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Seven infectious bronchitis virus genotypes including South American-origin G1-11 and Asian-origin GVI-1 circulated in southern African poultry from 2010 to 2020

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ABSTRACT

Infectious bronchitis virus (IBV) affects the respiratory, urogenital and reproductive systems of chickens and causes major economic losses. Biosecurity and vaccinations are used to limit the disease's impact, and identifying the circulating strains is important for selecting appropriate vaccines. The partial spike (S1) genes of 364 IBVs, isolated from commercial chickens in Botswana, Eswatini, Namibia and South Africa from 2010 to 2020, were phylogenetically analyzed. Seven genotypes were identified: 184 viruses (50,5 %) were classified as genotype GI-19 (QX) and 78 (21,4 %) were GI-1 (Mass/H120). Thirty-nine (10,7 %) were genotype GI-13 (4/91), 29 (8,0 %) were GVI-1 (TC07–2), 19 (5,2 %) were GI-23 (Variant 2), and 13 (3,6 %) were GI-11 (UFMG/G-Brazil). Two (0.5 %) viruses belonged to the GIV-1 (DE/072/92) genotype. Genotype GI-11 had not been reported outside South America before but has evidently circulated in South Africa for at least a decade. Similarly, genotype GVI-1, previously thought to be restricted to Asia, has been present in southern Africa since at least 2010. Prior to 2013, only Mass and H120 vaccines were permitted to be used in South Africa, but since 2013 793/B (GI-13), QX (GI-19), 4–91 (GI-13) and Variant 2 (GI-23) live attenuated vaccines were permitted. Accordingly, the four IBV variants we identified were putative recombinants of genotypes G1–1 and G1–19, G1–13 and G1–19, or G1–13 and unknown IBV strains, but these variant viruses did not spread extensively or persist in the region. The phylogenetic evidence points to imported contaminated poultry and poultry products as the source of new IBV genotypes in southern Africa.

1. Introduction

Infectious bronchitis (IB), caused by infectious bronchitis virus (IBV), is a highly contagious and costly disease of poultry of all ages, which occurs worldwide. The virus is transmitted between chickens in respiratory droplets and by fecal ingestion. It replicates in the epithelial cells of the respiratory, digestive and urogenital tracts, causing severe respiratory disease, depressed feed intakes, reduced egg production and malformed eggs (Gallardo et al., 2010). During the initial respiratory tract infection, the tracheal cilia are destroyed, predisposing the host to secondary infections with opportunistic pathogens like *Escherichia coli* and mycoplasmas, with increased deaths (Jackwood, 2012). Although vaccines are available, IB continues to be a severe economic problem in poultry production because many different serotypes of IBV exist and vaccines are not cross-protective (Cavanaugh and Naqi, 1997).

IBV is a single-stranded, positive sense RNA virus of the family *Coronaviridae*, genus *Gammacoronavirus*, known for their ability for rapid replication, high mutation rates, and homologous genomic RNA recombination, that generates extensive genetic diversity (Jackwood, 2012). The genome of ~ 27.6 kb encodes several non-structural proteins which are essential for viral replication such as the RNA-dependent RNA polymerase (RdRp), and structural proteins. The structural proteins include the spike (S) protein, matrix (M) protein, envelope (E) protein, and nucleocapsid (N) protein (Cavanagh, 2007). The glycosylated S protein trimer projects from the surface of the viral membrane to facilitate virus binding and entry to host cells (Niesters et al., 1987; Koch et al., 1990) and is the major inducer of neutralizing antibodies (Cavanaugh and Naqi, 1997; Winter et al., 2008). In the infection

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process, the S protein is enzymatically cleaved into an amino-terminal S1 subunit of ~535 amino acids and a carboxyl-terminal S2 subunit of ~627 amino acids. Most of the antigenic variability that determines the serotype, as well as the receptor binding site, resides in the S1 subunit. The heterogeneity in S1 is largely contained within three hypervariable regions (HVRs) located at amino acids 38–67, 91–141 and 274–387 (Cavanagh et al., 1988; Moore et al., 1997). IBV serotypes may differ by 20 % to 25 % at the genomic scale, and up to 50 % of amino acids in the S1 protein (Cavanagh et al., 2005), therefore the analysis of the complete or partial S1 gene nucleotide sequence, encompassing the HVRs, is conventionally used to classify IBV strains phylogenetically (Valastro et al., 2016) for the purposes of surveillance, and vaccine selection (Jackwood and de Wit, 2013). Eight genotypes are currently described (GI- GVIII). GI includes 31 genetic lineages (G1- to G1–31) while each of the other genotypes has only one lineage. The GI-1 and G1–13 lineages represent the old Massachusetts (Mass) and 793B lineages, respectively which are distributed globally and most commonly used as live attenuated vaccines, while other lineages are restricted to specific regions of the world (Valastro et al., 2016; Chen et al., 2017; Ma et al., 2019; Domanska-Bilcharz et al., 2020; Brown et al., 2020; Bali et al., 2022).

