

Viral Co-Infections Involving Influenza A: Changes Observed During the 2009 Pandemic Season

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Abstract

Objectives: Our objective is to highlight and focus on the viral etiology of influenza-like-illness (ILI), to compare the viral co-infections during 2009 pandemics and other influenza seasons, and to evaluate if respiratory viruses had an effect on the spread of A(H1N1)2009 or vice versa.

Methods: Between 15 July and 31 December 2009 upper respiratory tract specimens from ILI patients were prospectively examined by viral culture and/or A (H1N1)2009 PCR. On samples of 620 critically-ill patients, another 16 respiratory viruses were detected by combined mono plex/duplex PCR. This algorithm was also used during two preceding influenza seasons, while in later seasons multiparameter PCR on customized Taqman Array Cards were used. Five influenza/winter seasons (October 1st March 31st) were evaluated.

Results: From July to December 2009, 4,101 patients presented with ILI, 48.4% of them were hospitalized and 15.1% were admitted to ICU. In total, 4,895 samples of different episodes were analysed. A(H1N1)2009 was cultured from 781 samples (33.0%), of which 2.9% showed viral co-infections. More than 54% of the ICU patients had a viral infection; 13.1% of them were co-infected. Co-infections were found in 7% and 18.4% of A(H1N1)2009-positive samples, and A(H1N1)2009-negative samples respectively.

Overall, rhinoviruses (hRV) were the most frequently involved viruses in co-infections, closely followed by Parainfluenza viruses (PIV). The opposite was noticed in the ICU patients, where PIV was followed by both hRV and adenoviruses in equal numbers. Comparing the same period in previous/later seasons (1 October to 31 March) reveals significant lower percentages ($P < 0.0001$) of viral co-infections with and without the presence of circulating influenza A (IA) strain during the winter 2009-2010, both in culture and PCR. In season 2007-2008 (seasonal A/H1N1) and 2008-2009 (seasonal A/H3N2 predominantly), 34.2% and 36.7% of IA infected samples respectively, were co-infected, compared to 28.7% and 25.8% in 2012-2013 and 2013-2014 respectively, considering respiratory samples examined by multi parameter PCR. Looking at viral culture, a significant decrease of co-infections from 12.9% to 4% of IA infected samples from pre-pandemic to pandemic season was observed ($P < 0.0001$).

Conclusions: During the pandemic season of 2009, A (H1N1)2009 circulated in a dominant way, and patients with ILI were less co-infected than in surrounding flu seasons. This could partly be explained by the fact that the flu season started earlier than expected, when other respiratory viruses were less prevalent, but more decisive the new recombinant strain was less likely associated with other viruses. A(H1N1)2009 circulated preferentially isolated in its first season but lost its dominance in subsequent years.

Introduction

In Central- and North-America, six months after its emergence, the novel pandemic swine-origin influenza virus A (H1N1)2009 had spread worldwide, requiring implementation of national pandemic control plans in many countries [1,2].

In Europe, a combination of containment procedures and atmospheric conditions, such as dry and warm weather might have been responsible for the delayed and sporadic circulation and transmission of the virus. In the Southern hemisphere the pandemic occurred mainly in regions with a more temperate climate [3,4]. The proportion of hospitalized A (H1N1)2009 cases observed in EU countries started to increase since mid-June.

One month later, there were already 8,936 confirmed cases of A(H1N1)2009 reported by 28 EU countries, 67% of which by the United Kingdom [5].

In Belgium, the first case was identified on the 12th of May, followed by sporadic cases clearly linked to international traveling. From mid July 2009 on, an increasing number of indigenous cases were detected all over the country after several big international music festivals. Between week 40 and 49 the number of flu cases surpassed the epidemic threshold, with a peak in week 44. An adjuvant pandemic vaccine (Pandemrix®) was available after the peak incidence in October and was administered in priority to risk groups (defined as health care workers, pregnant women, obese patients, children below 6 months of age and chronically ill patients) [6]. The Belgian surveillance system estimated that 214,531 people were infected, 733,000 could have benefited from a vaccine and 19 patients died in conditions attributable to A(H1N1)2009 infection [7]. They clearly saw a higher number of ILI consultations in Brussels compared to Flanders and Wallonia, without obvious reason.

Although A (H1N1)2009 infection had several unique features which included rapid transmissibility, fast growth and high morbidity in patients of risk groups, the clinical characteristics did not significantly differ from previous influenza seasons and mainly consisted of influenza like illness (ILI). In addition to influenza viruses A/B (IA & IB) responsible for 5 to 15% of the total upper respiratory tract infections worldwide during annual epidemics [8], ILI can be attributed to a broad range of other respiratory viruses, such as rhinoviruses (hRV), Parainfluenza viruses (PIV1, PIV2, PIV3, PIV4), human metapneumoviruses (hMPV) and respiratory syncytial viruses (RSVA, RSVB). Even though the exact viral etiology of ILI has been extensively investigated, limited and conflicting information is available on the possible role of viral co-infections on clinical severity, especially during the 2009 pandemic [9-13]. However, some recent data suggest

that some viruses alone or in co-infection (e.g. A(H1N1)2009 and RSV), could be independently correlated with severity of infection (i.e. length of hospitalization, ICU admission, respiratory failure requiring mechanical ventilation etc...) [12,14]. This highlights the importance of diagnosing all causal pathogens during an ILI episode, in addition to therapeutic purposes when antiviral drugs are available.

Facing the lack of clinical presentation specificity, determination of the viral agent(s) involved requires an extensive work-up. Over the past decade, several nucleic acid amplification tests, including multiplex Real-Time Polymerase Chain Reaction (RT-PCR) and micro-array assays have shown high reliability in detecting the presence of one or more viruses in respiratory tract samples [10,15]. These assays have demonstrated superior sensitivities and specificities compared to traditional methods such as direct fluorescent-antibody assays, shell-vial culture, and Rapid Antigen Tests (RAT) [16].

In this study, we describe viral pathogens yielded from respiratory samples of patients suffering from ILI and attending the emergency room, outpatient clinics and all types of hospitalization wards (including ICU) in seven hospitals during 2009 pandemics in Brussels. Furthermore, we were able to evaluate the frequency of viral co-infection over 5 influenza seasons (between October 1st and March 31st) with observation of a substantial reduction of viral co-infections in general and IA-associated specifically in the winter 2009-2010, as compared with other influenza seasons.

Methods

Study Design

Between 15 July and 31 December 2009, respiratory specimens from 4,101 patients with ILI, attending seven public hospitals -emergency rooms or policlinics (4 university and 3 general hospitals)-were prospectively examined by RAT, viral culture and/or PCR (see Table 1 for stratification) in a licensed laboratory for medical microbiology. For each successive ILI episode (separated by at least 8 weeks) one sample was included, so patients could be included more than once. In total, 4,895 samples were included in the study period. Detection of a virus was recorded only once for each patient. ILI was defined as a patient suffering from at least one general symptom (fever >38.0°C, asthenia, myalgia, chills or headache) and one respiratory symptom (cough, dyspnoea/tachypnea, wheezing, rhinitis or pharyngitis). A pre-existing comorbidity was defined as a chronic condition requiring long term medication or medical follow-up. Co morbidities were listed based upon CDC A(H1N1)2009 guidelines [17]. The type of viral diagnostics was defined in advance according to patients' clinical condition (Table 1).

