Public Health 230 (2024) 157–162

Contents lists available at ScienceDirect

Public Health

journal homepage: www.elsevier.com/locate/puhe

Original Research

Real-time investigation of an influenza A(H3N2) virus outbreak in a refugee community, November 2022

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ARTICLE INFO

Article history:
Received 9 September 2023
Received in revised form
2 February 2024
Accepted 27 February 2024
Available online 29 March 2024

Keywords:
Acute respiratory infection (ARI)
Influenza-like illness (ILI)
Refugee
Outbreak investigation
A(H3N2)
Attack rate
Phylogenetic analysis
Differential virological diagnosis

ABSTRACT

Objectives: To report epidemiological and virological results of an outbreak investigation of influenza-like illness (ILI) among refugees in Northern Italy.
Study design: Outbreak investigation of ILI cases observed among nearly 100 refugees in Northern Italy unvaccinated for influenza.
Methods: An epidemiological investigation matched with a differential diagnosis was carried out for each sample collected from ILI cases to identify 10 viral pathogens (SARS-CoV-2, influenza virus type A and B, respiratory syncytial virus, metapneumovirus, parainfluenza viruses, rhinovirus, enterovirus, parechovirus, and adenovirus) by using specific real-time PCR assays according to the Centers for Disease Control and Prevention (CDC) protocols. In cases where the influenza virus type was identified, complete hemagglutinin (HA) gene sequencing and the related phylogenetic analysis were conducted.
Results: The outbreak was caused by influenza A(H3N2): the attack rate was 83.3% in children aged 9–14 years, 84.6% in those aged 15–24 years, and 28.6% in adults ≥25 years. Phylogenetic analyses uncovered that A(H3N2) strains were closely related since they segregated in the same cluster, showing both a high mean nucleotide identity (100%), all belonging to the genetic sub-group 3C.2a1b.2a.2, as those mainly circulating into the general population in the same period.
Conclusions: The fact that influenza outbreak strains as well as the community strains were genetically related to the seasonal vaccine strain suggests that if an influenza prevention by vaccination strategy had been implemented, a lower attack rate of A(H3N2) and ILI cases might have been achieved.

Introduction

Influenza A viruses (IAVs) are segmented single-stranded, negative-sense RNA viruses belonging to the Orthomyxoviridae family. Based on major differences in the surface proteins hemagglutinin (HA) and neuraminidase (NA), IAVs are divided into several subtypes; A(H3N2) and A(H1N1) are currently the two main IAV subtypes circulating in the human population.[1,2] From year to year, these subtypes undergo minor antigenic changes, termed antigenic drift, and, as a consequence of this evolutionary mechanism, they are responsible for yearly epidemics with substantial morbidity, mortality, and health care costs.[3]

Even if human seasonal influenza is a vaccine-preventable disease, it has a global burden of about 3–5 million cases of severe illness and about 290.000–650.000 respiratory deaths.[3] IAVs are airborne viruses, mainly spread from person to person by inhalation of virus-loaded droplets or via direct or indirect contact with infected individuals.[1,4] They generally cause highly contagious acute respiratory illnesses, and, as for other respiratory viruses, people who live or work in close contact with or in facilities with many other residents are more likely to develop infection.[1,4–6]

Household transmission studies have shown that once one household member is infected with influenza, the risk of infection in a household contact can be up to 38%, and the delay between

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https://doi.org/10.1016/j.puhe.2024.02.027

onset in index and secondary cases is around 3 days.[7,8] A recent study, comparing the risk of influenza virus infection among household contacts in pre- and post-pandemic periods, showed that the adjusted relative risk of A(H3N2) virus infection in 2021/2022 was 2.31 (95%CI, 1.86–2.86) compared with prepandemic seasons.[9] Additionally, several IAV outbreaks involving hospital settings,[10–12] long-term residential care facilities,[13–15] nursing homes,[16,17] military barracks,[18–20] and prisons[21,22] have been largely described. Migrant centres, including refugee centres and asylum seekers centres, are usually open or semi-open crowded communities in which the spread of infectious diseases is likely to happen.[23] The refugee's population is usually made up of young and healthy individuals, and the youngest residents usually show a higher susceptibility in viral transmission. Among infectious diseases, viral respiratory infections can cause large outbreaks and greater morbidity in refugee centres, particularly in consideration of the conditions they experienced during migration and during the hosting of refugee holding facility.[23] Epidemiological investigations are necessary to understand viral transmission routes during a respiratory illness outbreak, such as influenza outbreaks, but molecular characterisation of viral strains can yield insights into transmission dynamics and the description of contacts in order to take proper and targeted control measures.[24]

