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Real-time, MinION-based, amplicon sequencing for lineage typing of infectious bronchitis virus from upper respiratory samples

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Abstract. Infectious bronchitis (IB) causes significant economic losses in the global poultry industry. Control of IB is hindered by the genetic diversity of the causative agent, infectious bronchitis virus (IBV), which has led to the emergence of several serotypes that lack complete serologic cross-protection. Although serotyping requires immunologic characterization, genotyping is an efficient means to identify IBVs detected in samples. Sanger sequencing of the *S1* subunit of the spike gene is currently used to genotype IBV; however, the universal *S1* PCR was created to work from cultured IBV, and it is inefficient at detecting multiple viruses in a single sample. We describe herein a MinION-based, amplicon-based sequencing (AmpSeq) method that genetically categorized IBV from clinical samples, including samples with multiple IBVs. Total RNA was extracted from 15 tracheal scrapings and choanal cleft swab samples, randomly reverse transcribed, and PCR amplified using modified *S1*-targeted primers. Amplicons were barcoded to allow for pooling of samples, processed per manufacturer's instructions into a 1D MinION sequencing library, and then sequenced on the MinION. The AmpSeq method detected IBV in 13 of 14 IBV-positive samples. AmpSeq accurately detected and genotyped both IBV lineages in 3 of 5 samples containing 2 IBV lineages. Additionally, 1 sample contained 3 IBV lineages, and AmpSeq accurately detected 2 of the 3 lineages. Strain identification, including detection of different IBVs from the same lineage, was also possible with this AmpSeq method. Our results demonstrate the feasibility of using MinION-based AmpSeq for rapid and accurate identification and lineage typing of IBV from oral swab samples.

Key words: genotype; infectious bronchitis virus; MinION; nanopore sequencing; rapid sequencing; RNA.

Introduction

Infectious bronchitis (IB), which is caused by infectious bronchitis virus (IBV; *Avian coronavirus*), is one of the most important diseases of poultry, causing severe economic losses worldwide.[8] Clinical signs of disease are diverse and include respiratory distress, severe ocular discharge, poor body weight gain, decreased egg production, flushing (renal disease), and occasionally mortality in chickens.[7] IB is often complicated by secondary bacterial (e.g., *Escherichia coli*, *Mycoplasma* sp.) and viral infections (e.g., influenza A virus, Newcastle disease virus, infectious laryngotracheitis virus).[43] Lack of cross-protection among IBV serotypes is a challenge to controlling IB[15]; therefore, control of IB relies heavily on serotype-specific live attenuated vaccines.[8] Collectively, the presence of multiple IBVs in a single sample, emergence of variant IBVs, and high genetic diversity of IBV can complicate the diagnosis of IB and illustrate the need for enhanced testing.[15]

IBV is an enveloped, pleomorphic gammacoronavirus with an unsegmented, single-stranded, positive-sense, 26–27.8-kb, RNA genome that encodes the nonstructural polyproteins, 1a and 1b, and several structural proteins: spike (S), envelope (E), membrane (M), and nucleocapsid (N).[38,41] In addition, 2 accessory genes, expressing 3a, 3b and 5a, 5b, respectively, have also been described.[6,14,38] The S protein is highly glycosylated, and post-translational cleavage leads to 2 subunits: S1 and S2.[10,48] Besides acting as the viral attachment protein, the S1 protein is a major target of neutralizing antibodies.[7] As with many attachment proteins that are targets of virus-neutralizing antibodies, the S1 subunit is highly diverse with

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almost 50% of the amino acids differing among IBV serotypes.[2,21,38] Such variation leads to important biological differences between IBV serotypes and the emergence of novel variants. More than 60 serotypes of IBV have been reported, but the most common serotypes of IBV in North America are Arkansas, Connecticut (Conn), Delaware 072, Georgia 08 (GA08), Georgia 98 (GA98), and Massachusetts (Mass).[16] This genetic diversity leads to the emergence of new serotypes and a lack of complete cross-serotype protection by vaccines.[29] The correlation of IBV genotypes and serotypes of IBV has been reported[45]; therefore, accurate genotypic identification of IBV is an important step to identify IBV in clinical respiratory cases, ensure selection of proper IBV vaccines for use in vaccination programs, and to better understand the epidemiology of this global virus.

One comprehensive classification scheme for IBV uses *S1* gene sequence–based phylogeny of IBV, and identified 6 genotypes (I–VI), 32 subgenotypic lineages, and a number of inter-lineage recombinants in global strains of IBV.[44] Among the 6 genotypes, genotype I (GI) is the most diverse group of viruses, with 27 unique lineages.[44] As such, sequencing of the S1 subunit provides important information regarding the classification of IBV within samples.

The genetic classification of IBV has relied on genotype-specific, reverse-transcription real-time PCR (RT-rtPCR) assays, serotype-specific *S1* RT-rtPCR,[32,40] and/or pan-IBV *S1* RT-PCR assays coupled with Sanger sequencing.[8,31,49] Genotype-specific RT-rtPCR assays are limited to short fragments, which may miss important changes in the *S1* gene (~1.6 kb)[44] outside the short target. Additionally, given that the target is short, the primers lie within the variable regions of the *S1* gene and may require a different assay for each genotype. In contrast to the genotype-specific primers, the pan-IBV *S1* primers[1,17,22,23,25,33] only require one reaction to determine the presence of IBV; however, they have a relatively low sensitivity and are typically used only on egg-cultured virus, which adds an additional, time-consuming step, and many diagnostic laboratories do not have specific pathogen–free embryos readily available. Additionally, genotyping the pan-IBV results requires cloning of PCR products to detect multiple IBV subpopulations in the sample,[30] which is inefficient when potentially dealing with multiple (3 or 4) IBV types (i.e., "type" denoting genotype, lineage, and/or sublineage classification of an IBV). As such, the development of a pan-IBV sequencing method to rapidly determine the genotype(s) in samples would aid in lineage typing and tracking of IBV circulating in poultry flocks.