Table I: Viral Testing Algorithm during Pandemic Season

	Viral Ag Test	Viral culture	rtRT-PCR Influenza A & subtyping (H1N1)pdm2009	Multiparameter rtPCR*
Every stable patient - AMBULATORY n= 1908 respiratory samples; 1496 patients	X	X		
Every patient at risk for complications - HOSPITALIZED n= 2367 respiratory samples; 1985 patients	X	X	X	
Every critical ill patient - ICU n= 620 respiratory samples; 620 patients			only subtyping if IA+ in multiparameter rtPCR	X
	4275	4275	2367	620
* Molecular screening by several monoplex & duplex in-house rtPCRs for AdV, hEV, hRV, Influenza A/B, RSV-A/B, hMPV, PIV-1-4, hCoV-229E, hCoV-OC43, hCoV-NL63, hBoV				

For patients at risk of complications [1] or presenting with a severe illness who required >48h hospitalization, a Specific Real-Time RT-PCR assay for the detection of IA, and, if positive, additional sub typing for A(H1N1)2009 was performed on a daily basis (except Sunday). In case the patient was admitted to ICU (n=620), additional multi parameter PCRs for the detection of 16 respiratory viruses were applied.

Criteria for severe clinical presentation were a temperature <35°C or >39°C, a heart rate ≥120/min, a respiratory rate ≥30/min, respiratory distress with oxygen need, a systolic arterial pressure <90 mmHg or an altered consciousness.

Beside age and sex, predisposing factors for complicated disease such as obesity, pregnancy, chronic respiratory disease, diabetes mellitus, immunosuppression and chronic cardiac disease were registered [1]. To compare the circulation of the pandemic strain in 2009 with the circulation of influenza strains in other seasons, we considered 2 preceding (2007-08, 2008-09) and 2 following influenza seasons (2012-13, 2013-14), with the season 2009-10, each time from October 1st till March 31st to objectively compare the same seasonal time periods. Critically-ill patients were examined using multi parameter molecular tests

Respiratory Specimen Acquisition and Handling.

Nasopharyngeal aspirates (NPA) or flocked swabs (MicroReologics, Copan, Brescia, Italy) from throat or nasopharynx were used, according to the instructions of the manufacturer. The swabs were placed in 1 mL of homemade viral transport medium (VTM, for details see Annex I) and samples were transported at room temperature, submitted at the Virology Laboratory of Saint-

Peter University Hospital and held at 4°C prior to processing.

Viral Culture

Within 24h after sampling, three classical cell lines (LLC-MK2, Vero, MRC5) were inoculated (Annex I). Viral culture was performed on 4,275 samples.

Molecular Assays

I. Target Sequence Selection and Primer and Probe Design

Specific primers and probes were selected and designed by using the Primer Express software, version 2.0 (Applied Bio systems (AB), Foster City, CA) according to sequences available from public databases (National Centre for Biotechnology Information; NCBI). The oligonucleotide concentration which gives lowest threshold cycle (C_t) value and maximum amplification efficiency was selected (Supplement 1).

II. DNA Extraction

Genomic DNA and RNA were isolated using the MagNA Pure LC (Roche Diagnostics) instrument by using the Total Nucleic Acid Isolation Kit - Large Volume. 25µL of Phocid Distemper virus was spiked into each sample prior to extraction as an inhibition and amplification control.

III. Real-Time PCRs

An in house Real-Time (rt) Reverse Transcriptase (RT) PCR (rtRT-PCR InflA) targeting the matrix protein-coding gene, was

used for the detection of IA, able to detect all subtypes including A (H1N1)2009. For specific detection of the circulating variant, two monoplex rtRT-PCR assays according to the Centres of Disease Control (CDC) protocol were initially used (from 15/7/2009-31/8/2009): SW InfA PCR (swInfA) and SW H1 PCR (swH1), further called as RT-PCR A/H1N1 [18]. From September on, A (H1N1)2009 subtyping was based only on swInfA detection, due to numerous observed discrepancies in laboratories worldwide and the suspicion of mismatches in the swH1 primer, probe binding regions and of existing variants of the pandemic strain [19].

Five reactions were set up as duplex rtPCRs: human bocavirus (hBoV)-human adenovirus (hAdV), IA-RSVA, IB-PIV2, RSVB-hMPV (hMPV-1A,1B,2A,2B) and hRV-PIV3 duplexes. The six remaining rtRT-PCR reactions (human corona viruses: hCoV-229E, -NL63, -OC43, PIV1 and PIV4, hEV [19]. were set up as single plex PCRs. For concentrations of primers and probes see Supplement 1. In total, on 2,367 samples a separate IA rtRT-PCR with sub typing of A (H1N1)2009 was performed. On 158 IA-positive ICU samples (in multi parameter rtPCR) a sub typing PCR was directly performed supplementary. And also, on IA-positive cell cultures (without parallel rtRT-PCR-result for A (H1N1)2009 or with negative IA RT-PCR result) this typing step was performed in batch retrospectively, once weekly, to stratify the samples. During seasons 2012-13 and 2013-14, all samples of

critical-ill patients were prospectively tested once for respiratory pathogens with an in house customized TAC (Taqman™ Array micro-array Card) respiratory panel which includes rtPCR testing for the same viral pathogens by rtPCR (Annex I).

Data Analysis

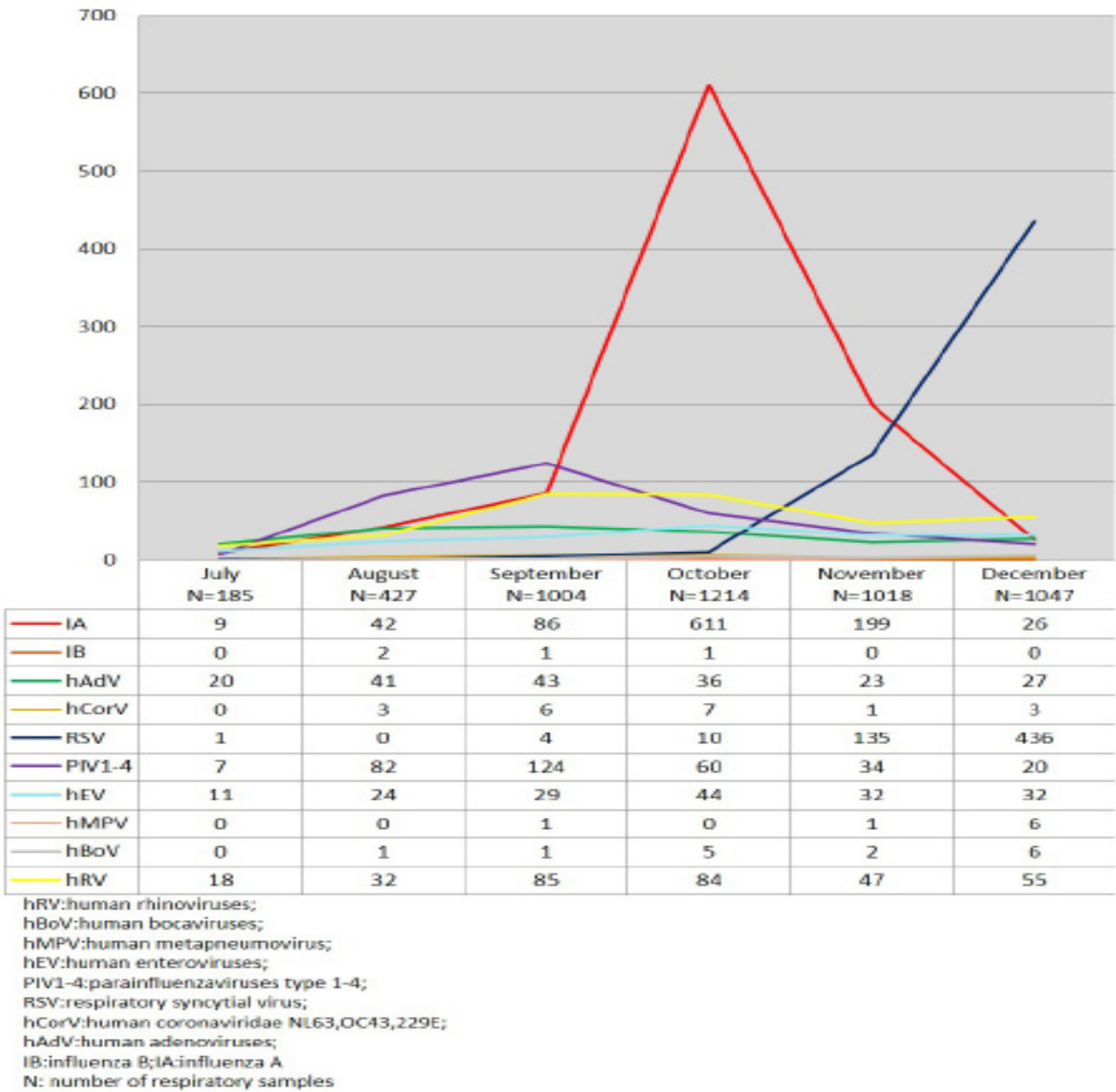
Statistical analyses were performed using Graph Pad Prism Software (Inc, 2003, San Diego, USA). Chi square or Fischer's exact test were used to compare non-continuous variables and Mann Whitney test to compare continuous variables. A two-tailed p-value less than 0.05 were considered as statistically significant.