South Africa has the largest and most developed commercial poultry industry in the southern African region (SAPA, 2022), where IBV has been present since the 1970′s (Morley and Thompson, 1984). Biosecurity measures and vaccination are applied to control the disease, and up until 2013 only Mass and H120 live attenuated vaccines (genotype G1–1) were permitted. Limited data is available for the IBV strains that are found in southern Africa, where a single previous study in South Africa in 2011–2012 identified Mass-type (G1–1) and QX-like (G1–19) viruses (Knoetze et al., 2014). In the present study, IBVs which circulated in commercial chicken flocks in South Africa, Botswana, Eswatini and Namibia from 2010 to 2020 were isolated, sequenced and phylogenetically classified/compared to gain a better understanding of the epidemiology of IBV in the region.

2. Materials and methods

2.1. Virus isolation

Clinical samples from commercial flocks with suspected IBV infection were submitted by clients to Deltamune (Pty) Ltd, Pretoria, South Africa, for diagnosis. Organ swabs were placed directly into 9mLs of sterile virus isolation medium (VIM) consisting of 10 mL Antibiotic x Antimycotic solution (Gibco, Thermo Fisher Scientific, Waltham, Massachusetts, USA), 2 mL of gentamycin (Sigma-Aldrich, Saint Louis, USA), 2.5 g of bovine serum albumin (Roche Diagnostics GmbH, Mannheim, Germany) and 500 mL of phosphate buffered saline (PBS) (Gibco, Thermo Fisher Scientific, Waltham, Massachusetts, USA), pH 7.0 - 7.4. Epithelial cell scrapings and exudates from tracheas and caecal tonsils were added to VIM. Organ samples were macerated before being added to VIM. Tissue and swab suspensions were vortexed, clarified by centrifugation at 4000 rpm for 10 min, and filtered through a 0.22 μm filter. The filtrates were inoculated into six 9–11-day-old specific pathogen free (SPF) hens' eggs (Avi-farms Pty Ltd, Pretoria, South Africa) via the allantoic sac route. Any embryo mortalities that occurred during the 7-day incubation period (excluding any first day nonspecific deaths) were harvested. The diagnosis of IBV was made based on embryo morphology and confirmed with IBV-specific real-time reverse transcription PCRs (RT-qPCR) (Callison et al., 2006). Allantoic fluids were aliquoted in 1.0 mL sterile cryotubes and stored at −80 °C.

2.2. RNA extraction and reverse transcription PCR (RT-PCR)

Total nucleic acids were extracted from the allantoic fluids using a QIAcube HT automated 96-well extraction machine (Qiagen, Hilden, Germany) with IndiSpin QIAcube HT Pathogen kits (Indical Bioscience, Leipzig, Sachsen, Germany). The nucleic acid eluates in 50 μl volumes were stored at −20 °C until testing. Primers S17F and S18R, that target the first 720 nucleotides of the S1 gene and encompass the HVRs (Gallardo et al., 2010), were used at 10 pmol concentrations in conventional RT-PCR reactions. Reaction mixes in 20 μl volumes consisted of 2X Phusion Flash Master Mix, 0.3 μl Moloney Murine Leukemia Virus (MMLV) reverse transcriptase enzyme, 0.2 μl of RNAse Inhibitor (all ThermoFisher Scientific, Waltham, Massachusetts), and 5 μl of the test sample nucleic acid. The thermal cycling conditions were as previously described (Gallardo et al., 2010). RT-PCR products were electrophoretically separated and visualized in 1 % agarose gels stained with ethidium bromide. Amplicons of the anticipated size of ~745 bp were extracted with a QIAquick Gel extraction kit (Qiagen, Hilden, Germany), according to the recommended procedure. DNAs were quantified with a NanoDrop spectrophotometer and submitted to Inqaba Biotechnical Industries (Pty) Ltd. in Pretoria for Sanger DNA sequencing.

2.3. Sequence and phylogenetic analysis

DNA sequences were visualized in Chromas Lite software (Version 2.6.6) (Technelysium Pty Ltd), and edited where necessary (visual base calling). Forward and reverse sequences for each sample were assembled into a consensus sequence in BioEdit software (Version 7.2.5) (Hall, 1999). Reference sequences for IBV genotypes (Bali, et al., 2022; Houta et al., 2021) were downloaded from the National Center for Biotechnology Information (NCBI) nucleotide database (https://www.ncbi.nlm.nih.gov/nucleotide). The closest relatives to the sequenced cases identified by Basic Local Alignment Search Tool (BLAST) homology searches were retrieved from the GenBank nucleotide sequence database (https://blast.ncbi.nlm.nih.gov/Blast.cgi). All sequences were then aligned using MAFFT Version 7 (https://mafft.cbrc.jp/alignment/server/index.html) (Katoh, et al., 2019; Kuraku, et al., 2013). Maximum likelihood phylogenetic trees were reconstructed via the Ultrafast method in IQ-TREE (Version 2.2.2) (Trifinopoulos, et al., 2016) with 1000 bootstrap replicates (http://iqtree.cibiv.univie.ac.at/) and were visualized in the Figtree (Version 1.4.4). Sequences of the partial S1 genes were deposited in Genbank under accession numbers OR727107 to OR727111 and OR742726 to OR742766. Where multiple sequences had 100 % nucleotide sequence identity, a single representative sequence was submitted to Genbank.

