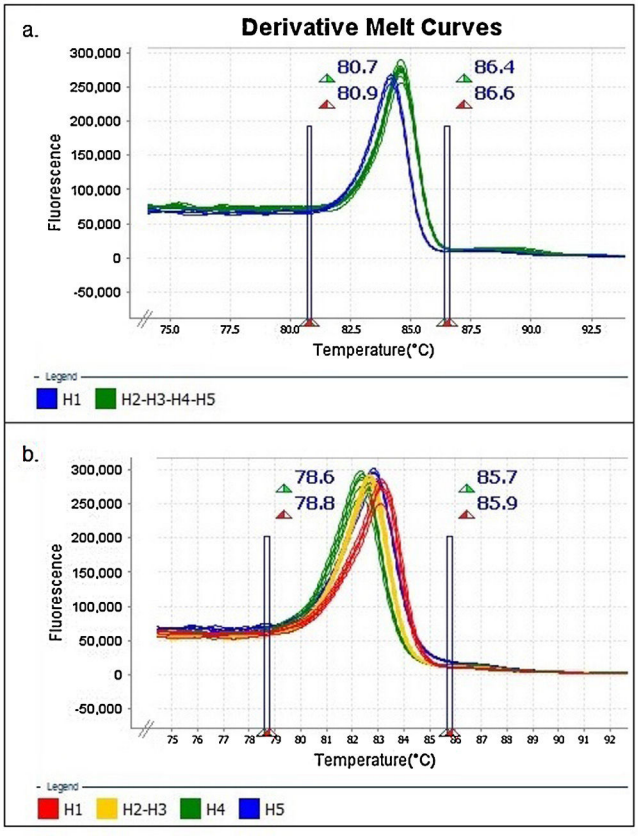

Figure 1 Pre- and post-melting limits around the Tm peak in the amplification of A. Z1 fragment and B. Z2 fragment.

The results from this assay were analyzed with High-Resolution Melt Software v3.0.1.

The Tm values obtained for each sample of the Z1 and Z2 fragments were analyzed with a nested ANOVA test to calculate the variability between replicates.

Concordance between HRMA and sequencing

Sixty-four ILTV field samples and two vaccines (Table 1) were previously characterized and their haplotypes were determined by sequencing[8]. Each DNA sample was seeded in triplicate together with clones H1 to H5 as controls. HRMA was performed on the Z2 fragment following the PCR protocol described above in the HRMA haplotype test. The characterization results obtained by sequencing and HRMA were compared and the concordance between both methodologies was determined using Cohen's κ coefficient[6], according to the following formula:

$$\kappa = \frac{Po - Pe}{1 - Pe}$$

where:

Po = proportion of observed matching between sequencing and HRMA in the establishment of a haplotype. Matching determinations/total of determinations.

Pe = proportion of expected matching occurring by chance.

Characterization of new field strains by HRMA

The setup of all the HRMA protocol parameters was checked by using five selected positive samples for ILTV that were collected between 2013 and 2015 (Table 1). Additionally, a sequencing analysis was performed on PCR amplicons (1279 bp) of the *US5* gene containing the Z1 and Z2 regions. These PCR fragments were purified using the QIAquick PCR purification kit (Qiagen Inc.), according to the manufacturer's recommendations. A bi-directional DNA sequencing was performed by the Sanger technique (3500xL Genetic Analyzer, Applied Biosystems™), by using primers IgJ-Fw and IgJ-Rv[13] (Table 3 in supplementary material), and

Table 2 Nucleotide base combinations generating each haplotype and HRMA expected profiles.

Haplotype	Positions of variable sites					HRMA expected profiles	
	Z1 fragment		Z2 fragment			Z1	Z2
	461	484	832	878	894		
1	A	T	A	C	G	H1	H1
2	A	C	A	T	G	H2-4-5	H2-3
3	T	C	A	T	G	H3	H2-3
4	A	C	A	T	A	H2-4-5	H4
5	A	C	G	T	G	H2-4-5	H5

internal primers gJ-ForS622 and gJ-RevS500. Finally, sequences were aligned, edited and analyzed using BioEdit Sequence Alignment Editor Software[15].

Results

Comparison of HRM sensitivity using different diluents

Amplification plots and standard curves from the Z1 and Z2 fragments were generated in two different diluents: nuclease-free water and matrix (Suppl. Table 5).

