

Severe Influenza Outbreak in Western Ukraine in 2009 – a molecular-epidemiological study

Janusz Kocik¹, Marcin Niemcewicz², Matthew Johns³, Kurt Jerke⁴, Aleksander Michalski², Anna Bielecka¹, Krzysztof Lasocki², Jerzy Gaweł², Agata Bielawska-Drózd², Justyna Joniec², Marcin Kołodziej², Grzegorz Graniak², Mariusz Goniewicz⁵, Leszek Kubiak¹

¹ Military Institute of Hygiene and Epidemiology, Department of Epidemiology, Warsaw, Poland

² Biological Threat Identification and Countermeasure Centre, Pulawy, Poland

³ Armed Forces Health Surveillance Center, Department of Global Outbreak Alert and Response & IHR Implementation, Division of Global Emerging Infections Surveillance, USA

⁴ Landstuhl Regional Medical Center Laboratory, Landstuhl, Germany

⁵ Emergency Medicine Unit, Medical University of Lublin, Poland

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Abstract

Introduction: In the autumn of 2009 the authors participated in a humanitarian operation in Western Ukraine by undertaking an epidemiological investigation of an influenza-like-illness (ILI) in the L'viv Oblast region. Mobile biological survey teams took samples from civilian patients with severe acute respiratory distress syndrome, rapid transportation of the samples, and their molecular analysis in Poland to provide accurate results.

Objective: The aim of the study was the molecular and epidemiological analysis of the biological samples collected.

Material and Methods: Real-time reverse transcriptase polymerase chain reaction (real-time RT-PCR), multiplex PCR techniques, traditional Sanger Sequencing and classical viral culture methods were used.

Results: Among the 124 influenza-like illness cases, ~50% (58) were positive for influenza A virus in WHO-CDC molecular assay, including subtyping. The specimens were further analyzed to confirm results and determine the genetic sequence. Phylogenetically, the nucleotide similarity of both the Ukraine specimens and reference A/California/7/2009 (pH1N1) was 99.2–99.3%. Oseltamivir resistance was not registered. HA1 region characterization showed an overall protein identity of 98.5–99.4%.

Conclusions: An unexpected high contribution of influenza A was confirmed among ILI patients, as well as a very limited number of other detected viruses, indicate that the 2009 epidemic in western Ukraine was strongly related to novel influenza A/H1N1. The importance of swift sharing of information and reference laboratories networking in surveillance, as well as serving governments and international agencies in pursuing adequate actions, should be stressed.

Key words

influenza A [H1N1], influenza-like illness, outbreak, gene sequencing

INTRODUCTION

Infections caused by influenza viruses are well-known for being associated with significant morbidity and mortality [1]. The evolution of human influenza A viruses includes antigenic shift and antigenic drift [2, 3]. Variation in influenza viruses is caused by the accumulation of point mutations in the haemagglutinin (HA) and neuraminidase (NA) genes, including substitutions, deletions and insertions, which produce genetic variation in influenza viruses (antigenic drift). In general, antigenic drifts are related to seasonal epidemics and often reduce the effectiveness of the previous seasonal vaccines [4]. An antigenic shift occurs when an animal influenza A virus is transmitted without reassorting from an animal reservoir to humans, or when a progeny virus with a new HA (with or without a new NA) arises as a result of genetic reassorting between animal and human influenza A viruses [2, 5, 6]. Antigenic shifts in HA subtypes are associated with pandemics [4].

The novel influenza A/H1N1 arouses serious concern because the genetic material of the new virus comprises previously detected swine, human and avian influenza type A fragments [triple reassorting]. Moreover, the World Health Organization (WHO) declared influenza A/H1N1 to be a global pandemic – Stage 6 [7] and the first public health emergency of international concern under the International Health Regulations (2005). In preparation for this, during the past 5–10 years the 194 WHO Member States have developed pandemic preparedness strategies with national plans for pandemic preparedness [8, 9]. These plans and strategies include the core concepts and themes of early detection of novel viruses to achieve a rapid and effective response [10].

