Following human infections with novel avian influenza A(H7N9) viruses in China, the European Centre for Disease Prevention and Control, the World Health Organization (WHO) Regional Office for Europe and the European Reference Laboratory Network for Human Influenza (ERLI-Net) rapidly posted relevant information, including real-time RT-PCR protocols. An influenza RNA sequence-based computational assessment of detection capabilities for this virus was conducted in 32 national influenza reference laboratories in 29 countries, mostly WHO National Influenza Centres participating in the WHO Global Influenza Surveillance and Response System (GISRS). Twenty-seven countries considered their generic influenza A virus detection assay to be appropriate for the novel A(H7N9) viruses. Twenty-two countries reported having containment facilities suitable for its isolation and propagation. Laboratories in 27 countries had applied specific H7 real-time RT-PCR assays and 20 countries had N9 assays in place. Positive control virus RNA was provided by the WHO Collaborating Centre in London to 34 laboratories in 22 countries to allow evaluation of their assays. Performance of the generic influenza A virus detection assay and H7 and N9 subtyping assays was good in 24 laboratories in 19 countries. The survey showed that ERLI-Net laboratories had rapidly developed and verified good capability to detect the novel A(H7N9) influenza viruses.

Introduction

On 31 March 2013, Chinese authorities announced the identification of a novel reassortant A(H7N9) influenza virus isolated from three unlinked fatal cases of severe respiratory disease in eastern China. A few small clusters had been detected but no sustained human-to-human transmission had been observed [1]. The Chinese Center for Disease Control and Prevention (CCDC) subtyped and sequenced the novel viruses and showed them to be low-pathogenic viruses of avian origin [2]. This is the first time that human infection with avian influenza A(H7N9) virus and human deaths due to a low-pathogenicity avian influenza virus have been identified [3]. As of 24 January 2014, 225 laboratory-confirmed human cases including 55 deaths had been reported from eight neighbouring provinces, two municipalities, the Hong Kong Special Administrative Region and Taiwan [4].

Detailed genetic sequence data from human, avian and environmental specimens and isolates of the novel avian influenza A(H7N9) viruses have been made available through the Global Initiative on Sharing All Influenza Data (GISAID) EpiFlu database and the International Nucleotide Sequence Database Collaboration (INSDC). These data suggest that multiple reassortment events have taken place, potentially involving wild birds [2,5-7]. The six RNA segments encoding the internal proteins of the outbreak virus are closely related to avian A(H9N2) viruses recently isolated from poultry in China, while, the segment encoding haemagglutinin (HA) belongs to the Eurasian A(H7) avian influenza virus lineage, and the segment for neuraminidase (NA) is most similar to those present in avian A(H11N9) and A(H7N9) viruses [2,5-7]. However, the nearest matches found for HA and NA are considerably less related than the nearest matches found for the six RNA segments encoding the internal proteins. This distinguishes the outbreak viruses from previously isolated avian influenza A(H7N9) viruses, including those reported in birds in Europe. The sequence diversity observed between different isolates of the novel influenza A(H7N9) virus, compared with historical data, suggests circulation of
the virus in birds before recent multiple introductions to humans [8]. The reservoir for this novel infection remains unknown, but the virus has been detected in domestic birds at live markets in eastern China [9].

It is recognised that real-time RT-PCR assays are at the forefront of influenza virus detection, with generic assays based on the matrix (M) gene for identification of influenza A, and specific assays for the HA and NA genes for identification of the different subtypes [10]. According to a survey conducted in July 2011, the majority of European national influenza reference laboratories (31 laboratories in 25 countries) are using generic RT-PCR tests based on the influenza A virus M gene [11], which have the potential to detect also the novel A(H7N9) viruses.

To assist European laboratories in verifying and ensuring their diagnostic capability to detect and identify the novel avian influenza A(H7N9) viruses, the
European Centre for Disease Prevention and Control (ECDC), jointly with the World Health Organization (WHO) Collaborating Centre for Reference and Research on Influenza (WHO CC) in London, other members of the European Reference Laboratory Network for Human Influenza (ERLI-Net) and the WHO Regional Office for Europe (WHO/Europe), has released a technical briefing note on diagnostic preparedness in Europe for detection of the novel avian influenza A(H7N9) viruses [12]. The briefing note provides a list of considerations to ensure European-wide diagnostic capability, an update on currently available methods used for molecular detection of human infection with the novel avian influenza A(H7N9) virus by real-time RT-PCR, a table of validation criteria for A(H7) HA molecular assays, and information on positive controls for RT-PCR assays.