2.4. Whole genome sequencing and assembly

Whole transcriptome amplification libraries were prepared from 14 μl volumes of the total nucleic acids extracted from allantoic fluid, using a Sigma-Aldrich Complete Whole Transcriptome Amplification (WTA2) kit (Merck KGaA, Darmstadt, Germany), according to the recommended protocol. The PCR products were submitted to the Central Analytical Facility and the University of Stellenbosch for sequencing in an Ion Torrent Ion GeneStudio S5 Prime System (ThermoFisher Scientific, Waltham, MA, USA). In CLC Genomics Workbench v24.0.2 (Qiagen, Aarhus, Denmark), IBV genomes were assembled from the quality-trimmed sequence reads using a combination of *de novo* contig assembly and assembly-to-reference approaches, using IBV reference genomes with the accession numbers PP228887, MK937833, MN548289, OM912684, KU356856 and KU900739 as scaffolds. Open reading frames were identified and checked in BioEdit (Version 7.2.5) software. Genome sequences were deposited in Genbank under accession numbers PQ738163 to PQ738166.

2.5. Recombination analysis

To detect any potential recombination events, the S1 gene sequences (~1600 bp) were extracted from the assembled genomes. BLAST searches of 200 bp sections across the length of each S1 gene retrieved the closest relatives from the Genbank nucleotide sequence database. After excluding the duplicates, the final multiple sequence alignment

was analyzed using the RDP, GENECOV, BootScan, MaxChi, Chimaera, SiScan and 3Seq methods in the Recombination Detection Program (RDP) version 4.101 (Martin et al., 2021). Putative recombination events supported by at least four methods with P values below 1×10^{-7} were considered valid.

3. Results

Field IBVs from South Africa ($n = 379$), Namibia ($n = 7$), Botswana ($n = 1$) and Eswatini ($n = 1$) were isolated (Supplementary Table 1), the partial S1 gene sequences were deduced, and phylogenetically classified into genotypes. Twenty-four isolates (6,2 %) contained coinfections of more than one virus genotype, where the DNA sequencing electropherograms showed overlapping peaks, and were not evaluated further. The remaining 364 isolates were classified into seven defined genetic lineages (Fig. 1). One hundred and eighty-four 184 (50,5 %) viruses, including one each from Botswana and Eswatini, were identified as GI-19 (QX) genotype strains and 78 (21,4 %) as the GI-1 (Mass/H120) genotype. Thirty-nine (10,7 %) isolates, including two from Namibia, were identified as the GI-13 (4/91) genotype, twenty-nine (8,0 %), including five from Namibia, as the GVI-1 (TC07–2) genotype, nineteen (5,2 %) as the GI-23 (Variant 2 or Var 2) genotype, and thirteen (3,6 %) as the GI-11 (UFMG/G - Brazil) genotype. Two (0.5 %) viruses were closely related to the GIV-1 (DE/072/92) genotype. Each of these genotypes are discussed below.

Genotype GI-1 (Mass/H120-like)

The GI-1 lineage contains the earliest known IBV strains. Discovered in the USA in the 1930′s, it is the most widely distributed lineage because