Support during the pandemic. Inadequate vigilance will seriously delay overall detection and treatment efforts. An effective multi-sector approach to surveillance, contact tracing, isolation measures, as well as optimal distribution of resources for pharmaceutical and non-pharmaceutical countermeasures are the cornerstone of effective mitigation strategies [11, 12]. Military support, as part of the multi-sector approach, may be necessary for the safety and security of the country given that public health emergencies have historically

Address for correspondence: Janusz Kocik, Military Institute of Hygiene and Epidemiology, Department of Epidemiology, 4 Kozielska, 01-163 Warsaw, Poland
E-mail: jkocik@wihe.waw.pl

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resulted in threats to security and stability. Military personnel are extensively trained to work in the crisis management environment, have the ability to deal with varied tasks in sub-optimal conditions, mobilise resources quickly and effectively, and are used for widespread deployment in the context of very complex emergencies or crises.

In the autumn of 2009, a serious influenza-like illness epidemic occurred in the Ukraine. Uncertain pathogenicity of the novel stain, an increasing number of cases, as well as shortages in prophylaxis, treatment and diagnostic resources, resulted in the President of the Ukraine seeking international support. The authorities of Poland answered positively. Among other activities during this operation, the Military Institute of Hygiene and Epidemiology (MIHE) was engaged to provide diagnostic capabilities. This task was accomplished in close scientific cooperation between MIHE and the US Armed Forces Health Surveillance Center, and the Global Emerging Infectious Surveillance and Response System. The increasing global threat from the highly pathogenic H5N1 avian influenza in 2005 resulted in AFHSC-GEIS dramatically enhancing their global influenza surveillance network and developing new ways to monitor for sudden changes in influenza activity. AFHSC-GEIS has implemented long-term initiatives to increase influenza surveillance through its global network of partners. Enhancement of influenza laboratory capacity, increased number of sentinel sites and the number and coverage of countries in which surveillance is conducted were the primary areas of focus at this time [13].

METHODOLOGY

On 4–5 November 2009, two Biological Survey Teams (BSTs) from MIHE, together with the Polish Armed Forces Epidemiological Response Center, were delegated to support the humanitarian operation in western Ukraine to investigate the influenza-like-illness (ILI). The BSTs were composed of a military physician, a diagnostician and a field epidemiologist. The main goal of this two-day mission was the military mobile sampling in cases of severe acute respiratory distress syndrome (ARDS) among groups of civilian patients in the L'viv Oblast region, rapid transportation of the samples to a reference laboratory in Poland. The locations for sampling were four city hospitals in L'viv (two general hospitals, one for infectious diseases and one for tubercular and pulmonary diseases). Selection criteria for patients were respiratory failure, severe/moderate disease, ICU patients, and those with pneumonia with complications.

For epidemiological analysis, all patients were divided into five groups: age 0–6 – infants and preschool children, 7–18 – children and adolescents, 19–40 – young age adults, 41–70 middle-aged and older adults, and over 70 – elderly people.

Influenza A/B and parainfluenza rule-out laboratory analysis was performed in less than 48 hours at the MIHE in Poland, and the test results sent to Ukrainian clinics. Comprehensive second-tier analysis against respiratory viruses was carried out in the regional laboratory of the AFHSC-GEIS network at the Landstuhl Regional Medical Center (LRMC) in Germany. Additionally, genetic sequence analysis were carried out in the laboratory of the School of Aerospace Medicine (USAFSAM) in San Antonio, Texas, USA. Genetic and predicted antigenic information resulting from the analysis could potentially

contribute to the seasonal Northern and Southern Hemisphere vaccine component selection.

MATERIALS AND METHODS

Nasal wash samples were collected from 124 patients with ARDS in accordance with US DoD GEIS sampling procedure [14] using transportation kits provided. Subsequently, the samples, suspended in transportation medium, were delivered to the MIHE's Biological Threats Identification and Countermeasure Center, aliquoted and stored at -70°C.