To complement the technical briefing note, we conducted a questionnaire-based survey with the objective of assessing the capability (but not assessing the detection capacity in terms of numbers over time) of EU/EEA countries to detect and subtype the novel avian influenza A(H7N9) viruses, given the possibility of their spread to Europe. Subsequently, all influenza reference laboratories were offered positive control material by WHO CC London to verify and to report on the experimental sensitivity of their RT-PCR assays.

### Methods

The survey questionnaire was developed jointly by ECDC, the ERLI-Net coordination team and WHO/Europe upon request of the European Commission. It was distributed to all ECDC influenza surveillance laboratory contact points (ERLI-Net laboratories) in May 2013. The questionnaire asked questions related to the current capability of the countries to detect the novel A(H7N9) viruses and their prediction, based on influenza RNA sequence analysis, regarding the need to update their detection primer sets. It also included questions asking for details of the primer sets in use and for the countries’ capability to isolate and propagate influenza A(H7N9) viruses.

### Results

#### Sequence-based analysis questionnaire

Thirty-two of 36 laboratories in 29 Member States of the European Union and European Economic Area (EU/EEA) responded to the questionnaire within a month (Table 1).

Thirty laboratories in 27 countries predicted that their M gene-based generic detection assay for influenza A virus would also detect the novel A(H7N9) viruses (Table 2). One laboratory considered their generic influenza A RT-PCR detection assay inappropriate for detecting the novel virus. One laboratory indicated use of a commercial influenza A detection assay, but not

---

**Table 2**

Results of the survey on detection of the novel avian influenza A(H7N9) virus, May 2013 (n=32 laboratories in 29 countries)

<table>
<thead>
<tr>
<th>Capability</th>
<th>Countries n</th>
<th>Laboratories n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Generic detection assay for influenza A is predicted to detect influenza A(H7N9)</td>
<td>27</td>
<td>30</td>
</tr>
<tr>
<td>Have BSL3 laboratory facilities that they can use for culture of this virus</td>
<td>27</td>
<td>29</td>
</tr>
<tr>
<td>Have isolation capability</td>
<td>22</td>
<td>24</td>
</tr>
<tr>
<td>Influenza A(H7) subtyping available</td>
<td>27</td>
<td>29</td>
</tr>
<tr>
<td>Influenza A(N9) subtyping available</td>
<td>20</td>
<td>22</td>
</tr>
</tbody>
</table>

BSL: biosafety level.
knowing the primer sequences, was unable to predict its diagnostic capability.

It is recommended by WHO that the novel A(H7N9) viruses be propagated in biosafety level (BSL) 3 facilities [14]; 29 laboratories in 27 countries reported having such facilities. Seven countries (Bulgaria, Cyprus, Estonia, Ireland, Malta, Romania and Slovenia) and Wales (United Kingdom (UK)), indicated that they would not propagate the novel A(H7N9) virus in their laboratories because they lacked capability in their BSL3 facilities.

Twenty-nine influenza reference laboratories in 27 countries indicated having a real-time RT-PCR assay for H7 subtyping in place. Nine laboratories in eight countries had implemented subtyping assays based on H7 primers and probes developed by CCDC [15] or Corman et al. [16] or their own primer/probe sets based on sequence alignments. Fourteen laboratories in 13 countries had more than one H7 subtyping assay in place which showed some variation (within 1 log; cycle threshold (Ct): 3.2) in the sensitivity of detection of the novel avian influenza A(H7N9) viruses. Eight laboratories in eight countries had implemented the complete protocol of the United States Centers for Disease Control and Prevention (US CDC) [17]. Four were using other alternative assay protocols than the ones listed here, for example those of Slomka et al. [18].

Twenty-two laboratories in 20 countries had set up N9 subtyping at the time of the survey (May 2013). Ten laboratories in nine countries indicated that they had not yet tested their protocol or did not have this test. Thirteen countries had chosen to use the CCDC [15] assay with the primers and probes for N9 from that protocol. Seven laboratories in six countries had developed their own assay, and three laboratories used the primers and probes described by Corman et al. [16]. One country indicated that they would sequence the NA gene instead of setting up a specific subtyping real-time RT-PCR assay for N9.