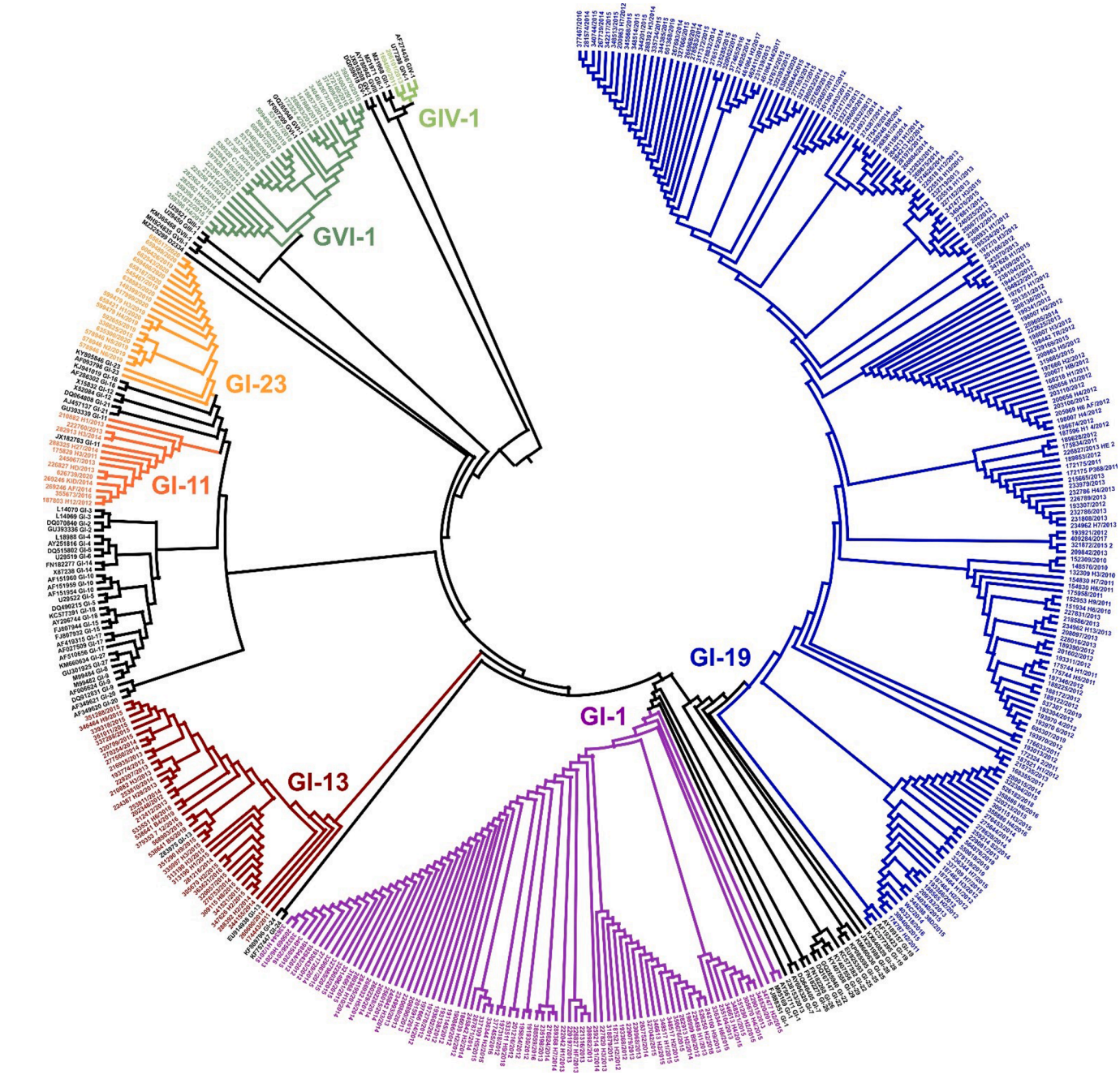

Fig. 1. Genotyping Maximum Likelihood phylogenetic tree based on 434 nucleotides of the infectious bronchitis virus S1 gene. Reference strains are in black and southern African viruses sequenced in this study are coloured.

the extensive use of live attenuated vaccines produced from it (Valastro et al., 2016). That 21 % of the isolates recovered from South African flocks were identified as GI-1 was unsurprising, given the wide application of Mass and H120 vaccines that have been used in the country since the mid-1990′s (Supplementary Figure 1). Most of these isolates are likely to be reisolated vaccines, but strain 259214_S1/2014, isolated from breeder birds in the North West province, formed an outgroup with no close relatives in the sequence database. The complete genome of this variant virus was sequenced, and subsequent recombination analysis of the S1 gene putatively identified a genotype GI-19 virus as a major parent and genotype GI-1 virus as the minor parent (Table 1).

Genotype GI-11 (UFMG/G Brazil-like)

The GI-11 lineage has until now been reported to be restricted to South America. It emerged in Brazil in the 1950s, where it continues to predominate, and has spread in Argentina, Uruguay and Colombia (Marandino and Pérez, 2021; Ramirez-Nieto et al., 2022). The only live attenuated vaccines approved for use in Brazil are genotype GI-1 Mass and H120 strains (Saraiva et al., 2018), yet *in vivo* studies showed that these vaccines provide inadequate protection against some of the Brazilian variants (Cook et al., 1999; Di Fabio et al., 2000). Thirteen IBV isolates collected from South African broiler or layer flocks, from the Gauteng, North West, Mpumalanga, Western Cape and KwaZulu-Natal provinces between 2011 and 2020 were identified as the Brazilian GI-11 genotype in this study (Supplementary Figure 2). The South Africa isolates formed a monophyletic clade nested within Brazilian viruses (supported by a bootstrap value of 99 %), pointing to a single introduction event which predates 2011, with continuous but low-prevalence circulation until 2020 (Fig. 2). No genotype GI-11 variant vaccine is currently registered for use in South Africa.

Genotype GI-13 (793B-like)

