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Rabies in an Arctic fox on the Svalbard archipelago, Norway, January 2011

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We report a case of rabies in an Arctic fox. In January 2011 a fox attacked dogs belonging to a meteorological station in the Svalbard archipelago, Norway. Rabies virus was detected in the fox's brain post-mortem. The dogs had been vaccinated against rabies and their antibody levels were protective. Post-exposure prophylaxis was administered to staff at the station. Rabies vaccination is recommended for inhabitants and visitors to the Arctic who may be in contact with wild animals.

Case of rabies in an Arctic fox

On 4 January 2011 an Arctic fox (*Vulpes lagopus*) attacked a group of four dogs on the small island of Hopen (76.30° N, 25.01° E) located in the south-eastern part of the Svalbard archipelago, Norway, in the Arctic.

The dogs belong to the staff of four persons operating a meteorological station on this island and are primarily kept to warn against and scare off migrating polar bears. During the attack, two dogs were bitten before they killed the fox (Figure). The staff suspected that the animal might be infected with rabies virus because of its aggressive behaviour. No staff had been bitten by the fox, however three had handled the dead body.

The Governor of Svalbard was immediately informed about the incident. After consultation with the competent authority, the Norwegian Food Safety Authority, the Governor approved helicopter transport of the carcass to the main island of Svalbard, Spitsbergen, and from there to the Norwegian Veterinary Institute (NVI) in Oslo, which is the reference laboratory for animal rabies in Norway.

Investigations undertaken

A necropsy of the fox was performed at the biosafety level 3 laboratory of NVI and brain samples were collected for lyssavirus examination. The fox was a thin, young adult male. Its eyes lay deep in the orbit and the nictitating membranes showed pronounced protrusion. The stomach contained small amounts of seaweed and white hairs. The content of the small intestine was sparse and the colon contents were dark and watery, indicating diarrhoea.

Seven samples from different parts of the brain were analysed by direct fluorescent antibody test (FAT) and reverse transcription polymerase chain reaction (RT-PCR). All samples were positive for rabies virus. A 571 base pair fragment of the viral nucleoprotein (N) gene was sequenced: a BLAST search against the GenBank database showed 99% identity with rabies virus isolates from the Arctic [1,2]. The results were confirmed by the Friedrich-Löeffler Institute, Germany, the World Organisation for Animal Health (OIE) reference laboratory for rabies.

Measures implemented

On the evening of 7 January health officials at the Longyearbyen hospital at Svalbard were informed that the fox was infected with rabies. The staff at the meteorological station had not previously been vaccinated against rabies. The dogs had been vaccinated (a mandatory requirement), but as their rabies-specific antibody titres were unknown, and given the risk of staff members being bitten when handling the animals, it was decided that post-exposure prophylaxis should be administered to all four staff. Human rabies immune globulin was transported overnight from the mainland. After consultation with the Norwegian Institute of Public Health, the following regime was adopted: On the first day of immunisation (four days after the incident) the person having been in closest contact with the dead fox was given one dose of immunoglobulin plus one dose of vaccine, and the other three staff members were given two doses of vaccine. This was followed by one dose after three, eight and 15 days. This regime for vaccination is in accordance with the new recommendations from the Advisory Committee on Immunization Practices at the United States Centers for Disease Control and Prevention [3] and the World Health Organization (WHO) [4].

The dogs had been vaccinated against rabies at annual intervals, with booster shots as recently as 28

December 2010. An inactivated vaccine (Rabisin, Merial) was used, providing a protective immune response by stimulating antibody production against glycoprotein surface antigens of rabies virus. The antibody response following a booster dose normally peaks a week following injection and stays at a high level. To confirm an adequate level of antibodies, blood samples taken from the dogs on 12 January were tested by the OIE-prescribed fluorescent antibody virus neutralisation assay. All the dogs had an antibody titre of >0.5 international units (IU)/ml (National Veterinary Institute, Uppsala, Sweden), which is the level accepted by OIE and WHO as indicating a protective response. For the dogs bitten by the rabid fox, the risk of contracting disease is considered negligible. The OIE recommends an observation period of 45 days for vaccinated dogs post exposure. The dogs at the meteorological station have therefore been isolated and contact between staff and dogs will be limited during this period.

Rabies in the Arctic

Rabies is regarded as an endemic disease throughout most parts of the Arctic, and several epidemics have been reported during the last 40–50 years in arctic Canada, Russia and Greenland [5]. The Arctic fox is the main host of the virus, and the same arctic virus variant seems to infect the Arctic fox throughout the area it inhabits. How rabies is maintained in the fox population remains largely unknown. On Svalbard, rabies was diagnosed for the first time in 1980 during an outbreak in the Arctic fox population [6]. In 1980 to 1999, a total of 25 animals were diagnosed with rabies on the islands, including three reindeer (*Rangifer tarandus platyrhynchus*) and one ringed seal (*Pusa hispida*). No further cases had been reported until the present case. Mainland Norway remains rabies free.

Concluding remarks

Rabies occurs sporadically in wildlife in the Arctic. Surveillance of the disease is important to obtain more information about the epidemiology and risks of human and animal exposure. Dead foxes and other animals should not be touched with naked hands, but secured in a plastic bag for laboratory examination. Any dead animal should be reported immediately to the Governor of Svalbard. Rabies in foxes is not characterised by specific gross lesions at necropsy, but signs of dehydration, atypical stomach content, low food intake and low body weight should raise suspicion of this disease and especially if the animal had shown aggressive behaviour.

The Governor maintains a register of all domestic animals in the islands and they are mandatorily vaccinated against rabies as a prophylactic measure to protect the resident animal population and reduce the risk of human exposure. Transmission to humans has never been documented. However, visitors to the region, particularly hunters and wildlife explorers, should be aware of the risk of rabies. Given the risk, rabies vaccination is recommended for people in the region who may be in contact with wild animals. In this remote and sparsely populated area, it is particularly important that immunoglobulin and vaccines are readily available, to keep the time between exposure and immunisation to a minimum. Following this incident, rabies immunoglobulin and vaccine are now stored at the local hospital in Longyearbyen. In addition, local health authorities at Svalbard will more actively promote pre-exposure rabies vaccination to persons at risk.

Figure

A rabid Arctic fox attacks a dog at Hopen meteorological station, Svalbard, Norway, January 2011

Photo: Ragnar Sønstebø, Hopen meteorological station.

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Effectiveness of trivalent seasonal and monovalent influenza A(H1N1)2009 vaccines in population with major chronic conditions of Navarre, Spain: 2010/11 mid-season analysis

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We defined a cohort of people with major chronic conditions (152,585 subjects) in Navarre, Spain, using electronic records from physicians, to obtain 2010/11 mid-season estimates of influenza vaccine effectiveness. The adjusted estimates of the effectiveness of the 2010/11 trivalent influenza vaccine were 31% (95% confidence interval (CI): 20–40%) in preventing medically attended influenza-like illness, and 58% (95% CI: 11–80%) in preventing laboratory-confirmed influenza. Having received the monovalent influenza A(H1N1)2009 vaccine in the 2009/10 season had an independent preventive effect against medically attended influenza-like illness (17%, 95% CI: 1–30%), and having received both vaccines had 68% (95% CI: 23–87%) effectiveness in preventing laboratory-confirmed influenza.

Introduction

Because the influenza vaccine composition is adapted every season to the circulating viruses, its effectiveness varies. Estimates of the effectiveness of the vaccine during the influenza season help guiding health interventions aimed at reducing the impact of influenza in the population [1]. In the absence of randomised trials evaluating the efficacy of this vaccine, observational studies are of interest to verify if the expected effect has been achieved [1-3]. A multi-centre European study (I-MOVE: Influenza Monitoring Vaccine Effectiveness in Europe) was launched in 2008, including cohort and case-control studies in several settings. As part of this project, a cohort study is being conducted in Navarre, Spain [1].

During the early 2010/11 season, the influenza A(H1N1)2009 virus was the predominant circulating influenza virus [4]. It is therefore expected that both the trivalent 2010/11 seasonal vaccine, which includes this virus, [5] and the monovalent influenza A(H1N1)2009 vaccine [6] may provide some protection. Several studies have reported high effectiveness of the monovalent pandemic vaccine in preventing influenza A(H1N1)2009 during the 2009/10 season [7-11]. The aim of this study was to provide early estimates of the effectiveness of the 2010/11 seasonal vaccine and the influenza A(H1N1)2009 vaccine administered during the 2009/10 season in preventing medically attended influenza-like illness (MA-ILI) and laboratory-confirmed influenza during the 2010/11 season. The study was restricted to the population with major chronic conditions, since vaccination with both influenza vaccines was recommended for this group.

Methods

Study population and data collection

We conducted a prospective cohort study based on electronic records of physicians and laboratories and a nested case–control analysis of swabbed patients in the region of Navarre, Spain. This cohort included all non-institutionalised persons covered by the Regional Health Service (95% of the population of the region) with known pre-existing major chronic conditions (heart disease, lung disease, renal disease, cancer, diabetes, cirrhosis, dementia, stroke, immunodeficiency and body mass index of 40 or greater). The Navarre Ethical Committee for Medical Research approved the study protocol. The present study analysed the cases registered from 24 October 2010 (first week in which influenza virus was detected in the region) to 22 January 2011.

The seasonal influenza vaccination campaign took place from 11 October to 26 November 2010, although

a very small number of doses were still administered after that period. The trivalent inactivated non-adjuvanted vaccine (Sanofi Pasteur MSD) was used for all subjects. Monovalent influenza A(H1N1)2009 vaccine had been administered exclusively from November 2009 to January 2010, using the MF59-adjuvanted vaccine from Novartis (Focetria) for children up to the age of 17 years and for adults aged 60 years and older, the AS03-adjuvanted vaccine from GlaxoSmithKline (Pandemrix) in adults between 18 and 59 years of age, and the non-adjuvanted vaccine from Sanofi Pasteur (Panenza) for pregnant women. All these vaccines were offered free of charge to individuals with major chronic conditions and other populations with specific indications. Precise instructions for registering each dose were given to all vaccination points. For the present study, influenza vaccine status was obtained from the online regional vaccination register that is updated by the healthcare centres of the Regional Health Service. Subjects were considered to be protected 14 days after vaccine administration.

