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Genotyping of Newcastle disease virus (NDV) isolated from Egyptian field strains using Pyrosequencing

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Abstract

Newcastle disease (ND) is a highly contagious avian disease, that poses considerable threat to the poultry industry worldwide, Newcastle Disease Virus (NDV) is the a member of serotype 1 avian paramyxoviruses (APMV-1)that causes respiratory and neurological disease in chickens and other species of birds, in this study a method based on RT-PCR and pyrosequencing analysis has been developed, to rapidly diagnose and pathotype NDV directly in clinical specimens, A pair of degenerated primers was designed to amplify a portion of the fusion (F) gene responsible for virulence and used to test 14 specimens collected from Egyptian farms in 2016 and 2017. The subsequent pyrosequencing reaction identified a 30-bp region encompassing the cleavage site. all samples were pyrosequenced and results were compared and confirmed by the Sanger sequencing procedure, which is traditionally performed for NDV pathotyping, this method was able to genotype 10 VVNDV and 4 classic NDV. The pyrosequencing reaction provided high quality results in real time and proved to be more rapid and cost-efficient than the classical sequencing procedure, indicating it as a possible valid alternative to the currently used diagnostic assays for NDV.

***Key words**: NDV, RT-PCR, Fusion gene (F gene), pyrosequencing, pathotyping.*

Introduction

Newcastle disease (ND) is caused by Newcastle disease virus (NDV), a member of avian paramyxovirus (APMV), which belongs to the genus Avulavirus, sub-family Paramyxovirinae, family Paramyxoviridae, and order Mononegavirales **(Mayo, 2002).** Its single-stranded RNA genome is composed of 6 genes, -NP-P-M-F-HN-L, encoding 6 major proteins nucleoprotein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin-neuraminidase (HN), and large protein (L), respectively. The hemagglutinin-neuraminidase (HN) and the fusion (F) proteins are both glycoproteins expressed at the surface of the enveloped virus. **(Madadger *et al*., 2013).** The most susceptible avian species to this disease are chickens **(Rezaeianzadeh *et al*., 2011)**. Several pathotypes (asymptomatic enteric, lentogenic, mesogenic, viscerotropic velogenic, or neurotropic velogenic) of NDV are recognized depending on the clinical symptoms observed in chickens (**OIE. 2012**). A virulent and virulent strain may also be distinguished on the basis of the cleavage site sequence of their F protein. During replication, the fusion gene is translated into a precursor protein, F0, which must be cleaved by host cell proteases into F1 and F2 subunits for viral particles to become infectious (**Morrison *et al*., 1993**). Most virulent strains exhibit the consensus sequence ^{112} (R/K) RQ (R/K) R*F^{117} at the cleavage site of the F0 precursor, in contrast to ^{112}(G/E) (K/R) Q (G/E) R*L^{117} in a virulent viruses (**Collins *et al*., 1993; OIE. 2012**) , so the fusion protein cleavage site has been deemed as responsible for the pathogenicity of NDV **(de Leeuw *et al.,* 2005)**and target to identify and type

lentogenic and velogenic strains (**Fuller *et al*., 2009; Yacoub *et al*., 2012).**

Detection of Newcastle disease virus (NDV) from recent outbreaks affecting poultry farms usually done using Rapid molecular detection and pathotyping approaches, including, A Real -time PCR assay that uses different TaqMan probes **(Aldous *et al.,* 2001; Farkas *et al.,* 2009; Wise *et al.,* 2004; Yacoub *et al.,* 2012)**; SYBR Green real-time PCR-based methods **(Pham *et al.,* 2005a; Tan *et al.,* 2009, 2004)**; loop mediated isothermal amplification (LAMP) **(Pham *et al.,* 2005b)**. These techniques show both advantages and limitations, including false negative results generated by primer or probe mismatches resulting from genetic variability of NDV (**Cattoli *et al*., 2009; Wise *et al*., 2004).** The pyrosequencing method presents a recent and new DNA-based approach for sequence identification and typing (**Langaee and Ronaghi, 2005).** Using this technique, 30–40 nucleotides of the target PCR-amplified products are sequenced based upon there lease of a pyrophosphate molecule (PPi), which occurs when a nucleotide is incorporated in the newly synthetized strand. This molecule (PPi) triggers an enzymatic reaction, resulting in the release of a fluorescent signal detected by the instrument Andis proportional to the amount of PPi released **(Ronaghi, 2001)**. The reaction is very efficient in producing short amplified fragments when compared with the classical sequencing method (Sanger-based) (**Cristian *et al.,* 2013)**. However, the amplified PCR products destined for pyrosequencing must be of good quality to obtain optimal sequencing data.