Here, we describe an influenza A(H3N2) outbreak in a refugee community in Northern Italy (Valle Imagna), including a detailed genetic characterisation of identified viral strains in order to highlight the importance of an integrated epidemiological and sequenced-based investigation to track and characterise epidemic clusters.

Methods

Between November 6th, 2022, and November 9th, 2022, a sharp increase of acute respiratory infection (ARI) cases among residents of two reception centres, one housing 20 refugees (A) and one housing 100 refugees (B), both situated in the province of Bergamo, Lombardy, Northern Italy, came to the attention of the Health Promoting Agency (HPA) of Bergamo (ASST Papa Giovanni XXIII). These two centres are accommodating refugees who originate from an orphanage on the outskirts of Mariupol, Ukraine. Centre B, located in the municipality of Rota Imagna, is home to 100 individuals, including minors and educators, who share facilities, food, and clothing. The youngest residents sleep in 15-bed dormitories with shared bathrooms, while the older ones sleep in 4-bed rooms with en-suite bathrooms. The children attend different schools based on their age, whether it is primary, secondary or e-learning. Communal areas are available for socialising, playing games and watching TV, as well as a designated dining room. Centre A, located in the municipality of Pontida, accommodates 20 children and is comparably smaller and less crowded than Centre B. The children share bedrooms, bathrooms, and toys and attend the same primary school but different classes. Currently, both communities do not have family groupings. It is noteworthy that the food provided is being cooked by volunteers in the kitchens of each house. The two cities are nearly 10 km away from each other and are placed in a pre-Alpine valley in the province of Bergamo, a city of the Lombardy region in Northern Italy.[25] The valley lies between 45° 48′ 51.33″ north latitudes and 9° 30′ 21.21″ east longitudes, with a total geographical area of 108.64 km^2. This valley is located within the hydrographic basin of the Imagna stream, which extends in a north-west/south-east direction from the slopes of Serrada Mount to the confluence of the Imagna stream into the Brembo river. Valle Imagna Community includes 16 municipalities, most of them located between 500 and 700 m above sea level. This community has a total population of 32,274 inhabitants.[25]

On being alerted of a possible outbreak of acute respiratory infection, the surveillance team of the HPA started an epidemiological investigation in order to collect data and biological samples to track this outbreak. Cases were then better defined as influenza-like illnesses (ILIs) following the ILI case definition of the European Centre for Disease Prevention and Control: patients of any age presenting with sudden onset of symptoms including at least one respiratory symptom (cough, sore throat and/or shortness of breath) and at least one systemic symptom (fever/feverishness, malaise, headache and/or myalgia).[26]

