RESEARCH ARTICLE

Novel real-time PCR-based patho- and phylotyping of potentially zoonotic avian influenza A subtype H5 viruses at risk of incursion into Europe in 2017

MM Naguib¹², A Graaf¹, A Fortin³, C Luttermann⁴, U Wernery⁵, N Amarin⁶, HA Hussein⁷, H Sultan⁸, B Al Adhadh⁹, MK Hassan², M Beer¹, I Monne³, TC Harder¹

- 1. Institute of Diagnostic Virology, Friedrich Loeffler Institute, Greifswald-Riems, Germany
- 2. National Laboratory for Veterinary Quality Control on Poultry Production, Animal Health Research Institute, Giza, Egypt
- 3. Istituto Zooprofilattico Sperimentale delle Venezie, Padua, Italy
- 4. Institute of Immunology, Friedrich Loeffler Institute, Greifswald-Riems, Germany
- 5. Central Veterinary Research Laboratory (CVRL), Dubai, United Arab Emirates
- 6. Boehringer Ingelheim, Dubai, United Arab Emirates
- 7. Faculty of Veterinary Medicine, Cairo University, Giza, Egypt
- 8. Birds and Rabbits Medicine Department, Faculty of Veterinary Medicine, Sadat City University, Egypt
- 9. Central Veterinary Laboratory, Ministry of Agriculture, Baghdad, Iraq

Correspondence: Timm Harder (timm.harder@fli.bund.de)

Citation style for this article: Naguib MM, Graaf A, Fortin A, Luttermann C, Wernery U, Amarin N, Hussein HA, Sultan H, Al Adhadh B, Hassan MK, Beer M, Monne I, Harder TC. Novel realtime PCR-based patho- and phylotyping of potentially zoonotic avian influenza A subtype H5 viruses at risk of incursion into Europe in 2017. Euro Surveill. 2017;22(1):pii=30435. DOI: http://dx.doi.org/10.2807/1560-7917.ES.2017.22.1.30435

Article submitted on 30 November 2016 / accepted on 20 December 2016 / published on 05 January 2017

Since November 2016, Europe witnesses another wave of incursion of highly pathogenic avian influenza (HPAI) A(H5) viruses of the Asian origin goose/ Guangdong (gs/GD) lineage. Infections with H5 viruses of clade 2.3.4.4b affect wild bird and poultry populations. H5 viruses of clades 2.2, 2.3.1.2c and 2.3.4.4a were detected previously in Europe in 2006, 2010 and 2014. Clades 2.2.1.2 and 2.3.2.1.c are endemic in Egypt and Western Africa, respectively and have caused human fatalities. Evidence exists of their cocirculation in the Middle East. Subtype H5 viruses of low pathogenicity (LPAI) are endemic in migratory wild bird populations. They potentially mutate into highly pathogenic phenotypes following transmission into poultry holdings. However, to date only the gs/ GD H5 lineage had an impact on human health. Rapid and specific diagnosis marks the cornerstone for control and eradication of HPAI virus incursions. We present the development and validation of five real-time RT-PCR assays (RT-qPCR) that allow sequencing-independent pathotype and clade-specific distinction of major gs/GD HPAI H5 virus clades and of Eurasian LPAI viruses currently circulating. Together with an influenza A virus-generic RT-qPCR, the assays significantly speed up time-to-diagnosis and reduce reaction times in a OneHealth approach of curbing the spread of gs/ **GD HPAI viruses.**

Introduction

Influenza A viruses constitute a virus species in the family Orthomyxoviridae. They harbour single-stranded negative-sense RNA arranged into eight genomic segments. Members of this species which infect avian hosts (avian influenza viruses, AIV) are grouped into 16 (H1 to H16) and 9 (N1 to N9) subtypes, respectively, based on phylogenetic and antigenic properties of their haemagglutinin (HA) and neuraminidase (NA) envelope glycoproteins [1]. Different species of aquatic wild birds are the natural reservoirs for all AIV subtypes. Novel subtypes and gene constellations continue to evolve in aquatic wild birds or in infected poultry populations by genetic reassortment during infection of a single host cell with two or more distinct AIV genotypes. In addition to reassortment, the intrinsically error-prone influenza virus genome replication machinery promotes the generation of quasi-species that can be shaped by directional selection pressures, e.g. following host species switches or by specific herd immunity. In the latter case, antigenic drift variants are selected that may escape immunity by very few amino acid substitutions in the HA [2].

Based on their virulence in galliform poultry (e.g. chicken, turkey), AIV are distinguished into groups of highly pathogenic (HP) and low pathogenic (LP) phenotypes [3]. Correct AI diagnosis includes determining the HA subtype and, in case of subtypes H5 or H7, also the pathotype. So far, HPAI phenotypes detected in the field (i.e. 'free' natural environment), were only described among AIV of subtypes H₅ and H₇ [4]. Some of these viruses including those of the HPAI H₅ goose/ Guangdong (gs/GD) lineage that emerged in southern China in 1996, have zoonotic potential and are sporadically transmitted from infected birds to humans [5,6]. HPAI viruses of the gs/GD lineage have continued to circulate and evolved into numerous clades. Viruses

FIGURE 1

Evaluation of detection limits and precision of pathotyping and phylotyping quantitative reverse transcription PCRs compared with a generic matrix (M) gene RT-qPCR^a



Cq: cycle of quantification; HPAI: highly pathogenic avian influenza; LPAI: low pathogenic avian influenza; M: matrix; RT-qPCR: quantitative reverse transcription PCR.

The detection limit was determined based on triplicate analyses of serial 10-fold dilutions of target RNA of reference viruses: HPAI H5: A/duck/Egypt/AR236-A3NLQP/2015 (H5N1); LPAI H5: A/tack/Egypt/AR236-A3NLQP/2015 (H5N1); clade 2.3.2.1: A/quail/Dubai/AR3445-2504.3/2014 (H5N1) and clade 2.3.2.4: A/turkey/Germany-MV/R2472/2014 (H5N8) (lower panel). A cut-off value of Cq 38 was chosen to calculate limits of detection and confidence intervals thereof.

^a Described in [29].

FIGURE 2

Pathotyping and phylotyping of virus isolates and clinical samples of potentially zoonotic Eurasian avian influenza A subtype H5 viruses by quantitative reverse transcription PCRs



Cq: cycle of quantification; HPAI: highly pathogenic avian influenza; LPAI: low pathogenic avian influenza; M: matrix; RT-qPCR: quantitative reverse transcription PCR.

Sample numbers in A and B refer to Table 3. Cq values obtained for each sample by M1.2 RT-qPCR are shown as black dots (panels A and B); Cq values obtained for each sample by the specific RT-qPCRs are depicted as follows: Panel A red lozenges – HPAI H5, green crosses - LPAI H5; panel B blue lozenges - clade 2.2.1.2, purple triangles - clade 2.3.2.1, ochre Xs - clade 2.3.4.4.; panel C compares categorised Cq values obtained for all samples by M1.2 RT-qPCR (black box-and-whiskers) and the specific RT-qPCRs (colours as described for panels A and B).

of three major phylogenetic clades (2.2.1.2, 2.3.2.1 and 2.3.4.4) as well as of three further minor clades (1.1.2, 2.1.3.2 and 7.2) have become endemic in poultry populations in several countries in Asia, Africa and the Middle East [7]. Occasionally, spillover transmission from infected poultry may cause infection and viral spread in wild birds with increased mortality in some species. Infected migratory wild birds may spread such viruses across wider distances and act as the source of transmission back to poultry [7,8].

Europe has experienced several incursions by viruses of the gs/GD lineage over the past decade; both wild birds and poultry were affected but no human cases were reported [9]. This is in sharp contrast to Egypt and Asian countries where the endemicity of HPAI H5 viruses in poultry is associated with repeated spillover transmission to and infection of humans. In fact, the majority of human HPAI H5 cases worldwide were registered in Egypt [10,11]. Moreover, a new major clade, designated 2.2.1.2, evolved along with transient spread of an escape mutant-based lineage, 2.2.1.1, in this country [12].

Further potentially zoonotic gs/GD viruses of clade 2.3.2.1c are widespread in Central and Southern Asia and they were sporadically detected along the European Black Sea coast as well as in the Middle East [13-15]. In addition, viruses of this clade have caused major outbreaks among poultry in several Western African countries with ongoing virus circulation to date [16]. Interestingly, 2.3.2.1c viruses have not (yet) been reported from Egypt. Since 2010, another gs/GD cluster, termed 2.3.4.4, has evolved in eastern China

TABLE 1

Primers and probes designed for differentiating pathotype and phylotype of Eurasian wild bird and goose/Guangdong origin potentially zoonotic avian influenza A subtype H5 viruses

Primer/Probe ID	Target	Sequence (5' to 3')	Location	Amplicon size	Accession number ^a	
H5_HP_EA_F1		CCTTGCDACTGGRCTCAG	984-1001		EDI6 (75 (O	
H5_HP_EA_F2	НОЛІНЕ	TCCTTGCAACAGGACTAAG	983-1001	100		
H5_HP_EA_probe	III AI IIS	FAM- AAGAARAAARAGAGGACTRTTTGGAGCT-BHQ-1		109	LF104/540	
H5_HP_EA_R		GTCTACCATTCCYTGCCA	1092-1075			
H5LP-EA_F		CCCAAATACGTGAAATCAGAT	955-975			
H5LP1_EA_probe	LPAI H5	FAM-CCAAATAGYCCTCTYGTYTCT-BHQ-1	1052-1072	133	EPI356413	
H5LP-EA_R		GCC ACC CTC CTT CTA TAA AG	1088-1069			
H5_2.2.1.2_Fw		CATTTTGAGAAAATTCAGATCATT	376-399			
H5_2.2.1.2_probe	Clade 2.2.1.2	FAM-TCCATACCARGGAAGATCCTCCTTT-BHQ-1	451-474	161	EPI573250	
H5_2.2.1.2_Rev		GGTATGCATCGTTCTTTTGG	537-517			
H5_2.3.2.1_F		GAGATTGGTACCAAAAATAGCC	669-690			
H5_2.3.2.1_probe	Clade 2.3.2.1	FAM-ACGGGCAAAGTGGCAGGATAGATTTC-BHQ-1	707-732	146	EPI603577	
H5_2.3.2.1_R		CAATGAAATTTCCATTACTCTCG	815-793			
H5_2.3.4.4_F_A		ATACCAGGGAGCATCCTCA	484-502			
H5_2.3.4.4_F_B	Clade	le ATACCAGGGAACGCCCTCC				
H5_2.3.4.4_probe	2.3.4.4	FAM-TCGTTCTTTTTGATGAGCCATACCACA-BHQ-1	540-560	114	EP1554605	
H5_2.3.4.4_R_A] [ATTATTGTAGCTTATCTTTATTGTC	598-574			
H5_2.3.4.4_R_B		ATTATTGTAGCTTATCTTTATTGTT	598-574	1		

gs/GD: goose/Guangdong; HA: haemagglutinin; ID: identity.

^a Accession number used to describe the position of the oligonucleotide along the HA gene. Sequences were obtained from GenBank at the National Center for Biotechnology Information (NCBI) or the EpiFlu database of the Global Initiative on Sharing Avian Influenza Data (GISAID).

and on the Korean peninsula [17]. These viruses have revealed a strong propensity to reassort with other influenza subtypes giving rise to novel HPAI sub- and genotypes including influenza A(H5N6) and A(H5N8). The latter subtype has proven to be highly mobile and was carried by infected wild birds to Europe and the North American continent in late 2014 [8,18]. In November 2016, HPAI H5N8 viruses of the 2.3.4.4 clade re-emerged on a large scale in wild birds in several central European countries and caused considerable mortality especially among diving duck species; sporadic incursions into poultry holdings were documented as well [19]. At the same time, this lineage was also detected in poultry in Israel [20].

Eurasian-origin LPAI subtype H5 viruses distantly related to the gs/GD lineage are routinely detected in aquatic wild bird populations with peak incidences during the autumn migration period [21]. Spillover of LPAI virus into poultry may cause notifiable outbreaks and bears the risk of the de novo generation of HP phenotypes following spontaneous mutations [3]. No human LPAI H5 virus infections have been reported so far. Continuous co-circulation in poultry and sporadic spillover into migratory wild bird populations of different endemic HPAI H5 virus lineages poses constant risks of new incursions into Europe by migrating wild birds or in association with (illegal) poultry trading practices [9]. Furthermore, co-circulation of various HPAI lineages with different antigenic properties potentiates problems of control and eradication. Given the zoonotic propensities of some of the H5 viruses, tight control of infections in poultry is essential to curtail risks of human infections and further spread [22,23]. Molecular diagnosis including patho- and phylotyping of the relevant AIV is an important prerequisite for effective control measures.

We developed rapid diagnostic solutions on the basis of quantitative reverse transcription real-time PCR assays (RT-qPCR), to pathotype, without sequencing, gs/GD lineage HPAI and Eurasian LPAI H5 subtype viruses, and to distinguish HPAI gs/GD viruses of clades 2.2.1.2, 2.3.2.1 and 2.3.4.4, including viruses of the ongoing 2016 epizootic in Europe.

TABLE 2

Reference viruses used to determine analytical specificity of five PCR assays to detect potentially zoonotic avian influenza subtype H5 viruses

Reference virus		Accossion	Patho- and	PCR method ^b						
		number of HA ^a	Phylotype	HPAI H5	LPAI H5	Clade 2.2.1.2	Clade 2.3.2.1	Clade 2.3.4.4		
1	A/turkey/Turkey/1/2005 (H5N1)	KF042153	HP Clade 2.2	Pos	Neg	Pos	Neg	Neg		
2	A/chicken/Egypt/0879-NLQP/R737/2008 (H5N1)	GQ184238	HP Clade 2.2.1. 1	Pos	Neg	Neg	Neg	Neg		
3	A/chicken/Egypt/NLQP7FL-AR747/ 2013 (H5N1)	EPI557170	HP Clade 2.2.1.2	Pos	Neg	Pos	Neg	Neg		
4	A/duck/Egypt/AR236-A3NLQP/2015 (H5N1)	EPI573260	HP Clade 2.2.1.2	Pos Neg		Pos	Neg	Neg		
5	A/turkey/Egypt/AR238-SD177NLQP/2014 (H5N1)	EPI573268	HP Clade 2.2.1.2	Pos Neg		Pos	Neg	Neg		
6	A/peregrine falcon/Dubai/AR3430/2014 (H5N1)	EPI603553	HP Clade 2.3.2.10	Pos	Neg	Neg	Pos	Neg		
7	A/quail/Dubai/AR3445–2504.3/2014 (H5N1)	EPI603577	HP Clade 2.3.2.10	Pos	Neg	Neg	Pos	Neg		
8	A/duck/Bangladesh/D3-AR2111/2013 (H5N1)	SAc	HP Clade 2.3.2.1a	Pos	Neg	Neg	Pos	Neg		
9	A/turkey/Germany/AR2485–86/2014 (H5N8)	EPI552746	HP Clade 2.3.4.4a	Pos	Neg	Neg	Neg	Pos		
10	A/turkey/Germany-MV/AR2472/2014 (H5N8)	EPI544756	HP Clade 2.3.4.4a	Pos	Neg	Neg	Neg	Pos		
11	A/tufted duck/Germany/AR8444/2016 (H5N8)	EP1859212	HP Clade 2.3.4.4b	Pos	Neg	Neg	Neg	Pos		
12	A/chicken/Indonesia/R132/2004 (H5N1)	EPI354072	HP Clade 2.1.1	Pos	Neg	Neg	Neg	Neg		
13	A/chicken/Indonesia/R134/2003 (H5N1)	AM183669	HP Clade 2.1.1	Pos	Neg	Neg	Neg	Neg		
14	A/chicken/Indonesia/R6o/2005 (H5N1)	AM183670	HP Clade 2.1.1	Pos	Neg	Neg	Neg	Neg		
15	A/Vietnam/1194/2004 (H5N1)	GQ149236	HP Clade 1.1	Pos	Neg	Neg	Neg	Neg		
16	A/chicken/GXLA/1204/2004 (H5N1)	AM183671	HP Clade 2.4	Pos	Neg	Neg	Neg	Neg		
17	A/chicken/Vietnam/P41/2005 (H5N1)	AM183672	HP Clade 1.1	Pos	Neg	Neg	Neg	Neg		
18	A/chicken/Vietnam/P78/2005 (H5N1)	AM183673	HP Clade 1.1	Pos	Neg	Neg	Neg	Neg		
19	A/common teal/Germany/Wv1378– 79/2003 (H5N2)	HF563058	LP	Neg	Pos	Neg	Neg	Neg		
20	A/duck/Germany/R1789/2008 (H5N3)	CY107849	LP	Neg	Pos	Neg	Neg	Neg		
21	A/turkey/Germany/AR915/2015 (H7N7)	SAc	H ₇ N ₇	Neg	Neg	Neg	Neg	Neg		
22	A/chicken/Egypt/AR754–14/2013 (H9N2)	EPI557457	H9N2	Neg	Neg	Neg	Neg	Neg		
23	A/chicken/Sudan/AR251–15/2014 (IBV)	KX272465	IBV	Neg	Neg	Neg	Neg	Neg		
24	A/chicken/Egypt/AR254–15/2014 (NDV)	SAc	NDV	Neg	Neg	Neg	Neg	Neg		

Cq: cycle of quantification; HA: haemagglutinin; HP: highly pathogenic; HPAI: highly pathogenic avian influenza; IBV: infectious bronchitis virus; LP: low pathogenic; LPAI: low pathogenic avian influenza; NDV: Newcastle disease virus; Neg: negative; Pos: positive; RT-qPCR: quantitative reverse transcription PCR; SA: sequences available.

