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Research article

Emerging highly pathogenic H5 avian influenza viruses in France during winter 2015/16: phylogenetic analyses and markers for zoonotic potential

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Several new highly pathogenic (HP) H5 avian influenza virus (AIV) have been detected in poultry farms from south-western France since November 2015, among which an HP H5N1. The zoonotic potential and origin of these AIVs immediately became matters of concern. One virus of each subtype H5N1 (150169a), H5N2 (150233) and H5N9 (150236) was characterised. All proved highly pathogenic for poultry as demonstrated molecularly by the presence of a polybasic cleavage site in their HA protein – with a sequence (HQRRKR/GLF) previously unknown among avian H5 HPAI viruses – or experimentally by the in vivo demonstration of an intravenous pathogenicity index of 2.9 for the H5N1 HP isolate. Phylogenetic analyses based on the full genomes obtained by NGS confirmed that the eight viral segments of the three isolates were all part of avian Eurasian phylogenetic lineage but differed from the Gs/Gd/1/96-like lineage. The study of the genetic characteristics at specific amino acid positions relevant for modulating the adaptation to and the virulence for mammals showed that presently, these viruses possess most molecular features characteristic of AIV and lack some major characteristics required for efficient respiratory transmission to or between humans. The three isolates are therefore predicted to have no significant pandemic potential.

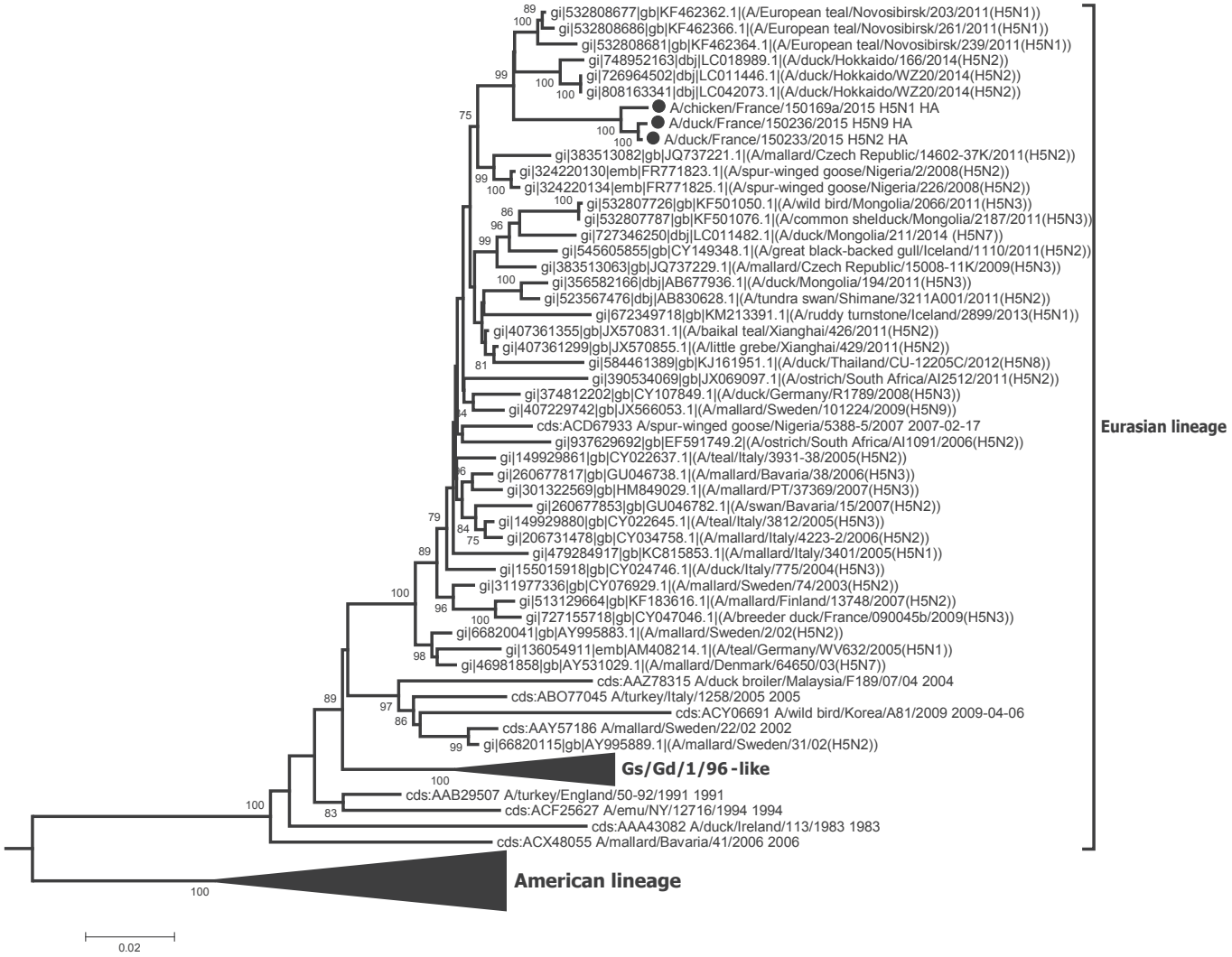
Introduction

On 24 November 2015, a highly pathogenic (HP) H5N1 avian influenza (AI) outbreak was confirmed by the French National Reference Laboratory (NRL) for avian influenza, in backyard layers and chickens in the Dordogne department, south-western France. From November 2015 to August 2016, a total of 80 highly pathogenic viruses have been identified, belonging to three different subtypes (H5N1, H5N2, H5N9).

In France, before 2015, the last H5 HPAI event was limited to wild swans *(Cygnus olor)* and mallards *(Anas platyrhynchos)*, in the eastern part of France during spring 2006 and summer 2007. It occurred almost concomitantly with outbreaks in wild birds and/or poultry in central Europe. The French HPH5 viruses isolated in 2006 and 2007 belonged to the A/goose/Guangdong/1/1996 (Gs/Gd/1/96-like) lineage (clade 2.2 subgroup), as did the 2007 viruses from central Europe) [1-5]. Until 2014, H5N1 HPAI viruses belonging to the Gs/Gd/1/96-like lineage have been maintained in south-east Asia, the Middle East and Egypt, in different locations and their haemagglutinin (HA) genes evolved continuously into a wide range of clades and subclades. They were reintroduced in West Africa and eastern Europe in 2015, and reassorted extensively, which in 2014 led to the emergence of new H5N6 and H5N8 HPAI subtypes (known as clade 2.3.4.4) in south-east Asia. The 2.3.4.4 H5N8 HPAI viruses spread to Europe (mainly the northern part) and to North America in late 2014. They are currently widespread in wildlife and poultry farms in the European Union (EU). In North America, they further reassorted as H5N2 and H5N1 HPAI, by incorporating neuraminidase (NA) genes of AIVs belonging to the American lineage. In the EU, recent reports also indicate reassortment as H5N5 HPAI

Figure 1

Phylogenetic tree of the H5 gene sequences, three avian influenza H5 viruses, France, November 2015



HA: haemagglutinin; HPAI: highly pathogenic avian influenza.

The complete coding sequences of the three H5 HPAI virus isolated from poultry in France were aligned with their closest related sequences as detected by BLAST, together with other sequences selected as representatives for more distant clusters. Then, the neighbour-joining method based on the Kimura-2 parameter model was applied using the MEGA 6 software [26] to obtain a phylogenetic tree with 1,000 bootstrap replicates. Only bootstrap values higher than 75% are shown. The HA sequences of French viruses are indicated with black dots. The tree was rooted by an H2 sequence.

[6]. However, no Gs/Gd/1/96-like viruses have been detected in France before November 2015, except for the 2006 and 2007 outbreaks mentioned above [7-12].

In contrast, low pathogenic (LP) H5N1, as well as H5N2 and H5N3 viruses, were detected in France in poultry, predominantly in domestic ducks, on several occasions [13-15], albeit seldom considering the fact that antibodies against H5 were regularly detected in sera of apparently healthy domestic ducks and geese during annual serological surveys [16-19].

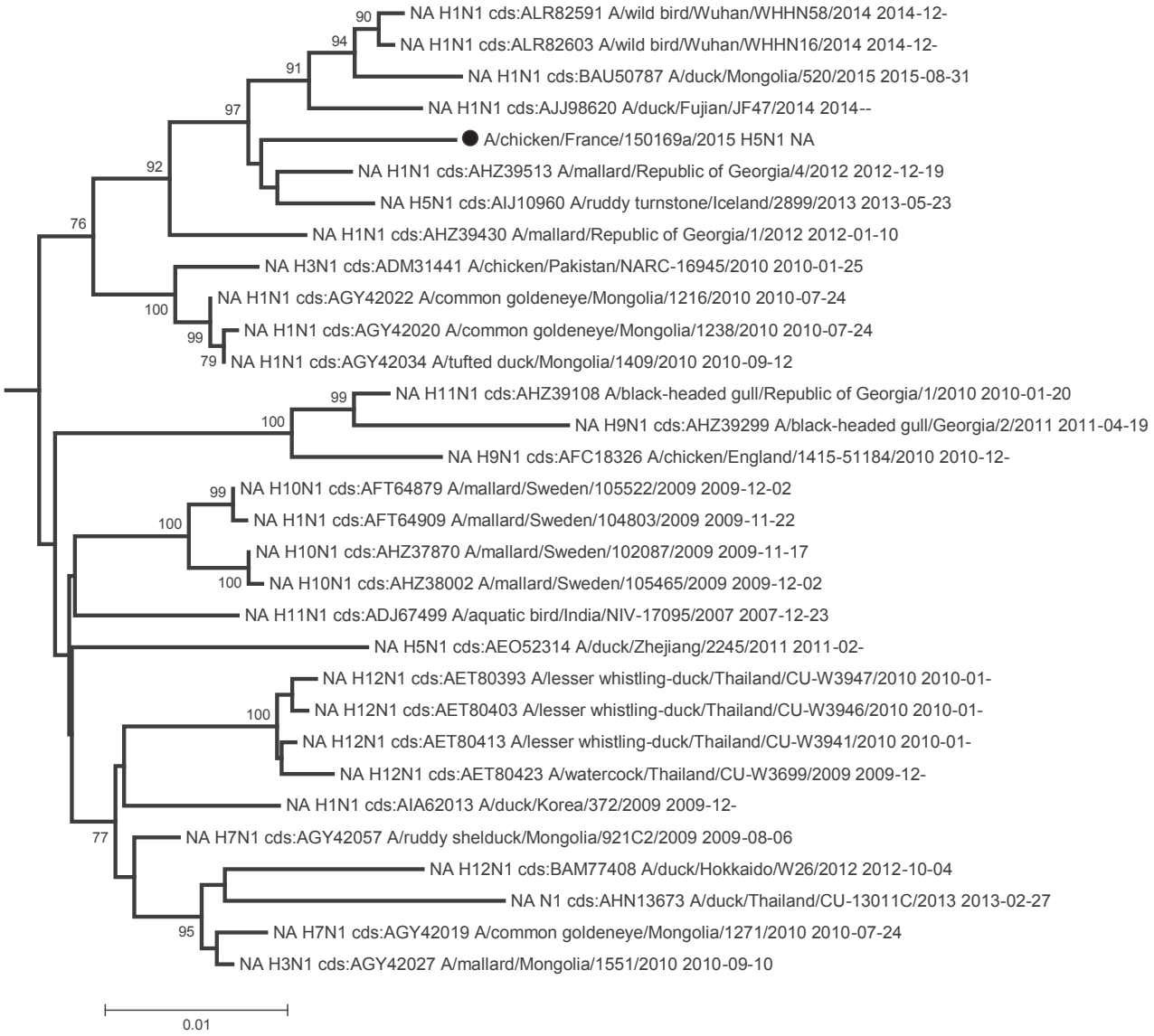
The relationships between the H5 HPAI viruses detected in November 2015 and other H5 HPAI and LPAI viruses, including H5N1 viruses previously detected in France and worldwide, as well as their zoonotic potential and origin were immediately matters of concern. Based on whole genome sequences established by next generation sequencing (NGS), this paper focuses on the phylogenetic relatedness of these newly isolated viruses and on their genetic characteristics at specific amino acid positions already reported as relevant for cross-species transmission, adaptation to and virulence for mammals, including humans.