Data regarding age, gender, date of onset of first symptoms, and vaccination history (particularly vaccines against respiratory diseases such as COVID-19 and influenza) were recorded. In addition, when possible, a respiratory sample from the upper respiratory tract (nasal or nasopharyngeal swab) was collected from each refugee who fits the ILI case definition. Collected swabs were placed into sterile screw-capped tubes with viral transport media (VTM) and transported at a controlled temperature to the regional reference laboratory (Department of Biomedical Sciences for Health, University of Milan) of the Lombardy region within the Italian influenza surveillance network (InfluNet).[27] At the virology laboratory, RNA was extracted by using the semi-automated extractor QIAcube Connect (QIAGEN) with the QIAamp Viral RNA Mini kit (QIAGEN), following the manufacturer's instructions. A one-step real-time RT-PCR was performed to detect the human ribonuclease P (RNP) gene as an endogenous control in order to check the extraction performance.[28] A differential diagnosis was carried out for each sample to identify 10 viral pathogens (SARS-CoV-2, influenza virus type A and B, respiratory syncytial virus, metapneumovirus, parainfluenza viruses, rhinovirus, enterovirus, parechovirus, and adenovirus) by using specific real-time PCR assays according to the Centers for Disease Control and Prevention (CDC) protocols. Briefly, SARS-CoV-2 RNA was detected by using a one-step real-time RT-PCR targeting different portions of the nucleocapsid gene.[29] A multiplex real-time RT-PCR assay was carried out to simultaneously detect influenza virus types A and B by using specific primer/probe sets targeting the matrix and the non-structural genes, respectively.[28] IAV-positive specimens were further subtyped by one-step RT-PCR assays targeting the hemagglutinin (HA) gene in order to discriminate between A(H1N1) and A(H3N2).[28] Respiratory syncytial virus (RSV) and metapneumovirus (MPV) were detected by a multiplex one-step real-time RT-PCR assay targeting the matrix and the fusion protein gene, respectively.[30,31] Rhinovirus (RV) was detected by using a one-step real-time RT-PCR assay targeting the 5′ non-coding region.[32] A multiplex real-time RT-PCR assay was carried out to simultaneously detect the enterovirus (EV) and human parechovirus (hPeV) genome by using specific primer/probe sets targeting the 5′ untranslated region.[33] Specific one-step real-time RT-PCR assays were conducted to detect parainfluenza viruses (PIV) by targeting the hemagglutinin-neuraminidase and the nucleocapsid protein genes.[34] Adenovirus (AdV) DNA was detected by amplifying a portion of the exon gene by real-time PCR assay.[35]

Results

Overall, 120 ILI cases were reported in all individuals in the reception centres: all 20 individuals in the reception centre A and all 100 individuals in the reception centre B. The first ILI cases experienced the same symptoms, including cough, sore throat, shortness of breath, fever/feverishness, malaise, headache, and/or myalgia, starting on November 6th. Fortunately, they fully recovered within two days, and no one required testing. Cases in

reception centre B were observed between November 8th and November 9th, 2022. All the ILI cases underwent a medical examination on November 10th, 2022; at that time, all residents of the reception centre A (N = 20) were already asymptomatic, while those of reception centre B (N = 100) still had symptoms. For these latter group, a more in-depth epidemiological investigation was conducted, and a nasal-pharyngeal swab (NPS) was collected from 95 symptomatic subjects; all the respiratory samples were collected from health care professionals. Among the 95 ILI cases from the centre B, 55% (52/95) were males, and their age ranged from 9 to 74 years (median age: 14 years; interquartile range, IQR: 4 years). In detail, 50.5% (48) of ILI cases were aged 5–14 years, 39 (41.1%) were aged 15–24 years, 7.4% (7) were 25–64 years, and 1% (1) was ≥65 years (Table 1).

All subjects had fever and respiratory symptoms; 10.5% (10/95) also had tonsillitis which was observed only in the youngest (5–14 and 15–24 years). The 95 residents of reception centre B with ILI lived together, sharing dormitories, toilets, a dining room and all other common areas. It is also of great interest that two children and two workers from reception centre B visited reception centre A in the days before (November 7th) the start of the ILI outbreak in reception centre B, probably acting as spreaders of the infection. Subjects with an age ranging from 9 to 14 years (50.5%; 48/95) also attended the same educational establishment. Two children and two workers of reception centre B visited reception centre A in the days before (November 7th) the ILI outbreak onset in reception centre B.

Virological tests revealed the presence of the IAV genome in 75 out of 95 individuals (78.9%); all of them were A(H3N2). In detail, the attack rate of A(H3N2) was computed by considering only individuals with virological investigation, resulted 83.3% (N = 40) in children aged 5–14-year old individuals, 84.6% (N = 33) in 15–24 years' individuals and 28.6% (N = 2) in adults aged ≥25 years (Table 1). None of them had been immunised with the 2022/2023 influenza vaccine. None of them required hospitalisation.