^a Sequences were obtained from GenBank at the National Center for Biotechnology Information (NCBI) or the EpiFlu database of the Global Initiative on Sharing Avian Influenza Data (GISAID).

^b Positive results: Cq value in similar range as with influenza A virus generic M RT-qPCR; negative results: Cq>40. ^c Sequenced in the frame of the current study; sequences available from the authors upon request.

Methods

Virus isolates and clinical samples

A total of 24 reference virus isolates were obtained from the virus repositories at the Friedrich Loeffler Institute, Greifswald-Riems, Germany, or were provided by the National Laboratory for quality control on poultry production in Giza, Egypt, and by the Central Veterinary Research Laboratory (CVRL) in Dubai, United Arab Emirates (see also first table under Results).

Moreover, 106 field samples were included. These were obtained from holdings of different poultry sectors and wild birds from countries in Western Europe (Germany), the Middle East (Egypt, Iraq, United Arab Emirates) and Western Africa (Burkina Faso, Cameroon, Ghana, Ivory Coast, Niger), for HPAI viruses in the period between

TABLE 3A

Pathotyping and phylotyping of different potentially zoonotic HPAI and LPAI influenza A subtype H5 virus isolates and field samples collected from poultry and wild bird species in different countries, 2013–2016

Distant Unique Number Number <th< th=""><th>No</th><th>Comple ID</th><th rowspan="2">Type of sample</th><th rowspan="2">Accession Number ª</th><th rowspan="2">Clade</th><th colspan="6">PCR results</th></th<>	No	Comple ID	Type of sample	Accession Number ª	Clade	PCR results					
1 Nonlower (Symplency) Nonlower (Symplency)	NU.					M1.2	HPAI H5	LPAI H5	2.2.1.2	2.3.2.1	2.3.4.4
1 AladicalizygriptingPackAlatysizype Isolate Empirys 192 18	1	A/chicken/Egypt/NLQP33SD-AR748/2013	Isolate	EPI557178	HP 2.2.1.2	27.25	30.72	Neg	27.43	Neg	Neg
1 Nat/Algopy/INOPO/SADY/OPO/SADY/SID Ideal Persons Per	2	A/chicken/Egypt/NLQP2AL-AR749/2013	Isolate	EPI557186	HP 2.2.1.2	27.65	30.53	35.56	27.94	Neg	Neg
4 Acidami(gpu)(Alge)(x)(x)(x)(x)) India Pip2. Pip2. <td< td=""><td>3</td><td>A/duck/Egypt/NLQP27SG-AR750/2013</td><td>Isolate</td><td>EPI557194</td><td>HP 2.2.1.2</td><td>28.01</td><td>30.01</td><td>Neg</td><td>27.41</td><td>Neg</td><td>Neg</td></td<>	3	A/duck/Egypt/NLQP27SG-AR750/2013	Isolate	EPI557194	HP 2.2.1.2	28.01	30.01	Neg	27.41	Neg	Neg
§ Alckach/gep/(Alcy3Ac) Index Pison Pison Pison Pis	4	A/chicken/Egypt/NLQP639V-AR752/2013	Isolate	EPI557202	HP 2.2.1.2	24.11	29.58	Neg	23.32	Neg	Neg
6 Alcolant/group/Adsystype Indiantes Birson	5	A/chicken/Egypt/NLQP20SL-AR751/2013	Isolate	EPI557210	HP 2.2.1.2	28.90	32.90	Neg	29.30	Neg	Neg
1NumberUP	6	A/chicken/Egypt/NLQP139V-AR753/2013	Isolate	EPI557218	HP 2.2.1.2	33.32	34.13	Neg	33.51	Neg	Neg
	7	A/quail/Egypt/BSU5514-AR2219/2014	Field sample	EPI557138	HP 2.2.1.2	15.12	22.12	Neg	15.47	Neg	Neg
9NutworkpayNutwo	8	A/chicken/Egypt/AR234-FAOF8NLQP/2014	Field sample	EPI573250	HP 2.2.1.2	28.60	31.95	Neg	28.75	Neg	Neg
inAnckank/gyn/Agon/actoIndiameIndi	9	A/turkey/Egypt/AR235-S240NLQP/2014	Field sample	EPI573252	HP 2.2.1.2	22.56	27.94	35.77	22.16	Neg	Neg
11Alcoken/gryn/ksyn/sondField and Field andSANo.	10	A/chicken/Egypt/AR3690A/2016	Field sample	SAb	HP 2.2.1.2	18.01	18.97	35.29	18.61	Neg	Neg
12Alcoken/regurd/synycondField and Field sameSA <td>11</td> <td>A/chicken/Egypt/AR3706/2016</td> <td>Field sample</td> <td>SAb</td> <td>HP 2.2.1.2</td> <td>19.27</td> <td>22.98</td> <td>Neg</td> <td>20.13</td> <td>Neg</td> <td>Neg</td>	11	A/chicken/Egypt/AR3706/2016	Field sample	SAb	HP 2.2.1.2	19.27	22.98	Neg	20.13	Neg	Neg
13Alcoken/regur/Arygn/sonicField sameSAVBA2.BA3	12	A/chicken/Egypt/AR3707/2016	Field sample	SAb	HP 2.2.1.2	23.39	27.66	Neg	23.71	Neg	Neg
10.Alcoken/regy/Argy/argy/argy/argyField same5A*6A*8	13	A/chicken/Egypt/AR3737/2016	Field sample	SAb	HP 2.2.1.2	19.53	24.16	Neg	20.58	Neg	Neg
19.Klicken/Egyn(Asy3)/2004Field sample5A*197.219.2019.50<	14	A/chicken/Egypt/AR3741/2016	Field sample	SA ^b	HP 2.2.1.2	20.25	23.08	35.04	21.71	Neg	Neg
16.19.1	15	A/chicken/Egypt/AR3753/2016	Field sample	SAb	HP 2.2.1.2	21.22	26.21	35.55	23.10	Neg	Neg
17Nione curlew/Dubal/Ab34A-e30.2014IsolateIsolateIP A3.2.1IA	16	A/seagull/Dubai/AR3443–2504.1/2014	Isolate	EPI603554	HP 2.3.2.1	15.62	16.32	Neg	Neg	15.72	Neg
18.A/ack/ack/ans/ans/asys/asys/asys/asys/asys/asys/a	17	A/stone curlew/Dubai/AR3444-2504.2/2014	Isolate	EPI603569	HP 2.3.2.1	13.81	14.72	Neg	Neg	14.70	Neg
99 A/chicken/Ghana/is/lRag88-a/2005 Spleen KUg7130 IP 2,3.2.1 26.2.0 24.4.7 Neg N	18	A/duck/Ivory_Coast/15VIR2742-1/2015	Spleen and caecum	NA	HP 2.3.2.1	31.79	23.56	Neg	Neg	23.93	Neg
200 A/chicken/Ghan/sSVIR2589=n/2005 Cloacal sawb KU97130 IP 2-3.2.1 S2.50 S2.68 Neg Neg 24.37 Neg 21 A/chicken/Niger/sVIR2606-1/2/035 Tacheal swb KU971320 IP 2-3.2.1 2,508 Nog Neg 24.37 Neg 23 A/chicken/Niger/sVIR260-1/2/035 Grgan KU97130 IP 2-3.2.1 30 9.03 Neg 4.02 4.02 24 A/chicken/Ghan/s/GNR4304-2/2016 Organ SA ¹ HP 3.2.2 1.05 1.562 Neg 1.69 1.20 1.20 1.20 Neg 1.20	19	A/chicken/Ghana/15VIR2588–4/2015	Spleen	KU97137	HP 2.3.2.1	22.72	24.47	Neg	Neg	18.07	Neg
11 A/chicken/Niger/sVIR2o60-3/2015 Tracheal samb KU97130 PP 3.2 25.08 3.08 Neg Neg 2.0.3 Neg 23 A/chicken/Niger/sVIR2o60-3/2015 Swab KU97130 PP 3.2 2.0.8 1.0.9 Neg PA 2.0.3 Neg PA 2.0.4 Neg PA 2.0.4 Neg Neg PA 2.0.4 Neg Neg PA 2.0.5 Neg Neg PA 2.0.9 PA 20 A/chicken/chana/sOUR.4304-42/2016 Organ SA HP 3.2.2. 2.0.5 Neg Neg PA PA <	20	A/chicken/Ghana/15VIR2588–10/2015	Cloacal swab	KU971357	HP 2.3.2.1	26.24	26.80	Neg	Neg	24.61	Neg
22A/chicken/kiger/ks/lRaco6-s/2otsSimabKSupsKUp sizeBP 2.3.2.121.0521.09NegNeg20.01Neg23A/domestic_bird/Burkina_Fasor/sty/Rr7A-2A/2015GyrganKUp sizesHP 2.3.2.121.9130.83NegNeg24.01Neg24A/domestic_bird/Burkina_Fasor/sty/Rr7A-2A/2015OrganSA'HP 2.3.2.121.9130.83NegNeg18.44Neg26A/chicken/Chana/toVIR-4304-2/2016OrganSA'HP 2.3.2.115.5115.62NegNeg18.93Neg27A/chicken/Chana/toVIR-4304-2/2016OrganSA'HP 2.3.2.121.022.04NegNeg18.93Neg28A/chicken/fana/toVIR-4304-2/2016OrganSA'HP 2.3.2.123.0318.14NegNeg19.93Neg29A/duck/Cameroon/toVIR-3791-21/2016Lungand trachaSA'HP 2.3.2.123.0513.12NegNeg19.93Neg30A/chicken/traq/ARS282/2016Field sampleSA'HP 2.3.2.128.050.65Neg26.09Neg26.09Neg26.09Neg26.09Neg26.09Neg19.9310.25NegNeg26.09Neg26.09Neg26.09Neg26.09Neg26.09Neg26.09Neg26.09Neg26.09Neg26.09Neg26.09Neg26.09Neg26.09Neg26.09Neg26.09Neg </td <td>21</td> <td>A/chicken/Niger/15VIR2060–12/2015</td> <td>Tracheal swab</td> <td>KU971309</td> <td>HP 2.3.2.1</td> <td>25.50</td> <td>25.08</td> <td>Neg</td> <td>Neg</td> <td>24.37</td> <td>Neg</td>	21	A/chicken/Niger/15VIR2060–12/2015	Tracheal swab	KU971309	HP 2.3.2.1	25.50	25.08	Neg	Neg	24.37	Neg
23A/domestic_bird/Burkina_Faso/stylRry74_2A/2015OrganKU971500HP 2.3.c.121.021.00NegNeg24.01Neg24A/domestic_bird/Burkina_Faso/stylRry74_23/2015OrganSA*HP 2.3.c.123.3722.49NegNeg14.2115.515.61NegNeg14.2115.2	22	A/chicken/Niger/15VIR2060–5/2015	Swab	KU971326	HP 2.3.2.1	23.08	21.99	Neg	Neg	20.35	Neg
24.A/domeslic_bird/Burkina_Faso/syURarya-3/2015OrganKU971500HP 2.3.2.121.0131.01NegNegNeg18.4.7Neg25.A/chicken/Ghana/ts/UR-43042/2016OrganSA*HP 2.3.2.123.224.22NegNeg12.09Neg26.A/chicken/Ghana/ts/UR-43043/2016OrganSA*HP 2.3.2.124.2226.45NegNeg12.09Neg28.A/chicken/Ghana/ts/UR-43043/2016OrganSA*HP 2.3.2.124.2226.45NegNeg19.29Neg29.A/chicken/fana/Ags28/2016Unagand tracheaSA*HP 2.3.2.123.0711.12NegNeg29.57Neg30.A/chicken/traq/Ags28/2016Field sampleSA*HP 2.3.2.131.7031.12Neg29.57Neg31.A/chicken/traq/Ags28/2016Field sampleSA*HP 2.3.2.123.7029.69Neg28.6928.6934.A/chicken/traq/Ags28/2016Field sampleSA*HP 2.3.2.123.6929.69Neg28.6928.69Neg34.A/chicken/traq/Ags28/2016Field sampleSA*HP 2.3.2.128.6927.64Neg28.6928	23	A/domestic_bird/Burkina_Faso/15VIR1774–24/2015	Swab	KU971508	HP 2.3.2.1	21.05	29.03	Neg	Neg	24.01	Neg
25 A/chicken/Ghana/16VIR-4304r/2016 Organ SA ³ HP 2.3.2.1 23.37 22.49 Neg Neg 18.44 Neg 26 A/chicken/Ghana/16VIR-430442/2016 Organ SA ³ HP 2.3.2.1 21.25 I.56 Neg Neg 18.29 Neg 27 A/chicken/Ghana/16VIR-430442/2016 Organ SA ³ HP 2.3.2.1 21.27 Value Neg Neg Neg 18.29 Neg 28 A/chicken/Ghana/16VIR-430442/2016 Lungand trache SA ³ HP 2.3.2.1 23.00 I.81 Neg Neg Neg 19.29 Neg 30 A/chicken/Iraa/A82542/2016 Eleid sample SA ³ HP 2.3.2.1 28.05 Neg Neg 28.05 Res 38.0 Neg 28.05 Res 38.0 Nether/Iraa/A8254/2016 Field sample SA ³ HP 2.3.2.1 28.05 Neg 28.05 Res 38.0 Neg 28.05 Res 38.0 31 A/chicken/Iraa/A8254/2016 Field sample SA ³ </td <td>24</td> <td>A/domestic_bird/Burkina_Faso/15VIR1774–23/2015</td> <td>Organ</td> <td>KU971500</td> <td>HP 2.3.2.1</td> <td>21.91</td> <td>30.83</td> <td>Neg</td> <td>Neg</td> <td>24.72</td> <td>Neg</td>	24	A/domestic_bird/Burkina_Faso/15VIR1774–23/2015	Organ	KU971500	HP 2.3.2.1	21.91	30.83	Neg	Neg	24.72	Neg
26 A/chicken/Ghana/tóVIR-430a-2s/2016 Organ SA ^a HP 2.3.2.1 24,22 24,62 Neg Neg 15.8 Neg 27 A/chicken/Ghana/tóVIR-430a-4/2016 Organ SA ^a HP 2.3.2.1 24,22 24,42 Neg Neg 15.88 Neg 28 A/chicken/Iraq/ASp362/2016 Lungan Irachea SA ^a HP 2.3.2.1 23.00 18.4 Neg 17.92 Neg <td>25</td> <td>A/chicken/Ghana/16VIR-4304–1/2016</td> <td>Organ</td> <td>SAb</td> <td>HP 2.3.2.1</td> <td>23.37</td> <td>22.49</td> <td>Neg</td> <td>Neg</td> <td>18.44</td> <td>Neg</td>	25	A/chicken/Ghana/16VIR-4304–1/2016	Organ	SAb	HP 2.3.2.1	23.37	22.49	Neg	Neg	18.44	Neg
27 A/chicken/Ghana/16VIR-430a-42/2016 Organ SA ^b HP 2.3.2.1 24.22 20.45 Neg Neg 18.28 Neg 28 A/chicken/Ghana/16VIR-430a-9/2016 Organ SA ^b HP 2.3.2.1 23.00 Neg Neg Neg 19.33 Neg 29 A/chicken/Iraq/AS282/2016 Lungand trachea SA ^b HP 2.3.2.1 23.00 Neg Neg 19.93 Neg 30 A/chicken/Iraq/AS282/2016 Field sample NA HP 2.3.2.1 28.70 Neg Neg 49.00 Neg 36.50 Ne	26	A/chicken/Ghana/16VIR-4304–25/2016	Organ	SAb	HP 2.3.2.1	15.51	15.62	Neg	Neg	12.09	Neg
28A/chicken/isqlasql-g/2016OrganSA ^b HP 2.3.2.121.7921.90NegNeg19.31Neg29A/duck/Cameron/isGNR-3791-21/2016Lungand tracheaSA ^b HP 3.3.2.128.0018.14NegNeg17.98Neg30A/chicken/iraq/RA5282/2016Field sampleNAHP 3.3.2.128.7031.22NegNeg28.70NegNeg28.70NegNeg28.70NegNeg28.70NegNeg28.70NegNeg28.70NegNeg28.70NegNeg28.70NegNeg28.70NegNeg28.70NegNeg28.70Neg28.70NegNeg28.70NegNeg28.70 <td>27</td> <td>A/chicken/Ghana/16VIR-4304–42/2016</td> <td>Organ</td> <td>SAb</td> <td>HP 2.3.2.1</td> <td>24.22</td> <td>20.45</td> <td>Neg</td> <td>Neg</td> <td>18.28</td> <td>Neg</td>	27	A/chicken/Ghana/16VIR-4304–42/2016	Organ	SAb	HP 2.3.2.1	24.22	20.45	Neg	Neg	18.28	Neg
29A/duck/Cameroon/i6VIR-3791-21/2016Lungand trachenSA ⁵ HP 2.3.2.12.3.0018.1.4NegNeg1.7.98Neg30A/chicken/Iraq/AR528/2016Field sampleNAHP 2.3.2.123.7828.70Neg3.703.10Neg3.703.10Neg3.703.10Neg3.703.10Neg3.703.10Neg3.703.10Neg3.703.703.703.70NegNeg3.70NegNeg3.70NegNeg3.70NegNeg3.70NegNeg3.70NegNeg3.70NegNeg3.70NegNeg3.70NegN	28	A/chicken/Ghana/16VIR-4304–9/2016	Organ	SAb	HP 2.3.2.1	21.79	21.90	Neg	Neg	19.13	Neg
30A/chicken/traq/ARs282/2016Field sampleNAHP 2.3.2.182.7.882.8.0Neg9.9.9Neg9.9.9Neg31A/chicken/traq/ARs283/2016Field sampleSA°HP 2.3.2.128.1128.013.0.03.102Neg8.0.0Neg32A/chicken/traq/ARs286/2016Field sampleSA°HP 2.3.2.128.0128.053.0.0Neg26.00Neg9.00Neg26.00Neg33A/chicken/traq/ARs287/2016Field sampleSA°HP 2.3.2.120.053.0.02Neg3.0.02Neg3.0.0Neg9.00Neg3.0.0Neg3.0.0Neg3.0.0Neg3.0.0Neg3.0.0Neg3.0.0Neg3.0.0Neg3.0.0Neg3.0.0NegNeg3.0.0Neg3.0.0Neg3.0.0Neg3.0.0Neg3.0.0Neg3.0.0Neg3.0.0Neg3.0.0Neg3.0.0Neg3.0.0Neg3.0.0Neg3.0.0Neg3.0.0Neg3.0.0NegNeg3.0.0NegNeg3.0.0NegNeg3.0.0NegNegNeg3.0.0NegNegNegNeg2.0.0NegNe	29	A/duck/Cameroon/16VIR-3791–21/2016	Lungand trachea	SAb	HP 2.3.2.1	23.00	18.14	Neg	Neg	17.98	Neg
31A/chicken/traq/AR5283/2016Field sampleNAHP 2.3.2.131.031.12NegNeg35.00Neg32A/chicken/traq/AR5286/2016Field sampleSA ^b HP 2.3.2.128.0128.0130.00Neg28.00Neg33A/chicken/traq/AR5287/2016Field sampleSA ^b HP 2.3.2.129.0229.00NegNeg28.00Neg34A/chicken/traq/AR5292/2016Field sampleNAHP 2.3.2.128.6028.01Neg31.00NegNeg	30	A/chicken/Iraq/AR5282/2016	Field sample	NA	HP 2.3.2.1	28.78	28.20	Neg	Neg	29.57	Neg
32A/chicken/traq/AR5286/2016Field sampleSA ^{\oveen} HP 2.3.c.128.c.928.c.9Me28.c.6Neg33A/chicken/traq/AR5287/2016Field sampleSA ^{\oveen} HP 2.3.c.129.c9Z9.o9NegNeg28.o9Neg34A/chicken/traq/AR529/2016Field sampleSA ^{\oveen} HP 2.3.c.129.c9Z9.o9NegNeg28.o9Neg35A/chicken/traq/AR529/2016Field sampleSA ^{\oveen} HP 2.3.c.128.c9Z9.c9NegNeg28.c928.c137A/turkey/Germany/AR299/2014Field sampleSA ^{\oveen} HP 2.3.c429.c9Z.44NegNeg28.c338A/turkey/Germany/AR290/2014Field sampleSA ^{\oveen} HP 2.3.c429.c9Z.44NegNeg28.c339A/turkey/Germany/AR290/2014Field sampleSA ^{\oveennotice} HP 2.3.c429.c9Z.44NegNeg28.c340A/turkey/Germany/AR250/2014Field sampleSA ^{\oveennotice} HP 2.3.c429.c5Z.50NegNeg28.c341A/turkey/Germany/AR250/2014Field sampleSA ^{\oveennotice} HP 2.3.c429.c5Z.50NegNeg28.c342A/turkey/Germany/AR250/2014Field sampleSA ^{\oveennotice} HP 2.3.c426.55Z.50Z.68NegNeg25.c143A/turkey/Germany/AR250/2014Field sampleSA ^{\oveennotice} HP 2.3.c426.55Z.50Z.68Neg <t< td=""><td>31</td><td>A/chicken/ Iraq/AR5283/2016</td><td>Field sample</td><td>NA</td><td>HP 2.3.2.1</td><td>31.70</td><td>31.12</td><td>Neg</td><td>Neg</td><td>35.02</td><td>Neg</td></t<>	31	A/chicken/ Iraq/AR5283/2016	Field sample	NA	HP 2.3.2.1	31.70	31.12	Neg	Neg	35.02	Neg
33A/chicken/traq/ARs287/2016Field sampleSAbHP 2.3.2.128.0527.08NegNeg26.09Neg34A/chicken/traq/ARs291/2016Field sampleSAbHP 2.3.2.130.3230.2836.32Neg31.55Neg35A/chicken/traq/ARs292/2016Field sampleNAHP 2.3.2.130.3330.2836.32Neg31.55Neg36A/chicken/traq/ARs292/2016Field sampleSAbHP 2.3.2.128.0528.21NegNeg28.03Neg37A/turkey/Germany/AR2499/2014Field sampleSAbHP 2.3.4.427.7826.4836.71NegNeg28.0338A/turkey/Germany/AR250/2014Field sampleSAbHP 2.3.4.432.2130.65NegNeg28.3040A/turkey/Germany/AR250/2014Field sampleSAbHP 2.3.4.430.6827.92NegNeg28.3041A/turkey/Germany/AR250/2014Field sampleSAbHP 2.3.4.430.6827.92NegNeg28.2142A/turkey/Germany/AR250/2014Field sampleSAbHP 2.3.4.430.5236.35NegNeg28.2143A/turkey/Germany/AR250/2014Field sampleSAbHP 2.3.4.430.5236.35NegNeg28.2144A/turkey/Germany/AR250/2014Field sampleSAbHP 2.3.4.431.6030.6336.82NegNeg30.7145A/turkey/Germany/AR250/2014 </td <td>32</td> <td>A/chicken/Iraq/AR5286/2016</td> <td>Field sample</td> <td>SAb</td> <td>HP 2.3.2.1</td> <td>28.21</td> <td>28.10</td> <td>36.50</td> <td>Neg</td> <td>28.16</td> <td>Neg</td>	32	A/chicken/Iraq/AR5286/2016	Field sample	SAb	HP 2.3.2.1	28.21	28.10	36.50	Neg	28.16	Neg