Influenza surveillance is based on automatic reporting of cases from all primary healthcare centres. Cases of MA-ILI are defined according to the International Classification of Primary Care version 2 (code R80) [12]. Two laboratories perform influenza testing in the region and provided the data for virological surveillance. All hospitalised patients with ILI or other acute respiratory diseases were swabbed for influenza virus testing. In addition, through a sentinel network composed of a representative sample of primary healthcare physicians covering 16% of the population, nasopharyngeal and pharyngeal swabs were taken from all patients

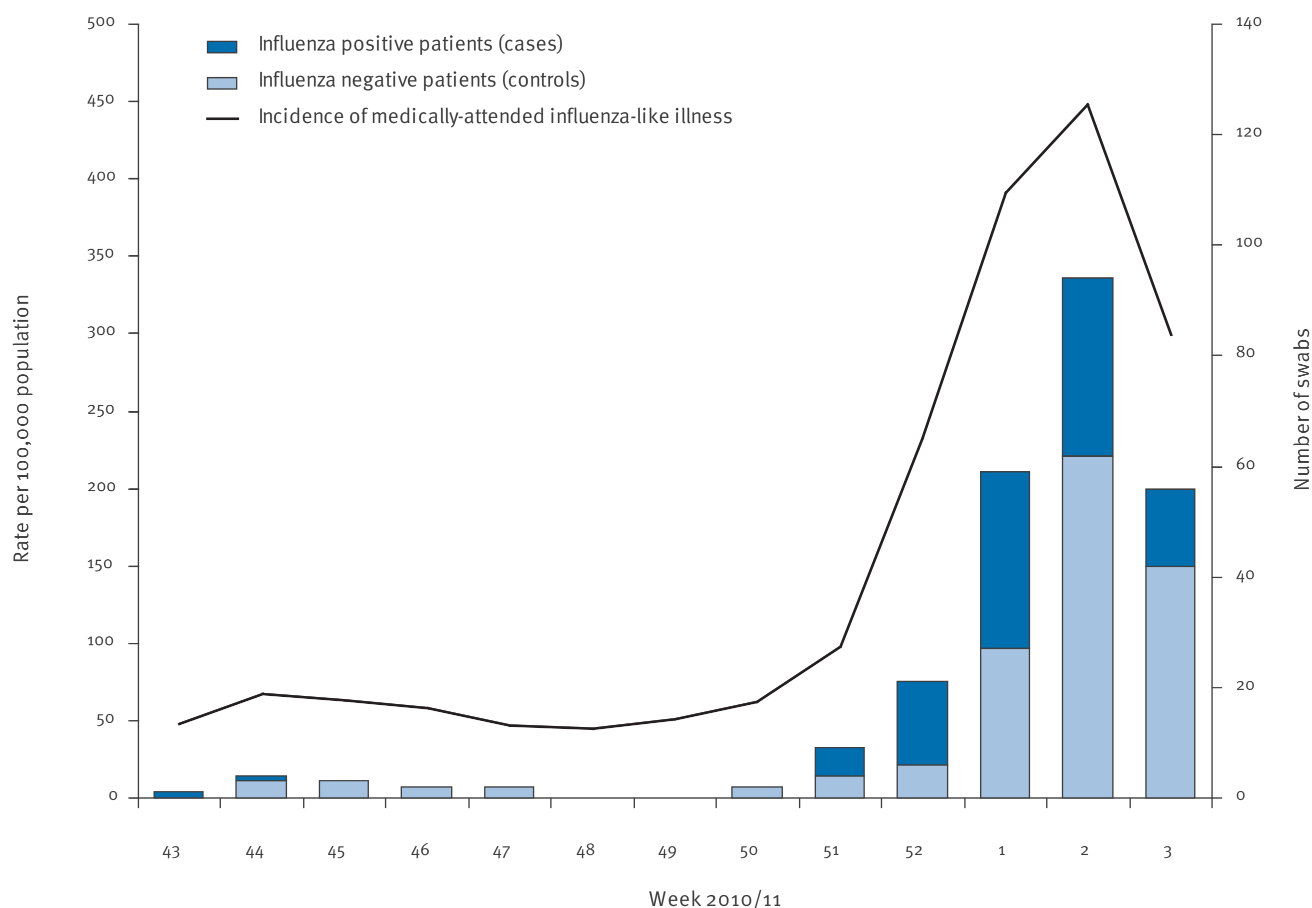
Table 1

Population with major chronic conditions included in the cohort study and vaccine coverage by age group, Navarre, Spain, 2010/11 (n=152,585)

Age group	Population (number)	Seasonal vaccine 2010/11 coverage (%)	Pandemic vaccine in 2009/10 coverage (%)	Both vaccines coverage (%)
1 to 59 years	81,407	11.3	7.7	4.2
≥ 60 years	71,178	60.0	26.2	22.5
Total	152,585	34.0	16.4	12.7

Figure 1

Weekly incidence of medically attended influenza-like illness and swabbed patients (n=253) according to influenza virus test result in the population with major chronic conditions, Navarre, Spain, 24 October 2010–22 January 2011



with MA-ILI, after obtaining verbal informed consent. Swabs were processed by RT-PCR assay and virus culture. Positive samples were characterised as influenza A (H1 and H3) and B virus using immunofluorescence and RT-PCR. Real-time RT-PCR for detection of the influenza A(H1N1)2009 virus was performed for all swabs.

From the electronic primary healthcare records we obtained the following baseline variables: sex, age, migrant status, district of residence, major chronic conditions, number of outpatient visits during the previous 12 months, and children in the household.

Study design and statistical analysis

In the cohort analysis, the incidence rates of MA-ILI in primary health care were compared in vaccinated and unvaccinated persons. Cox regression models were used to obtain MA-ILI-adjusted hazard ratios (HRs) for influenza vaccination status. Calendar time was used as the underlying time variable, with exit time as the date of MA-ILI diagnosis, death, or 22 January 2011

Table 2

Estimates of the effect of the 2010/11 seasonal influenza vaccine and influenza A(H1N1)2009 vaccine in preventing medically diagnosed influenza-like illness in the population with major chronic conditions, Navarre, Spain, 24 October 2010–22 January 2011 (n=152,585)

	Person-years	Cases	Crude hazard ratio (95% CI)[a]	Adjusted hazard ratio (95% CI)[b]
Analysis 1				
Seasonal vaccine 2010/11				
Yes	10,828	296	0.36 (0.32-0.42)	0.69 (0.60-0.80)
No	26,569	1,736	Reference	Reference
Pandemic vaccine 2009/10				
Yes	6,102	172	0.78 (0.66-0.92)	0.83 (0.70-0.99)
No	31,295	1,860	Reference	Reference
Analysis 2				
Seasonal and pandemic vaccines	4,108	100	0.30 (0.25-0.37)	0.59 (0.47-0.73)
Only seasonal vaccine 2010/11	6,720	196	0.35 (0.30-0.41)	0.69 (0.58-0.81)
Only pandemic vaccine 2009/10	1,994	72	0.72 (0.57-0.91)	0.81 (0.64-1.03)
Unvaccinated	24,575	1,664	Reference	Reference

CI: confidence interval.

[a] Cox regression model including vaccination status for 2010/11 seasonal and pandemic influenza A(H1N1)2009 vaccines.

[b] Cox regression model adjusted for sex, age group, major chronic conditions, outpatient visits during baseline period (tertiles within each age stratum), urban/rural residence, migrant status and children in the household, and stratified by age (1-14; 15-59; ≥60 years) and health district.

Table 3

Estimates of the effect of the 2010/11 seasonal influenza vaccine and influenza A(H1N1)2009 vaccine in preventing laboratory-confirmed influenza in the population with major chronic conditions, Navarre, Spain, 24 October 2010–22 January 2011 (n=253)

	Cases/controls	Crude odds ratio (95% CI) [a]	Adjusted odds ratio (95% CI) [b]
Analysis 1			
Seasonal vaccine 2010/11			
Yes	22 / 78	0.32 (0.17-0.60)	0.42 (0.20-0.89)
No	78 / 75	Reference	Reference
Pandemic vaccine 2009/10			
Yes	16 / 51	0.69 (0.33-1.41)	0.78 (0.35-1.73)
No	84 / 102	Reference	Reference
Analysis 2			
Seasonal and pandemic vaccines	10 / 43	0.22 (0.10-0.47)	0.32 (0.13-0.77)
Only seasonal vaccine 2010/11	12 / 35	0.32 (0.15-0.67)	0.45 (0.19-1.03)
Only pandemic vaccine 2009/10	6 / 8	0.70 (0.23-2.12)	0.88 (0.25-3.18)
Unvaccinated	72 / 67	Reference	Reference

CI: confidence interval.

[a] Logistic regression model including 2010/11 seasonal and pamdemic influenza A(H1N1)2009 vaccination status.

[b] Logistic regression analysis adjusted for sex, age (1-14; 15-59; ≥60 years), children in the household, urban/rural residence, healthcare setting (primary healthcare, emergency room, hospitalisation) and date (Week 43–49 2010; Week 50 2010–Week 1 2011; Week 2–3 2011).

(end of this mid-season analysis), whichever came first. Vaccination status for the 2010/11 seasonal trivalent inactivated vaccine was included in the analyses as a time-dependent variable. The models were stratified by health district and age (1-14, 15-59, ≥60 years) because patients younger than 15 years are cared for by paediatricians and the vaccine coverage is higher among those aged 60 or older. Other potential confounders were adjusted for in the models, with age in intervals of 10 years and the number of outpatient visits categorised in tertiles within each age stratum.

From the cohort population, all outpatients and hospitalised patients who were swabbed during the study period were included in a case–control analysis that compared seasonal vaccination status in patients in whom any influenza virus was detected (cases) and those who were negative for influenza (controls). Crude and adjusted estimators of the effect were quantified by odds ratios (ORs) with their 95% confidence intervals (CI), calculated using logistic regression models.

The effects of the seasonal vaccine and the pandemic influenza A(H1N1)2009 vaccine were evaluated as independent variables in one model, and as a combined variable (unvaccinated, only seasonal vaccine, only pandemic vaccine, or both vaccines) in a different model. Vaccine effectiveness was estimated as a percentage: (1–HR)×100 or (1–OR)×100.