Methods

Three AIV subtypes were detected in south-western France during the winter 2015/16 (H5N1, H5N2, H5N9).

Figure 2

Phylogenetic trees of the neuraminidase gene sequences (N1 segment), three avian influenza H5 viruses, France, November 2015



HA: haemagglutinin; HP: highly pathogenic; M: matrix; NA: neuraminidase; nt: nucleotide.

The complete coding sequence of the three different neuraminidases of French H5 HP viruses were aligned with their closest related sequences as detected by BLAST, together with other sequences selected as representatives for more distant clusters. The neighbour-joining method based on the Kimura-2 parameter model was applied with the MEGA 6 software [26] to obtain a phylogenetic tree with 1,000 bootstrap replicates. Only bootstrap values higher than 75% are shown. The NA of French H5HP viruses are indicated by a black dot. The neuraminidase sequences from human viruses (H5N1 and H9N2) were not represented in the N1 and N2 trees respectively (nt distances between these sequences and the French sequences are too high).

One representative of each subtype is described in the present paper.

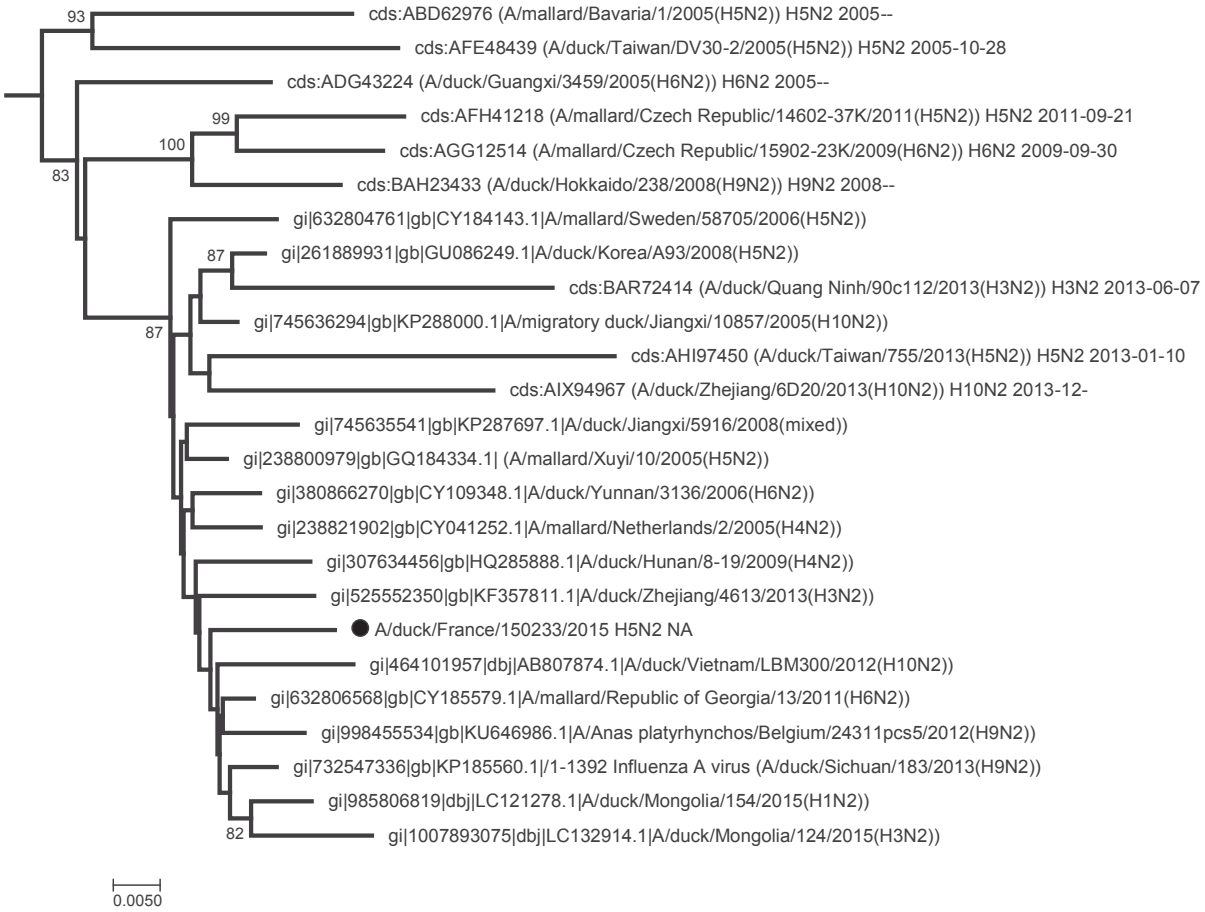
Outbreak description

The first virus was isolated from an affected chicken in a suspected outbreak of avian influenza virus infections (150169a; H5N1) declared to the local French animal health services on 19 November 2015. The affected flock was a backyard of 32 layer hens and broiler chickens, 9 to 10 months of age, located in Biras, Dordogne department. Die-off without any previous clinical signs caused a mortality rate of 69%. Post-mortem examination performed at the local veterinary diagnosis laboratory showed sub-cutaneous oedema of the head, neck and breast.

The second virus investigated here (150233; H5N2) was obtained from a duck on a farm located in Manciet,

Figure 3

Phylogenetic trees of the neuraminidase gene sequences (N2 segment), three avian influenza H5 viruses, France, November 2015



HA: haemagglutinin; HP: highly pathogenic; M: matrix; NA: neuraminidase; nt: nucleotide.

The complete coding sequence of the three different neuraminidases of French H5 HP viruses were aligned with their closest related sequences as detected by BLAST, together with other sequences selected as representatives for more distant clusters. The neighbour-joining method based on the Kimura-2 parameter model was applied with the MEGA 6 software [26] to obtain a phylogenetic tree with 1,000 bootstrap replicates. Only bootstrap values higher than 75% are shown. The NA of French H5HP viruses are indicated by a black dot. The neuraminidase sequences from human viruses (H5N1 and H9N2) were not represented in the N1 and N2 trees respectively (nt distances between these sequences and the French sequences are too high).

Gers departement, maintaining 8,300 ducks for fattening and fatty liver production, where 3% mortality was observed on 8 December 2015.

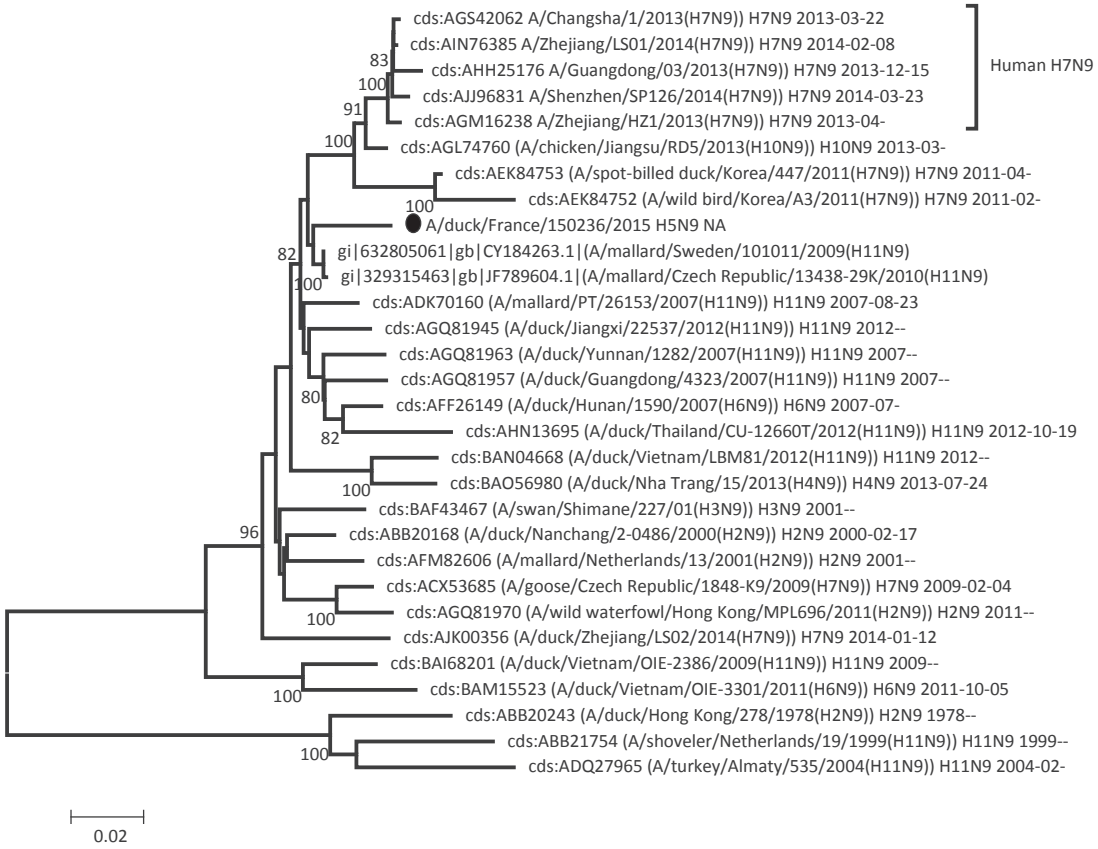
The third virus (150236; H5N9) was from a duck in Arrosès, Pyrénées-Atlantiques departement, where a flock of 500 ducks raised for fatty liver production experienced a 5% mortality on 9 December 2015.