34A/chicken/Iraq/AR5291/2016Field sampleSA ^b HP 2.3.2.129.2929.09NegNeg28.09Neg35A/chicken/Iraq/AR5292/2016Field sampleNAHP 2.3.2.130.8330.2836.32Neg31.15Neg36A/chicken/Iraq/AR5292/2016Field sampleSA ^b HP 2.3.2.128.6028.21NegNeg28.53Neg37A/turkey/Germany/AR299/2014Field sampleSA ^b HP 2.3.4.427.6726.4836.71NegNeg24.6138A/turkey/Germany/AR250/2014Field sampleSA ^b HP 2.3.4.429.5927.44NegNegNeg28.5339A/turkey/Germany/AR250/2014Field sampleSA ^b HP 2.3.4.430.0827.92NegNegNeg28.5340A/turkey/Germany/AR250/2014Field sampleSA ^b HP 2.3.4.430.0827.92NegNeg25.9241A/turkey/Germany/AR250/2014Field sampleSA ^b HP 2.3.4.430.5236.36NegNeg25.9242A/turkey/Germany/AR250/2014Field sampleSA ^b HP 2.3.4.426.5736.36NegNeg25.9243A/turkey/Germany/AR250/2014Field sampleSA ^b HP 2.3.4.426.5736.36NegNeg26.5744A/turkey/Germany/AR250/2014Field sampleSA ^b HP 2.3.4.426.5726.0536.36NegNeg26.5745 <td< td=""><td>33</td><td>A/chicken/Iraq/AR5287/2016</td><td>Field sample</td><td>SAb</td><td>HP 2.3.2.1</td><td>28.05</td><td>27.08</td><td>Neg</td><td>Neg</td><td>26.80</td><td>Neg</td></td<>	33	A/chicken/Iraq/AR5287/2016	Field sample	SAb	HP 2.3.2.1	28.05	27.08	Neg	Neg	26.80	Neg
35 A/chicken/Iraq/AR5292/2016 Field sample NA HP 2.3.2.1 30.3 30.28 36.32 Neg 31.15 Neg 36 A/chicken/Iraq/AR5296/2016 Field sample SA ^b HP 2.3.2.1 28.60 28.21 Neg Neg 28.53 Neg 37 A/turkey/Germany/AR2499/2014 Field sample SA ^b HP 2.3.4.4 27.78 26.48 36.71 Neg Neg 24.61 38 A/turkey/Germany/AR2499/2014 Field sample SA ^b HP 2.3.4.4 29.59 27.44 Neg Neg Neg 28.50 39 A/turkey/Germany/AR250/2014 Field sample SA ^b HP 2.3.4.4 30.08 27.92 Neg Neg Neg 25.67 41 A/turkey/Germany/AR250/2014 Field sample SA ^b HP 2.3.4.4 30.52 28.21 Neg Neg Neg 25.21 42 A/turkey/Germany/AR250/2014 Field sample SA ^b HP 2.3.4.4 26.55 25.02 36.88 Neg Neg 28.57 43 A/turkey/Germany/AR250/2014 Field sample <td< td=""><td>34</td><td>A/chicken/Iraq/AR5291/2016</td><td>Field sample</td><td>SAb</td><td>HP 2.3.2.1</td><td>29.29</td><td>29.09</td><td>Neg</td><td>Neg</td><td>28.09</td><td>Neg</td></td<>	34	A/chicken/Iraq/AR5291/2016	Field sample	SAb	HP 2.3.2.1	29.29	29.09	Neg	Neg	28.09	Neg
36 A/chicken/Iraq/AR5296/2016 Field sample SA ^b HP 2.3.2.1 28.60 28.21 Neg Neg 28.55 Neg 37 A/turkey/Germany/AR2499/2014 Field sample SA ^b HP 2.3.4.4 27.78 26.48 36.71 Neg Neg 24.61 38 A/turkey/Germany/AR2500/2014 Field sample SA ^b HP 2.3.4.4 29.59 27.44 Neg Neg Neg 28.50 39 A/turkey/Germany/AR2501/2014 Field sample SA ^b HP 2.3.4.4 30.08 27.92 Neg Neg Neg 25.67 41 A/turkey/Germany/AR2502/2014 Field sample SA ^b HP 2.3.4.4 30.52 28.21 Neg Neg Neg 25.67 42 A/turkey/Germany/AR250/2014 Field sample SA ^b HP 2.3.4.4 26.15 25.02 36.88 Neg Neg 25.21 43 A/turkey/Germany/AR257/2014 Field sample SA ^b HP 2.3.4.4 26.03 30.06 37.3 Neg Neg 28.57 45 A/turkey/Germany/AR2591/2014 Field sample	35	A/chicken/Iraq/AR5292/2016	Field sample	NA	HP 2.3.2.1	30.83	30.28	36.32	Neg	31.15	Neg
37A/turkey/Germany/AR2499/2014Field sampleSA ^b HP 2.3.4.427.7826.4836.71NegNeg24.6138A/turkey/Germany/AR2500/2014Field sampleSA ^b HP 2.3.4.42.9592.7.44NegNegNeg2.8.2039A/turkey/Germany/AR2501/2014Field sampleSA ^b HP 2.3.4.43.0.82.7.92NegNegNeg2.8.2140A/turkey/Germany/AR2502/2014Field sampleSA ^b HP 2.3.4.43.0.82.9.22NegNeg2.5.9241A/turkey/Germany/AR2502/2014Field sampleSA ^b HP 2.3.4.43.0.522.8.21NegNeg2.5.9242A/turkey/Germany/AR2502/2014Field sampleSA ^b HP 2.3.4.42.6.52.5.023.6.8NegNeg2.5.1243A/turkey/Germany/AR2502/2014Field sampleSA ^b HP 2.3.4.42.6.93.0.603.7.3NegNeg2.8.1744A/turkey/Germany/AR2501/2014Field sampleSA ^b HP 2.3.4.42.6.93.0.603.7.3NegNeg2.8.1745A/turkey/Germany/AR2501/2014Field sampleSA ^b HP 2.3.4.42.6.93.0.603.7.3NegNeg2.8.1746A/turkey/Germany/AR337/2014Field sampleSA ^b HP 2.3.4.42.6.93.0.603.0.83.6.8Neg2.9.1747A/turkey/Germany/AR3381/2014Field sampleSA ^b HP 2.3.4.42.6.9NegNeg<	36	A/chicken/Iraq/AR5296/2016	Field sample	SA ^b	HP 2.3.2.1	28.60	28.21	Neg	Neg	28.53	Neg
38A/turkey/Germany/AR250/2014Field sampleSA ^b HP 2.3.4.429.5927.44NegNegNeg25.2039A/turkey/Germany/AR250/2014Field sampleSA ^b HP 2.3.4.430.65NegNegNeg28.3040A/turkey/Germany/AR250/2014Field sampleSA ^b HP 2.3.4.430.6528.21NegNeg25.2741A/turkey/Germany/AR250/2014Field sampleSA ^b HP 2.3.4.420.5525.0236.88NegNeg25.2742A/turkey/Germany/AR250/2014Field sampleSA ^b HP 2.3.4.421.6930.0336.30NegNeg28.2143A/turkey/Germany/AR251/2014Field sampleSA ^b HP 2.3.4.421.6930.0637.13NegNeg28.5745A/tarkey/Germany/AR251/2014Field sampleSA ^b HP 2.3.4.421.6930.0637.03NegNeg28.5745A/turkey/Germany/AR337/2014Field sampleSA ^b HP 2.3.4.421.6930.0630.88NegNeg23.1746A/turkey/Germany/AR338/2014Field sampleSA ^b HP 2.3.4.426.5325.65NegNeg23.1747A/turkey/Germany/AR338/2014Field sampleSA ^b HP 2.3.4.426.5325.26NegNeg23.1748A/turkey/Germany/AR338/2014Field sampleSA ^b HP 2.3.4.426.5325.26NegNeg24.5150A/t	37	A/turkey/Germany/AR2499/2014	Field sample	SAb	HP 2.3.4.4	27.78	26.48	36.71	Neg	Neg	24.61
39 A/turkey/Germany/AR2501/2014 Field sample SA ^b HP 2.3.4.4 32.21 30.65 Neg Neg 28.30 40 A/turkey/Germany/AR2502/2014 Field sample SA ^b HP 2.3.4.4 30.08 27.92 Neg Neg Neg 25.67 41 A/turkey/Germany/AR2502/2014 Field sample SA ^b HP 2.3.4.4 30.52 28.21 Neg Neg 25.92 42 A/turkey/Germany/AR2502/2014 Field sample SA ^b HP 2.3.4.4 26.15 25.02 36.88 Neg Neg 25.21 43 A/turkey/Germany/AR2502/2014 Field sample SA ^b HP 2.3.4.4 26.05 30.66 Neg Neg 28.01 44 A/turkey/Germany/AR2502/2014 Field sample SA ^b HP 2.3.4.4 28.09 30.06 37.13 Neg Neg 28.01 45 A/turkey/Germany/AR2502/2014 Field sample SA ^b HP 2.3.4.4 28.09 30.06 37.33 Neg Neg 28.57 45 A/turkey/Germany/AR2502/2014 Field sample SA ^b HP 2.3.4.4 <	38	A/turkey/Germany/AR2500/2014	Field sample	SAb	HP 2.3.4.4	29.59	27.44	Neg	Neg	Neg	25.20
40 A/turkey/Germany/AR2502/2014 Field sample SA ^b HP 2.3.4.4 30.08 27.92 Neg Neg 25.67 41 A/turkey/Germany/AR2503/2014 Field sample SA ^b HP 2.3.4.4 30.52 28.21 Neg Neg Neg 25.92 42 A/turkey/Germany/AR2502/2014 Field sample SA ^b HP 2.3.4.4 26.15 25.02 36.88 Neg Neg 25.92 43 A/turkey/Germany/AR2502/2014 Field sample SA ^b HP 2.3.4.4 26.15 25.02 36.88 Neg 28.01 44 A/turkey/Germany/AR2502/2014 Field sample SA ^b HP 2.3.4.4 26.05 30.06 37.31 Neg Neg 28.01 44 A/turkey/Germany/AR2501/2014 Field sample SA ^b HP 2.3.4.4 26.03 30.06 37.31 Neg Neg 23.07 45 A/turkey/Germany/AR2307/2014 Field sample SA ^b HP 2.3.4.4 26.35 25.10 Neg Neg 23.12 48 A/turkey/Germany/AR3381/2014 Field sample SA ^b HP 2.3.4.4	39	A/turkey/Germany/AR2501/2014	Field sample	SAb	HP 2.3.4.4	32.21	30.65	Neg	Neg	Neg	28.30
41 A/turkey/Germany/AR2503/2014 Field sample SA ^b HP 2.3.4.4 30.52 28.21 Neg Neg 25.92 42 A/turkey/Germany/AR2562/2014 Field sample SA ^b HP 2.3.4.4 26.15 25.02 36.88 Neg Neg 25.92 43 A/turkey/Germany/AR2574/2014 Field sample SA ^b HP 2.3.4.4 26.09 30.60 37.13 Neg Neg 28.01 44 A/turkey/Germany/AR2591/2014 Field sample SA ^b HP 2.3.4.4 28.09 30.60 37.13 Neg Neg 28.01 45 A/turkey/Germany/AR2917/2014 Field sample SA ^b HP 2.3.4.4 28.09 30.60 37.13 Neg Neg 28.07 46 A/turkey/Germany/AR2917/2014 Field sample SA ^b HP 2.3.4.4 26.39 26.16 Neg Neg 23.07 47 A/turkey/Germany/AR3372/2014 Field sample SA ^b HP 2.3.4.4 26.39 25.10 Neg Neg 23.40 49 A/turkey/Germany/AR3381/2014 Field sample SA ^b HP 2.3.4.4	40	A/turkey/Germany/AR2502/2014	Field sample	SAb	HP 2.3.4.4	30.08	27.92	Neg	Neg	Neg	25.67
42A/turkey/Germany/AR256/2014Field sampleSAbHP 2.3.4.426.1525.0236.88Neg25.2143A/turkey/Germany/AR2574/2014Field sampleSAbHP 2.3.4.427.4930.2336.36NegNeg28.0144A/turkey/Germany/AR2591/2014Field sampleSAbHP 2.3.4.428.0930.0637.13NegNeg28.5745A/teal/Germany/AR291/2014Field sampleSAbHP 2.3.4.431.6030.0836.82NegNeg23.0746A/turkey/Germany/AR3372/2014Field sampleSAbHP 2.3.4.426.3324.85NegNeg23.0747A/turkey/Germany/AR3372/2014Field sampleSAbHP 2.3.4.426.3325.10NegNeg23.0748A/turkey/Germany/AR3381/2014Field sampleSAbHP 2.3.4.426.3525.26NegNeg23.0749A/turkey/Germany/AR3382/2014Field sampleSAbHP 2.3.4.426.3525.26NegNeg24.8550A/turkey/Germany/AR3382/2014Field sampleSAbHP 2.3.4.426.3525.26NegNeg26.0651A/duck/Germany/AR3457/2014Field sampleSAbHP 2.3.4.420.2526.18NegNeg26.0651A/duck/Germany/AR3457/2014Field sampleSAbHP 2.3.4.420.2628.13NegNeg26.0651A/duck/Germany/AR3457/2014Field sampleSA	41	A/turkey/Germany/AR2503/2014	Field sample	SAb	HP 2.3.4.4	30.52	28.21	Neg	Neg	Neg	25.92
43 A/turkey/Germany/AR2574/2014 Field sample SA ^b HP 2.3.4.4 27.49 30.23 36.36 Neg 28.01 44 A/turkey/Germany/AR2591/2014 Field sample SA ^b HP 2.3.4.4 28.09 30.06 37.13 Neg Neg 28.57 45 A/teal/Germany/AR291/2014 Field sample SA ^b HP 2.3.4.4 31.60 30.08 36.82 Neg Neg 28.57 46 A/turkey/Germany/AR391/2014 Field sample SA ^b HP 2.3.4.4 26.33 24.85 Neg Neg 23.07 47 A/turkey/Germany/AR3372/2014 Field sample SA ^b HP 2.3.4.4 26.33 24.85 Neg Neg 23.07 47 A/turkey/Germany/AR337/2014 Field sample SA ^b HP 2.3.4.4 26.35 25.05 Neg Neg 23.07 49 A/turkey/Germany/AR3381/2014 Field sample SA ^b HP 2.3.4.4 26.45 26.18 Neg Neg 24.18 50 A/turkey/Germany/AR3382/2014 Field sample SA ^b HP 2.3.4.4 29.26 28.13 <t< td=""><td>42</td><td>A/turkey/Germany/AR2562/2014</td><td>Field sample</td><td>SA⁵</td><td>HP 2.3.4.4</td><td>26.15</td><td>25.02</td><td>36.88</td><td>Neg</td><td>Neg</td><td>25.21</td></t<>	42	A/turkey/Germany/AR2562/2014	Field sample	SA⁵	HP 2.3.4.4	26.15	25.02	36.88	Neg	Neg	25.21
44 A/turkey/Germany/AR2591/2014 Field sample SA ^b HP 2.3.4.4 28.09 30.06 37.13 Neg 28.77 45 A/teal/Germany/AR2917/2014 Field sample SA ^b HP 2.3.4.4 31.60 30.08 36.82 Neg Neg 35.41 46 A/turkey/Germany/AR3917/2014 Field sample SA ^b HP 2.3.4.4 26.33 24.85 Neg Neg 23.07 47 A/turkey/Germany/AR3372/2014 Field sample SA ^b HP 2.3.4.4 26.39 25.10 Neg Neg 23.07 48 A/turkey/Germany/AR3381/2014 Field sample SA ^b HP 2.3.4.4 26.35 25.26 Neg Neg 23.07 49 A/turkey/Germany/AR3381/2014 Field sample SA ^b HP 2.3.4.4 26.85 25.26 Neg Neg 23.07 49 A/turkey/Germany/AR3382/2014 Field sample SA ^b HP 2.3.4.4 20.85 25.16 Neg Neg 24.08 50 A/turkey/Germany/AR3383/2014 Field sample SA ^b HP 2.3.4.4 29.26 28.13 Neg <	43	A/turkey/Germany/AR2574/2014	Field sample	SAb	HP 2.3.4.4	27.49	30.23	36.36	Neg	Neg	28.01
45 A/teal/Germany/AR2917/2014 Field sample SA ^b HP 2.3.4.4 31.60 30.08 36.82 Neg 35.41 46 A/turkey/Germany/AR3372/2014 Field sample EP155372 HP 2.3.4.4 26.33 24.85 Neg Neg 23.07 47 A/turkey/Germany/AR3376/2014 Field sample SA ^b HP 2.3.4.4 26.39 25.10 Neg Neg 23.07 48 A/turkey/Germany/AR3381/2014 Field sample SA ^b HP 2.3.4.4 26.53 25.26 Neg Neg 23.02 49 A/turkey/Germany/AR3381/2014 Field sample SA ^b HP 2.3.4.4 26.55 25.26 Neg Neg 23.02 49 A/turkey/Germany/AR3382/2014 Field sample SA ^b HP 2.3.4.4 26.45 26.18 Neg Neg 24.18 50 A/turkey/Germany/AR3382/2014 Field sample SA ^b HP 2.3.4.4 29.26 28.13 Neg Neg 26.06 51 A/duck/Germany/AR3457/2014 Field sample SA ^b HP 2.3.4.4 30.29 29.30 Neg Neg <t< td=""><td>44</td><td>A/turkey/Germany/AR2591/2014</td><td>Field sample</td><td>SAb</td><td>HP 2.3.4.4</td><td>28.09</td><td>30.06</td><td>37.13</td><td>Neg</td><td>Neg</td><td>28.57</td></t<>	44	A/turkey/Germany/AR2591/2014	Field sample	SAb	HP 2.3.4.4	28.09	30.06	37.13	Neg	Neg	28.57
46 A/turkey/Germany/AR3372/2014 Field sample EPI553172 HP 2.3.4.4 26.33 24.85 Neg Neg 23.07 47 A/turkey/Germany/AR3376/2014 Field sample SAb HP 2.3.4.4 26.39 25.10 Neg Neg Neg 23.07 48 A/turkey/Germany/AR3376/2014 Field sample SAb HP 2.3.4.4 26.39 25.10 Neg Neg Neg 23.12 48 A/turkey/Germany/AR3381/2014 Field sample SAb HP 2.3.4.4 26.85 25.26 Neg Neg 23.40 49 A/turkey/Germany/AR3382/2014 Field sample SAb HP 2.3.4.4 26.65 25.26 Neg Neg 24.85 50 A/turkey/Germany/AR3383/2014 Field sample SAb HP 2.3.4.4 29.26 28.13 Neg Neg 26.06 51 A/duck/Germany/AR3457/2014 Field sample SAb HP 2.3.4.4 30.29 29.30 Neg Neg 26.36 52 A/duck/Germany/AR345/2014 Field sample SAb HP 2.3.4.4 20.92 23.15 Neg	45	A/teal/Germany/AR2917/2014	Field sample	SA ^b	HP 2.3.4.4	31.60	30.08	36.82	Neg	Neg	35.41
47 A/turkey/Germany/AR3376/2014 Field sample SA ^b HP 2.3.4.4 26.39 25.10 Neg Neg Neg 23.12 48 A/turkey/Germany/AR3381/2014 Field sample SA ^b HP 2.3.4.4 26.39 25.10 Neg Neg Neg 23.12 49 A/turkey/Germany/AR3381/2014 Field sample SA ^b HP 2.3.4.4 26.85 25.26 Neg Neg Neg 23.40 49 A/turkey/Germany/AR3382/2014 Field sample SA ^b HP 2.3.4.4 29.26 28.13 Neg Neg Neg 24.18 50 A/turkey/Germany/AR3383/2014 Field sample SA ^b HP 2.3.4.4 29.26 28.13 Neg Neg Neg 26.06 51 A/duck/Germany/AR3457/2014 Field sample SA ^b HP 2.3.4.4 30.29 29.30 Neg Neg Neg 28.34 52 A/duck/Germany/AR3457/2014 Field sample SA ^b HP 2.3.4.4 20.98 23.15 Neg Neg 20.70 53 A/duck/Germany/AR3465/2014 Field sample SA ^b <	46	A/turkey/Germany/AR3372/2014	Field sample	EPI553172	HP 2.3.4.4	26.33	24.85	Neg	Neg	Neg	23.07
48 A/turkey/Germany/AR3381/2014 Field sample SA ^b HP 2.3.4.4 26.85 25.26 Neg Neg Neg 23.40 49 A/turkey/Germany/AR3381/2014 Field sample SA ^b HP 2.3.4.4 26.85 25.26 Neg Neg Neg 23.40 50 A/turkey/Germany/AR3382/2014 Field sample SA ^b HP 2.3.4.4 29.26 28.13 Neg Neg Neg 24.18 50 A/turkey/Germany/AR3383/2014 Field sample SA ^b HP 2.3.4.4 29.26 28.13 Neg Neg Neg 26.06 51 A/duck/Germany/AR3457/2014 Field sample SA ^b HP 2.3.4.4 30.29 29.30 Neg Neg 28.34 52 A/duck/Germany/AR3465/2014 Field sample SA ^b HP 2.3.4.4 23.98 23.15 Neg Neg Neg 20.70 53 A/duck/Germany/AR3470/2014 Field sample SA ^b HP 2.3.4.4 23.18 Neg Neg Neg 20.70 53 A/duck/Germany/AR3470/2014 Field sample SA ^b HP 2.3.4.4	47	A/turkey/Germany/AR3376/2014	Field sample	SA ^b	HP 2.3.4.4	26.39	25.10	Neg	Neg	Neg	23.12
49 A/turkey/Germany/AR3382/2014 Field sample SA ^b HP 2.3.4.4 27.64 Z6.18 Neg Neg Neg Z4.18 50 A/turkey/Germany/AR3382/2014 Field sample SA ^b HP 2.3.4.4 29.26 28.13 Neg Neg Neg 26.06 51 A/duck/Germany/AR3457/2014 Field sample SA ^b HP 2.3.4.4 30.29 29.30 Neg Neg Neg 28.34 52 A/duck/Germany/AR3465/2014 Field sample SA ^b HP 2.3.4.4 23.98 23.15 Neg Neg Neg 20.70 53 A/duck/Germany/AR3470/2014 Field sample SA ^b HP 2.3.4.4 23.98 23.15 Neg Neg Neg 20.70	48	A/turkey/Germany/AR3381/2014	Field sample	SA ^b	HP 2.3.4.4	26.85	25.26	Neg	Neg	Neg	23.40
50 A/turkey/Germany/AR3383/2014 Field sample SA ^b HP 2.3.4.4 29.26 28.13 Neg Neg Neg 26.06 51 A/duck/Germany/AR3457/2014 Field sample SA ^b HP 2.3.4.4 30.29 29.30 Neg Neg 28.33 52 A/duck/Germany/AR3465/2014 Field sample SA ^b HP 2.3.4.4 23.98 23.15 Neg Neg Neg 20.70 53 A/duck/Germany/AR3470/2014 Field sample SA ^b HP 2.3.4.4 22.15 23.89 Neg Neg Neg 20.70	49	A/turkey/Germany/AR3382/2014	Field sample	SA ^b	HP 2.3.4.4	27.64	26.18	Neg	Neg	Neg	24.18
51 A/duck/Germany/AR3457/2014 Field sample SA ^b HP 2.3.4.4 30.29 29.30 Neg Neg 28.34 52 A/duck/Germany/AR3465/2014 Field sample SA ^b HP 2.3.4.4 23.98 23.15 Neg Neg 20.70 53 A/duck/Germany/AR3470/2014 Field sample SA ^b HP 2.3.4.4 22.15 23.89 Neg Neg 20.70	50	A/turkey/Germany/AR3383/2014	Field sample	SA ^b	HP 2.3.4.4	29.26	28.13	Neg	Neg	Neg	26.06
52 A/duck/Germany/AR3465/2014 Field sample SA ^b HP 2.3.4.4 23.98 23.15 Neg Neg 20.70 53 A/duck/Germany/AR3470/2014 Field sample SA ^b HP 2.3.4.4 22.15 23.89 Neg Neg 21.78	51	A/duck/Germany/AR3457/2014	Field sample	SA ^b	HP 2.3.4.4	30.29	29.30	Neg	Neg	Neg	28.34
53 A/duck/Germany/AR3470/2014 Field sample SA ^b HP 2.3.4.4 22.15 23.89 Neg Neg 21.78	52	A/duck/Germany/AR3465/2014	Field sample	SA ^b	HP 2.3.4.4	23.98	23.15	Neg	Neg	Neg	20.70
	53	A/duck/Germany/AR3470/2014	Field sample	SA⁵	HP 2.3.4.4	22.15	23.89	Neg	Neg	Neg	21.78

