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**Evaluation of full S1 gene sequencing of classical and variant infectious bronchitis viruses extracted from allantoic fluid and FTA cards**

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**ABSTRACT**

Sequence variability in the S1 gene determines the genotype of infectious bronchitis virus (IBV) strains. A single RT‑PCR assay was developed to amplify and sequence the full S1 gene for six classical and variant IBVs (M41, D274, 793B, IS/885/00, IS/1494/06 and Q1) enriched in allantoic fluid (AF) or the same AF but inoculated onto Flinders Technology Association (FTA) cards. Representative strains from each genotype were grown in SPF eggs and RNA was extracted from AF. Full S1 gene amplification was achieved using primer A and primer 22.51. Products were sequenced using primer A, 1050+, 1380+ and SX3+ to obtain short sequences covering the full gene. Following serial dilutions of AF, detection limits of the partial assay were higher than those of the full S1 gene.  Partial S1 sequences exhibited higher than average nucleotide similarity percentages (79%; 352bp) compared to full S1 sequences (77%; 1,756bp), suggesting that full S1 analysis allows greater strain differentiation. For IBV detection from AF inoculated FTA cards, four serotypes were incubated for up to 21 days at three temperatures; 4 oC, 24 oC and 40 oC. RNA was extracted and tested with partial and full S1 protocols. Through partial sequencing, all IBVs were successfully detected at all sampling points and storage temperatures. In contrast, using full S1 sequencing was not possible to amplify the gene beyond 14 days or when stored at 40oC. Data presented shows that for full S1 sequencing, a substantial amount of RNA is needed. Field samples collected onto FTA cards are unlikely to yield such quantity or quality.

**Keywords:** Infectious bronchitis virus, spike gene, sequencing, allantoic fluid, FTA card

**Abbreviations:** Infectious bronchitis virus (IBV), Infectious bronchitis (IB), Flinders Technology Association (FTA), allantoic fluid (AF)

**INTRODUCTION**

Infectious bronchitis (IB) is an acute viral disease caused by infectious bronchitis viruses (IBVs) that mainly infect the respiratory system in broiler and layer chicken flocks (Cavanagh, 2005). Infection causes economic losses due to high mortality, poor body weight gain (Ignjatovic & Sapats, 2000), decreased egg production (reaching up to 70%), and reduced egg quality (increased eggshell pigment) in layers (Muneer *et al.*, 2000). Further complications can occur in some cases, including cystic oviducts (Benyeda *et al.*, 2009) and false layer syndrome (Crinion & Hofstad, 1972). Certain IBVs, including several Australian strains, have been associated with renal complications such as nephritis (Mahmood *et al.*, 2011) and digestive complications, such as proventriculitis (Toffan *et al.*, 2013).

IBV is a single stranded RNA virus (approximately 27.6kb in size) belonging to the family *Coronaviradae*. It contains four structural proteins: spike protein (S), small membrane envelope protein (E), membrane protein (M), and nucleoprotein (N). The spike protein comprises 1,145 amino acids and undergoes post translational cleavage to form S1 and S2 subunits (Jackwood & de Wit, 2013). The functions of spike include attachment to host cells, neutralization of antibodies and initiation of protective immunity (Johnson *et al.*, 2003). Furthermore, it is the most variable of the IBV proteins, with most mutations and recombination events mapping to the S1 region (Kingham *et al.*, 2000), which can lead to the emergence of new variant strains (Zhang *et al.*, 2015).

The relationship between antigenic differences among IBV strains has been investigated (Jackwood & de Wit, 2013), and previous work has shown that partial S1 sequences have a stronger correlation with protective relatedness between strains, rather than antigenic relatedness (Ladman *et al.*, 2006). In addition, variation in the nucleotide sequence of S1 may lead to alterations in the virus categorisation, such as receptor binding abilities (Jackwood & de Wit, 2013).