Detection, subtyping and molecular pathotyping

For each outbreak, RNA was extracted (RNeasy mini kit, Qiagen) from cloacal and oropharyngeal pools of five samples from dead birds. Samples were tested with the screening M-gene and H5 rRT-PCRs [20] by district laboratories. H5-positive samples were sent to NRL for further characterisation. The nucleotide (nt) sequences encompassing the cleavage site in the HA genes were amplified by the J3/B2a or Kha1/Kha3 RT-PCRs [21]. Similarly, a portion of the NA gene was amplified by Pan NA RT-PCR [22]. PCR products were sequenced using the PCR primers. Subtype determination was performed by BLAST against sequences of the Influenza virus resource database [23]. The theoretical pathotype of the viruses was inferred from the cleavage site sequences according to the World Organisation for Animal Health (OIE) / Food and Agriculture Organisation (FAO) Network of expertise on animal Influenza (OFFLU) [24].

Figure 4

Phylogenetic trees of the neuraminidase gene sequences (N9 segment), three avian influenza H5 viruses, France, November 2015



HA: haemagglutinin; HP: highly pathogenic; M: matrix; NA: neuraminidase; nt: nucleotide.

The complete coding sequence of the three different neuraminidases of French H5 HP viruses were aligned with their closest related sequences as detected by BLAST, together with other sequences selected as representatives for more distant clusters. The neighbour-joining method based on the Kimura-2 parameter model was applied with the MEGA 6 software [26] to obtain a phylogenetic tree with 1,000 bootstrap replicates. Only bootstrap values higher than 75% are shown. The NA of French H5HP viruses are indicated by a black dot. The neuraminidase sequences from human viruses (H5N1 and H9N2) were not represented in the N1 and N2 trees respectively (nt distances between these sequences and the French sequences are too high).

Virus isolation and in vivo characterisation

Virus isolation was performed from individual tracheal and/or cloacal swabs, on 9-day-old specific pathogen free (SPF) eggs, and was positive after the first passage. Viral pathogenicity was confirmed through determination of the HA cleavage site sequence and in vivo by inoculation of the H5N1HP 150169a isolate to 6.5-week-old SPF chickens, to establish the intravenous pathogenicity index (IVPI). All methods were conducted according to the OIE manual of standards for diagnostic tests and vaccines [25]. The IVPI experiment was performed according to international standards and was approved by the French Agency for Food, Environmental and Occupational Health and Safety (Anses)/National veterinary school of Alfort (ENVA)/Paris-est Créteil University (UPEC) Ethics Committee (no.14–060–18/11/14–6).

Library preparation, whole genome sequencing and NGS data analysis

Total RNA (170–200ng) was extracted from infected allantoic fluid and was treated with DNase, then was depleted from rRNA. cDNA libraries were prepared using the Ion Total RNA-Seq Kit (Life Technologies, Carlsbad, California, United States (US)) according to a protocol adapted from supplier's instructions (available upon request from the authors). The cDNA libraries were enriched then sequenced using the Ion Proton Sequencer and an Ion PI Chip v2 (Life Technologies). The resulting reads were cleaned with the Trimmomatic 0.32 software, then a Bowtie 2 alignment was performed on avian influenza genome references. The reads were down-sampled to fit a global coverage estimation of 80 x and were submitted to the SPAdes 3.1.1 de novo assembler. The de novo contigs were then

Table 1

GenBank accession number, full reference and percent nucleotide identity of the complete coding sequences of the eight genome segments of three avian influenza H5 viruses with their closest genetic relatives, France, November 2015

Segments	Closest relatives of H5N1 150169a virus	Closest relatives of H5N2 150233 virus	Closest relatives of H5N9 150236 virus
PB2	KM213385 A/ruddy turnstone/ Iceland/2899/2013(H5N1) 98,2	KF918334 A/Italy/3/2013(H7N7) 97,5	KM213385 A/ruddy turnstone/ Iceland/2899/2013(H5N1) 97,9
PB1	CY183997 A/mallard/ Sweden/133546/2011(H10N4) 98,4	KP137828 A/harbour seal/ Germany/1/2014(H10N7)) 97,5	CY041344 A/common eider/ Netherlands/1/2006(H3N8) 97,2
PA	CY185470 A/common teal/Republic of Georgia/1/2011(H3N8) 98,2	CY184282 A/mallard/ Sweden/100878/2009(H11N9) 98,3	KF874480 A/wild waterfowl/Dongting/ C2383/2012(H1N2) 98,4
HA	KF462362 A/european teal/ Novosibirsk/203/2011(H5N1) 96,4	KF462362 A/European teal/ Novosibirsk/203/2011(H5N1)) 96,8	KF462362 A/European teal/ Novosibirsk/203/2011(H5N1) 96,6
NP	CY185468 A/common teal/Republic of Georgia/1/2011(H3N8) 98,5	CY165689 A/mallard/ Sweden/93211/2009(H4N6) 97,3	CY046143 A/mallard/ France/061054/2006(H5N3) 96,7
NA	AIJ10960 A/ruddy turnstone/ Iceland/2899/2013(H5N1) 98,2	CY185579 A/mallard/Republic of Georgia/13/2011(H6N2) 98,1	CY184263 A/mallard/ Sweden/101011/2009(H11N9) 97,8
M	CY185341 A/mallard/Chany/425/2009(H4N6) 99	CY183800 A/mallard/ Sweden/64476/2007(H10N4) 98,4	CY183800 A/mallard/ Sweden/64476/2007(H10N4)) 98,5
NS	GQ907290 A/bar headed goose/ Mongolia/143/2005(H12N3) 97,8	KF260032 A/common teal/Hong Kong/ MPD322/2007(H11N9) 97,4	KF2600329 A/common teal/Hong Kong/ MPD322/2007(H11N9) 97,3

HA: haemagglutinin; NA: neuraminidase.

submitted to BLAST on a local nt database. For each segment the best matches were selected for a Bowtie 2 alignment, which produces very clean and robust 5' and 3' ends, contrary to de novo assemblies of viral genomes for which 5' and 3' ends are sometimes incomplete. Finally, the de novo assemblies and the alignment on the references were compared and the strict identities of the de novo and aligned sequences were assessed.

For each virus, the consensus of the eight avian influenza segments was submitted to the GenBank database (accession numbers KU310444 to KU310451, KX014875 to KX014882 and KX014883 to KX014890 for isolates 150169, 150233 and 150336, respectively).

Phylogenetic analysis

For each segment, the sequences were aligned with most of the closest full-length related sequences, as obtained by BLAST, and with genetic sequences selected as representative of the segment genetic diversity. Then, the neighbour-joining method based on the Kimura-2 parameter model was applied using the MEGA 6 software [26] to obtain phylogenetic trees with 1,000 bootstrap replicates.

In silico analysis of molecular markers for transmission, replication and/or virulence in mammalian hosts

For the in silico prediction of the zoonotic potential of the new French viruses, the deduced amino acid sequences of viral proteins were analysed to search for the presence of residues previously known to be associated with increased transmission, replication, and/or virulence in mammalian hosts. The analysis was based on the inventory provided by the US Centers for Disease Control and Prevention (CDC) [27] and on a recent review by Neumann et al. [28].

Results

Virus isolation and in vivo pathotyping

Allantoic fluids with a haemagglutinating activity were collected from the inoculated embryonated eggs after the first passage of the three samples.

Table 2

Percent nucleotide identity of the eight complete coding sequences between the three avian influenza H5 viruses, France, November 2015

	PB2	PB1	PA	HA	NP	M	NS
150169a (H5N1) to 150233 (H5N2)	96.6	94.5	93.1	98.9	95.5	95.6	98.6
150169a (H5N1) to 150236 (H5N9)	97.5	94.9	94.4	98.8	92.3	95.9	98.7
150233 (H5N2) to 150236 (H5N9)	96.4	93.3	93.4	99.8	91.6	99.5	99.2

HA: haemagglutinin; NA: neuraminidase.

Isolates obtained from the allantoic fluids were further identified by HI test as H5 influenza viruses which were designated A/chicken/France/150169a/2015, A/duck/France/150233/2015 and A/duck/France/150236/2015 for the H5N1, H5N2 and H5N9 isolates, respectively. Because the HA genes of the three isolates were so similar, and in order to reduce the number of animal experiments, only the first virus isolate, the 150169a H5N1 virus, was tested in vivo for pathogenicity. Following intravenous inoculation, mortality was observed on the first day (3 of 10 birds died) and all inoculated birds had died on the second day. The IVPI was 2.9, close to its maximum value of 3, and higher than the regulatory threshold value of 1.2 required to declare the isolate highly pathogenic [25].

Sequence analysis

Tables 1 and 2 present the closest genetically related avian influenza sequences, as identified by BLAST, and the percent nt identities between the three newly determined genomes, respectively. Although the three French HA sequences were close (nucleotide percent identities from 98.9 to 99.8), and shared a polybasic HQRRKR/GLF cleavage site not previously recognised in avian H5 HP viruses [24], their internal genes proved more distant with percent identities ranging from 93.1 to 99.5, and even more distant for the NA genes which belonged to the N1, N2 and N9 NA subtypes.

Phylogenetic analyses of the eight segments of the French viruses confirmed that they were all part of the avian Eurasian lineage, as illustrated for the HA and NA genes in Figures 1, 2, 3, 4.

The PB2 gene of the three French viruses belonged to a cluster including viruses from Europe, Gerogia, Iceland and notably one H9N2 virus previously detected in France (GenBank accession number: CY080412) and one avian HP H7N7 virus detected from Italian poultry workers with conjunctivitis [29].

For the PB1 gene, the sequence of the 150169a H5N1 virus was grouped mainly with viruses from Georgia, Mongolia and Sweden. The 150233 H5N2 virus sequence was grouped with a Belgian H5N2 and Chinese sequences and a sequence of an H10N7 virus isolated from a German harbor seal [30], whereas the sequence of the 150236 H5N9 virus was associated with sequences from various subtypes of AIVs from Sweden and the Netherlands.

The sequences of the PA segments clustered either with those of AIVs from Georgia, the Czech Republic, and the H10N7 virus from harbour seals [30] (150169a H5N1 isolate), or with virus sequences from Sweden and Norway (150233 H5N2 isolate), or with a group of sequences from Asian and Georgian viruses (150236 H5N9 isolate).

The HA phylogenetic analysis showed that the sequences of three French H5 HP clustered with those from Japanese, Mongolian and Russian viruses, but were not closely related to those of the Gs/Gd/1/96-like clades which all grouped in a separate cluster with a 100% bootstrap value (Figure 1).

