Multiple novel non-canonically transcribed sub-genomic mRNAs produced by avian coronavirus infectious bronchitis virus

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Abstract
Coronavirus sub-genomic mRNA (sgmRNA) synthesis occurs via a process of discontinuous transcription involving complementary transcription regulatory sequences (TRSs), one (TRS-L) encompassing the leader sequence of the 5′ untranslated region (UTR), and the other upstream of each structural and accessory gene (TRS-B). Several coronaviruses have an ORF located between the N gene and the 3′-UTR, an area previously thought to be non-coding in the Gammacoronavirus infectious bronchitis virus (IBV) due to a lack of a canonical TRS-B. Here, we identify a non-canonical TRS-B allowing for a novel sgmRNA relating to this ORF to be produced in several strains of IBV: Beaudette, CR88, H120, D1466, Italy-02 and QX. Interestingly, the potential protein produced by this ORF is prematurely truncated in the Beaudette strain. A single nucleotide deletion was made in the Beaudette strain allowing for the generation of a recombinant IBV (rIBV) that had the potential to express a full-length protein. Assessment of this rIBV in vitro demonstrated that restoration of the full-length potential protein had no effect on viral replication. Further assessment of the Beaudette-derived RNA identified a second non-canonically transcribed sgmRNA located within gene 2. Deep sequencing analysis of allantoic fluid from Beaudette-infected embryonated eggs confirmed the presence of both the newly identified non-canonically transcribed sgmRNAs and highlighted the potential for further yet unidentified sgmRNAs. This HiSeq data, alongside the confirmation of non-canonically transcribed sgmRNAs, indicates the potential of the coronavirus genome to encode a larger repertoire of genes than has currently been identified.

INTRODUCTION
Coronaviruses are members of the order Nidovirales, and possess the largest genomes of the currently identified RNA viruses. There are four genera, Alpha-, Beta-, Gamma- and Deltacoronavirus, all of which contain coronaviruses that pose a significant threat to human health, animal health and welfare, as well as food security. Notable members of the genus Betacoronavirus include the human pathogens severe acute respiratory syndrome coronavirus (SARS-CoV) and Middle East respiratory syndrome coronavirus (MERS-CoV), and the recent zoonotic virus SARS-CoV-2, the causative agent of COVID-19. Infectious bronchitis virus (IBV), the prototype of the genus Gammacoronavirus, is the aetiological agent of infectious bronchitis; an acute, highly contagious and economically important disease of poultry (reviewed elsewhere [1, 2]). As with all members of the family Coronaviridae, IBV possesses a large, ~27 kb, non-segmented, positive-sense, ssRNA genome, which is capped at the 5′ end and polyadenylated at the 3′ end [3]. General genome organization is shared between all coronaviruses, with the 5′
Transmission of the structural and accessory genes occurs via a process of discontinuous transcription during negative-strand synthesis; a model unique to the members of the order *Nidovirales*, and first proposed by Sawicki and Sawicki in 1995 [8]. The model relies on the presence of two complementary TRSs, one located at the 5′ end of the genome, in the leader sequence, part of the 5′-UTR (TRS-L), and the second upstream of the gene, within the body of the genome (TRS-B). During minus-strand sgRNA synthesis, each TRS-B may act as a stop signal for the transcription-replication complex and facilitate a template switch through hybridization to the TRS-L. The result of this process is a nested set of negative-sense sgRNAs that act as templates for the generation of positive-sense sgRNAs with co-terminal 5′ and 3′ ends. Each sgRNA contains a common 5′ leader sequence and terminal 3′ sequence. With the exception of the smallest, all sgRNAs are structurally polycistronic and possess the genetic information for several proteins. In the majority of instances, only the 5′ most proximal ORF is translated and, therefore, coronavirus sgRNAs are functionally monocistronic, although some virus- and gene-specific exceptions do occur. In IBV, for example, gene 3 encodes proteins 3a, 3b and 3c (E), with 3a and 3b translated by leaky ribosomal scanning and 3c translated using an internal ribosomal initiation site [9].

The process of discontinuous transcription is controlled by interactions of both the TRS-L and the TRS-B. The presence of a TRS-B upstream of each structural and accessory gene is essential for the transcription of the relevant sgRNA. The sequence of the TRSs is largely conserved across the coronavirus family and such is possible to use a bioinformatics approach to screen full genomic sequences to identify possible sgRNAs and subsequent ORFs. For IBV, the proposed consensus TRS is CUUAACAA; however, there are naturally occurring variations of the TRS-B sequence suggesting flexibility and that an exact consensus sequence is not an absolute requirement for the TRS-L and TRS-B to perfectly match. For example, for the apathogenic strain Beaudette and the pathogenic strain M41, the TRS-B for the S gene is CUGAAACAA. In addition, gene 5 of the M41 strain is preceded by a TRS-B of CUUAAUAA, and gene 3 of the Beaudette strain utilizes the same sequence as the S gene. Brown et al. have also identified variations in putative TRS-Bs of turkey coronavirus (TCoV) [10]. The matter is further complicated by the presence of functional non-canonical TRS-Bs. Bentley et al. identified the presence of a sgRNA, relating to gene 4b, which is transcribed from a shortened non-canonical TRS-B of only 3 nucleotides, CAA [6]. This shortened TRS-B was found to hybridize to nucleotide positions 6, 7 and 8 of the TRS-L, with the remaining TRS in the sgRNA derived from TRS-L. The possibility of IBV generating such transcripts was first suggested by Stern and Kennedy in 1980 [11]. Such transcripts are not unique to IBV, and have previously been described in other coronaviruses including SARS-CoV and mouse hepatitis virus (MHV) [12, 13].

Two recent publications have described the detection of a non-canonicaly transcribed sgRNA in the IBV strain Beaudette, located at the very 3′ terminus of the genome, between the N gene and the poly(A) tail [7, 14]. Additionally, Dinan et al. identified ribosomes bound to the transcript suggesting protein translation [7]. The resulting prospective protein in the Beaudette strain, however, is prematurely truncated due to a single point mutation that results in a premature stop codon. In the present study, we similarly describe detection of a non-canonically transcribed sgRNA correlating to the 3′ extremity of the genome across multiple strains of IBV including Beaudette, as well as TCoV. In an effort to study potential protein products of the 3′ transcript, we utilized a well-established reverse genetics system to generate two Beaudette-based rIBVs. The first rIBV has a single nucleotide deletion that removes the premature stop codon, thereby allowing a full-length polypeptide comparable to other avian coronaviruses to be produced, and the second rIBV contains a His-tag added to the N-terminus of this full-length polypeptide. In this study we also identified a gene 2-derived transcript, denoted 2′, that has previously been reported both in IBV [6] and other coronaviruses [15, 16]. We confirmed the presence of the internal gene 2 and the 3′ terminal sgRNAs by performing deep-sequence analysis on allantoic fluid harvested from IBV-infected embryonated eggs and report on the site of leader-body fusions. Our findings highlight the potential for further unidentified non-canonically transcribed sgRNAs produced by IBV and further demonstrate the potential for coronaviruses to encode an additional repertoire of as yet unidentified genes.

**METHODS**

**Cells and viruses**

Primary chicken kidney (CK) cells and *ex vivo* tracheal organ cultures (TOCs) were prepared from 2 to 3-week-old...
specific pathogen free (SPF) Rhode Island Red (RIR) chickens as described elsewhere [17, 18]. Continuous cells lines Vero and DF1 were provided by the cell culture department at The Pirbright Institute. Embryonated RIR hens’ eggs at 10 days of age were provided by the Poultry Production Unit at The Pirbright Institute.

IBV strains M41–CK [19], Beau-R [20] and Beau-CK [21], all of the Massachusetts (Mass) serotype, GI-1 lineage [22], have been described previously. IBV strain CR88 is used as a vaccine against 793B strains, also known as 4/91, which are all of the GI-13 lineage [22]. D1466, a member of the GI-1 lineage first detected in The Netherlands in the late 1970s, was a gift from Dr Franz Davelaar of the Doorn Institute, Holland [22, 23]. H120 is a widely used vaccine of the Mass serotype, GI-1 lineage [22, 24]. Italy-02, a member of the GI-21 lineage [22], originated from Italy and was a gift from Professor Elena Catelli of the University of Bologna, Italy. The QX strain of the GI-19 lineage [22] used in this study was isolated in the UK [25, 26] and has since been used to generate a vaccine [27]. All isolates of IBV and rIBV were propagated in 10-day-old RIR SPF embryonated hens’ eggs. Allantoic fluid was clarified by low-speed centrifugation at 24 to 48 h.p.i. (hours post-infection) and subsequently assayed to titrate progeny virus by plaque assay in CK cells.