More than 25 serotypes have been recognized to date, often differing by up to 50% at the amino acid level for S1 gene (Gelb *et al.*, 2005). Only a minor amino acid sequence alteration, at the very least of a 5% change (25-30 amino acids) in the S1 subunit, is required to affect the protection status conferred by vaccinations of the same serotype (Cavanagh, 2003). As a result, low cross protection is provided by different vaccine virus serotypes (Kuo *et al.*, 2010).

In recent years, sequence analysis has become a common method to identify strain types, typically being carried out on specific nucleotide regions of the S gene (Cavanagh, 2003) or the N gene (Williams *et al.*, 1992). Previous studies have focused on analysing different nucleotide regions of the S1 gene (between 228 to 380bp) (Wang & Tsai, 1996; Al-Shekaili *et al.*, 2015) leading to difficulties when comparing genotype analysis between studies.

For many years, the Massachusetts (Mass) serotype, originally isolated in Massachusetts in the 1930s, was the only recognized serotype (via virus neutralisation). Jungherr *et al.,* (1956) reported a Connecticut IBV type, which was not cross protected or cross neutralised against the Mass IBV strain (Jungherr *et al.*, 1956). During the 1960s, the Australian nephropathogenic strains were first diagnosed and connected to uraemia (Cumming, 1963), including Australia/N1/62 which is considered to be the first strain isolated in the continent (Jackwood, 2012). In 1984, researchers in the Doorn Institute, The Netherlands first isolated the Dutch strain D207 from Mass vaccinated flocks (Davelaar *et al.*, 1984). The 793B serotype was identified in the 1990s (Parsons *et al.*, 1992), with sequence data distinct from Dutch and Mass strains (Cavanagh, 2005). The QX genotype was first described in 1996 in China, and was associated with severe nephritis, proventriculitis, tracheitis, decreased egg production and false layer syndrome (De Wit *et al.*, 2011). Later, another variant, known as Q1, which can also cause proventriculitis (Yu *et al.*, 2001), was detected from layer flocks showing respiratory signs, While certain genotypes like 793B and Mass have global distribution (Cook *et al.*, 1996), others are considered to have regional importance, such as IS/885/00 (Meir *et al.*, 2004) and IS/1494/06 (GenBank accession number EU780077).

Recently, there has been growing interest in complete S1 sequencing, with reports from Thailand, Sweden and India (amongst others) aiming to further understand the molecular characterisation of IBV (Pohuang *et al.*, 2011; Abro *et al.*, 2012; Kamble *et al.*, 2016). In addition to the classical IBVs, recombinant viruses have been identified in the field. Sequencing the full S1 gene, rather than a partial segment, will aid in the identification of such variants. In addition, it was suggested during the 8th International Symposium on avian Corona-Pneumoviruses and complicating pathogens (2014), that the full S1 gene should be sequenced to determine the genotype of IBV isolates.  Work in Italy has outlined the benefits of sequencing the full S1 gene and classification of IBV strains into a number of distinct lineages (Valastro *et al.*, 2016). An earlier study aimed to obtain full S1 sequences by using two primers to amplify and sequence around 1,700 base pairs (bp) from the S1 gene, after purifying and digesting with restriction enzymes (Kwon *et al.*, 1993). The same primers were used in another study (Abdel-Moneim *et al.*, 2006), while a separate investigation looked into the differences in S1 and partial N gene sequences (Huang *et al.*, 2004).

