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New real time and conventional RT-PCRs for updated molecular diagnosis of infectious bronchitis virus infection (IBV) in chickens in Egypt associated with frequent co-infections with avian influenza and Newcastle Disease viruses

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A B S T R A C T

In Egypt, currently two geographically restricted genotypes of the infectious bronchitis *coronavirus* (IBV) are circulating with detrimental effects for poultry industry. A sensitive real-time RT-PCR assay targeting the IBV nucleocapsid gene (N) was developed to screen clinical samples for presence of IBV. Conventional RT-PCRs amplifying hypervariable regions (HVRs 1–2 and 3) of the IBV S1 gene were developed and amplificates used for nucleotide sequence-based typing of IBV field strains in Egyptian chickens directly from clinical samples.

In total, fifty samples from poultry comprising swabs, tissues, and allantoic fluid were examined. Twenty eight samples from chickens showed IBV-positive results. Genetic analysis of the HVRs 1–2 of seven samples revealed closest amino acid homology of 83.3-89.7% in four viruses and 96.1–97.7% in the others to the previously described Egyptian variant II (EG/12197B/2012), while all seven samples shared >98.2% amino acid homology at the HVR3 locus with that genotype.

In addition, in most of samples a high degree of co-infections with highly pathogenic AIV H5N1, low pathogenic H9N2, and Newcastle disease was found. Mixed infections in this study were detected in 19 out of 28 IBV positive samples. This indicates an intricate situation in Egyptian poultry populations with unknown putative synergistic effects on pathogenicity and spread of these pathogens. Effective control measures including vaccination may be severely compromised.

1. Introduction

Avian infectious bronchitis virus (IBV) is a member of the genus *Gammacoronavirus* in the *Coronaviridae* family (King et al., 2011). IBV induces an acute, highly contagious infectious disease of chicken. IBV is globally distributed and responsible for huge economic losses in the poultry industry. IBV was first reported in North Dakota, USA, as a novel respiratory disease affecting chickens (Schalk and Hawn, 1931). IBV infects initially the respiratory tract; for some IBV strains further virus spread may involve kidneys and oviduct causing reduction of growth rate, decreased performance and reduction of egg quality and quantity (Cavanagh, 2003). Also, some strains showing a shift of tissue tropism may cause proventriculitis (Yu et al., 2001).The infection is spread by aerosols, direct contact and indirectly through contaminated fomites (Ignjatovic and Sapats, 2000).

IBV harbors an unsegmented RNA genome of positive polarity which is approximately 27.6 kb in size and codes for four structural proteins: the spike (S) glycoprotein, the membrane (M) glycoprotein, the nucleocapsid (N) phosphoprotein, and the envelope (E) protein (Spaan et al., 1988). The N protein is a major structural protein, and highly conserved among different IBV serotypes The spike (S) glycoprotein, an integral membrane protein, is another major structural protein; it is cleaved post translationally into the

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S1 (N terminal part) and S2 fragments. Mature S proteins trimerize and form the globular head (S1) and the stalk domain (S2) of the viral peplomer spikes (Belouzard et al., 2012; Cavanagh, 2007). The S1 protein carries the receptor binding site and thus plays an important role in both tissue tropism and induction of protective immunity (Belouzard et al., 2012; Wickramasinghe et al., 2011). Numerous distinct serotypes have been described which differ by 20–25% and sometimes up to 50% in the S1 protein sequence (Adzhar et al., 1997). In particular, three hypervariable regions (HVRs; amino acid residues 38–67, 91–141 and 274–387) along the S1 gene are affected that elicit neutralizing and serotype-specific antibodies (Cavanagh et al., 1988; Koch et al., 1990; Moore et al., 1997). Variation in these epitopes has been implicated in escape from vaccine-induced immunity (Belouzard et al., 2012).Genotypes of IBV are classified based on the genetic variation of the S gene encoding the spike protein, in particular its S1 fragment (Belouzard et al., 2012; Cavanagh, 2007; Valastro et al., 2016).

Consequently, cross protection between these different serotypes is limited (Cavanagh, 2005; Jackwood, 2012; Kuo et al., 2010; Wickramasinghe et al., 2014). The S1 gene therefore is used for IBV strain differentiation (Gough et al., 1992; Kingham et al., 2000). New S1 genotypes of IBV that often show antigenic variation and, hence, define new serotypes, appear to emerge frequently in different parts of the world (Jackwood, 2012). A number of mutation processes account for the emergence and evolution of multiple serotypes (Cavanagh et al., 1992; Jackwood, 2012) including point mutations, insertions, deletions, and also recombination between strains (Adzhar et al., 1997; Hewson et al., 2014).

In Egypt, highly pathogenic avian influenza viruses (HPAIV) of subtype H5N1 and co-circulating low pathogenic AIV H9N2 have established endemic status (Naguib et al., 2015). Various chicken flocks are suffering from respiratory disease caused, at least in part, by infection with IBV and NDV (Abd El Rahman et al., 2015). Over the last decades, different genotypes of IBV have been recognized in Egypt which were related to the Massachusetts, D3128, D274, D-08880 and 4/91 genotypes (Abdel-Moneim et al., 2006; Jackwood, 2012; Sheble et al., 1986). In 2001, a unique Egyptian variant (type isolate: Egypt/Beni-Suef/01), closely related to an Israeli variant strain II, was identified in different chicken farms and classified as Egyptian variant I (Abdel-Moneim et al., 2002). In 2011, yet another genotype, Egyptian variant II (CK/Eg/BSU-2,3/2011), was isolated, and representatives of these genotypes have been co-circulating since with the previously detected classical (vaccine like) and variant IBV genotypes as mentioned above (Abdel-Moneim et al., 2012).

