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# Rate and influence of respiratory virus co-infection on pandemic (H1N1) influenza disease<sup>☆</sup>

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## KEYWORDS

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Respiratory disease

**Summary Objectives:** Many patients with influenza have more than one viral agent with co-infection frequencies reported as high as 20%. The impact of respiratory virus copathogens on influenza disease is unclear. We sought to determine if respiratory virus co-infection with pandemic H1N1 altered clinical disease.

**Methods:** Respiratory samples from 229 and 267 patients identified with and without H1N1 influenza respectively were screened for the presence of 13 seasonal respiratory viruses by multiplex RT-PCR. Disease severity between coinfecting and mono-infected H1N1 patients were quantified using a standardized clinical severity scale. Influenza viral load was calculated by quantitative RT-PCR.

**Results:** Thirty (13.1%) influenza samples screened positive for the presence of 31 viral copathogens. The most prominent copathogens included rhinovirus (61.3%), and coronaviruses (16.1%). Median clinical severity of both mono-infected and coinfecting groups were 1. Patients coinfecting with rhinovirus tended to have lower clinical severity (median 0), whereas non-rhinovirus co-infections had substantially higher clinical severity (median 2). No difference in H1N1 viral load was observed between coinfecting and mono-infected groups.

**Conclusions:** Respiratory viruses co-infect patients with influenza disease. Patients coinfecting with rhinovirus had less severe disease while non-rhinovirus co-infections were associated with substantially higher severity without changes in influenza viral titer.

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## Introduction

Respiratory tract disease is a major cause of morbidity and mortality throughout the world accounting for approximately 4 million deaths worldwide.<sup>1,2</sup> While viruses such as influenza, parainfluenza, respiratory syncytial virus (RSV), and adenovirus are well described as major respiratory pathogens, advancements in molecular and genomic techniques have increased detection and identification of new respiratory viruses.<sup>3–11</sup> There is also renewed attention on respiratory viruses including the human rhinovirus and coronaviruses as there is mounting evidence that these viruses can also lead to clinically severe disease.<sup>5,12–18</sup>

Currently, the majority of practitioners approach respiratory viral infections assuming a single-agent etiology. However, our group and others have realized that a substantial number of patients with respiratory tract disease have more than one viral pathogen.<sup>19–21</sup> The frequency of respiratory virus co-infections varies widely in the literature but is often reported between 10 and 20%<sup>22–25</sup> and in one report as high as 60%.<sup>26</sup> This is understandable as many respiratory viruses circulate at similar times often with a winter time predominance in temperate climates. Recently, Brunstein et al. provided statistical evidence that co-infection with certain pathogens occurs more frequently than expected if co-infection was random.<sup>25</sup> Influenza has often been identified with co-infecting viruses.<sup>20</sup> Several groups speculate that early spread of influenza may be altered by co-circulation of traditional seasonal viruses.<sup>27–30</sup>

There is increasing evidence in the literature for the importance of polymicrobial infections. Virus–virus interactions have been recognized both directly, indirectly and through immunological interactions.<sup>31</sup> Yet the clinical relevance of respiratory virus copathogens in association with disease is unclear. Several studies have reported viral co-infections being associated with increased morbidity. These studies show greater hospitalization rates and admission to intensive care associated with co-infection.<sup>24,32,33</sup> However, this remains controversial as several other reports describe co-infections having no increase in patient morbidity.<sup>13,23,34–38</sup>

One explanation for the disagreement is the assumption that disease severity associated with dual infection is independent of the copathogen involved. Aberle et al. found that dual infections with rhinovirus and RSV was associated with reduced IFN- $\gamma$  response and a more severe clinical course than other co-infection combinations.<sup>22</sup> In animal models, specific dual respiratory infections resulted in either enhanced clinical manifestations or viral interference.<sup>39–41</sup> These findings suggest certain copathogen pairings may be more clinically relevant than others.

There remain gaps in our knowledge and understanding of respiratory virus co-infections. Previous investigations of influenza co-infection either combine viral copathogens together in analysis<sup>20</sup> or do not address clinical outcomes.<sup>26–30,42</sup> Studies quantifying the effect of viral co-infection are lacking yet such lines of investigation will be paramount to our understanding of viral pathogenesis. In this study we identify the prominent viral copathogens occurring with pandemic H1N1 influenza and compare the resulting clinical disease. This is

the first study which critically looks at how specific copathogens alter resulting influenza disease and the effect on influenza viral load.