In 12 out of 75 (16%) A(H3N2)-positive cases, co-detections with other study viruses were observed: in 3 cases a double infection with AdV, in 7 cases with RV, in one case a triple infection with AdV and RV and in another case with EV and PIV. The rate of viral co-detections in A(H3N2)-positive cases differed with respect to the age group; in fact, 27.5% (11/40) of IAV samples from ILIs aged 5–14 years and 3% (1/33) of ILIs aged 15–24 years presented a viral co-detection. No co-detection was observed in the other age groups of 25–64 and ≥65 years (Table 1). Among the IAV-negative patients, 15% (3/20) of samples were positive for other study viruses: AdV, RV, and a co-detection of RV and EV, respectively. For the remaining 17 IAV-negative patients, no aetiology of the respiratory disease for the investigated viruses could be defined.

The complete HA gene sequencing and the related phylogenetic analysis were conducted in order to characterise the study A(H3N2) strains. Particularly, 25% (19/75) of A(H3N2)-positive samples underwent HA gene amplification and Sanger sequencing, as previously described.[36] All selected samples were successfully amplified and sequenced. All edited and assembled HA nucleotide sequences of this study were submitted to GISAID database[37] under the following accession numbers: EPI_ISL_18045081-99. After the alignment of the HA study sequences with reference sequences, phylogenetic trees were inferred by means of the neighbour-joining method and the Kimura 2-parameter model, with 1000 bootstrap replicates, using the bioinformatic programme MEGA6.[38]

As shown in Fig. 1, the A(H3N2) strains of this study clustered together (bootstrap: 99%), sharing a mean intra-group nucleotide identity of 100% (range: 99.8–100%), which was calculated by using the sequence identity matrix tool of BioEdit software.[39] A pairwise distance analysis within the cluster sequences was also conducted using the bioinformatic programme MEGA6[38] and showed a mean p-distance of 0.0003. Four sequences showed a different nucleotide within the coding region of the HA gene: A153T, A362G, A464C and A951T, respectively. The intra-group comparison of the predicted amino acid sequences of this study, obtained by the Toggle translation tool implemented in BioEdit,[39] revealed that in two cases (A153T and A951T), a synonymous mutation was observed; instead, in the other two cases (A362G and A464C), there was a missense mutation, resulting in the amino acid substitutions K121R and T155N. However, the overall mean intra-group amino acid similarity was 100%, ranging from 99.8% to 100%.

The phylogenetic analysis of the study strains with other field strains and reference sequences revealed that all the study sequences belonged to the clade 3C.2a, sub-clade 3C.2a1b, and the genetic sub-group 3C.2a1b.2a.2, which includes both the 2022/2023 A(H3N2) circulating strains identified by our laboratory during the influenza surveillance activities in a period spanning from August 2022 to November 2022 and the 2022/2023 vaccine strain (A/Darwin/9/2021, EPI_ISL_2233240) for the Northern hemisphere. Comparing the HA gene sequences of the study A(H3N2) strains with those of the 2022/2023 A(H3N2) circulating strains, a mean nucleotide identity of 99.6% (range: 98.0–100%) and amino acid similarity of 99.6% (range: 97.8–100%) were observed. The mean pairwise distance between the two groups was 0.003. The comparison between the study strains and the vaccine strain (A/Darwin/9/2021) showed a mean nucleotide identity of 98.6% (range: 98.5–98.6%) and a mean amino acid similarity of 98.4% (range: 98.3–98.4%). The predicted HA amino acid sequences revealed several substitutions between the vaccine strain and the study A(H3N2) viruses; particularly, the amino acid substitutions E50K, F79V, T135A, I140K, S256H, N186D, G225D and S262N characterised the study strains as well as the majority of 3C.2a1b.2a.2 HA sequences compared to the vaccine strain sequence.

Discussion

This study reports the results of the epidemiological and virological examination as part of an outbreak investigation identified among residents of a refugee's community located in a small valley in Northern Italy. Through the epidemiological investigations, we defined the involved ILI cases and the epidemic period, but it was

Table 1
Distribution of ILI cases, influenza A(H3N2) attack rate and viral detection by age group. ILI, influenza-like illness.