Results

Vaccine effectiveness in preventing medically attended influenza-like illness

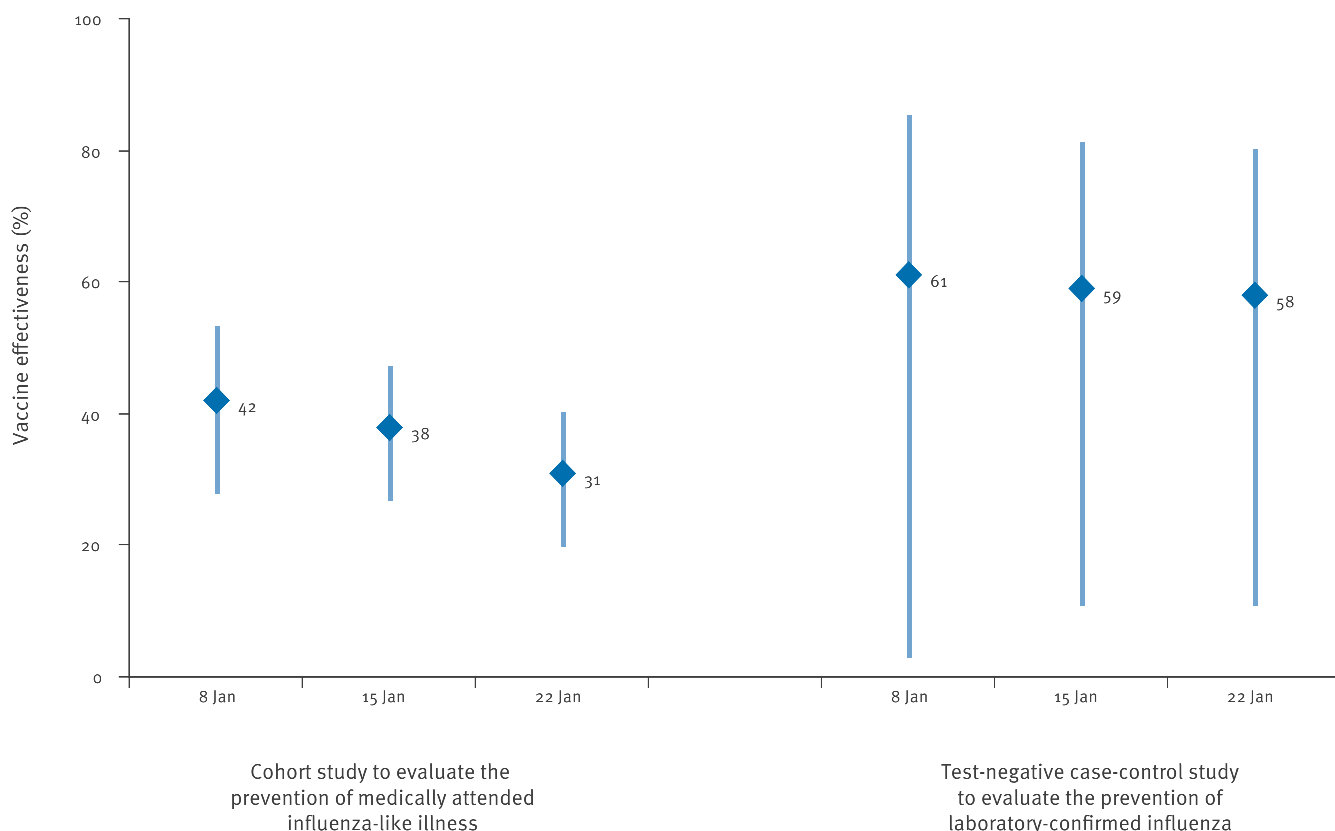
A total of 152,585 persons had major chronic conditions registered at baseline and were included in the cohort study, with 46.6% aged 60 years old or older. The seasonal influenza vaccine coverage for 2010/11 was 34.0%, and 16.4% had received the influenza A(H1N1)2009 pandemic vaccine in 2009/10 (Table 1).

From week 43 of 2010 (first influenza virus detection in the season) to week 3 of 2011, 2,032 cases of MA-ILI were diagnosed among the 152,585 cohort subjects in primary care centres, with the highest incidence in week 2 of 2011 (Figure 1). Eighty-nine of these patients were swabbed by sentinel physicians, and 51 (57%) of them were found positive for influenza virus.

The incidence rate was 27 per 1,000 vaccinated person-years with the seasonal vaccine as opposed to 65 per 1,000 unvaccinated person-years ($p<0.001$). In the adjusted Cox regression model the seasonal vaccine effectiveness against MA-ILI was 31% (HR=0.69; 95% CI: 0.60–0.80), and the effectiveness of the monovalent pandemic vaccine was 17% (HR=0.83; 95% CI: 0.70–0.99). As compared with unvaccinated

Figure 2

Effectiveness of the 2010/11 seasonal influenza vaccine in preventing medically attended influenza-like illness and laboratory-confirmed influenza in the population with major chronic conditions, Navarre, Spain[a]



[a] Preliminary estimates obtained for the periods from 24 October 2010 to 8, 15 and 22 January 2011, respectively.

individuals, having received both vaccines provided a 41% reduction in the incidence of MA-ILI (HR=0.59; 95% CI: 0.47–0.73) (Table 2).

Vaccine effectiveness in preventing laboratory-confirmed influenza

During the study period swabs were analysed from 253 cohort patients who had MA-ILI (n=89) or were treated in hospitals for acute respiratory infection (n=164), and had major chronic conditions (Figure 1). A total of 100 cases (39.5%) were confirmed for influenza: 97 were positive for the influenza A(H1N1)2009 virus, one for influenza A(H3N2) and two for influenza B. There were 22 laboratory-confirmed cases in patients who had received the 2010/11 seasonal vaccine. Their mean age was 66 years (range: 52–84 years) and 10 of them had also been vaccinated with monovalent influenza A(H1N1)2009 vaccine. In the cases with vaccine failure the time from seasonal vaccination to diagnosis ranged 57 to 91 days. At baseline, 10 of these cases had lung diseases, nine had diabetes mellitus, seven had cardiovascular diseases, five had cancers, four had renal diseases and one had liver disease.

Compared with the influenza-negative controls, cases were less likely to have received the influenza seasonal vaccine (OR=0.32; 95% CI: 0.17–0.60). In the logistic regression analysis adjusting for sex, age (1-14; 15-59; ≥60 years), living with children, living in an urban/rural area, healthcare setting (primary healthcare, emergency room, hospitalisation) and date (Week 43–49 2010; Week 50 2010–Week 1 2011; Week 2–3 2011), seasonal influenza vaccination was associated with a 58% lower probability of a positive swab (OR=0.42; 95% CI: 0.20–0.89). The pandemic influenza vaccine showed a lower, not statistically significant, protective effect against laboratory-confirmed influenza (OR=0.78, 95% CI: 0.35–1.73). The interaction term between both vaccines was not significant (p=0.95). Compared with not being vaccinated, having received both vaccines provided 68% protection against laboratory-confirmed influenza (OR=0.32; 95% CI: 0.13–0.77) (Table 3).

Early estimates of influenza vaccine effectiveness
Effectiveness estimates made at the end of week 1 and 2 of 2011, when the numbers of influenza cases were still increasing, produced similar results (Figure 2). It is worth noticing the progressive decrease in the estimates of effectiveness in preventing MA-ILI, which coincides with a reduction in the percentage of swabs positive for influenza.

Discussion

The mid-season results of this study show a moderate protective effect of the 2010/11 seasonal influenza vaccine in preventing laboratory-confirmed influenza and MA-ILI during the 2010/11 seasonal period in a high-risk population. In these analyses, receipt of the monovalent influenza A(H1N1)2009 pandemic vaccine in the previous season also showed a small preventive effect. Influenza A(H1N1)2009 virus was found in 97% of the laboratory-confirmed influenza cases and was included in both vaccines, which is consistent with the observed protection. The greatest protective effect was seen in people who had received both vaccines, which could be interpreted as a dose-response effect. Similar findings have been reported in a mid-season analysis in the United Kingdom [13].

This moderate effect is in contrast with the more pronounced protection reported for the 2009/10 season [7-11]. In addition, we detected a number of vaccine failures in persons with laboratory-confirmed influenza. Unlike the pandemic vaccine administered in 2009/10, the 2010/11 seasonal vaccine used in Navarre was not adjuvanted and this could explain a slightly lower immune response. The antigenic drift of the circulating virus could produce a certain degree of mismatch with the vaccine virus, although virological surveillance does not support this so far [14]. Factors such as advanced age or some immunodepression may be more common among people with major chronic conditions, which would explain a poor response to the vaccine. The reduced effect of the monovalent pandemic vaccine in this season can be explained by the loss of immune response more than a year after its administration.

The results presented here are preliminary and may have limited statistical power for some analyses. Therefore the final results for the season may be different. Cohort studies can be affected by biases if those who are vaccinated tend to have poorer health status or if, on the contrary, they tend to take better care of their health than the unvaccinated [15-16], but our analyses were controlled for the most frequently recognised confounders [17]. All the analyses were restricted to the population with major chronic conditions in whom vaccination was indicated. Calendar time was used as the underlying time variable in the Cox regression analysis to control for its possible confounding effect. The case–control analysis only included laboratory-confirmed cases and compared them with controls recruited in the same healthcare settings before patient and physician knew the laboratory result, a fact that reduced selection bias.

The analyses of the vaccine effectiveness against two outcomes, in the same place and period, provide complementary information. The effectiveness of 58% in preventing laboratory-confirmed influenza can be considered the best estimate of the actual protective effect of the trivalent 2010/11 seasonal vaccine. The effectiveness of 31% in preventing primary care-attended ILI describes the effect as seen in the clinical practice, where only a part of MA-ILI are confirmed for influenza virus (57% in the study period). That the results obtained using two designs for two different outcomes were consistent reinforces their validity.

Differences between unadjusted and adjusted estimates were greater in the cohort analysis than in the case–control comparison. The test-negative

case–control analysis provides a better comparability since cases and controls were recruited in the health-care system under similar circumstances. However, the comparability in the population-based cohort analysis requires a good control of confounding factors.

Conclusion

Our study shows that it is feasible to provide early estimates of influenza vaccine effectiveness during the season from cohort studies based on healthcare databases. These results support a moderate protective effect of the 2010/11 seasonal vaccine and a low residual effect of last season's monovalent pandemic vaccine against influenza disease in the high-risk population in the 2010/11 season. These results highlight the importance of annual immunisation against influenza of high-risk populations and complementing it with other preventive initiatives such as promotion of basic hygiene measures and avoiding contact with influenza cases.