HA: haemagglutinin; HP: highly pathogenic; HPAI: highly pathogenic avian influenza; ID: identity; LP: low pathogenic; LPAI: low pathogenic avian influenza; NA: sequence not available; Neg: negative; SA: sequence available.

^a Sequences were obtained from GenBank at the National Center for Biotechnology Information (NCBI) or the EpiFlu database of the Global Initiative on Sharing Avian Influenza Data (GISAID).

^b Sequenced in the frame of the current study; sequences available from the authors upon request.

2013 and 2016. Samples consisted mainly of oropharyngeal and/or cloacal swabs and tissues samples (n = 70) or AIV isolated from such samples (n = 36) (see also second table under Results).

A subsection of the 106 clinical samples (n=13) was provided as dried material on Whatman FTA card (Sigma Aldrich, Germany). Samples from Western African countries were exclusively assayed at the Istituto Zooprofilattico Sperimentale delle Venezie, Padua, Italy.

Design of primers and probes

Primers were chosen based on alignments of the HA H5 gene of a selection of influenza A virus sequences submitted over the past 10 years to GenBank at the National Center for Biotechnology Information (NCBI) or to the EpiFlu database of the Global Initiative on Sharing Avian Influenza Data (GISAID). Selected sequences represented Eurasian LP viruses and HP isolates and clades of the gs/GD lineage that were detected in Europe, the Middle East and Western Africa during the past decade. Selection of primers to amplify a small fragment of the HA gene spanning the endoproteolytic cleavage site aimed at being broadly inclusive so as to target as many of the published LP Eurasian H₅ HA sequences as possible and to distinguish them from HP viruses of the gs/GD lineage. The probes were placed directly onto the cleavage site in the attempt to specifically bind to sequences encoding either monoor polybasic patterns that distinguish LP and HP pathotypes, respectively (Table 1).

At first, sets of primers and probes were designed to detect and discriminate between HP and LP biotypes, i.e. Eurasian H₅ viruses encoding a monobasic or a polybasic HA cleavage site. In addition, four different sets of primers and probes were developed to differentiate between gs/GD clades 2.2.1.2, 2.3.2.1 and 2.3.4.4 (A and B). Pre-selected primers were then screened in silico for their specificity properties using Shannon entropy plots implemented in the Entropy One soft-(http://www.hiv.lanl.gov/content/sequence/ware ENTROPY/entropy_one.html). Oligont (oligont) were selected so as to retain full specificity for the selected clade and to maximise entropy against all other clades. Basic physical properties of oligont were checked using the online web interface Oligo Calculator version 3.27. The finally chosen oligont are shown in Table 1. Detailed results of the in silico analyses are available on request from the authors.

One-step quantitative reverse transcription PCR assays

All reactions were performed using the AgPath-ID One-Step RT-qPCR kit (Thermofisher, scientific, United States) as follows: Reverse transcription at 45 °C for 10 min, initial denaturation at 95 °C for 10 min, 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 μ l reaction mixture using 15 pmol of each forward and reverse primers and 5 pmol probe per reaction. For each parameter a separate reaction was used. Cycling was performed on a Biorad CFX96 Real-Time cycler (BioRad, Germany). Fluorescent signals were collected during the annealing phase, and the amplification data were analysed using Bio-Rad CFX Manager 3 software accessing automated fluorescence drift correction for baseline adjustment.

Nucleotide sequencing and clade assignment

Patho- and phylotyping results obtained by newly developed RT-qPCRs were counter-checked by nt (nt) sequencing of the entire or parts of the HA gene of the respective isolates/clinical samples. Amplification of the HA gene was performed using primers published previously [24] and primers recommended in the European Union Diagnostic Manual for AI in a one-step RT-PCR [25]. In addition, amplificates of the HPAI H5 and LPAI H5 RT-qPCRs were used for sequencing purposes as well. Products were size-separated in agarose gels, excised and purified using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). Purified PCR products were used for cycle sequencing reactions (BigDye Terminator v1.1 Cycle Sequencing Kit, Applied Biosystems, California, United States) the products of which were purified using NucleoSEQ columns (Macherey-Nagel GmbH and Co, Düren, Germany) and sequenced on an ABI PRISM 3130 Genetic Analyzer (Life Technologies, Darmstadt, Germany).