This study is aimed at providing novel molecular diagnostic tools that can be used to detect and characterize IBV genotypes circulating on chicken farms in Egypt. An N gene specific real time RT-PCR (RT-qPCR) specifically tailored to detect IBV circulating in Egypt complements with previously published RT-qPCRs targeting another IBV ORF e.g., (Callison et al., 2006). In addition, conventional RT-PCRs for nucleotide sequence analysis of the S1 gene HVRs have been developed and validated.

2. Materials and methods

2.1. Viruses

Fifty field samples including tracheal swabs, allantoic fluid (from isolation attempts), and tissues (kidney and trachea) were obtained in the frame of routine veterinary measures from commercial poultry farms showing severe respiratory problems and/or performance losses in Egypt between 2012 and 2014 (Table S1). Detailed information on species, type, age and mortality is presented in table S1. The locations of the farms are depicted in table S1 at the Governorate level. Swab samples from alive birds were jointly obtained according to standard procedures by authorized veterinarians of the National Laboratory for Veterinary Quality Control on Poultry Production (NLQP, Ministry of Agriculture, Giza) and the Faculty of Veterinary Medicine, Beni-Suef University (BSU), Egypt. Tissues were collected from birds that had died spontaneously. Following initial examination at these institutions samples were submitted to the Friedrich-Loeffler-Institut, Germany.

2.2. RNA extraction and molecular diagnosis

Viral RNA was extracted using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA was eluted in 70 μL nuclease-free water, aliquoted at 10 μL and stored at −20 °C until use. By using a newly developed RT-qPCR (see below), presence of N-specific IBV RNA was examined and positive samples were further subjected to two conventional RT-PCRs specific for hypervariable regions (HVRs) of the S1 gene of IBV (see below).

In addition, all samples were screened for presence of the influenza A virus matrix (M) gene (Fereidouni et al., 2012), and positive samples were further subtyped using RT-qPCRs specific for subtypes H5 and H9 (Monne et al., 2008). Also, samples were examined for NDV RNA using an RT-qPCR specific for the avian paramyxovirus-1 matrix gene (Wise et al., 2004). PCR reactions for AIV and NDV were performed in 25 μL volumes using SuperScript® III One-Step RT-PCR kit with Platinum® Taq DNA Polymerase (Invitrogen) on a CFX96 thermocycler machine (Bio-Rad). Pathotyping of H5- and NDV-positive samples was achieved by sequencing of the HA (Naguib et al., 2015) or F gene cleavage sites, respectively (Aldous et al., 2001).

2.3. Development and validation of IBV-specific PCRs

Primers were selected based on alignments of the N and S genes of a selection of avian gamma-coronavirus sequences available from Genbank (NCBI). Pre-selected primers were then screened in silico for their binding properties to IBV and Shannon entropy plots using Entropy One software (http://www.hiv.lanl.gov/content/sequence/ENTROPY/entropy_one.html) were produced to confirm the specificity of the primers and probes for different IBV genotypes (Fig. 1). Shannon entropy analysis for IBV-specific primers and probes targeting a fragment of the nucleocapsid (N) ORF was carried out using an alignment of 500 sequences of the N ORF of representative IB viruses from different lineages. Entropy values of primers used for amplification of the HVR of the S1 gene was performed on alignments of sequences representing various genotypes including Egyptian variants I and II, different Israeli variants, QX viruses as well as different vaccine strains. The oligonucleotides finally designed are shown in Table 1. Furthermore, the specificity of the newly developed RT-qPCR was evaluated using different IB reference viruses: M41, Ma5, H120, H52, Beaudette, QX, QX-like, CR88-121, 4/91, D880, D274, and the Egyptian variants I (Eg/IBV1) and II (IBV-EG/1212B-2012). Turkey coronavirus, porcine epidemic diarrhea virus, transmissible gastroenteritis virus, equine torovirus, bovine and canine coronaviruses, in addition to AIV subtypes H5N1, H9N2 and NDV were used to further define specificity.

The optimized thermal cycling conditions for the newly developed RT-qPCR specific for the IBV N gene fragment using the AgPath-ID One-Step RT-PCR (Thermofisher) kit were as follows: A reverse transcription step was carried out at 45 °C for 10 min, followed by an initial denaturation step at 95 °C for 10 min, and 40 cycles of PCR amplification at 95 °C for 30 s, 58 °C for 15 s, and 72 °C for 15 s in a 25 μL reaction mixture using 12.5 pmol of each forward, 30 pmol of the reverse primer and 10 pmol probe per reaction.

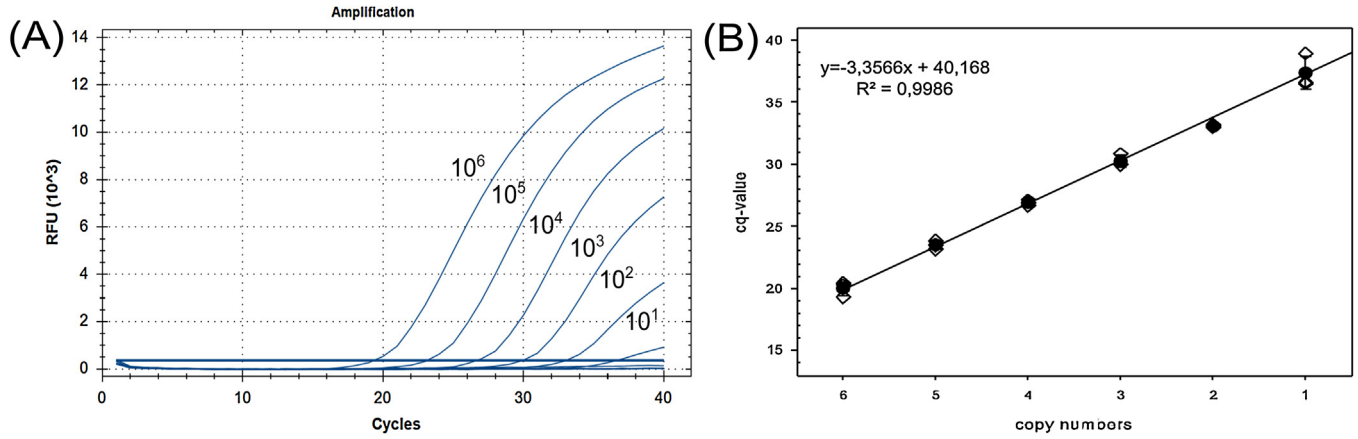

Fig. 1. Analysis of the detection limit of the IBV N-specific RT-qPCR based on serial ten-fold dilutions of transcribed viral RNA ranging from 10^6 to 10^0 copies/reaction (a). Linear regression analysis was constructed revealing the correlation coefficients (R2) and the slope value (b).