Third-generation sequencing technology has been used for the detection of viral nucleic acids and sequencing ultra-long DNA molecules.[34] The MinION nanopore sequencer (Oxford Nanopore Technologies [ONT], Oxford, UK), a new DNA sequencing technology that allows for rapid, in-house, real-time detection and differentiation of IBV lineages, may be cost-effective and useful in the field.[19] Amplicon-based sequencing (AmpSeq) has also been used to amplify specific regions of Newcastle disease,[3,13] infectious laryngotracheitis,[39] Zika,[34] Ebola,[35] and influenza A viruses,[46] by simple RT-PCR and then sequencing on the MinION device. Real-time data analysis, the lack of significant start-up cost investment or maintenance expenses, simultaneous and sequential multiplexing unique to MinION, and the ability to sequence long DNA molecules so that primers are in conserved regions while the product contains the variable region are the features that make the use of this technology highly feasible in disease diagnosis.

Accurate identification of IBV genotypes from samples, including the detection of multiple IBV types from a single sample, is crucial to respiratory disease diagnosis, selection of appropriate IBV vaccines, and epidemiologic studies. Therefore, our aim was to create a single, amplicon-based protocol to sequence the IBV *S1* gene and develop a sequence analysis workflow to identify IBV types from clinical swab samples. Our method provides a useful assay for IBV and a model for the development of future AmpSeq assays.

Materials and methods

Samples

Clinical swab samples (n = 15) were obtained from samples submitted to the Poultry Diagnostic & Research Center, University of Georgia (Athens, GA; Table 1). Trachea and choanal cleft palate swabs were collected from commercial chickens at 5 d of age (also corresponds to 5 d post spray vaccination in the hatchery with a commercial IBV GA08 vaccine and 5 d post placement in the chicken house). Briefly, the birds were swabbed with sterile polyester swabs (Puritan, Guilford, ME), and swabs were placed in 500 µL of phosphate-buffered saline without calcium and magnesium (cellgro; Corning, Manassas, VA). Swabs in tubes were stored on ice immediately following collection through delivery to the lab.

IBV RT-rtPCR assays

Total RNA was extracted from each of the swab samples (QIAamp viral RNA mini kit; Qiagen, Hilden, Germany) as per the manufacturer's instructions, aliquoted, and stored at −80°C until further use. These samples were tested previously for IBV and the GA08 serotype of IBV. Briefly, a pan-IBV reverse-transcription quantitative PCR (RT-qPCR) assay[4] was used to detect IBV in general, and then an IBV strain–specific RT-rtPCR assay (in-house validated set of primers) was used to detect GA08 in the samples (AgPath-ID one-step RT-PCR kit; Applied Biosystems 7500 Fast; Thermo Fisher Scientific, Waltham, MA).

MinION cDNA synthesis

For MinION complementary DNA (cDNA) synthesis, RNA was extracted from 500 µL of each clinical sample (Trizol-LS;

Table 1. Background information of clinical samples collected from broiler chickens.

Sample	Run	Study	Pan-IBV RT-qPCR (Cq)	GA08 (GI-L27)-specific RT-rtPCR (Ct)
1	1	Vaccine study-flock A	30.9	38.0
2	1	Vaccine study-flock A	24.3	29.4
3	1	Vaccine study-flock A	25.5	Neg
4	1	Vaccine study-flock A	27.0	Neg
5	1	Vaccine study-flock A	Neg	Neg
6	1	Vaccine study-flock B	NT	27.0
7	1	Vaccine study-flock B	NT	24.3
8	1	Vaccine study-flock B	NT	Neg
9	1	Vaccine study-flock B	NT	Neg
10	1	Vaccine study-flock B	NT	Neg
11	2	Respiratory disease	18.8	NT
12	2	Respiratory disease	19.1	NT
13	2	Respiratory disease	17.5	NT
14	2	Respiratory disease	18.1	NT
15	2	Respiratory disease	19.2	NT

Cq = quantification cycle; Ct = cycle threshold; Neg = negative; NT = not tested; RT-qPCR, RT-rtPCR = reverse-transcription quantitative and real-time PCR, respectively.

Thermo Fisher Scientific) per the manufacturer's directions. A reaction mixture of 8 µL of total RNA, 1 µL of random primers, and 1 µL of dNTPs was incubated at 65°C for 5 min, chilled on ice for at least 1 min, followed by addition of 10 µL of cDNA synthesis mix including SuperScript III reverse transcriptase (Thermo Fisher Scientific) according to the manufacturer's instructions. The reaction was incubated at 25°C for 10 min, then at 50°C for 50 min for cDNA synthesis. The reaction was terminated at 85°C for 5 min, and then chilled on ice. To remove residual RNA, the cDNA solution was incubated with RNase H at 37°C for 20 min.

MinION amplicon synthesis

A universal *S1* primer set,[27] tailed with the MinION universal adapter sequence of 22 nucleotides (underlined, Table 2) to allow barcoding of amplicons, was used for targeted amplification of the IBV *S1* gene for IBV. The PCR reaction mixture (Expand high fidelity PCR system; Roche Diagnostics, Mannheim, Germany) was composed of 10 µL of cDNA, 1 µL of 10 µM forward primer, 1 µL of 10 µM reverse primer, 2.5 µL of 10× Expand high fidelity buffer, 1 µL of Expand high fidelity enzyme mix, and 1 µL of 10 mM dNTPs, to a final volume to 25 µL with 9 µL of nuclease-free water. The following thermocycling conditions were used for amplicon synthesis: denaturation at 95°C for 2 min; 30 cycles of 94°C for 1 min, 45°C for 2 min, and 72°C for 2 min; and a final extension at 72°C for 10 min. PCR products were electrophoresed in 1.5% agarose with SYBR Safe DNA gel stain (Invitrogen, Carlsbad, CA) for visual evaluation. Amplified DNA was purified (Agencourt AMPure XP beads; Beckman Coulter, Brea, CA) at 1.6:1 (volumetric bead:DNA) and quantified (dsDNA high sensitivity assay kit; Qubit 3.0 fluorimeter; Biotium, Fremont, CA).