Results

I. Sample Distribution

We received 4,895 unique respiratory samples (after exclusion of 645 replicated samples) between July and December 2009, corresponding to 4,101 patients with medically-attended ILI episodes, including 954 (19.5%) throat flocked swabs, 2,176 nasopharyngeal aspirates (44.5%), and 1765 nasopharyngeal flocked swabs (36.1%). Distribution of the samples over the study period is shown in (Annex II and Figure 1). Delivery time before processing of specimens ranged from 45 minutes to 36h (median time 8h45). Overall, 2,371 samples yielded a respiratory virus (viral prevalence of 48.4% in our cohort).

Figure 1: Respiratory viruses collected over the study period (15 July -31 December 2009)



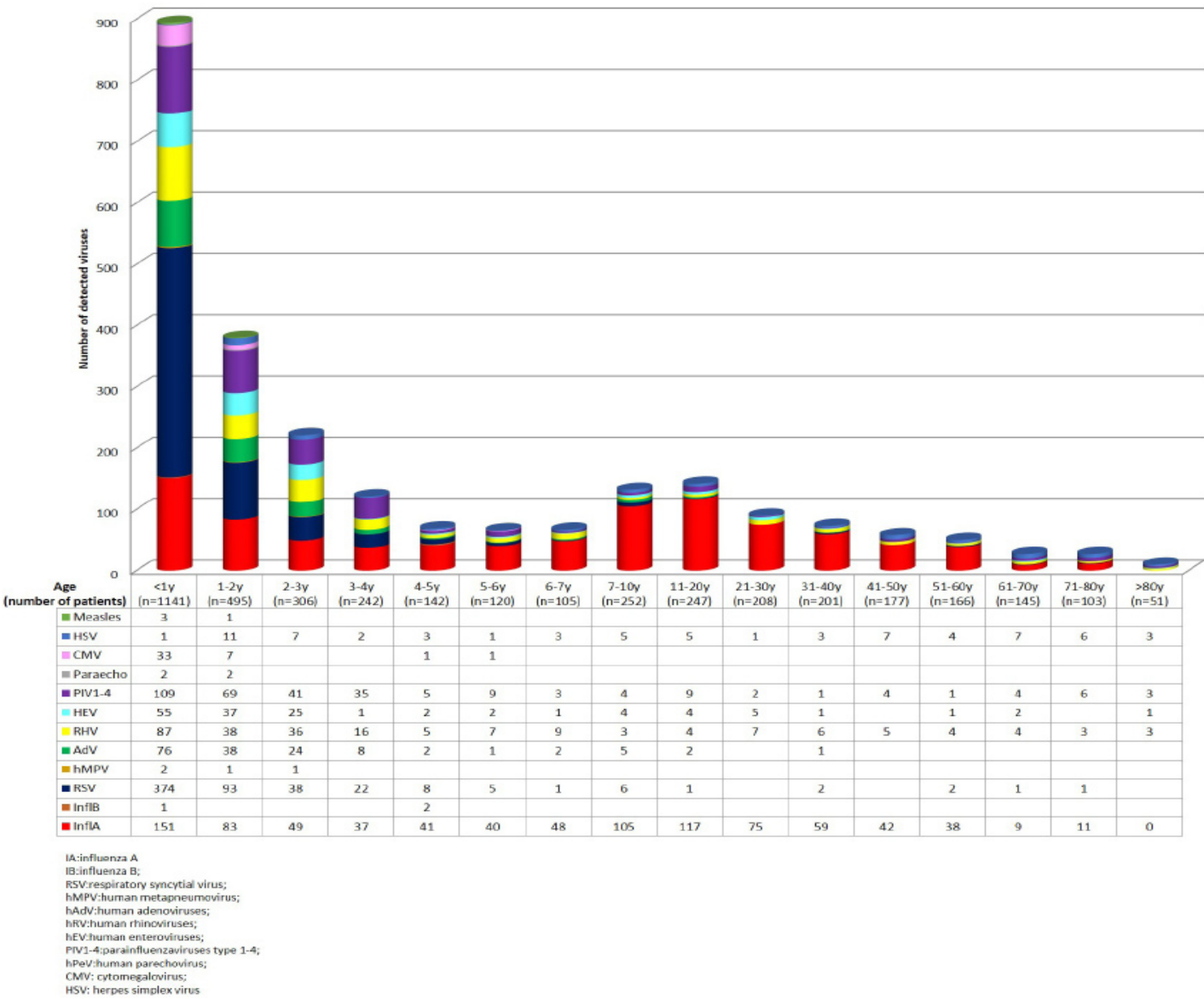
For the comparison of viral co-infection between different seasons all unique samples from medically-attended ILI-patients were included from the 1st of October until the 31st of March to cover the classic influenza season.

II. Description of the Study Population

The samples came from 2,102 males and 1,999 females. Out of the whole cohort, 22.1% of patients were infected with A (H1N1)2009 equally distributed according to gender (p-value =0.73).

As presented in (Figure 2), age distribution of patients harbouring respiratory viruses ranged from 1 day to 94.9 years old with a median age of 1.4 years [interquartile range (IQR) of 0-8.25 years] in the global cohort and 0.8 years [IQR 0-43 years; range 0-86 years] In the ICU group. Overall, 57%, 12%, 24% and 7% of all included respiratory samples were taken from children <5y of age, children between 5-10y, patients from 11-60 years of age and patients above 60 years, respectively.

Figure 2: Age distribution of the patients harbouring respiratory viruses



Children <5 years accounted for the major part (72.4%) of the 2,371 virus-positive samples; whereas only 2.7% of positive samples came from patients older than 60 years (11.0% and 13.9% of positives was taken from people between 5-10y and 11-60y respectively). Overall, pandemic H1N1 strain explained 21.9% (361/1,645), 77.2% (193/250), 80.1% (331/413) and 31.7% (20/63) of

the total proven viral episodes in the <5y, 5-10y, 11-60y and >60 y age categories, respectively. As illustrated in Figure 2, the prevalence of other respiratory viruses also significantly varied according to age groups.

In children <4 years, the main isolated viruses were RSV and PIV; together with IA they were responsible for 50.4% of infections. While pandemic H1N1 infections predominated in age groups from 2-3y to 71-80y, RSV exceeded the prevalence of IA in age group < 1 y (32% of children RSV+) and 1-2 y, and of the patients >80y none was infected with A(H1N1)2009. Between 61-94y, 21% (63/299) was virally infected, 2/3 of them due to non-IA viruses (mainly hRV, HSV, PIV, hEV& RSV).

Overall, age-specific notification rate was highest in the age subcategory “11-20y” where 47.4% of patients was infected with A(H1N1)2009, followed by age subcategory “6-7y” where 45.7% of patients showed infection with the pandemic strain.