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Impact of the 2009 influenza A(H1N1) pandemic on public health workers in the Netherlands

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A cross-sectional study was undertaken to analyse the impact of the 2009 influenza A(H1N1) pandemic on frontline public health workers in the Netherlands and to consider its implications for future pandemics. A structured, self-administered questionnaire was made available online (26 March to 26 May 2010) for frontline public health workers employed by the communicable disease departments of the public health services in the Netherlands (n=302). A total of 166 questionnaires (55%) were completed. The majority of respondents reported an increased workload, perceived as too busy (117 respondents, 70.5%) or extreme (13 respondents, 7.8%). Most respondents were not anxious about becoming infected (only seven were regularly concerned). The overall compliance with the control measures was good. The case definition was strictly applied by 110 of the 166 respondents (66%); 56 of 141 (39.7%) consistently consulted the Preparedness and Response Unit within a centralised assessment system, while 68 of 141 (48.2%) consulted the unit only at the beginning of the pandemic. Of 145 respondents with available data, 128 (88.3%) always used personal protective equipment. Reported adherence to the advice to discuss the various isolation measures with patients and their contacts was between 71% and 98.7%. Our study shows that the surveyed frontline public health workers considered the workload to be high during the first 3.5 months of the pandemic and their level of anxiety about becoming infected was reported to be low. During the pandemic, these workers were able to accommodate what they considered to be an excessive workload, even though initially their assignments were unfamiliar to them.

Introduction

On 25 April 2009, the World Health Organization (WHO) declared the outbreak of influenza A(H1N1)2009 to be a public health emergency of international concern [1]. On 11 June 2009, WHO raised the pandemic alert level to phase 6, thereby acknowledging a worldwide pandemic [2]. In the Netherlands, influenza A(H1N1) 2009 virus infection became mandatorily notifiable on 29 April 2009, as a group A disease. This group consists of diseases that pose a very serious threat to public health and thus require national control decisions and coordination. Physicians and staff in laboratories that suspect or confirm a group A disease in a patient need to notify the regional public health service, which then reports anonymised patient data to the Centre for Infectious Disease Control of the National Institute for Public Health and the Environment (RIVM). Within the Centre for Infectious Disease Control, the Preparedness and Response Unit is responsible for coordinating disease control and implementing national control policies. During the pandemic, the unit worked closely with the local public health services.

From 30 April to 15 August 2009, infection with influenza A(H1N1)2009 virus was reported in 1,473 cases nationwide [3]. The policy for carrying out active case finding was defined on 29 April 2009. Patients were classified according to the national case definition, which is based on the European Union case definition [4]. The epidemiological criteria within the case definition changed frequently as the affected areas with sustained human-to-human transmission changed.

The assessment and management of each case (including the need for sampling, classification according to the case definition, assessment of the risk of infection in close contacts, provision of antiviral drug prophylaxis, monitoring of home isolation procedures for cases and their contacts and informing them about the isolation measures and the need for them) was done by frontline public health workers from the public health services together with an expert from the Preparedness and Response Unit (until 29 June 2009), within a centralised assessment system. Initially, samples were taken from all patients with suspected influenza A(H1N1)2009 virus infection and their contacts and antiviral drugs were given if the diagnosis was confirmed. From 15 June 2009, antiviral drugs were also administered to probable cases (i.e. without confirmation of the diagnosis). Personal protective equipment (FFP2 masks, gloves, gown and goggles) was provided for health professionals who took the samples. After

10 July 2009, FFP1 masks and gloves were considered sufficient, as there was increasing evidence that these would prevent droplet transmission. After 22 July 2009, general practitioners were responsible for assessing and managing individual cases and when clusters of cases appeared, they contacted public health service professionals.

As the number of cases increased rapidly during the summer and the clinical picture proved to be relatively mild [5], the notification procedure was adjusted on 15 August 2009. From then, only hospitalised patients or deaths due to influenza A(H1N1)2009 were notified to the public health service. This approach was consistent with the WHO pandemic plans stating that where there is widespread community transmission, containment strategies requiring control measures for each individual case should be replaced by mitigation strategies [6]. In the Netherlands, between 24 April 2009 and 24 June 2010, a total of 2,196 patients with influenza A(H1N1)2009 virus infection were hospitalised and 63 died. Of the deceased patients, 53 had an underlying disease [7].

It is known that communicable disease outbreaks can have a substantial impact on healthcare workers [8], as a result of increased workload, uncertainty about the pathogenicity of the causative agent and anxiety about becoming infected [9,10]. However, there is limited knowledge on the impact of a pandemic on healthcare workers, as the most recent pandemic was the 1968 influenza pandemic [11]. During the 2009 influenza pandemic, public health workers were requested to function as the first-line filter in assessing, sampling and treating cases, meaning that they had to perform new tasks that required additional skills – tasks that interfered with their usual daily routine. Our goal was therefore to assess the consequences of the 2009 influenza A(H1N1) pandemic on frontline public health workers (public health physicians, public health nurses and health department managers) employed by a public health service in the Netherlands in order to contribute to a knowledge base for optimising response strategies in future infectious disease outbreaks.

Methods

Study population

In the Netherlands, there are 28 public health services employing 302 frontline public health workers (119 public health physicians, 166 public health nurses and 17 health department managers). The smallest public health service has a catchment area of 216,403 inhabitants, the largest has 1,245,516.

Questionnaire development and administration

A structured, self-administered questionnaire was developed on the basis of a literature study (using MEDLINE) and 11 in-depth interviews with frontline public health workers (the search strategy and results of the literature study and interviews are available from the authors on request). The questionnaire was tested in a pilot study – to assess its feasibility and completeness – involving two public health workers, two policy advisors from the Preparedness and Response Unit and seven regional public health consultants. After revision, based on the results of the pilot study, the final questionnaire was made available online to the 302 frontline public health workers from 26 March to 26 May 2010. A hyperlink was sent to them by the Preparedness and Response Unit, along with a request to complete the questionnaire.

The questionnaire addressed the first months of the pandemic (29 April to 15 August 2009). Several topics were covered: 12 questions addressed the characteristics of the respondents (profession, sex, age, whether there were children in household, years of work experience, previous experience of working in an infectious disease outbreak, amount of days worked per week, amount of overtime worked, whether they had had direct contact with a confirmed case, whether they had had an infection with influenza A(H1N1)2009 virus, whether they assumed that they had been infected with influenza A(H1N1)2009 virus during work and whether any family members had been infected); other questions were related to perceived workload (n=10), anxiety about becoming infected (n=4) and compliance with the control measures (n=7). The 10 questions for measuring workload were a validated set of questions [12] that are often used to measure workload in medium or small businesses.

At the start of the questionnaire, a detailed timeline was displayed, showing all control measures taken, to facilitate the respondents' recall.

Variables

We composed overall scales for two variables: perceived workload (Cronbach's alpha of 0.886) and anxiety of becoming infected (Cronbach's alpha of 0.799). The validated set of questions on workload used a four-point Likert scale (1 = never; 2 = sometimes; 3 = regularly; 4 = always) and consisted of questions such as 'were you working under time pressure?' and 'did you have to work extra hard to finish your work?' The questions were combined to create the variable perceived workload, which reflected the retrospectively reported perceived workload. Workload was categorised as a relaxed (10–14 points), normal (15–20 points), too busy (21–30 points) and extreme (31–40 points).

For the second variable (anxiety of becoming infected), responses to statements concerning home isolation measures were dichotomised: neutral responses (neither agreed nor disagreed) were excluded from the analysis.

To increase our understanding of the differences between the public health services, three other variables were created. The variable 'degree of urbanisation' was created based on data from Statistics Netherlands (CBS) [13]. The variable 'catchment area'

was based on data received from the Dutch association of public health services (GGD Nederland) (categories: regions with 200,000–500,000 inhabitants, those with 500,001–900,000 and those with 900,001–1,200,000). The variable 'objective workload' was based on the number of cases for which the respondents had consulted the Preparedness and Response Unit within the centralised assessment system of each public health service (categories: 0–40 cases, 41–80 cases and 81–120 cases).

Data analysis

Data were analysed using SPSS v. 18.0. Descriptive statistics (frequencies) were generated. Means were calculated for the answers given on the Likert scale. Differences in means were assessed by Student's t-tests. Differences in proportions were assessed by chi-square test. A p value of ≤0.05 was considered statistically significant. Cronbach's alpha was used to assess whether various questions could be combined: the cut-off value was 0.6. Statements with responses ranging from 'totally disagree' to 'totally agree' were recoded 1 to 5 and four-point scales were recoded 1 to 4. Parametric and non-parametric tests and analysis of covariance (ANCOVA) for regression analysis were used when appropriate. Non-responder analysis was performed for sex and profession.

Results

Of the 302 public health workers contacted, 166 completed the questionnaire (response rate: 55%). Responses were received from all 28 public health services. The proportion of responders among the public health physicians was higher than the proportion of responders among the public health nurses (p=0.023). The general features of the respondents are listed in Table 1.

Non-responder analysis showed that the male–female ratio was not significantly different between responders and non-responders (p=0.221).

Workload

Of the 166 respondents, 117 (70.5%) reported that they were too busy, 13 (7.8%) had an extreme workload, while 36 (21.7%) had a normal or a relaxed workload, during the first months of the pandemic (29 April 2009 to 15 August 2009) (Figure).

A higher perceived workload was associated with a higher degree of urbanisation of the public health service (ANCOVA F-value (1, 162)=9,223, p=0,003) and with regularly working overtime (F(2, 162)=4,687, p=0.010). There were no differences in perceived workload between respondents who worked full-time (4–5 days per week) and those who worked part-time (1–3 days per week).

Anxiety about becoming infected

The level of anxiety about becoming infected during the pandemic was relatively low among the respondents: 100 (60.2%) had no fear of infection at all, 59 (35.5%) were sometimes worried about infection and seven (4.2%) were regularly afraid of becoming infected. Having children (p=0.030) and having doubts about the effectiveness of personal protective measures taken (p=0.044) increased the level of anxiety regarding infection.

Compliance with control measures

We measured how consistently the respondents had applied the criteria for the case definition that was issued to identify suspected patients from whom sampled had to be taken. We also measured the amount of consultation with the centralised assessment system for the final classification of patients, the extent of use of personal protective equipment during sampling and home visits and whether the workers informed patients and contacts about the isolation measures.

Case definition

Of the 166 respondents, 110 (66.3%) reported that they had always strictly followed the case definition, while 50 (30.1%) had only occasionally followed the case definition (Table 2). The main reasons for not following the case definition were that there was already sustained transmission of the influenza A(H1N1)2009 virus in many other countries not included in the case definition (56.6%), that patients or general practitioners applied pressure on the respondents (15%) or because the respondents felt that the criteria defining a contact were too strict (9.6%). Respondents who were public health physicians followed the case definition less strictly than those who were public health nurses (p=0.000) and compliance was lower in male respondents compared with female respondents (p=0.002).