As already observed with PB2, the NP sequences of 150169a (H5N1 virus) and 150233 (H5N2 virus) were grouped with those of viruses from Europe, Georgia and Iceland, again including the avian H7N7 isolated from poultry workers (A/Italy/3/2013) [29], whereas the NP gene of the French 150236 H5N9 HP was closely related with many other sequences from French H5 viruses isolated from 2006 to 2007 and with sequences from Dutch viruses.

The N1 gene sequence of 150169a H5N1 was related to sequences from viruses from Egypt, Georgia or Iceland. The Egyptian N1 sequence was from the avian H1N1 subtype, and not from H5N1 viruses with a zoonotic potential (Figure 2). The neuraminidase sequence from the 150233 H5N2 virus was close to an Asian N2 gene found in combination with several HA genes (H1, H3, H4, H5, H6, H9 and H10) and to a sequence from a Belgian H9N2 virus (Figure 3). The N9 sequence of isolate 150236 proved closest to several Swedish H11N9 sequences (Figure 4), and, although within a group with a significant bootstrap value, only distantly related with sequences from H7N9 viruses with a zoonotic potential.

For the M (matrix) gene sequences, only one large Eurasian cluster, including the three H5 HP viruses, exhibited a bootstrap value higher than 75%. This group included only four sequences of viruses from mammalian hosts, the aforementioned H7N7 and H10N7 viruses, as well as two H2N2 and H4N1 viruses detected in muskrat and swine, respectively [29-31].

Finally, the phylogenetic analysis of the three NS gene sequences indicated that they all belonged to a large cluster inside the A allele group, which contained sequences from African, Asian and European viruses, with only one sequence from a virus from a mammalian host, the swine H4N1 virus [31].

Table 3

Amino acid residues in different genes of highly pathogenic H5 viruses, at positions previously identified to promote transmission, replication (in vitro or in vivo) or pathogenicity in mammalian hosts, or associated with decreased sensitivity to antivirals, France, 2015[a]

Protein	Aa substitution[b]	Aa present in the French H5 viruses	The most represented residue for European AIV (% of this residue)[c]	Comments[d]	PMID[e]
HA	D94N	N94	N94 (62.5)	Increased binding to alpha 2-6 receptor	19020946
	S159N; T160A	N159; A160	N159 (81.4); A160 (97.6)	Increased binding to alpha 2-6 receptor	20427525;19116267
	S239P	P239	P239 (99.4)	Slightly increased binding to alpha 2-6 receptor (aa 239 corresponds to aa 235 in H5 numbering)	21637809
	T160A	A160	A160 (97.6)	Increased airborne transmission in ferrets; increased binding to alpha2-6 receptor (aa 160 corresponds to aa 156 in H5 numbering)	22723413; 20427525
	Multibasic cleavage site	HQRRKR/GLF	Same sequence not found in other European viruses	Multiple basic residues in H5 viruses that are highly pathogenic for avian hosts (but unique sequence)	Same sequence not found in other European viruses
PB2	I63T	I63	I63 (99.2)	Decreased pathogenicity in mice in association with PB1 T677M	21367983
	L89V; G309D; T339K; R477G; I495V; A676T	V89; D309; K339; G477; V495; T676	V89 (99.7); D309 (99.5); K339 (91.7); G477 (100); V495 (99.4); T676 (98.3)	Increased polymerase activity in mouse cells	19393699
	R368Q; Q447H	R368; Q447	R368 (91.9); Q447 (99.9)	Reduced virulence (lethality in mice) and conferred histologic alteration in the lungs, liver and brain of ferrets	16533883; 15681421
PB1	K207R	K207	K207 (100)	Decreased polymerase activity	17553873
	Y436H	Y436	Y436 (99.9)	Decreased virulence in ducks, mice and ferrets	17553873
	V473L	V473	V473 (99.5)	Decreased polymerase activity in mammalian cells and mice	22090209
	T677M	T677	T677 (99.9)	Increased polymerase activity in vitro; reduced replication efficiency; decreased virulence in mice in association with PB2 I63T	21367983
PB1-F2	N66S	S66 (150169a); N66 (150233); PB1-F2 truncated (150236)	N66 (84,8); S66(15,1)	Increased replication efficiency in mice	21852950
PA	T515A	T515	T515 (99.8)	Decreased polymerase activity	17553873
	R266H; T515S	R266 T515	R266 (99.8) T515 (99.8)	Reduced polymerase activity in vitro	20211480
M1	N30D	D30	D30 (99.9)	Increased virulence in mice	19117585
	T215A	A215	A215 (99.9)	Increased virulence in mice	19117585
NS1	P42S	S42	S42 (66.0)	Increased virulence (lethality in mice and the systemic spread of infection); affected IFN pathway	18032512
	E92D	D92	D92 (99.8)	Cytokine resistance using antiviral activity assay	12195436
	L103F; I106M	F103; M106	F103 (65.7); M106 (99.8)	Increased virulence compared to WT in mice	19052083; 21593152
	N205S	S205	S205 (64.9)	Implicated in high virulence in ferrets	20862325
	227-230 (presence of PDZ ligand domain)	Amino acid motif (ESEV)	Amino acid motif (ESEV) (>80)	Amino acid motif (ESEV) increased virulence and pathogenicity in mice	18334632
NS2	T48A	A48	A48 (66.2)	Implicated in high virulence in ferrets	20862325

Aa: amino acids; AIV: avian influenza viruses; HA: haemagglutinin; IFN: Interferon; M: matrix; NA: neuraminidase; WT. : wild type

[a] Markers for mammalian host adaptation or antiviral resistance were retrieved from [27] and the automated annotation of the studied sequence using the Influenza Research database [37].

[b] H3 numbering for HA gene and numbering from the first methionine residue for other genes. Only positions where the sequence of French H5 HP viruses is consistent with a tropism for mammalian hosts are shown. For a more complete version with all positions shown, see ANSES table [12].

[c] Percentage based on all complete protein sequences from viruses (all subtypes included except for the H5 and N1 genes) isolated in Europe and available in the Influenza Research database [37].

[d] Comments on the biological effect of the studied set of mutations as per [27,37], except authors' comment for the HA cleavage site.

[e] PMID numbers correspond to PubMed identifiers of cited references.

Molecular markers for transmission, replication and/or virulence in mammalian hosts

The profiles of the three newly isolated viruses proved extremely similar in this respect, with only one position in the PB1-F2 protein differing between the three viruses.

Among the positions in the HA at which amino acid substitutions have been reported as potential determinants of host-range or of virulence for mammals, five were found in the HA of the new viruses (N94, N159, A160, P239, polybasic cleavage site). However, the HA of the isolated H5 HP viruses did not show the Q226L and G228S substitutions in the receptor-binding site of the HA that result in a switch of receptor binding preference from SAα2,3Gal to SAα2,6Gal [28].

Among the PB2, PB1 and PA polymerase complex proteins, PB2 has been shown to harbour major determinants of host-restriction and adaptation. None of these two major substitutions were found in any of the viruses investigated here. In PB1, substitutions shown to increase virulence in mice (V3A; N328K; N375S) or to contribute to airborne transmission in mammals (H99Y; I368V) were not present in the three new H5 HPAI viruses [32]. As shown in Table 3, at nine positions in PB2, three positions in PA and four positions in PB1 for which substitutions were shown to result in reduced polymerase activity or decreased virulence in mammals, the viruses exhibited residues typical of the vast majority (91.7%–100%) of European AIV. No amino acid changes associated with increased polymerase activity, virulence or transmission in mammals were present in the nucleoprotein. In the M protein, the viruses isolated in France since November 2015, as more than 99% of European AIV, harboured amino acids D30 and T215 associated with increased virulence for mice.

In the PB1-F2 of the 150169a H5N1 virus, the N66S substitution is observed as in HPAI H5N1 (Gs/Gd/1/96-like) and the 1918 pandemic virus. This substitution was not observed in the 150233 H5N2 or 150236 H5N9 viruses, the latter apparently exhibiting a truncated PB1-F2 ORF with a premature stop codon at position 26.

The NS1 protein is a major antagonist of the antiviral host responses. Among substitutions in NS1 associated with enhanced interferon antagonistic activity or that contribute to increased virulence in mammals, the P42S, E92D, L103F and I106M, and N205S mutations were present in the NS1 of these three French HP viruses as in the majority (›65%) of European AIV. Furthermore, the ESEV PDZ-ligand domain, identified as an important virulence determinant, was present at the C-terminus of NS1 of the three French H5 viruses, as for more than 80% of European AIV.

Based on known substitutions in the M2 or the N1 that confer resistance or reduced susceptibility to antivirals, it could be considered that the French viruses are sensitive to both M2-blockers (amantadine, rimantadine) and neuraminidase inhibitors (zanamivir, oseltamivir, peramivir).

Discussion

The unusual cleavage site corresponding to HP viruses observed in the November/December 2015 AIV circulation episode indicated that the acquisition of multiple basic residues did not occur by insertions as observed in the H5 HA from Gs/Gd/96-like viruses, but rather by substitution. Between positions 1009 to 1035 of the H5 encoding region, at least five nt substitutions were observed between the new French H5 HP and the closest H5 sequences. In addition, the full genome sequencing by NGS of the three French H5 HP virus isolates confirmed the presence of three different NA subtypes. The existence of reassortment events can be directly inferred from the finding that three different neuraminidases (N1, N2 and N9) were associated with very similar and original H5 HP sequences. This result seems to be confirmed by the phylogenetic analyses of the internal genes. Indeed, the internal genes of the three H5 HP viruses were not always directly closely related (higher percent identities in Table 1, as compared with Table 2). This demonstrates that the three viruses were not simply derived from a single H5HP ancestor through reassortment events leading to the acquisition of three different neuraminidases. More detailed analyses of the reassortment events will be made when other full genomes are sequenced, including non HP influenza viruses detected in the same area during the virological surveillance of the epizootic, which could have acted as partners in the reassortment events. Unfortunately, this research has been postponed due to the need to investigate the subsequent 2016/17 AIV circulation episode (due to H5N8 HPAI); in spite of intense virological surveillance, this investigation did not detect circulation of the 2015/16 H5 HPAI viruses.

Overall, based on the analysis of the sequences, only few residues that may increase transmission, replication and/or virulence in mammalian hosts were detected in the viruses analysed here. These residues were shared by the majority of other contemporary AIVs. To what extent the observed substitutions in the HA (N94, N159, A160, P234 and poly basic cleavage site) could contribute to the ability to bind the human SAα2,6Gal receptors in addition to avian SAα2,3Gal receptors needs to be evaluated in receptor-binding assays [33]. Such feature could potentially contribute to the ability of the virus to bind to both the upper and lower respiratory tract in humans. However, as for other European AIV including HPAI viruses, the polymerase complex proteins, the NP and the M1 of these viruses lack the major features associated with increased efficiency of replication in mammals. This does not preclude the possibility that under particular circumstances e.g. massive exposure or individual genetic susceptibility, infection in humans might occur

and result in severe infections. Indeed, these viruses exhibit a multibasic cleavage site in their HA, that provides potential for systemic spread, and determinants in PB1-F2 and NS1 associated with an increased virulence in mammals and/or with the ability to antagonise the antiviral host response more efficiently.