Out of our global cohort, 3,065 (74.7%) patients belonged to an officially declared WHO risk group for pandemic influenza. A total of 2,605 patients (63.5%; corresponding to 2987/4895 infectious episodes) required hospitalization out of which 2,474 (94.97%) harbored 1 or more co morbidities/risk factors listed above. This proportion even increased when focusing on ICU admitted cases, where 608/620 (98.1%) patients belonged to

One or more not mutually exclusive risk groups. Pregnant women in particular represented 27% and 13% of 16-42 years old female patients admitted to medical wards or ICU, respectively.

When analysing patients with a confirmed A(H1N1)2009 infection, 53.7% belonged to an officially declared WHO risk group.

There was no clinically relevant difference between the median ages of the described tested population (0.8y in ICU & 1.4y in whole study population) and the median ages of examined patient groups in the 4 other influenza seasons (Table 2), namely 0.6y; 0.7y; 0.5y and 1.7y for 2007-2008; 2008-2009; 2012-2013 and 2013-2014 respectively [IQR were 0-1.5y; 0-1.8y; 0-2.7y and 0-11.5y respectively].

III. Severe Cases and Outcome

In 337/620 (54.4%) ICU patients, a respiratory viral pathogen could be identified, which was A(H1N1)2009 in 47% of them. Overall 25.5% of all ICU patients were infected with the pandemic strain (Figure 3).

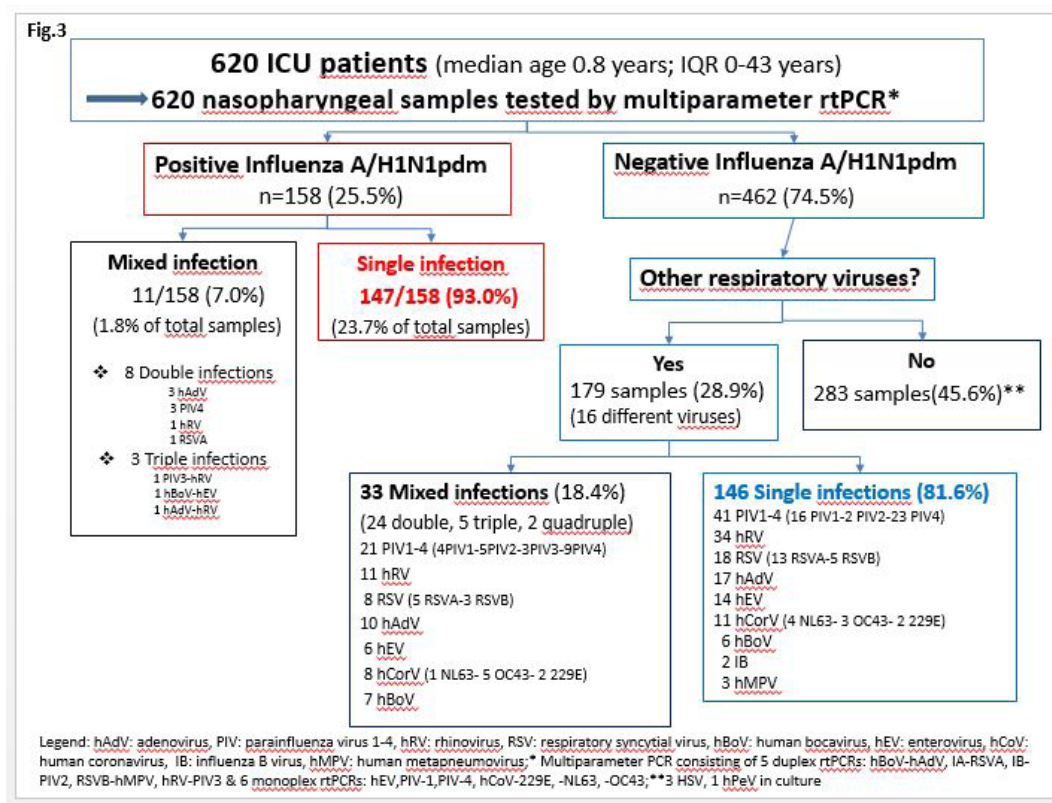


Figure 3: ICU patients and the distribution of their viral infections.

During the study period, 51 A(H1N1)2009 infected patients died leading to an in-hospital fatality rate possibly related to influenza A of 5.6%. One or more underlying co morbidities were present in most of these patients (49/51), including five children (4 with a chronic neuromuscular disorder, 1 with severe immunosuppression). The main co morbidities in adults were immunosuppressant (66%), chronic cardiac disease (49%), chronic respiratory disease (44%), and morbid obesity (32%). Two previously healthy patients died: a 43 years old man and a 28 years old pregnant woman in whom no other attributable cause of death could be found apart from A(H1N1)2009 infection. Only 4 co-infections were identified among the IA-related fatal cases, involving hAdV twice, hRV once and PIV3+hRV once. These patients had no statistical different risk on fatality compared with patients suffering from IA-mono infection ($P=0.7475$; OR: 1,216; 95% CI: 0.3391 - 4.359), but the numbers are too low for conclusions.

IV. Respiratory Virus Circulation Over the Pandemic Period

Figure 1 shows the temporal evolution of isolation of IA and other viruses. From the 2,371 positive samples, the four most detected viral species were A(H1N1)2009 (40.1%) with a peak in October, RSV (24.3%) with a peak in December, PIV1-4 (13.8%) with a peak in September and hRV (13.5%) with a peak in September-October.

V. Viral Co Infection Rate

Viral culture

Overall, 85 of the 1988 positive samples (15/7-31/12/2009)

from non-critical ill patients (4.30%) were infected by multiple viruses, as assessed by viral culture. But for a fair comparison with other seasons we looked specifically at the viral winter season (1/10/2009-31/3/2010), where 108 of 2487 positive samples (4.34%) were virally co-infected. This number of viral co-infections is significantly fewer compared to the 2007-08 and 2008-09 seasons harbouring 6.95% and 6.70% of co-infection rate in this population, respectively ($P=0.0004$, $X^2=15.48$, 2). Children <5 years were affected by the vast majority of co-infections (82.5 %), especially among those aged <3 years (76.5% of all co-infections in culture). As for co-infections involving specifically A(H1N1)2009, they were only detected in 2.9% of all described ILI patients, and in 3.99% in the whole winter season 2009-10, which was significantly lower than observed with IA strains from previous seasons 2007-08 and 2008-09, 12.7% and 6.4%, respectively ($P<0.0001$, $X^2=37.24$, 2).

Molecular assays

A similar trend was observed when analysing samples from 620 ICU patients, tested by multi parameter PCRs. Out of the 337 virus containing samples, 44 (13.1%) showed mixed viral infection compared to global viral co-infection prevalence's of 27.23%, 21.83%, 29.1% and 26.4% in 2007-08, 2008-09, 2012-13 and 2013-14 seasons, respectively ($P<0.0001$, $X^2=33.84$, 4). During the whole 2009-10 winter period, there were 43 co-infected samples out of the 322 positives (13.35%) within 550 ICU patients (Table 2). The majority of them suffering from co-infection were again <3 years old (88.2%). Within the ICU population, only 7% of A(H1N1)2009-positive samples contained another virus, whereas in the A(H1N1)2009-negative group, the co-infection rate was significantly higher and reached 18.4% ($P=0.0018$, $X^2=9.733$, 1; OR 0.3311; 95%CI: 0.1612 - 0.6801) (Figure 3).