Full genome sequencing of viral stocks

As required for 454 pyosequencing, stocks of each of CR88, D1466, H120, QX and Italy-02 were grown in order to obtain sufficient purified RNA. To generate these stocks, twenty 10-day-old embryonated SPF RIR chicken eggs per virus were incubated with 100 µl IBV-infected allantoic fluid. After 24 h incubation, embryos were candled and culled by refrigeration for a minimum of 4 h. Allantoic fluid from each viral group was pooled and centrifuged to clarify supernatant.

Partial purification of IBV and isolation of viral RNA for deep sequencing were performed as previously described [28]. Briefly, IBV-infected allantoic fluid was purified by ultracentrifugation and the resulting virus pellet was resuspended in TRIzol reagent (Invitrogen, Thermo Fisher Scientific). RNA was extracted from supernatant as per the manufacturer’s instructions and quantified by NanoDrop assay. Samples were then sent to the Centre for Genomic Research (CGR), University of Liverpool, UK, for library preparation and sequencing. cDNA libraries were generated according to the GS FLX Titanium cDNA Rapid Library Preparation Method Manual [29] and sequenced using a GS FLX with Titanium series and run protocol.

The consensus sequence of the 5’ end of the genome for QX, D1466, H120, CR88 and Italy-02 was determined from RNA extracted from IBV-infected CK cells using a 5’ RACE system (Invitrogen by Life Technologies), using IBV-specific primers 5’-TGTCTGCTCAATAC-3’ for the reverse transcription step, and 5’-AGAAGTACCCCCAACGC-3’ for the amplification of d-tailed cDNA step. PCR products were cloned using a TOPA TA cloning kit for sequencing (Invitrogen by Life Technologies), and the sequences of two clones were determined per virus. Sanger sequencing was performed by Source BioScience, Oxford.

Consensus sequences were assembled for each IBV strain using the mira assembler version 3.4.0.1 [30] with genome, accurate, 454 and no vector screen settings. Contigs from the initial mira assemblies were viewed, edited and ordered as necessary, based on known virus consensus sequences using the Gap4 and Gap5 programs from the Staden packages [31, 32]. The sequences were deposited in GenBank under accession numbers MN548285 for CR88, MN548286 for D1466, MN548287 for H120, MN548288 for Italy-02 and MN548289 for QX.

IBV/rIBV infection of CK cells for intracellular RNA preparation

Confluent CK cells in six-well plates (approximately 1.2×10⁶ cells per well) were washed once with 1x PBS and inoculated with 500 µl IBV (at the highest titre possible) per well. After incubation at 37 °C for 4 h in 5% CO₂, the inoculum was removed and replaced with 3 ml BES (Sigma)-containing medium [33]. Infected cells were then further incubated until extensive cytopathic effect was observed, approximately 24 h.p.i., at 37 °C in 5% CO₂.

IBV/rIBV infection of CK cells for assessment of viral growth kinetics

Confluent CK cells, seeded in six-well plates, were infected with either 1×10⁴ p.f.u. or 1×10⁵ p.f.u. virus and incubated at 37 °C for 1 h with 5% CO₂. The inoculum was removed and the cells washed twice with 1x PBS, after which 3 ml BES-containing medium was added per well. Virus-containing supernatant was harvested at 1, 24, 48, 72 and 96 h.p.i., and subsequently screened for progeny virus by titration in CK cells.

IBV infection of ex vivo TOCs for assessment of intracellular RNA or ciliary activity

For the assessment of intracellular RNA, TOCs were washed once with 1x PBS and inoculated with 500 µl IBV/rIBV (at the highest titre possible) per tube in triplicate. After incubation at 37 °C for 1 h, 1 ml TOC culture medium was added [17]. Infected TOCs were incubated at 37 °C, at 8 revolutions h⁻¹ for a further 23 h. For TOCs being assessed for ciliary activity as described elsewhere [34, 35], incubation was for a further 95 h, with ciliary activity assessed at 24 h intervals using an EVOS digital inverted microscope. For this assay, TOCs were infected with 1×10⁵ p.f.u. or mock infected with medium in replicates of ten.

Preparation of RNA from CK cells, TOCs and allantoic fluid

Intracellular RNA was extracted from IBV-infected and mock-infected CK cells and ex vivo TOCs at 24 h.p.i. using an RNeasy mini kit (Qiagen). The manufacturer’s protocol for animal cells, spin with homogenization using a TissueLyser II (Qiagen) for 30 s at 25 Hz, was used. RNA
was extracted from allantoic fluid using an RNeasy mini kit, utilizing the RNA clean-up protocol. All RNA samples were stored at −20°C.

**Leader-body junction reverse transcription PCR (RT-PCR) analysis**

Intracellular RNA was reverse transcribed using Superscript III (Life Technologies) with either virus-specific primers (Table 1) or random primer 5′-GTTTCCAGTCAGAT CNNNNNNNNNNNNN-3′ (Sigma). The resulting cDNA was amplified by PCR using *Taq* polymerase (Life Technologies) with either a universal forward primer (5′-CTATTACACTAGCCCTTGCGC-3′) or a virus-specific primer (Table 1) located in the leader sequence of the 5′ end of the IBV genome. This was paired with either a universal primer (5′-GCTCTAACTCCTATAGCCCT-3′) or virus-specific primer (Table 1) located at the far 3′ end of the genome, directly preceding the poly(A) tail. PCRs were performed using a thermal cycler (2720 Thermal Cycler; Applied Biosystems) with the following protocol: 95°C for 2 min; followed by 25–35 cycles of 95°C for 30 s, 49°C for 30 s and 72°C for 2 min; and a final cycle of 72°C for 10 min. PCR products were cloned using either a TOPO TA cloning kit for sequencing with a pCR4-TOPO vector or a plasmid preparation kit (Qiagen). Both the plasmid preparations and PCR products were sequenced by Source BioScience, Oxford or GATC, Germany. Approximately 25–50 colonies from each transformation were screened.

**Northern blot analysis**

mRNA was isolated from intracellular RNA extracted from CK cells at 24 h.p.i. using a Poly(A) Purist MAG kit (Ambion), following the manufacturer’s protocol. Northern blot analysis was carried out using a NothernMax-Gly kit (Ambion). Briefly, after denaturation at 50°C with an equal volume of glyoxal loading dye for 30 min, mRNA was separated in a 1.1% low-electroendosmosis (LE) agarose gel. The mRNA was transferred to a BrightStar-Plus positively charged nylon membrane via capillary action for 90 min and cross-linked by UV exposure (using the auto crosslink function of a Stratalink UV Cross-linker; Stratagene). The membrane was pre-hybridized with ULTRAhyb buffer for 30 min at 42°C, before overnight incubation at 42°C with a DNA probe specific for the 3′ end of the genome (forward primer, 5′-CAACAGGCCTTTTGAAGAAGA-3′, within the N gene and reverse primer located in the 3′-UTR, 5′-GCTCTAACTCCTATAGCCCT-3′). The membrane was washed and the blot developed using a BrightStar Biodetect kit (Ambion), following the manufacturer’s protocol.