However, the viruses did not exhibit the combinations of mutations found to be required for respiratory droplet transmission in ferrets [32,34,35]. These include (i) mutations in the HA that allow H5 HA binding to the SAα2,6Gal receptor as observed in human influenza viruses (N224K, Q226L, G228S); (ii) mutations resulting in the loss of a glycosylation site at position 158–160 in the HA that favours binding to the human SAα2,6Gal receptors; (iii) mutations at the HA trimer interface (H110Y; T318I) that increase the stability of the HA and result in a reduction of the optimal pH at which the conformational change required for fusion occurs; (iv) mutations in PB2 (E627K and D701N substitutions were considered as major determinants of adaptation to mammals [35,36]) and PB1 (H99Y) that ensure efficient viral replication in mammalian cells.

Hence, even in the very unlikely event of human infection with the 2015/16 H5 HPAIV, further human-to-human transmission is not anticipated and the pandemic potential of these viruses can be considered to be negligible.

Conflict of interest

None declared.

Authors' contributions

KO, ALP, CGC, CG, CA, MOLB, MCP, EL, CC: Samples processing, RT-PCR, pathotyping, egg isolation at NRL.

FXB, AS, EN, AF, NE: Design and supervision.

EH, HQ, FT, YB: NGS sequencing and analyses.

FXB: Phylogenetic analysis.

HG, PD: Screening samples (local veterinary diagnosis laboratory).

EN, SVDW, YB, VJ, FXB, NE: Manuscript writing.

PM: Table and manuscript revision.

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Research article

Multi-laboratory validation study of multilocus variable-number tandem repeat analysis (MLVA) for *Salmonella enterica* serovar Enteritidis, 2015

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Multilocus variable-number tandem repeat analysis (MLVA) is a rapid and reproducible typing method that is an important tool for investigation, as well as detection, of national and multinational outbreaks of a range of food-borne pathogens. *Salmonella enterica* serovar Enteritidis is the most common *Salmonella* serovar associated with human salmonellosis in the European Union/European Economic Area and North America. Fourteen laboratories from 13 countries in Europe and North America participated in a validation study for MLVA of *S.* Enteritidis targeting five loci. Following normalisation of fragment sizes using a set of reference strains, a blinded set of 24 strains with known allele sizes was analysed by each participant. The *S.* Enteritidis 5-loci MLVA protocol was shown to produce internationally comparable results as more than 90% of the participants reported less than 5% discrepant MLVA profiles. All 14 participating laboratories performed well, even those where experience with this typing method was limited. The raw fragment length data were consistent throughout, and the inter-laboratory validation helped to standardise the conversion of raw data to repeat numbers with at least two countries updating their internal procedures. However, differences in assigned MLVA profiles remain between well-established protocols and should be taken into account when exchanging data.

Introduction

The global public health impact of non-typhoidal salmonellosis is high, with an estimated 93.8 million illnesses, of which 80.3 million are estimated to be food-borne [1].

The ability to rapidly identify the primary sources of bacterial contamination using genetic subtyping is critical in the investigation of food-borne infections. If common outbreak sources can be determined in a timely fashion, further *Salmonella* infections can be prevented.

Multilocus variable-number tandem repeat (VNTR) analysis (MLVA) is a rapid, inexpensive and reproducible high-resolution typing method that has become an increasingly popular tool for the investigation, as well as detection, of national and multinational outbreaks of a range of foodborne pathogens [2-6].The method is based on multiplex PCR amplification of repetitive DNA elements organised in tandem within the genome (tandem repeats), followed by concurrent fragment size analysis of the resulting amplified polymorphic

Table 1

Reference strains for MLVA of *Salmonella enterica* serovar Enteritidis (adapted from Hopkins et al. [12])

Calibration strain	SENTR7 (SE9, STTR9)		SENTR5 (SE5, STTR5)		SENTR6 (SE2, ENTR20)		SENTR4 (SE1, ENTR13)		SE-3	
	Length in bp[a]	Number of TRs	Length in bp[a]	Number of TRs	Length in bp[a]	Number of TRs	Length in bp[a]	Number of TRs	Length in bp[a]	Number of TRs
HPA001	135	3	265	9	173	4	119	4	308	1
HPA002	135	3	301	15	180	5	119	4	320	2
HPA003	126	2	277	11	180	5	112	3	320	2
HPA004	135	3	289	13	180	5	119	4	309	1
HPA005	135	3	271	10	187	6	119	4	309	1
HPA006	117	1	265	9	194	7	112	3	320	2
HPA007	126	2	295	14	208	9	112	3	320	2
HPA008	126	2	277	11	215	10	112	3	320	2
HPA009	126	2	233	12	229	12	112	3	320	2
HPA010	126	2	235	4	208	9	126	5	0	NA
HPA011	126	2	247	6	187	6	126	5	308	1
HPA012	126	2	253	7	229	12	133	6	308	1
HPA013	126	2	259	8	201	8	126	5	320	2
HPA014	126	2	271	10	236	13	126	5	308	1
HPA015	126	2	301	15	201	8	140	7	0	NA
HPA016	126	2	253	7[b]	194	7	147	8	0	NA

bp: base pairs; MLVA: multilocus variable-number tandem repeat analysis; NA: no amplification at this locus; TR: tandem repeat.

[a] Length of fragment as determined by sequencing, which may differ from the size determined by capillary electrophoresis.

[b] Sequence of first three TRs is GACCAC-GACCAC-GGCCAT.

regions. The latter are detected using capillary electrophoresis (CE) where an internal size standard is included for each sample. *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) MLVA, using five loci known to demonstrate inter-strain variability, has previously been validated successfully during inter-laboratory comparisons [7,8]. The resulting protocol [9] is used by countries in the European Union (EU) and European Economic Area (EEA) that report molecular data to The European Surveillance System (TESSy) [10].

However, *Salmonella enterica* serovar Enteritidis (*S.* Enteritidis) remains the most commonly reported serovar within the EU/EEA. In 2013, it was responsible for 39.5% of *Salmonella* infections in humans, followed by *S.* Typhimurium (20.2%) [11]. Due to the lack of genetic variation within the serovar Enteritidis population, previous molecular methods, such as pulsed-field gel electrophoresis (PFGE), lack the necessary discrimination for informing outbreak investigations. Thus the utility of MLVA has come to the fore.

A nine loci MLVA scheme for this serovar was originally developed by Hopkins et al. in 2011 [12], and has found widespread popularity within the EU. The Hopkins publication concluded that selecting fewer loci could also provide adequate discrimination, and exclusion of loci that showed minimal diversity left five specific loci remaining, all with relatively short repeats at 6–12 base pairs (bp). The Hopkins' scheme nomenclature follows the same basic tenet as for *S.* Typhimurium MLVA [13] i.e. it is based on the actual number of repeats in each locus and the MLVA profile is described as a string of five numbers.

The publication of the Hopkins protocol triggered the independent development of many different protocols for *S.* Enteritidis MLVA by individual laboratories. The production of comparable data between laboratories is crucial for the usefulness of typing for foodborne pathogens, thus, there was a need to harmonise the current MLVA methodologies for *S.* Enteritidis and reach consensus with regard to nomenclature, comparability and meaningful interpretation of data.

Using recommendations provided by previous MLVA harmonisation studies [7,14], the objective of the present study was to test whether comparable *S.* Enteritidis MLVA results could also be obtained between different laboratories, often using different equipment. Study participants were provided with a suggested MLVA protocol but were not obliged to use this and could follow any in-house protocols that already existed within their laboratory. However, they were all asked to analyse the same five loci, in the same order and report the number of tandem repeats found at each locus.

Methods

This international, inter-laboratory comparison of MLVA results was largely based upon the recommendations of Nadon et al. [14] for intra- and inter-laboratory validation of MLVA schemes and was carried out using a set of calibration strains to redress any laboratory or equipment set-up-dependent discrepancies between sequenced and measured fragment lengths. Following the initial set-up and normalisation of fragment sizes for the calibration set, 14 laboratories from 13 different countries participated in an inter-laboratory validation of MLVA for *S.* Enteritidis using a blinded set of 24 strains with known allele sizes.

Participants

Fourteen laboratories (A–N), 12 from EU/EEA countries and two from North America (Canada and the United States (US)), participated in the validation, most using a scheme routinely used in their own laboratory for *S.* Enteritidis MLVA. Although largely a European initiative, it was important to ensure global comparability of typing results and therefore participants from Canada and the US were invited to take part in this study. The participants comprised 13 national public health laboratories and one national public health and food safety laboratory. Participants' experience in *S.* Enteritidis MLVA varied from having only recently set up the method to having performed extensive validations of the method over the years.

Bacterial isolates

Using differing CE platforms and chemistries is known to yield different fragment sizes which in turn may affect the interpretation of the correct number of tandem repeats as determined by sequencing. To overcome this, each laboratory was firstly required to calibrate their own equipment using a set of 16 reference strains with sequenced alleles [12]. Strains were selected from Public Health England's (PHE) collection of isolates to provide a good coverage of the range of alleles known to exist at each locus. The five loci chosen were SENTR4, SENTR5, SENTR6, SENTR7 and SE-3 [15]; alternate names [16,17], bp lengths and number of tandem repeats are shown in Table 1. These *S.* Enteritidis strains enabled laboratories to normalise their raw fragment data to actual fragment sizes.

A further set of 21 isolates were chosen as a blinded validation set from ca 2,000 *S.* Enteritidis previously MLVA-typed at PHE (Table 2). The MLVA profiles for these are stored within a BioNumerics database at PHE and the validation set was selected to represent a wide range of the known allelic diversity at each of the five loci. Three of the isolates were included in duplicate to test the reproducibility and repeatability of the method making a total of 24 blinded isolates (ECDC1-ECDC24).