Table 1
Sequence and genome location of oligonucleotides developed and used in this study.

Primer Name	Sequence (5′-3′)	Gene	Location	Ref
IBV-pan_FW-1	CAG TCC CDG ATG CNT GGT A	Nucleocapsid	26129–26147[a]	This study
IBV-pan_FW-2	CAG TCC CDG ACG CGT GGT A	(N)	26129–26147[a]	
IBV-pan_RV	CC TTW SCA GMA ACM CAC ACT		26230–26211[a]	
IBV-pan_Probe	ACT GGA ACA GGA CCD GCC GCT GAC CT	Spike (SP1)	26158–26183[a]	
IBV-HVR1-2-FW	GTK TAC TAC TAC CAR AGT GC		79–98	This study
IIBV-HVR1-2-RV	GAA GTG RAA ACR AGA TCA CCA TTT A		533–509)[b]	
IBV-HVR3-FW	TAC TGG TAA TTT TTC AGA TGG		564–584 [c]	Adzhar et al. (1997),
IIBV-HVR3-RV	CAG AYT GCT TRC AAC CAC C		946–928 [c]	modified this study

[a] IBV isolate H120 (GU393335).
[b] Eg/CLEVB-1/IBV/012 (JX173489).
[c] Eg/12120s/2012 (KC533684).

This new diagnostic tool was compared to a previously published RT-qPCR targeting IBV ORF1a; thermal cycling conditions of this method were described before (Callison et al., 2006). For the conventional RT-PCRs targeting the S1 HVRs 1–3 primer concentrations of 12.5 pmol each were used per reaction. The SuperScript® III One-Step RT-PCR kit with Platinum-Taq was used with the following cycling condition: 45 °C for 10 min, followed by an initial denaturation step at 95 °C for 10 min followed by 35 PCR amplification cycles were run at 95 °C for 15 s, 52 °C for 15 s, and 68 °C for 30 s with a final extension step of 68 °C for 5 min.

2.4. Production of RNA run-off transcripts as a copy-based standard for the IBV N-specific RT-qPCR

An amplificate was produced from IBV strain M41 using the N-specific RT-qPCR primer set and cloned into the Topo TA cloning Dual Promoter kit (Invitrogen) containing T7 and Sp6 promoter sequences. RNA was transcribed with T7 RNA polymerase (Promega) from *BamH*I linearized plasmids according to the manufacturer's instructions. Transcribed RNA was further purified and quantified. The detection limit of the RT-qPCR assays was determined by testing in triplicate serial ten-fold dilutions of the in-vitro transcribed viral RNA ranging from 10^6 to 10^0 RNA copies/µl/reaction, and standard curves were produced. A Cq value <40 was considered positive. RNA copy number was calculated based on the DNA/RNA copy number calculator online tool http://endmemo.com/bio/dnacopynum.php

2.5. Coronaviral sequence and phylogenetic analyses

For amplification of the HVR 1 and 2 of the S1 gene of IBV, forward and reverse primers were designed in the frame of this study, while those for the HVR3 were modified from Adzhar et al. (1997) (Table 1). Amplificates were size-separated by agarose electrophoresis, excised and purified from gels using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). Purified PCR products were used directly for cycle sequencing reactions (BigDye Terminator v3.1 Cycle Sequencing Kit, Applied Biosystems). The reaction products were purified using NucleoSEQ columns (Macherey-Nagel GmbH ® 3100 Genetic Analyzer (Life Technologies).

The obtained S1 HVR sequences were assembled and edited using Geneious software, version 7.1.7 (Kearse et al., 2012). Alignment and identity matrix analyses were performed using MAFFT (Katoh and Standley, 2013) and BioEdit (Hall, 1999). Sequences generated in this study were deposited in the GenBank database, and assigned accession numbers are shown in Table S1. Sequences of other viruses required for further analyses were extracted from public databases. Phylogenetic analyses were based on manually edited alignments of the full-length open reading frames. For maximum likelihood analysis of phylogenetic relationship, a best fit model was chosen first on which further calculations and an ultrafast bootstrap equivalent analysis was based. The IQ-tree software version 1.1.3 was used for all operations (Minh et al., 2013; Nguyen et al., 2015). Trees were finally viewed and edited using FigTree v1.4.2 software (http://tree.bio.ed.ac.uk/software/figtree/) and Inkscape 0.48.

3. Results

3.1. Performance characteristics of quantitative (N-specific) and conventional (S1-specific) RT-PCRs for IBV detection

Shannon entropy plots, used to evaluate the specificity of the designed primers, predicted a broad reactivity against different

Table 2
Comparison of the analytical specificity of two real time RT-qPCRs targeting UTR (Callison et al., 2006) or N (this study) using a panel of IBV and other coronaviruses as well as other avian viral pathogens.