Flinders Technology Association (FTA) cards have been utilised for a number of IBV studies looking to detect the virus from clinical or laboratory samples (Moscoso *et al.*, 2005; Ganapathy *et al.*, 2015). While the cards are useful for transporting genomic material worldwide, the quality and quantity of RNA eluted varies depending on the initial amount of IBV RNA, storage duration and temperature (Moscoso *et al.,* 2005), and the extraction methodology. Previous work has outlined that for IBV, a partial S1 amplification and sequencing from FTA card extraction is successful to a viral concentration of 100.2 EID50/ml (Awad *et al.*, 2012). For IBV, it has been shown that RNA can be successfully extracted from field samples embedded onto FTA cards, and following RT-PCR and sequencing, genotypes can be determined based on the partial S1 gene (Ganapathy *et al*., 2015; Ball *et al.*, 2016).  Moscoso *et al.* (2005) and Awad *et al.* (2014) also demonstrated the effects of different temperature storage of IBV inoculated FTA cards (Moscoso *et al*., 2005; Awad *et al.*, 2014). However, to date, the feasibility of full S1 gene sequencing from FTA cards has not been examined.

The aim of this study was to develop and evaluate a single RT-PCR assay in order to amplify and sequence the full S1 gene of six common IBV genotypes grown in allantoic fluid. The sensitivity of the scheme was investigated using RNA extracted from allantoic fluid, both directly and after inoculation onto FTA cards, and field samples submitted on FTA cards.  The influence of storage temperatures on detectability of these IBV strains, through full and partial S1 PCR and sequencing were also assessed.

**MATERIALS AND METHODS**

**Virus strains**

The following virulent IBV strains were used in this study: 793B (106.4 CD50/ml), M41 (106.3 CD50/ml), D274 (106.75 CD50/ml), QX (106.3 CD50/ml), Q1 (105.5 CD50/ml), IS/1494/06 (106.3 CD50/ml) and IS/885/00 (106.3 CD50/ml) (Ganapathy *et al.*, 2012; Chhabra *et al.*, 2015). Viruses were passaged in embryonated specific-pathogen free (SPF) chicken eggs and titrated in tracheal organ cultures (TOCs) to determine the median ciliostatic dose per ml (CD50/ml) (Cook *et al.*, 1976).

**RNA extraction from allantoic fluid and FTA cards**

***Allantoic fluid***: Viral RNA was extracted using the QIAamp viral RNA Mini Kit (Qiagen, UK) according to the manufacturer’s instructions from 100 µl of neat and diluted allantoic fluid.

***Extraction from FTA cards***: FTA cards (WhatmanTM)were inoculated with 100µl of ten-fold diluted allantoic fluid for each virulent strain. The inoculated FTA cards were stored at three different temperatures; 4oC, 40oC and room temperature, defined as 22-26oC. For each sampling point (1, 2, 3, 7, 14, 21 days post inoculation) [dpi], a single, complete FTA circle from each dilution and each temperature was removed using sterile scissors, placed into 1000 µl of TE buffer ((10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0) (Thermo Fisher Scientific, USA)) and incubated for 10-15 minutes (Sakai *et al.*, 2015). After vortexing, 100 µl was taken and processed for RNA extraction using the QIAamp viral RNA Mini Kit (Qiagen, UK) according to manufacturing instructions.

**IBV RT-PCR and sequencing**

***Partial S1 RT-PCR:*** Extracted RNA was used for both partial and full S1 amplifications. For partial S1 amplification,RT-PCR targeting a 393bp partial sequence of the S1 gene was conducted (Ganapathy *et al.,* 2015; Ball *et al*., 2016), followed by gel electrophoresis.

***Full S1 RT-PCR:*** For full S1 amplification,reverse transcription was conducted using the primer 22.51 (5’ GAACGTCTAAAACGACGTGTTCC 3’) (Table 1). The reaction mix comprised of 5x first strand buffer [250 mM Tris-HCl (pH 8.3), 375 mM KCl, 15 mM MgCl2; Invitrogen], dithiothreitol (DTT) [100 mM; Invitrogen], dNTPs (dATP, dCTP, dGTP, dTTP) [10mM], RNasin ribonuclease inhibitor [20 U/µl; Promega], Superscript III Reverse Transcriptase [200U/µl; Invitrogen], oligonucleotide [10 pmol], sterile distilled water [2.13 µl] and 0.5 µl of template RNA. Reverse transcription conditions were: 48° C for 45 min, and 94° C for 5 min. Generated cDNA was then amplified in a one-step PCR using primer A and primer 22.51 (Table 2). The PCR mixture included Supermix [22 mM Tris-HCl (pH 8.4), 55 mM KCl, 1.65 mM MgCl2, 220 µM dNTPs, 22 U/ml recombinant Taq DNA polymerase, and stabilizers; Invitrogen], forward and reverse primers [10 pmol] and 0.5 µl of cDNA. Cycling conditions were: 35 cycles of denaturation at 94° C for 60s, annealing at 54° C for 30s, extension at 72°C for 60s, and a final extension at 72° C for 10 min. Products were visualised on an agarose gel.