Age group (years)	ILI cases		A(H3N2) attack rate		Other viruses[a] in A(H3N2)-positive ILI cases		Other viruses[a] in A(H3N2)-negative ILI cases	
	No.	%	No.	%	No.	%	No.	%
5-14	48	50.5%	40	83.3%	11	27.5%	1	2.1%
15-24	39	41.1%	33	84.6%	1	3.0%	0	0%
25-64	7	7.4%	2	28.6%	0	0%	2	28.6%
>65	1	1%	0	0%	0	0%	0	0%
Total	**95**	**100%**	**75**	**78.9%**	**12**	**16%**	**3**	**3.1%**

[a] Including: SARS-CoV-2, respiratory syncytial virus, metapneumovirus, parainfluenza viruses, rhinovirus, enterovirus, parechovirus and adenovirus.

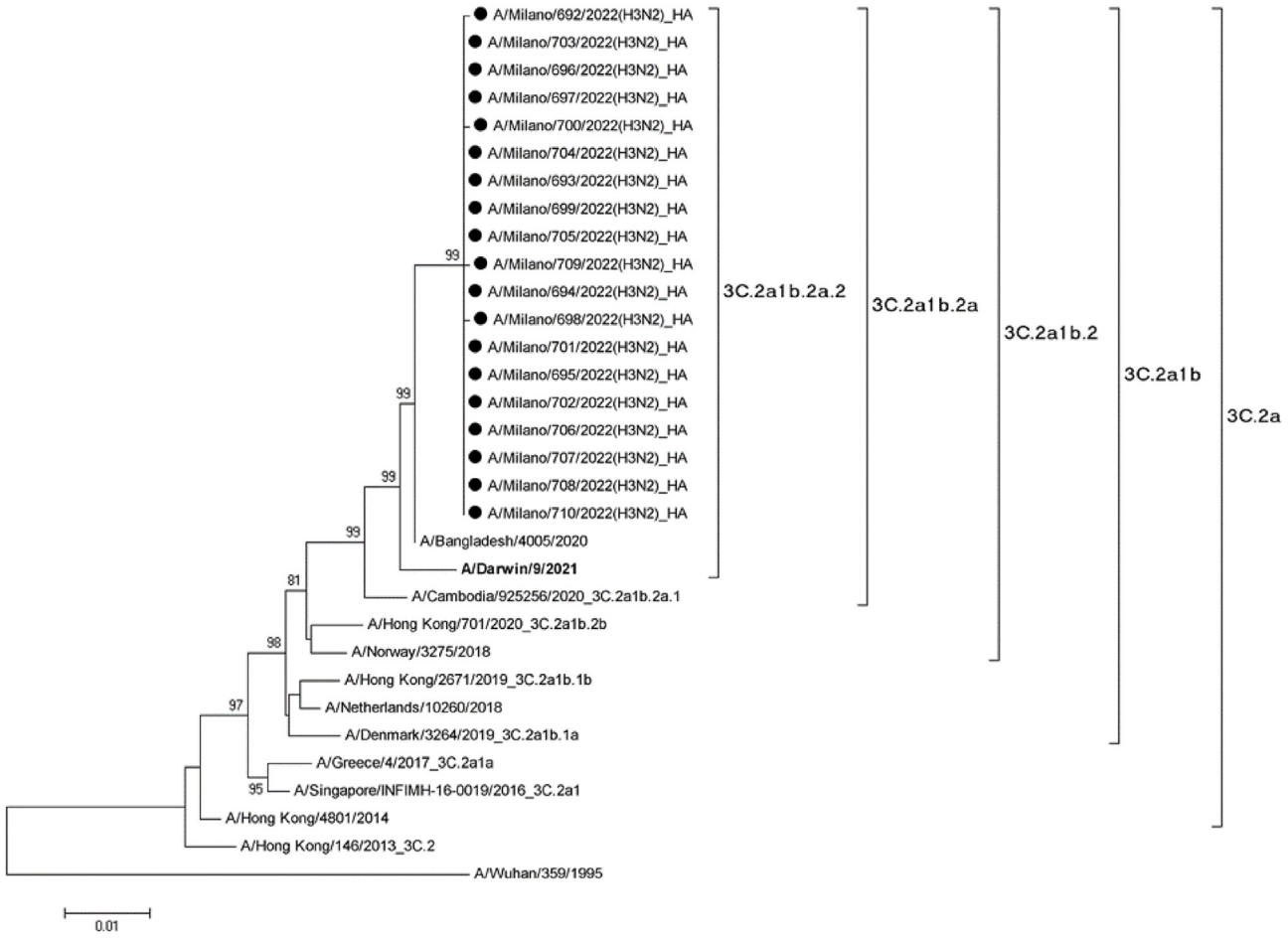

Fig. 1. Phylogenetic tree of the 19 HA nucleotide sequences of study A(H3N2) strains (named as A/Milano and marked with black dots, ●) and reference strains (N = 13), including the 2022/2023 vaccine strain for the Northern Hemisphere (A/Darwin/9/2021; in bold). HA, hemagglutinin.

only through the molecular investigations that we were able to prove that the outbreak of acute respiratory infection was caused by the A(H3N2) influenza virus. In fact, among the 95 symptomatic ILI cases identified in 2 days at the study reception centre and undergone virological analyses, the A(H3N2) positive rate was 79%. The multitarget assays involved in this outbreak investigation were useful not only to promptly identify the causative agent of the outbreak but also to have a wider virological and epidemiological overview of a specific geographic area over a given period of time. In fact, besides A(H3N2), we identified the presence of at least one other of the study viruses (SARS-CoV-2, respiratory syncytial virus, metapneumovirus, parainfluenza viruses, rhinovirus, enterovirus, parechovirus, and adenovirus) in 13% of the respiratory samples collected from the individuals involved in the outbreak, and considering the ILI cases (21%) not linked to the A(H3N2) outbreak, 3% of them were positive for other respiratory viruses (rhinovirus, adenovirus and enterovirus), whereas for 18% of ILIs the aetiology remained undefined. It should be noted that only virological investigations were conducted during this outbreak investigation. Therefore, it was not possible to determine whether ILI cases that tested negative for influenza were positive for other microbiological pathogens (such as bacteria, other viruses, or fungi) that were not included in the outbreak investigation.