Centralised assessment

Of 141 respondents, 56 (39.7%) reported that until 29 June 2009 they always consulted the Preparedness and Response Unit for centralised assessment, 68 (48.2%) consulted the unit only at the beginning of the pandemic, while 17 sometimes (n=14, 9.9%) or never (n=3, 2.1%) consulted the unit (Table 2). Reasons for non-compliance were that they found it unnecessary (38.3%), time consuming (22.7%) or that the assessments were sometimes contradictory or divergent from the advice specified in the case definition (9.9%). Female respondents consulted the unit less often than male respondents (p=0.008). The compliance of respondents who regularly worked overtime was reduced compared with those who did not (p=0.024).

Personal protective equipment

Personal protective equipment was always used by 128 of 145 respondents (88.3%), regularly by 15 (10.3%) and only sometimes by two (1.4%) (Table 2). The extent of use of personal protective equipment was higher in female respondents (p=0.037) and in those who had been working at a public health service for one to 10 years (p=0.034).

Table 1

General characteristics of questionnaire respondents during 29 April to 15 August 2009, Netherlands (n=166)[a]

Characteristic	Percentage of respondents[b]	Number of respondents
Sex		
Female	66	110
Male	34	56
Profession		
Public health physician	46	77
Public health nurse	51	85
Health department manager	2	4
Age (years)		
<25	4	7
26–35	26	44
36–45	26	44
46–55	32	54
56–65	10	17
Children in household (n=165)		
Yes	50	83
No	50	82
Number of years of work experience		
<1	7	12
1–5	38	63
6–10	30	49
>11	25	42
Previous work experience in an infectious disease outbreak		
Yes	49	81
No	51	85
Number of working days per week		
1	8	14
2	11	19
3	29	48
4	28	47
5	23	38
Working overtime		
Regularly	62	103
Sometimes	34	57
Never	4	6
Having had direct contact with a confirmed case		
Yes	76	127
No	23	39
Had had an influenza A(H1N1)2009 virus infection		
Yes, laboratory confirmed	1	2
Considered as likely	19	32
No	79	132
Infected with influenza A(H1N1)2009 virus during work (n=34)		
Yes	3	1
Considered as likely	27	9
No	62	21
Did not know	8	3
Family member with laboratory-confirmed influenza A(H1N1)2009 virus infection (n=136)		
Yes	7	9
No	70	95
Did not know	23	32

[a] Unless otherwise indicated.

[b] The percentages in some categories do not total 100% due to rounding.

Informing patients and contacts about isolation measures

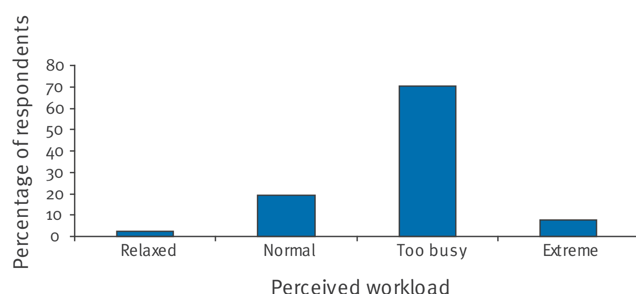
Of 121 respondents, 86 (71.1%) had always told patients that they should wear a mask indoors. Of 156 respondents, 154 (98.7%) had always informed patients about the need for social distancing and 142 of 149 respondents (95.3%) reported that they had informed patients that they were not supposed to leave their home while they were still ill. Further, 145 of 149 respondents (97.3%) had always provided patients with a leaflet containing a summary of the information about isolation measures (Table 2).

Working overtime was associated with increased compliance with informing patients that they were not supposed to leave their home while ill (p=0.048) and providing patients with the information leaflet (p=0.002). The confidence of respondents regarding the effectiveness of the home isolation measures was positively associated with informing patients about wearing a mask indoors (p=0.006) and about social distancing (p=0.004) and informing them that they were not supposed to leave their home while ill (p=0.044).

The perceived workload, anxiety of becoming infected and compliance with control measures were not influenced by the number of inhabitants within the catchment area of the public health service or by the number of cases for which consultation within the centralised assessment system of each public health service with the Preparedness and Response Unit was carried out (objective workload).

Figure

Perceived workload of questionnaire respondents during 29 April to 15 August 2009, Netherlands (n=166)



Discussion and conclusions

This study is one of the first systematic evaluations of the impact of the 2009 influenza A(H1N1) pandemic on public health services. The low level of anxiety of public health workers about becoming infected with the influenza A(H1N1)2009 virus is in stark contrast to that reported during outbreaks of other infectious diseases, such as severe acute respiratory syndrome (SARS) [9,14-18] and the degree of anxiety experienced by the public during the first months of the 2009 influenza A(H1N1) pandemic in the Netherlands [19]. The low level of anxiety in our study may be explained by the fact that the course of illness in the pandemic was mild [5]. This knowledge, which became increasingly clear during the pandemic, might have influenced the health workers' perception of their own health risks and thus might have diminished any anxiety and stress. It has been reported in studies mainly involving experience with SARS that several factors were associated with

Table 2

Compliance of questionnaire respondents with control measures during 29 April to 15 August 2009, Netherlands (n=166)[a]

Compliance with control measures	Percentage of respondents[b]	Number of respondents
Applying case definition (n=166)		
Strictly followed	66	110
Sometimes followed	30	50
Did not know	4	6
Centralised assessment (n=141)		
Always	40	56
Only at the beginning of the pandemic	48	68
Sometimes	10	14
Never	2	3
Use of personal protective equipment (n=145)		
Always	88	128
Regularly	10	15
Sometimes	1	2
Informing patients and contacts about isolation measures[c]		
Wearing a mask indoors (n=121)	71	86
Social distancing (n=156)	99	154
Not leaving home (n=149)	95	142
Additional information (n=149)	97	145

[a] Unless otherwise indicated.

[b] The percentages in some categories do not total 100% due to rounding.

[c] Multiple responses possible.

anxiety, fear or psychological distress, such as direct contact with patients [14,20] and years of working experience [10]. However, in our study population, only having children in the household and having doubts about the effectiveness of the personal protective equipment had an effect on anxiety levels. Such associations have also been reported elsewhere [14,20]. However, we found no association between the length of work experience and the level of anxiety regarding infection.

Our study shows that during the first months of the pandemic, compliance with control measures was good. Confidence in the appropriateness of personal protective measures to reduce transmission can lower the level of anxiety, as was observed by Nickel *et al*. during the SARS outbreak [20]. We believe that confidence in the appropriateness of the personal protective measures further strengthened compliance of the respondents in our study, as Cabana *et al*. reported that having trust in recommended control measures makes a professional more likely to comply with control measures or to emphasise the importance of the measures to patients [21]. In our study, the majority of the surveyed health professionals used personal protective equipment for house visits, even though only a minority was concerned about getting infected. Interestingly, respondents who were less compliant had been working at a public health service for either less than one year or more than 10 years. Therefore, efforts to increase compliance should be focused primarily on these groups.

Previous studies have shown that, during the SARS outbreak, 53–66% of the healthcare workers had an increased workload [9,10,22]. Similarly, in our study, the workload was reported to be very high to extremely high. However, we are not able to compare the workload during the pandemic with that in the period before it, as workload has not been systematically assessed for these groups of professionals outside outbreak periods. The increased workload was partially due to carrying out tasks that normally do not belong to the regular work of public health services, such as systematic sampling of patients, and prescribing and distributing antiviral drugs, which are rather the domain of general practitioners and pharmacists. Given that the pandemic demanded prolonged exertion from most frontline public health workers, including tasks that required new skills, it is likely that the maximum response capacity of public health services was reached. Such a high workload could probably not have been maintained for a longer period of time and workload can therefore become an issue in future outbreaks of diseases with high severity and involving a high number of cases. Therefore, the importance of thorough preparedness plans needs to be emphasised. These plans should consider ways to increase numbers of staff at short notice.

In our study, although the level of anxiety about infection among the respondents was low during the pandemic, our results showed that confidence in the appropriateness of personal protective measures to reduce transmission can lower the level of anxiety. Thus preparedness plans should include strategies that increase the confidence of public health workers in infection control measures. Adequate and timely information on such measures has been reported to be a major factor affecting health professionals' confidence in them [23]. In the light of these findings, we support the view that information about the choice and rationale for infection control measures, together with the expected efficacy, should be made available to health professionals at the very beginning of a crisis or outbreak, to increase their confidence in the measures and thus reduce concerns about possible infection. Furthermore, new insights from research or daily practice should prompt timely adjustments of the measures to increase credibility and stimulate adherence.

We believe that our findings are applicable to other European countries with a similar structure of communicable disease control. A pandemic may be seen as the ultimate test for public health response capacity. Our study shows the importance of thorough preparedness for crisis situations due to infectious disease outbreaks and its implications extend beyond the 2009 influenza A(H1N1) pandemic. To the best of our knowledge, the impact of the 2009 pandemic on healthcare workers has not been previously investigated. However, an initial response of healthcare institutions regarding experiences, barriers and perceived future needs was studied by Lautenbach *et al*., who concluded that revision of preparedness plans seems to be necessary, including items related to workload and education [24]. We also consider preparedness and planning for an optimal response and surge capacity an important subject of concern for the future, given the likelihood that severe outbreaks and communicable disease threats will occur again [25-29] and will be a serious burden on the public health system.

One limitation of our study is that data were collected nine months after the beginning of the 2009 pandemic and therefore could be subject to recall bias. A detailed timeline was displayed on the questionnaire, to aid the respondents' memory, but recall bias could lead, for example, to underestimation of the level of anxiety about becoming infected during the first months of the pandemic. Nevertheless, our results show that the pandemic had a substantial impact on the surveyed public health workers and that this was still felt nine months later.

A second aspect that should be considered is that in our study, the proportion of responders among the public health physicians was higher than the proportion of responders among the public health nurses. This is not surprising, considering the fact that in the Netherlands public health physicians carry final responsibility for

the management of public health issues and are more likely to consult the Preparedness and Response Unit than public health nurses would. Or it may be that the questionnaire was of greater interest to public health physicians than to public health nurses, as it dealt with issues regarding strategies used during outbreaks. Therefore, our results may be more applicable to public health physicians than to public health nurses.