In Egypt, velogenic strains of NDV were isolated and clustered as class II genotype VII sub -genotype d and closely related to Middle East isolates **(Radwan *et al*., 2013).** The predominant NDV isolates circulating among chickens are virulent and associated with outbreaks in commercial poultry farms and backyard reared chickens in Egypt **(Osman *et al*., 2014)**. In this study 14 samples were pyrosequenced and results were compared and confirmed by the Sanger sequencing procedure, which is traditionally performed for NDV pathotyping.

Materials and Methods

Sample collection:

Tracheal, cloacal swabs and organs (Brain, trachea, lungs, proventriculus, small intestine and pancreas) from fourteen chicken farms were collected from different Egyptian governorates that showed respiratory, nervous signs and diarrhea.

Detection of NDV by RRT-PCR

The viral genomic RNAs of 14 sample were extracted from the infective cloacal and tracheal swabs and also from organs using QiAmp Viral RNA Mini kit (QiagenGmbH, Hilden, Germany) according to the manufacturer's instructions, RRT-PCR was carried out using a commercial kit Quanitnova probe RT-PCR kit (Qiagen, GmbH). Primers used NDV-F (F+4829) 5′-GGT GAG TCT ATC CGG ARG ATA CAA G-3′, NDV-R (F-4939) 5′-TCA TTG GTT GCR GCA ATG CTC T-3′ and probe- (F+4894) 5'-FAM-AAG CGT TTC TGT CTC CTT CCT CCA-BHQ-3' (**Creelan *et al*., 2002; Ausvetplan, 2006**). RRT-PCR was conducted in the Stratagene 3005P MXpro Real-Time PCR System (Stratagene, USA) .

RT-PCR and partial sequence of fusion gene using Sanger method:-

PCR amplification was performed by using Qiagen One Step Enzyme Mix according to the manufacturer's instructions, using the following primer set.

NDV-M2 **F** TGG AGC CAA ACC CGC ACC TGCGG

NDV-F2 **R** GGA GGA TGT TGG CAG CATT **Mase *et al*., (2002).**

Gel containing DNA band of the expected size (766bP for partially sequenced samples was excised and purified with the QIAquick Gel Extraction Kit (Qiagen) according to the manu-

facturer instruction .Purified RT-PCR products were sequenced using Big dye Terminator V3.1 cycle sequencing kit (Perkin-Elmer, Foster city, CA) and Applied Biosystems 3500xl genetic analyzer (ABI, USA). Sequences similarities and relationships of the partial (14 samples) in this study were compared with previously published NDV vaccine and references strains available in the public database (BLAST, NCBI, USA). Amino acid phylogenetic relationship was constructed for the sequenced samples and other vaccine, reference and Egyptian strains previously published and available on the Genbank database using MEGA version 6 **(Tamura *et al*., 2013).** A comparative analysis of deduced amino acids and nucleotides sequences of the sequenced fusion gene were created using the CLUSTAL W Multiple Sequence Alignment Program, version 1.83 of MegAlign module of Laser gene DNA Star software.

Pyrosequencing:

In this study we design a new set of primers capable of identifying a 30 bp region covering the cleavage site. The forward primer was biotinylated ,making it suitable for the pyrosequencing assay. The conserved sequence downstream of the 30 bp region was used to design the reverse PCR primer, which served also as the sequencing primer .The 203bp fragment obtained using the newly designed primers was amplified using the Qiagen One-step RT-PCR kit, according

to the manufacturer's instructions (Qiagen, Hilden, Germany). Extracted RNA (5) was added to 45 of reaction mix containing a final primer concentration of 200 nM each .A 30 min retro-transcription step at 50 ◦C was followed by a15 min denaturation at 95 ◦C and then by 45 cycles of 94 ◦C for 30 s,64 ◦C for 30 s and 72 ◦C for 40 s.