For pathotyping, deduced amino acid sequences of the endoproteolytical cleavage site of the HA gene were inspected and compared with the molecular pathotyping database provided by OFFLU [26]. Assignment of nt sequences to the gs/GD HPAI H5 virus clade system was performed by use of clade prediction tool implemented in the Influenza Research Database [27].

Results

Analytical specificity of pathotyping and phylotyping quantitative reverse transcription PCR assays

The specificity of the assays was evaluated with viral RNA from representative influenza A subtype H5 viruses that had been phylotyped based on full-length HA nt sequence analysis (Table 2). Furthermore, non-H5 subtypes, i.e. H9N2 and H7N7, as well as non-influenza avian viruses i.e. avian infectious bronchitis virus (IBV) and Newcastle disease virus (NDV) were employed (Table 2), and none of them was detected by any of the specific PCRs.

In the initial evaluation of the specificity of the pathotyping RT-qPCR assays carried out using two reference viruses: HPAI A/chicken/Egypt/AR236/2015 (H5N1, clade 2.2.1.2) and LPAI A/turkey/Germany/R2025/2008 (H5N3), specific reactivity exclusively with the homopathotypic virus was evident. In a second step, assays were extended to the full range of 24 reference viruses yielding a similar sharp distinction between HP and LP cleavage sites (Table 2).

TABLE 3B

Pathotyping and phylotyping of different potentially zoonotic HPAI and LPAI influenza A subtype H5 virus isolates and field samples collected from poultry and wild bird species in different countries, 2013–2016