**Construction of a plasmid containing modified ORF 7**

The plasmids pGPT-BeauR-ORF7rep and pGPT-BeauR-ORF7rep-HIS were generated by GeneArt. Briefly, to construct pGPT-BeauR-ORF7rep, gene synthesis was carried out generating a sequence relating to Beau R [National Center for Biotechnology Information (NCBI) GenBank accession number AY311317] genome positions 26714–27540 with nucleotide T27141 deleted. To construct pGPT-BeauR-ORF7rep-HIS, gene synthesis was used to generate the above fragment with nucleotides encoding a His-Tag comprised of six histidine residues inserted at position 27114 immediately after the start codon, enabling ORF 7 to express a His-Tag on the N-terminus of the protein. This genome segment was then cloned into plasmid pGTP-NEB193, which is used as a donor molecule in the IBV reverse genetics system [20].

| Virus (strain) | Forward primer (5′-3′) | Reverse primer (5′-3′) |
|---------------|------------------------|----------------------|
| TCoV          | CTATCATACTAGCCCTTGCC   | GCTCTAACGCTATAGCCCT  |
| Beaudette     | CTATTACACTAGCCCTTGCC   | TGCTCTAACGCTATAGCCCT |
| M41           | CTATCACAAGCTTTGCGC     | TGCTCTAACGCTATAGCCCT |
| H120          | CTTGACACTAGCCCTTGCC    | TTCTCTAACGCTATAGCCCT |
| Italy-02      | CATACACACTAGCTTTGCGC   | GTCTCTAACGCTATAGCCCT |
| QX            | CATACTACACTAGCTTTGCGC  | TGCTCTAACGCTATAGCCCT |
| CR88          | CATACTACACTAGCTTTGCGC  | TGCTCTAACGCTATAGCCCT |
| D1466         | CATACTACACTAGCTTTGCGC  | TTTTTTGAACCTATAGCCCT |
Generation of a recombinant vaccinia virus containing modified IBV cDNA and the subsequent recovery of infectious IBV

The modified region of the IBV cDNA encoded in plasmids pGPT-BeauR-ORF7rep and pGPT-BeauR-ORF7rep-HIS were introduced into the full-length IBV cDNA Beau-R held within a vaccinia virus genome by homologous recombination using the transient dominant selection system described previously [33, 36, 37]. Infectious rIBVs containing the correctly modified sequence were then recovered [20, 33, 37] and passaged twice in CK cells. A stock of each rIBV was grown in 10-day-old SPF embryonated hens’ eggs, which was used for all subsequent experiments.

Deep sequencing of Beau-CK infected allantoic fluid

Beau-CK infected allantoic fluid was harvested and clarified by low-speed centrifugation for 10 min. The resulting supernatant was then subjected to ultracentrifugation at 236,880 g for 1 h on a 30% sucrose gradient and the pellet resuspended in 300 μL RLT buffer. Total RNA was then extracted using a Qiagen RNeasy kit, following the manufacturer’s protocol.

RNA was checked for quality using a Bioanalyzer 2100 RNA Pico kit (Agilent), quantitated using a Qubit (Life Technologies) and normalized for a 100 ng input. Sequencing library preparation was performed using a NEBNext directional Ultra RNA-Seq kit (NEB). Library quality control (QC) was performed using the Bioanalyzer 2100 DNA 1000 kit and Qubit prior to pooling. The library pool was quantified using a NEBNext Illumina library quantitation kit (NEB) before being diluted and loaded onto a single lane of an Illumina HiSeq 4000.

Analysis of deep sequencing data

Resulting reads were quality assessed using FastQC [38] with quality filtering then performed with Trim Galore! version 0.6.2 [39] using the following parameters: input as paired reads, minimum sequence length = 100, quality score cut-off = 30, trim 15 bp from 5′ end of R1 and R2 read. Host subtraction was performed by aligning passed quality filter (PQF) reads to the galGal6 reference genome with a de novo consensus sequence for Beau-CK generated from non-host derived reads using SPAdes. All quality-filtered reads were then aligned to the Beau-CK genome using BBMap [40] using default parameters. Chimeric reads were identified by initially performing an alignment with bwa [41] with reads flagged as chimeric exported and subsequently aligned using BBMap.

PQF reads possessing leader sequence were identified by searching for the sequence TTTAAAATCTAGC, the reverse complement of GCTAGATTATTAA, a sequence located within leader of Beau-CK at nucleotide positions 44–56 (NCBI GenBank accession number AY311317). To prevent duplicate matches (in forward and reverse reads), read pairs were merged using pear (Paired-End reAd merger) [42] prior to searching. The resulting.fasta sequences were trimmed to remove the 5′ sequence upstream (relative to the viral genome) up to and including the leader search sequence and aligned to the Beau-CK reference using bwa. Coverage was reported using SAMTools (version 1.4) [43]. Data has been deposited in the Sequence Read Archive (SRA), under accession number PRJNA577406.

RESULTS

Identification of a putative sgmRNA located downstream of the N gene

The analysis of the full-length genome sequences of several IBV strains has identified a potential ORF at the 3′ end of the genome between the end of N and the poly(A) tail in what is considered as a hypervariable region of the IBV 3′-UTR [7, 14]. Britton and Cavanagh (in 2008) had previously considered an ORF in this area due to the presence of an AUG start codon, but discounted the possibility of another gene due to the apparent lack of a TRS-B [44]. For the IBV strain Beaudette, a canonical TRS-B of either CUUAAACAA, CUGAAACAA or CUUAUAA cannot be identified in the genome between the middle of the N gene and 5′ of the putative ORF, which raised the possibility that the mechanism of transcription was either similar to that of gene 4b [6], which uses a shortened non-canonical TRS-B, or that a TRS-B of an unidentified sequence was being utilized. To determine whether a sgmRNA was produced during IBV infection that relates to the 3′ end of the genome, primary CK cells were inoculated with the rIBV Beau-R [20]. Northern blot analysis of the infected cell lysate identified a potential sgmRNA, smaller than the sgmRNA encoding the N protein (Fig. 1a).

Interestingly, on a 1.1% agarose gel this smaller putative sgmRNA runs in front of the loading dye, so it is likely that it had either run off the gel or been removed when the gel was trimmed for the RNA transfer process in previous experiments. In addition, the relatively low abundance of the mRNA compared to the other sgmRNAs produced by the structural and accessory genes raises the possibility that this band has previously been assumed to be background and the result of non-specific binding, similarly to the way 4b had previously been described [6]. In the time that this paper has been in preparation, both Dinan et al. (2019) [7] and An et al. (2019) [14] have both also identified a potential sgmRNA located between the N gene and the 3′-UTR.

Confirmation of the presence of a sgmRNA

To confirm that the IBV-derived RNA observed on the Northern blot did relate to a novel IBV sgmRNA, intracellular RNA was extracted from CK cells infected with Beau-R or M41-CK. Leader-body junction RT-PCR was then used to amplify sgmRNAs. The primer set chosen, forward primer (5′-CTATTACACTAGCCTTGCGC-3′) located in the leader sequence and reverse primer (5′-GCTCTAATCTTACTAGGCT-3′) located upstream of the poly(A) tail, would potentially amplify all sgmRNAs present. Due to the nature of PCRs, however, smaller templates would be preferentially amplified. A PCR product of ~2000 bp was produced from both Beau-R and M41-CK infected cells, whilst a PCR product of 500–650 bp was consistently produced from Beau-R infected cells but not those infected with M41-CK.
(Fig. 1b). Cloning of the Beaudette- and M41-derived PCR products, followed by sequence analysis, confirmed that the PCR products related to sgRNAs due to the presence of the IBV leader sequence. Sequence alignments showed that the products of around 2000 bp related to the sgmRNA encoding the N gene from both M41-CK and Beau-R infected cells. The smaller PCR product (~500–650 bp) is also produced in Beau-R, but does not occur in M41-CK infected cell lysate. (c) Sequencing of leader-body PCR products. Sequencing of this smaller band confirms a match to the 3′ end of the virus genome (Beau-R NCBI GenBank accession number AJ311317), but the sequence also possesses a leader sequence corresponding to the 5′ end of the genome and, hence, has the characteristics of a sgmRNA. Reads do not confirm an alignment to the TRS with perfect homology and instead match only 5 nucleotides (TAACA). (d) Pairwise alignment of Beau-R and M41-CK sequences after the N stop codon. An alignment between the Beau-R and M41-CK sequences from genome position 27100–27607 (relative to Beau-R NCBI GenBank accession number AJ311317) reveals a 185 nucleotide deletion in M41-CK versus Beau-R, as well as three single nucleotide polymorphisms (shown in red font). The deletion includes part of the prospective TRS-B and the sgmRNA sequence. In (c) and (d), the coloured boxes indicate: red, N stop codon; blue, nucleotide matches to the canonical TRS (CTTAACA); green, prospective ORF 7 start codon.