Pyrosequencing primers:-

F: CCTTGGTGATTCTATCCGTAGG
R: CTGCCACTGCTAGTTGIGATAATCC
S: CACTGCTAGTTGTGATAATC

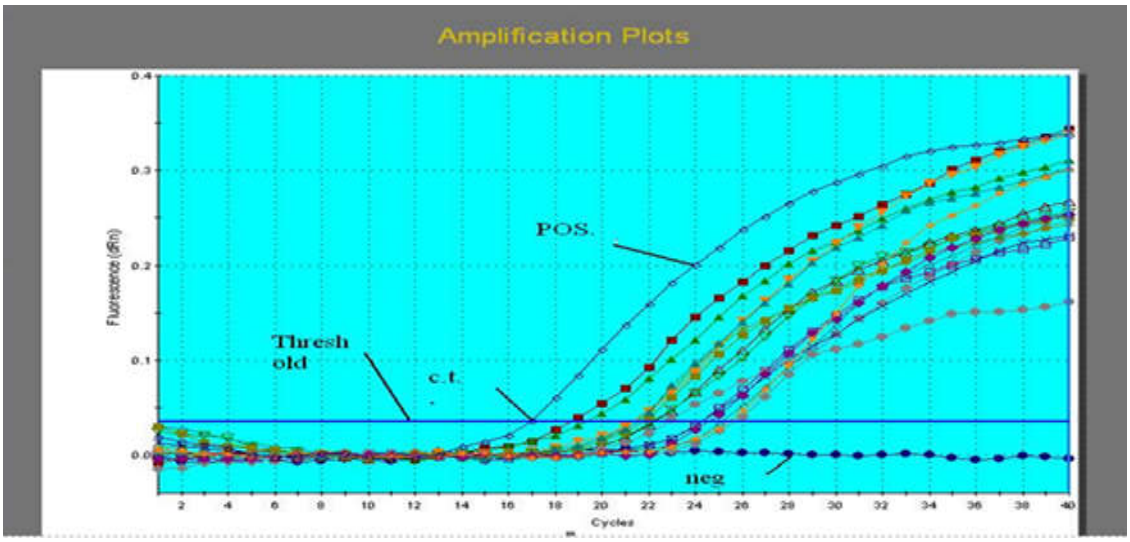
Pyrosequencing reactions were performed using the Pyromark ID platform using Pyro Gold reagents according to the manufacturer's instructions (Biotage, Uppsala, Sweden). Assay performances were compared to the Sanger sequencing results.

Results

Detection and amplification of NDV by RRT -PCR

All collected samples (14) are positive by RRT -PCR using specific primers and probe for NDV with threshold cycle between 18.66 and 26.66 .

Figure (1). Positive results of 14 samples examined by specific primers and probe for NDV