Library preparation and MinION sequencing

The amplicons obtained from the tailed *S1* primer set were then used to prepare MinION-compatible DNA libraries. Briefly, each of the amplicons was diluted to 0.5 nM and amplified using barcoding primers (1D PCR barcoding amplicon kit; ONT) and LongAmp Taq 2× master mix (New England Biolabs, Ipswich, MA) with the following conditions: 95°C for 3 min; 15 cycles of 95°C for 15 s, 62°C for 15 s, and 65°C for 80 s; and a final extension at 65°C for 80 s. The barcoded amplicons were bead purified (1:1.4, bead:solution), pooled (run 1: pool of 10 samples; run 2: pool of 5 samples) into a single tube, end prepped (NEBNext Ultra end repair/dA-tailing module, New England Biolabs), bead purified (1:1, bead:solution), and ligated to the sequencing adapters (Ligation sequencing kit 1D, catalog SQK-LSK108; ONT), all per ONT directions. Final DNA libraries were bead purified (0.4:1, bead:solution), eluted in 15 µL of elution buffer, and sequenced with the MinION sequencer (ONT). A new flow cell (FLO-MIN106 R9.4; ONT), stored at 4°C prior to use, was allowed to equilibrate to room temperature for 10 min and then primed with running buffer as per the manufacturer's instructions. The pooled DNA libraries were prepared by combining 12 µL of the library pool with 2.5 µL of nuclease-free water, 35 µL of running buffer with fuel, and 25.5 µL of library loading beads. After the MinION platform quality-control run, the DNA library was loaded into the MinION flow cell via the SpotON port. The standard 48-h 1D sequencing protocol was initiated using the MinKNOW software v.5.12. For a more rapid run, run 1 sequencing was allowed to continue for 2 h until 42,940 reads were obtained. For a deeper run, run 2 was allowed to continue for ~6 h until ~156,000 reads were obtained.

Table 2. Details of PCR primer sets used to detect infectious bronchitis virus (IBV) in samples.

Primer name	Primer sequence	Target gene	Sequencing	Amplicon length (bp)	Targeted genotype and lineage
IBV-Universal S1[4]					
IBV-S1-adap Fwd	5′-TTTCTGTTGGTGCTGATATTGCTGAACCTGAACAAAAGAC-3′	*S1*	Nanopore	~1,672	All genotypes and lineages
IBV-S1-adap Rev	5′-ACTTGCCTGTCGCTCTATCTTCCCATAAGTAACATAAGGRCRA-3′				
IBV-Conn					
IBV_FJ904716.1Conn_A_F_20473	5′-AGACCACCACCTAATGGTTGGCA-3′	*S1*	Sanger	378	GI-L1 lineage
IBV_FJ904716.1Conn_A_R_20889	5′-AGAGGTGTAAACAAGATCACCA-3′				
IBV GA98					
IBV-GA98 _F101	5′-GGCCTCCTAATGGATGGCATA-3′	*S1*	Sanger	305	GIV-L1 lineage
IBV_GA98_R101	5′-TAATGACTGGCAGCGCTAAG-3′				

The underlined sequences are Oxford Nanopore Technology adapter sequences for multiplexing samples in a single sequencing run.

Building customized BLAST databases for AmpSeq analysis

First, a lineage-typing database, containing 32 IBV *S1* gene sequences (1 sequence from each of 32 lineages in the 6 genotypes)[44] and the chicken genome (GCF_000002315.4_Gallus_gallus-5.0), was constructed (hereafter, IBV-lineage-typing database). A second database was constructed with all of the avian coronavirus *S1* sequences (n = 7,328) available in NCBI (as of 2017.09.08) and the chicken genome (hereafter, All-IBV database). Prior to database construction, all sequences were dustmasked (NCBI C++ ToolKit, https://ncbi.github.io/cxx-toolkit/), and each IBV sequence was assigned a unique taxonomy ID as a species hierarchically under the genus *Gammacoronavirus* to allow easy sorting. The local BLAST (https://blast.ncbi.nlm.nih.gov/) databases were compiled using default settings. The IBV-lineage-typing database was used to assign IBV lineages to each read, as appropriate. The All-IBV database was used to assign a taxonomic ID to each of the IBV reads from within IBV lineage read clusters. The IBV-lineage-typing database, which contains only one sequence per lineage, is required because Centrifuge (computational tool for taxonomic classification of individual reads) divides the score for any given read by the number of hits that have an equal score. Given that some lineages are overrepresented in GenBank (e.g., Mass strains), this results in the scores of those reads (e.g., Mass reads) being divided by a large number, effectively reducing the score for any single alignment. However, because there is only one read per lineage in the IBV-lineage-typing database, it has insufficient diversity to determine if there is more than one strain of the same lineage present in a sample. Thus, the All-IBV database provides the diversity required for that analysis.