However, unlike gene 4b in which only 3 nucleotides (CAA) of the TRS of the sgmRNA match the genome sequence [6], 5 nucleotides (UAACA) matched in the newly identified sgmRNA. Therefore, the TRS-B was determined to consist of nucleotides UAACA, which align with positions 3 to 7 in the TRS-L (Fig. 1c). This finding supports reports by both An et al. (2019), who identified a TRS-B of UAACA [14], and Dinan et al. (2019), who identified a putative TRS-B of UAACAU [7]. Sequence alignment of the 3′-UTR for Beau-R and M41-CK identifies that the deletion seen in the latter starts 2 bp into the newly identified TRS-B (Fig. 1d), suggesting that the 5 nucleotides comprising this TRS-B are required for the transcription of the newly identified sgmRNA. Considering the
identification of a functional TRS-B, and the location of the ORF that is separate to gene 6 (N gene), the newly identified sgRNA and corresponding ORF will be referred to as gene 7.

A sgRNA relating to gene 7 is expressed from several IBV strains and TCoV

To establish whether the newly identified sgRNA was specific to the Beaudette strain or was present in other strains of IBV, full genome sequencing of five strains of IBV was undertaken using 454 pyrosequencing technology. The strains investigated included vaccine strains CR88 and H120, as well as field isolates D1466, Italy-02 and QX.

Investigation of a variety of both field and vaccine IBV strains by leader-body junction RT-PCR and Northern blot analysis identified sgRNA 7 in several other viruses (Fig. 2a, b), including the vaccine strains H120 and CR88 and pathogenic strains D1466, Italy-02 and QX. Leader-body junction RT-PCR followed by sequence analysis demonstrated that there is variability in the number of nucleotides that constitute the TRS-B (Fig. 2c). Both the genomes of Beau-CK and QX contain a TRS-B of UAACA, which aligns with nucleotides 3 to 7 of the TRS-L, CUUAACAA. This also confirms the molecular clone Beau-R is comparable to the parental virus Beau-CK (Figs 1c and 2c). Unlike the Beaudette strains, the TRS-B for CR88 reads UUAACA aligning to positions 2 to

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**Fig. 2.** A gene 7 transcript is produced by diverse strains of IBV. (a) Leader-body PCR analysis of infected CK cell whole-cell lysate. Amplification of sgRNAs by PCR and subsequent separation by agarose-gel electrophoresis produces two bands in the instance of Beau-CK, D1466, QX, H120 and CR88, and three bands in the instance of Italy-02. All six viruses produce bands corresponding to gene 6 (N protein, ~2000 bp) and gene 7 (~500–600 bp). The larger band in Italy-02 relates to gene 5. (b) Northern blot analysis of infected whole-cell lysate. A clearly distinguishable band is present at the bottom of the gel for H120, Beau-R, Beau-CK and CR88 that corresponds to sgRNA 7. Confirmatory to Fig. 1(b), M41-CK does not have a band corresponding with sgRNA 7. All five viruses have bands corresponding to the other sgRNAs as anticipated. However, there is a consistent pattern of bands between genes 6 and 7 across the IBVs that cannot be explained. (c) Sequence analysis of leader-body PCR products. All sequences are indicative of sgRNAs; however, the TRS-B and the site of leader-body switch is not consistent. Genome positions for each IBV strain are indicated on the left (NCBI GenBank accession numbers: AJ311317, M5548285, M5548286, M5548287, M5548288, M5548289). Beau-CK and QX both have a partial match to the canonical 8 nucleotide TRS like Beau-R (TAACA). CR88 has a partial match of 6 nucleotides (TAAACA) versus the canonical TRS. Sequencing of D1466 resulted in a fluctuation (orange) as to where the leader-body junction for sgRNA 7 is situated, but possess a TAACA TRS. H120 and Italy-02 possess the canonical 8 nucleotide TRS. The distance between the N stop codon (red) and the TRS for gene 7 is longer in H120 and Italy-02 versus the other studied IBVs. Red, N stop codon; blue, nucleotide matches to the canonical TRS; green, prospective ORF 7 start codon; orange, single nucleotide difference in D1466 leader/body junction.
7 of the TRS-L, and the TRS-B in the genomes of Italy-02 and H120 have the highest sequence homology with the TRS-L, differing only at position 8. Interestingly, both H120 and Italy-02 have leader-like sequence located immediately upstream of the TRS-B, resulting in a greater number of nucleotides between the N stop codon and the TRS-B than the other strains assessed. The pathogenic strain D1466 also presents a more complex scenario as in this assay two populations of sgmRNA 7 were identified. One population contains a TRS of CUUAACA, which similarly to Beaudette had nucleotides 3–7 matching the TRS-B. The other population contained a TRS of CGUAACA, differing at position 2, with this nucleotide appearing to be derived from the genome sequence at position 27079, rather than the leader sequence at position 37. In the latter population, it appears that the recombination event between TRS-L and TRS-B has occurred between positions 1 and 2 of the TRS-L, with position 1 of the TRS derived from TRS-L and positions 2–7 from the genome, TRS-B (Fig. 2c). Despite the variation, all the TRSs for the sgmRNA 7, in all the IBV strains assessed, possessed a common core sequence (CS) of UAACA, supporting the notion that the TRS-B is universally UAACA, and also suggesting this could be the minimum requirement for effective transcription.

The leader-body junction analysis was initially carried out using universal primers with RNA extracted from infected CK cells. To ensure there was no RT-PCR/priming error, primers were re-designed to be strain specific (Table 2). Intracellular RNA was then extracted from infected CK cells, embryonated eggs and TOCs and the leader-body junction RT-PCRs were carried out utilizing the strain-specific primers. The results of this analysis matched those results detailed in Fig. 2; therefore, demonstrating that the sgmRNA was not an artefact of virus replication in cell culture (data not shown).

To determine whether the presence of sgmRNA 7 is potentially common to other gammacoronaviruses, its presence in another avian Gammacoronavirus, TCoV, was investigated. Total RNA was extracted from TCoV-infected turkey embryos and leader-body junction RT-PCR analysis was carried out to confirm the presence of a PCR product that related to a sgmRNA at the 3′ of the genome, after the N gene (Fig. 3). Sequence analysis of the TCoV-derived PCR products identified a PCR product that was analogous to the IBV sgmRNA 7, which contained a leader sequence fused to the 3′ end of the TCoV genome via a TRS-B of UAACA.

### Table 2. Quantifying transcript sequence origin for each sgmRNA in Beau-CK

| sgmRNA (products) | TRS-L | Genome Transcript sequence | Instances (percentage) |
|-------------------|-------|---------------------------|------------------------|
| 2 (S)             | CTTAACAA | CTGAACAA | CTTAACAA, CTGAACAA | 3240 (53.4%), 2754 (45.4%) |
| 2*                | CTTAACAA | CCAAACAA | CTTAACAA, CTTAACAA | 398 (90.7%), 35 (8.0%) |
| 3 (3a/3b/E)       | CTTAACAA | CTGAACAA | CTTAACAA, CTGAACAA | 1777 (61.5%), 1099 (38.0%) |
| 4 (M)             | CTTAACAA | CTTAACAA | CTTAACAA           | 4148 (99.0%) |
| 4b (4b/4c)        | CTTAACAA | GTGACCAA | CTTAACAA, CTTAACAA | 922 (96.0%), 19 (2.0%) |
| 5 (5a/5b)         | CTTAACAA | CTTAACAAAAACTTAACAA | CTTAACAAAAACTTAACAA, CTTAACAAAAACTTAACAA | 400 (3.0%), 12500 (95.2%) |
| 6 (N)             | CTTAACAA | CTTAACAA | CTTAACAA           | 35826 (99.7%) |
| 7 (7)             | CTTAACAA | AGTAACAT | CTTAACAT, CTAACAT  | 90 (82.6%), 18 (16.5%) |
were derived from the TRS-B, with the template switch during sgmRNA synthesis occurring between positions 5 and 6.