In conclusion, during the pandemic, the frontline public health workers surveyed in the Netherlands showed they were able to accommodate a substantially increased workload, even though initially their assignments were unfamiliar.

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A multiplex one-step real-time RT-PCR assay for influenza surveillance

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For surveillance purposes real-time PCR assays for influenza viruses had to be adapted to the pandemic influenza A(H1N1)2009 strain. We combined published primers and probes for influenza A, influenza B and an internal amplification control with a detection system for influenza A(H1N1)2009 to set up a rapid, reliable, simple and cost-effective high-throughput multiplex one-step real-time RT-PCR. The workflow also includes automated sample preparation for high-throughput screening. The lower limit of detection of the multiplex assay was 3.5×10^2 RNA copies per PCR reaction. The diagnostic sensitivity of the multiplex assay was 87.7%, but increased to 99.4% for influenza-positive samples yielding C_t values of less than 34 cycles in the respective diagnostic assay. High specificity was confirmed by sequencing and correct detection of 15 reference samples from two quality assurance studies. The multiplex PCR was introduced for surveillance of samples from a network of general practitioners and paediatricians in Bavaria, Germany during the influenza pandemic of 2009. Comparison with surveillance data from reported cases proved the reliability of the multiplex assay for influenza surveillance programmes.

Introduction

In April 2009, a novel influenza A(H1N1) virus emerged [1] that could not be detected by routine diagnostic assays for subtyping seasonal influenza A(H1N1) viruses. Therefore, accurate and reliable diagnostic tests for the new influenza A strain had to be established to screen patients with influenza-like illness (ILI) for the 2009 pandemic influenza virus [2-9]. At the onset of the pandemic, public health control measures, namely the isolation of patients and suspected cases to limit the spread of the virus, were guided by the results of these tests [10].

In October 2009, mass vaccination programmes with different pandemic influenza vaccines were implemented globally. In Germany, about 6 million people were vaccinated from the end of October to the end of December 2009. At that stage of the pandemic the World Health Organization (WHO), the European Centre for Disease Prevention and Control (ECDC) and the Robert Koch Institute in Germany (RKI) recommended strengthening the influenza surveillance. This surveillance should persist throughout the whole year and include the new influenza strain as well as seasonal influenza strains, because co-circulation was reported and also expected in the future. At that time, no multiplex real-time RT-PCR assay was available for the simultaneous detection of seasonal influenza A, influenza B and pandemic influenza A(H1N1)2009 viruses. Published diagnostic assays focused more on subtyping of influenza viruses using microarrays and sequencing [11-14]. However, these tests are not suitable for high-throughput routine diagnostic screening.

For large scale surveillance of ILI patients cost effective and time-saving methods for the detection of influenza viruses are needed. The multiplex real-time RT-PCR assay described here provides a diagnostic tool for the fast, simultaneous and reliable diagnosis of influenza A and B viruses with validated and well established real-time PCR protocols with minor modifications, and includes an officially recommended real-time PCR protocol for simultaneous subtyping of the pandemic influenza A(H1N1)2009 virus.

Methods

Specimen collection

For specificity and sensitivity testing as well as for the evaluation of the multiplex assay different panels of clinical samples and reference material were used in this study:
The specificity of the PCR protocol for subtyping pandemic influenza A(H1N1)2009 virus was assessed by sequencing 50 PCR products from clinical samples collected in the beginning of the pandemic in May 2009.

We tested the specificity of the multiplex assay with the following samples: influenza A/Bavaria/63/2009 (a pandemic influenza (H1N1)2009 virus) in six consecutive dilutions, influenza A/Brisbane/59/2007 (H1N1) in four dilutions, influenza A/Brisbane/10/2007 (H3N2), influenza A/chicken/Germany/R3294/2007 (H5N1) in two dilutions, influenza A/whooper swan/Germany/R65-2/2006 (H5N1) and influenza B/Brisbane/60/2008.

Table 1

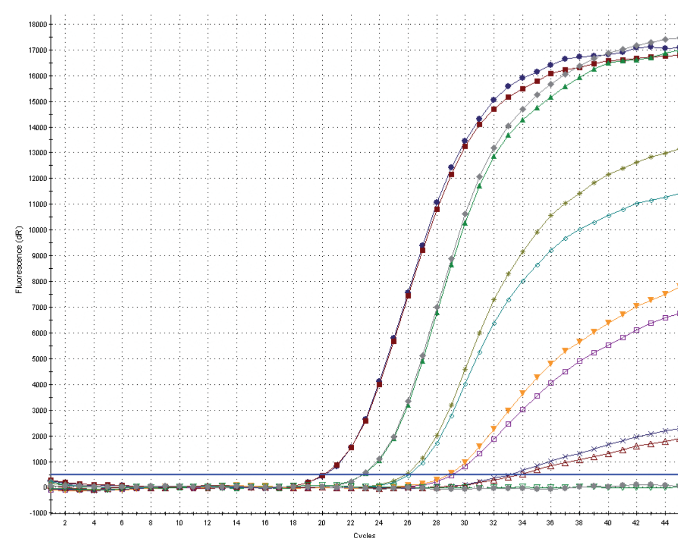
Primers and probes used in the multiplex one-step real-time RT-PCR assay for the detection of different influenza virus strains

	Primers and probes	Sequence (5'→3')	Working concentration	Reference
Influenza A	InfA M+25	AGATGAGTCTTCTAACCGAGGTCG	400 nM	15
	InfA M-124	TGCAAAAACATCTTCAAGTCTCTG	400 nM	
	InfA M-124-mod	TGCAAAGACACTTTCCAGTCTCTG	400 nM	
	InfA M + 64-FAM	6FAM-TCAGGCCCCCTCAAAGCCGA-BBQ	200 nM	
Influenza A(H1N1)2009	Flu Sw H1 F236	TGGGAAATCCAGAGTGTGAATCACT	400 nM	9
	Flu Sw H1R318	CGTTCCATTGTCTGAACTAGRTGTT	400 nM	
	Flu Sw H1 TM298-TEX	TEX-CCACAATGTAGGACCATGAGCTTGCTGT-BBQ	200 nM	
Influenza B	InfB BP-13	GAGCACAATTGCCTACCTGC	400 nM	16
	InfB BMP102	CCACCGAACCAACAGTGTAAT	400 nM	
	InfB BMP-72-CY5	CY5-AGATGGAGAGGCAGCGAACTAGC-BBQ	200 nM	
Internal amplification control	IAC EGFP-12-F	TCGAGGGCGACACCCTG	400 nM	18
	IAC EGFP-10-R	CTTGTACAGCTCGTCCATGC	400 nM	
	IAC EGFP-HEX	HEX-AGCACCCAGTCCGCCCTGAGCA-BBQ	200 nM	

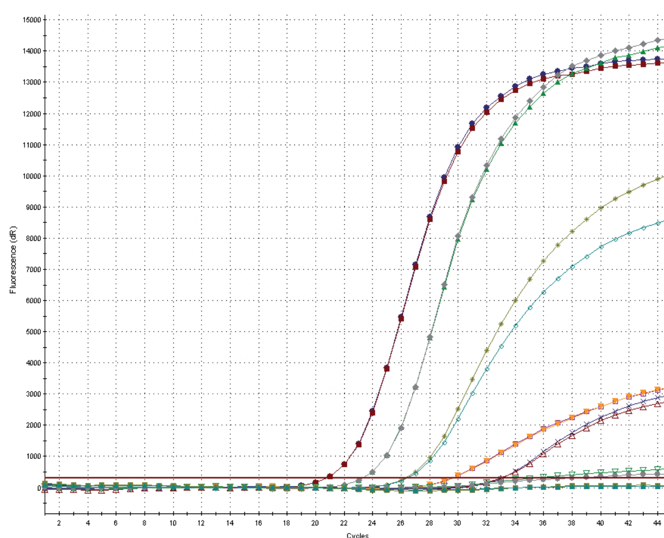
Figure 1

Typical RT-PCR amplification curves for influenza viruses

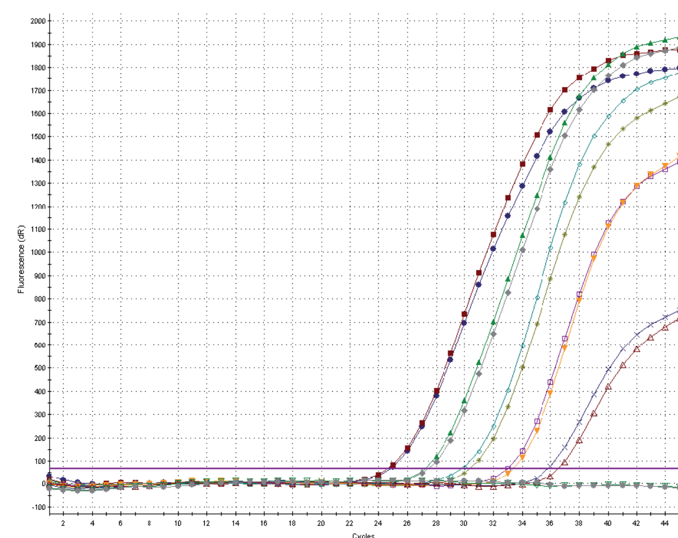
A: Seasonal influenza A virus detected in the FAM channel



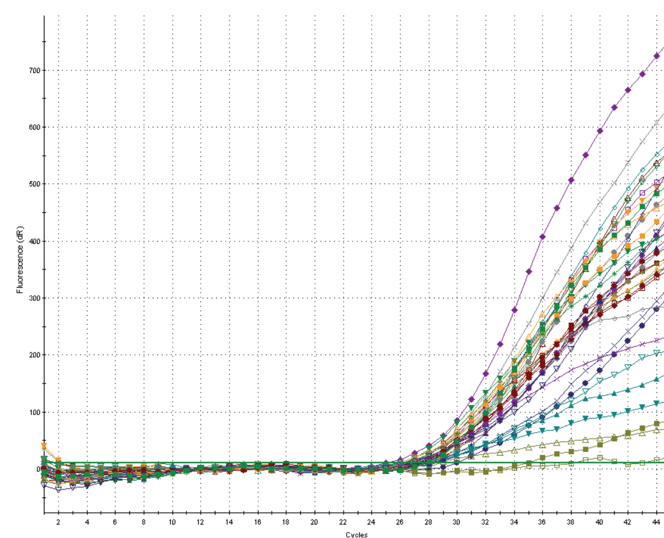
B: Pandemic influenza A(H1N1)2009 virus detected in the TEX channel



C: Influenza B virus detected in the CY5 channel



D: HEX channel showing the internal amplification control



All viruses in dilution series of 10 ng RNA to 1 pg RNA.