***Partial and full S1 sequencing*:** Positive amplicons (partial or full) were purified (ExoSAP-IT, Affymetrix, Santa Clara, CA, USA) and sent for commercial Sanger sequencing (Source BioScience). For partial sequencing, the positive sense SX3+ primer (Worthington *et al.*, 2008) was utilised. For full S1 sequencing, the following primers were used; primer A (Pohuang *et al.,* 2011), SX3+ (Worthington, *et al*., 2008), 1050+ and 1380+ (Falchieri *et al.*, 2013) (Table 1). Sequences were then concatenated into a single contig using ChromasPRO v1.7.3. An additional assay was used for the amplification and sequencing of QX for inclusion with analysis (Falchieri *et al.,* 2013). In brief, the primer RT QX negative (5’‑CATCTTTAACGAACCATCTGG-3’) was used for the RT-stage. For amplification, the primers QX S1 start positive (5’-CCAGTTGTGAATTTGAAGAAAGAACAAAAGACCGACTTAG-3’) and QX S1 end negative (5’-CGAACCATCTGGTTCAATACAAAATCTGC-3’) were used in one step PCR amplification.

***Phylogenetic analysis:*** Reads for the full S1 sequencing were initially examined for their quality and contigs were assembled from each sequencing primer (Primer A, 1050+, 1380+ and SX3+) using ChromasPRO v1.7.3 (http//technelysium.com.au/). Sequences were then trimmed to cover the full S1 gene. Partial S1 sequences [SX3+ (Cavanagh *et al.,* 1999)] were processed using the same methodology. BLAST comparisons against the NCBI database (GenBank) were carried out to confirm strain identity. Alignments for all S1 sequences and reference genomes were carried out in MEGA6 (Tamura *et al.*, 2013), with phylogeny comparisons using the maximum likelihood calculation with 1000 bootstrap replicates. Reference strains used were D274 (X15832), 793B (AF093794), IS/885/00 (AY279533), IS/1494/06 (EU780077), Q1 (AF286302), QX (KF297571), M41 (AY561711), H120 vaccine C (KU736750), H120 vaccine D (KU736751), Ma5 vaccine (KU736747), Beaudette strain (DQ001336), Ark-DPI (KX529820), IBV Quebec strain (AF349621), IBV T strain (AY775779) and Connecticut vaccine (EU283058).

**Field samples**

Twenty-five tissues and swab samples collected from flocks in the Middle East, which were previously confirmed as IBV positive were used (Ganapathy *et al*., 2015).  These samples were imprinted onto FTA cards. RNA was extracted from these samples and subjected to the full S1 RT-PCR described above.

**RESULTS**

**Detection limits for partial *versus* full S1.**

Results showed that the minimum detection limits for the full S1 gene from virus enriched allantoic fluid were 10-2 for all strains except for M41, which was 10-3 (103.5 CD50/ml). In contrast, the detection limits for the partial S1 detection were at 10-7 for all strains except for Q1, which was 10-6 (100.5 CD50/ml) (Table 2; supplementary Figure 1).