Table II: Evolution of viral coinfections overall and influenza A (IA)-associated coinfections in ICU patients over 5 different influenza seasons (detected with multiparameter molecular assays*)

Dominant IA strain	Season	Samples	Mono-infection IA	Co-infection IA	Viral coinfections overall
		N	N	N (%)	%**
Seasonal A/H1N1	2007-2008	405	27	14 (34.2)	27.2%
Seasonal A/H3N2	2008-2009	531	19	11 (36.7)	21.8%
Pandemic A/H1N1pdm	2009	620	147	11 (7.0)	13.1%
Pandemic A/H1N1pdm	2012-2013*	1104	102	41 (28.7)	29.1%
Seasonal A/H3N2	2013-2014*	1915	69	24 (25.8)	26.4%

*70% of IA strains A/H1N1pdm2009 vs 30% A/H3N2; *60% seasonal A/H3N2 vs 40% A/H1N1pdm2009; *real-time PCRs for detection of 16 viruses; **% of all virus-positive samples

Looking at the seasons 2007-08, 2012-13 and 2013-14, no statistical difference was seen between viral co-infection numbers in IA-positive and IA-negative samples from ICU-patients (2007-08: $P=0.27$, $X^2=1.217$, 1; 2012-13: $P=0.8889$, $X^2=0.01951$, 1; 2013-2014: $P=0.8896$, $x^2=0.01925$, 1). In 2008-09, in contrast to the following (pandemic) season, significantly more IA-positive samples were virally co-infected than IA-negative samples ($P=0.038$, $X^2=4.307$, 1; OR: 2,316; 95%CI: 1.030 - 5.206).

The percentages of viral co-infections in critically-ill IA-infected patients in 4 surrounding seasons differ significantly from what was observed during the pandemic season ($P<0.0001$, $X^2=32.88$, 4). (Table 3)

Table III: Distribution of Influenza A and Influenza B infections over 5 flu seasons & during pandemic peak* in Belgium

Flu season (1/10/20xx-31/3/20xx)	Influenza A strain (IA)	Samples N	Influenza A positive N (%)	Influenza B positive N (%)
2007-2008	Seasonal A/H1N1	4838	236 (4.9)	78 (1.6)
2008-2009	Seasonal A/H3N2	5094	512 (10.1)	61 (1.2)
2009 pandemic peak*	A/H1N1pdm2009	4895	950 (19.4)	4 (0.0008)
2009-2010	A/H1N1pdm2009	5591	853 (15.3)	13 (0.002)
2012-2013	A/H1N1pdm & seasonal A/H3N2 ^a	1104	143 (13.0)	126 (11.4)
2013-2014	Seasonal A/H3N2 & A/H1N1pdm ^b	1915	93 (4.9)	3 (0.2)

*Pandemic peak from 15/7/2009 - 31/12/2009, study period

^a 30% of circulating IA strains concerned seasonal A/H3N2, 70% A/H1N1pdm2009

^b 60% of all IA-positive samples was A/H3N2, 40% A/H1N1pdm2009

VI. Testing Performances

Throat versus nasopharyngeal flocked swabs

In 272 patients, throat and nasopharyngeal swab obtained simultaneously could be both analysed separately. Of them, 65 throats and 91 nasopharyngeal samples were positive in viral culture. A number of samples was additionally positive by multi parameter PCR examination (12 and 13 in throat and nasopharyngeal groups, respectively). Overall, the nasopharyngeal swabs showed significantly greater sensitivity in detecting any respiratory virus compared to the throat swabs (38.2% versus 28.3%, $p=0.018$). Considering viral distribution (Supplement 2), hRV, A(H1N1)2009, and PIV predominated in the nasopharynx, whereas AdV, hEV and RSV similarly occurred in both sites. Only the nasopharyngeal swabs of these 272 patients were included in final epidemiologic analysis. (Supplement 2)

Culture versus real-time RT-PCR for the detection of influenza A (H1N1)v2009

The pandemic strain A(H1N1)2009 largely predominated among circulating influenza viruses (94.04% and 91.6% in culture and PCR assays, respectively). Out of 1993 samples for which both PCR and culture were simultaneously performed, A(H1N1)2009 was detected by RT-PCR in 641 (32.2%) samples but only in 532 (26.7%) by culture. The sensitivity of viral culture compared to PCR was 83.0% and increased to 87.8% if only NPA were considered.

The mean time to positivity of culture for A(H1N1)2009 on LLC-MK2 cells was 3.1 days (CI 95%: 2.1 - 7.3 d), and the mean time to result for RT-PCR IA was 1.3 day (CI 95%: 6 hours-3.6 d).

In previous seasons with a circulating seasonal IA strain, growth was seen after a mean time of 6.1 days and 4.4 days in 2007-08 and 2008-09 respectively.

Discussion

In this study, we provide an extensive description of viral epidemiology during the first pandemic wave of influenza A(H1N1)2009 by examining 4,895 samples of 4,101 ILI patients of all age groups, attending the Emergency department or polyclinics from seven hospitals in Brussels area. We were thereby able to demonstrate that co-infection rate during medically-attended ILI was clearly decreased when patients were infected by the pandemic strain, compared to other viruses or other influenza seasons. We also observed that the pandemic influenza variant circulated quite differently its first season compared to previous influenza strains, not only considering the targeted population (adolescents/young adults without risk factors), but also considering its impact on circulation of other respiratory viruses and its specific and rapid growth pattern in vitro.

Viral respiratory infections often show a yearly or biennial appearance during the winter months, such as observed with IA/IB, RSV, PIV and hMPV. Whereas these latter viruses are quite easily detected with rapid antigen assays, others are more challenging to diagnose in routine but could potentially be involved in co-infections and contribute to morbidity. So far, there have been several publications discussing the relative importance of mixed viral infections among ILI, particularly in combination with IA [14,20,21,22,]. However, findings and conclusions remain divergent. While some authors observed an increased severity of respiratory illness in children when infected with two or more viruses [13,23], others supported exactly the opposite [22]

or even didn't find out any association between co-infections and severity of respiratory illness [24]. Discrepancy of these findings may be partly explained by a large heterogeneity in study design, included populations (age range, comorbidity, or illness severity), geographical location, seasonal timing (influencing virus circulating) and method of viral detection (traditional culture and direct immunofluorescence assays versus molecular assays, variable performance characteristics of commercial kit versus in house molecular tests etc...). Among others, Esposito et al reviewed community-acquired pneumoniae etiologies in children and highlighted that the association of hRV with either RSV or hMPV could increase severity [25]. Furthermore, Goka et al. concluded from a large study including all age groups in North West England that co-infection with RSV and hAdV was associated with increased risk of admission to ICU, even though their results did not reach statistical significance. They further reported that co-infection with seasonal IA and IB was associated with a higher risk of ICU admission or death [14]. During the first pandemic wave in Argentina, Torres et al also observed that viral co-infection with RSV was associated to increased mortality in pediatric ICU, illustrating the critical scenario of two virulent pathogens circulating in parallel in the Southern hemisphere [26]. Luckily RSV peaked only at the end of the first pandemic wave in Belgium and Europe, so that we did not encounter much morbidity of RSV-A(H1N1)2009 co-infections. Unfortunately, our study could not assess whether co-infection resulted or not in a higher severity of illness, for two main reasons: firstly, rate of co-infections involving the pandemic strain was very low among ICU patients, irrespective of the outcome (11/620 IA-associated co-infections in total, fatality rate 4/11 vs. 47/147 among persons with multiple or single H1N1 infections, respectively, $P=0.7475$) and the patients could not be matched for underlying co morbidities. Any conclusions on the pathogenicity of each respiratory virus are therefore difficult to draw. Secondly, the use of multi parameter PCR was restricted to critically-ill patients (due to the lack of reimbursement in Belgium), rendering any comparison between general wards and ICU patients poorly reliable in terms of co-infection numbers.

Although the exact influence of viral co-infection on pathogenicity remains unclear, some viruses seem more prone to arise in co-infections than others. hRV is regardless of age groups in general the most prevalent virus in mixed viral infections, followed by hBoV, hCoV and hMPV. But during annual flu seasons influenza viruses are not infrequently encountered in viral co-infection neither. In this study, it was clear that in addition to almost complete exclusion of seasonal influenza strains [27], the pandemic variant, during its first circulation, was associated with a remarkably low rate of viral co-infection compared to other viruses overall and to other flu seasons. Indeed, only 7% and 2.9% of IA-positive samples harbored multiple pathogens compared to 18.4% and 5.5% in IA-negative specimens, as obtained by multi parameter PCR and viral culture respectively. This observation remained true irrespective of patient's status (critically ill or not, age, risk factors etc...) or included period. In absolute numbers, more A (H1N1)2009 positive samples were seen in the group

below 5y (highest number in toddlers <1y) than in age group 11-60y. In this age group one should expect a huge number of viral co-infection, but even there most of the IA+ samples showed mono-infection in contrast to other seasons.