To share viruses with a WHO CC and the WHO GISRS, 22 laboratories in 19 countries reported use of the WHO shipment fund for shipments to WHO CC London in the influenza season and during emerging outbreaks. Four countries used their own budgets with additional WHO shipment funding, and eight laboratories from eight countries used only their own budget. Three countries and laboratories indicated use of Quality Assurance Exercises and Networking on the Detection of Highly Infectious Pathogens (http://www.quandhip.info/) for shipments, in addition to the WHO shipment fund, and none of the responding laboratories indicated further need for financial support for sample shipment.

### Table 3

<table>
<thead>
<tr>
<th>Positive control (vRNA) dispatch</th>
<th>Number of laboratories</th>
<th>Countries represented</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acknowledged receipt of vRNA</td>
<td>32</td>
<td>22</td>
</tr>
<tr>
<td>Tested primer/probe protocols with the vRNA</td>
<td>24</td>
<td>19</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Number of laboratories reporting (number of assays carried out)</th>
<th>Number of countries</th>
<th>Number of different assays</th>
<th>Product size range</th>
<th>Number of reports on end point titrations (range of end points reported)^(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M gene (generic influenza A assay)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>19^a</td>
<td>16</td>
<td>8</td>
<td>77–205</td>
<td>6 (10^−7–10^−9)</td>
</tr>
<tr>
<td>H7:HA gene</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24 (33)^b</td>
<td>19</td>
<td>16</td>
<td>52–254</td>
<td>10 (10^−7–10^−9)</td>
</tr>
<tr>
<td>N9-NA gene</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16 (17)^d</td>
<td>15</td>
<td>6</td>
<td>107–153</td>
<td>4 (10^−4–10^−7)</td>
</tr>
</tbody>
</table>

HA: haemagglutinin; M: matrix protein; NA: neuraminidase.

^a The number of reports that included end point titrations is given, with the titration range for the assays in brackets.

^b The most commonly used assay (six of 19 reports) was the InfA primer set from the United States Centers for Disease Control and Prevention (US CDC) in Atlanta [17]. The other assays were developed locally.

^c Seven laboratories in different countries reported using more than one primer set: Austria (n=2), Belgium (n=2), Germany (n=4), Italy (n=2), Luxembourg (n=2), the Netherlands (n=2), and Norway (n=2), generating 33 reports.

^d Germany tested two primer sets, and 12 of the 17 reports were for the CCDC [15] N9 primer set.
The 24 laboratories (19 countries) that subsequently reported their results on detecting the novel influenza A(H7N9) vRNA standard in their real-time RT-PCR protocols, had correctly predicted their capability to detect the novel virus (Table 3). Nineteen laboratories reported on generic influenza A virus detection, all used real-time RT-PCR assays based on the M gene. Eight different assays were employed that generated product sizes in the range 77–205 nt, and six of 19 reports employed the US CDC InFA primer set (106 nt PCR product). The six end point titration results all showed good sensitivity (five were positive for dilutions in the range $10^{-7}$–$10^{-8}$ and one laboratory showed positivity up to $10^{-9}$), five of them with the US CDC InFA primer set.

H7 detection was reported by all 24 laboratories (Table 3). Sixteen different H7 assays were employed, generating PCR fragments in the range 52–254 nt. The primer sets spanned five different regions in the HA gene, two in HA1, one spanning the HA1/2 cleavage site, and two in HA2. Seven laboratories in different countries reported on more than one primer set, resulting in 33 individual primer set reports, 19 of which employed the following primer sets: CCDC HA1 [15] (n=10), Slomka et al. HA2 [18] (n=4), Corman et al. HA2 [16] (n=3), and US CDC HA2 [17] (n=2). The remaining reports used primers developed in-house or modifications of the primers listed here above, making them more specific for the novel avian influenza A(H7N9). The 10 H7 end point titrations, all of which showed good sensitivity (nine were positive for dilutions in the range $10^{-7}$–$10^{-8}$ and one laboratory showed positivity up to $10^{-9}$), were using among others the CCDC HA1 [15] (n=3), Slomka et al. HA2 [18] (n=2), Corman et al. HA2 [16] (n=2) and the US CDC HA2 [17] (n=1) primer sets.