Virus Strain	RT-qPCR N	RT-qPCR ORF1a
IBV-M41	+[a]	+
IBV-Ma5	+	+
IBV-H120	+	+
IBV-H52	+	+
IBV-QX	+	+
IBV-QX-like	+	+
IBV-CR88-121	+	+
IBV-4/91	+	+
IBV-D880	+	+
IBV-Beaudette	+	+
IBV-D274	+	+
IBV-Egyptian Var I	+	+
IBV-Egyptian variant II	+	+
Turkey coronavirus	+	+
Porcine Epidemic Diarrhea	–[b]	–
Transmissible Gastroenteritis	–	–
Equine Torovirus	–	–
Canine Coronaviruses	–	–
Bovine Coronaviruses	–	–
H5N1	–	–
H9N2	–	–
NDV	–	–

[a] positive, Cq values obtained with the two RT-qPCRs did not differ by more than 1–2 values indicating similar analytical sensitivity.
[b] negative; Cq values > 40.

IBV genotypes circulating in the Middle East (Fig. S1). Considerable sequence variability was evident despite selecting regions with pronounced overall conservation. Thus, primers were degenerated at key positions as shown in Fig. S1 to allow broader, yet specific, target hybridization.

The specificity of RT-qPCR primer set was evaluated by examining different avian respiratory viruses circulating in poultry in Egypt including AIV H5N1, H9N2 and NDV as well as different coronaviruses including a panel of IBV reference strains as listed in the materials section. This comprises all IBV genotypes reported to be circulating in Egypt. No amplification was detected for H5N1-, H9N2- and NDV-specific RNA (Table 2). As predicted by Shannon entropy plots, a distinctly specific amplification of IBV RNA but not of other coronaviruses, with the exception of turkey coronavirus, was observed with the N-specific RT-qPCR primer set. Genomic RNA from IBV isolate (M41) was used as template for initial validation experiments. Analytical specificity and sensitivity of the newly developed RT-qPCR compared favorably with the 5′UTR −specific RT-qPCR of Callison et al. (2006) (Table 2) which also does not differentiate between IBV and turkey coronaviruses. Run-off RNA transcripts of the IBV N gene fragment were used to evaluate analytical sensitivity of the IBV-N-specific RT-qPCR. Investigations using copy-based RNA run-off transcripts showed a detection limit of that PCR close to 10 copies per assay. The Cq values corresponding to 10 RNA copies were 37 ± 0.5. The standard curves as shown in Fig. 1 depict a dynamic linear range across at least 6 log units of RNA copies. Linear regression analysis revealed excellent reproducibility characteristics (Fig. 1). Based on these results the N-specific RT-qPCR was deemed fit as a screening tool for IBV infections in clinical poultry samples from Egypt.

The two primer sets designed or modified to amplify IBV S1 HVR1-2 and 3 produced a band of 454-bp covering HVR 1 and HVR 2 (set 1), or a 382-bp band covering HVR3 (set 2) as predicted from an alignment of all different serotypes and variants detected in Egypt so far (Fig. S2). The specificity of the two HVR primer sets was further confirmed using reference strains compromising different genotypes circulating in Egypt as mentioned in the Material section. An amplificate of 1010 bp was also generated using the forward primer for HVR1-2 and the reverse primer of HVR3; this amplificate covers all three HVRs of the S1 gene (Fig. S2). A trade-off had to be observed between a shorter PCR product which is less vulnerable to be affected by target RNA degradation in clinical samples and a longer one yielding more sequence information (Moss and Thein, 1998). Therefore, a decision was taken to generally amplify the three S1 HVRs in two separate PCRs when examining clinical samples. Generally, a sequenceable product was generated with these S1 RT-PCRs from clinical samples if the Cq value obtained by use of the N-specific screening RT-qPCR was lower than 32 (equivalent to approximately 100 RNA target copies). Thus, this method gives maximum flexibility that can be adjusted according to the sample quality (virus isolate versus clinical sample).

3.2. Detection of IBV and other avian respiratory viruses in clinical samples from poultry in Egypt

A total of 50 field samples were examined by IBV N-specific RT-qPCR. 28 samples yielded positive signals for presence of IB viruses (Table 3). In the same sample set other avian respiratory viruses were detected by further RT-qPCRs specific for the hemagglutinin gene of AIV subtypes H9 (n = 24), and H5 AIV (n = 6), or specific for the M gene of NDV (n = 13). Molecular pathotyping for the H5 viruses revealed presence of highly pathogenic strains of clade 2.2.1 as genetically characterized in a previous study (see also (Naguib et al., 2015)). For ND, three velogenic viruses all clustering with genotype VII were detected while the remaining eight were lentogenic vaccine strains. In two IBV-positive samples also H5 HPAIV was found; sixteen IBV positive samples also harbored H9 AIV, and seven IBV positive samples also contained NDV. Only nine samples showed solely IBV infection without AIV or NDV. Furthermore, AIV and/or NDV were detected in several IBV negative samples. Details of the various co-infections are shown in Table 3.

3.3. Genetic and phylogenetic characterization of Egyptian IBV from clinical samples

Sequenceable HVR fragments were generated from seven IBV positive samples (shown in bold-face in Table S1). No RT-PCR amplicates were obtained from two further samples that showed Cq values <32 in IBV RT-qPCR; failure to amplify IBV S1-specific RNA from these samples may be related to advanced RNA degradation. The sequences obtained were added to an alignment that had also been used for the in silico analysis of the HVR primers: The sequences derived from three samples (AR2206, AR2185 and AR2211) revealed a high similarity of about 96.1-97.7% at the amino acid (AA) level with Egyptian IBV variant II (e.g., strain EG/1297B/2012, Genbank accession number: KC533683.1) but only 72.1-72.9% to Egyptian variant I (Eg/Beni-Suef/01, GenBank accession number: AF395531) within their HVRs 1 and 2. The remaining four viruses (AR2189, AR2198, AR2211 and AR2212) showed 83.3-89.7% AA identity in those regions with Egyptian variant II viruses (Table 4a). This indicates that two groups of viruses can be distinguished within Egyptian variant II with respect to HVR 1, 2 sequences. All seven samples revealed 98.2–100% AA identity with Egyptian variant II for the HVR 3 and 88.4-89.3% identity compared to Egyptian variant I (Eg/Beni-Suef/01 GenBank accession number: JX174183). All sequences obtained from seven clinical samples are remarkably distinct (more than 20% AA difference) to classical vaccine strains (Ma5, M41 and H120) that are used in Egypt. The seven field-type sequences were also grossly distinct from other vaccine strains currently or previously used in Egypt (4/91, CR88 or D274) (Table 4b).