In the MIHE, all 124 specimens were subjected to PCR analysis (one step real-time PCR, LightCycler PCR, Roche; QIAamp Viral RNA Mini Kit, Qiagen) to confirm the presence of influenza A or novel influenza A genetic material, as well as additional assays for influenza B and parainfluenza type 1,2,3. Specimens were also investigated by the xTAG respiratory panel (influenza A, influenza A subtype H1, influenza A subtype H3, influenza B, respiratory syncytial virus (RSV), human metapneumovirus (hMPV), rhinovirus, and adenovirus) on the Luminex multiplexing platform in the US Army Public Health Command – European Region laboratory at the LRMC. Both types of assays for influenza included subtyping (simultaneous detection of specific sequences characteristic for influenza).

After front-end testing at the LRMC regional laboratory, the samples were then sent to the main reference laboratory for the AFHSC-GEIS global network in USAFSAM. USAFSAM in consultation with the LRMC determined the resulting initial testing algorithm for a subset of 20 of the 58 specimens. The LRMC provided real-time RT-PCR cycle threshold yields with values of less than 30 for 10 specimens from the group of 28 specimens positive for novel influenza A/H1N1 virus. These 10 samples, along with randomly-selected samples from the group with indeterminate novel influenza A/H1N1 virus results, and 5 samples negative for respiratory viruses at the LRMC, were concurrently processed for sequence analysis and cultured for influenza. The remaining 38 samples were archived, pending review of the initial sequence and culture results.

Full-length segment 7 of influenza A (matrix gene and M2 proton ion channel gene) were sequenced for 6 of the 20 specimens. The haemagglutinin (HA) gene and portions of the neuraminidase (NA) gene from cultured viruses were sequenced using dry terminator, Sanger-based methods.

Sequence data was constructed and analyzed using multiple software programmes. Sanger sequencing of the NA gene was performed to detect the presence of genetic sequences, which had indicated resistance to the antiviral drug Oseltamivir. Full-length HA sequence analysis of the HA1 region of the HA gene was performed for 8 isolates, which were further characterized in an un-rooted neighbour-joining phylogenetic tree comparing protein identity to the vaccine reference strain, A/California/7/2009 (pH1N1)-like virus.

Phylogenetic trees were constructed based on the continuous nucleotide sequences aligned with ClustalX. Genetic distances were calculated applying Kimura's two-parameter method using MEGA3.1, and used to construct neighbour-joining (NJ) trees. Confidence values for the tree topologies were evaluated by bootstrap analysis of 1,000 pseudo-replicate datasets. HA ribbon models were produced using first approach mode in SWISS-MODEL Workspace [15, 16, 17, 18].

RESULTS

The cohort studied was dominated by young adults, while infants and preschool children and the older groups of patients were underrepresented. The number of cases and incidence per 100,000 of the population by age range is represented in Table 1. The highest incidence rate was noted in the group aged 19–40, the young adults.

Table 1. Number of cases and incidence per 100,000 population by age range in L'viv Oblast.

	Age Range						Total	
	0-6	7-18	19-40	41-70	70+	Un-known	(N)	(%)
Gender (F/M)	2/2	7/3	37/26	19/17	2/2	4/3	124	100
A/H1N1								
Number of cases (N)	2	4	23	11	2	2	44	35.48
Incidence per 100,000	0.08	0.16	0.90	0.43	0.08	0.08	1.72	
Influenza Typ A								
Number of cases (N)	2	0	6	3	2	1	14	11.29
Incidence per 100,000	0.08	0.00	0.24	0.12	0.08	0.04	0.55	
Not Influenza Typ A								
Number of cases (N)	0	6	34	22	0	4	66	53.23
Incidence per 100,000	0.00	0.24	1.33	0.86	0.00	0.16	2.59	

The real time PCR screening showed that Influenza type A accounted for almost 50% of all cases – 22 positive results of novel influenza A/H1N1, 22 presumptive positive for novel influenza A/H1N1 and 14 presumptive Influenza A (Fig. 1). The non-influenza A samples were subject to further analysis against influenza B virus and parainfluenza types 1, 2, 3, and proved to be negative.