IA-related co-infections most frequently included PIV (PIV-2 epidemics observed during the study period), hAdV and hRV. Looking at other published data, our observation was similar to the study of Lees EA [20] reporting a co-infection rate of only 7.4% when involving the pandemic strain. Furthermore, authors from Israel also corroborated our findings of lower viral co-infection rates during the first pandemic season [9]. However, our data contrast with the 13.1% co-infections described by Esper et al. [21] as well with data from France reporting higher co-infection rates (up to 19%) in A(H1N1)2009-positive samples [28]. Surprising was therefore the conclusion of the French authors claiming that the presence of hRV in the nasopharynx, though being normally not seldom associated with IA, reduced the likelihood of co-detection with A(H1N1)2009 and that hRV circulation delayed the spread of the pandemic flu [29]. In our study, hRV infections in Belgium showed a parallel increase in time with A(H1N1)2009 infections from August to September 2009 (Figure 1) and seemed not to have had an important impact on A(H1N1)2009 circulation in Brussels and surroundings. Once the pandemic peaked in October 2009, hRV stayed at a steady level as did hAdV and hCoV, while hEV arose and PIV decreased significantly with more than 50%. Probably slight geographical and atmospheric differences between the two countries could be responsible for this delay of appearance of A(H1N1)2009 in France. As for hRV, the pattern of RSV circulation was not much influenced by the early occurrence of flu pandemics in Belgium neither; with a peak around week 49-50 [this study; 30], in contrast to the delayed RSV epidemics observed in some countries [9]. Moreover, RSV remained by far more prevalent than A(H1N1)2009 in children <2 years similarly to other winter seasons.

Further, we were intrigued by the difference we observed in the same epidemiological context (region, population, sample types) during previous and following flu seasons. During the pandemic 2009, the co-infection rate within the group of IA-positive samples was significantly decreased in comparison with rates measured when seasonal A/H1N1 and A/H3N2 were predominating in 2007-08 and 2008-09, respectively ($P < 0.0001$). Furthermore, the total viral co-infection rate we measured in the pandemic season (including all respiratory viruses with/without IA) was also significantly lower than during previous influenza seasons, even when calculation was extended till the end of March 2010 to include the whole winter season ($P=0.0001$). Mixed viral infections in ICU globally predominated among children below 5y of age, which could be expected, because early in life children acquire 3-8 viral upper respiratory tract infections each year [30].

As found in the literature, an advanced age did not appear to be a major risk factor associated with influenza A(H1N1)2009 infection with only 7.2% positive patients above 60 years, and none above 80y old. This low incidence could be due to cross-reactive antibodies from previous exposure to IA. It was shown

that IA antibodies cross-react with A(H1N1)2009, and they were detected in up to one third of healthy adults >60 years [31]. In the Southern hemisphere, pandemic IA showed two incidence peaks, one in children ≤5y old and the second in individuals between 20-29 years [4,32]. However, here only one large incidence peak was seen: between 6 and 60 years old >70% of positive patients were infected by A(H1N1)2009, this feature being the single major epidemiologic signature of a pandemic. IA prevalence was highest in the age category “11-20” where 47.4% of patients were infected with the pandemic variant. Another Belgian study showed that IA mostly affected the 6-15 years old age group, comparable with our findings [33].

In our study as in others [21,28], hRV was the virus most frequently involved in co-infection overall, regardless of the presence of the pandemic strain. Debate is ongoing about the exact role played by hRV in virus-virus co-infections. Whereas hRV is often co detected with other viral pathogens as shown above [31], other authors have suggested that this virus possesses a competitive relationship with other viruses. In a study of 1,742 specimens Brunstein et al. reported a number of instances of suspected pathogen co suppression between specific viral combinations, particularly between single-stranded RNA-viruses [32]. It was described that hRV may render the host less likely to be infected by other viruses, for a certain time. On another hand, Esper et al. [21] found that hRV co-infection had little impact on severity of influenza disease; in fact, such patients had a lower median clinical severity score, while the opposite was true for non-hRV co-infections.

Focusing on ILI etiology, more than 40% of our samples were positive for at least one virus and A(H1N1)2009 was the most common virus causing ILI overall with a global prevalence of 22.1%. These results were in line with previous reports on viral etiologies of ILI during the A(H1N1)2009 pandemic, with incidences of viral infection ranging from 37% to 89%, depending on the study design [10,11,34]. In the ICU group 54.4 % of the samples were positive for at least one virus and 25.5% for A(H1N1)2009, similar to reports from the literature [35].

In conclusion, our study confirmed that during the 2009 flu pandemics, the novel A(H1N1)2009 strain was the most prevalent virus responsible for medically-attended ILI, except inside extreme age groups (infants and the elderly). Thanks to extensive epidemiological data collected, it was demonstrated that the pandemic strain during its first season was remarkably associated with a reduced likelihood of coincidental viral infection as compared to other respiratory viruses or other flu seasons. Much less co-infections were indeed detected in ILI episodes at that time, regardless of age and patient’s status. This unique feature could probably partly be due to the early circulation of the virus, but secondly due to changes in viral interferences related to the first circulation of A(H1N1)2009, since influenza A related co-infections increased again during consecutive flu seasons. The reasons why some viral strains are prone to arise in mono-infection as well as whether the presence of multiple viral pathogens

together influence ILI pathogenicity and severity remain to be

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Conflict of interest

The authors declare that they have no conflict of interest.

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Annex I. Technical Details of Samples Collection, Treatment and Analysis

Respiratory Specimen acquisition and handling

VTM was prepared as follows: 12.5 g of Difco Veal Infusion Broth (VIB, Becton & Dickinson, Erembodegem, Belgium) was dissolved in 500 mL of distilled water and the solution was autoclaved at 121°C for 15 minutes. After cooling, 2.5 g of Bovine Serum Albumin-Fraction V (Sigma-Aldrich, Diegem, Belgium) was dissolved in Sterile VIB and filtrated through a 0.45 µm filter and added to the rest of the VIB to a final concentration of 0.5%. VTM was supplemented with gentamicin, vancomycin and amphotericin B to a final concentration of respectively 333.33µg/mL, 66.66µg/mL and 33.33µg/mL. After reception, 3 mL of VTM was added to the respiratory sample and vortexed for 30 seconds. The samples were subsequently divided into two aliquots, 0.5 mL, to which an additional 2 mL of VTM was added, for virus culture, and 1 mL for molecular testing.

Viral Culture

Daily, three classical cell lines (LLC-MK2, Vero and MRC5) were inoculated, with respectively 300 µL, 150 µL and 150 µL of the diluted sample and subsequently incubated in a 10% CO₂ atmosphere at 36°C. The presence of a virus was determined by routine microscopic examination after 2, 3, 5, 7, 10, 14, and 21 days of incubation. The culture medium was replaced weekly.

Molecular assays

Target Sequence Selection and Primer and Probe Design

Specific primers and probes were selected and designed by using the Primer Express software, version 2.0 (Applied Biosystems (AB), Foster City, CA) according to sequences available from public databases (National Center for Biotechnology Information; NCBI). The oligonucleotide sequences, PCR products lengths, locations, and GenBank accession numbers of the corresponding target genes are displayed in Supplement 1. As indicated in this table, some of the primers reveal a degenerated code. This was a requirement for the detection of viral subspecies differing from each other by single nucleotides. All primers and probes included in this study were synthesized by Eurogentec (Seraing, Belgium) and Applied Biosystems. Prior to experimental testing, the primers and probe sequences were assessed for specificity by comparing them to sequences of other prokaryotic and eukaryotic organisms by using standard nucleotide-nucleotide BLAST (NCBI) alignment software. None of the selected oligonucleotide displayed significant homologies to any other sequences. Then, the optimal concentration of oligonucleotides used in real-time PCR was assessed. The oligonucleotide concentration which gives the lowest threshold cycle (Ct) value and maximum amplification efficiency was selected (Supplement 1).