## Materials and methods

### Sample collection

From September through November 2009, corresponding to the time of the major H1N1 pandemic wave, we archived respiratory specimens which screened positive for influenza. For comparison, we selected influenza negative samples collected during this same period. All samples originated from the emergency department, inpatient wards, intensive care units and hospital affiliated primary care outpatient clinics. Samples were submitted to the Core Laboratory at the discretion of the primary medical teams for influenza testing. Influenza screening was performed by either direct immunofluorescence assay (DFA) or real time RT-PCR using standardized techniques.<sup>43,44</sup> All samples were confirmed by RT-PCR using primer sets targeting pandemic H1N1.<sup>43</sup>

### RNA extraction, reverse transcription

Nucleic acid from respiratory samples were extracted using either QIAcube (QIAamp Viral RNA Kit, QIAGEN, Valencia, CA) or MagMAX™-96 Total NA Isolation Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. Random hexamer primers (Invitrogen Carlsbad, CA) were used to create a cDNA library for each specimen. Reverse transcription reactions were performed with M-MLV RT (Invitrogen, Carlsbad, CA) according to the manufacturer's specifications.

### Respiratory virus screening

Each cDNA was subsequently screened for the presence of common respiratory viruses including, human parainfluenza 1–3 (HPIV 1–3), human metapneumovirus (MPV), respiratory syncytial virus (RSV), rhinovirus (RV), adenovirus (AdV), and human coronaviruses (HCoV) 229E, OC43, and NL63. Primers and probe sets used for multiplex real-time RT-PCR detection have been described.<sup>45</sup> We screened respiratory samples under conditions as described with the exception that chlamydia, mycoplasma, and enterovirus primer sets were omitted. In addition, recently recognized respiratory viruses including coronavirus HKU1, WU Polyomavirus, and human bocavirus were screened using conventional RT-PCR in separate reactions using primer and reaction conditions described.<sup>9,15,46</sup>

### Clinical severity score

Medical records of H1N1 positive individuals were reviewed and recorded on a standard collection form. The clinical severity was scored by criteria as described by Martinello et al.<sup>47</sup> This clinical severity score (CSS) was chosen as it is designed specifically around respiratory disease. The CSS ranges from 0 to 6. Two points are assigned if the patient

required mechanically ventilator support during the illness, and 1 point is assigned for each of the following: hospital admission, prolonged hospitalization ( $\geq 5$  days), severe hypoxia  $\leq 87\%$ , and use of supplemental oxygen. H1N1 mono and coinfecting individuals were then compared for clinical severity. We defined severe respiratory disease as patients with CSS  $> 3$ .

Sample collection and chart analysis was approved by the University Hospital-Case Medical Center IRB.

### Quantitative real time PCR

Quantitative analysis was performed on a StepOne Plus Taqman Real Time PCR (Applied Biosystems, Branchburg, NJ) using TaqMan Universal PCR Master Mix (Applied Biosystems, Branchburg, NJ), 2ul of cDNA sample, and primers/probes targeting the H1N1 matrix gene.<sup>43</sup> A reference standard was prepared using a cDNA fragment of the H1N1 matrix gene and human RNase P amplified by conventional RT-PCR, gel purified (QIAquick, Qiagen, Valencia, CA), and quantified using a spectrophotometer (Beckman Coulter, Brea, CA).

### Statistical analysis

Statistical analysis was performed using R software (version 2.11.1).<sup>48</sup> Student *t*-test was used to analyze the mean log<sub>10</sub> viral copy numbers between monoinfected and coinfecting groups. Chi-square test of independent proportions was used to analyze clinical severity between mono and coinfecting patients. A two-tailed *p* value  $< 0.05$  was considered significant.

### Results

A total of 229 H1N1 influenza positive samples and 267 H1N1 negative samples were screened for the presence of common respiratory viruses. Influenza samples originated from 133 (58.0%) adults (age  $\geq 18$  yrs) and 96 (42.0%) children (age  $< 18$  yrs) with a median age of 20.3 yrs. Thirty (13.1%) influenza samples screened positive for the presence of 31 viral copathogens including 19 (61.3%) rhinovirus, 5 (16.1%) coronaviruses, 2 (6.4%) adenovirus, 2 (6.4%) human parainfluenza type 2 (HPIV2), and 1 (3.2%) each for WU polyomavirus, HPIV1 and RSV (Table 1). One pediatric patient was identified having co-infection with both HPIV2 and coronavirus HKU1 in addition to influenza. Neither hMPV nor HCoV NL63 were detected. With the exception of RSV and HPIV1, The proportion of patients positive for all tested viruses was similar for patients with and without influenza H1N1 infection. Median age for coinfecting individuals was 19.2 yrs and included 17 adults (median age 37.7 yrs) and 13 children (median age 7.4 yrs). More adults had rhinovirus co-infection than children (12 vs. 7 respectively).