The samples were provided for two external quality assurance studies (organised by INSTAND e.V., Germany in 2009/10. In addition, the oseltamivir-resistant strain influenza A/Berlin/58/2008 (H1N1) was provided by the national reference centre for influenza at the RKI in Berlin.

The analytical sensitivity (limit of detection) of the multiplex real-time RT-PCR assay was determined using plaque-quantified influenza A/Hamburg/05/2009 (H1N1) virus with a concentration of 3.5×10^5 PFU/ml [15]. A 10-fold dilution series of extracted RNA was generated from 3.5×10^5 to 3.5 plaque-forming units per ml (PFU/ml) and analysed in triplicate in the FAM-channel (matrix gene) as well as the ROX channel (HA gene) of the multiplex PCR assay. To compare the sensitivity of the multiplex and each single assay, RNA was prepared from egg cultures of an early case of pandemic influenza A(H1N1)2009 in Bavaria, detected on 29 April 2009 and confirmed by the national reference centre for influenza at the RKI, as well as from cell cultures of reference material: influenza A/Bayern/89/2007 (H1N1), influenza A/Sydney/5/1997 (H3N2) and influenza B/Brisbane/60/2008. RNA was analysed in 10-fold dilution series in nuclease-free water containing background calf thymus DNA (Type I fibres, Sigma-Aldrich) in a concentration of 100 ng/µl. RNA dilutions were prepared from 100 ng to 1 pg per PCR reaction.

For evaluation of the multiplex one-step real-time RT-PCR assay and to determine diagnostic sensitivity,

Table 2

PCR efficiencies of the single assays compared to the multiplex assay

	E single	E multiplex
Influenza A	112.9%	112.9%
Influenza A(H1N1)2009	103.1%	105.5%
Influenza B	92.0%	120.0%

E: PCR efficiency

Figure 2

Detection rate of the multiplex PCR for influenza viruses in samples with different C_t values in the respective diagnostic assay

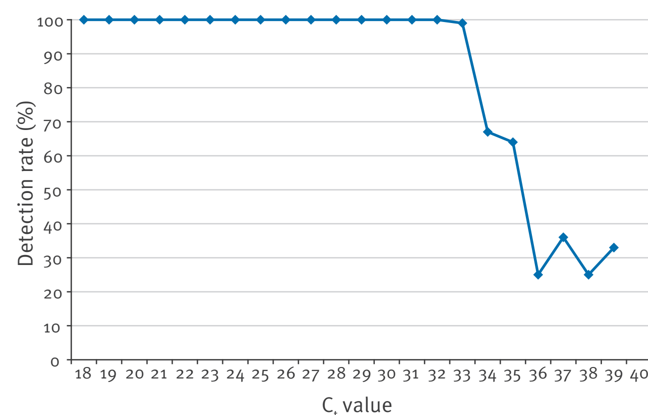


Table 3

Sensitivity, specificity, positive predictive value and negative predictive value of the multiplex assay

	All single assays					C_t≤34 in the single assays					C_t>34 in the single assays				
	Samples	Sensitivity	Specificity	PPV	NPV	Samples	Sensitivity	Specificity	PPV	NPV	Samples	Sensitivity	Specificity	PPV	NPV
Seasonal influenza A	47	78.7	100	100	90.0	31	100	100	100	100	16	37.5	100	100	90.0
Seasonal influenza B	50	76.0	100	100	88.2	28	96.4	100	100	98.9	22	50	100	100	89.1
Pandemic influenza A(H1N1)2009	130	95.4	98.9	99.2	93.7	116	100	98.9	99.1	100	14	57.1	98.9	88.9	93.7
Overall	227	87.7	99.6	99.5	90.6	175	99.4	98.9	99.4	98.9	52	48.1	98.9	96.2	76.7

NPV: negative predictive value; PPV: positive predictive value.
Numbers are also shown for samples with Ct-values below and above 34.

we used clinical samples obtained from ILI patients during the influenza season 2008/09 and the 2009 influenza pandemic in Bavaria. ILI was defined by sudden onset with fever (>38.5°C), cough, sore throat and myalgia and/or headache. We had previously tested the samples with diagnostic real-time RT-PCR assays for seasonal influenza A and B [16,17] and for influenza A(H1N1)2009 [2,9]. The panel consisted of 317 samples: 90 influenza-negative samples, 47 samples positive for seasonal influenza A(H3N2) and A(H1N1), 50 samples positive for influenza B viruses as well as 130 samples positive for influenza A(H1N1)2009 virus. Original specimens included nasopharyngeal and throat swabs in viral transport medium. After screening for influenza, the remaining RNA was stored at -80°C until testing with the multiplex assay.

Nucleic acid extraction

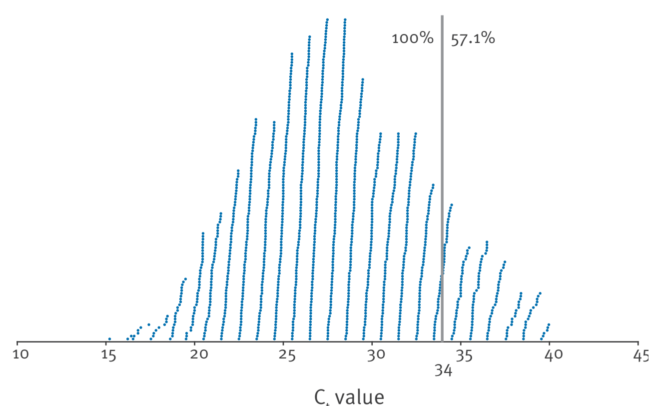
Viral nucleic acid was extracted using the QIAamp Virus Bio Robot 9604 kit (Qiagen) adapted to the robot Hamilton Microlab Star (Hamilton) for large numbers of samples or the Viral RNA mini kit (Qiagen) for small numbers of samples. From our routine diagnostic analyses we know that the extraction method has no influence on the results.

Internal amplification control

Commercially available heterologous *in vitro*-transcribed RNA (INTYPE IC-RNA Labordiagnostik, Leipzig, Germany) was used as PCR inhibition control. This *in vitro* transcript has proven its robustness in previous multiplex real-time RT-PCR assays [18]. The stock solution ($8x10^5$ copies/µl) of the *in vitro*-transcribed RNA was stored at -80°C, and the working dilutions of $1x10^5$ copies/µl were stored at -20°C.

Figure 3

C_t values of samples positive for pandemic influenza A(H1N1)2009 in the diagnostic PCR, Bavaria, 27 April–9 November 2009 (n=1,322)



Each dot represents one sample. The performance of the multiplex assay was retrospectively calculated for the first wave of the 2009 influenza pandemic. The resulting detection rates of the multiplex assay for influenza A(H1N1)2009 in the validation study for samples below (100%) and above (57.1%) a C_t value of 34 are shown. The multiplex assay would have detected at least 93.6% of the positive samples.

Multiplex real-time RT-PCR assay

Different published primers and probes of real-time RT-PCR assays specific for influenza A, influenza B and pandemic influenza A(H1N1)2009 viruses were tested to determine whether they could be used together in a multiplex assay. We show here only those primer and probe sets that performed well when combined in preliminary tests.

In order to minimise the risk of PCR product contamination, we introduced one-step RT-PCR protocols using the commercially available QuantiTect Virus +ROX Vial kit (Qiagen) including QuantiTect Virus No Rox (NR) Mastermix and QuantiTect virus RT. For specific detection of influenza A, influenza A(H1N1)2009, influenza B and the internal amplification control (IAC), we used primers and probes of published or officially recommended real-time PCR systems (Table 1): a previously published real-time RT-PCR assay [16] for influenza A viruses targeting the matrix gene, with an optimised reverse primer (InfA M-124-mod) for reliable detection of pandemic influenza A(H1N1)2009 (recommended by the national reference centre for influenza at the RKI); an officially recommended real-time PCR system with primers and a TaqMan probe for the specific detection of influenza A(H1N1)2009 [9] targeting the HA gene, and a real-time RT-PCR assay for the detection of influenza B [17] targeting the matrix gene, with a slightly modified reverse primer that has been routinely applied for years for routine diagnosis in our laboratory. The detection system for the internal amplification control has been described previously for multiplex real-time PCR assays [18].

All primers and probes were synthesised by TIB Molbiol. For the three influenza single target real-time RT-PCR assays a 25 µl PCR reaction was prepared containing: 400 nM of each forward and reverse primer (see Table 1), 100 nM of TaqMan probe, 1x QuantiTect virus reverse transcription mix, 1x QuantiTect virus NR mastermix, 4U RNase inhibitor (Invitrogen) and 5 µl RNA extract.

For optimisation of the multiplex assay all primer concentrations were titrated from 100 to 500 nM and all probe concentrations from 100 to 300 nM. Fluorescence filter sets for 6-carboxyfluorescein (FAM), hexachloro-6-carboxy-fluorescein (HEX/VIC), Texas Red (TEX/ROX) and a cyanine dye (CY5) were used simultaneously. The influenza A- and B-specific probes were labelled with FAM and CY5, respectively. The probe specific for pandemic influenza A(H1N1)2009 was labelled with TEX. The IAC probe was labelled with HEX. All four TaqMan probes were labelled with Black Berry Quencher (BBQ) as quencher dye. For the multiplex real-time PCR assay optimised probe concentrations were applied (see Table 1).

For single and multiplex real time PCR thermal cycling was performed on MX3000P and MX3005P real-time PCR instruments (Agilent Technologies) under the fol-

lowing conditions: 20 min at 50°C; 10 min at 95°C; 45 cycles of 15 s at 95°C and 45 s at 60°C.

Efficiency of the multiplex assay

PCR efficiencies were determined for influenza A, influenza B and pandemic influenza A(H1N1)2009 in each single assay as well as the individual channels of the multiplex assay. PCR efficiency was calculated according to the PCR amplification formula $E = 10^{(1/slope)}-1 \times 100\%$, E being the PCR efficiency.

Results

Optimisation of the multiplex assay

Primer titration from 100 to 500 nM as well as probe titration from 100 to 300 nM indicated an optimal primer concentration of 400 nM and an optimal probe concentration of 200 nM for all four assays in the multiplex real-time RT-PCR (see Table 1). Higher or lower concentrations did not alter the sensitivity of the multiplex assay significantly (results not shown).