MLVA was performed by each of the participants largely using their own protocols adapted from previously published assays [12,18,19]. All countries used a single multiplex PCR except three countries that used two separate multiplex PCR, two of which used the PulseNet protocol [18,19] and one an in-house protocol targeting five loci. Annealing temperatures ranged from 55 °C to 60 °C and were individually optimised

Table 2

Validation strain panel for the five-locus *Salmonella enterica* serovar Enteritidis multilocus variable-number tandem repeat analysis

Validation strain[a]	MLVA		
	Fragment sizes[b]	Profile (TRs)	Number of laboratories identifying incorrectly (incorrectly identified locus)
ecdc_1	131–297–176–118–317	3–15–5-4–2	0
ecdc_2	122–273–176–111–318	2–11–5-3–2	0
ecdc_3	131–285–176–118–305	3–13–5-4–1	Strain excluded
ecdc_4	131–267–183–118–305	3–10–6-4–1	1 (SENTR4)
ecdc_5	113–261–190–111–317	1–9-7–3-2	0
ecdc_6	122–291–204–111–317	2–14–9-3–2	0
ecdc_7	122–273–211–111–317	2–11–10–3-2	0
ecdc_8	122–231–204–125–0	2–4-9–5-NA	2 (SENTR5, SE-3)
ecdc_9	122–243–183–125–305	2–6-6–5-1	0
ecdc_10	122–249–226–133–305	2–7-12–6-1	1 (SENTR5)
ecdc_11	121–291–190–111–318	2–14–7-3–2	0
ecdc_12	121–260–196–117–317	2–9-8–4-2	0
ecdc_13	121–267–183–111–318	2–10–6-3–2	0
ecdc_14	131–255–176–118–305	3–8-5–4-1	2 (SENTR7, SENTR5)
ecdc_15	130–279–169–118–305	3–12–4-4–1	0
ecdc_16	112–273–190–111–317	1–11–7-3–2	0
ecdc_17	121–267–197–124–317	2–10–8-5–2	0
ecdc_18	121–297–203–110–317	2–15–9-3–2	0
ecdc_19	130–237–176–111–305	3–5-5–3-1	0
ecdc_20	122–279–161–111–317	2–12–3-3–2	0
ecdc_21	131–273–175–118–305	3–11–5-4–1	Strain excluded
ecdc_22	112–273–190–111–317	1–11–7-3–2	0
ecdc_23	121–267–197–124–317	2–10–8-5–2	1 (SENTR4)
ecdc_24	130–237–176–111–305	3–5-5–3-1	0

MLVA: multilocus variable-number tandem repeat analysis; NA: no amplification at this locus; TR: tandem repeat.

[a] MLVA target alleles were sequenced for validation strains 1–10 and 20.

[b] Length of fragment as determined by capillary electrophoresis at Public Health England using ABI 3730 DNA Analyzer (Applied Biosystems, Foster City, California, US), order of alleles SENTR7; SENTR5; SENTR6; SENTR4; SE-3.

Three samples were duplicated and therefore have identical profiles: ecdc_16 and ecdc_22; ecdc_17 and ecdc_23: ecdc_19 and ecdc_24.

for each laboratory. Primer concentrations were also individually optimised as per the recommendation of Nadon et al. [14].

Twelve of 14 laboratories used Applied Biosystems Genetic Analyzer (ABI) platforms (Foster City, California, US) for CE, one laboratory used the Beckman Coulter platform (Brea, California, US) and the remaining laboratory used both systems.

Allele assignment

For the validation set, at the five respective loci in the order SENTR7; SENTR5; SENTR6; SENTR4; SE-3, each laboratory was requested to report the number of tandem repeats found and the fragment sizes used to determine them. Where no predominant peak was present at a locus, this was considered to be a null allele and reported as NA (no amplification at this locus).

Participants were free to use any local method for calculation of the number of repeat units from their obtained fragment sizes. A number of laboratories used a compensation matrix in Excel format originally developed for MLVA of *S.* Typhimurium by Larsson et al. [9] while others adopted the use of binned datasets with an expected range of fragment sizes suggested for each set of tandem repeats. The latter approach relied upon look-up tables with the allele size range being well-characterised for each of the five loci.

Comparability analysis

The inter-laboratory comparability of the *S.* Enteritidis MLVA method was considered as adequate if more than 80% of the participating laboratories reported less than 5% discrepant MLVA type assignment for the blinded set of validation strains [14].

Table 3

Capillary electrophoresis platforms, size markers, dye sets and proportion of loci reported as expected in the *Salmonella* Enteritidis MLVA inter-laboratory validation study, Europe[a], 2015 (n = 14 participating laboratories)

Laboratory	Size marker	Dye set	Capillary electrophoresis	MLVA score[b] (%)
A	GeneScan 600 LIZ	ABI G5	ABI 3130	100.0
B	GeneScan 600 LIZ	ABI G5	ABI 3130xl	100.0
C	GeneFlo 625 ROX	ABI D	ABI 3730xl	100.0
D	GeneFlo 625 ROX	ABI D	ABI 3130xl	99.1
E	GeneScan 1200 LIZ	ABI G5	ABI 3130xl	100.0
F	GeneScan 600 LIZ	ABI G5	ABI 3730xl	100.0
G	GeneFlo 625 ROX	ABI D	ABI 3730	99.1
H	GeneScan 600 LIZ	ABI G5	ABI 3500	98.2
I	CEQ DNA Size Standard Kit600	D2, D3, D4	Beckman Coulter GeXP	99.1
J	GeneFlo 625 ROX	ABI D	ABI 3130XL	100.0
K	Roche LIZ1200	Unknown	ABI 3730	99.1
L	GeneScan 600 LIZ	ABI G5	ABI 3130	100.0
M	GeneScan 600 LIZ	ABI G5	ABI 3500xL	100.0
N	CEQ 600-bp DNA size standard	D2, D3, D4	Beckman Coulter GeXP	100.0
N	GeneFlo 625 ROX	ABI D	ABI 3500	99.1

MLVA: multilocus variable-number tandem repeat analysis.

[a] Fourteen laboratories from 11 European Union and European Economic Area countries and two laboratories from North America (Canada and the United States).

[b] Percentage of loci correctly assigned out of a total of 110.

Results

Of the 14 participating laboratories, eight reported expected profiles for all 22 validation strains and their 110 loci (Tables 2 and 3). Five reported expected profiles for 21 out of 22 validation strains and 109 of their 110 loci, although one of these laboratories reported all loci as expected when using another sequencing platform. One laboratory reported expected profiles for 20 out of 22 validation strains and 108 of their 110 loci.

Two validation strains were excluded from the result analysis. Eight laboratories reported double peaks or finding two distinct MLVA profiles for ECDC3, and four laboratories reported more than one allele at the second locus, SENTR5, for ECDC21. As so many participants reported issues with these two strains, it is probable that they contained a mixed population. Those laboratories with greater experience of the MLVA process were still able to ascertain the correct profiles for these strains following purification and analysis of multiple colonies.

Sporadic deviations from the expected results in single loci were reported by six participants. For ECDC8, laboratory D reported one TR at the last locus, SE-3, while all other participants recorded the expected result of no amplification at this locus. For this same strain laboratory H recorded an additional two TRs at locus SENTR5; i.e. six TRs instead of the expected four. This was due to a conversion error in their results tables as the fragment size they recorded equated to four TRs and not six. Laboratory H also recorded an additional TR at locus SENTR5 for ECDC10. Again this would appear to be a conversion error as the correct fragment size for seven TRs was recorded. Laboratory K reported one less TR at locus SENTR7 for ECDC14, corresponding to a fragment size of 130.6 bp. For a fragment of this size, the result should have been recorded as three TRs and not two TRs so this was also likely a conversion error. Furthermore, Laboratory N reported six TRs instead of the expected eight TRs for SENTR5 locus of ECDC14. Laboratory G was the only participant to report a mixed population for ECDC4. For the two MLVA profiles they recorded for this strain, one profile equated to ECDC7 while the other profile was similar to that of ECDC4 apart from the loss of a TR repeat at locus SENTR4 i.e. three TRs instead of the expected four. Additionally, although Laboratory M reported what appeared to be a mixed population for ECDC11, they were still able to report the correct final MLVA profile.

Laboratory I initially reported difficulty using the calibration strains which resulted in a large number of erroneous results for all 24 validation strains. This was these participants' first experience at setting up a MLVA protocol for *S.* Enteritidis and they were one of the few laboratories using a Beckman Coulter platform. Following feedback about these problematic results, Laboratory I carried out further optimisation of their PCR and CE protocols before resubmitting their results. This new set of results corresponded much more accurately to the expected results for the validation strains. Apart from the previously mentioned problems for ECDC3 and ECDC21, Laboratory I were unable to correctly amplify

a fragment for SENTR4 of ECDC23. However, they did report this fragment correctly for ECDC17 which was the duplicate isolate of ECDC23.

Laboratory F initially reported consistently higher repeat numbers for SENTR7. However, these issues were resolved after adjusting the ranges for repeat number assignment using the calibration strain set.

Comparison to PulseNet protocol for *S.* Enteritidis MLVA

Importantly, during this validation study it was noted that there were differences between the five-loci MLVA protocol [12] and the PulseNet protocols [18,19] in two alleles: compared with PulseNet results, the five-loci protocol gave consistently one less repeat number for SENTR4, and two repeat numbers less for SE-3. However, the issue was purely related to the result analysis since the raw data (measured fragment lengths) gave consistent results if the result analysis i.e. assignment of TR numbers was changed (raw data from the reference and validation strains obtained with PulseNet protocol analysed using conversion tables for the five-loci protocol).

Discussion

The *S.* Enteritidis MLVA protocol targeting five loci was shown to produce internationally comparable results during the inter-laboratory validation study. More than 90% of the participating laboratories reported less than 5% discrepant MLVA profiles for the blinded set of validation strains. All 14 participating laboratories performed well, even those where experience was initially lacking in MLVA and fragment analysis technology. The most critical phase was the conversion of raw fragment length data to repeat numbers, an issue that the present inter-laboratory validation helped to standardise.

Following the proof-of-concept study published for *S.* Typhimurium MLVA [7], this study has likewise shown the efficacy of using calibration strains for MLVA of *S.* Enteritidis to minimise any differences in laboratory set-ups. While the general idea for multi-laboratory validation is not new [12,14,20], to our knowledge this is the first international, inter-laboratory study to verify the concept for this particular serovar.

Despite the wide variation in laboratory protocols, CE chemistries and level of experience in MLVA methods, all 14 participants demonstrated that they could correctly identify MLVA profiles with a minimum of 98% correct allele assignments for the validation strain set. Thirteen of the participants returned correct assignments for practically all of the 110 targeted alleles.

Even with the lack of a standardised data analysis system, all laboratories were able to obtain comparable results for virtually all of the loci tested within the validation set. Six laboratories reported sporadic deviations from the expected results in single loci. In one of these laboratories, the MLVA method for *S.* Enteritidis had only recently been set up. Had they gained more experience in this method and made more rigorous TR assignments, this laboratory would have also likely identified all 110 alleles correctly. Likewise, for the other five laboratories with sporadic deviations, the importance of critically assessing data for each individual locus in comparison to the results corresponding to the other TR numbers in the same locus is highlighted, e.g. where SENTR5 is known to comprise of a 6 bp TR and a fragment size of 237 bp represents five TRs, then a fragment size of 231 bp should logically represent one less repeat i.e. four TRs. The conversion errors might be due to human error when converting raw data into TR numbers, but likely the absence of consolidated procedures for this critical step also plays an important role. To avoid the possibility of human errors, automated processing of the raw data to repeat numbers via dedicated software can be helpful. In addition, regular External Quality Assessments (EQAs) for MLVA for *S.* Enteritidis should be set up at the EU/EEA level to ensure that data remain comparable and consistent.