Supplementary table 5 displays slope and regression coefficient (R^2) values together with the amplification efficiency (Eff%) obtained for the quantification standard curves by using StepOne™ Software v2.3. In both cases, either efficiency or R^2 showed values with an excellent adjustment between the Ct and the numbers of DNA copies.

Multiple linear regression between both diluents showed no significant differences ($p < 0.05$) between the regression lines, as evidenced by the statistical similarity of slopes and intercepts.

Based on the results obtained, the optimal concentration range was between 10^2 and 10^9 DNA copies/μl ($9.00 < Ct < 32.5$). These concentrations allowed a reliable analysis of the Z1 and Z2 fragments.

Identification of haplotypes by HRMA

An *in silico* analysis of the Z1 fragment indicated that there should be three different curve profiles containing the five haplotypes grouped as follows: one curve for haplotypes 2, 4 and 5 (H2-4-5) and the other two for haplotype 1 (H1) and haplotype 3 (H3), respectively. On the other hand, the four expected curve profiles for the Z2 fragment should correspond to H1, H2-3, H4 and H5. Therefore, a proper determination of the haplotype of an ILTV sample needs a combined analysis of the curve profiles of both fragments (Table 2).

Upon analyzing the Z1 fragment, it was observed that all samples were classified as variants. In the case of the field samples, the assignment of haplotype identity was unsuccessful with this region.

Similar results were obtained when using different concentrations (copies/μl) of DNA template and with the two evaluated dilution media (nuclease-free water and elution buffer) (Figs. 2A and B). Furthermore, as expected, the shape of the curves was irregular in the case of the clones with low concentration (10^2 copies/μl and a $Ct > 30$, Fig. 2A).

In the Z2 fragment analysis, clones with 10^4 copies/μl ($Ct < 30$) showed four different curve profiles that clearly distinguished haplotype 1, haplotypes 2 and 3 together, haplotype 4 and haplotype 5, as expected according to the sequence of this fragment (Fig. 2D). The average percentage of confidence of the software in assigning the haplotypes was: 93.3 for H1, 99.0 for H2-3, 99.5 for H4 and 81.4 for H5.

Although the nucleotide sequences did not change in this analysis, a low copy number of clones from this region (10^2 copies/μl and a $Ct > 30$) reduced the possibility of characterizing the samples and increased the occurrence of discordance in the curve profiles (variants) (Fig. 2C).

Because no additional information could be extracted from the Z1 fragment, any further analysis henceforth only contained the Z2 fragment in order to discriminate between H1, H2-3, H4 and H5.

No differences were detected using water or buffer as diluents in any of the assays.

HRMA repeatability assay

Intra-plate as well as inter-plate repeatability assays showed no significant difference between replicates. The tested clones presented the same profile in both assays, with no significant difference of Tm values between replicates. Tm values with nested ANOVA showed a variability coefficient below 0.1.

Concordance between HRMA and sequencing

Of the 66 analyzed samples, only one yielded an unexpected haplotype. Its profile showed it as a variant, even though it had been assigned as haplotype 3 by sequencing (Table 1). Further DNA extraction was performed to this sample to improve this result. However, the analysis over this fresh DNA remained inconclusive.

Cohen's κ coefficient was calculated to determine the concordance between HRMA and sequencing. Samples with haplotypes 2 and 3 were grouped together in sequencing results for a fair comparison with HRMA, taking into account that the Z2 fragment analysis exhibited one curve profile for both haplotypes. This κ value was 97.49%, thus indicating a