GI-13, first described in the UK in 1991, can be traced back to an isolate from Morocco in 1987, originally called the "793B" type or "4/91″ type (Valastro et al., 2016). There was marked genetic heterogeneity among the 39 South African isolates classified as genotype GI-13. Various commercial attenuated live vaccines of this type have been registered for use in South Africa since 2013. The number of genotype GI-13 viruses isolated as a proportion of cases peaked in 2015, but declined thereafter (Fig. 2), possibly as a result of vaccine use. Sub-clades (a) to (d) were designated in Supplementary Figure 3 for discussion purposes only. Sub-clade (a) contained only Chinese strains along with seventeen South African IBVs isolated from 2012 to 2015, mainly broilers or layers from the North West, Free State and Gauteng provinces, and one case from Mpumalanga in 2012. Five of the eight South African viruses in Sub-clade (b) shared high sequence homology with IBVs originating from a wide range of countries such as India, Poland, China and Mexico, and likely represent reisolated vaccines. In sub-clade (b) a smaller group of viruses was rooted with a Chinese strain (MT766966), to which isolates 538641/2019 (broilers, Namibia) and 568903/2019 (breeders, Mpumalanga province) appeared to be closely related. Longer branches within this cluster linked an isolate from Thailand in 2015 (MG190989) with two distinct viruses, Ukrainian strain IBVUkr27–11 (KJ135013) from 2011, and isolate 238153/2013, a South African virus isolated from a grandparent flock in the KwaZulu-Natal province. The complete genome of 238153/2013 was sequenced and two possible recombination events were identified in the S1 gene. The first had breakpoints at nucleotides 63–297 where the major parent was a genotype GI-19 virus and the minor parent a genotype G1–1 strain, and the second was located at the 3′-end of S1, at nucleotide 1470, where the major parent was a genotype GI-13 virus and the minor parent a GI-19 virus (Table 1). Sub-clade (c) contained two South African IBVs, 244155/2014 (broilers, Free State province), apparently closely related to strain Polish strain D2353/2013/PL (MT984600), and JQ946056, the IBVAR 223A vaccine from Israel. The latter was the first "793/B" vaccine registered for use in South Africa, in 2013. Another vaccine strain, namely 260600/2014 was sequenced in this study and also fell within sub-clade (c). Eleven South African IBVs clustered closely together in sub-clade (d), and they were mainly isolated from broilers in Gauteng, but also the North West and Free State provinces, from 2014 to 2016. Their closest relative was strain Spain/99/327 (DQ386097), previously classified as "Spanish Genotype 1" (Dolz et al., 2008). Finally, isolate 174443/2011 (layers, Western Cape province), formed an outgroup to all the GI-13 local and international viruses. The complete genome was sequenced, and two potential recombination breakpoints were detected in the S1 gene, the first at nucleotides 379–624 where the major parent was a genotype GI-13 strain and the minor parent was an unknown virus, and the second breakpoint at position 1063–1211 was associated with a major parent from genotype G1–13 and a minor parent from genotype GI-19 (Table 1). BLAST analysis of the S1 gene of variant 174443/2011 retrieved a top hit with 88.53 % nucleotide sequence homology (GI-13

Table 1
Recombination in the S1 genes of variant IBVs detected in South Africa.

Isolate	Breakpoint positions		Major parent			Minor parent			Probability value	Detection methods RGBMCS3
	Begin	End	Strain	Genotype	Similarity	Strain	Genotype	Similarity		
259214/2014 (GI-1 variant)	1490	undetermined	IBV298	GI-1	96.4 %	05/P2-D5/Tr3	GI-19	96.8 %	5.681×10^{-19}	+ + - + - + -
238153/2013 (GI-13 variant)	63	297	KF809784 gCoV/Ck/ Poland/548/ 2004	GI-19	80 %	ON081494 IBV298	GI-1	98.3 %	2.140×10^{-18}	+ + + + + + +
	1470	undetermined	MK581205 IBVUkr27–11	GI-13	98.7 %	KF809784 11,518/ Kiskunmajsa/ 2010/HU	GI-19	99.3 %	7.247×10^{-21}	+ + + + + + +
174443/2011 (GI-13 variant	379	624	KJ135013 IBVUkr27–11	GI-13	95.1 %	MT984585 unknown	unknown		2.278×10^{-7}	+ + + + + - +
	1063	1212	KJ135013 IBV/ck/MEX/ 2826/21			D535/4/FR/05				
			OM912701	GI-13	89 %	OM525803	GI-19	96 %	5.547×10^{-17}	+ + + + + + +
565876/2019 (GI-19 variant)	613	702	D535/4/FR/05 OM525803	GI-19	98.4 %	IBVUkr27–11 KJ135013	GI-13	100 %	2.318×10^{-19}	+ + - + + - +

RGBMCS3: RDP, GENECOV, BootScan, MaxChi, Chimaera, SiScan, 3Seq.