The optimised multiplex real-time RT-PCR assay in a 25 µl PCR reaction volume was composed as follows: 400 nM of all primers and 200 nM of each of the four TaqMan probes, 1x QuantiTect virus RT mix, 1x QuantiTect virus NR mastermix, 4U RNase inhibitor, 0.25 µl IAC RNA (2.5×10^4 copies) and 5 µl RNA extract. Thermal cycling was performed on MX3000P and MX3005P under the same conditions as the individual single assays. The optimised multiplex real-time RT-PCR assay is shown in Figure 1 for 10-fold dilution series of viral RNA from 10 ng to 1 pg RNA.

Specificity of the multiplex assay

The specificity of the diagnostic assays for influenza A and influenza B has previously been tested and confirmed [16,17]. Therefore it was not further tested during multiplex optimisation. We checked the specificity of the PCR for pandemic influenza A(H1N1)2009 virus that was unpublished at the time [9] by sequencing the 80 bp amplicons (HA gene) of positive pandemic influenza A(H1N1)2009 samples. All fifty sequenced PCR products were 100% identical to published sequences of pandemic influenza A(H1N1)2009 proving the high specificity of the assay. The specificity of the multiplex assay was confirmed in two official external quality assurance studies (INSTAND e.V., Germany) comprising 15 samples of six different influenza strains, which were tested in duplicate. No cross-reactivity was observed in any of the 15 samples, and all specific targets showed strong positive signals. Furthermore the oseltamivir-resistant strain influenza A/Berlin/58/2008 (H1N1) was tested and correctly identified by the multiplex real-time RT-PCR assay.

Analytical sensitivity of the multiplex assay

With plaque-quantified influenza A/Hamburg/05/2009 (H1N1) we found a linear dynamic range from 10^5 to 10^2 genome equivalents. The detection limit was below 3.5×10^2 PFU/ml for the matrix gene as well as the HA gene. Testing of each RNA concentration of the influenza A (Sydney/5/1997 (H3N2) and B (Brisbane/60/2008) reference material in triplicates yielded a sensitivity of 10 pg per PCR reaction for each detection system in the single assays as well as in the multiplex assay and detected RNA extracted from influenza-infected cell cultures (seasonal influenza A(H3N2) and B) and from egg cultures (influenza A(H1N1)2009) with equal sensitivity.

Efficiency of the multiplex assay

The real-time PCR runs of the sensitivity tests for influenza A (Sydney/5/1997 (H3N2), influenza A/Hamburg/05/2009 (H1N1) and influenza B (Brisbane/60/2008) were applied for the determination of the PCR efficiencies in the multiplex real-time PCR compared to the individual single real time PCR assays. The PCR efficiencies of the single real-time PCR assays in comparison to the individual channels of the multiplex PCR assay are shown in Table 2. The PCR efficiency of each individual assay was determined as between 92% to 120% for the individual assays. The PCR efficiencies of the respective single assay were comparable to the PCR efficiency in the multiplex assay. The influenza B assay had a PCR efficiency of 92% in the single assay while in the multiplex assay the PCR efficiency was 120%, which was considered as acceptable for a screening assay.

Evaluation of the multiplex assay with samples of ILI patients

A total of 317 stored RNA samples from the respiratory tract of ILI patients that had previously been tested with diagnostic real-time RT-PCR assays, were retrospectively tested with the multiplex assay. The overall diagnostic sensitivity of the multiplex assay was 87.7%, specificity was 99.6% and positive (PPV) and negative predictive values (NPV) 99.5% and 90.6%, respectively, compared to the respective diagnostic assay. Ninety samples had been negative in all diagnostic assays. Of those 90, 89 were also negative when we tested them in the multiplex assay, but one sample yielded a positive result for pandemic influenza A(H1N1)2009 in the multiplex assay (C_t value 35).

Of 175 influenza-positive samples with C_t values under 34 in the respective diagnostic assay, 174 were confirmed by the multiplex assay, with positive signals for seasonal influenza A (31/31), influenza B (27/28) and pandemic influenza A(H1N1)2009 (116/116) viruses. The influenza B-positive sample that was missed in the multiplex PCR had had a C_t value of 34 in the diagnostic PCR. In samples that had C_t values above 34 in the respective diagnostic assay, the reliability of detection with the multiplex assay was lower: 25 of 52 influenza samples overall, with 6 of 16 seasonal influenza A, 11 of 22 influenza B, and 8 of 14 influenza A(H1N1)2009 (Figure 2 and Table 3). The sensitivity of detection of influenza A(H1N1)2009 was slightly lower with the primers targeting the matrix gene (116/130; 89.2%) than with primers targeting the HA gene (124/130; 95.4%) especially in samples that had been only weakly

positive in the respective diagnostic PCR (C_t values>34). The IAC was positive in all influenza-negative samples, indicating that failure to detect influenza virus was not due to inhibition.

Based on the detection rates of this evaluation we calculated that the multiplex assay would have correctly identified at least 1,238 of the 1,322 (93.6%) influenza A(H1N1)2009-positive samples (Figure 3), which were analysed at the Bavarian Health and Food Safety Authority between 27 April and 9 November 2009 using the diagnostic assays. The C_t values were between 20 and 32 for 1,025 of these samples.

The multiplex assay was introduced as the sole screening test into laboratory influenza surveillance in Bavaria on 10 November 2009. Until 16 April 2010, 310 of 1,228 nasopharyngeal and throat swabs of ILI patients tested positive for influenza A(H1N1)2009 using this assay. The results reflected the epidemic curve of reported cases of influenza A(H1N1)2009 in November 2009 in Bavaria.

The IAC was negative in five throat swabs which all tested negative for influenza viruses. After 10-fold dilution of the sample, the IAC was positive in all five samples. The negative results of three of these samples were confirmed negative when retested in dilution in the multiplex assay, while two were positive for pandemic influenza A(H1N1)2009.

Discussion

We report on a multiplex one-step real-time RT-PCR assay for the simultaneous detection of seasonal influenza A and B as well as influenza A(H1N1)2009 viruses. The assay was optimised for multiplex real-time PCR from published, validated and well established PCR protocols with minor modifications. The multiplex assay proved to be as specific as the respective diagnostic PCR assay. Only one sample tested negative in the diagnostic assays but positive for influenza A(H1N1)2009 in the multiplex assay in two replicates. We ran out of patient material and could not retest the sample with the diagnostic assay. As we detected only a low positive signal, neither a false positive result of the multiplex assay due to contamination, nor a false negative result of the diagnostic assay could be ruled out. A PCR inhibition control was successfully integrated into the assay for accurate interpretation of negative results. Interestingly, two samples positive for pandemic influenza A(H1N1)2009 would have been missed without the IAC. Dilution of the RNA before PCR successfully abolished the inhibitory effect. As we used the *in vitro*-transcribed RNA as an amplification control we could not control for inhibitory effects due to the extraction protocol.

We consider our multiplex assay that has shown its functionality in a high number of patient samples a useful tool for general public health laboratories. In contrast to the evaluation of other published assays [5,7,19], we have tested our multiplex assay on a very large number of clinical samples, including a high number of positive samples. In our analysis of patient samples, the diagnostic sensitivity of the multiplex PCR was slightly lower than that of the respective diagnostic assays, even if RNA dilution series of reference material showed equal sensitivity when determining the detection limit of the multiplex assay in comparison to the single assays. This might be explained by degradation due to storage of weakly positive patient RNA samples for up to one year, whereas dilution series were performed with freshly isolated RNA from reference material for the single as well as the multiplex assays. The overall sensitivity was 87.7%, but was 99.4% for samples with moderate and high viral loads (C_t value>34). In a situation with population-wide screening in which patients with acute ILI yielding high viral loads are tested, we consider the slightly lower sensitivity acceptable. The assay has been validated for routine diagnosis of influenza and is used for large scale surveillance of influenza activity. While the pandemic subtype was reliably recognised during the 2009 pandemic, specificity and sensitivity of the multiplex assay was also shown for seasonal, avian and an oseltamivir-resistant virus. The assay is used to monitor influenza viruses throughout the whole year. By introducing the multiplex assay we were able to lower costs by saving reagents and working time. Furthermore we reduced sample turnaround time in comparison to the diagnostic PCR assays.

Diagnostic tools for surveillance are applied for the general identification of influenza viruses. Although mutation of the pandemic influenza A(H1N1)2009 virus was rare in the 2009 pandemic [20], we also addressed this possibility by including conserved regions (matrix genes) as PCR target. Our multiplex assay is capable to both identify the circulating pandemic strain (HA gene) and screen for other influenza A and B viruses (matrix genes). These should be further subtyped to confirm other seasonal influenza A subtypes or to detect changes in the circulating strain.

Chen *et al.* [21] also published a multiplex real-time RT-PCR assay for the simultaneous detection and subtyping of influenza viruses including the pandemic influenza AH1N1(2009), that has been evaluated on a high number of patient samples. Compared with our one-step real-time RT-PCR assay, this assay is based on a two-step real-time RT-PCR.

The 2009 pandemic is a reminder for public health laboratories to monitor influenza activity not only during the season of influenza circulation, but during the whole year. Our assay proved to be a convenient, rapid, reliable and cost effective way to meet this requirement.

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European Parliament adopts resolution on the Tuberculosis Vaccine Initiative (TBVI)

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On 3 February 2011 the European Parliament adopted a resolution which highlights the role that the European Union (EU) can play in meeting the Millennium Development Goal (MDG) to halt and begin to reverse the incidence of tuberculosis (TB) by 2015. While progress has been made, the disease still causes almost 2 million deaths every year, with mortality especially high in developing countries and among sufferers of HIV/AIDS [1,2].

But TB is still a danger to European citizens as well, because of the worrying emergence of strains of tuberculosis resistant or highly resistant to treatment. In addition, the cost of TB treatment alone in the EU is EUR 2 billion per year [3].

According to the European Parliament, only a vaccination programme involving a large-scale vaccination campaign could have a positive impact in achieving the MDG to reduce prevalence and death rates after 2015, and in particular the elimination of TB by 2050.

MEPs highlight the central role that can be played by Tuberculosis Vaccine Initiative (TBVI), an independent, not-for-profit organisation that develops new vaccines, with the aim of making them globally accessible and affordable. According to the resolution, the TBVI's work should be included in the practical implementation of the Europe 2020 Strategy [4] and a much greater pharmaceutical research effort should be put in tackling diseases such as TB.

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