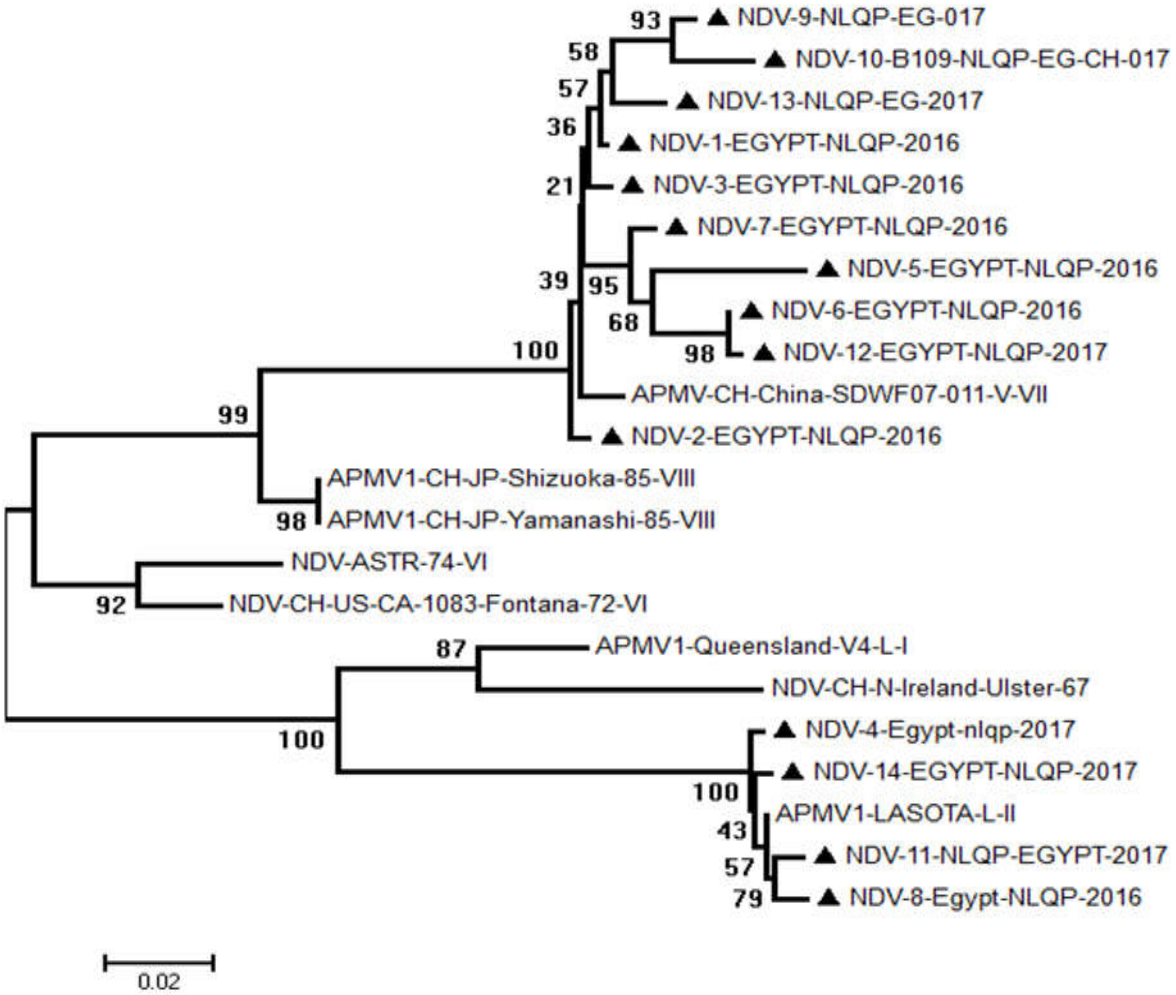


Also All collected samples (14) are positive by RT- PCR using specific primers for partial amplification of fusion gene showing specific bands at 766 bp on agarose gel.

Sequence and phylogenetic analysis

Sequence analysis revealed that (10) examined samples are closely related to the very virulent strains of NDV (Genotype VIId) and are compared with other strains published on Gene Bank on NCBI website indicate that all of them were closely related to Chicken/China/SDWF07/2011 strain with identity (98%-99%)**,** Analyses of these samples indicate presence of motif ^{112}RRQKRF^{117} in hypervariable region which is indicative of velogenic character of NDV strains**,** (four) samples are closely related to vaccinal NDV strains (Lasota, Hitchner, clone 30 and Avenu) used in Egypt which genetically genotyped as lentogenic strains of NDVs (class II genotype II).

Fig. (2): Phylogenetic characterization of Amino acid sequence



Phylogenetic tree of partially sequenced NDV strains with other Egyptian and vaccinal strains and other strains from Middle East.

Fig. (3): Partial Amino Acid Sequence of hypervariable region of fusion protein Deduced Amino acid sequence using sanger method