Leader-body junction analysis on RNA extracted from BeauR-l-CTGAACAA infected CK cells for characterization of sgmRNA 7 identified the transcript possessed a CUGAACU sequence (Fig. 4), confirming that the template switch included the TRS-L mutation. This would indicate that the template switch occurs after position 3 on the canonical TRS. Unfortunately, BeauR-l-CTTAACAT could not be used in this study as the nucleotide in position 8 in the TRS-B for the sgmRNA 7 is already a U.

**Potential protein encoded by the novel ORF**

Analysis of the genomes from the IBV strains investigated, as well as TCoV, demonstrated a large degree of variability in the predicted protein sequences of 73 aa associated with sgmRNA 7 (Fig. 5). However, for both Beaudette and D1466, the predicted proteins are truncated when compared to the putative proteins from the other IBVs and TCoV. The Beaudette-encoded peptide consists of 10 aa and the D1466-encoded peptide of 53 aa. As previously mentioned, the genome of M41 does not encode sgmRNA 7 due to a deletion in this part of the genome (Fig. 1). Therefore, it is reasonable to assume that any potential protein encoded by sgmRNA 7 is non-essential for virus replication. Conversely, it is possible that expression of the protein may be detrimental to virus proliferation in cell culture considering that Beaudette, a virus which has been passaged over 300 times in various systems, has mutated to result in a severely prematurely terminated polypeptide.

Using a reverse genetics system previously described by Casais et al. [20], a single nucleotide was deleted in Beau-R in order to cause a frameshift removing the associated premature stop codon (Fig. 6a). Two rIBVs were produced from independent homologous recombination events and independent rescue events, denoted BeauR-ORF7rep A and BeauR-ORF7rep...
Fig. 5. Alignment of ORF 7 prospective proteins in different IBV strains and TCoV using MView. Gene 7 sgRNA sequences were screened for the presence of an AUG start codon. A 53–77 aa protein sequence was identified in TCoV and all IBVs with exception to Beau-R, which has a highly truncated 10 aa protein sequence. A single T deletion in Beau-R at position 27141 (BeauR-ORF7rep) results in a frameshift mutation and the potential to generate a 73 aa product.

To establish whether other IBV strains were capable of expressing sgRNA 2*, total RNA was extracted from CR88, QX, Italy-02, H120, D1466 and M41-CK infected CK cell lysates and investigated by leader-body junction RT-PCR analysis. No RT-PCR product relating to Beaudette sgRNA 2* was identified for any of the other IBV strains (data not shown). Sequence alignment of the IBV genomes, corresponding to the Beaudette sgRNA 2* region, showed that all of the other IBV strains investigated, except for CR88, did not possess a TRS-B of AACAA (Fig. 7c), possibly accounting for the lack of sgRNA 2* expression. Notably, all the other strains, except CR88, contained an A to C substitution at position 5 of the Beaudette TRS-B, which could suggest that a TRS-B of AACA is not sufficient to enable synthesis of sgRNA 2*. Interestingly, CR88 contains a TRS-B of AACAA, comparable to the Beaudette strain; however, despite several attempts by both leader-body junction RT-PCR analysis and Northern blot analysis an RNA relating to sgRNA 2* could not be identified in CR88 infected cells. Further analysis of the genome sequence highlights that whilst CR88 and Beau-R share identical TRS-B sequences, there are nucleotide differences located in both the immediate 5' and 3' flanking regions.

B, both of which have the potential to express a full-length protein of 73 aa (Fig. 5) from sgRNA 7. In an effort to detect this protein, two further viruses were generated, similarly from independent lineages, BeauR-ORF7rep-HIS 7 and BeauR-ORF7rep-HIS 14, both of which contain a His-Tag comprised of six histidine residues located at the N-terminal end of the repaired polypeptide. The 5' end of ORF 7 was chosen for the His-tag modification instead of the 3' end due to the proximity of the latter to the secondary RNA structures present in the 3'-UTR that regulate RNA synthesis [45–48].

The growth kinetics of both isolates of BeauR-ORF7rep and BeauR-ORF7rep-HIS were analysed in CK cells, and were found to be comparable to Beau-R (Fig. 6b, c). Of note, the titres for BeauR-ORF7-rep B were lower than Beau-R at both the 48 and 96 h.p.i. time points (P<0.05; Fig. 6b), although the difference in titres was minimal. Ex vivo TOCs were infected with BeauR-ORF7rep A and BeauR-ORF7rep B, and the ciliary activities assessed over a 96h period in 24h intervals (Fig. 6d). Both isolates did cause ciliostasis similar to the parental virus; however, the process appeared to be delayed with lower ciliary activities compared to Beau-R in comparison to the two rIBVs at 48 h.p.i. (P<0.005) (Fig. 6d). The observation that both rIBVs could cause ciliostasis alongside the growth kinetic assays completed in CK cells indicated that any potential protein encoded by ORF 7 is not detrimental to viral replication in vitro nor in ex vivo TOCs.

To determine whether both isolates of BeauR-ORF7rep and BeauR-ORF7rep-HIS produced modified sgRNA 7s for potential expression of the ORF 7 protein or His-tagged version, total RNA was extracted from infected CK cell lysate. Leader-body junction RT-PCR analysis confirmed that BeauR-ORF7rep expressed the modified sgRNA 7; however, BeauR-ORF7rep-HIS did not (Fig. 6e). Sanger sequence analysis of an RT-PCR product to analyse the modified genome, utilizing RNA from the same cell lysate, demonstrated that the genome of the BeauR-ORF7rep-HIS virus retained the His-Tag modification located at the 5' end of ORF 7. The results of the leader-body junction RT-PCR analysis indicate that the His-Tag modification appears to prevent the expression of the sgRNA 7, indicating a possible role for sequences flanking the TRS-B in the regulation of sgRNA expression.

Investigation of a non-canonically transcribed sgRNA within gene 2

Previous work involving Northern blot analysis of CK cells infected with the rIBV Beaux-Δ-IR revealed the presence of an additional RNA species, denoted 2*, that related to a putative sgRNA site located within gene 2 (S) [6]. An investigation, utilizing leader-body junction RT-PCR analysis of RNA extracted from Beau-R and Beau-CK infected CK cells, identified an RT-PCR product whose sequence contained the IBV leader sequence fused to the IBV genome within the S gene, at position 21249 of the genome, via a non-canonical TRS-B of AACA accounting for positions 4 to 8 of the TRS-L (Fig. 7a, b). Interestingly the sequence analysis also identified a TRS-like sequence of CUUACCA located immediately adjacent to the TRS-B (Fig. 7b).

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which may be responsible for why CR88 does not produce a sgmRNA 2*.

Detection of Beau-CK transcripts in infected allantoic fluid

The identification of up to three non-canonically transcribed sgmRNAs, 2*, 4b and 7 from IBV, highlighted the possibility for IBV to generate a larger number of yet unidentified gene products. In an effort to investigate whether there is potential for even more sgmRNAs, a HiSeq dataset was generated to screen for the presence of additional putative sgmRNAs. The HiSeq dataset was generated by HiSeq analysis of allantoic fluid harvested from Beau-CK infected SPF embryonated hens' eggs. A total of 185,992 reads aligning to two distinct, non-consecutive positions of the genome (termed chimeric reads) were identified. Separately, a total of 73,846 reads possessing the IBV 5' leader sequence were identified after merging quality-filtered reads using PEAR [42]. Peaks in coverage for both chimeric-mapped reads and leader-possessing reads correspond to sgmRNAs, with 2 (S),
3, 4 (M), 5 and 6 (N) clearly identified. Peaks corresponding to the non-canonically transcribed sgmRNAs 2*, 4b and 7 were also identified (Fig. 8). Additional peaks relating to either/both chimeric reads and reads containing the IBV 5′ leader sequence across the Beaudette genome, including some originating within gene 1, indicate the potential for previously unidentified sgmRNAs and putative associated proteins.