Phylogenetic analysis confirmed the clustering of the seven IBV positive samples with Egyptian variant II sequences (Fig. 2). Within HVR 1, 2 sequences, however, the existence of two distinct

Table 3
RT-qPCRs reveal frequent co-infections in Egyptian poultry samples with avian influenza (AIV), Newcastle Disease (NDV) and infectious bronchitis viruses (IBV).

	Sample ID	IBV	AIV_M	AIV-H5	AIV-H9	NDV	Collective results
1	EG/AR2206-14/2012	30,13	–	–	–	–	IB
2	EG/AR2207-14/2012	–	–	–	–	–	–
3	EG/AR2208-14/2012	33,22	–	–	–	–	IB
4	EG/AR2164-14/2013	–	–	–	–	–	–
5	EG/AR2165-14/2013	–	17,26	–	18,58	–	H9
6	EG/AR2166-14/2013	–	37,5	–	–	16,46	ND(L[a]),AI(NT[b])
7	EG/AR2167-14/2013	–	17,31	–	19,49	–	H9
8	EG/AR2168-14/2013	–	36,92	–	–	37,48	ND(NT),AI(NT)
9	EG/AR2170-14/2013	–	–	–	–	22,16	ND(L)
10	EG/AR2177-14/2013	–	–	–	–	–	–
11	EG/AR2178-14/2013	–	34,95	36,19	–	17,24	H5,ND(V[c])
12	EG/AR2195-14/2013	–	–	–	–	–	–
13	EG/AR2199-14/2013	36,09	38,31	–	–	–	IB,AI(NT)
14	EG/AR2200-14/2013	33,41	29,13	–	31,25	30,39	IB,ND(L),H9
15	EG/AR2201-14/2013	33,96	27,17	–	29	–	IB,H9
16	EG/AR2202-14/2013	34,29	24,19	–	27,26	–	IB,H9
17	EG/AR2203-14/2013	–	23,25	–	26,43	–	IB,H9
18	EG/AR2205-14/2013	34,57	37,33	–	–	24,3	IB,ND(V),AI(NT)
19	EG/AR2183-14/2013	–	23,86	–	24,25	–	H9
20	EG/AR2184-14/2013	35,55	27,04	–	27,35	–	IB,H9
21	EG/AR2185-14/2013	28,26	24,05	–	23,87	–	IB,H9
22	EG/AR2186-14/2013	33,22	23,76	–	23,93	38,26	IB,H9,ND(NT)
23	EG/AR2187-14/2013	34,34	22,9	–	22,31	38,52	IB,H9,ND(NT)
24	EG/AR2188-14/2013	34,18	31,31	–	30,81	–	IB,H9
25	EG/AR2189-14/2013	28,41	–	–	–	–	IB
26	EG/AR2190-14/2013	30,56	27,12	35,69	26,36	27,56	IB,H5,H9,ND(V)
27	EG/AR2191-14/2013	31	29,73	–	28,5	–	IB,H9
28	EG/AR2192-14/2013	32,15	24,08	–	19,81	–	IB,H9
29	EG/AR2193-14/2013	33,93	–	–	–	–	IB
30	EG/AR2194-14/2013	–	27,16	–	25.61	–	H9
31	EG/AR2196-14/2013	–	27,69	–	27.29	–	H9
32	EG/AR2197-14/2013	34,29	–	–	–	–	IB
33	EG/AR2204-14/2013	33,7	–	–	–	–	IB
34	EG/AR2181-14/2013	–	–	–	–	–	–
35	EG/AR2182-14/2013	–	–	–	–	–	–
36	EG/AR2198-14/2013	28,25	–	–	–	–	IB
37	EG/AR2209-14/2014	37,59	25,98	36	25,58	–	IB,H5,H9
38	EG/AR2210-14/2014	–	–	–	–	–	–
39	EG/AR247-15/2014	–	–	–	–	31,72	ND(NT)
40	EG/AR2211-14/2014	30,75	–	–	–	–	IB
41	EG/AR2212-14/2014	31,11	26,37	–	24,91	–	IB,H9
42	EG/AR2213-14/2014	32,68	38,03	–	–	–	IB,H5
43	EG/AR2214-14/2014	35,25	–	–	–	–	IB
44	EG/AR2215-14/2014	33,18	26,06	–	26,57	36.58	IB,H9,ND(NT)
45	EG/AR2216-14/2014	–	22,99	–	24,35	–	H9
46	EG/AR2217-14/2014	34,12	27,28	–	27	–	IB,H9
47	EG/AR2218-14/2014	–	28,64	26,38	–	–	H5
48	EG/AR2219-14/2014	–	17,79	14,84	–	–	H5
49	EG/AR250-15/2014	29,18	23	–	24,01	27,62	IB,H9,ND(L)
50	EG/AR2220-14/2014	–	26	22,49	33,5	35,14	H5,H9,ND(L)

– = Negative.
[a] L: lentogenic.
[b] NT Not typeable due to very low virus loads.
[c] Velogenic.

groups was confirmed (Fig. 2A). Interestingly, one group of HVR 1, 2 sequences was closely related to vaccine strain 1494 (Fig. 2A, black dot) which, in turn, belonged to Egyptian variant I in terms of HVR 3 sequences. The other group was closely related to vaccine strain D274 whose HVR 3 was closely related to Mass-like vaccine strains.