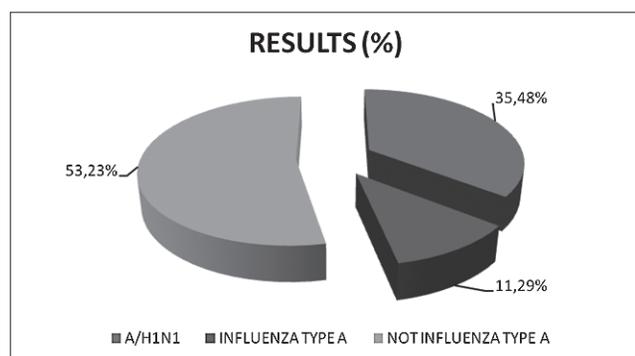


Figure 1. Summary of molecular screening results

The 58 positive samples were sent to the LRMC and consequently to the USAFSAM for respiratory virus differentiation. The results indicated that of the 58 specimens, 31 were positive for novel influenza A/H1N1 virus (pH1N1), 12 were indeterminate for novel influenza A/H1N1, and 15 were negative for respiratory viruses. These positive results were inter-laboratory consistent for 43 tested samples (positive, indeterminate, presumptive results). Another 15 were not confirmed. All the remaining samples of non-influenza A/B or non-parainfluenza type 1,2,3 were reconfirmed as being negative, while 5 of them proved to be positive for rhinovirus genetic material.

Initial sequence and culture results for the 20 selected specimens. The USAFSAM Public Health Epidemiology

Laboratory performed additional testing and molecular analysis to further characterize the pH1N1 influenza viruses detected at LRMC laboratory. Full-length segment 7 of influenza A (matrix gene and M2 proton ion channel gene) was successfully sequenced for 6 of the 20 specimens. The successfully sequenced specimens were all from the group of specimens which previously tested positive for novel influenza A/H1N1 virus. Good quality sequence data could not be obtained from any of the specimens that had tested indeterminate for novel influenza A/H1N1 virus or negative for respiratory viruses. Phylogenetically, the nucleotides from the successfully sequenced segment 7 for all six specimens aligned with segment 7 of A/California/7/2009 (pH1N1) exhibited an overall nucleotide similarity of 99.2–99.3% (Fig. 2). A Blastn analysis of A/Ukraine/4898/2009 resulted in a $\geq 99.8\%$ match with specimens from Scandinavia and Russia.

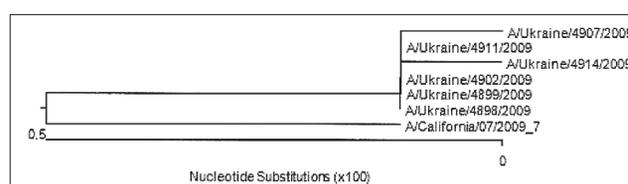


Figure 2 – Phylogenetic tree of obtained sequences of segment 7 aligned to segment 7 of influenza A/California/7/2009

Tissue culture analysis for influenza A was conducted concurrently with sequencing segment 7 of influenza A from 20 selected specimens. Nine of the specimens were cultured positive for influenza A. No influenza A was detected in any of the specimens that were indeterminate for novel influenza A/H1N1 virus or negative for respiratory viruses.

Given that sequencing was successful in 6 of 20 of the selected nasal wash specimens, and culture was successful on 9 of the 20 specimens, additional sequence analysis of the full hemagglutinin gene, as well as portions of the neuraminidase gene, was performed on all culture isolates. It should be noted that although both sequencing and influenza culture results were obtained only from the group of specimens received as positive for novel influenza A/H1N1 virus, there was one specimen – A/Ukraine/4911 pH1N1 – successfully sequenced, but not cultured. Overall, of the 10 specimens positive for novel influenza A/H1N1 virus at the LRMC, all were either culture positive for influenza A or produced sequence data indicating that the novel influenza A/H1N1 virus was present.

Relevant sequencing data from culture isolates. Sanger sequencing of the neuraminidase gene was performed to detect the presence of the genetic sequences that result in a change from histidine to tyrosine at amino acid 274 (H274Y, N2 numbering), previously shown to lead to the loss of oseltamivir binding to neuraminidase, rendering the virus resistant to this class of antivirals. While other resistance markers may be present and actual *in vivo* resistance may vary, all isolates exhibited H274 (histidine at amino acid 274), which does not correspond to Oseltamivir resistance.