Sixteen laboratories reported on N9 detection (Table 3). Six assays were employed (PCR product size range: 107–153 nt), two of which were the initial and modified versions of the CCDC assay. Twelve reports employed one of the CCDC N9 primer sets. Of the four N9 end point titrations, three employed a CCDC primer set (PCR product size 107 nt), while the fourth employed an assay developed in-house (RIVM, the Netherlands, product size: 125 nt); both N9 assays were less sensitive than the generic (M gene) and H7 assays, with end points at dilutions in the range $10^{-6}$–$10^{-7}$. One laboratory (NIC, Norway) obtained good sensitivity through end point titration ($10^{-7}$–$10^{-9}$ for M and H7 but still only $10^{-6}$–$10^{-7}$ for N9) with real-time RT-PCRs set up using an AgPath-1D One-Step RT-PCR kit (Life Technologies) and run on Rotor-Gene 3000 or 6000 thermocyclers (Qiagen); this may be due to dilution of the vRNA standard with water containing carrier RNA as supplied with the QIAamp vRNA extraction kit (Qiagen).

A wide variety of RT-PCR kits and thermocycler platforms were used across the reporting laboratories for detection and subtyping of A(H7N9) viruses. Most laboratories used one-step RT-PCR kits from Life Technologies (TaqMan Fast Virus 1-Step, SuperScript III Platinum One Step qRT-PCR, AgPath-1D One-Step RT-PCR) or Qiagen (One-Step RT-PCR, Quantifast Probe RT-PCR), while some laboratories employed two-step systems (e.g. QuantiTect Reverse Transcription kit, Qiagen) developed in-house, and one laboratory reported on generic influenza A detection as part of a multiplex assay. The various assays were implemented on Stratagene (MX3005), ABI (7300, 7500, 7500 FAST, 7900 HT), Rotor-Gene/Qiagen (Q, 3000, 6000), Roche (LC480) and Bio-Rad (CFX96) thermocyclers.

**Discussion**

ERLI-Net laboratories had built up detection capability for the novel influenza A(H7N9) viruses within approximately two months from the first reports of this virus. Most ERLI-Net laboratories had developed or applied specific H7 and/or N9 real-time RT-PCR assays to identify the novel A(H7N9) viruses: 27 countries have an H7 assay and 21 an N9 assay in place in their influenza reference laboratories. Overall, 28 of 31 laboratories in 27 countries reported an ability to subtype A(H7) viruses, with the remaining three laboratories proposing to send their non-subtypeable viruses to WHO CC London.

Overall, laboratories in EU/EEA countries appear to be well prepared for the detection and identification of the novel avian A(H7N9) influenza virus, because they either can detect the viruses themselves or, if not, have a mechanism in place to forward the viruses to WHO CC for characterisation. Furthermore, it is likely that the H7-specific HA2 primer set from the US CDC will be adopted by more laboratories, as it is now available through the Influenza Reagent Resource (https://www.influenzareagentresource.org #FR-1258). Due to the high genetic diversity in the HA of influenza viruses of the A(H7) subtype, it has not been possible to design a universal primer/probe set of the required specificity and sensitivity to detect all avian influenza A(H7) viruses. However, both the US CDC H7-HA2 primer set [17] and the set by Slomka et al. [18] have been evaluated and are capable of detecting Eurasian H7 avian influenza viruses typically infecting poultry in Europe that have the potential to cause zoonoses.

From the survey responses and results reported on the use of an A/Anhui/1/2013(H7N9) vRNA standard, it is apparent that ERLI-Net laboratories across EU/
EEA countries have a range of assays available that are suitable for detecting the M, HA and NA genes of the novel A(H7N9) influenza virus. With current capabilities, these novel avian influenza A(H7N9) viruses would be detected in the majority of EU/EEA countries on submission of a sample to a national influenza reference laboratory for characterisation. However, as these A(H7N9) viruses are likely to evolve, sequence-based comparison of primer/probe sets with circulating H7N9 viruses should be part of a continuous monitoring practice in all influenza reference laboratories, with modification of set(s) as required. Despite such monitoring it is clear that, even when the same detection algorithm, equipment and laboratory protocols are used, the human factor plays a role in laboratory detection, and assay performance can only be verified through external quality assessment (EQA) and clinical validation [19]. ERLI-Net undertook an EQA in autumn 2013 that included an A(H7N9) virus in the panel; the results are pending.