In our study, the majority of ILI cases involved in the A(H3N2) influenza outbreak were children and adolescents who lived together, sharing most of the environments of everyday life: the young age and the close-contact life are two major conditions well described as risk factors for spreading of respiratory pathogens.[4,5,7,9,40] Moreover, the absence of vaccination may have greatly amplified the spread of the infection in only two days, as observed by other authors in vulnerable settings including hospital wards and long-term care facilities,[10,11,13] although it is fortunate to report that no hospitalisations were necessary here. The sequencing results showed that the viral strains identified from residents of the study reception centre were closely related since they segregated in the same cluster, showing both a high mean nucleotide identity (100%) and a high mean amino acid similarity (100%). Moreover, the mean genetic distance here obtained among outbreak strains was lower than the value obtained by comparing the study strains with other circulating strains (0.0003 vs. 0.003); once more, this result highlights the strong genetic relationship among the study strains. However, a certain evolutionary pressure of the A(H3N2) strains was observed even among the study sequences. The influenza viruses' continuous ability to mutate, especially for A(H3N2) viruses, has already been proven in previous studies.[36,41] In the present study, despite the high similarity identified among the study sequences, the presence of nucleotide mutations, some of them also translated into amino acid substitutions, in four study sequences can underline a possible, albeit limited, intra-host selection of A(H3N2) viruses.

Even if the study sequences clustered together and had a certain genetic distance compared to field strains, the A(H3N2) influenza strains causing the outbreak here described are genetically similar to those circulating in the community during the same period; in

fact, they showed a mean nucleotide identity and a mean amino acid similarity greater than 98%, all of them belonging to the same genetic sub-group 3C.2a1b.2a.2. Moreover, the study strains as well as the majority of field strains had the same major amino acid substitutions compared to the 2022/2023 vaccine virus. These observations allowed us to speculate that outbreak strains do not evolve from one common ancestor but most likely originate from a strain circulating within the community.