4. Discussion

Controlling IB in Egypt remains a challenging task due to the wide circulation of at least two different genotypes and emerging of novel strains which may pose risks of IBV vaccination failure. There also is a lack of consistent biosecurity levels in some intense production regions which opens the door to the possibility of mixed infection with other respiratory viruses as well as bacterial pathogens like *Mycoplasma* spp. and *E. coli*.

Therefore, rapid molecular diagnostic tools covering the broad spectrum of IBV circulating in Egypt are required as well as subsequent genotype identification. The IBV N-specific RT-qPCR and HVR1-3-specific RT-PCR assays developed and adapted in this study are shown to provide a reliable sensitive and specific approach for screening of suspect samples as well as for downstream genetic characterization of viruses. The HVR-specific PCRs allowed for genotyping directly from clinical samples omitting the need to isolate virus, provided a well preserved RNA sample quality and sufficient viral loads. Partial S1 gene analysis of the three HVRs of seven IB viruses detected and characterized in this study showed that they were closely related genetically and phylogenetically to the currently circulating Egyptian variant II. In particular, HVR3 of these viruses formed a monophyletic cluster (Fig. 2B). Analyses of HVRs 1 and 2, however, revealed two distinct phylogenetic groups (Fig. 2A). Thus, there seem to exist two populations of Egyp-

Table 4
Comparison of nucleotide and deduced amino acid sequences of the S1 HVR1-2 (a) and HVR3 (b) of IBV from Egyptian field samples with reference and vaccine strains of different IBV serotypes.

(A)

HVR1-2-Nucleotide HVR1-2-Amino acid	EG_AR2206-14_2012	EG_AR2185-14_2013	EG_AR2189-14_2013	EG_AR2198-14_2013	EG_AR2211-14_2014	EG_AR2212-14_2014	EG_AR250-15_2014	IBV_EG_12197 B_2012	IBV_EG _Beni-Seuf_01	IBV_IS_ 1494_06	IBV_IS_885	IBV_4_91_ attenuated	IBV_ CR88121	IBV_strain _H120	IBV_ D274	IBV_ QXIBV
EG_AR2206-14_2012		98.1	86.8	84.4	97.8	85.8	94.9	99.2	72.5	84.7	71.8	71.7	73.6	69.1	90.6	71.5
EG_AR2185-14_2013	**96.9**		87.4	84.9	98.7	86.3	95.4	98.4	72.5	85.2	71.8	72.5	74.4	69.3	90.6	72.0
EG_AR2189-14_2013	**84.1**	**84.1**		94.4	87.9	95.2	88.2	86.6	74.3	95.4	72.6	75.5	76.0	76.5	87.1	72.3
EG_AR2198-14_2013	**84.1**	**84.1**	**88.9**		85.5	98.7	89.0	84.1	74.1	97.0	72.3	74.1	74.9	73.9	84.7	69.6
EG_AR2211-14_2014	**97.7**	**96.9**	**84.9**	**84.9**		86.8	95.4	98.1	73.0	85.8	72.3	72.3	74.1	69.3	90.1	72.3
EG_AR2212-14_2014	**84.9**	**84.9**	**90.5**	**96.9**	**85.7**		89.2	85.5	74.3	97.3	72.6	74.1	75.2	73.3	86.0	69.3
EG_AR250-15_2014	**90.5**	**90.5**	**84.1**	**90.5**	**90.5**	**89.7**		94.6	72.7	87.6	71.0	72.3	74.9	70.4	91.4	71.5
IBV_EG_12197B_2012	**97.7**	**96.1**	**83.3**	**83.3**	**96.9**	**84.1**	**89.7**		72.5	84.4	71.8	72.0	73.9	69.1	90.3	71.7
IBV_EG _Beni-Seuf_01	**72.1**	**72.1**	**68.9**	**70.5**	**72.9**	**70.5**	**70.5**	**72.9**		73.3	95.1	70.8	69.5	67.1	69.5	67.6
IBV_IS_1494_06	**84.1**	**84.1**	**92.1**	**92.9**	**84.9**	**94.5**	**87.3**	**83.3**	**69.7**		71.8	74.1	75.2	72.8	85.8	69.9
IBV_IS_885	**72.9**	**72.9**	**69.7**	**71.3**	**73.7**	**71.3**	**71.3**	**73.7**	**95.3**	**70.5**		70.7	69.3	66.7	69.4	66.9
IBV_4_91_attenuated	**71.3**	**71.3**	**70.5**	**71.3**	**71.3**	**71.3**	**69.7**	**72.1**	**68.1**	**70.5**	**87.2**		93.6	71.7	73.1	73.6
IBV_CR88121	**72.9**	**72.9**	**72.9**	**73.7**	**72.9**	**73.7**	**72.9**	**73.7**	**64.1**	**73.7**	**61.7**	**87.2**		71.5	74.7	73.3
IBV_strain_H120	**61.7**	**61.7**	**68.1**	**63.3**	**61.7**	**63.3**	**61.7**	**62.5**	**62.6**	**64.1**	**72.1**	**61.7**	**64.1**		72.0	73.6
IBV_D274	**87.3**	**86.5**	**85.7**	**83.3**	**86.5**	**84.1**	**88.1**	**86.5**	**69.7**	**85.7**	**68.8**	**72.1**	**72.9**	**64.9**		72.0
IBV_QXIBV	**66.5**	**65.7**	**68.9**	**65.7**	**67.3**	**66.5**	**64.9**	**67.3**	**68.1**	**67.3**	**87.2**	**68.8**	**68.0**	**67.3**	**67.3**	

(B)