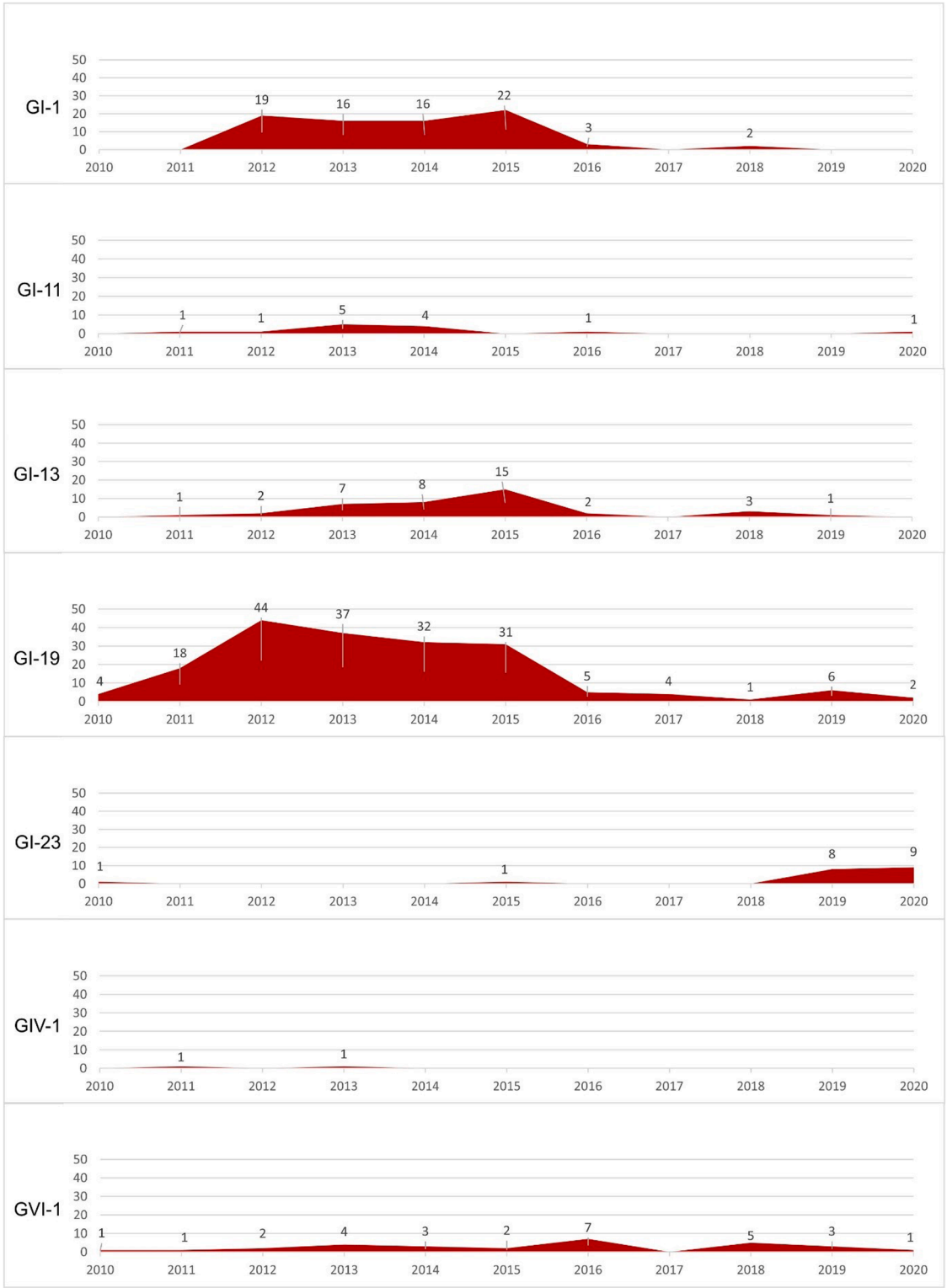

Fig. 2. Relative numbers of Infectious Bronchitis Virus genotypes isolated in southern Africa from 2010 to 2020.

strain FR-CR88061–88; AJ618986), and at the full genome level the closest relative was 90.77 % identical (strain ck/CH/LHB/111268; KJ425491). This virus does not meet the criteria for classification as a new IBV lineage (Valastro et al., 2016), as at least two additional two related viruses collected from at least two distinct outbreaks would be required, and together they would need to form a statistically-supported monophyletic cluster with at least 13 % uncorrelated pairwise distances.

Genotype GI-19 (QX-like)

The GI-19 lineage is believed to have originated in China decades ago before spreading to other Asian countries, Europe, and Africa (Franzo et al., 2017). Of the 184 South African isolates identified as GI-19, 18 (92 %) formed a monophyletic clade that spanned the period 2010–2018 (Supplementary Figure 4). The number of field isolates in the region peaked in 2012 but began to decline after 2015 (Fig. 2), possibly because of the use of live attenuated QX-like vaccines that were registered in the country from 2013. Strains isolated after 2018 appeared to be more

recent introductions, with multiple sources. A cluster of eight South African IBV isolates from broilers in the Free State, Gauteng, Western Cape and North West provinces from 2014–2016, were identical in sequence to European commercial live attenuated QX vaccine strain L1148 (KY933090) (Listorti et al., 2017), consistent with this vaccine's registration for local use in 2013. Two other strains, 336344/2015 (broilers, Gauteng province) and 337109/2015 (broilers, Free State province) appeared to share a recent common ancestor with IBVs from Greece in 2013 (MT984599) and Romania in 2011–2012 (MT984595, MT984602). Further down the tree, 550819/2019 (broilers, Western Cape province) was identical in sequence to a strain from China in 2011 (KC795604), but was located in the same cluster as isolate 229081/2013 (Gauteng province) and an Italian virus from 2013 (MK491751). Isolates 564876/2019 (broilers, location unknown) and 579119/2019 (broilers, Eastern Cape province) formed outgroups to all GI-19 viruses and were selected for whole genome sequencing and further analysis. During Ion Torrent data analysis, it became apparent that 579119/2019 contained a second genotype in the minority of sequence reads, and we were thus unable to complete the assembly and analysis. The complete genome of the other GI-19 variant, 564876/2019 was determined, and recombination analysis detected a breakpoint at positions 613–702 where the major parent was genotype G1–19 and the minor parent was a genotype GI-13 strain (Table 1), which accounts for the outgroup positioning of this variant in the phylogenetic tree.

Genotype GI-23 (IB Var 2-like)

Strains of the GI-23 lineage were detected since 1998 in Israel and remained restricted to the Middle East region for a period (Valastro et al., 2016), but in recent years it has spread to several other countries in Asia, Africa, Europe and South America (Trevisol, et al., 2023; Finger et al., 2024). According to the data from this study, GI-23 was present in South Africa as early as 2010 (Supplementary Figure 5). Isolate 149, 399_2010 from broilers in the Western Cape province, was closely related (99 %) to isolates collected during the 2018–2019 period in Poland (MZ666025, MZ666060, MZ666059 and MZ666056). Vaccines for GI-23 were only registered in South Africa in 2015, therefore 149399/2010 was most likely a field strain. The incidence of GI-23 began to increase in South Africa in 2019–2020 (Fig. 2), affecting broilers in the North West, Free State, Gauteng and Western Cape provinces. The South African genotype G1–23 strains did not form a regional cluster but instead were interspersed with multiple highly similar strains that circulated in Turkey, Poland and Romania during the same period.