A second analysis was performed on the HiSeq dataset assessing the usage of specific TRSs for the known sgmRNAs (Table 2). This assessment demonstrated there is fluidity in the recombination events between the TRS-Bs and TRS-L, with variability in the recombination locations identified. The Beaudette sgmRNA 2 (S gene) TRS-B has the sequence CUG AACAA, meaning there is a mismatch at position 3 with the TRS-L, CUU AACAA. HiSeq analysis has shown that approximately 50% of the chimeric reads identified a sgmRNA 2 possessing a TRS containing a G at position 3 and 50% possess a U. This indicates there is the potential for variability in the location of the recombination event between two TRSs, with the recombination event between the S gene TRS-B and TRS-L occurring between positions 1 and 2 or 2 and 3 of TRS-B to include the G at position 3 in sgmRNA 2. In order to allow the U nucleotide at position 3, recombination would need to occur at a point somewhere between positions 3 to 8 of TRS-B. A similar occurrence was observed, from the HiSeq data, for sgmRNA 3 (gene 3), which has a TRS-B of CUGAACAA; approximately 60% of the sgmRNAs contain a U at position 3 and ~40% a G.

The sgmRNAs that utilize non-canonical TRS-Bs also demonstrate variable locations of the recombination events between the associated TRS-B and TRS-L; however, these
also show a clear preference. For sgRNA 4b (gene 4b) the genome reads GUCACCAA with the TRS-B identified as CAA. HiSeq analysis showed that the majority of sgRNA 4b contained a TRS of CUUAACAU, but there is a very small population, ~2%, that contain CUUACCAC, a difference at nucleotide position 5. A similar situation was identified with sgRNA 7, ~80% of sgRNA-derived reads contained a TRS of CUUAACAU and ~16% contained CGUACAA; the TRS-B has been identified as UAACA. This observed plasticity in the location of the recombination event observed between the TRS-I and a TRS-B suggests that the presence of a fixed sequence TRS-B is not sufficient alone to regulate transcription, and that other regulatory factors are involved.

**DISCUSSION**

This work has identified and investigated additional examples of sgRNAs transcribed from non-canonical TRS-Bs within the gammacoronavirus IBV genome, and further highlights the potential for coronavirus genomes to encode additional ORFs. Interestingly, whilst sgRNA 2* and associated ORF 2*, are likely to be Beaudette-specific and not a shared feature of IBV strains, a similarly located ORF has been identified in SARS-CoV and in porcine respiratory coronavirus [12, 49, 50].

This report identifies another non-canonically transcribed sgRNA, which like sgRNA 4b, is seemingly universally shared between IBV strains. This sgRNA, designated sgRNA 7, is transcribed from a non-canonical TRS-B, UAACA, and contains a putative ORF located at the 3′ end of the genome immediately downstream of the N gene and upstream of the 3′-UTR: an area previously reported not to encode an ORF due to the lack of apparent TRS-B [51]. As this paper was in preparation, two further publications have identified this sgRNA in the Beaudette strain of IBV, transcribed from a TRS-B of UAACAU or a putative TRS-B of UAACAU [7, 14]. However, this report furthers the findings of both Dinan et al. [7] and An et al. [14] by (i) confirming the TRS-B of UAACA in the Beaudette strain and (ii) identifying the presence of sgRNA 7 and associated ORF in several other IBV strains, both vaccine and field isolates, including CR88 and QX, respectively. Interestingly, there is variability in the sequence of the TRS-B between the strains of IBV, with the genome of CR88 containing a 6 nucleotide TRS-B of UAACAU and Italy-02 a 7 nucleotide TRS-B of CUUACA. Of particular note, An et al. [14] stated that H120 did not express sgRNA 7 due to the lack of a TRS-B of UAACA; however, this report has demonstrated that H120 does transcribe sgRNA 7, but utilizes an extended TRS-B of 7 nucleotides, CUUACA. The TRS-B is, therefore, only 1 nucleotide short of being considered a canonical TRS-B and, interestingly, immediately upstream of the TRS-B is a leader-like sequence, also observed in the Italy-02 genome. We also demonstrated that the M41-CK strain does not encode sgRNA 7 due to a 185 nucleotide deletion that removes the 3′ terminal CA of the TRS-B, as well as the majority of the genome sequence encoding the associated ORF.

Further comparison of other Mass serotype strains indicates that there is some variability between isolates. Whilst vaccine strains H52 and H120 share an identical ORF 7 sequence (Fig. 9), other Mass isolates differ in this region. Identical to the Beaudette strain, a Mass isolate from 1979 (NCBI GenBank accession number FJ904722) contains a single nucleotide insertion within ORF 7, which results in a premature stop codon truncating the polypeptide to 10 aa. Mass isolates from 1965, 1972 and 1985 (NCBI GenBank accession numbers FJ904720.1, FJ904721 and FJ904723.1) possess a shortened TRS of TAA and do not have an AUG start codon, like the M41-CK strain used in this study (Fig. 9a). Interestingly, a Mass isolate from 2006 (NCBI GenBank accession...

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**Fig. 9.** The presence of gene 7 varies between strains of the Massachusetts serotype. (a) The nucleotide sequences of different Mass strains were compared over the start of gene 7. Red, N stop codon; blue, nucleotide matches to the canonical TRS; green, prospective ORF 7 start codon. (b) A comparison of the amino acid sequences. NCBI GenBank accession numbers: AJ311317.1, MK728875.1, FJ904720.1, FJ904721, FJ904722, FJ904723.1, FJ904713, EU817497.1 and M5648287.
number FJ904713) contains the TAACA TRS like Beau-CK, but encodes a different sgmRNA 7 sequence (Fig. 9b). All of these Mass isolates were isolated from broiler chickens with clinical signs of disease [52], so the variation in ORF 7 cannot be linked to pathogenicity.

In alpha- and betacoronaviruses, each TRS is thought to contain a CS that is conserved between the TRS-L and TRS-B. Flanking the CS is a number of nucleotides, located at the 5′ and 3′ ends of a TRS that varies between TRS-Bs [15, 53]. Research has shown that sequence homology between the CS-L and CS-B is proportional to the abundance of sgmRNA observed [15, 16]. In all the IBV strains assessed in this report, sgmRNA 7 would appear to have a CS of UAACA; however, this CS is not shared between all the TRS-Bs in the genome. HiSeq data generated from Beau-CK infected allantoic fluid identified that only nucleotide A at position 4 and CA at positions 6 and 7, respectively, of the IBV TRS-L, CUUAACAA, are shared (Table 2). As a note, the TRS-B of sgmRNA 4b only contains CAA, position 6 to 8 of the TRS-L [54], but there is an A nucleotide located at position 4 that corresponds with both TRS-L and the body of the genome.

What is interesting regarding sgmRNA 7 is that nucleotides CA at positions 6 and 7 of the TRS-L, CUUAACAA, are those that are missing from the TRS-B in the M41-CK genome. This perhaps indicates that these nucleotides are required for the expression of sgmRNA 7.

Regardless of the dynamics and regulation of transcription, there is precedent for a functional accessory gene to be located between the N gene and the 3′-UTR, with such genes extensively studied in the alphacoronaviruses transmissible gastroenteritis virus (TGEV) and feline infectious peritonitis virus (FIPV) [55–58], deletion of which does not affect replication in vitro but does affect pathogenicity in vivo [57, 59]. In FIPV, the sgmRNA 7 encodes two proteins 7a and 7b, 101aa/10kDa and 207aa/24kDa, respectively. Similarly to TGEV, it has been demonstrated that the 7ab cluster is essential for virulence in vivo, but not always for replication in vitro, with deletion knock-out viruses able to replicate well in feline FCWF cells but not peripheral blood monocytes [56, 58, 60]. The function of sgmRNA 7 in IBV, and whether a protein is produced from the sgmRNA, remains undetermined. An et al. [14] reported that there was no AUG start codon present, and concluded that sgmRNA 7 was non-coding [14]. Our report contradicts these findings by identifying an AUG start codon present in all IBV strains investigated, except for some Mass viruses including M41-CK, located 2 nucleotides from the 3′ end of the TRS-B. This proximity of the TRS-B to the AUG start codon is unusually close; the distances between the TRS-B and the AUG in Beau-R for genes 2, 3, 4, 5 and 6 are 52, 23, 77, 20/6 and 93 nucleotides, respectively.