AmpSeq IBV lineage and type identification

A schematic diagram of the workflow of MinION data analysis is presented in Figure 1. For nanopore sequencing data, pre-processing steps were performed to prepare data for downstream analysis. Briefly, nanopore reads (FAST5) were basecalled using Albacore v.2.02 (https://github.com/Albacore/albacore) with the following parameters (read_fast5_basecaller.py -i /Input_reads_fast5/ –recursive -t 4 -s /Output_files –flowcell FLO-MIN107 –kit SQK-LSK108 -o fastq). Porechop (https://github.com/rrwick/Porechop) was used for adapter-trimming (default setting), barcode-based demultiplexing (default settings), and to trim an additional 21 nucleotides representing the *S1* primer sequences (porechop -i Input_file.fastq –extra_end_trim 21 -b ./output_demultiplexed/). After barcode and adapter removal, reads were analyzed with a script-based, 2-step data analysis protocol, which includes centrifuge-kreport as taxonomic read classifier[24] using the sequences in the above-mentioned BLAST databases. Briefly, basecalled reads (FASTQ) from individual barcoded samples were used as an input. First, the basecalled reads were aligned to the IBV-lineage-typing database using BLASTn and reads were clustered based on the read sequence alignment to the respective prototype sequence of IBV lineage. These read clusters were used to interpret the presence of IBV genotypes and lineages in the samples. For the identification of IBV types, the lineage-based read clusters were individually aligned to the All-IBV database, which produced subclusters of reads. Each of these read subclusters potentially represents a different sequence of IBV and was further used for interpretation. Knowing that MinION sequencing has a high sequencing error rate in individual reads, further steps were added in the data analysis algorithm to obtain a more accurate consensus sequence. Therefore, these read subclusters were mapped, using Geneious mapper in Geneious software v.11.1.3. (Biomatters, Auckland, New Zealand), to the IBV-lineage-typing database (a reference FASTA file) to obtain consensus sequences from each subcluster. A minimum threshold for the number of reads per consensus sequence was not set because in 2 samples (sample 4 in run 1 and sample 12 in run 2), only 1 and 5 IBV reads were obtained, respectively; therefore, consensus sequences were built from all of the available IBV reads per subcluster. The consensus sequences

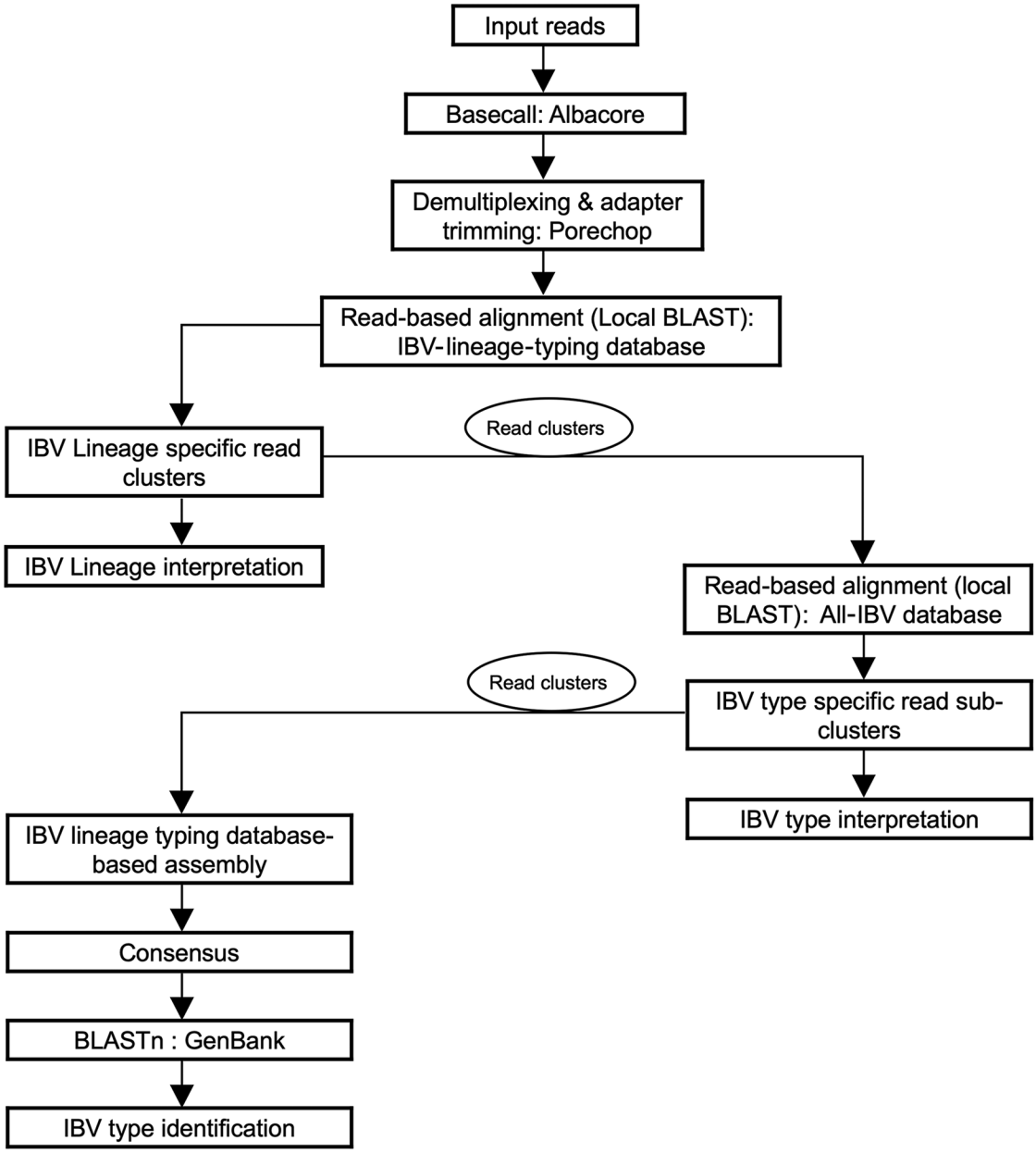

Figure 1. A schematic diagram of the workflow of MinION data analysis.

were compared to GenBank using BLASTn. To select the "top hit" from BLASTn output, sequence search results were ordered by "sequence identity" and then sequence alignments were evaluated for "minimum mismatches" and "coverage" of query or subject sequence. For each sequence, the BLASTn output with the highest query or subject coverage and the fewest mismatches was used as "top hit" in the final results.