Genotype GIV-1 (USA lineage-like)

Genotype GIV-1 is a North American indigenous lineage typified by Delaware variant DE072, which was first described in the early 1990′s in USA broiler flocks. Initially this variant caused severe respiratory infections but later became associated with decreased egg production in layers (Valastro et al., 2016; Rafique et al., 2024). In the present study two isolates from broiler flocks were phylogenetically classified as genotype GIV-1 (Supplementary Figure 6), one in the North West province in 2011 (169490/2011) and the other from Mpumalanga in 2013 (206101/2013). The closest relatives were Canadian strains (accession numbers KJ196131, KJ196165 and KJ196193) isolated between 2003 and 2009 (Martin et al., 2014). No North American-lineage vaccines are used in South Africa. The GVI-1 genotype was only detected for a very limited period in South Africa and wasn't isolated after 2013 (Fig. 2).

Genotype GVI-1 (Asian TC07–2 -like)

Genotype GVI-1 reportedly emerged in Asia where the prototype strain TC07–2 was initially detected in China, subsequently spread to South Korea, Vietnam and Japan, but was also identified in Columbia in 2003 (Ren et al., 2019; Rafique et al., 2024). Our results show that GVI-1 has been present in southern Africa since at least 2010 (Supplementary Figure 7), and no GVI-1 vaccines are registered for use in South Africa. Twenty-nine of the isolates we sequenced, including five from Namibia, were classified as genotype GVI-1, with three distinct sub-clades, labeled (a), (b) and (c), evident within the southern African isolates. Sub-clade GVI-1(a) contained five viruses isolated from Namibian broiler flocks from 2013 to 2016, plus breeders in South Africa's Gauteng province (321872/2015), layers in the Eastern Cape province (2235677/2013) and broilers in Mpumalanga province (233942_H5/2013). Sub-clade GVI-1(b) included the viruses isolated from broilers or breeder birds in multiple provinces of South Africa (Northwest, Western Cape, and Gauteng), from 2018–2020. Sub-clade GVI-1(c) similarly contained isolates from broiler and layer flocks of multiple provinces in South Africa, including Free State, Gauteng and Mpumalanga, from 2010–2016. Given the substantial genetic diversity in the southern African GVI-1 viruses, indicated by long branches in the phylogenetic tree, these viruses may have descended from a common progenitor which was introduced to the region prior to 2010, that subsequently diversified into the three genetically-distinct groups we identified. Alternatively, there have been at least three separate introductions of genotype GVI-1 strains into the region, with no publicly-available sequences for the international ancestors.

4. Discussion

IBV is highly adept at mutating to evade host immunity, which over several decades of expanding intensive poultry production and widespread vaccination, gave rise to a large number of serotypes and genetic lineages (Cavanagh and Naqi, 1997). Biosecurity in combination with vaccination is used to limit the impact of IB on flocks, and identifying the circulating strains is important to selecting appropriate vaccines (de Wit et al., 2011). Public data on the IBV strains that circulate in southern African poultry is limited, and a single previous study in South Africa in 2011–2012 identified only Mass-type (GI-1) and QX-like (GI-19) viruses (Knoetze et al., 2014). The present study is the largest of its kind in the subregion thus far, where in addition to the aforementioned genotypes, genotype GI-11, GI-13, GI-23, GI-IV and GI-VI viruses were also identified. A surprising and novel finding was that the Brazilian genotype (GI-11), which has not been reported outside of the South American continent up until now, has evidently been circulating in South Africa for quite some time. Similarly, our results showed that genotype GVI-1, previously thought to be restricted to Asia (Rafique et al., 2024), has been present in southern Africa since at least 2010.

Vaccination with live attenuated vaccines reduces the incidence of IB (Lian et al., 2021), indeed our results showed genotype GI-19-related outbreaks which peaked in 2012 in South Africa, began to decline in 2013, coinciding with the first registration and use of live attenuated QX vaccines, and that genotype GI-13-related outbreaks which peaked in 2015, similarly declined after variant vaccines were first approved for use in 2013. The use of live attenuated vaccines, however, also increases the risk of recombination with field strains, and the frequency of gene recombination between vaccine strains and field strains has increased over the past decades (Lim et al., 2012; Lian et al., 2021). Prior to 2013, only Mass and H120 vaccines were permitted to be used in South Africa, but since 2013 793/B (GI-13), QX (GI-19), 4–91 (GI-13) and Var-2 (GI-23) live attenuated vaccines were approved. Accordingly, four of the IB variants we identified were putative recombinants of genotypes GI-1 and GI-19, GI-13 and GI-19, or GI-13 and unknown IBV strains, but these variant viruses did not spread extensively or persist in the region.