Charts for 196 (98.0%) monoinfected and 30 (100%) coinfecting patients were available for chart review and clinical severity was calculated. Median clinical severity of both monoinfected and coinfecting groups were 1 (Table 2). However, influenza patients coinfecting with rhinovirus tended to have lower clinical severity (median 0), whereas non-rhinovirus co-infections had substantially higher clinical

**Table 1** Presence of respiratory viruses in H1N1 and non H1N1 positive respiratory samples.

Virus	H1N1 Positive	H1N1 Negative
	(N = 229)	(N = 267)
	# (%)	# (%)
RhinoVirus	19 (8.3%)	35 (13.1%)
Coronaviruses <sup>a</sup>	5 (2.2%)	3 (1.1%)
Adenovirus	2 (0.9%)	6 (2.2%)
HPIV1	1 (0.4%)	16 (5.9%)
HPIV2 <sup>a</sup>	2 (0.9%)	1 (0.4%)
HPIV3	0 (0.0%)	1 (0.4%)
WU Polyoma	1 (0.4%)	0 (0.0%)
RSV	1 (0.4%)	13 (4.9%)
HBoV1	0 (0.0%)	2 (0.7%)
Total <sup>a</sup>	30 (13.1%)	77 (28.8%)

<sup>a</sup> 1 Patient coinfecting with HPIV2 and coronavirus.

severity (median 2). As a group, coinfecting individuals had a higher proportion of severe disease (CSS  $> 3$ ) compared to influenza mono-infection (26.7% vs 11.2%,  $P < 0.05$ ), the majority of which is derived from non-rhinovirus co-infections (62.5%). Coinfecting adults and children had severe disease at similar rates (29.4% vs 23.0%). The majority of influenza mono-infecting patients with severe disease were adults (17, 15.2%) compared with only 5 (6.0%) children.

Influenza patients with rhinovirus co-infection tended to have less admission to the hospital (52.6% vs. 69.8%) and oxygen use (21.1% vs. 31.0%) (Table 3). Non-rhinovirus co-infections had significantly higher ventilator use (45.5% vs. 8.5%) and severe hypoxia (36.4% vs. 9.0%) compared to influenza mono-infecting patients ( $P < 0.05$ ). Coronavirus co-infection had a surprisingly high CSS (Median 4) with 80% being admitted to the hospital and requiring oxygen use as well as 60% requiring mechanical ventilation.

Influenza viral load was calculated by quantitative RT-PCR (Fig. 1). No significant difference in mean viral load was observed between coinfecting or mono-infecting groups ( $P = 0.36$ ). Influenza titer originating from samples coinfecting with rhinovirus was similar to influenza samples coinfecting with other viruses.

### Discussion

There are several reports where viral interactions have measurable outcomes on the course of infections. However, these interactions are relatively unexplored given the number of viral illnesses which occur. Most investigations focus on lifelong viral infections such as HIV, Epstein-Barr, and hepatitis C. Due to the relatively short duration of viral respiratory infections, co-infection is thought to be less of a concern.<sup>31</sup> We are only now beginning to recognize the diversity of the nasopharyngeal microbiome in the normal and diseased states.<sup>49,50</sup> With this comes the realization that co-detection of pathogens may become the norm, making our understanding of clinically relevant pathogen pairings essential. This is one of the first investigations to quantify clinical severity of influenza disease based on specific copathogens.

**Table 2** Clinical severity scores (CSS) in H1N1 mono and coinfecting patients.

	Median severity score	Severe disease (CSS >= 3)
H1N1 Monoinfection (N = 196)	1	22 (11.2%)
H1N1 Co-infection (N = 30)	1	8 (26.7%)
Rhinovirus (N = 19)	0	3 (15.8%)
Non-Rhinovirus (N = 11)	2	5 (45.4%)
<sup>a</sup> Coronavirus (N = 5)	4	3 (60.0%)
HPIV1 (N = 1)	1	0 (0.0%)
<sup>a</sup> HPIV2 (N = 2)	2	1 (50.0%)
RSV (N = 1)	6	1 (100%)
WU Polyoma (N = 1)	5	1 (100%)
Adenovirus (N = 2)	1	0 (0.0%)

<sup>a</sup> 1 Patient coinfecting with HPIV2 and coronavirus.

Our co-infection rate of 13.1% is similar to those reported in other studies.<sup>24,38,42</sup> Difference in patient population, time of sample collection, selection of pathogens to be screened and screening methodologies all contribute to variance in co-infection rates between studies. However, with more sensitive diagnostic methods becoming widely used combined with identification of previously unrecognized viral pathogens, we should expect the number of influenza co-detections to increase.