Where other differences were noted, they only occurred as single locus variants rather than gross deviations from the expected MLVA profile. The initial discrepancy in MLVA allele assignment in Laboratory I was caused mainly by difficulties in optimising the PCR and the lack of any significant prior experience in fragment analysis with the Beckman Coulter platform.

Although it has been previously recorded that some VNTRs are not entirely stable [21,22], Bertrand et al. have shown that there were no variations over time for the five MLVA loci chosen for *S.* Enteritidis following numerous serial passages of the organism [23]. From this present study, although it would appear that the stability of the number of tandem repeats in the MLVA loci is not in question, it is also not entirely unexpected to occasionally find a single locus variant among a large set of alleles. Within the blinded panel of validation strains, three isolates were represented twice to test for reproducibility and repeatability of the method. All laboratories correctly identified the replicates apart from one laboratory that could not verify a fragment for SENTR4 of ECDC23. This may have been due to the previously mentioned challenges this laboratory experienced trying to establish the methodology in the absence of deep-rooted knowledge or workflows for their MLVA system.

Based on previous studies, the discriminatory power of MLVA for *S.* Enteritidis has limitations. Bertrand et al. [23] concluded that one single MLVA profile represented more than a quarter of 1,498 *S.* Enteritidis strains isolated during 2007–2012 in Belgium. The most common MLVA types can be further divided in subgroups using phage typing and PFGE [12,23,24]. This indicates that MLVA should not be relied upon as a single typing method but complementary methods should be used in parallel for prevalent MLVA types. Furthermore, since MLVA schemes for *Salmonella* are serovar-specific, the

method cannot fully replace PFGE. Subtyping methods based on next generation sequencing technologies show enormous potential. They have been shown to produce epidemiologically robust data also for *S.* Enteritidis with a superior discriminatory power compared with MLVA [24,25], but data standardisation and common nomenclature need to be agreed upon before the results can be used routinely for international comparisons [26,27]. Until then, MLVA could have a role in providing a common international strain nomenclature and providing an adequate typing method for laboratories that do not foresee moving to whole genome sequencing technology in the near future.

Even with the above-mentioned limitations, MLVA has already been shown to be a good candidate for performing *S.* Enteritidis surveillance at EU/EEA level [3], and it can only be beneficial to further this development to additional pathogens and on a global scale. Both PulseNet International and ECDC have already published suggested operating procedures for *S.* Typhimurium MLVA [9,28]. In addition, PulseNet International have also published MLVA protocols for *S.* Enteritidis [18,19] and verotoxigenic *Escherichia coli* O157 [29]. As discovered during our study, the five-loci MLVA protocol [12] and the PulseNet protocols for *S.* Enteritidis assign repeat numbers differently for loci SENTR4 and SE-3 although the raw data from the two protocols are consistent. This is due to the fact that PulseNet currently assigns alleles based on the calculated copy number, not the actual sequenced copy number. This should be remembered when exchanging data during international outbreak investigations to ensure a rapid, cooperative response, which is important for source tracing, particularly with the global food markets of today where cross-border action may be required [30].

Subtyping of *S.* Enteritidis is important for outbreak detection and timely provision of information for surveillance programmes such as TESSy and PulseNet International. The use of the nomenclature in this study is currently widely accepted within the EU/EEA as unambiguous when applied to MLVA of *S.* Enteritidis. As demonstrated by our study, even when multiple, only partially overlapping protocols are used in many different countries around the world, it is still possible to exchange data without rigid standardised methodology and equipment. To facilitate the set-up in laboratories with no experience in the method, the European Centre for Disease Prevention and Control (ECDC) has published a standardised protocol for *S.* Enteritidis MLVA [31]. *S.* Enteritidis MLVA data collection for EU/EEA countries has been started in TESSy in June 2016, enabling EU/EEA-wide analysis of *S.* Enteritidis MLVA data and multi-country cluster detection.

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Conflict of interest

None declared.

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Towards elimination of hepatitis B and C in European Union and European Economic Area countries: monitoring the World Health Organization's global health sector strategy core indicators and scaling up key interventions

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The World Health Organization 'Global Health Sector Strategy on Viral Hepatitis 2016–2021' aimed at the elimination of viral hepatitis as a public health threat provides a significant opportunity to increase efforts for tackling the epidemics of hepatitis B and hepatitis C virus infections across Europe. To support the implementation and monitoring of this strategy, core epidemiological and programmatic indicators have been proposed necessitating specific surveys, the systematic collection of programmatic data and the establishment of monitoring across the care pathway. European Union and European Economic Area countries already made progress in recent years implementing primary and secondary prevention measures. Indeed, harm reduction measures among people who inject drugs reach many of those who need them and most countries have a universal hepatitis B vaccination programme with high coverage above 95%. However, while a further scaling up of prevention interventions will impact on incidence of new infections, treating those already infected is necessary to achieve reductions in mortality. The epidemiological, demographic and socio-political situation in Europe is complex, and considerable diversity in the programmatic responses to the hepatitis epidemic exists. Comprehension of such issues alongside collaboration between key organisations and countries will underpin any chance of successfully eliminating hepatitis.

Background

It is estimated that ca 4.7 million people living in European Union (EU) and European Economic Area (EEA) countries are chronically infected with the hepatitis B virus (HBV) and 5.6 million have been infected with the hepatitis C virus (HCV). Both are major causes of chronic liver disease, liver cirrhosis and hepatocellular carcinoma [1]. The resulting burden of disease presents a public health challenge for national health systems. While the incidence of new infections has declined in many European countries due to implementation of effective vaccination programmes (against hepatitis B) and prevention strategies targeting transmission through injecting drug use and healthcare, modelling suggests that morbidity and mortality will continue to increase [2,3]. Indeed, deaths from hepatitis now exceed those from HIV and tuberculosis combined and latest published estimates show that 96,000 people die each year in EU/EEA countries from HBV and HCV-related liver disease [4].

In May 2016, the World Health Assembly adopted the first 'Global Health Sector Strategy (GHSS) on Viral Hepatitis' aimed at eliminating viral hepatitis as public health threat [5]. The concept of elimination for these infections is based on reducing the incidence of chronic infections and the associated mortality, with the World Health Organization (WHO) setting global targets for reducing the incidence of chronic infections by 90% and mortality by 65% by 2030. Achieving these targets will require significant scaling-up of key interventions, including hepatitis B childhood vaccination, birth-dose vaccination or other means to prevent mother-to-child transmission, improved systems to assure safe blood transfusions/blood products, injection safety, interventions aimed at preventing transmission among people who inject drugs, and increased testing with linkage to care and treatment.

Table

Core indicators for the World Health Organization's monitoring and evaluation framework for hepatitis B and hepatitis C virus elimination 2016–2021

Indicator number	Indicator name
C1	Prevalence of chronic HBV and HCV infection
C2	Infrastructure for HBV and HCV testing
C3	a. Coverage of timely hepatitis B vaccine birth dose (within 24 hours) and other interventions to prevent mother-to-child transmission of HBV b. Coverage of third-dose hepatitis B vaccine among infants
C4	Needle–syringe distribution
C5	Facility level injection safety
C6	People living with HCV and/or HBV diagnosed
C7	a. Treatment coverage for hepatitis B patients b. Treatment initiation for hepatitis C patients
C8	a. Viral suppression for chronic hepatitis B patients treated b. Cure for chronic hepatitis C patients treated
C9	a. Cumulated incidence of HBV infection in children 5 years of age b. Incidence of HCV infection
C10	Deaths from hepatocellular carcinoma, cirrhosis and liver diseases attributable to HBV and HCV infection

HBV: hepatitis B virus; HCV: hepatitis C virus.
Source: [6].

To support the implementation and monitoring of this strategy, a framework with 10 core indicators has been proposed by WHO, which include a mix of epidemiological and programmatic indicators (Table) [6].

The process and criteria for selecting the indicators are described in detail in the WHO technical report [6]. In this paper we provide an overview of the current situation across EU/EEA countries in the context of the global WHO indicators to highlight gaps in programmatic responses and challenges in achieving elimination in Europe.

The European situation

The WHO Regional Office for Europe (WHO/Europe), in consultation with the Member States and partner organisations, has developed an action plan to guide the implementation of the GHSS in the European Region [7]. This regional plan was launched following endorsement by the Regional Committee in September 2016 and provides the structural framework for countries to use when organising their responses. It includes regional targets, some of which are more ambitious than the global targets in recognition of already existing prevention and control efforts in the Region and the capacity of existing systems to further impact on the epidemics. The plan refers to the WHO monitoring and evaluation framework with 10 core indicators as a tool intended to facilitate the generation, collection and analysis of standardised data for the monitoring of the response on the national and Regional level (Table).

The European Centre for Disease Prevention and Control (ECDC) and the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA), both EU agencies, are well placed to provide technical support to assist EU/EEA countries develop tailored national plans for achieving the WHO targets. In 2016, the two agencies, in collaboration with WHO/Europe, assessed the availability of data for each of the core indicators and concluded that current data sources in most EU/EEA countries are insufficient, particularly for assessing the epidemiological burden and for monitoring the different steps along the cascade of care [8]. Further collaboration with the countries and clinical associations will be required to improve data sources. Regular seroprevalence surveys and sentinel-site surveys will be required to determine (i) estimates of prevalence and incidence, (ii) the attributable fraction of liver cirrhosis and hepatocellular carcinoma cases related to HBV and HCV infections and (iii) the size of the undiagnosed population [6]. The systematic collection of programmatic data related to testing and to prevention and treatment coverage will also need to be conducted.

While some EU/EEA countries have well-developed data systems providing comprehensive epidemiological information on hepatitis B and C to support local policy initiatives, there is variation between countries [9]. In an attempt to address such differences and standardise notification data, ECDC implemented in 2011 an enhanced surveillance system to facilitate the collection of data on newly diagnosed cases. Recognising the limitations of routine notification data to provide a clear epidemiological overview of the numbers and groups affected by infection, EMCDDA and ECDC have started work to collect and collate seroprevalence data from key risk groups and the general population using standardised methodologies and will publish this information when available.