Conclusions

This capability assessment would not have been possible without the prompt actions of the Chinese authorities and the CCDC who rapidly deposited sequence data in the GISAID database and provided virus for culture and RNA extraction to WHO CC London. Feedback from ERLI-Net laboratories indicates that EU/EEA countries have good detection capabilities for these novel avian influenza A(H7N9) viruses. Generally, this study illustrates the importance of having a coordinated laboratory network such as ERLI-Net, with a direct link to the WHO GISRS for virus and reagent sharing, and the usefulness of timely responses to sequence-based analysis surveys as well as testing of performance and proficiency to inform a regional risk management response.

A large diversity of assays and platforms for influenza detection and diagnosis are available in the European health sector, reflecting prevailing local conditions. Nevertheless, good technical performance can be achieved, even though a lack of detailed knowledge of primer and probe binding sites in commercial kits makes it difficult to predict their match with the viral target genes. The mechanism described here (a survey including sequence-based analysis followed by practical assessment) is likely to be necessary every time a new variant of influenza virus with pandemic potential emerges. During such surveys, clear technical communication channels both within and between countries, ERLI-Net/ECDC, WHO/Europe and WHO CC London, are a crucial part of preparedness and response.

In influenza reference laboratory networks, the existing pathways for specimen referral to the WHO CCs and annual EQAs have proven useful tools in ensuring good seasonal influenza surveillance. The same pathways can be used in an emergency. For EU/EEA countries, an additional element in an emergency response is enhanced communication between ECDC, WHO/Europe, WHO CC London and other ERLI-Net virology experts, to ensure high quality and rapid technical support for the ERLI-Net laboratories. The network benefits from WHO CC functions through the distribution of positive controls for RT-PCR and support in the validation of protocols. Larger network laboratories, such as Public Health England Colindale, can support the network in the clinical validation of the detection assays. However, a sequence-based computational assessment of the detection platforms is not enough, and EQA of the assays is crucial to ensure the field validation of primers and reagents. This ERLI-Net model could be applied to other dedicated communicable disease networks to assess the performance of their schemes for sample referral, setup of detection assays and validation of the assays for emerging infectious disease events.

The current response to the emergence of influenza A(H7N9) has demonstrated a good preparedness in European influenza reference laboratories. Nevertheless, a number of areas can be improved: (i) how best to assess the detection assays used in the primary diagnostic laboratories, (ii) how well evaluated and clinically validated a detection protocol should be before it is shared with the network laboratories, (iii) where to post all technical material so that it can be easily found, (iv) how to speed up the distribution of positive controls, (v) the best way to communicate rapidly within the network, (vi) how the questionnaire results are followed up with the laboratories, and (vii) how the required training, based on EQA results, is delivered. Overall, there is a need to decide on a standard operation procedure for emergency responses in the network, so that all parties know their role(s) and timeline(s) for response and what is expected from them when a novel virus emerges. As the influenza A(H7N9) situation is still evolving, there is no reason for complacency, and preparedness at the European level will continue to be monitored. Gene sequences from the most recent influenza A(H7N9) zoonotic infections available in GISAID, as of 24 January 2014, indicate that the low levels of genetic drift observed are unlikely to adversely affect the detection capabilities that have been developed in EU/EEA countries.

Authors’ contributions

EB, RD, JMcC were responsible for the analyses of returned data and drafting of the manuscript. These authors and the others (JE, AM, DPa, DPe, MS and MZ) took part in discussions relating to the development and drafting of guidance for ERLI-Net/NIC laboratories and the sequence-based questionnaire, and critical review of the present manuscript.

Conflict of interest

None declared.
Acknowledgements

The authors thank all persons in EU/EEA countries and their human influenza reference laboratories that responded to the sequence-based analysis questionnaire and the request for feedback on the use of positive control vRNA from a representative novel avian influenza A(H7N9) virus shared with GISRS by WHO CC Beijing. The work of the WHO CC was supported by MRC programme U17512723.

References

c laboratoryBioriskManagementH7N9_10May13.pdf