Image of the start of the s	No	Comple ID	Type of sample	Accession Number ª	Clade	PCR results					
>1 Alleianty (many) (Man	NO.					M1.2	HPAI H5	LPAI H5	2.2.1.2	2.3.2.1	2.3.4.4
j Symbol	54	A/wild-duck/Germany/AR8603/2016	Field sample	SAb	HP 2.3.4.4b	22.51	22.90	Neg	Neg	Neg	21.14
	55	A/greyleg goose /Germany/AR8604/2016	Field sample	SAb	HP 2.3.4.4b	22.41	22.30	Neg	Neg	Neg	20.26
jp>Alterior subjeemanyAbogenersFind sumeSAMPersonal <td>56</td> <td>A/greater scaup/Germany/AR9090/2016</td> <td>Field sample</td> <td>SAb</td> <td>HP 2.3.4.4b</td> <td>27.29</td> <td>31.71</td> <td>Neg</td> <td>Neg</td> <td>Neg</td> <td>29.61</td>	56	A/greater scaup/Germany/AR9090/2016	Field sample	SAb	HP 2.3.4.4b	27.29	31.71	Neg	Neg	Neg	29.61
jalAlgentersong/AssorptionField<	57	A/greater scaup/Germany/AR9091/2016	Field sample	SA⁵	HP 2.3.4.4b	28.95	34.40	Neg	Neg	Neg	31.74
90Agree henolycemany/Absony/conkField sample5A*HP 2.3.4.060.32.3.6MeNe8.2.4.060Agreents exany/Germany/Absony/concField sample5A*HP 2.3.4.06.0.22.4.0MeMe2.8.0NeMe2.8.0NeMe2.8.0NeMe2.8.0NeMe2.8.0NeMe2.8.0NeMe2.8.0NeMe2.8.0NeMe2.8.0NeMe2.8.0NeMe2.8.0NeMe2.8.0NeMe2.8.0NeMe2.8.0NeMe2.8.0NeMe2.8.0NeMe2.8.0NeMe2.8.0NeMeMe2.8.0NeMeMe2.8.0NeMeMe2.8.0NeMeMe2.8.0NeMeMeMe2.8.0NeMeMeMe2.8.0NeMe<	58	A/greater scaup/Germany/AR9092/2016	Field sample	SA ^b	HP 2.3.4.4b	23.85	25.70	Neg	Neg	Neg	26.05
inSynchronic NameField sampleSAVHP2.3.4.06.0.2.0.5.Ref.HP2.3.4.05.0.7.6.0.5.HP2.3.4.05.0.7.6.0.5.7.10.7.5.4.05.0.7.10.7.5.4.05.0.7.5.10.7.5.4.05.0.7.5.10.7.5.4.05.0.7.5.10.7.5.7.5.10.7.5.7.5.10.7.5.7.5.7.5.10.7.5.7.5.7.5.10.7.5.7.5.7.5.7.5.10.7.5.7.5.7.5.7.5.7.5.7.5.7.5.7.5.7.5.7.	59	A/grey heron/Germany/AR9093/2016	Field sample	SA ^b	HP 2.3.4.4b	20.10	22.62	Neg	Neg	Neg	22.44
61Mirrather sampl(Aspong)2noisPield samplePield s	60	A/greater scaup/Germany/AR9094/2016	Field sample	SA ^b	HP 2.3.4.4b	16.31	20.62	Neg	Neg	Neg	18.92
orAnothern pinal i Caranay Abgog/ 2004Pield sampleNew	61	A/greater scaup/Germany/AR9095/2016	Field sample	SAb	HP 2.3.4.4b	20.99	22.79	Neg	Neg	Neg	21.75
9Abea space/errany/Abog/2abíField sampleSPA*IP3-AuIP3NetNe	62	A/northern pintail /Germany/AR9096/2016	Field sample	SA⁵	HP 2.3.4.4b	22.83	28.12	Neg	Neg	Neg	23.95
6xAltering gull (Gramar)/AlgogN2030Field sampleSAVIP 3.4, al.Val. <th< td=""><td>63</td><td>A/bean goose/Germany/AR9097/2016</td><td>Field sample</td><td>SA⁵</td><td>HP 2.3.4.4b</td><td>22.97</td><td>24.88</td><td>Neg</td><td>Neg</td><td>Neg</td><td>24.92</td></th<>	63	A/bean goose/Germany/AR9097/2016	Field sample	SA⁵	HP 2.3.4.4b	22.97	24.88	Neg	Neg	Neg	24.92
63Mathe som Germany/Abgog/andField sampleSAVHP 3.4.4.4H2.1.4.4U.2.1.5H2.1.4.4H2.1.4.4U.2.1.5H2.1.4.4U.2.1.5U.2.1.	64	A/herring gull /Germany/AR9098/2016	Field sample	SAb	HP 2.3.4.4b	20.40	22.35	Neg	Neg	Neg	23.64
64.6.10kkin/Germany/Abyai/2005Frield sample5.34HP 3.4.4.31.032.05.3MegMegMegA.9270Alcikan/Germany/Abyai/2005Frield sampleSAVHP 3.4.4.312.23.2.33.1.3MegMegMegMeg3.2.360Alcikan/Germany/Abyai/2005Frield sampleSAVHP 3.4.4.312.3.48.0.8MegMe	65	A/mute swan/Germany/AR9099/2016	Field sample	SA⁵	HP 2.3.4.4b	21.12	25.83	Neg	Neg	Neg	22.75
90A/chicken/Germany/Abgua/2006Field sampleSA ^N HP 23.4.4.21.2121.31NegNeg21.3268A/chicken/Germany/Abgua/2006Field sampleSA ^N HP 23.4.4.1.321.53NegNeg25.4070A/chicken/Tath/23/2098GislolateCAPSitesLPAI 15K91.24Neg9.00Neg	66	A/chicken/Germany/AR9140/2016	Field sample	SAb	HP 2.3.4.4b	21.08	20.55	Neg	Neg	Neg	22.92
64. AlchickenGermany/Abgua/2016 Field sample SA ¹ HP 2.34b 21.30 Nag Nag Nag S.2.32 69 AlchickenGermany/Abgua/2006 Field sample SA ¹ HP 3.34b 21.30 Nag Nag Nag S.2.32 Alchicken/Germany/Wayap-Stk/Zoog Isolate CAPSis64 LPAH H5bl 2.6.00 Nag LAP Nag <	67	A/chicken/Germany/AR9141/2016	Field sample	SAb	HP 2.3.4.4b	21.21	20.13	Neg	Neg	Neg	23.12
96 A/chkken/Germany/Agnia/2x06 Field sample SA ^A PA 2, a, b S. 20.9 Neg Neg Neg 0 A/bicken/Italy/3z/9398 Isolate CAP8 IAN 185 3-00.0 Neg 2,00 Neg Neg Neg 23 A/mallar/Germany/Waya-yr/X/2x04 Isolate NA I/Al H35 2.9.0 Neg 1.9.0 Neg Neg 24 A/mallar/Germany/Waya-yr/X/2x04 Isolate NA I/Al H35 2.6.0 Neg 2.9.0 Neg Neg Neg 25 A/mallar/Germany/Ry12/0x06 Isolate SA ^A I/Al H35 2.6.2 Neg	68	A/chicken/Germany/AR9143/2016	Field sample	SAb	HP 2.3.4.4b	32.27	31.51	Neg	Neg	Neg	35.49
700Nchlacken/taly/22/1998IsolateIcAPs BickIcAPs Bick <td>69</td> <td>A/chicken/Germany/AR9144/2016</td> <td>Field sample</td> <td>SAb</td> <td>HP 2.3.4.4b</td> <td>21.89</td> <td>20.89</td> <td>Neg</td> <td>Neg</td> <td>Neg</td> <td>25.92</td>	69	A/chicken/Germany/AR9144/2016	Field sample	SAb	HP 2.3.4.4b	21.89	20.89	Neg	Neg	Neg	25.92
1 1	70	A/chicken/Italy/22/1998	Isolate	CAP58165	LPAI H5N9	12.34	Neg	19.06	Neg	Neg	Neg
22 Amallard/Germany/WuAg-6/2004 Isolate NA LPAI H5N2 29.57 Neg Neg Neg 73 Amallard/Germany/WuAg-77K/2004 Isolate NA LPAI H5N2 20.66 Neg 3.63 Neg Neg Neg 74 Amallard/Germany/Rys1/2005 Isolate NA LPAI H5N3 26.26 Neg 3.02 Neg Neg Neg 74 Amallard/Germany/Rys1/2008 Isolate SA ¹ LPAI H5N3 20.24 Neg 20.25 Neg Neg Neg Neg 75 Amallard/Germany/Rys1/2008 Isolate SA ¹ LPAI H5N3 23.68 Neg 2.6.22 Neg Neg Neg 75 Afulter/Germany/Rys1/2008 Isolate SA ¹ LPAI H5N3 2.5.7 Neg 2.6.2 Neg Neg Neg 76 Afutre/Germany/Rsty2/2008 Isolate SA ¹ LPAI H5N3 2.5.7 Neg 2.6.2 Neg Neg Neg 80 Afutre/Germany/R	71	A/mallard/Germany/Wv1349-51K/2003	Isolate	CAP58164	LPAI H5N3	26.00	Neg	14.79	Neg	Neg	Neg
7.3 A/mallard/German/Wwq4-7/K/2004 Isolate NA LPAI HSN 20,4 Neg Aeg Neg Neg Neg 7.4 A/ostrich/German/R5-10/2006 Isolate HF50037 LPAI HSN 26,50 Neg 26,50 Neg Neg Neg 7.5 A/mallard/German/R5-10/2006 Isolate SA' LPAI HSN 30,6 Reg 30,28 Neg Neg 7.6 A/mallard/German/R571/2008 Isolate SA' LPAI HSN 20,48 Neg 24,50 Neg Neg Neg 7.8 A/mallard/German/R571/2008 Isolate SA' LPAI HSN 25,61 Neg 24,92 Neg Neg Neg 8.0 A/turkey/German/R55/2008 Isolate SA' LPAI HSN 24,33 Neg 24,92 Neg Neg Neg 8.1 A/turkey/German/R55/2008 Isolate SA' LPAI HSN 24,33 Neg 24,92 Neg Neg Neg 8.3 A/turkey/German/R201	72	A/mallard/Germany/Wv476/2004	Isolate	NA	LPAI H5N2	29.87	Neg	29.5	Neg	Neg	Neg
7.4 Alostrich/Germany/Rsy-io/2006 Isolate HF563057 LPAI HSN 26.60 Neg Zeing Neg Neg 75 A/mallard/Germany/Rsys/2006 Isolate NA LPAI HSN 26.41 Neg 2.61 Neg Neg Neg 76 A/mallard/Germany/Rzys/2008 Isolate SA* LPAI HSN 20.64 Neg Jos Neg Neg Neg 78 A/mallard/Germany/Rzys/2008 Isolate SA* LPAI HSN 20.64 Neg Alos Neg A.ge Alos Neg Alos Neg Neg Alos Neg Alos Neg Alos Neg Neg <td>73</td> <td>A/mallard/Germany/Wv474–77K/2004</td> <td>Isolate</td> <td>NA</td> <td>LPAI H5N2</td> <td>29.64</td> <td>Neg</td> <td>34.81</td> <td>Neg</td> <td>Neg</td> <td>Neg</td>	73	A/mallard/Germany/Wv474–77K/2004	Isolate	NA	LPAI H5N2	29.64	Neg	34.81	Neg	Neg	Neg
75 A/mallard/Germany/R255/2006 Isolate NA LPAI H5M3 26.24 Neg 27.61 Neg Neg Neg 76 A/mallard/Germany/R731/2008 Isolate SA* LPAI H5M3 30.36 Neg 30.28 Neg Neg Neg Neg 77 A/mallard/Germany/R731/2008 Isolate SA* LPAI H5M3 23.68 Neg 30.28 Neg Neg Neg 79 A/turkey/Germany/R355/2008 Isolate SA* LPAI H5M3 23.60 Neg 24.01 Neg Neg Neg 80 A/turkey/Germany/R55/12008 Isolate SA* LPAI H5M3 24.36 Neg Neg Neg 81 A/turkey/Germany/R2014/2008 Isolate SA* LPAI H5M3 24.37 Neg Neg Neg 82 A/turkey/Germany/R2014/2008 Isolate SA* LPAI H5M3 24.33 Neg Neg Neg 84 A/turkey/Germany/R2014/2008 Isolate SA* LPAI H5M3 15.63 Neg Neg Neg 85 A/turkey/Germany/R201	74	A/ostrich/Germany/R5–10/2006	Isolate	HF563057	LPAI H5N3	26.80	Neg	26.19	Neg	Neg	Neg
A A Isolate SA ¹ LPAI HSM3 30.5 Neg Neg Neg 77 A/mallard/Germany/R771/2008 Isolate SA ¹ LPAI HSM3 29.24 Neg 30.88 Neg Neg Neg 78 A/mallard/Germany/R771/2008 Isolate SA ¹ LPAI HSM3 23.68 Neg 24.50 Neg Neg Neg 78 A/mallard/Germany/R550/2008 Isolate NA LPAI HSM3 23.68 Neg 24.50 Neg Neg 70 A/turkey/Germany/R550/2008 Isolate NA LPAI HSM3 23.50 Neg 24.61 Neg Neg 84 A/turkey/Germany/R551/2008 Isolate NA LPAI HSM3 35.69 Neg 30.38 Neg Neg 84 A/turkey/Germany/R2016/2008 Isolate SA ¹ LPAI HSM3 18.32 Neg 16.92 Neg Neg 85 A/turkey/Germany/R201/2008 Isolate SA ¹ LPAI HSM3 18.32 Neg </td <td>75</td> <td>A/mallard/Germany/R2557/2006</td> <td>Isolate</td> <td>NA</td> <td>LPAI H5N3</td> <td>26.24</td> <td>Neg</td> <td>27.61</td> <td>Neg</td> <td>Neg</td> <td>Neg</td>	75	A/mallard/Germany/R2557/2006	Isolate	NA	LPAI H5N3	26.24	Neg	27.61	Neg	Neg	Neg
77 A/mallard/Germany/R771/2008 Isolate SA ¹ LPAI H5M3 29.24 Neg 30.28 Neg Neg 78 A/mallard/Germany/R771/2008 Isolate SA ¹ LPAI H5M3 25.27 Neg 24.50 Neg Neg Neg 79 A/turkey/Germany/R550/2008 Isolate NA LPAI H5M3 25.17 Neg 24.50 Neg Neg Neg 80 A/turkey/Germany/R551/2008 Isolate NA LPAI H5M3 23.50 Neg 24.16 Neg Neg Neg 81 A/turkey/Germany/R51/2008 Isolate SA ³⁺ LPAI H5M3 25.50 Neg 29.25 Neg Neg Neg 83 A/turkey/Germany/R501/2008 Isolate SA ³⁺ LPAI H5M3 15.31 Neg 15.92 Neg Neg Neg 84 A/turkey/Germany/R201/2008 Isolate SA ³⁺ LPAI H5M3 15.32 Neg 16.92 Neg Neg 87 A/turkey/Germany/R201/2008	76	A/mallard/Germany/R731/2008	Isolate	SA⁵	LPAI H5N3	30.36	Neg	32.30	Neg	Neg	Neg
Namilard/Germany(R72/2008 Isolate SA ^b LPAI H5/H3 23.68 Neg Leg Neg Neg 79 A/turkey/Germany/R1550/2008 Isolate NA LPAI H5/H3 23.61 Neg 2.4.25 Neg Neg Neg 80 A/turkey/Germany/R1551/2008 Isolate NA LPAI H5/H3 23.25 Neg 2.4.26 Neg Neg Neg 81 A/turkey/Germany/R355/2008 Isolate SA ^A LPAI H5/H3 2.7.3 Neg 2.9.25 Neg Neg Neg 81 A/turkey/Germany/R357/2008 Isolate SA ^A LPAI H5/H3 2.7.3 Neg 2.9.9 Neg Neg Neg 82 A/turkey/Germany/R201/2008 Isolate SA ^A LPAI H5/H3 18.32 Neg 16.90 Neg	77	A/mallard/Germany/R771/2008	Isolate	SA⁵	LPAI H5N3	29.24	Neg	30.28	Neg	Neg	Neg
7 A/turkey/Germany/Rs50/2008 Isolate NA LPAI H5N3 25.77 NB 26.22 NB NB NB 80 A/turkey/Germany/Rs55/2008 Isolate NA LPAI H5N3 24.03 NB 24.21 NB NB NB NB A/turkey/Germany/Rs55/2008 Isolate SA ¹⁺ LPAI H5N3 27.43 NB 24.21 NB NB NB NB A/turkey/Germany/Rs51/2008 Isolate SA ¹⁺ LPAI H5N3 27.43 NB 29.25 NEg NEg NEg 81 A/turkey/Germany/Raot4/2008 Isolate SA ¹⁺ LPAI H5N3 27.43 NB 29.25 NEg NEg NEg 82 A/turkey/Germany/Raot4/2008 Isolate SA ¹⁺ LPAI H5N3 18.32 NEg 16.92 NEg NEg NEg 84 A/turkey/Germany/Raot6/2008 Isolate SA ¹⁺ LPAI H5N3 18.32 NEg 16.92 NEg NEg 86 A/turkey/Germany/Raot6/2008 Isolate	78	A/mallard/Germany/R772/2008	Isolate	SA⁵	LPAI H5N3	23.68	Neg	24.50	Neg	Neg	Neg
No. Alturkey/Germany/Ra551/2008 Isolate NA LPAI H5N3 24.03 Neg 24.91 Neg Neg 81 Alturkey/Germany/Ra551/2008 Isolate SA ¹⁺² LPAI H5N3 23.50 Neg 24.91 Neg Neg 82 Alturkey/Germany/Ra521/2008 Isolate SA ¹⁺² LPAI H5N3 27.43 Neg 29.25 Neg Neg Neg 83 Alturkey/Germany/Ra51/2008 Isolate SA ¹⁺ LPAI H5N3 27.43 Neg 21.99 Neg Neg Neg 84 Alturkey/Germany/Ra51/2008 Isolate SA ³⁺ LPAI H5N3 18.33 Neg 16.92 Neg Neg 86 Alturkey/Germany/Ra51/2008 Isolate SA ³⁺ LPAI H5N3 14.35 Neg 16.52 Neg Neg 87 Alturkey/Germany/Ra50/2008 Isolate SA ³⁺ LPAI H5N3 14.35 Neg Neg Neg Neg 88 Alturkey/Germany/Ra50/2008 Isolate SA ³⁺	79	A/turkey/Germany/R1550/2008	Isolate	NA	LPAI H5N3	25.17	Neg	26.22	Neg	Neg	Neg
Alturkey/Germany/Rsj5/2008 Isolate SA* LPA HS/3 Z3_50 Neg LPA Neg Neg Neg 82 A/turkey/Germany/Rsi57/2008 Isolate SA* LPA HS/3 Z4,3 Neg 2.9.5 Neg Neg Neg 83 A/turkey/Germany/Rz014/2008 Isolate SA* LPA HS/3 Z4,33 Neg 2.9.9 Neg Neg Neg 84 A/turkey/Germany/R2016/2008 Isolate SA* LPA HS/3 18.13 Neg 15.96 Neg Neg Neg 85 A/turkey/Germany/R2016/2008 Isolate SA* LPA HS/3 18.13 Neg 16.92 Neg Neg Neg 87 A/turkey/Germany/R201/2008 Isolate SA* LPA HS/3 14.16 Neg 16.92 Neg Neg Neg 90 A/turkey/Germany/R201/2008 Isolate SA* LPA HS/3 14.16 Neg 1.6.93 Neg Neg Neg 91 A/turkey/Germany/R202/2008	80	A/turkev/Germany/R1551/2008	Isolate	NA	LPAI H5N3	24.03	Neg	24.91	Neg	Neg	Neg
N. J.	81	A/turkev/Germany/R1557/2008	Isolate	SA ^{b a}	LPAI H5N3	23.50	Neg	24.16	Neg	Neg	Neg
h h	82	A/turkev/Germany/R1612/2008	Isolate	NA	LPAI H5N3	27.43	Neg	29.25	Neg	Neg	Neg
Jurkey/Germany/R2015/2008 Isolate SA ^b LPAI H5/N 15.65 Neg Neg Neg 84 Alturkey/Germany/R2015/2008 Isolate SA ^b LPAI H5/N 15.66 Neg Neg Neg 85 A/turkey/Germany/R2017/2008 Isolate SA ^b LPAI H5/N 18.32 Neg 16.92 Neg Neg 87 A/turkey/Germany/R2015/2008 Isolate SA ^b LPAI H5/N 14.16 Neg 16.92 Neg Neg 88 A/turkey/Germany/R2019/2008 Isolate SA ^b LPAI H5/N 14.16 Neg 16.37 Neg Neg Neg 90 A/turkey/Germany/R2012/2008 Isolate SA ^b LPAI H5/N 12.71 Neg 16.34 Neg Neg Neg 91 A/turkey/Germany/R2021/2008 Isolate SA ^b LPAI H5/N 12.71 Neg 16.34 Neg Neg Neg 92 A/turkey/Germany/R2021/2008 Isolate SA ^b LPAI H5/N 12.71	83	A/turkev/Germany/R2014/2008	Isolate	SA⁵	LPAI H5N3	24.33	Neg	21.99	Neg	Neg	Neg
Normal/Network/network/network/network/Network/	84	A/turkey/Germany/R2015/2008	Isolate	SAb	LPAI H5N3	15.69	Neg	30.13	Neg	Neg	Neg
b Lurkey/Germany/R201/2008 Isolate SA ^b LPAI H5N3 18.32 Neg Neg Neg 87 A/turkey/Germany/R2018/2008 Isolate SA ^b LPAI H5N3 14.16 Neg 16.92 Neg Neg Neg 88 A/turkey/Germany/R2019/2008 Isolate SA ^b LPAI H5N3 14.55 Neg 16.63 Neg Neg 89 A/turkey/Germany/R2020/2008 Isolate SA ^b LPAI H5N3 12.71 Neg 13.51 Neg Neg 90 A/turkey/Germany/R2022/2008 Isolate SA ^b LPAI H5N3 12.71 Neg 13.18 Neg Neg Neg 91 A/turkey/Germany/R2022/2008 Isolate SA ^b LPAI H5N3 12.23 Neg 1.80 Neg Neg Neg 92 A/turkey/Germany/R2022/2008 Isolate SA ^b LPAI H5N3 22.52 Neg 20.39 Neg Neg 93 A/turkey/Germany/R2024/2008 Isolate SA ^b <td< td=""><td>85</td><td>A/turkey/Germany/R2016/2008</td><td>Isolate</td><td>SAb</td><td>LPAI H5N3</td><td>18.13</td><td>Neg</td><td>15.96</td><td>Neg</td><td>Neg</td><td>Neg</td></td<>	85	A/turkey/Germany/R2016/2008	Isolate	SAb	LPAI H5N3	18.13	Neg	15.96	Neg	Neg	Neg
Arturkey/Germany/R2018/2008 Isolate SA ^b LPAI H5N3 14.16 Neg Neg Neg 87 A/turkey/Germany/R2018/2008 Isolate SA ^b LPAI H5N3 14.15 Neg 16.05 Neg Neg 88 A/turkey/Germany/R2019/2008 Isolate SA ^b LPAI H5N3 19.38 Neg 16.73 Neg Neg 90 A/turkey/Germany/R2021/2008 Isolate SA ^b LPAI H5N3 12.71 Neg 13.51 Neg Neg Neg 91 A/turkey/Germany/R2021/2008 Isolate SA ^b LPAI H5N3 12.63 Neg 13.18 Neg Neg Neg 92 A/turkey/Germany/R2021/2008 Isolate SA ^b LPAI H5N3 12.63 Neg 17.07 Neg Neg Neg 93 A/turkey/Germany/R2023/2008 Isolate SA ^b LPAI H5N3 22.52 Neg 16.06 Neg Neg 94 A/turkey/Germany/R2027/2008 Isolate SA ^b LPAI H5N3	86	A/turkev/Germany/R2017/2008	Isolate	SAb	LPAI H5N3	18.32	Neg	16.92	Neg	Neg	Neg
Alturkey/Germany/R2019/2008 Isolate SA ^b LPAI H5N3 11.455 Neg Neg Neg 89 A/turkey/Germany/R2019/2008 Isolate SA ^b LPAI H5N3 11.455 Neg 16.84 Neg Neg Neg 90 A/turkey/Germany/R2021/2008 Isolate SA ^b LPAI H5N3 12.71 Neg 13.51 Neg Neg Neg 91 A/turkey/Germany/R2021/2008 Isolate SA ^b LPAI H5N3 12.63 Neg 13.18 Neg Neg Neg 92 A/turkey/Germany/R2022/2008 Isolate SA ^b LPAI H5N3 19.37 Neg 17.07 Neg Neg Neg 93 A/turkey/Germany/R202/2008 Isolate SA ^b LPAI H5N3 14.70 Neg 16.26 Neg Neg 94 A/turkey/Germany/R202/2008 Isolate SA ^b LPAI H5N3 14.70 Neg 16.66 Neg Neg 95 A/turkey/Germany/R202/2008 Isolate EPI356412	87	A/turkev/Germany/R2018/2008	Isolate	SAb	LPAI H5N3	14.16	Neg	16.05	Neg	Neg	Neg
Alturkey/Germany/R2020/2008IsolateSA*LPAI H5N317.5017.50NegNegNeg90A/turkey/Germany/R2021/2008IsolateSA*LPAI H5N312.71Neg13.51NegNegNeg91A/turkey/Germany/R2022/2008IsolateSA*LPAI H5N312.63Neg13.18NegNegNeg92A/turkey/Germany/R2023/2008IsolateSA*LPAI H5N319.37Neg17.07NegNegNeg93A/turkey/Germany/R2024/2008IsolateSA*LPAI H5N322.52Neg20.39NegNeg94A/turkey/Germany/R2025/2008IsolateSA*LPAI H5N322.44Neg25.22NegNeg95A/turkey/Germany/R2026/2008IsolateSA*LPAI H5N314.70Neg16.66NegNeg96A/turkey/Germany/R2027/2008IsolateSA*LPAI H5N317.80Neg16.66NegNeg97A/mallard/Germany/R2027/2008IsolateEPI356412LPAI H5N317.80Neg14.37NegNeg98A/duck/Germany/R2027/2008IsolateEPI356412LPAI H5N319.38Neg14.37NegNegNeg99A/turkey/Germany/R2027/2008IsolateEPI356412LPAI H5N311.98Neg14.37NegNeg99A/duck/Germany/AR1965/2013Field sampleSA*LPAI H5N32.02Neg2.15NegNe	88	A/turkev/Germany/R2019/2008	Isolate	SAb	LPAI H5N3	14.55	Neg	16.84	Neg	Neg	Neg
Operation	89	A/turkey/Germany/R2020/2008	Isolate	SAb	LPAI H5N3	19.38	Neg	16.73	Neg	Neg	Neg
Dr. Dr. Markey/Deckary/Deckary/Deckary Dr. Markey/Deckary/Deckary Dr. Markey/Deckary Dr.D. Markey/Deckary Dr.D. Markey/Deckary	90	A/turkey/Germany/R2021/2008	Isolate	SAb	LPAI H5N3	12.71	Neg	13.51	Neg	Neg	Neg
A/Lurkey/Germany/R2023/2008 Isolate SA ^b LPAI H5N3 19.37 Neg 17.07 Neg Neg Neg 93 A/Lurkey/Germany/R2023/2008 Isolate SA ^b LPAI H5N3 22.52 Neg 20.39 Neg Neg Neg 94 A/Lurkey/Germany/R2024/2008 Isolate SA ^b LPAI H5N3 22.52 Neg 20.39 Neg Neg Neg 95 A/Lurkey/Germany/R2025/2008 Isolate SA ^b LPAI H5N3 14.70 Neg 16.26 Neg Neg Neg 96 A/Lurkey/Germany/R2027/2008 Isolate SA ^b LPAI H5N3 14.70 Neg 16.26 Neg Neg Neg 97 A/mallard/Germany/R2027/2008 Isolate SA ^b LPAI H5N3 11.98 Neg 14.37 Neg Neg Neg Neg 97 A/mallard/Germany/R2892-94/2009 Isolate SA ^b LPAI H5N3 26.62 Neg 24.37 Neg Neg Neg 98 A/duck/Germany/AR1965/2013 Field sample SA ^b LPAI H5N3 26.62<	91	A/turkey/Germany/R2022/2008	Isolate	SAb	LPAI H5N3	12.63	Neg	13.18	Neg	Neg	Neg
A/Lurkey/Germany/R2024/2008IsolateSAbLPAI H5N322.52Neg20.39NegNeg93A/Lurkey/Germany/R2025/2008IsolateSAbLPAI H5N322.52Neg25.22NegNegNeg94A/Lurkey/Germany/R2025/2008IsolateSAbLPAI H5N322.44Neg25.22NegNegNeg95A/Lurkey/Germany/R2026/2008IsolateSAbLPAI H5N314.70Neg16.26NegNegNeg96A/Lurkey/Germany/R2027/2008IsolateSAbLPAI H5N317.80Neg16.06NegNegNeg97A/mallard/Germany/R2892-94/2009IsolateEPI356412LPAI H5N311.98Neg14.37NegNegNeg98A/duck/Germany/AR1965/2013Field sampleNALPAI H5N326.62Neg27.25NegNegNeg99A/turkey/Germany/AR1892/1/2014Field sampleSAbLPAI H5N329.20Neg34.01NegNegNeg100A/duck/Germany/AR192015Field sampleSAbLPAI H5N329.20Neg31.02NegNegNeg101A/swan/Germany/AR398/2015Field sampleSAbLPAI H5N320.67Neg32.91NegNegNeg102A/duck/Germany/AR395/15-1/2015Field sampleSAbLPAI H5N327.66Neg32.69NegNeg103A/duck/Germany/AR3264/1/2015Field sampleS	92	A/turkey/Germany/R2023/2008	Isolate	SAb	LPAI H5N3	19.37	Neg	17.07	Neg	Neg	Neg
yNietrey/Germany/Rotra/LoosInstanceSNLinyLinyLinyLinyNiesNiesNiesNiesNiesNiesNiesNiesNies94A/turkey/Germany/R2025/2008IsolateSAbLPAI H5N314.70Neg16.26NegNegNeg95A/turkey/Germany/R2027/2008IsolateSAbLPAI H5N314.70Neg16.26NegNegNeg97A/mallard/Germany/R2027/2008IsolateSAbLPAI H5N317.80Neg16.06NegNegNeg98A/duck/Germany/R2892-94/2009IsolateEPI356412LPAI H5N311.98Neg14.37NegNegNeg99A/turkey/Germany/AR1965/2013Field sampleNALPAI H5N326.62Neg27.25NegNegNeg99A/turkey/Germany/AR1892/1/2014Field sampleSAbLPAI H5N329.20Neg34.01NegNegNeg100A/duck/Germany/AR11/2015Field sampleSAbLPAI H5N427.45NegNegNegNeg101A/swan/Germany/AR11/2015Field sampleSAbLPAI H5N329.20Neg34.01NegNegNeg102A/goose/Germany/AR398/2015Field sampleSAbLPAI H5N226.74Neg31.02NegNeg103A/duck/Germany/AR3264/1/2015Field sampleSAbLPAI H5N327.65Neg34.01NegNegNeg <td>03</td> <td>A/turkey/Germany/R2024/2008</td> <td>Isolate</td> <td>SAb</td> <td>I PALH5N3</td> <td>22.52</td> <td>Neg</td> <td>20.30</td> <td>Neg</td> <td>Neg</td> <td>Neg</td>	03	A/turkey/Germany/R2024/2008	Isolate	SAb	I PALH5N3	22.52	Neg	20.30	Neg	Neg	Neg
yqNytarkey/Germany/R2026/2008IsolateSNLPAI H5N3LPAI H5N314.70Neg16.26NegNegNeg96A/turkey/Germany/R2027/2008IsolateSA ^b LPAI H5N317.80Neg16.06NegNegNeg97A/mallard/Germany/R2027/2008IsolateEPI356412LPAI H5N317.80Neg14.37NegNegNeg98A/duck/Germany/R2027/2009IsolateEPI356412LPAI H5N326.62Neg27.25NegNegNeg99A/turkey/Germany/AR1965/2013Field sampleNALPAI H5N329.20Neg21.15NegNegNeg99A/turkey/Germany/AR1921/2014Field sampleSA ^b LPAI H5N329.20Neg34.01NegNegNeg100A/duck/Germany/AR12015Field sampleSA ^b LPAI H5N329.20Neg34.01NegNegNeg101A/swan/Germany/AR11/2015Field sampleSA ^b LPAI H5N427.45Neg31.02NegNegNeg102A/goose/Germany/AR398/2015Field sampleSA ^b LPAI31.09Neg33.69NegNegNeg103A/duck/Germany/AR2853/15-1/2015Field sampleSA ^b LPAI H5N327.06Neg32.91NegNegNeg104A/duck/Germany/AR264/1/2015Field sampleSA ^b LPAI H5N327.06Neg26.25NegNegNeg <td< td=""><td>0/</td><td>A/turkey/Germany/R2025/2008</td><td>Isolate</td><td>SA^b</td><td>I PALH5N3</td><td>22.44</td><td>Neg</td><td>25.22</td><td>Neg</td><td>Neg</td><td>Neg</td></td<>	0/	A/turkey/Germany/R2025/2008	Isolate	SA ^b	I PALH5N3	22.44	Neg	25.22	Neg	Neg	Neg
ysNytatkey/Germany/R2027/2008IsolateSNENN (N)HysNegNegNegNegNeg96A/turkey/Germany/R2027/2008IsolateSAbLPAI H5N317.80Neg16.06NegNegNeg97A/mallard/Germany/R2027/2008IsolateEPI356412LPAI H5N311.98Neg14.37NegNegNeg98A/duck/Germany/R2892-94/2009IsolateEPI356412LPAI H5N326.62Neg27.25NegNegNeg99A/turkey/Germany/AR1965/2013Field sampleSAbLPAI H5N329.20Neg21.15NegNegNeg100A/duck/Germany/AR12015Field sampleSAbLPAI H5N329.20Neg34.01NegNegNeg101A/swan/Germany/AR111/2015Field sampleSAbLPAI H5N427.45Neg31.02NegNegNeg102A/goose/Germany/AR398/2015Field sampleSAbLPAI31.09Neg33.69NegNegNeg103A/duck/Germany/AR1231/1/2015Field sampleNALPAI H5N327.06Neg32.91NegNegNeg104A/duck/Germany/AR2853/15-1/2015Field sampleSAbLPAI H5N327.06Neg26.25NegNegNeg105A/goose/Germany/AR3264/1/2015Field sampleSAbLPAI H5N327.06Neg26.25NegNegNeg106A/duck/Germany/AR3	05	A/turkey/Germany/R2026/2008	Isolate	SA ^b	I PALH5N3	1/1.70	Neg	16.26	Neg	Neg	Neg
yoNyatkay/retringNya	06	A/turkey/Germany/R2027/2008	Isolate	SAb	I PALH5N3	17.80	Neg	16.06	Neg	Neg	Neg
y/ik/indicatek/indicatek/indicatek/indicatek/indicatek/indicatek/indicate98A/duck/Germany/AR1965/2013Field sampleNALPAI H5N326.62Neg27.25NegNegNeg99A/turkey/Germany/AR1892/1/2014Field sampleSA ^b LPAI H5N329.20Neg21.15NegNegNeg100A/duck/Germany/AR1/2015Field sampleSA ^b LPAI H5N329.20Neg34.01NegNegNeg101A/swan/Germany/AR11/2015Field sampleSA ^b LPAI H5N427.45Neg31.02NegNegNeg102A/goose/Germany/AR398/2015Field sampleSA ^b LPAI31.09Neg33.69NegNegNeg103A/duck/Germany/AR1231/1/2015Field sampleSA ^b LPAI H5N226.74Neg32.91NegNegNeg104A/duck/Germany/AR3264/1/2015Field sampleSA ^b LPAI H5N327.06Neg26.25NegNegNeg105A/goose/Germany/AR3264/1/2015Field sampleSA ^b LPAI H5N327.06Neg26.25NegNegNeg104A/duck/Germany/AR3264/1/2015Field sampleSA ^b LPAI H5N327.06Neg26.25NegNegNeg105A/goose/Germany/AR3264/1/2015Field sampleSA ^b LPAI H5N223.17Neg35.50NegNegNeg106A/wild hird/G	97	A/mallard/Germany/R2892-94/2009	Isolate	EPI356412	LPAI H5N3	11.98	Neg	14.37	Neg	Neg	Neg
youNotech Germany/AR1890/12013NegNegNegNegNegNeg99A/turkey/Germany/AR1892/1/2014Field sampleSAbLPAI H5N220.03Neg21.15NegNegNeg100A/duck/Germany/AR1/2015Field sampleSAbLPAI H5N329.20Neg34.01NegNegNeg101A/swan/Germany/AR11/2015Field sampleSAbLPAI H5N427.45Neg31.02NegNegNeg102A/goose/Germany/AR398/2015Field sampleSAbLPAI31.09Neg33.69NegNegNeg103A/duck/Germany/AR1231/1/2015Field sampleSAbLPAI H5N226.74Neg32.91NegNegNeg104A/duck/Germany/AR2853/15-1/2015Field sampleSAbLPAI H5N327.06Neg26.25NegNegNeg105A/goose/Germany/AR3264/1/2015Field sampleSAbLPAI H5N327.06Neg26.25NegNegNeg106A/wild bird/Germany/AR3264/1/2015Field sampleSAbLPAI H5N327.06Neg35.50NegNegNeg	08	A/duck/Germany/AR1065/2012	Field sample	ΝΔ		26.62	Neg	27.25	Neg	Neg	Neg
100A/duck/Germany/AR1/2015Field sampleSA*LPAI H5N329.20NegMegNegNeg101A/swan/Germany/AR11/2015Field sampleSA*LPAI H5N329.20Neg34.01NegNegNeg102A/goose/Germany/AR398/2015Field sampleSA*LPAI H5N427.45Neg31.02NegNegNeg103A/duck/Germany/AR1231/1/2015Field sampleSA*LPAI H5N226.74Neg32.91NegNegNeg104A/duck/Germany/AR2853/15-1/2015Field sampleSA*LPAI H5N327.06Neg26.25NegNegNeg105A/goose/Germany/AR32641/2015Field sampleSA*LPAI H5N234.47Neg35.50NegNegNeg106A/wild bird/Germany/AR232/2015Field sampleSA*LPAI H5N223.17Neg23.68NegNeg	90	A/turkey/Germany/AR1802/1/2014	Field sample	SAb		20.02	Neg	21.15	Neg	Neg	Neg
101A/swan/Germany/AR111/2015Field sampleSA*LPAI H5N429.20Neg54.01NegNegNeg102A/goose/Germany/AR398/2015Field sampleSA*LPAI31.09Neg33.69NegNegNeg103A/duck/Germany/AR1231/1/2015Field sampleNALPAI H5N226.74Neg32.91NegNegNeg104A/duck/Germany/AR2853/15-1/2015Field sampleSA*LPAI H5N327.06Neg26.25NegNegNeg105A/goose/Germany/AR3264/1/2015Field sampleSA*LPAI H5N234.47Neg35.50NegNegNeg106A/wild bird/Germany/AR221/2015Field sampleSA*LPAI H5N223.17Neg23.68NegNeg	100	A/duck/Germany/AR1/2015	Field sample	SA ^b		20.20	Neg	3/1.01	Neg	Neg	Neg
102A/goose/Germany/AR398/2015Field sampleSAbLPAI31.09Neg33.69NegNegNeg103A/duck/Germany/AR1231/1/2015Field sampleNALPAI H5N226.74Neg32.91NegNegNeg104A/duck/Germany/AR2853/15-1/2015Field sampleSAbLPAI H5N327.06Neg26.25NegNegNeg105A/goose/Germany/AR3264/1/2015Field sampleSAbLPAI H5N234.47Neg35.50NegNegNeg106A/wild bird/Germany/AR223/2015Field sampleSAbLPAI H5N223.17Neg32.91NegNegNeg	101	A/swan/Germany/AR111/2015	Field sample	SAb		27.//5	Neg	31.02	Neg	Neg	Neg
102N/good/seminity/Mgy0/201311cd sample3ALFAI31.09NegNegNegNegNeg103A/duck/Germany/AR1231/1/2015Field sampleNALPAI H5N226.74Neg32.91NegNegNeg104A/duck/Germany/AR2853/15-1/2015Field sampleSA ^b LPAI H5N327.06Neg26.25NegNegNeg105A/goose/Germany/AR3264/1/2015Field sampleSA ^b LPAI H5N234.47Neg35.50NegNegNeg106A/wild bird/Germany/AR221/2015Field sampleSA ^b LPH FN223.17Neg23.68NegNeg	102	A/goose/Germany/AR208/2015	Field sample	SVP	Ι ΡΔΙ	21.00	Neg	22 60	Nea	Nea	Neg
104 A/duck/Germany/AR2853/15-1/2015 Field sample SAb LPAI H5N3 27.06 Neg 26.25 Neg Ne	102	A/duck/Germany/AR1231/1/2015	Field sample	ΝΔ		26.74	Neg	32 01	Neo	Neo	Neo
105 A/goose/Germany/AR3264/1/2015 Field sample SAb LPAI H5N2 34.47 Neg 35.50 Neg Neg Neg 106 A/wild bird/Germany/AR3264/1/2015 Field sample SAb LPAI H5N2 34.47 Neg 35.50 Neg Neg Neg	104	A/duck/Germany/AR2853/15-1/2015	Field sample	SΔb		27.06	Neg	26.25	Neo	Neo	Neo
106 A/wild hird/Germany/AR221/2015 Field sample SAb IPHEN2 22.17 Neg 22.48 Neg Neg Neg	105	A/goose/Germany/AR3264/1/2015	Field sample	SAb		34.47	Neg	35.50	Neg	Neg	Neg
	106	A/wild bird/Germany/AR221/2015	Field sample	SA ^b	LP H5N3	22.17	Neg	23.48	Neg	Neg	Neg