Full-length haemagglutinin (HA) sequences were obtained for 8 isolates; however, all single amino acid changes resided within the HA1 domain, the primary immunogenetic factor. Blastp analysis of HA protein homologs suggests overall that all the Ukraine isolates shared over 99% similarity with

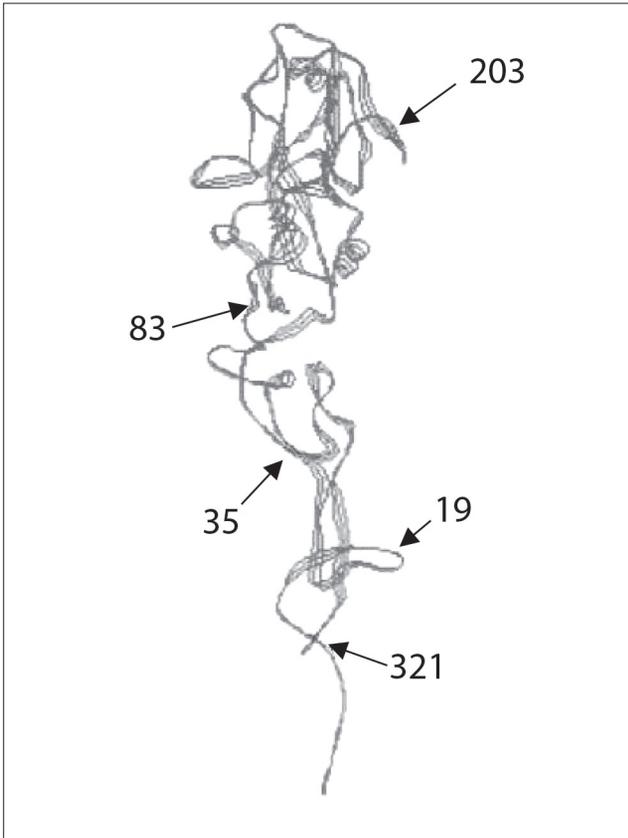


Figure 3. HA ribbon model of strain A/Ukraine/4902/2009 (98,5% overall HA1 amino acid homology) contained the following amino acid changes (V19I, D35G, P83S, S203T, I231V- marked with arrows) with respect to A/California /7/2009 (pH1N1) virus

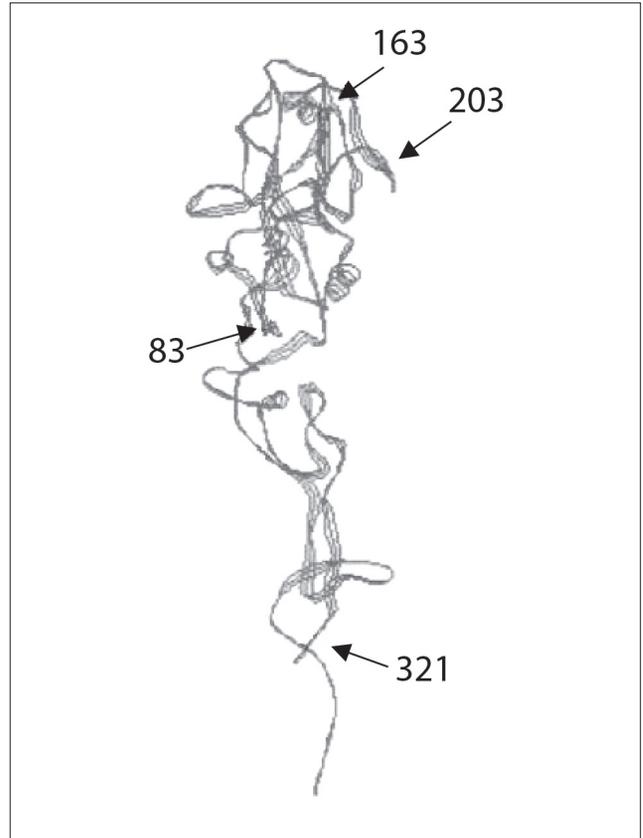


Figure 4. HA ribbon model of strain A/Ukraine/4903/2009 (99,1% overall HA1 amino acid homology) contained the following amino acid changes (P83S, K163Q, S203T, I231V- marked with arrows) with respect to A/California /7/2009 (pH1N1) virus

isolates collected from the USA, Asia, Canada, Mexico and Europe.