RT-PCR coupled with Sanger sequencing for lineage analysis

For Sanger sequencing amplicon synthesis, cDNA from the MinION library preparation (as described above) was amplified using the following primer sets (Table 2). First, a primer set based on Connecticut sequence NCBI FJ904716.1 was used to amplify genotype I–lineage 1 (GI-L1) viruses (e.g., Conn and Mass serotypes). Second, a primer set based on GA98 sequence (NCBI AF274439.1) was used to amplify genotype IV–lineage 1 (GIV-L1) viruses (e.g., Georgia 1998 and Delaware 072 serotypes). Primer sets were designed using NCBI Primer-BLAST.[51] The PCR reaction mixture (Expand high fidelity PCR system; Roche Diagnostics) is composed of 10 µL of cDNA, 0.5 µL of 10 µM forward primer, 0.5 µL of 10 µM reverse primer, 2.5 µL of 10× buffer, 0.5 µL of enzyme mix, and 0.5 µL of 10 mM dNTPs, then made to the final volume to 25 µL with nuclease-free water. The following

thermocycling conditions were used for amplicon synthesis: denaturation at 95°C for 1 min; 35 cycles of 94°C for 30 s, 55°C (ConnA primer set) or 50°C (GA98 primer set) for 30 s, and 72°C for 45 s; and a final extension of 72°C for 5 min. PCR products were visually inspected after electrophoresis in 1.5% agarose gel, and the correctly sized bands were cut out for DNA purification (Qiagen PCR purification kit) and quantified (dsDNA high sensitivity assay kit; Qubit 3.0 fluorimeter; Biotium). Briefly, purified amplicons were inserted into plasmids, and ligation reactions (10 µL) were set up as per the manufacturer's instructions (pGEM-T Easy vector system; Promega). After ligation, 3 µL of ligation mixture was transformed to JM109 competent cells by heat shock. Individual bacterial colonies were checked with PCR, and the positive bacterial colonies were plated on lysogeny broth agar–ampicillin plates at 37°C for 16 h. The plasmids were extracted from these positive bacterial colonies (QIAprep spin miniprep kit; Qiagen) and submitted for bidirectional, commercial (Genewiz, South Plainfield, NJ) Sanger sequencing.

Sanger sequencing analysis

For Sanger sequencing data, the chromatogram from each sample was manually checked, and primer sequences were trimmed in MEGA 6.0.[42] Forward and reverse sequences from multiple clones were aligned using MEGA 6.0 software, and consensus sequences were compared to GenBank using BLAST (as of 2018.03.12) and the top hit from the BLASTn output was selected, as described above for MinION sequencing data, to identify the IBV type in samples.

Sanger and MinION sequence pairwise identity

For each of the samples in run 1, a pairwise nucleotide identity comparison between the IBV partial *S1* gene sequences obtained from Sanger sequencing and MinION sequencing was performed. Briefly, the final AmpSeq consensus sequences from identical IBV types in each of the samples were aligned using ClustalW in MEGA6,[42] and this alignment was used for pairwise nucleotide identity using MEGA6.[42]

Results

RT-rtPCR assays

Five samples from run 1 had been tested previously with a pan-IBV RT-qPCR assay,[4] and 4 were positive for IBV. Samples with pathogenic IBV strains (run 2, $n = 5$) were tested with the pan-IBV RT-qPCR, and all 5 samples were IBV positive (Table 1). Additionally, samples were also tested previously with a GA08-specific RT-rtPCR (in-house validated set of primers used in the Poultry Diagnostic & Research Center), which was used on all samples from run 1 (including 5 samples that were tested previously by the pan-IBV RT-qPCR), and 4 of 10 samples were positive. Samples 3 and 4, which were positive with the pan-IBV RT-qPCR assay, were negative with the GA08-specific RT-rtPCR assay, indicating that these samples contained IBV serotypes other than GA08 and required further testing. Sample 5 tested negative with both assays (Table 1).

MinION sequencing and lineage identification (run 1)

PCR amplicons obtained directly from 10 clinical swab samples with vaccine IBV serotypes were barcoded, pooled, and sequenced on the MinION device (run 1). A total of 38,661 reads were successfully basecalled from the entire sequencing run (42,940 total reads). After demultiplexing, reads per barcode ranged from 831 to 4,114. A total of 14,845 reads were not assigned to any of the used barcodes, and 128 reads were discarded because of middle adapters. The nanopore reads were queried against the IBV-lineage-typing database to determine if the samples contained IBV (Fig. 1). This AmpSeq protocol detected IBV reads in 8 of 10 samples. The number of IBV reads per sample (IBV-positive sample) ranged from 56 to 944. The MinION-negative samples included sample 5, which was consistently negative with RT-rtPCR and RT-PCR assays (and thus interpreted as IBV negative), and sample 1, which had the highest cycle threshold (Ct) values in the pan-IBV RT-qPCR (Table 1).

Sequencing data were further analyzed to determine if multiple IBV genotypes or lineages could be detected. Lineage 1 and lineage 27 from genotype I (GI-L1, GI-L27), and lineage 1 from genotype IV (GIV-L1), were the detected lineages in the samples. Results from the individual RT-rtPCR assay for GA08 (GI-L27), and RT-PCR assays for GIV-L1 and GI-L1, confirmed the presence of multiple IBV genotypes and lineages in 6 of the 9 IBV-positive samples (1–4, 6, 7); AmpSeq results confirmed multiple genotypes and lineages in 4 (2, 4, 6, 7) of those 6 samples (AmpSeq failed to detect any IBV in 1, and failed to detect GIV-L1 in 3). The presence of a single lineage was confirmed by PCR-based assays and AmpSeq in 3 samples (8–10; Table 3).