HVR3- Nucleotide HVR3- Amino acid	EG_AR2206-14_2012	EG_AR2185-14_2013	EG_AR2189-14_2013	EG_AR2198-14_2013	EG_AR2211-14_2014	EG_AR2212-14_2014	EG_AR250-15_2014	IBV_EG _12197 B_2012	IBV_EG _Beni -Suef_01	IBV_IS/ 1494/06	IBV_IS/ 885	IBV_4/91	IBV_ CR88121	IBV_strain _H120	IBV_ D274	IBV_ QXIBV
EG_AR2206-14_2012		98.5	98.8	98.2	97.6	98.5	98.5	98.5	88.7	87.8	89.0	80.7	79.5	80.4	81.9	81.0
EG_AR2185-14_2013	**98.2**		99.1	98.5	99.1	99.4	99.4	99.4	88.4	87.5	88.1	80.7	79.5	80.7	81.6	81.0
EG_AR2189-14_2013	**98.2**	**98.2**		98.2	98.2	99.1	99.1	98.5	88.4	87.5	88.4	80.7	79.5	80.4	81.9	81.0
EG_AR2198-14_2013	**97.3**	**98.2**	**97.3**		98.2	98.5	98.5	98.5	89.3	88.4	88.7	81.0	79.8	79.8	81.9	80.4
EG_AR2211-14_2014	**98.2**	**100**	**98.2**	**98.2**		98.5	98.5	98.5	88.4	87.5	87.8	81.0	80.1	80.1	81.6	80.4
EG_AR2212-14_2014	**97.3**	**99.1**	**97.3**	**97.3**	**99.1**		99.4	98.8	88.4	87.5	88.1	80.7	79.5	80.7	82.2	81.0
EG_AR250-15_2014	**97.3**	**99.1**	**97.3**	**97.3**	**99.1**	**98.2**		98.8	88.4	87.5	88.1	80.7	79.5	80.7	81.6	81.0
IBV_EG_ 12197B_2012	**98.2**	**100**	**98.2**	**98.2**	**100**	**99.1**	**99.1**		89.0	88.1	88.7	80.7	79.5	80.1	81.6	81.0
IBV_EG_ Beni-Suef_01	**88.4**	**89.3**	**88.4**	**89.3**	**89.3**	**88.4**	**88.4**	**89.3**		98.2	89.3	81.6	79.8	80.1	82.2	80.4
IBV_IS/1494/06	**87.5**	**88.4**	**87.5**	**88.4**	**88.4**	**87.5**	**87.5**	**88.4**	**96.5**		90.4	83.2	81.4	81.1	82.2	80.1
IBV_IS/885	**89.3**	**89.3**	**89.3**	**89.3**	**89.3**	**89.3**	**88.4**	**89.3**	**87.5**	**88.4**		82.0	80.2	80.5	79.5	80.4
IBV_4/91	**81.3**	**82.2**	**81.3**	**82.2**	**82.2**	**81.3**	**81.3**	**82.2**	**82.2**	**83.1**	**81.3**		98.2	77.8	82.8	81.0
IBV_CR88121	**79.5**	**80.4**	**79.5**	**80.4**	**80.4**	**79.5**	**79.5**	**80.4**	**81.3**	**82.2**	**79.5**	**94.7**		76.6	82.8	79.2
IBV_strain_H120	**78.6**	**80.4**	**78.6**	**79.5**	**80.4**	**79.5**	**79.5**	**80.4**	**77.7**	**77.7**	**75.9**	**73.3**	**71.5**		81.9	77.4
IBV_D274	**83.0**	**83.0**	**82.1**	**83.0**	**83.0**	**83.9**	**82.1**	**83.0**	**82.1**	**82.2**	**80.4**	**81.3**	**81.3**	**76.8**		80.1
IBV_QXIBV	**82.1**	**83.0**	**82.1**	**83.0**	**83.0**	**82.1**	**82.1**	**83.0**	**84.8**	**84.9**	**84.0**	**84.0**	**83.1**	**75.9**	**83.0**	

Boldface indicates results of the analyses of deduced amino acid sequences.