Dinan et al. [7] have also shown the IBV ORF 7 AUG is occupied by ribosomes on RNA isolated from Beau-R infected CK cells, indicating that sgmRNA 7 is translated [7]. Unfortunately, our efforts to His-Tag the full-length Beaudette ORF 7 protein were unsuccessful with the His-Tag sequence insertion disrupting the transcription of sgmRNA 7. Whilst this was disappointing, it has provided evidence of the importance of the flanking regions located at the 3′ end of this TRS-B. An et al. also demonstrated the importance of the 3′ flanking sequence, particularly the sequence AAUGG at position 27110–27114 [14]. Mutations of this motif to UUCC completely disrupted sgmRNA 7 expression; although it must be noted these mutations also disrupted the start codon. The His-Tag sequence insertion detailed in this report also disrupts the AAUGG motif by trans-locating the G at position 27114 to 27132 of the genome. RNA modelling using the RNAstructure tools webserver version 6.0.1 (2018) [61] suggests a change in RNA structure as a consequence of the 18 nucleotide His-Tag sequence insertion. RNA structure has been shown to play a regulatory role in coronavirus sgmRNA synthesis and in particular the interaction of distally located motifs [53, 62, 63]. It is also likely that flanking regions surrounding the TRS-B and/or RNA structure play a role in the expression of sgmRNA 7*, as despite the presence of a TRS-B, CR88 does not express this sgmRNA, indicating that other required regulatory factors are not present or are not active within the genome.

To assess whether the IBV genome could potentially encode additional undefined ORFs, a HiSeq dataset generated from Beau-CK infected allantoic fluid was investigated. This analysis highlighted that there are several chimeric RNAs consisting of the IBV leader sequence fused to different regions throughout IBV genome, including within the replicase gene (gene 1), all representing potential sgmRNAs. Whether these sequences account for biologically relevant sgmRNAs remains an unanswered question; however, this is not the first report to identify additional chimeric reads through sequencing analysis [64, 65]. Of particular note is the investigation of the arterivirus simian haemorrhagic fever virus, which identified an additional 51 functional TRS-Bs, greatly increasing the number of potential ORFs, with a number of the newly identified TRS-Bs located within gene 1 [64]. Analysis of this dataset has also highlighted that the location of the recombination event between the TRS-L and TRS-B is not fixed, which could indicate for the role of secondary regulating factors.

In conclusion, this report has provided further evidence for the presence of an ORF transcribed from non-canonical TRS-B located between the N gene and 3′-UTR in several strains of the gammacoronavirus IBV, denoted ORF 7. Investigation of the newly identified sgmRNA, as well as the investigation of sgmRNA 2*, indicates roles of the 5′ and 3′ flanking sequences of the TRS-B in the transcription of sgmRNAs. The HiSeq data generated in the report also identifies that there is variation in the location of the recombination event between the TRS-L and TRS-B, and additionally highlights the possibility of the IBV genome encoding further ORFs likely transcribed at low frequency from non-canonical TRS-Bs. The coronavirus replication cycle is complex and not fully understood and it is, therefore, important for future research to investigate these novel ORFs transcribed from non-canonical TRS-B sequences and to assess their role in IBV replication.
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Conflicts of interest
The authors declare that there are no conflicts of interest.

References
1. Cavanagh D. Coronavirus avian infectious bronchitis virus. Vet Res 2007;38:281–297.
2. Cook JKA, Jackwood M, Jones RC. The long view: 40 years of infectious bronchitis research. Avian Pathol 2012;41:239–250.
3. Boursnell MEG, Brown TDK, Foulds UJ, Green PF, Tomley FM et al. Completion of the sequence of the genome of the coronavirus avian infectious bronchitis virus. J Gen Virol 1987;68:57–77.
4. Brierley I, Boursnell MEG, Binns MM, Bilimoria B, Blok VC et al. An efficient ribosomal frame-shifting signal in the polymerase-encoding region of the coronavirus IBV EMBJO 1987;6:3779–3785.
5. Brierley I, Digard P, Inglis SC. Characterization of an efficient coronavirus ribosomal frameshifting signal: requirement for an RNA pseudoknot. Cell 1989;57:537–547.
6. Bentley K, Keep SM, Armesto M, Britton P. Identification of a noncanonically transcribed subgenic mRNA of infectious bronchitis virus and other gammacoronaviruses. J Virol 2013;87:2128–2136.
7. Dinan SM, Keep S, Bickerton E, Britton P, Firth AE et al. Comparative analysis of gene expression in virulent and attenuated strains of infectious bronchitis virus at subcodon resolution. J Virol 2019;93:e00714–19.
8. Sawicki SG, Sawicki DL. Coronaviruses use discontinuous extension for synthesis of subgenom-length negative strands. Adv Exp Med Biol 1995;380:499–506.
9. Liu DX, Inglis SC. Internal entry of ribosomes on a tricistronic mRNA encoded by infectious bronchitis virus. J Virol 1992;66:614–615.
10. Brown PA, Touzain F, Brind F, Goulam AM, Courtillon C et al. First complete genome sequence of European turkey coronavirus suggests complex recombination history related with US turkey and guinea fowl coronaviruses. J Gen Virol 2016;97:110–120.
11. Stern DF, Kennedy SIT. Coronavirus multiplication strategy. I. Identification and characterization of virus-specific RNA. J Virol 1980;34:665–674.
12. Hussain S, Pan J, Chen Y, Yang Y, Xu J et al. Identification of novel subgenomic RNAs and noncanonical transcription initiation signals of severe acute respiratory syndrome coronavirus. J Virol 2005;79:5288–5295.
13. Zhang X, Liu R. Identification of a noncanonical signal for transcription of a novel subgenomic mRNA of mouse hepatitis virus: implication for the mechanism of coronavirus RNA transcription. Virology 2000;278:75–85.
14. An H, Cai Z, Yang Y, Wang Z, Liu DX et al. Identification and formation mechanism of a novel noncoding RNA produced by avian infectious bronchitis virus. Virology 2019;582:176–180.
15. Sola I, Moreno JL, Zúñiga S, Alonso S, Enjuanes L. Role of nucleotides immediately flanking the transcription-regulating sequence core in coronavirus subgenomic mRNA synthesis. J Virol 2008;82:2506–2516.
16. Zúñiga S, Sola I, Alonso S, Enjuanes L. Sequence motifs involved in the regulation of discontinuous coronavirus subgenomic RNA synthesis. J Virol 2004;78:980–994.
17. Hennion R. The preparation of chicken tracheal organ cultures for virus isolation, propagation, and titration. In: Maier HJ, Bickerton E, Britton P (eds). Coronaviruses: Methods and Protocols (Methods in Molecular Biology vol. 1282).New York: Springer; 2015. pp. 51–56.
18. Hennion R, Hill G. The preparation of chicken kidney cell cultures for virus propagation. In: Maier HJ, Bickerton E, Britton P (eds). Coronaviruses: Methods and Protocols (Methods in Molecular Biology vol. 1282).New York: Springer; 2015. pp. 57–62.
19. Darbyshire JH, Rowell JG, Cook JKA, Peters RW. Taxonomic studies on strains of avian infectious bronchitis virus using neutralisation tests in tracheal organ cultures. Arch Virol 1979;61:227–238.
20. Casais R, Thiel V, Siddell SG, Cavanagh D, Britton P. Reverse genetics system for the avian infectious coronavirus bronchitis virus. J Virol 2001;75:12359–12369.
21. Cavanagh D, Davis PJ, Pappin DJC, Binns MM, Boursnell MEG et al. Coronavirus IBV: partial amino terminal sequencing of spike polypeptide S2 identifies the sequence Arg-Arg-Phe-Arg-Arg at the cleavage site of the spike protein propolypeptide of IBV strains Beaudette and M41. Virus Res 1986;4:133–143.
22. Valastro V, Holmes EC, Britton P, Fusaro A, Jackwood MW et al. S1 gene-based phylogeny of infectious bronchitis virus: an attempt to harmonize virus classification. Infect Genet Evol 2016;39:349–364.
23. Daveelaar FG, Kouwenhoven B, Burger AG. Occurrence and significance of infectious bronchitis virus variant strains in egg and broiler production in the Netherlands. Vet Q 1984;6:114–120.
24. Bijlenga G, Cook JKA, Gelb J, de Wit JJ. Development and use of the H strain of avian infectious bronchitis virus from the Netherlands as a vaccine: a review. Avian Pathol 2004;33:550–557.
25. Gough RE, Cox WJ, de B Welchman D, Worthington KJ, Jones RC. Chinese QX strain of infectious bronchitis virus isolated in the UK. Vet Rec 2008;162:99–100.
26. Worthington KJ, Currie RJW, Jones RC. A reverse transcriptase-polymerase chain reaction survey of infectious bronchitis virus genotypes in Western Europe from 2002 to 2006. Avian Pathol 2008;37:247–257.
27. Geerligs HJ, Boelm GJ, Meinders CAM, Stuurman BGE, Symons J et al. Efficacy and safety of an attenuated live QX-like infectious bronchitis virus strain as a vaccine for chickens. Avian Pathol 2011;40:93–102.
28. Keep SM, Bickerton E, Britton P. Partial purification of IBV and subsequent isolation of viral RNA for next-generation sequencing. Methods Mol Biol 2015;1282:109–112.
29. Roche, GS. FLX Titanium DNA Rapid Library Preparation Method Manual: Mannheim; Roche, 2008.
30. Chevreux B, Wetter T, Suhai S. Genome sequence assembly using trace signals and additional sequence information. Comput Sci Biol 1999;99:45–56.
31. Bonfield JK, Whitwham A. Gap5 – editing the billion fragment sequence assembly. Bioinformatics 2010;26:1699–1703.
32. Staden R, Beal KF, Bonfield JK. The Staden package, 1998. Methods Mol Biol 2000;132:115–130.
33. Keep SM, Bickerton E, Britton P. Transient dominant selection for the modification and generation of recombinant infectious bronchitis coronaviruses. Methods Mol Biol 2015;1282:115–133.
34. Cook JKA, Orbell SJ, Woods MA, Huggins MB. Breadth of protection of the respiratory tract provided by different live-attenuated infectious bronchitis vaccines against challenge with infectious bronchitis viruses of heterologous serotypes. Avian Pathol 1999;28:477–485.
35. Cavanagh D, Elus MM, Cook JKA. Relationship between sequence variation in the S1 spike protein of infectious bronchitis virus and the extent of cross-protection in vivo. Avian Pathol 1997;26:63–74.
36. Britton P, Evans S, Dove B, Davies M, Casais R et al. Generation of a recombinant avian coronavirus infectious bronchitis virus using transient dominant selection. J Virol Methods 2005;123:203–211.
37. Bickerton E, Keep SM, Britton P. Reverse genetics system for the avian infectious coronavirus bronchitis virus. In: Perez DR (ed).
Reverse Genetics of RNA Viruses: Methods and Protocols, Methods in Molecular Biology (vol. 1602). New York: Springer; 2017. pp. 83–102.