DNA Extraction

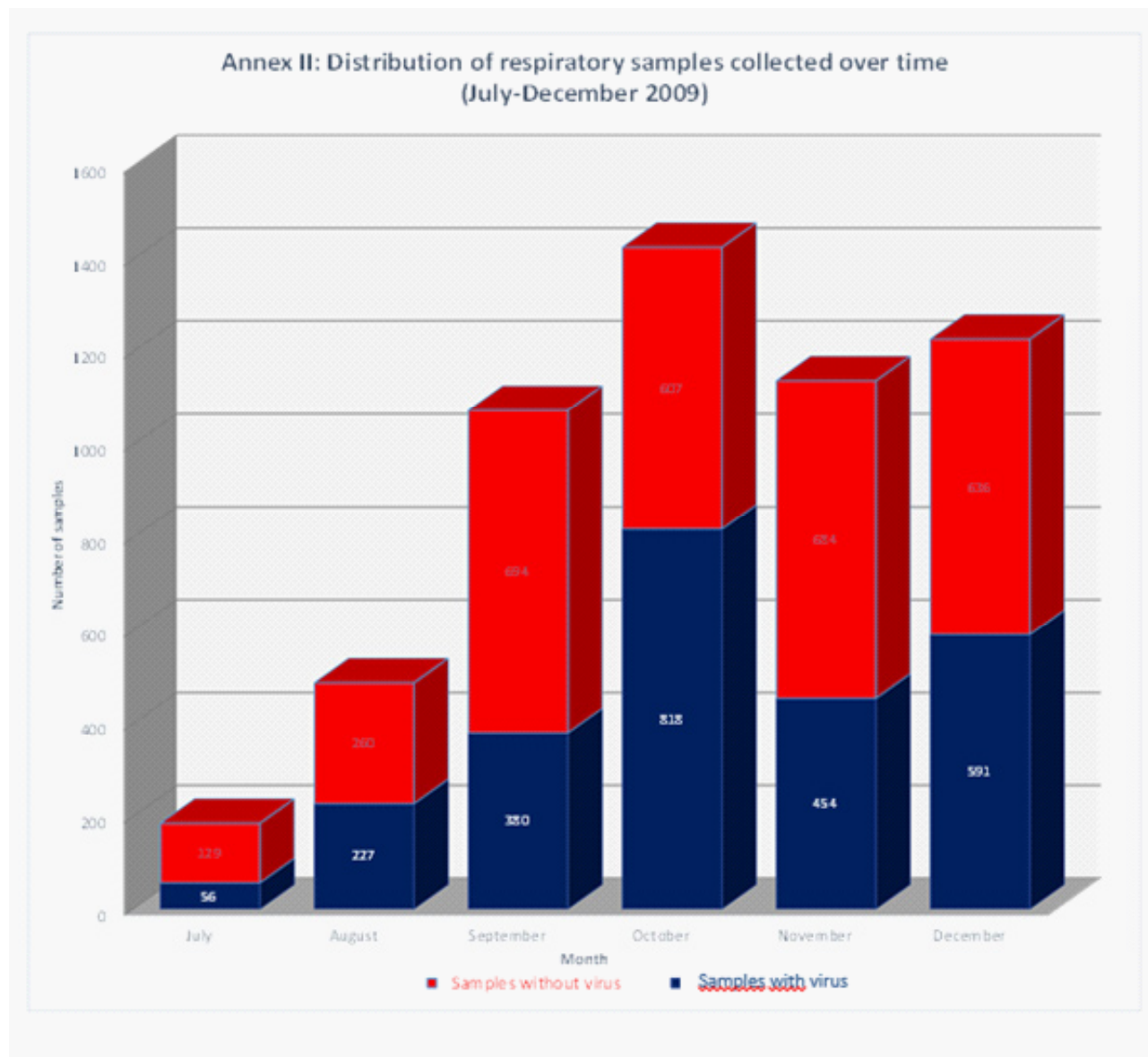
Genomic DNA and RNA were isolated using the MagNA Pure LC (Roche Diagnostics) instrument by using the Total

Nucleic Acid Isolation Kit - Large Volume. 25 µL of a nonhuman control virus (Phocid Distemper virus, (Kindly provided by dr. G. van Doornum, University of Rotterdam, The Netherlands), was spiked into each sample prior to DNA and RNA extraction. In total, for nucleic acid extraction, the input volume was 500 µL and the elution volume was 100 µL. The nucleic acid recovered was stored at -70°C until further testing.

Real-time PCRs

An in house reversed transcriptase real-time PCR (rtRT-PCR InflA), targeting the matrix protein-coding gene, was used for the detection of IA virus (Supplement 1). This one-step RT-PCR is able to detect all subtypes of IA Viruses including A (H1N1) pdm2009. For specific detection of the circulating pandemic variant, two multiplexed RT-PCR assays according to the Centers of Disease Control (CDC) protocol were initially used (from the 15th of July till the end of August 2009): the SW InflA PCR (swInflA) and the SW H1 PCR (swH1), further called as RT-PCR A/H1N1 [16]. From September on, A (H1N1) pdm2009 subtyping was based only on swInflA detection, due to the numerous observed discrepancies in laboratories worldwide and the suspicion of mismatches in the swH1 primer, probe binding regions and of existing variants of the pandemic strain [17]. Five reactions were set up as duplex PCRs. The reaction was composed of 12.5 µL of reaction mix, 50 to 900 nM concentrations of primers of each virus, 100 to 300 nM TaqMan probe of each virus (Supplement 1), and 10 µL of nucleic acid extract to a final volume of 25 µL. For the human Boca virus (hBoV) human Adenovirus (hAdV) duplex, the reaction mix consists of the Light Cycler 480 Probes Master (Roche Diagnostics). For IARSVA, IB-PIV2, RSVB-hMPV (hMPV 1A, 1B, 2A, 2B) and hRV-PIV3 duplexes, the reaction mix consists of 1x TaqMan EZ buffer (AB), 3 mM manganese acetate solution, 300 nM each dNTP, 2.5 U rTth DNA polymerase and 0.25 U Amperage UNG (AB). The six remaining RT-PCR reactions (229E, NL63, OC43, hEV, PIV1 and PIV4) were set up as single plex PCRs in a total volume of 25 µL containing 1X TaqMan A buffer (AB), 5.5 mM magnesium chloride, 300 nM each deoxyribonucleotide triphosphate (dNTP), 0.625 U AmpliTaq Gold DNA polymerase (AB), 6.25 U Multi Scribe reverse transcriptase (AB), 10 U RNase inhibitor (AB), 50 to 900 nM concentrations of primers, 100 to 200 nM TaqMan probe, and 10 µL of nucleic acid extract. The concentrations of primers and probes are specified in Supplement 1. The mixtures were then prepared in 96-well optical micro titer plates (Roche, Vilvoorde, Belgium), centrifuged for 1 min at 250 X g and amplified by using the following cycling parameters: for duplex PCRs performed on the Light Cycler 480 (Roche Diagnostics, Vilvoorde, Belgium): 2 min at 50°C, 30 min at 60°C, 5 min at 95°C, and 50 cycles of 20 s at 95°C and 60 s at 62°C. For single plex PCRs performed on iCycler (Bio-rad, Diegem, Belgium): 30 min at 48°C, 5 min at 95°C, and 50 cycles of 15 s at 95°C and 60 s at 60°C. During the weekends, PCRs were not performed, and samples that arrived on Friday after 1 p.m. were examined on Monday morning. During seasons 2012-2013 and 2013-2014, samples of critical-ill patients in ICU were prospectively tested for respiratory pathogens with an in house customized TAC (Taqman™ Array micro-array Card) respiratory panel which includes testing for the following pathogens: IA (H1,