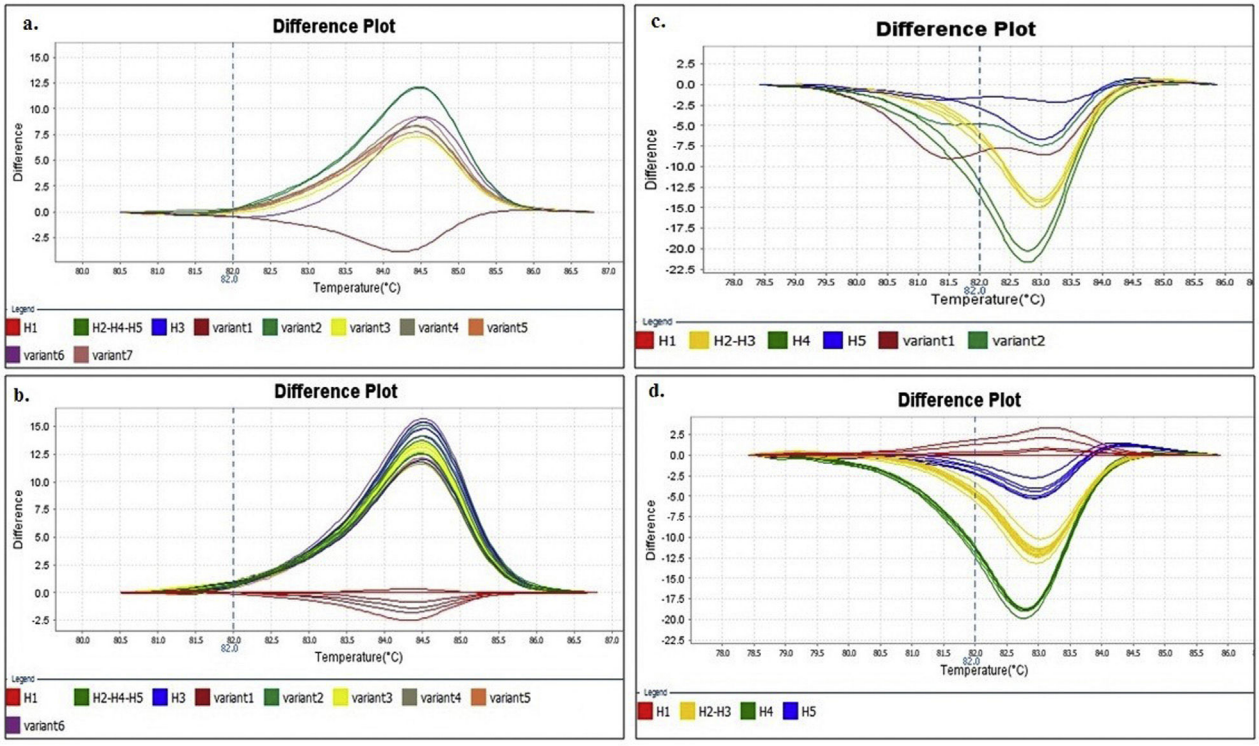

Figure 2 Denaturing profiles obtained after amplification. A. Z1 fragment with 10^2 copies/μl of clones diluted in nuclease-free water and B. 10^4 copies/μl diluted in nuclease-free water and elution buffer. C. Z2 fragment with 10^2 copies/μl of clones diluted in nuclease-free water and D. 10^4 copies/μl diluted in nuclease-free water and elution buffer.

high percentage of coincidence between both methodologies under these conditions.

Characterization of new field strains by HRMA

Field strains named ER13_14, ER13_21, ER13_22, ER15_37 and ER15_38 were characterized by HRMA (Table 1) to determine the haplotype and to test the conditions that were set up for this HRM analysis. Subsequently, they were sequenced to verify the identity determined by the haplotype profile.

The Z2 fragment analysis by HRMA showed profiles corresponding to haplotype 5 in four samples (ER13_21, ER13_22, ER15_37 and ER15_38) and to H2-H3 in another sample (ER13_14).

The sequence analysis confirmed the identity of all analyzed samples.

Discussion

In this study, we optimized an HRMA specific for ILTV that would allow a faster and more economical characterization of field strains than the classical sequencing technique. HRMA is simple, economical and accurate and these characteristics make it a valuable tool as a screening method prior to the sequence analysis.

Instead of amplifying the 1279-bp fragment of the *US5* gene studied by Craig and collaborators[8], we designed primers to amplify shorter regions to ensure maximum sensitivity, in accordance with the recommendation of amplicon size for a better discrimination[25].

Some critical points must be taken into consideration when performing HRMA. For instance, the accuracy of the results depends to a large extent on the quality of the real-time amplification[25]. Those samples that amplify late or fail to reach a high signal plateau in the PCR phase can result in inconclusive or low-resolution HRMA data.

With this fact in mind, we assayed different factors that could possibly affect the quality of the DNA and, hence, the reliability of the results, in order to optimize the technique.