Finally, the fact that influenza outbreak strains as well as the community strains are genetically related to the seasonal vaccine strain suggests that if an influenza prevention by vaccination strategy had been implemented, a lower attack rate might have been achieved. Although it is pivotal to examine asymptomatic individuals and contacts of those who have become symptomatic during an outbreak investigation from a public health perspective to reduce the magnitude of the epidemic wave, this study only tested individuals who showed ILI symptoms from centre B. It is important to note that individuals from centre A had already resolved their ILI symptoms at the time of the medical investigation.

Influenza vaccines are available for all subjects upto 6 months of age, and seasonal vaccination is recommended to protect high-risk groups, such as toddler, the elderly, people with chronic medical conditions, pregnant women, and immunocompromised individuals, from influenza-related complications and death.[3] Moreover, this important preventive measure should always be promoted to reduce the morbidity of the influenza illness in all the contexts in which people live in close contact and, therefore, where a viral airborne transmission is more likely to occur, such as in reception centres for refugees like the one here studied. New preventive approaches are based on the observation that much of the influenza transmission takes place in children's daycare facilities and amongst school-age children and adolescents: immunising these groups of population, in addition to the older population, may reduce overall influenza transmission and protect those in the risk groups.[42]

Through this study, we are highlighting the importance of an integrated epidemiological and sequence-based investigation to track and characterise an epidemic cluster. By matching epidemiological and molecular investigations, we were able to distinguish and profile the A(H3N2) influenza outbreak among residents of a reception centre in a small valley in Northern Italy.

Some limitations should be acknowledged in the study of this A(H3N2) outbreak: information on ILI cases and vaccination status among workers in the reception centres and eventual visitors was not collected, and we were not able to virologically investigate respiratory samples from ILI cases living in the reception centre A.

In conclusion, the epidemiological investigation is the primary tool for defining an outbreak by gathering information on the cases involved, including their magnitude, temporal distribution, and spatial spread. However, only molecular investigations using virological tests and sequence-based typing with phylogenetic analyses of microorganisms, performed in advanced virology laboratories, can provide information to define outbreak clusters, better characterise the pathogen involved, and implement control measures in a timely manner. This integrated approach should always be the best practice to pursue in order to detect and study outbreaks caused by infectious pathogens, such as the influenza virus. However, in addition to the benefits of this approach, it is important to introduce non-pharmaceutical interventions at both the individual and facility levels to reduce the risk of a respiratory virus outbreak in high-density settings. It is crucial to maintain a balanced approach that considers both pharmaceutical and non-pharmaceutical interventions. For example, providing non-dormitory accommodation could have prevented or at least mitigated the outbreak. Furthermore, when it is not feasible to isolate or reduce interaction among individuals living in a community, it is important to practice certain virtuous behaviours, such as frequent handwashing, thorough disinfection of common areas and surfaces, and wearing a high-quality mask to protect the airway. These measures should always be considered to prevent the spread of airborne-transmitted microorganisms. Adequate hygiene measures are certainly important in reducing their transmission. However, vaccination with high immunisation coverage remains the primary tool for influenza prevention and control, particularly for vulnerable individuals and in high-risk settings.

Author statements

Ethical approval

The study was conducted in accordance with the Declaration of Helsinki. This study was performed according to the Institutional Review Board guidelines concerning the use of biological specimens for scientific purposes in compliance with Italian law (Art.13 D.Lgs. 196/2003). Approval from an ethics committee and informed consent (either written or verbal) for influenza detection, typing and molecular characterisation were not required since data and samples from individuals were collected and analysed anonymously within the National Influenza Surveillance Programme and they were managed according to the Good Laboratory Practice procedure.

Funding

This research was partially supported by EU funding within the Next Generation EU-MUR PNRR Extended Partnership initiative on Emerging Infectious Diseases (Project no. PE00000007, INF-ACT).

Competing interests

Nothing to declare.

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