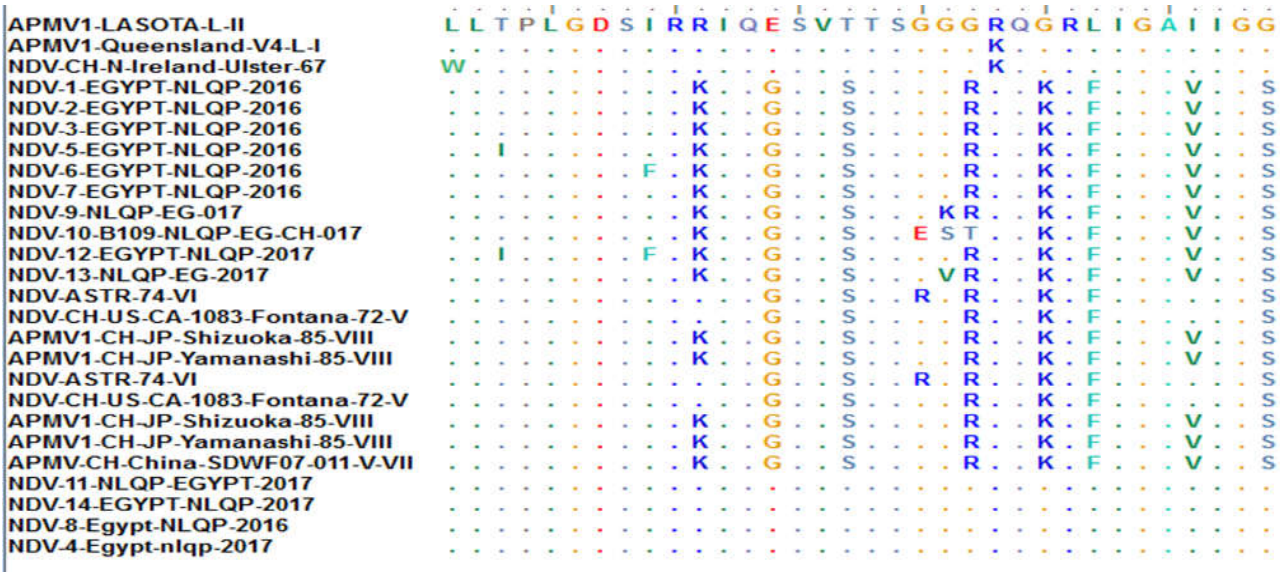
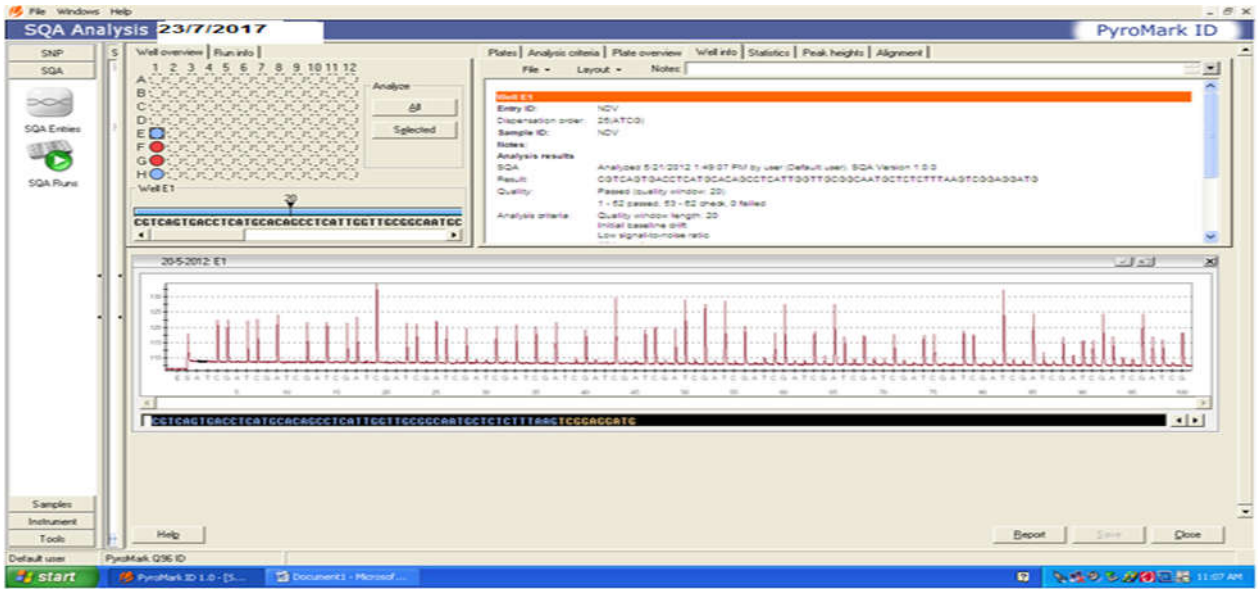


Table (1). Partial Amino Acid Sequence of hypervariable region of fusion protein using Pyrosequencing:-