HA: haemagglutinin; HP: highly pathogenic; HPAI: highly pathogenic avian influenza; ID: identity; LP: low pathogenic; LPAI: low pathogenic avian influenza; NA: sequence not available; Neg: negative; SA: sequence available.

^a Sequences were obtained from GenBank at the National Center for Biotechnology Information (NCBI) or the EpiFlu database of the Global Initiative on Sharing Avian Influenza Data (GISAID).

^b Sequenced in the frame of the current study; sequences available from the authors upon request.

Primers and probes for phylotyping RT-qPCR assays distinguishing three clades of gs/GD origin HPAIV H5 were placed within the HA1-fragment of the HA gene. This region encodes the receptor binding unit and harbours a number of neutralisation-relevant epitopes that are targets of antigenic drift. Hence, the HA1 fragment harbours regions that are the least conserved within the influenza A virus genome. Primer selection aimed at the inclusion of as many as possible distinguishing nt that would define exclusivity at the five most 3' positions while probes were placed so as to accommodate distinguishing nt in the centre of the oligont. In order not to compromise amplification efficacy, amplicon size was limited to 130 nt wherever possible given the above mentioned constraints for primers and probes. The finally chosen oligont are listed in Table 1 and provided specific detection exclusively of the homologous clade. No cross-reactivity among the other gs/GD clades examined was evident on basis of the used panel of reference viruses (Table 2). Also, no cross-reactivity was detected for any of the five assays against other influenza A viruses or other avian viral respiratory pathogens (Table 2).

Validation of the analytical sensitivity, limit of detection and precision

Detection limits of the assays were determined by testing 10-fold serial dilutions of viral RNA extracted from representative viruses of each of the three HPAI virus clades (2.2.1.2, 2.3.2.1 and 2.3.4.4), and of Eurasian H5 LPAI virus. Cycle of quantification (Cq) values were compared with a standard RT-qPCR for the matrix (M) gene of these viruses with a reported detection limit of 2 to 20 RNA copies/5 μ l [28]. Average values of three separate runs were computed and plotted using SigmaPlot V 11 software. Plotting these values revealed a linear relationship between the log of the viral RNA dilution and the Cq value for all assays and the kinetics of the assays and their sensitivity were determined to be very similar to the generic M gene RT-qPCR (M1.2 RT-qPCR [29]) (Figure 1).

The correlation coefficient of the standard curves was 0.99 for all assays, indicating a highly precise log–linear relationship between the viral RNA log dilution and the corresponding Cq-value (Figure 1). Based on these results the threshold distinguishing positive and negative was set at Cq=38.

Pathotyping and phylotyping of clinical samples of potentially zoonotic Eurasian avian influenza A subtype H5 viruses by quantitative reverse transcription PCR

In order to evaluate the diagnostic performance capacity of the developed assays, field samples (RNA extracted from swabs, tissues or FTA cards) and clinical virus isolates obtained during the period 2013 to 2016 (HPAI viruses) or 2003 to 2015 (LPAI viruses) were examined. The sample set was preselected on basis of a positive generic M-specific RT-qPCR.

Among the final set of 106 samples, the pathotyping RT-qPCRs sharply discerned two groups of 69 samples reacting only in the new HPAI H5 RT-qPCR while 37 samples reacted positive in the LPAI H5 RT-qPCR (Figure 2a; Table 3).

All pathotyping results matched the results obtained by nt sequence analysis of the HA cleavage site. However, in a few samples (two isolates, 10 clinical samples) of HP viruses, the LPAI H5 RT-qPCR also gave a weak positive signal (Cq>35). Compared with the LPAI H5 signal the HPAI H5 signal of these samples yielded Cq values 6–10 units lower on average ascertaining good diagnostic specificity. Depending on the clade, the HP phenotype was detected with equal (clade 2.3.2.1) or slightly reduced (clade 2.2.1.2) sensitivity; the LP H5 RT-qPCR appeared to be slightly less sensitive than the M PCR as far as clinical samples were concerned (Table 3; Figure 2a and c). Sequences across the cleavage sites of these samples are presented in a supplemental alignment (Figure 2).

In a next step, the samples that were designated HPAI H5-positive were subjected to the three phylotyping RT-qPCRs. Here, 15, 21 and 33 samples, respectively, were exclusively positive for either clade 2.2.1.2, 2.3.2.1 or 2.3.4.4 (Table 3). Thus, a clear cut clade assignment was possible for all gs/GD HP H5 samples. Results were counterchecked by feeding available HA sequences of these samples into the IRD clade prediction tool (www.fludb.org/brc/h5n1-Classifier.spg?met hod=ShowCleanInputPage&decorator=influenz): In all cases the same clade was assigned by sequence analysis and by PCR. In a final step also all LPAI H₅ samples were tested in the phylotyping RT-qPCRs and none of them cross-reacted. Regarding the sensitivity of these PCRs, the Cq values were compared with those of the generic M1.2-specific RT-qPCR (Figure 2b). For clade 2.2.1.2 and 2.3.2.1 the sensitivity was almost identical to the M PCR; for clade 2.3.4.4a, the clade-specific PCR proved to be slightly more sensitive while viruses of clade 2.3.4.4b were detected at a slightly lower sensitivity; detection of clade 2.3.4.4b viruses was slightly less sensitive than the M PCR (Figure 2b and c; Table 3) as far as clinical samples were concerned.

Rank Sum tests implemented in the SigmaPlot software package were performed and no statistically significant difference between the median Cq values of each specific assay and the M1.2 RT-qPCR assay was found (p>0,50) indicating that the newly developed RT-qPCRs display similar analytical sensitivity. Thus, the phylotyping RT-qPCRs allow a sensitive and highly specific detection and distinction of the three major gs/GD clades currently circulating in countries where the viruses were obtained from.

Discussion

Rapid molecular diagnosis including patho- and phylotyping is basis to enable measures aimed at repressing the spread of potentially zoonotic HPAI viruses. The TagMan PCR technology has proven reliable, versatile, and comparatively cost-effective in the generic detection and subtype differentiation of AIV [30]. Further differentiation of clades, lineages and pathotypes was previously nearly entirely based on nt sequencing approaches which require expensive equipment and are time consuming. In epidemiologically complex settings where different lineages and pathotypes of potentially zoonotic and notifiable infectious agents co-circulate, a more rapid and direct access to testing and results, e.g. by using RT-qPCRs, is desirable. Although RT-gPCRs are inferior to sequencing techniques in terms of retrievable data details, they are superior with respect to time-to-diagnosis and ease-ofuse. This concept which we used earlier for pathotyping of H₅N₁ [31], was here further extended and refined for the identification and discrimination of avian influenza A subtype H₅ viruses of different patho- and phylotypes. The focus was put on those clades of H₅ viruses (2.2.1.2, 2.3.2.1, 2.3.4.4) that had previously 'escaped' from Asia and were detected in western parts of Eurasia and in Africa.

Pathotyping of avian influenza A subtype H₅ viruses is mandatory from an animal health perspective. The pathotyping RT-qPCRs presented here reduce time-to-diagnosis to just three hours following sample receipt. To our knowledge this is the broadest and most detailed attempt of AIV pathotyping using RT-qPCR. The availability of highly sensitive pathotyping PCRs would also allow to detect mixtures of HP and LP H₅ viruses in the same sample; in fact, some of our HP-positive field samples also gave weak LP signals (Table 3, sample numbers 2, 9, 10, 14, 15, 30, 32, 35, 37, 42–5). Yet, LPAI pathotypes in these samples were detected at distinctly higher Cq values indicating either a minor population in a quasispecies of different pathotypes or expressing some cross-reactivity of LPAI primers and probe; in any case, the detection of HPAI genotypes as a major population in a set of field samples was always unequivocal. Further insight into the true nature of these mixtures would only be unravelled by deep sequencing approaches of those samples.

Rapid pathotyping enables rapid implementation of appropriate measures to prevent further spread of virus such as closure of poultry holdings and/or live poultry markets, culling of infected flocks etc. This impedes accumulation of potentially zoonotic AIV at the poultry-human interface which in turn lowers the risks of human infection.

Phylotyping of gs/GD HPAI H5 virus clades is important since each clade, and often also sublineages thereof, display distinct antigenic and pathogenetic properties. This has direct implications, as by assigning the matching clade, appropriate vaccines that ensure the closest antigenic match with the circulating viruses can be selected [32,33]. In particular, countries where gs/GD viruses have become endemic in poultry populations, rely on vaccination of poultry on a broad scale to suppress circulating viruses and to limit risks of human exposure [23]. However, it should be noted that mutant escape variants within these clades selected by vaccine-induced population immunity will not be detected as such by the assays, and in fact, such mutants may also be detected at lower sensitivity if primer and/ or probe binding sites are affected by mutations. Detection of variants will still depend on either nt sequencing or virus isolation/antigenic characterisation approaches but the newly developed assays will aid in selection of meaningful samples in this respect. In particular, samples that do not give conclusively similar Cq values in the generic and the specific assays should prompt in-depth analysis by nt sequencing.