**Nucleotide sequence and phylogenetic analysis**

The seven partial S1 sequences exhibited a higher average of nucleotide similarity (79%) when compared to the full S1 sequences (77%) with the range being 72-84% for both the partial andfull S1 (Table 3). This was true for both the classical and variant strains. M41 demonstrated a similarity of 77% (full) and 80% (partial) to D274, whereas the closely related IS/885/00 strain had 82% (full) and 83% (partial) similarity with IS/1494/06. Similarly, the Q1 strain had 77% (full) and 79% (partial) similarity with QX.

**Amino acid analysis**

Similar to the nucleotide sequences, full S1 amino acid sequences had a lower similarity compared to partial sequences overall (Table 4). Strain comparisons showed that M41 had 77% and 73% similarity to D274 and 793B respectively, compared to the partial amino acid sequence where similarity was higher (80% for both). IS/885/00 showed 82% (full) and 83% (partial) similarity compared with IS/1494/06. Similarly, Q1 had 77% (full) and 79% (partial) similarity compared with QX.

**Sensitivity of IBV detection following inoculation onto FTA cards**

IBV detection using both full and partial S1 RT-PCR varied between strains (Table 5). Partial RT-PCR detected all strains, at all temperatures, up to 21 dpi. However, the assay was the least sensitive for the FTA cards stored at 40oC at 21 dpi. For example, IS/1494/06 was only detected at the neat concentration for 40oC, compared to room temperature (10-3 dilution factor; 103.5 CD50/ml) and 4oC (10-4 dilution factor; 102.5 CD50/ml).

For the full S1 detection, we only detected IBV from the cards stored at 4oC and room temperature (Table 6). Additionally, the lowest dilution we identified IBV at was 10-1. No cards stored at 40oC were positive for the full S1 gene, and no IBV was detected at any temperatures past 14 dpi.

**Sensitivity of IBV detection from field samples**

Of the 25 tested samples, 76% were from tissue samples and 24% from swabs. We were able to amplify the full S1 gene for three samples (12%), whereas all samples were positive using partial S1 amplification.

**DISCUSSION**

The current study aimed to establish and test a universal RT-PCR scheme used for detection and sequencing of the full S1 gene of six globally distributed IBV strains (M41, 793B, D274, IS/1494/06, IS/885/00 and Q1).  Common practice for IBV research and diagnosis is to extract RNA for RT-PCR and sequencing.  Subsequently part of the S1 gene is analysed and cross-compared for differentiation between the strains (Wang & Huang, 2000). Many laboratories rely on a partial S1 gene assay, but a single full S1 gene assay capable of amplifying a number of common IBV serotypes has not been reported to date. One reason is that conserved sequences of approximately 20 nucleotides within the S1 gene are limited among genetically different IBV strains, decreasing the availability of applicable universal oligonucleotides (Adzhar *et al.*, 1996).

The full S1 protocol described in this study gave a lower average of S1 nucleotide similarity between genotypes when compared to the partial S1 assay, demonstrating that full S1 sequencing is a more accurate method to differentiate between strains. This is partly due to a single nucleotide variation affecting 0.3% similarity for a partial sequence (350bp), compared to 0.06% for a full S1 gene sequence (1,600bp). Such changes may cause an alteration in the virulent properties (Fang *et al.*, 2005).

For research and more widely for the diagnostic differentiation of IBVs, genotyping is commonly conducted using a partial sequence containing a hypervariable region (HVR) of S1. However, such an approach shows inconsistencies compared to full S1 genotyping (Mo *et al.*, 2013). Importantly, phylogenetic assessment in this study demonstrated that both the full and partial sequencing were able to distinguish individual genotypes. However, it should be noted that relying solely on partial sequences for phylogeny may give a false representation, as comparisons are dependent on the region sequenced. As a result, using the full S1 gene sequence demonstrates a better representation of strain differences (Valastro *et al.,* 2016). Due to this, using the full S1 sequence when possible would be advantageous and would allow for more accurate comparisons between studies.