Name	Pyrosequencing results	Genotyping
NDV-1-EGYPT-NLQP-2016	GSVSTSGGRRQKRF	Very virulent
NDV-2-EGYPT-NLQP-2016	GSVSTSGGRRQKRF	Very virulent
NDV-3-EGYPT-NLQP-2016	GSVSTSGGRRQKRF	Very virulent
NDV-4-Egypt-nlqp-2017	ESVTTSGGGRQGRL	classic
NDV-5-EGYPT-NLQP-2016	GSVSTSGGRRQKRF	Very virulent
NDV-6-EGYPT-NLQP-2016	GSVSTSGGRRQKRF	Very virulent
NDV-7-EGYPT-NLQP-2016	GSVSTSGGRRQKRF	Very virulent
NDV-8-Egypt-NLQP-2016	ESVTTSGGGRQGRLI	classic
NDV-9-NLQP-EG-017	GSVSTSGKRRQKRFI	Very virulent
NDV-10 -NLQP-EG-CH-017	GSVSTSESTRQKRFI	Very virulent
NDV-11-NLQP-EGYPT-2017	ESVTTSGGGRQGRLI	classic
NDV-12-EGYPT-NLQP-2017	GSVSTSGGRRQKRFI	Very virulent
NDV-13-NLQP-EG-2017	GSVSTSGVRRQKRFI	Very virulent
NDV-14-EGYPT-NLQP-2017	ESVTTSGGGRQGRLI	classic

Fig. (4): Pyro-mark SQA analysis of NDV:-



All the samples tested by pyrosequencing had been previously sequenced using the capillary ABI PRISM 3500 xl Genetic Analyzer (Applied Biosystem, Foster City, CA, USA). All pyrosequencing results showed complete agreement with those obtained by the Sanger sequencing method, indicating that both approaches provide comparable results in the discrimination between lentogenic and velogenic strains.

Discussion

The present study describes the development of a pyrosequencing-based method for the rapid identification and pathotyping of different NDV strains belonging to various genetic lineages and sub lineages **(Aldous *et al*., 2003).** Egypt is endemic for Newcastle disease virus (NDV) with continuous long-lasting outbreaks causing significant economic losses in the poultry industry due to high mortality which may reach 100% in very virulent strains of NDV, despite the intensive vaccination programs **(Mohamed *et al*., 2011; Radwan *et al*., 2013; and El Bagoury *et al*., 2015).** The use of different techniques for the determination of the virulence of ND viruses and for Phylogenetic studies has been reported **(Farkas *et al*., 2009)**, there is increasing use of such molecular techniques to detect NDV in clinical specimens with its advantage being the extremely rapid demonstration of the presence of virus **(OIE, 2009).**

Overall, the results presented here indicate that this system is a useful and practical alternative to the classical pathotyping of NDV evolution of NDV may be related to the accumulation of point mutations that induce amino acid substitution in the neutralizing epitopes as the N-linked glycosylation sites of the F protein **(Chambers *et al*., 1986).** F-Protein of virulent strains differ from those of a virulent virus by virtue of possessing a pair of dibasic amino acids at the carboxyl (C) terminus of F2 and phenylalanine at residue 117 which is the N-terminus of the F1 protein. The term 'multiple basic amino acids' refers to at least three arginine (R) or lysine (K) residues between residues 113 and 116 while a virulent viruses have Leu (leucine) at amino acid no.117 cleavage site in their fusion protein **(Collins *et al*., 1993; Zhu *et al*., 2010).**

Analysis of the deduced amino acid sequence and phylogenetic analysis of the NDV F-gene

of the strains isolated from Egypt in 2016 and 2017 suggests that 10 examined samples are closely related to genotype VIId circulating in the Middle Eastern countries and China, which is responsible for the continuing outbreaks of ND in Egypt **(Hussien *et al*., 2014).**

Samples that tested by pyrosequencing analysis were correctly identified as being either lentogenic or velogenic strains in relation to Sanger based sequencing, Compared to the rapid tests based on real time PCR, RT-PCR and other probe hybridization-based assays, this method can rapidly deduce an amino acid sequencing result, which is in line with one of the official pathotyping method accepted by the international organizations.

Furthermore, the cost of the pyrosequencing analysis is decreased since it does notneed terminator enzymes and time-consuming purification steps **(Dudley *et al*., 2012; Pizzuto *et al*., 2010; Timbs *et al*., 2012**).Another important practical feature of the protocol described related to the short fragment amplified (203bp) that allows for the generation of PCR products suitable for pathotyping in samples where DNA integrity is reduced or damaged **(Rasmussen and Morrissey, 2008).**However, to obtain the best amplified products and generate good quality pyrosequences, validation of the preliminary RT-PCR reaction step is crucial.

In conclusion all pyrosequencing results showed complete agreement with those obtained by the Sanger sequencing method, indicating that both approaches provide comparable results in the discrimination between lentogenic and velogenic strains.

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