It should be clearly stated that the assays presented here have limitations owed to the restricted geographical distribution of the targeted clades. The use of the newly developed PCRs in regions where viruses belonging to the targeted clades (2.2.1.2, 2.3.2.1c and 2.3.4.4) are reportedly absent is only recommended if immediate incursions with any of these clades are apprehended. Phylotyping indirectly may point towards zoonotic potential since different gs/GD lineages vary in their zoonotic propensity: Egyptian 2.2.1.2 viruses are characterised by increased affinity to human-like sialic acid receptors and have caused by far the largest number of human influenza A(H5N1) virus infections over the past decade [12]. For clade 2.3.2.1c viruses, repeatedly detected in the Middle East (excluding Egypt) and endemic in Western African countries, only few human cases have been recorded. The 2.3.4.4 viruses currently present in various parts of Europe have not provoked human infection so far [34].

Extended co-circulation of more than one gs/GD lineage in poultry and/or wild birds in a wider geographic region was repeatedly reported [35,36]. It is pivotal, for the above mentioned reasons, to detect incursions of distinct HPAI virus lineages in a timely manner. In this respect, the newly developed RT-qPCR assays were shown to be useful tools for an improved rapid and simple characterisation of patho- and phylotypes of Eurasian origin avian influenza A subtype H₅ viruses. The assays aid in speeding up diagnosis on clinical samples because the time consuming (initial) need of virus isolation and nt sequencing is avoided. Given the high substitution rate of HP H₅ influenza viruses frequent checks and, if required, updates of the primers and probes are recommended to ensure full specificity and sensitivity of the patho- and phylotyping RT-qPCRs. These PCRs are advantageous in particular for wild bird samples, especially those that contain LPAI viruses, often with low viral loads and therefore fail to yield replication-competent virus. With respect to HPAI virus, the renouncement from initial virus isolation improves biosecurity. However, the presented assays are not intended to replace virus isolation and antigenic characterisation as a means to detect emerging antigenic drift mutants. Nevertheless, they may aid in selection of appropriate samples for such tasks. Accurate phylotyping also facilitates selection of appropriate vaccines as it serves as an early warning for the incursion of new and antigenically possibly distinct phylotypes.

Conclusions

The assays reported here are primarily intended for screening purposes of avian samples; confirmatory assays, including nt sequence analyses and antigenic characterisation, are still required for new incursions and outbreak scenarios that feature an expansion of the geographic area and/or the range of affected species or poultry sectors. When used in the frame of on-going outbreaks, in particular in regions where vaccination is not used as a preventive measure, results of the patho- and phylotyping PCRs are deemed solid enough for reporting purposes and to justify the implementation of restriction measures. In such settings, similar to the current outbreaks of clade 2.3.4.4b HP H5N8 in Europe, the assays can be prioritised to running the HP and only one (i.e. the fitting) of the phylotyping PCRs on M1.2- and H5 PCR-positive samples. This significantly speeds up time-to-diagnosis and reduces reaction times in a OneHealth approach of repressing the spread of gs/GD HP AIV. Sequencing facilities, classically required for patho- and phylotyping, may not be available, and even not logistically accessible in many regions severely affected by H₅ HPAI incursions. The prospect of having sequencing-independent, TaqMan-based specific and sensitive typing assays, as described here, available in developing regions is expected to boost regional diagnostic capacities eventually leading to improved disease control.

Acknowledgements

The authors greatly acknowledge Dr Joseph Adongo Awuni from Accra Veterinary Laboratory, Veterinary Services Directorate, Ministry of Food and Agriculture, Accra (Ghana), Dr Germaine L. Minoungou from the Laboratoire National d'Elevage in Ouagadougou (Burkina Faso), Dr Zangui Ibrahima Mahaman Sani and Dr Abdou Alassane from the LABOCEL - Laboratoire Central de l'Elevage, Niamey (Niger), Dr Emmanuel Couacy-Hymann from the Laboratoire Central de Pathologie Animale, Bingerville (Côte d'Ivoire) and Abel Wade from the National Veterinary Laboratory (LANAVET), Yaounde (Cameroon) for submitting samples to the Istituto Zooprofilattico Sperimentale delle Venezie, Padova, Italy and for their interest in collaborating in the validation of the developed real-time RT-PCR assays. Field samples from Ghana, Côte d'Ivoire, Cameroon, Niger, Burkina Faso used to evaluate the diagnostic performance capacity of the developed assays were obtained through technical support provided by the United Nations Food and Agriculture Organisation (letter of agreement number 321149).

We wish to thank Aline Maksimov and Diana Wessler for excellent technical support. We are grateful to veterinarians and diagnosticians in Germany for submitting diagnostic samples.

We also gratefully acknowledge the authors, originating and submitting laboratories of the sequences from GISAID's EpiFlu Database used in this study; a detailed list of the respective sequences from the EpiFlu database is available on request from the authors.

Conflict of interest

None declared. One of the authors (N.A.) works for Boehringer Ingelheim, Dubai, United Arab Emirates.

Authors' contributions

Mahmoud M. Naguib, Annika Graaf and Timm Harder conceived the study. Andrea Fortin, Ulrich Wernery, Nadim Amarin, Hussein A. Hussein, Hesham Sultan and Basem Al Adhadh were involved in the collection, initial analysis and provision of viruses and field samples. Christine Luttermann conducted the Sanger sequencing analyses. Mahmoud M. Naguib, Annika Graaf and Andrea Fortin produced, analysed and interpreted data. Timm Harder and Mahmoud M. Naguib drafted the manuscript. Isabella Monne, Martin Beer and all co-authors critically analysed and revised the manuscript and provided final approval.

References

- Cheung TK, Poon LL. Biology of influenza a virus. Ann N Y Acad Sci. 2007;1102(1):1-25. DOI: 10.1196/annals.1408.001 PMID: 17470908
- 2. Webster RG, Govorkova EA. Continuing challenges in influenza. Ann N Y Acad Sci. 2014;1323(1):115-39. DOI: 10.1111/nyas.12462 PMID: 24891213
- 3. França MS, Brown JD. Influenza pathobiology and pathogenesis in avian species.Curr Top Microbiol Immunol. 2014;385:221-42. DOI: 10.1007/82_2014_385 PMID: 25015786
- 4. Wallace RG, Bergmann L, Kock R, Gilbert M, Hogerwerf L, Wallace R, et al. The dawn of Structural One Health: a new science tracking disease emergence along circuits of capital. Soc Sci Med. 2015;129:68-77. DOI: 10.1016/j. socscimed.2014.09.047 PMID: 25311784
- Malik Peiris JS. Avian influenza viruses in humans.Rev Sci Tech. 2009;28(1):161-73. DOI: 10.20506/rst.28.1.1871 PMID: 19618624
- 6. Capua I, Munoz O. Emergence of influenza viruses with zoonotic potential: open issues which need to be addressed. A review.Vet Microbiol. 2013;165(1-2):7-12. DOI: 10.1016/j. vetmic.2013.01.044 PMID: 23567150
- Smith GJ, Donis RO. World Health Organization/World Organisation for Animal HF, Agriculture Organization HEWG. Nomenclature updates resulting from the evolution of avian influenza A(H5) virus clades 2.1.3.2a, 2.2.1, and 2.3.4 during 2013-2014.Influenza Other Respi Viruses. 2015;9(5):271-6. DOI: 10.1111/irv.12324
- Lycett S, Bodewes R, Pohlmann A, Banks J, Bányai K, Boni MF, et al., Global Consortium for H5N8 and Related Influenza Viruses. Role for migratory wild birds in the global spread of avian influenza H5N8.Science. 2016;354(6309):213-7. DOI: 10.1126/science.aaf8852 PMID: 27738169
- Adlhoch C, Gossner C, Koch G, Brown I, Bouwstra R, Verdonck F, et al. Comparing introduction to Europe of highly pathogenic avian influenza viruses A(H5N8) in 2014 and A(H5N1) in 2005. Euro Surveill. 2014;19(50):20996. DOI: 10.2807/1560-7917.ES2014.19.50.20996 PMID: 25597538
- Abdelwhab EM, Hassan MK, Abdel-Moneim AS, Naguib MM, Mostafa A, Hussein IT, et al. Introduction and enzootic of A/ H5N1 in Egypt: Virus evolution, pathogenicity and vaccine efficacy ten years on. Infect Genet Evol. 2016;40:80-90. DOI: 10.1016/j.meegid.2016.02.023 PMID: 26917362
- World Health Organization (WHO). Cumulative number of confirmed human cases for avian influenza A(H5N1) reported to WHO, 2003-2016. Geneva: WHO; 2016. [Accessed 01 Jan 2017]. Available from: http://www.who.int/influenza/ human_animal_interface/H5N1_cumulative_table_archives/ en/.
- Arafa AS, Naguib MM, Luttermann C, Selim AA, Kilany WH, Hagag N, et al. Emergence of a novel cluster of influenza A(H5N1) virus clade 2.2.1.2 with putative human health impact in Egypt, 2014/15. Euro Surveill. 2015;20(13):2-8. DOI: 10.2807/1560-7917.ES2015.20.13.21085 PMID: 25860390
- 13. Tosh C, Nagarajan S, Kumar M, Murugkar HV, Venkatesh G, Shukla S, et al. Multiple introductions of a reassortant H5N1 avian influenza virus of clade 2.3.2.1c with PB2 gene of H9N2 subtype into Indian poultry. Infect Genet Evol. 2016;43:173-8. DOI: 10.1016/j.meegid.2016.05.012 PMID: 27174088

- 14. Bi Y, Chen J, Zhang Z, Li M, Cai T, Sharshov K, et al. Highly pathogenic avian influenza H5N1 Clade 2.3.2.1c virus in migratory birds, 2014-2015. Virol Sin. 2016;31(4):300-5. DOI: 10.1007/S12250-016-3750-4 PMID: 27405930
- Naguib MM, Kinne J, Chen H, Chan KH, Joseph S, Wong PC, et al. Outbreaks of highly pathogenic avian influenza H5N1 clade 2.3.2.1c in hunting falcons and kept wild birds in Dubai implicate intercontinental virus spread. J Gen Virol. 2015;96(11):3212-2. DOI: 10.1099/jgv.0.000274 PMID: 26350163
- Tassoni L, Fusaro A, Milani A, Lemey P, Awuni JA, Sedor VB, et al. Genetically Different Highly Pathogenic Avian Influenza A(H5N1) Viruses in West Africa, 2015. Emerg Infect Dis. 2016;22(12):2132-6. DOI: 10.3201/eid2212.160578 PMID: 27389972
- 17. Claes F, Morzaria SP, Donis RO. Emergence and dissemination of clade 2.3.4.4 H5Nx influenza viruses-how is the Asian HPAI H5 lineage maintained.Curr Opin Virol. 2016;16:158-63. DOI: 10.1016/j.coviro.2016.02.005 PMID: 26991931
- Lee DH, Torchetti MK, Winker K, Ip HS, Song CS, Swayne DE. Intercontinental Spread of Asian-Origin H5N8 to North America through Beringia by Migratory Birds.J Virol. 2015;89(12):6521-4. DOI: 10.1128/JVI.00728-15 PMID: 25855748
- ProMED-mail. Avian influenza H5N8 Germany. Archive. 2016;4641281(: 20161119):19. Available from: http://www. promedmail.org/post/20161119.4641281
- 20. ProMED-mail. Avian influenza H5N8 Israel. Archive. 2016;4628359(: 20161114):14. Available from: http://www. promedmail.org/post/4628359
- 21. Munster VJ, Baas C, Lexmond P, Waldenström J, Wallensten A, Fransson T, et al. Spatial, temporal, and species variation in prevalence of influenza A viruses in wild migratory birds. PLoS Pathog. 2007;3(5):e61. DOI: 10.1371/journal.ppat.0030061 PMID: 17500589
- 22. Peiris JS, Cowling BJ, Wu JT, Feng L, Guan Y, Yu H, et al. Interventions to reduce zoonotic and pandemic risks from avian influenza in Asia. Lancet Infect Dis. 2016;16(2):252-8. DOI: 10.1016/S1473-3099(15)00502-2 PMID: 26654122
- 23. Sims LD. Intervention strategies to reduce the risk of zoonotic infection with avian influenza viruses: scientific basis, challenges and knowledge gaps.Influenza Other Respi Viruses. 2013;7(Suppl 2):15-25. DOI: 10.1111/irv.12076 PMID: 24034479
- 24. Höper D, Hoffmann B, Beer M. Simple, sensitive, and swift sequencing of complete H5N1 avian influenza virus genomes.J Clin Microbiol. 2009;47(3):674-9. DOI: 10.1128/JCM.01028-08 PMID: 19109473
- 25. European Commission (EC). 2006/437/EC: Commission Decision of 4 August 2006 approving a Diagnostic Manual for avian influenza as provided for in Council Directive 2005/94/EC (notified under document number C(2006) 3477) (Text with EEA relevance). OJ L 237, 31.8.2006, p. 1-27. Brussels: EC; 2006. Available from: http://eur-lex.europa.eu/legal-content/EN/ TXT/?uri=CELEX%3A32006D0437
- 26. World Organisation for Animal Health/Food and Agriculture Organization of the United Nations (OIE/FAO). Network of expertise on animal influenza. Influenza A Cleavage sites. Paris/Rome: OIE/FAO; 2015. [Accessed o1 Dec 2016] Available from: http://www.offlu.net/fileadmin/home/en/resourcecentre/pdf/Influenza_A_Cleavage_Sites.pdf
- 27. Influenza research database (IRD). [Internet]. Highly Pathogenic H5N1 Clade Classification Tool. [Accessed 21 Nov 2016]. Available from: https://www.fludb.org/brc/ h5n1Classifier.spg?method=ShowCleanInputPage&decorator= influenza
- 28. Hoffmann B, Harder T, Lange E, Kalthoff D, Reimann I, Grund C, et al. New real-time reverse transcriptase polymerase chain reactions facilitate detection and differentiation of novel A/H1N1 influenza virus in porcine and human samples. Berl Munch Tierarztl Wochenschr. 2010;123(7-8):286-92.PMID: 20690540
- 29. Spackman E, Senne DA, Bulaga LL, Myers TJ, Perdue ML, Garber LP, et al. Development of real-time RT-PCR for the detection of avian influenza virus. Avian Dis. 2003;47(3) Suppl:1079-82. DOI: 10.1637/0005-2086-47.53.1079 PMID: 14575115
- 30. Hoffmann B, Hoffmann D, Henritzi D, Beer M, Harder TC. Riems influenza a typing array (RITA): An RT-qPCR-based low density array for subtyping avian and mammalian influenza a viruses.Sci Rep. 2016;6:27211. DOI: 10.1038/srep27211 PMID: 27256976
- Hoffmann B, Harder T, Starick E, Depner K, Werner O, Beer M. Rapid and highly sensitive pathotyping of avian influenza A H5N1 virus by using real-time reverse transcription-PCR.J Clin Microbiol. 2007;45(2):600-3. DOI: 10.1128/JCM.01681-06 PMID: 17182758
- 32. Ampofo WK, Azziz-Baumgartner E, Bashir U, Cox NJ, Fasce R, Giovanni M, et al. , WHO Writing Group. Strengthening

the influenza vaccine virus selection and development process: Report of the 3rd WHO Informal Consultation for Improving Influenza Vaccine Virus Selection held at WHO headquarters, Geneva, Switzerland, 1-3 April 2014.Vaccine. 2015;33(36):4368-82. DOI: 10.1016/j.vaccine.2015.06.090 PMID: 26148877

- 33. Wong S-S, Webby RJ. Traditional and new influenza vaccines. Clin Microbiol Rev. 2013;26(3):476-92. DOI: 10.1128/ CMR.00097-12 PMID: 23824369
- 34. European Centre for Disease Prevention and Control (ECDC). Outbreak of highly pathogenic avian influenza. A(H5N8) in Europe – 18 November 2016. Stockholm: ECDC; 2016. [Accessed 21 Nov 2016] Available from: http://ecdc.europa.eu/ en/publications/Publications/risk-assessment-avian-influenza-H5N8-europe.pdf
- 35. Kis Z, Jones J, Creanga A, Ferdinand K, Inui K, Gerloff N, et al. Real-time RT-PCR assay to differentiate clades of H5N1 avian influenza viruses circulating in Vietnam. J Virol Methods. 2013;193(2):452-8. DOI: 10.1016/j.jviromet.2013.06.023 PMID: 23850699
- 36. Abdelwhab SM, Erfan AM, Grund C, Ziller M, Arafa A-S, Beer M, et al. Simultaneous detection and differentiation by multiplex real time RT-PCR of highly pathogenic avian influenza subtype H5N1 classic (clade 2.2.1 proper) and escape mutant (clade 2.2.1 variant) lineages in Egypt. Virol J. 2010;7(1):260. DOI: 10.1186/1743-422X-7-260 PMID: 20929539

License and copyright

This is an open-access article distributed under the terms of the Creative Commons Attribution (CC BY 4.0) Licence. You may share and adapt the material, but must give appropriate credit to the source, provide a link to the licence, and indicate if changes were made.

This article is copyright of the authors, 2017.