Not all laboratories are able to isolate and propagate IBV. Instead, a number of countries are dependent on utilising FTA cards to transport potential IBV positive samples (such as tracheal or oropharyngeal swabs, tissues of lungs, caecal tonsil and kidneys) for diagnosis, as these cards are a safe method of storing and shipping genomic material (Ganapathy *et al.,* 2015). To date, there has been no published attempt to extract and sequence a full S1 genome from samples embedded onto FTA cards. This study showed that for RNA extracted from allantoic fluid or FTA cards, the partial S1 RT-PCR demonstrated greater detection limits compared to the full S1. This may be due to the significant difference in amplicon length: 0.39kb and 1.72kb for the partial and full S1 gene respectively. Successful molecular characterization of small sequence lengths (383 bp) of virus RNAs from FTA cards, including those that were stored under non favourable environmental conditions (at 41o C for at least 15 days), has been previously demonstrated (Moscoso *et al.,* 2005). In addition, recent work has shown difficulty in amplifying a viral nucleotide product greater than 900 bp, despite detecting a 290 bp product from the same sample after six months of storage (Sakai *et al.,* 2015). This work has shown that the partial assay was able to detect IBV RNA inoculated onto FTA cards for up to 21 dpi for all storage conditions, including at 40o C - a common summer temperature in many Middle East countries. In contrast, this was not possible for the full S1 gene (1,700 bp), even when applying the phenol chloroform method for extraction (Awad *et al.,* 2014). Furthermore, incubating the FTA cards in TE buffer overnight for further RNA elution accompanied with occasional vortexing resulted in no change in the amount of viral load when quantified using qRT-PCR. From data presented here, it appears that the sensitivity for the larger target (full S1) detection is affected by virus titre, and duration and temperature of storage.

Following the successful detection and sequencing of partial and full S1 genes of 793B, M41, D274, QX, Q1, IS/885/00 and IS/1494/06, an attempt to utilise the above S1 protocols for field samples collected via FTA cards from the Middle East (Ganapathy *et al.,* 2015) was carried out. From a total number of 25 partial S1 positive IBV samples, only three samples were positive via similarity to the full S1. This detection level reflects the low sensitivity of this assay when FTA card samples were stored in different conditions to this study. Further optimisation of the full S1 RT-PCR, including optimisation of RNA extraction from the FTA cards, may be necessary to increase detection limits. In our study, we attempted to mimic field practices, where oropharyngeal swabs or tissue samples collected at necropsy, or IBV-enriched allantoic fluids are embedded onto FTA cards, then stored and/or transported at different temperatures to local or international laboratories for detection of IBVs. It appears that temperatures up to 40° C, with storage for up to 21 days, has no negative impact on the recovery of sufficient RNA for a successful partial S1 RT-PCR and sequencing. However, it recovers inadequate RNA for a full S1 RT-PCR or sequencing.

In conclusion, the full S1 scheme presented in this study is mostly suitable for IBV enriched samples (e.g. allantoic fluid). Clinical samples (either swabs or tissues) that have been directly obtained from the field or *in vivo* experiments, including those imprinted on FTA cards, are unlikely to provide sufficient genome quantities for the full S1 detection. The partial S1 scheme was effective in detecting and differentiating IBV in enriched allantoic fluids and those embedded onto FTA cards.

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**Table 1.** Primers used for amplification and sequencing of IBV strains in this study.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Oligonucleotide** | **Sequence (5’-3’)** | **Sense** | **Length** | **Location (kb)** | **Reference** |
| **Primer A** | GCCAGTTGTTAATTTGAAAAC | Fa | 19 | 20.25  | (Pohuang, et al., 2011) |
| **Primer 22.51** | GAACGTCTAAAACGACGTGTTCC | Rb | 23 | 22.51  | In house |
| **1050+** | GGTTTAATTCCTTGTCAGTTTCTCTTACTTATGG | F | 34 | 21.36  | (Falchieri, etal., 2013) |
| **1380+** | GCTGCTAATTTTAGTTATTTAGCAGATGGTGG | F | 32 | 21.69  | (Falchieri,  et al., 2013) |
| **SX3+** | TAATACTGG C/T AATTTTTCAGA | F | 21 | 21.03  | (Worthington, et al., 2008) |