MinION consensus sequence evaluation for identification of IBV

The single-read cluster composed of <5 reads (run 1, sample 4, GA98) yielded a poor-quality consensus sequence, consistent with the known individual read error rate of MinION sequencing. High-quality consensus sequences were obtained from the other read clusters (>5 reads per cluster; Supplementary Table 2). Each of the obtained consensus sequences was compared to GenBank sequences using BLAST, which revealed >99% sequence identity to respective IBV sequences (Table 4). As described in the Sanger sequencing section, 2

Table 3. Detection of different lineages of infectious bronchitis virus in tracheal swab samples using RT-rtPCR, RT-PCR, and MinION sequencing.

	Lineage IDs*		Lineage totals	
Sample	RT-rtPCR or PCR with Sanger	MinION AmpSeq	All 4 RT-PCR assays†	MinION AmpSeq
1	GI-L27 | GIV-L1	Neg	2	0
2	GI-L27 | GIV-L1 | GI-L1	GI-L27 | GI-L1	3	2
3	GIV-L1 | GI-L1	GI-L1	2	1
4	GIV-L1 | GI-L1	GIV-L1 | GI-L1	2	2
5	Neg	Neg	0	0
6	GI-L27 | GIV-L1	GI-L27 | GIV-L1	2	2
7	GI-L27 | GIV-L1	GI-L27 | GIV-L1	2	2
8	GIV-L1	GIV-L1	1	1
9	GIV-L1	GIV-L1	1	1
10	GIV-L1	GIV-L1	1	1

RT-rtPCR = reverse-transcription real-time PCR.
* G-L = genotype and lineage (e.g., genotype I–lineage 27); Neg = negative.
†Pan-IBV RT-qPCR, GA08 serotype–specific RT-rtPCR, GA98 RT-PCR, and Conn RT-PCR.

samples (8 and 9) contained only 1 IBV type per the non-MinION assays, and the AmpSeq results were consistent with these findings. Non-MinION assays showed that 7 samples contained 2 or more IBVs per sample. Six samples (1, 3, 4, 6, 7, 10) contained 2 IBV types per sample (Table 4; Supplementary Tables 1, 2). Of those 6 samples, AmpSeq detected both IBV types in 4 samples (4, 6, 7, 10), 1 IBV type in 1 sample (3), and no IBV in 1 sample (1; as mentioned above, sample 1 was the sample with the highest Ct value). Of note, sample 10 contained 2 IBV types from the same lineage and AmpSeq was able to identify both types within this sample. Finally, the seventh multi-type sample (2) contained 4 IBV types per the non-MinION results, and the AmpSeq detected 2 of those 4 types. These data show that a coinfection of multiple IBV lineages existed in the above-mentioned samples, but a single RT-rtPCR, or Sanger sequencing of a single clone, may not have detected these coinfecting IBV lineages and multiple assays were required to detect all the IBV types. However, the AmpSeq protocol accurately detected multiple IBV lineages in 4 of 7 samples, with partial detection in 2 of the remaining 3 samples (Table 4). In samples that had the same IBV type, BLAST search of consensus sequences obtained from AmpSeq and Sanger sequencing identified the same (or highly related IBV type for samples 6 [GA98] and 9) in the NCBI nt database as per parameters described in the Methods section.

MinION sequencing and lineage identification (run 2)

To evaluate the utility of this protocol on clinical swab samples with pathogenic IBV variants, the IBV *S1* gene was amplified directly from 5 clinical tracheal scrapings, and the PCR amplicons were used to create MinION libraries. A total of 146,540 reads were successfully basecalled from the entire sequencing run (156,000 total reads). After demultiplexing, reads per barcode ranged from 4,285 to 41,131. A total of 24,297 reads were not assigned to any of the used barcodes, and 8,109 reads were discarded because of middle adapters in the basecalled reads. Real-time analysis of MinION data, which was obtained within 10 min of the sequencing, was sufficient for the detection of IBV. However, sequencing data obtained from the entire sequencing run was processed with the same protocol as described above. This AmpSeq protocol detected IBV reads in all of the 5 tested samples using all basecalled reads. The number of IBV reads per sample were 5–4,956. Additionally, the sequencing data analysis showed that the IBV reads belonged to GI-L17 and typed the detected IBV variant as DMV1639. After MinION sequencing results, these samples were later tested to confirm the presence of IBV variant by MDL_DMV1639 IBV variant-specific RT-rtPCR assay. All 5 samples were positive for the IBV MDL_DMV1639 variant of IBV (Table 5, Supplementary Table 2).

Non-MinION lineage-based and type-based analyses

Ten clinical swab samples (run 1) were processed for RT-PCR and Sanger sequencing to confirm the presence of IBV lineages. Based on the MinION results, primers targeting GIV-L1 and GI-L1 (GA98 and Conn primer sets, respectively) were created (Table 3). Using GIV-L1–specific primers showed that 9 of 10 samples were positive, with 1 sample (5) negative for IBV (which is consistent with the pan-IBV RT-qPCR results for this sample). The PCR products were cloned, and multiple (6–24) clones from each sample were submitted for Sanger sequencing and type-based analysis (Table 4). The consensus sequences from multiple clones obtained by using GIV-L1–specific primers showed the

Table 4. Consensus-based identification of infectious bronchitis virus types in clinical swab samples.