Isolates are characterized in an un-rooted, neighbour-joining phylogenetic tree (Fig. 5). Thus far, all novel influenza A/H1N1 virus specimens characterized by USAFSAM

exhibited an overall protein identity from 97.9–99.4%, compared to the A/California/7/2009 (pH1N1)-like virus. The 8 Ukrainian specimens that were successfully sequenced exhibited an overall protein identity of 98.5–99.4%. All 8 specimens (Fig. 3, 4) differ from the vaccine strain by an

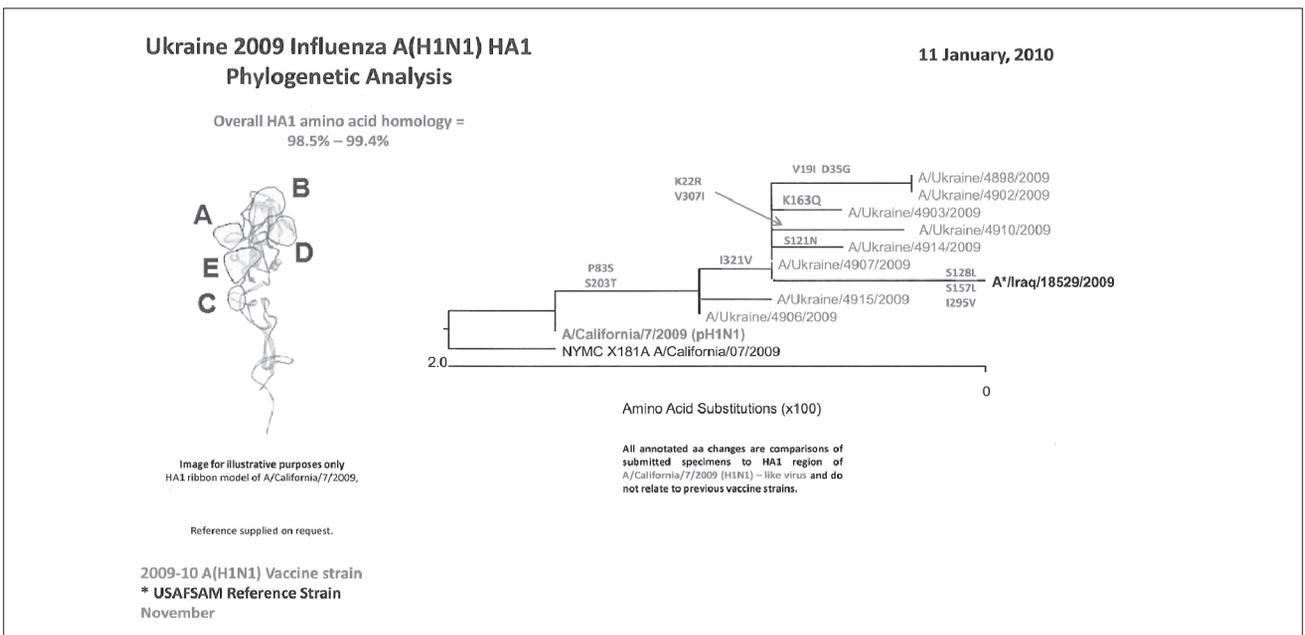


Figure 5. Un-rooted, neighbor-joining phylogenetic tree with HA ribbon model of A/California /7/2009 (pH1N1) virus (letters correspond to putative antigenic epitopes, which historically produced antibodies)

amino acid change at position 83 (proline to serine), and at position 203 (serine to threonine); 7/8 isolates had an additional change at position 321 (isoleucine to valine). These 3 primary differences are consistent with those seen in isolates collected from the USA, Europe, Asia, South America, the Middle East and Africa throughout the pandemic (Fig. 3, 4).