Andrews S. FastQC – a quality control tool for high throughput sequence data; 2010. https://www.bioinformatics.babraham.ac.uk/projects/fastqc/

Krueger F. Trim Galore! 2019. https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/

Bushnell B. BBMap: a fast, accurate, splice-aware aligner (Lawrence Berkeley National Laboratory report no. LBNL-7065E). 9th Annual Genomics of Energy and Environment Meeting, Walnut Creek, CA, USA, March 17–20; 2014

Li H, Durbin R. Fast and accurate short read alignment with Burrows-Wheeler transform. Bioinformatics 2009;25:1754–1760.

Zhang J, Kobert K, Flouri T, Stamatakis A. PEAR: a fast and accurate Illumina Paired-End reAd merger. Bioinformatics 2013:30:614–620.

Li H, Handsaker B, Wysoker A, Fennell T, Ruan J et al. The Sequence Alignment/Map format and SAMtools. Bioinformatics 2009;25:2078–2079.

Britton P, Cavanagh D. Nidovirus genome organization and expression mechanisms. In: Perlman S, Gallagher T, Snijder EJ (eds). Nidoviruses: Washington, DC: ASM; 2008. pp. 29–46.

Williams GD, Chang RY, Brian DA. A phylogenetically conserved hairpin-type 3′ untranslated region pseudoknot functions in coronavirus RNA replication. J Virol 1999;73:8349–8355.

de Haan CAM, Volders H, Koetzner CA, Masters PS, Rottier PJM. Coronaviruses maintain viability despite dramatic rearrangements of the strictly conserved genome organization. J Virol 2002;76:12491–12502.

Züst R, Miller TB, Goebel SJ, Thiel V, Masters PS. Genetic interactions between an essential 3′ cis-acting RNA pseudoknot, replicase gene products, and the extreme 3′ end of the mouse coronavirus genome. J Virol 2008;82:1214–1228.

Yang D, Leibowitz JL. The structure and functions of coronavirus genome 3′ and 5′ ends. Virus Res 2015;206:120–133.

Vaughn EM, Halbur PG, Paul PS. Sequence comparison of porcine respiratory coronavirus isolates reveals heterogeneity in the S, 3 and 3′-1 genes. J Virol 1995;69:3176–3184.

Callebaut P, Correa I, Pensaert M, Jiménez G, Enjuanes L. Antigenic differentiation between transmissible gastroenteritis virus of swine and a related porcine respiratory coronavirus. J Gen Virol 1998;69:1725–1730.

Britton P, Cavanagh D. Avian coronavirus diseases and infectious bronchitis vaccine development. In: Thiel V (ed). Coronaviruses: Molecular and Cellular Biology. Norfolk: Caister Academic Press; 2007. pp. 161–181.

McKinley ET, Jackwood MW, Hilt DA, Kissinger JC, Robertson JS et al. Attenuated live vaccine usage affects accurate measures of virus diversity and mutation rates in avian coronavirus infectious bronchitis virus. Virus Res 2011;158:225–234.

Sola I, Almazán F, Zúñiga S, Enjuanes L. Continuous and discontinuous RNA synthesis in coronaviruses. Annu Rev Virol 2015;2:265–288.

Bentley K, Armesto M, Britton P. Infectious bronchitis virus as a vector for the expression of heterologous genes. PLoS One 2013;8:e67875.

Tung FYT, Abraham S, Sethna M, Hung SL, Sethna P et al. The 9-kDa hydrophobic protein encoded at the 3′ end of the porcine transmissible gastroenteritis coronavirus genome is membrane-associated. Virology 1992;186:676–683.

Hajjema BJ, Volders H, Rottier PJM. Live, attenuated coronavirus vaccines through the directed deletion of group-specific genes provide protection against feline infectious peritonitis. J Virol 2004;78:3863–3871.

Cruz JLG, Sola I, Becares M, Alberca B, Planas J et al. Coronavirus gene 7 counteracts host defenses and modulates virus virulence. PLoS Pathog 2011;7:e1002090.

Dedeuwerdaer A, Olyslaegea DJ, Desmaraets LMB, Roukaerts IDM, Theuns S et al. ORF7-encoded accessory protein 7a of feline infectious peritonitis virus as a counteragent against IFN-α-induced antiviral response. J Gen Virol 2014;95:393–402.

Ortego J, Sola I, Almazán F, Ceriani JE, Riquelme C et al. Transmissible gastroenteritis coronavirus gene 7 is not essential but influences in vivo virus replication and virulence. Virology 2003;308:13–22.

Dedeuwerdaer A, Desmaraets LM, Olyslaegea DJ, Vermeulen BL, Dewerchin HL et al. The role of accessory proteins in the replication of feline infectious peritonitis virus in peripheral blood monocytes. Vet Microbiol 2013;162:447–455.

Mathews Group. RNAstructure tools, version 6.0.1; 2018. https://rna.urmc.rochester.edu/RNAstructureWeb/Servers/Predict1.Predict1.html

Moreno JL, Zúñiga S, Enjuanes L, Sola I. Identification of a coronavirus transcription enhancer. J Virol 2008;82:3882–3893.

van Vliet ALW, Smits SL, Rottier PJM, de Groot RJ. Discontinuous and non-discontinuous subgenomic RNA transcription in a nidovirus. EMBO J 2002;21:6571–6580.

Di H, Madden JC, Morantz JE, Tang H-Y, Graham RL et al. Expanded subgenomic mRNA transcription and coding capacity of a nidovirus. Proc Natl Acad Sci USA 2017;114:E8995–E8904.

Irigoien N, Firth AE, Jones JD, Chung BY-W, Siddell SG et al. High-resolution analysis of coronavirus gene expression by RNA sequencing and ribosome profiling. PLoS Pathog 2016;12:e1005473.

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