a F=Forward

b R=Reverse

**Table 2.** Partial and full S1 detection limits and corresponding CD50/ml values from diluted allantoic fluid.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  |  | **Allantoic fluid detection limits** |  |  |
| **IBV Strain** | **Titre (CD50/ml)** | Dilution factor | **Corresponding  CD50/ml**  |
| *Partial S1* | *Full S1* | *Partial S1* | *Full S1* |
| 793B | 106.38 | 10-7 | 10-2 | 100.62 | 104.062 |
| M41 | 106.33 | 10-7 | 10-3 | 100.67 | 104.067 |
| D274 | 106.75 | 10-7 | 10-2 | 100.25 | 104.025 |
| QX | 106.39 | 10-7 | 10-2 | 100.61 | 104.061 |
| Q1 | 105.5 | 10-6 | 10-2 | 100.50 | 103.050 |
| IS/885/00 | 106.5 | 10-7 | 10-2 | 100.50 | 104.050 |
| IS/1494/06 | 106.5 | 10-7 | 10-2 | 100.50 | 104.050 |

**Table 3.** Nucleotide identity (%) comparison of the seven IBV strains used in this study based on the full S1 and partial S1.

|  |
| --- |
| **Nucleotide identity (%) / Full S1** |
|   | *M41* | *D274* | *793B* | *IS/885/00* | *IS/1494/06* | *Q1* | *QX* |   |
| *M41* |   | 77 | 73 | 77 | 79 | 73 | 72 | *M41* |
| *D274* | 84 |   | 77 | 82 | 84 | 79 | 77 | *D274* |
| *793B* | 78 | 81 |   | 79 | 77 | 75 | 76 | *793B* |
| *IS/885/00* | 83 | 79 | 82 |   | 82 | 79 | 73 | *IS/885/00* |
| *IS/1494/06* | 81 | 79 | 79 | 83 |   | 82 | 81 | *IS/1494/06* |
| *Q1* | 81 | 84 | 79 | 83 | 82 |   | 77 | *Q1* |
| *QX* | 81 | 75 | 78 | 72 | 74 | 80 |   | *QX* |
|   | *M41* | *D274* | *793B* | *IS/885/00* | *IS/1494/06* | *Q1* | *QX* |   |
| **Nucleotide identity (%) / Partial S1** |

**Table 4.** Amino acid identity (%) comparison of the seven IBV strains used in this study based on the full S1 and partial S1.

|  |
| --- |
| **Amino acid identity (%) / Full S1** |
|   | *M41* | *D274* | *793B* | *IS/885/00* | *IS/1494/06* | *Q1* | *QX* |   |
| *M41* |   | 62 | 53 | 60 | 52 | 56 | 52 | *M41* |
| *D274* | 79 |   | 61 | 66 | 73 | 63 | 60 | *D274* |
| *793B* | 72 | 74 |   | 65 | 62 | 57 | 58 | *793B* |
| *IS/885/00* | 78 | 78 | 78 |   | 67 | 63 | 54 | *IS/885/00* |
| *IS/1494/06* | 76 | 79 | 75 | 85 |   | 55 | 61 | *IS/1494/06* |
| *Q1* | 77 | 84 | 76 | 82 | 83 |   | 56 | *Q1* |
| *QX* | 76 | 56 | 59 | 48 | 52 | 79 |   | *QX* |
|   | *M41* | *D274* | *793B* | *IS/885/00* | *IS/1494/06* | *Q1* | *QX* |   |
| **Amino acid identity (%) / Partial S1** |

**Table 5.** IBV detection limits (CD50/ml) using the partial-S1 assay from FTA cards following IBV strain inoculation and up to 21 days incubation at varying temperatures.