DISCUSSION

In April 2009, the World Health Organization (WHO) received an alert from the US CDC, based on reports from laboratories within the GEIS Network, that indicated the occurrence of the new influenza A(H1N1) virus [19]. A few days later, the WHO noticed that the current influenza outbreak was caused by a virus generated from a triple reassortment of human, swine and avian viruses [20, 21]. The scale of the threat was vast and the virus spread globally within months. The rapid decision by the WHO Director-General to announce a Level 6 Influenza Pandemic forced governments to evaluate their pandemic plans and to ensure appropriate responses [22, 23]. Sharing information and strains between different parties around the world enabled manufacturers to produce a new vaccine.

Lessons were learned from the 2009 outbreak during which a high proportion of severe ILI cases were observed in the L'viv Oblast region of the Ukraine. Contrary to seasonal influenza where the highest incidence rate was among infants, preschool children and the elderly, in this case it was noted among young adults (aged 19–40). The rapidity of rule-out screening should be stressed, even though the logistics of the sampling mission required the use of military resources designed specifically for international deployment. The capability to deploy mobile teams is limited to few civilian agencies, such as WHO. Rapid clinical diagnosis confirmation within 48 hours and feedback information allowed targeted treatment with antivirals. The well-described roles in the GEIS collaboration network allowed the adequate use of resources in the chain of demanded analyses. This clearly shows that the establishment of networks by services, even if partly twinning other efforts, adds to the universal preparedness.

The dominating results for influenza A (including novel A/H1N1) and the very limited number of other viruses, support the assumption that the novel virus was responsible for this epidemic. It is worth mentioning that the fragility of virus samples during prolonged storage and transport may lead to losses in diagnostic material and subsequent false negative results, or not fully confirmed results (indeterminate or presumptive), as was encountered during primary and confirmatory analysis. Since molecular analysis is very sensitive for quality of diagnostic material, an appropriate time-temperature regime should be implemented during sample transfer.

The results of the sequence analysis indicated that the Ukrainian novel influenza A/H1N1 virus sequenced specimens were very similar to the pH1N1 reference strain virus (A/California/7/2009 (pH1N1)-like virus) [23]. Segment 7 sequence analysis of the reference strain evidenced that the most highly conserved portion of the virus conferred with the adamantane resistance, suggesting a limited effectiveness of this group of influenza virus inhibitors [24, 25]. Moreover, no antigenic shift from the pH1N1 vaccine reference strain was observed [20]. In this case, none of them carried genetic

markers indicating oseltamivir resistance [26]. However, because of the high percentage of adamantane-resistance, as well as regional cases of oseltamivir resistance, influenza antiviral resistance surveillance is to be recommended [27, 28, 29, 30, 31]. Hence, the necessity for the permanent monitoring of drug resistance and the categorization of possible evolving viral proteins during pandemics is still justified [32, 33, 34].

Sequence analysis of the HA1 region of the haemagglutinin gene indicated that the Ukraine novel influenza A/H1N1 virus was very similar to the pH1N1 reference strain virus (A/California/7/2009 (pH1N1)-like virus). The differences were similar to those seen in other specimens from around the world that have been sequenced at the USAFSAM. Despite the fact that the ILI cases sampled demonstrated severe symptoms of the ailment, the influenza virus found in the Ukrainian specimens in 2009 did not possess the same mutation as the specimen(s) in Norway, and there is no information to suggest any correlation with increased morbidity or mortality [35].

CONCLUSIONS

Although detecting the global passage of a influenza virus by sequence analysis is complicated by skewed geographic and seasonal distributions in viral isolates, the lessons learnt in 2009 show that the quick-sharing of information and reference laboratories networking in surveillance over prevalence of the virus was critical for allowing governments and international agencies to undertake adequate actions and evaluate their effectiveness. Furthermore, the epidemic of the novel influenza A/H1N1v in western Ukraine was successfully traced, with an unusual incidence rate among young adults. The molecular screening results were confirmed by detailed sequencing analysis; therefore the diagnostic protocol applied was a useful tool for the rapid screening of influenza infections. However, despite the advances in technology, surveillance and pandemic planning is crucial and those involved should be aware of the dangerous scale of emerging pandemics, and the difficulty in their global control.

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