H3, H5, H7), IB, RSV A, RSV B, PIV 1 to 4, hAdV, hRV, hEV, hMPV, coronavirus (hCoV) (229E, HKU1, OC43, NL63, SARS), hBoV, cytomegalovirus (CMV), parainfluenza virus, mumps virus, measles virus, and nine bacterial respiratory pathogens. From each Respiratory patient sample, 78 µL of nucleic acid extract and 26 µL of Taqman Fast Virus 1-step mastermix (Life Technologies, Carlsbad, CA) were mixed and added to the TAC sample port. A reversed transcriptase RTPCR was performed on the Viia 7 (ThermoFisher, Carlsbad, CA) using following amplification protocol: 50°C for 5 min, 95°C for 20s, and 40 cycles of 95°C for 1s followed by 60°C for 20s. Multiple genetic targets per pathogen are being detected; sample adequacy and extraction/amplification inhibition is assessed. The system reports a cycle threshold for each positive PCR assay so that the load of the micro-organisms present in the clinical sample can be estimated. Based on the internal QC (Phocid Distemper Virus) data a %CV of 9.5% was registered, indicating a highly reproducible method.



Supplement 1: Optimized primers and probes for in house multiplex real-time PCR (2007-2010).

Virus type	Target gene	Amplicon length (bp)	Oligonucleotide sequence (5'-3')	Probe labeling (5'-3')	Concentration (nM)	Nucleotide positions	GenBank accession n°
IB	MT	95	GAG ACA CAA TTG CCT ACC TGC TT AGA AGA TGG AGA AGG CAA AGC AGA ACT AGC TTC TTT CCC ACC GAA CCA AC	Cy5 BHQ3	100 200 300	14-36 45-74 89-108	FJ461677
hAdV	HEX	147	ACT CCG CCC ACG CGC TMG ACA TG TTT GAG GTG GAT CCC ATG GAC GAG C GAG AAG GGB GTG CGC AGG TA	Cy5 BHQ3	200 100 900	2758-2780 2784-2806 2889-2904	EU128938
NLB3	N	78	GAA GCG TGT TCC TAC CAG AGA AAA TGT TAT TCA GTG CTT TGG TCC TCG TGA GAA TCC CCC ATA TTG TGA TTA AA	FAM BHQ1	600 200 900	708-728 732-761 763-785	EF081296
PIV2	HN	167	ACY CAT TGG TGT TAY ACY CA TGG AGA TTG YCT YGA TTT CAC GGC A CAG CTT TTG CGA TTG ATT CC	FAM BHQ1	900 300 600	7348-7368 7381-7402 7496-7515	AF533011
IA	MT	95	AAG ACC AAT CCT GTC ACC TCT GA TTT GTG TTC ACG CTC ACC GTG CC CAA AGC GTC TAC GCT GCA GTC C	FAM BHQ1	400 100 300	189-191 202-226 242-263	GQ161131
PIV3	HN	170	GGA CCA GGG ATA TAC TAY AAA ATC TGY AAC ACA ACT GGR TGT CCY GGG AA TTG ACC ATC CTY CTR TCT GAA	FAM BHQ1	900 200 900	7772-7792 7850-7878 7921-7941	EU424082
PIV1	HN	82	TGA TTT AAA CCC GGT AAT TTC TCA T ACG ACA ACA GGA AAT C CCT TGT TCC TGC AGC TAT TAC AGA	MGBNFQ BHQ1	300 100 300	7619-7643 7657-7672 7677-7700	AF457102
OC43	N	68	GCT CAG GAA GGT CTG CTC C TTC CAG ATC TAC TTC GCG CAC ATC C TCC TGC ACT AGA GGC TCT C	FAM BHQ1	900 200 50	29903-29921 29925-29949 29953-29970	FJ838087
PIV4	HN	76	ATG GTG GGA GAY ATT GCA AA ATA TAG CYA ATG TCG GAA TGA GYG CGT TCT TT CCA AGC CGA ACT TAA GYG TAA	FAM BHQ1	600 200 600	790-809 812-843 845-865	M32982
229E	N	82	CGC AAG AAT TCA GAA CCA GAG CCA CAC TTC AAT CAA AAG CTC CCA AAT G GGG AGT CAG GTT CTT CAA CAA	FAM BHQ1	200 100 300	355-375 379-406 416-436	DQ243982
RSVA	NP	84	AGA TCA ACT TCT GTC ATC CAG CAA CAC CAT CCA ACG GAG CAC AGG AGA T TTC TGC ACA TCA TAA TTA GGA GTA TCA AT	Cy5 BHQ3	900 250 600	42-65 69-92 97-125	DQ780565
RSVB	NP	129	CAC TCC CAA TTA TGA TGT GCA AAA CAA ACT ATG TGG TAT GCT ATT AAT CAC TGA TCC CTT CCT AAC CTG GAC ATA GC	Cy5 BHQ3	400 100 700	1188-1209 1219-1246 1292-1314	D00738
hBoV	NP1	76	TCG GGC TCA TAT CAT CAG GAA ACC CAA TCA GCC ACC TAT CGT CTT GCA TCA CTT GGT CTG AGG TCT TCG AA	FAM BHQ1	900 100 900	2554-2574 2576-2602 2607-2629	EU262979
HMPV	N	163	CAT ATA AGC ATG CTA TAT TAA AAG AGT CTC TGY AAT GAT GAG GGT GTC ACT GCG GTT G CCT ATT TCT GCA GCA TAT TTG TAA TCA G	FAM BHQ1	900 250 200	89-118 149-178 224-251	AB503857
hRV	5' UTR	131	TGT GAA GAS YCS CRT GTG TCC TCC GGC CCC TGA ATG CG GTA GTC GGT CCC ATC CC	FAM BHQ1	300 100 300	304-321 333-352 418-434	FJ042525

hEV	5' UTR	148	CCC TGA ATG CGG CTA ATC C		900	423-441	FJ461781
			AAC CGA CTA CTT TGG GTG TCC GTG TTT C	FAM BHQ1	250	508-533	
			ATT GTC ACC ATA AGC AGC CA		900	549-568	
PDV	HA	77	CGG GTG CCT TTT ACA AGA AC		800	44-62	FJ648456
			ATG CAA GGG CCA ATT CTT CCA AGT T	FAM BHQ1	250	64-86	
			TTC TTT CCT CAA CCT CGT CC		900	101-120	

Legend: Sequences are for the forward primer, probe, and reverse primer (top, middle, and bottom, respectively).

UTR: untranslated region.

MT: matrix protein.

HA: hemagglutinin.

HN: hemagglutinin/neuramidase.

FAM: 6-carboxyfluorescein.

BHQ1: black hole quencher.

MGBNFQ: minor groove binder nonfluorescent quencher.

M, A/C; B, T/C/G; Y, C/T; R, A/G; S, C/G.

IA: influenza A virus; IB: influenza B virus; hEV: enteroviruses; hAdV: adenoviruses; hRV: rhinoviruses; hMPV: human metapneumoviruses; hBoV: bocaviruses; NL63: coronavirus-NL63; OC43: coronavirus OC43; 229E: coronavirus 229E; PIV1-PIV4: parainfluenzaviruses 1-4; RSVA-B: respiratory syncytial viruses A & B.

Supplement 2: Evaluation of diagnostic sensitivity for respiratory viral detection of two different anatomical sites by comparing 272 parallel nasopharyngeal and throat samples.

	Throat swab (n)	Nasopharyngeal sample* (n)
Rhinovirus	1	11
Adenovirus	13	11
Influenza A virus	28	38
Influenza B virus	1	1
Enterovirus	7	4
Respiratory Syncytial virus	6	7
Parainfluenza virus 1-4	18	29
Herpes Simplex virus	3	3
*Nasopharyngeal aspirates and nasopharyngeal flocked swabs		