|  |  |
| --- | --- |
|   | **Strain** |
|  | *M41* |  | *793B* |  | *IS/1494/06* |  | *Q1* |
|  | **Temperature (oC)** |
| **DPI** | *4* | *40* | *Room**Temperature* |  | *4* | *40* | *Room**Temperature* |  | *4* | *40* | *Room**Temperature* |  | *4* | *40* | *Room**Temperature* |
| 1 | 103.33 | 103.33 | 103.33 |  | 103.38 | 103.38 | 102.38 |  | 102.5 | 102.5 | 102.5 |  | 101.5 | 102.5 | 101.5 |
| 2 | 103.33 | 104.33 | 103.33 |  | 102.38 | 103.38 | 102.38 |  | 102.5 | 102.5 | 102.5 |  | 101.5 | 102.5 | 101.5 |
| 3 | 101.33 | 103.33 | 102.33 |  | 102.38 | 103.38 | 102.38 |  | 104.5 | 104 | 102.5 |  | 101.5 | 102.5 | 101.5 |
| 7 | 102.33 | 105.33 | 103.33 |  | 103.38 | 104.38 | 103.38 |  | 103.5 | 104.5 | 104.5 |  | 102.5 | 102.5 | 102.5 |
| 14 | 101.33 | 103.33 | 102.33 |  | 102.38 | 103.38 | 103.38 |  | 102.5 | 105.5 | 101.5 |  | 102.5 | 102.5 | 101.5 |
| 21 | 101.33 | 103.33 | 102.33 |  | 102.38 | 103.38 | 103.38 |  | 102.5 | +a | 103.5 |  | 101.5 | 104.5 | 102.5 |

a Virus was only detected in the undiluted fluid

**Table 6.** IBV detection limits (CD50/ml) using the full S1 assay from FTA cards following IBV strain inoculation and up to 21 days of storage at different temperatures.

|  |  |
| --- | --- |
|   | **Strain** |
|  | *M41* |  | *793B* |  | *IS/1494/06* |  | *Q1* |
|  | **Temperature (oC)** |
| **DPI** | *4* | *40* | *Room**Temperature* |  | *4* | *40* | *Room**Temperature* |  | *4* | *40* | *Room**Temperature* |  | *4* | *40* | *Room**Temperature* |
| 1 | 105.33  | - b | 105.33  |   | 105.38  | - | 105.5  |   | 105.5  | -  | 105.5  |   | 104.5  | -  | 104.5  |
| 2 | 105.33  | -  | +a |  | 105.38  | -  | 105.5  |  | 105.5  | -  | 105.5  |  | 104.5  | -  | + |
| 3 | 105.33  | -  | + |  | 105.38  | -  | -  |  | 105.5  | -  | 105.5  |  | 104.5  | -  | -  |
| 7 | + | -  | -  |  | + | -  | -  |  | + | - | - |  | 104.5  | -  | -  |
| 14 | + | -  | -  |  | -  | -  | -  |  | - | - | - |  | -  | -  | -  |
| 21 | -  | -  | -  |   | -  | -  | -  |   | - | - | - |   | -  | -  | -  |

a + Virus was only detected in the undiluted fluid

b - No IBV was detected.

**List of figures**

**Figure 1.** Phylogenetic tree based on both the partial (grey circle) and full (black triangle) S1 nucleotide sequence among the IBV strains used in this study and reference strains. Analysis inferred using maximum likelihood analysis with Tamura 3-parameter and 1000 bootstrap replicates.

**Supplementary Figure 1. (A)** Sensitivity testing of partial S1 RT-PCR assay applied to 793B infected allantoic fluid. DNA marker (lane 1), serial dilutions of 793B infected allantoic fluid (lanes 2-9). **(B)** Sensitivity testing of full S1 RT-PCR assay applied to 793B infected allantoic fluid. DNA marker (lane 1), serial dilutions of 793B infected allantoic fluid (lanes 2-5).