From an epidemiological perspective, the prevalence of HBV and HCV is low-to-intermediate in most EU/EEA countries, but the situation is diverse and dynamic. National estimates of seroprevalence in the general population vary from 0.1% to 4.4% for HBV and from 0.1% to 5.9% for HCV [1]. Among key risk groups, prevalence estimates show similar variation. For the population of people who inject drugs (PWIDs) and former PWIDs in Europe, the prevalence of HCV is high, with 11 of the 16 countries with recent data reporting national estimates of over 40% [1]. Harm reduction programmes, especially those combining needle and syringe programmes (NSP) and opioid substitution treatment of people who inject opioids, as well as more recently, treatment with the new direct-acting antiviral drugs, may have the potential to contribute considerably to reducing transmission in many countries. In spite of this, prevalence rates found in national and subnational seroprevalence studies among PWID in most EU/EEA countries are high (›50%) [10], including among young and new injectors [11]. Reports suggest that only a small proportion of those infected with HBV or HCV are aware of their infection [2,12]. Among PWIDs, the proportion of those undiagnosed for HCV is likely to be very high, with estimates ranging from 24% to 76% [13]. This highlights a clear need to extend existing testing programmes.

Migrants, defined as individuals born outside their country of residence, contribute to the HBV and HCV prevalence pool. A recent analysis estimated that 1 to 2 million chronically HBV-infected migrants from endemic countries with a prevalence of over 2%, reside in the EU/EEA and account for 25% of all chronic HBV cases [14]. For HCV, estimates indicate that chronic infections among migrants account for 14% of all chronic infections [14].

Men who have sex with men (MSM) are a key risk group for current HBV and HCV transmission in most European countries. Vaccination has reduced HBV transmission, however, there have been increasing reports from European countries of acute HCV infections among HIV-infected MSM [15]. Reports of HCV infections among HIV-negative MSM have raised concern that HCV is an expanding epidemic among MSM [1].

Despite the emerging trends described above and high levels of infection among key risk groups, the incidence of HBV and HCV has declined slightly across Europe in recent years [2,12]. For HBV, this is demonstrated by the surveillance data reported to ECDC which have shown a steady decline in the rates of acute infections across EU/EEA countries, with rates in most countries now less than 1 case per 100,000 [16]. However, there remains considerable diversity between countries with notification rates for acute HBV cases in 2014 ranging from 0 cases in Malta to 3.2 per 100,000 in Bulgaria. While chronic viral hepatitis is known to be one of the leading causes of end-stage liver disease, estimation of the proportion of deaths from liver cirrhosis and hepatocellular carcinoma attributable to HBV and HCV infection is difficult due to scarcity of data [17].

Data on hepatitis B vaccination coverage are routinely collected by WHO and the United Nations Children's Fund (UNICEF) through Joint Reporting Form on Immunization [18]. Twenty-three of the 31 EU/EEA countries reported data on coverage with three doses of HBV vaccine among 1-year-olds in 2014. Of these 23 countries, 11 reported coverage of 95% or over [18]. EU/EEA countries offer the first dose at birth either as a general recommendation to all newborns (7/31) or targeted to newborns from mothers from groups at risk or mothers with HBV infection (24/31) [19].

In relation to the indicator on injection safety, there is no systematic data collection of facility level injection safety in EU/EEA countries, but evidence from the notification data submitted to ECDC indicates that nosocomial transmission remains an ongoing transmission route for both infections in some countries [16,20].

Data on the levels of testing and treatment in EU/EEA countries are currently not systematically collected at the EU/EEA level or even nationally in most countries, but the available published evidence of ad hoc reviews suggests that provision is suboptimal in many countries, with high numbers of infections undiagnosed and only a small proportion of those who have been diagnosed effectively treated [13,21].

Programmatic data relating to prevention programmes for HBV and HCV across EU/EEA countries, although incomplete, show similar levels of diversity. The data collected by EMCDDA on harm reduction measures targeting injecting drug users show considerable variation across the region with suboptimal levels in many countries. Indeed, while the data indicate that one in two problem opioid users in Europe receive opioid substitution treatment (OST), in some countries the fraction of high-risk opioid users receiving OST is less than 20% [10]. In 14 countries providing recent estimates of the size of the PWID population, the number of syringes distributed per year from specialised NSPs remains below 50 syringes per injector in three countries and only four countries were able to document coverage above the recommended threshold of 200 syringes/PWID/year [10].

In addition to current gaps in prevention programmes and the available data required to monitor the implementation of these programmes, EU/EEA countries face other challenges to the successful elimination of hepatitis B and C. While recent data indicate that injecting drug use is stable or declining in Europe, the prevalence of injecting drug use ranges between 1 and 9 cases per 1,000 population aged 15-64 years and is high (› 4 /1,000) in five countries [22]. Furthermore, a potentially large population of HCV infected ex-injectors might need to be included in future healthcare estimates [11].

The population of migrants coming from countries with high endemicity for HBV and HCV is dynamic and recent studies indicate that estimates of prevalence from the country of origin may not be a good proxy for prevalence in all migrant groups. The prevalence in migrant populations has been found to be lower, especially for hepatitis B, so the true extent of the burden among different migrant groups is unclear [14].

Interventions are further hampered as stigma and discrimination surround hepatitis B and C, migrants, MSM and injecting drug use. In some parts of eastern Europe, repression is the prevailing response to drug use, while across most of the EU, a balanced approach with public health and criminal justice elements is now common [23-25]. Indeed, stigma and discrimination are barriers to testing and treatment access among PWID. Stigma around hepatitis B infection has been shown to impact negatively on testing behaviour of some migrant groups [26].

The EU/EEA is mostly comprised of high income countries. However, resources dedicated to the prevention and control of hepatitis have been described as suboptimal [21] and in striving towards elimination and the necessary scaling up of services, this will need to be addressed. The current cost of antiviral drugs for curing hepatitis C remains high and this could undermine national efforts in impacting upon the growing disease burden. Indeed, while prevention measures are able to impact on the incidence of new infections [13,16], it is only through identifying and treating those already infected that a reduction in mortality will be possible. EU mechanisms such as the joint procurement of medical countermeasures [27] could be one option for countries to consider, to help reduce the costs of antiviral treatment, while continued advocacy by non-governmental organisations remains important. WHO has developed several tools to assist countries in their prevention and control efforts including global testing and treatment guidance and national planning toolkits [28-30]. ECDC and EMCDDA provide complementary tools, such as specific evidence-based recommendations for action, tailored to the EU context, and both agencies will continue to work in close collaboration with WHO to support countries in their efforts to scale up activities.

Further development of existing monitoring platforms and working to minimise the reporting burden for countries is important and prevention and control efforts for hepatitis could benefit from understanding some of the lessons learnt in relation to HIV in this area. Indeed, developing a standardised monitoring approach for interventions including diagnosis and treatment along the continuum of care, which is already established for HIV, could now be considered for hepatitis B and C. A recent review of operational interventions along the chronic viral hepatitis care continuum for people with diagnosed or undiagnosed chronic viral hepatitis demonstrated that a range of relatively simple, inexpensive operational interventions can substantially improve engagement and retention along the cascade of care, thereby optimising the implementation of screening, care, and treatment programmes [31].

Conclusions

The launch of a global strategy aimed at the elimination of viral hepatitis provides an opportunity to increase efforts aimed at tackling the HBV and HCV epidemics. European countries have already made progress in recent years implementing primary and secondary prevention measures. Indeed, measures aimed at reducing health-related harm among PWIDs, such as OST and NSP, now reach many of those who need them and most countries have in place a hepatitis B vaccination programme with high levels of coverage. These measures have had an impact on the epidemiology of HBV and HCV. However, the epidemiological, demographic and socio-political situation is complex in Europe and diversity and inequities in the programmatic responses to the epidemics exist. Stigma and discrimination are both important in Europe in relation to hepatitis B and C and efforts to reducing or eliminating stigma are essential if disease elimination is to be achieved. Comprehension of such issues alongside collaboration between key organisations and countries will underpin any chance of successfully eliminating hepatitis.

Conflict of interest

None declared.

Authors' contributions

All authors contributed to the paper. EFD conceived the idea for the paper, led its coordination and prepared the first draft of the article. DH, OM and AM reviewed and revised the draft. All authors read and approved the final manuscript.

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Letter to the editor: Increasing proportion of new HIV diagnoses in Ireland previously diagnosed elsewhere – potential impact on estimating incidence

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To the editor: In a recent issue of *Eurosurveillance*, Pharris et al. presented a very interesting study which estimated the HIV incidence and number of undiagnosed people living with HIV in the European Union/European Economic Area (EU/EEA) [1]. The 'Incidence Method' used was a CD4 cell count-based back-calculation method [2].

The use of CD4 counts raises important issues for HIV surveillance and interpretation of findings. The Irish surveillance system, for example, includes all persons newly diagnosed with HIV in Ireland, even if previously diagnosed elsewhere. An increasing proportion of newly-diagnosed HIV cases have previously been diagnosed HIV-positive in another country before arrival in Ireland, and have been receiving antiretroviral therapy (ART). In 2015, 27% (129/485) of newly-diagnosed HIV cases had a previous HIV diagnosis in another country, up from a range of 14%–18% for the years 2011 to 2014 [3]. By risk group, heterosexuals were the group with the highest proportion previously diagnosed HIV-positive (35%, 45/130), followed by men who have sex with men (MSM) (29%, 72/247) and people who inject drugs (PWID) (10%, 5/49). The majority (79%, 102/129) of those with a previous HIV diagnosis, transferred their HIV care to Ireland and 63% (81/129) had been receiving ART before arrival in Ireland.

We found that when looking at stage of infection, as per CD4 cell counts at diagnosis, the contribution of people who were previously diagnosed HIV-positive and on treatment can make a considerable difference to the findings. Of those newly diagnosed with HIV in Ireland in 2015, 45% (n=161) were late presenters (CD4 cell count at diagnosis less than 350 cells/µl or an AIDS defining illness at diagnosis), where information on CD4 count or AIDS defining illness at diagnosis was available (74%, 357/485). However, confining the analysis to those who were not reported to have a previous diagnosis abroad, 52% (126/243) presented late. Understandably, the proportion of people presenting late among those who had a previous HIV diagnosis was much lower (31%, 35/114). Consequently, the use of CD4 count data to estimate HIV incidence should be carefully considered in countries where surveillance data includes a significant proportion of people who have been previously diagnosed with HIV in another country, who have previously received ART, and who have transferred their care to Ireland.

Another issue for consideration is the best way to present HIV surveillance data to reflect the increasing proportion of people with prior HIV infection. This will be important in order to accurately measure the impact of HIV prevention strategies within countries and at European level. Due to increased access to testing and treatment worldwide and the mobility of populations in general, this number is likely to remain high. In our opinion this should be an important consideration for surveillance of HIV at both national and European levels.

Conflict of interest

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Authors' contributions

Both authors composed the letter.

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