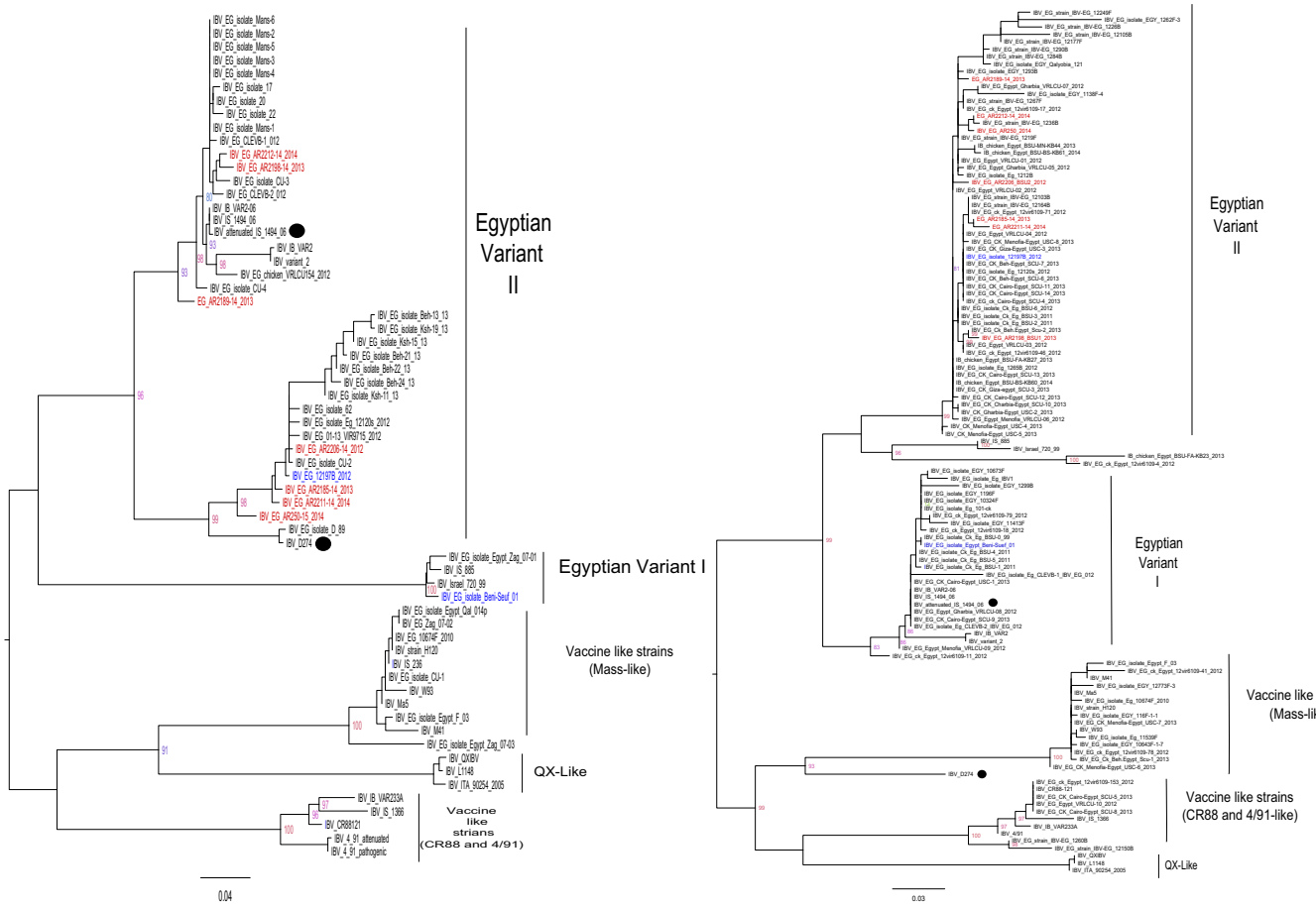

Fig. 2. Phylogenetic tree of S1 HVR 1, 2 (A) and 3 (B) sequences based on maximum likelihood calculations (IQTree software) under the best fit model according to the Akaike criterion (model: TVM + I + Γ). Numbers at nodes represent measures of robustness based on an ultrafast bootstrap approach implemented in IQTree. Viruses of the current study are shown in red, reference viruses are colored in blue. Black dots indicate vaccine strains D274 and 1494 which are, together with other strains, in use or planned for use in Egypt. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

tian variant II viruses which can be distinguished in their HVR1, 2 loci. The molecular mechanisms having caused this split remain to be elucidated. To confirm or rule out recombination full length S1 sequences will be required of a larger panel of Egyptian IB viruses. In addition, it remains to be clarified whether antigenic differences exist between the two clusters within Egyptian variant II. In this set of samples, no IBV of Egyptian variant I was detected; this seems to confirm previously published data from GenBank indicating a dominance of Egyptian variant II.

Infectious bronchitis, avian influenza and Newcastle disease are the three major causes of economic losses in the poultry industry; they are able to induce disease independently or in association with each other. Avian influenza H5N1 and H9N2 subtypes continue to circulate in Egypt since 2006 and 2011, respectively, causing many outbreaks in poultry farms (Naguib et al., 2015). Velogenic NDV circulating among chickens in Egypt resembles genotype VIId (Chicken/China/SDWF07/2011) and is associated with outbreaks in commercial poultry farms despite adherence to strict vaccination regimes (Radwan et al., 2013). Similar co-infections of avian influenza and velogenic NDV were observed also in neighboring countries such as Jordan and Libya (Kammon et al., 2015; Roussan et al., 2008). In addition, a recent similar single case was reported in Egypt (Hussein et al., 2014). An at least partial but not sterilizing, possibly vaccine-induced, specific immunity against these pathogens is likely to be at the basis of these observations. Continuing clinically disguised virus circulation in the presence of specific immunity not only fosters spread of these agents but also drives the development of viral escape mutants. It should be noted that the mortality rates described for the holdings do not correlate with the presence of IBV, HPAIV H5N1 or velogenic NDV (Table 3, e.g., holding 34). This investigation focused on IBV, AIV and NDV and did not consider further avian viral or bacterial pathogens. It is highly likely that also various bacterial co-pathogens contribute to and complicate the overall clinical picture.

The current situation of IB infections in Egypt seems to be the result of a continuing evolution starting with infections caused by Egyptian variant I since 2001, Egyptian variant II since 2011, and Mass-like strains since 2006 (Abdel-Moneim et al., 2012; Selim et al., 2013). Tracheal ciliostasis is one of the early and characteristic pathogenetic sequelae of IBV infection (Cook et al., 1976). Cileostatic respiratory epithelium is known to be more vulnerable for infections with further viral and bacterial co-pathogens and may dispose for infection with AIV and NDV even in the presence of (suboptimal) vaccine-induced immunity.

In conclusion, the continuous circulation of the Egyptian variant II IBV and co-infections with AIV and/or NDV severely complicate the epidemiology of viral respiratory infections of chicken in Egypt. Intensive surveillance is required for a better understanding of this situation. Moreover, molecular identification of circulating viruses and experimental vaccination-challenge studies are required to provide data for eventual updating of vaccine strains and to strategically strengthen application programs.

Conflict of interest

All authors declare that they have no conflict of interest.

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Ethical approval

Sample collection by veterinarians was achieved from poultry kept as commercial livestock in farms in Egypt. Obtaining swab samples from the trachea of poultry is minimal invasive and does not require any anesthesia of the animal; minimal restrainment is used for a very short time. Sampling, analysis and shipment of samples from Egypt to Germany was under the legal auspices of the National Animal Health and Research Institute, Giza, Egypt. Three co-authors of the manuscript are employees of this governmental institution.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jviromet.2017.02.018.

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