The S1 phylogenetic data showed that some IBVs, introduced to southern Africa pre-2010 from their known regions of origin continued to circulate, whereas other viruses were more recently introduced from North and South America, Europe and Asia, and that introductions of new IBV strains may be happening on a frequent basis. There is little information or research on how specific IBV strains that emerged in certain geographic regions eventually spread to other continents. Some authors have suggested that wild birds might play a role in disseminating IBVs across the world (de Wit et al., 2011). The natural host of IBV is the chicken, however, other Galliformes such as pheasants and partridges may also become infected with poultry-endemic IBV strains. Related *Gammacoronaviruses* have been isolated from other avian

species such as turkeys, teal, geese, pigeons, guinea fowl, ducks and other wild birds. However, these viruses are genetically and antigenically distinct from the IBV genotypes that infect chickens (Cavanagh et al., 2005; Marchenko et al. 2022). There is very little evidence for poultry endemic IBV strains in wild bird populations (Wille and Holmes, 2020), and in cases where such viruses, including vaccines, have been found in house crows, house sparrows or ducks amongst others, these were likely direct spill-over events from infected chicken premises (Wille and Holmes, 2020). Furthermore, onward transmission in other species, like ducks, is limited (Wille and Holmes, 2020) and the recent global upsurge in wild bird surveillance for clade 2.3.4.4B H5Nx high pathogenicity avian influenza viruses has failed to detect any co-infecting IBVs in migratory bird species thus far.

Two of the genotypes identified in South Africa, namely GIV-1 and GI-11, were geographically restricted to North or South America, respectively. There is no bird migration route across the Atlantic Ocean which links the African and American continents, but the USA and Brazil are the two single largest exporters of poultry products to South Africa. In 2021 Brazil accounted for 66.6 % of all poultry products (*i.e.*, meat, hatching eggs, day-old-chicks, and egg products) imported into South Africa, followed by the USA (15.6 %), European Union countries (8.8 %), Argentina (5.5 %), Canada (1.5 %) and others (2 %). South Africa only exports poultry products to neighboring southern African countries (SAPA, 2021). Because IB is not a controlled disease, there is no regulation to ensure that products are free of IBV before they are moved internationally (Leow et al., 2024), or that they originate from flocks free of IBV infection. The risk that IBV strains are being transmitted in these commodities is not negligible. For example, a genotype GI-19 (QX-like) virus was isolated from a chicken meat product imported into Malaysia from China, and cold chain practices prolong the survival of the virus in imported food products (Leow et al., 2024). Secondly, at a certain stage of infection there are high IBV titers in the oviduct, yet the ability for vertical transmission of IBV is largely dismissed in the scientific literature (Cook et al., 2012). In one study IBV was detected in the chorioallantoic fluid of live embryonated eggs, from hens that had been vaccinated with a live attenuated Mass vaccine (Pereira et al., 2016), so this possible transmission route warrants further research. Nonetheless, IBV transmission via surface contamination of the egg certainly occurs, as high titers of IBV are present in the cecal tonsils during infection (Bwala et al., 2018). Fortunately, IBV does not pose a risk to human health if consumed in an infected or contaminated poultry product, and is easily inactivated by heating at 56 °C for 15 min (Leow et al., 2024). The possibility that some commercial live attenuated IB vaccines may contain low-level contamination with other lineages should not be discounted either, especially if the seed strain was not deep sequenced by the manufacturer to verify its purity, although this is speculative and there are no such cases reported in the scientific literature.

Although South Africa is the largest poultry producer in the southern African region it remains a net importer of poultry products, because it cannot meet local demand. Increasing the production is constrained by factors such rising feed costs caused by droughts, rising cost input for transport, electricity and labor, and cheap or dumped brown chicken meat sold locally below the foreign cost of production through foreign government subsidies (SAPA, 2021; 2022). The devastating economic impacts of recurring outbreaks of high-profile diseases like high pathogenicity avian influenza on the South African poultry industry are quantified (SAPA, 2022), but the impact and costs of controlling "enzootic" diseases like IBV, are not. The size of the South African chicken production industry is roughly comparable to Canada's, as South Africa produces roughly 1.8 million tons of meat annually, and approximately 520 million dozen eggs (SAPA, 2022), in comparison to Canada's 1.3 million tons of chicken meat and 850 million dozen eggs (Puntsho, 2022). Notably, the impact of IB outbreaks on Canadian poultry industry was recently estimated to be around 207 billion Canadian Dollars annually (Puntsho, 2022). In conclusion, the phylogenetic evidence points to imported contaminated poultry and poultry products as the source of new IBVs in southern Africa. The costs of treating and preventing local IB outbreaks, in an already-constrained market, are likely substantial based on Canada's example. To protect local industries from new disease outbreaks, governments could require exporting countries to prove that poultry products originate from flocks that are free of IBV infection, or that consignments are free of IBV. Live attenuated IB vaccines will need to be monitored for purity and efficacy and updated as the field viruses continue to evolve, and new variants emerge.

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Institutional review board statement

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Informed consent statement

Not applicable.

CRediT authorship contribution statement

Christine Strydom: Writing – original draft, Investigation, Formal analysis, Data curation. **Celia Abolnik:** Writing – original draft, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.virusres.2025.199568.

Data availability

All the data generated have been detailed in Supplementary materials and sequence data was deposited in the Genbank database.

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