The HRMA performed on samples diluted alternatively in nuclease-free water or matrix showed that the presence of heterologous DNA and other cellular metabolites in the matrix did not interfere with the accuracy of the technique. The absence of both nonspecific amplification and/or alteration of the curve shape in HRMA is essential for the proper characterization of a pathogen.

Relative stabilities of native and denatured DNA may be altered in concentrated salt solutions. This effect is reflected in changes in the Tm of the DNA[24] and can result in low sensitivity, poor reproducibility and incorrect genotype calls. In the present study, we evaluated two different eluents that are commonly used in DNA extraction. No evident differences were observed between the curve behavior using nuclease-free water and the elution buffer (provided by the DNA extraction kit).

Another factor to consider is the concentration of the DNA template in the sample. There are different suggestions about the cutoff of the Ct that results in reliable curve

profiles: White and Potts[30] recommend employing this tool in those cases where the Ct value is below 35; however, the equipment manufacturer suggests using it with Ct values below 30. The influence of this parameter was assessed by analyzing two different concentrations (10^2 and 10^4 DNA copies/μl) of the haplotype clones, the obtained Ct values being 32.5 and 26.0, respectively.

At the highest clone concentration (Ct ≅ 26.0), the results of the analysis of the Z2 fragment yielded well defined curve profiles that were clearly distinguishable from each other. The profiles obtained at a lower clone concentration (Ct ≅ 32.5), however, showed more irregular curves. These altered curve profiles have led to classify these clones as variants, therefore, hindering the characterization of the samples into their corresponding haplotypes. Based on this result, HRMA would be reliable only to identify samples with a Ct value below 30.

Unexpectedly, an experimental limitation was evident during the adjustment of the technique with regard to the discrimination between the nucleotide transversion A/T at position 461 in the Z1 fragment. This position is responsible for differentiating haplotype 3 from 2, 4 and 5. Each transition and transversion has different variations in their Tm; the change A/T has the lowest variation in Tm, with a deviation lower than 0.25 °C[17]. Although the equipment used in this study makes fluorescence readings every 0.1 °C, we were unable to discriminate H3 from the other haplotypes within the Z1 fragment. In a comparison study of nine instruments capable of performing HRMA, the researchers reported certain variability between the predicted Tm value for a specific mutation and the measured one[17].

Another reason that could explain this limitation is the position of the mutation within the genomic region. Indeed, curve profiles could differ depending on the surrounding sequences of a nucleotide variant.

Sequencing is generally regarded as the gold standard for assessing sequence variation with an accuracy approaching 99.9%[26] and therefore the assessment of the concordance value between both techniques is essential. In terms of the studied genomic region (*US5*), and under the conditions used in this study, the concordance (97.49%) between HRMA and sequencing was high.

These results support the idea that HRMA can be applied as an effective screening tool for ILTV strains. Furthermore, although the analysis of the Z1 fragment was dismissed because of the impossibility to differentiate H2 from H3, the Z2 fragment analysis allowed us to distinguish between the other haplotypes and showed a very good concordance with sequencing. Taking these results into account, the use of sequencing for the characterization of some haplotypes (H1, H4 and H5) would not be necessary, although the differentiation of H2 and H3 would still require this standard method. Sequencing would also be essential to give a new identity in the event of the emergence of a new variant.

Altogether, the analysis of the Z2 fragment allowed us to identify haplotypes H1, H4 and H5 and therefore to reduce the number of samples to be sequenced.

Although in this study we used HRMA only to characterize field samples, we do not dismiss the possibility of using this technique as a diagnostic tool as well, because a wide range of DNA concentrations (10^2 to 10^9 DNA copies/μl) generate a proper amplification signal. To achieve this, firstly it will be necessary to determine if the Z2 fragment of *US5* is specific enough to make the diagnosis by comparing the results with the validated protocol for ILT detection[2]. This procedure would give us the advantage of detecting and characterizing some samples in just one step. In case of H2/H3, the sample could be purified and sequenced in a step forward. Therefore, as previously underlined by others[26,31], this procedure would be an available scanning technique that can be performed in the same container used for PCR amplification, consequently preventing possible environmental contamination.

In summary, HRMA showed its potential to replace sequencing for ILTV characterization. Although in this case it was not possible to differentiate H2 from H3, this tool showed a very good correlation to sequencing, thus providing the possibility of reducing workflow and the number of field samples to be sequenced.

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Conflict of interest

The authors declare that they have no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ram.2020.04.008.

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