	MinION AmpSeq (full *S1*)			PCR with Sanger (partial *S1*)		AmpSeq vs. Sanger
Sample	Consensus (bp)	Top hit in BLASTn search	Similarity (%)	Top hit in BLASTn search	Similarity (%)	Pairwise identity (%)
1	NA	NA	NA	DE072 vaccine (AF274435.1)	100	NA
	NA	NA	NA	No BLAST results, tested (and positive) by RT-rtPCR only (see Table 3)	NA	NA
2	1,628	Georgia 08 (GU301925.1)	99.7	No BLAST results, tested (and positive) by RT-rtPCR only (see Table 3)	NA	NA
	1,620	Conn/B6dpv contact (EU283059.1)†	99.6	Conn/B6dpvcontact (EU283059.1)†	99.8	100
	NA	NA	NA	DE072 vaccine (AF274435.1)	100	NA
	NA	NA	NA	GA/A9dpvvaccinated (EU283069.1)	100	NA
3	1,634	PDRC_110177 (KX529703.1)†	99.9	PDRC_110177 (KX529703.1)†	99.5	100
	NA	NA	NA	GA/A9dpvvaccinated (EU283069.1)	98.5	NA
4	1,639*	GA/A9dpvvaccinated (EU283069.1)†	92.1	GA/A9dpvvaccinated (EU283069.1)†	98.0	96.1
	1,621	Conn/B6dpvcontact (EU283059.1)†	99.8	Conn/B6dpv contact (EU283059.1)†	100	100
5	NA	NA	NA	NA	NA	NA
6	1,626	Georgia 08 (GU301925.1)	99.7	No BLAST results, tested (and positive) by RT-rtPCR only (see Table 3)	NA	NA
	1,623	GA/5416/99 (AF274440.1)	99.5	GA/A9dpvvaccinated (EU283069.1)	99.6	99.5
7	1,622	Georgia 08 (GU301925.1)	99.5	No BLAST results, tested (and positive) by RT-rtPCR only (see Table 3)	NA	NA
	1,623	GA/A9dpvvaccinated (EU283069.1)†	99.5	GA/A9dpvvaccinated (EU283069.1)†	99.0	99.5
8	1,625	GA/A9dpvvaccinated (EU283069.1)†	99.6	GA/A9dpvvaccinated (EU283069.1)†	99.0	100
9	1,627	DE072 vaccine (AF274435.1)†	99.9	DE072 vaccine (AF274435.1)†	98.7	100
10	1,625	GA/5416/99 (AF274440.1)	99.6	GA/A9dpvvaccinated (EU283069.1)	98.6	99.5
	1,625	DE072 vaccine (AF274435.1)†	99.9	DE072 vaccine (AF274435.1)†	98.6	99.0

*This read had high score from Centrifuge and hit only to GA98. The top 5 BLAST hits of the read were all GA98 viruses with similarity of 91–92%. The top hit of this single read was to EU283068.1 and was interpreted as correct identification.
†BLAST search had multiple (2–13) sequence alignments with the same number of mismatches and gaps with 100% coverage of the query or subject sequence. The accession numbers provided in this table are one of those top alignments.

presence of GA98 (top BLAST hit: "GA/A9dpvaccinated" [EU283069.1]) in 7 of 9 IBV-positive samples. DE072 was detected in samples 1 and 9 as the lone GIV-L1, but was also detected in 2 samples (2, 10) that also contained GA98 (Table 4); thus, these samples had 3 and 1 lineages, respectively (Table 3), but 4 and 2 IBV types, respectively (Table 4). By using the GI-L1–specific primer set, which amplified the Conn and Mass type of IBVs, 3 of 9 IBV-positive samples were positive (2 and 4 for Conn; 3 for Mass [PDRC_110177]; Table 4). Combining the lineage-based (RT-rtPCR and RT-PCR) and type-based (RT-PCR coupled with Sanger sequencing) analyses (Table 3, 4, respectively), only samples 8 and 9 contained a single IBV type; the other 7 samples were positive for 2–4 (e.g., 2 has four, and 4 has two) types of IBV (Tables 3, 4).

Pairwise sequence identity between Sanger and MinION consensus sequences

In all of the samples (run 1) in which a matching IBV type was identified by both AmpSeq and Sanger sequencing, the AmpSeq data showed high concordance with Sanger sequencing data. In one sample (4), only a single MinION read of GA98 was detected by AmpSeq and had only 96.1%

Table 5. Detection of infection bronchitis virus genotype and lineage using RT-rtPCR and MinION AmpSeq in tracheal swab samples (run 2).

Sample	Reads per consensus	Consensus (bp)	Top hit in BLASTn search	Accession	Similarity (%)	MinION genotype and lineage	DMV-1639 RT-rtPCR (Ct)
11	45	1,600	MDL_DMV1639_15-1103	KX529739.1	92.1	GI-L17	33.2
12	5	1,561	MDL_DMV1639_15-1328	KX529734.1	87.0	GI-L17	20.6
13	150	1,607	MDL_DMV1639_15-1328	KX529734.1	97.6	GI-L17	19.8
14	1,219	1,605	MDL_DMV1639_15-1328	KX529734.1	97.8	GI-L17	19.6
15	4,956	1,605	MDL_DMV1639_15-1328	KX529734.1	97.3	GI-L17	18.9

RT-rtPCR = reverse-transcription real-time PCR.

similarity to the respective, shorter sequence from Sanger sequencing, consistent with the reported single-read accuracy of MinION sequencing. All other samples had 99–100% pairwise identity across the partial *S1* fragment generated by Sanger sequencing (Table 4).

Discussion

The accurate detection of IBV as the cause of clinical respiratory disease is contingent on virus typing and differentiating live vaccine viruses from field strains.[11] It has been reported that IBV genotypes are well correlated with the serotypes of IBV[45]; therefore, accurate genotypic identification of IBV will be useful to identify vaccine and variant viruses in clinical samples. Rapid pan-IBV RT-qPCR and serotype-specific RT-rtPCR assays[4,23,36] have been used for serotype identification; however, positive results from RT-rtPCR is insufficient to determine the IBV genotype; thus, sequence analysis of the IBV *S1* gene is required. Use of partial *S1* gene sequences (450 bp) to type IBV is described.[29] However, increasing the length of sequenced *S1* gene (~1,620 bp) results in more data to be used for genotyping because more of the hypervariable region is covered.[17,27,45] For example, the AmpSeq protocol that we used was able to differentiate 2 genetically very similar (99.5% at *S1* gene of IBV genome) but serotypically different GIV-L1 IBVs, DE072 and GA98, within a single sample. Thus, the detection of highly diverse IBV genotypes, via the *S1* subunit, with a single and rapid sequencing protocol is desirable and the ability to detect multiple types of IBV in a single sample could improve IBV detection in clinical samples.

Currently, pan-IBV RT-qPCR is used to rapidly detect IBV from clinical samples for screening purposes,[4] but this requires additional serotype-specific RT-rtPCR assays to genotype positive samples, including samples containing more than one type of IBV. Additionally, if unidentified IBVs are in the sample, then current detection and characterization may also require egg culture followed by various PCR assays.[8] Our AmpSeq method detected IBV in 13 of 14 IBV-positive samples and detected all of the mixed IBVs in 4 of 7 samples containing 2 or more IBVs. Although mixed IBVs were not 100% identified (0 of 2 in sample 1; 2 of 4 in sample 2; and 1 of 2 in sample 3) in 3 samples with our AmpSeq method, detection of those mixed IBVs required several non-AmpSeq assays. Although there is room for improving our new AmpSeq method, it represents a promising, single-step assay that can be used without egg culture. Thus, it is another tool for the detection of IBV, especially in cases in which multiple IBVs may be present or when genotyping is especially important.

One area that is problematic for a test such as AmpSeq is the detection of all genotypes of IBV that may be present. Although AmpSeq did detect all genotypes in 4 samples, in 3 cases not all of the genotypes of IBVs were detected. A potential explanation of partial detection of multiple IBV genotypes could be the relative abundance of IBV genotypes in these clinical samples. It could also be that amplification of these IBV genotypes by serotype-specific RT-rtPCR assays is more efficient given the small targeted fragment size (e.g., 120 bp for PCR and ~1,600 bp for AmpSeq) and better primer alignment to the target (e.g., degenerate bases are used in the *S1* primers used for AmpSeq). One complicating factor of our current AmpSeq protocol is that the IBV target sequence[27] was not originally designed for high specificity, especially from clinical samples. As such, a high proportion of total reads were non-IBV reads (e.g., often mapping to the chicken genome, data not shown), consistent with the extra bands visible in the original report for these target sequences.[28] Future studies to reduce the proportion of chicken reads may increase the sensitivity of this assay. Additionally, it is possible that certain genotypes are better complemented to the *S1* primers than others and may outcompete those IBV genotypes for amplification in the AmpSeq protocol. Also, increasing the total number of reads collected by AmpSeq may improve the ability to detect all of the genotypes in a given sample. It is possible to allow the sequencing to continue longer to obtain more reads per sample. Overall, AmpSeq is a feasible test for IBV characterization, and work is ongoing to improve this new type of assay.

Cost and time efficiency of a sequencing protocol can be improved through the multiplexing of more samples in a single sequencing run.[3,47] In our study, samples (run 1 = 10, run 2 = 5) were simultaneously multiplexed (i.e., pooled and then sequenced in one run) while maintaining IBV genotyping from data collected. Given that the MinION flow cells were not exhausted, and can be washed and re-used, the

AmpSeq method also has the potential for sequential multiplexing. This would decrease the need to hold samples for weeks while waiting for the cost-optimal number of samples for simultaneous multiplexing. The single protocol nature of AmpSeq, the ability to obtain *S1* gene sequence results, real-time data analysis, and flexibility of testing design makes MinION-based AmpSeq a viable sequencing protocol for genotyping and lineage typing of IBV.

The advent of real-time, in-house, third-generation sequencing represents a transformative opportunity for diagnostic laboratories by offering the ability to more fully characterize PCR reactions beyond confirming amplicon size (e.g., routine electrophoresis), Sanger sequencing RT-PCR products, or by confirming a partial sequence through probe hybridization (e.g., probe-based RT-rtPCR). However, the interpretation of such large data sets represents a challenge to veterinary diagnosticians. Read-based classification software such as Centrifuge,[24] Kraken,[50] QIIME,[5] and Mothur[37] have been used to identify and profile microbial species; however, the high error rate[20] of nanopore reads translates to poor classification accuracy for many of these tools. Alternatively, de novo[3,26] or reference-based[18] assembly methods have been used in MinION and other deep-sequencing platforms. Using a strategy similar to other unbiased laboratory tests (e.g., standard bacterial cultures), an approach was developed to maximize usage of reads (i.e., reads are not discarded based on pre-set length or abundance requirements, similar to how a single colony may be interpreted as a significant result). This approach uses read-based classification against a database containing an equal number of representative IBV sequences per lineage to detect and classify the IBV reads based on their lineage, before conducting read-based classification of IBV reads by using all available IBV sequences in a separate database. Finally, lineage-clustered IBV read assignments are interpreted by a veterinary diagnostician to result in reads available for consensus building. The use of a final consensus alignment helps to overcome the individual error rate of MinION sequencing.[3] Similarly, the absence of predetermined metrics used in de novo assembly allows for the informed decision as to how many consensus sequences to build, a bioinformatics problem when dealing with clinical cases that can contain more than one type of IBV (e.g., similar to how there is not a predetermined number of significant bacterial colonies). Confirmatory follow-up tests (e.g., RT-PCR) may be needed when dealing with low numbers of reads in clinical samples; however, future testing of this new technology will allow for creating standards for such confirmatory testing.

Our results suggest that the application of MinION-based AmpSeq, specifically for detecting IBV genotypes and lineages from clinical samples within a few days, compared favorably to several days to weeks and multiple detection assays to culture and detect multiple IBV genotypes from a single sample. Thus, MinION-based AmpSeq coupled with data analysis workflow for identification, differentiation, and accurate prediction of IBV genotypes from clinical swab samples can be used as an adjunct to other established rapid detection assays[9,12] until extensive testing of this protocol is done to improve and validate AmpSeq for IBV identification. Furthermore, AmpSeq-based assays can be and are being applied to other viral pathogens,[3,37] demonstrating the power and utility of this method.

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Supplementary material

Supplementary material for this article is available online.

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