Similar to other investigations the predominant pathogen co-infecting with influenza was rhinovirus.<sup>24,26,42</sup> Rhinovirus has historically been considered ubiquitous but clinically mild. Yet a growing number of reports now challenge this notion.<sup>16–18</sup> Rhinovirus co-infections have been associated with worse disease when co-infecting with RSV.<sup>13</sup> Our findings demonstrate this is not true regarding influenza. Rhinovirus co-infection had little impact on influenza disease, in fact they had milder findings in several key areas. The opposite was true in patients with coronavirus/influenza co-infection. Comparable to other studies,<sup>38,42</sup> we observe the total number of influenza and coronavirus co-infections is low (1.12%). But we find that the resulting disease was substantially more severe. This highlights the importance of recognizing respiratory virus pairings as distinct clinical entities.

Viral interference of seasonal viruses (especially rhinovirus) with pandemic influenza has been suggested recently. Much of this is based on surveillance and inferred relationships of rhinovirus and influenza detection.<sup>27</sup> However our study demonstrates that influenza viral titer was not altered by viral co-infection suggesting that obstruction of influenza growth, either directly or indirectly, is not occurring. Explanation as to why disease severity was altered without changes in viral titer may lie in the resulting immunologic response. Investigation of host cytokine and cellular

response associated with co-infections is a logical next step.

We should recognize that the absence of specific co-infections may be as important as those which are observed. Both RSV and HPIV1 were significantly absent from influenza samples greater than expected if co-infection occurred by chance alone. This pattern is often noted with RSV, influenza and parainfluenza: when one of these epidemic respiratory viruses reached a peak the others seemed to be relatively inactive.<sup>29,52</sup> One wonders if the reasons behind this pattern involve direct or indirect viral interactions including changes in tissue permissiveness, viral replication, or host immunologic response.

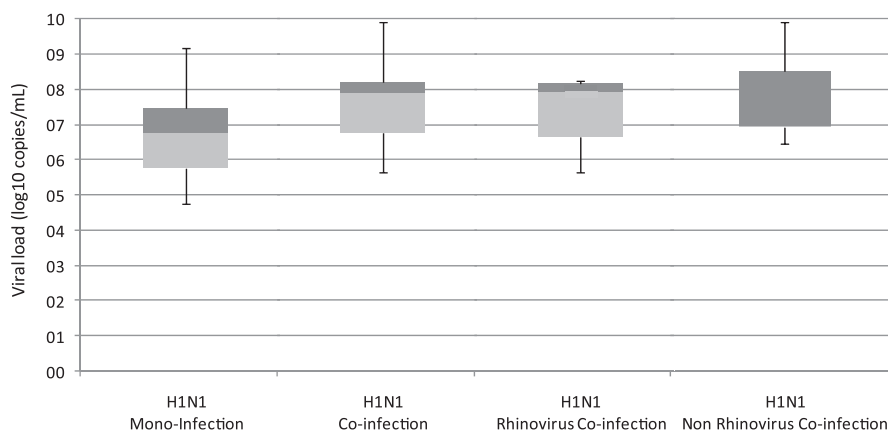
Our study has several limitations. First, not all possible viral pathogens were targeted. The co-infection rate may be even higher were we to expand the number of pathogens to include enterovirus, HPIV4, and other influenza virus types (B and C). Time to presentation also varied within the groups which may affect the sensitivity of viral detection. Advancing molecular modalities including MassTag PCR, ViroChip and Deep sequencing will ultimately provide a more complete picture of co-infection frequency. It has been well described that severe clinical illness in the 2009 H1N1 pandemic was associated with underlying co-morbidities including obesity, diabetes, or pregnancy. Collection and screening of samples as well as the use of the clinical severity score were independent of any patient's underlying co-morbidities. In addition to patient co-morbidities, the severity of illness may also be associated to other factors, such as delayed medical attention and lack of antiviral therapy. Larger studies that include multivariate analysis of these parameters should be undertaken.

It is important to note, the morbidity and mortality of the 2009 pandemic influenza virus differs from seasonal influenza viruses. Therefore, it is not clear that what

**Table 3** Breakdown of clinical severity score attributes between mono and coinfecting H1N1 patients.

	Vent	Admit	Admit ≥ 5d	O2 ≤ 87%	O2 Given
H1N1 Monoinfected (N = 199)	8.5%	69.8%	28.1%	9.0%	31.0%
H1N1 with co-infection (N = 30)	23.3%*	60.0%	26.7%	20.0%	33.3%
Non-rhinovirus co-infections (N = 11)	45.5%*	72.7%	27.3%	36.4%*	54.5%
Rhinovirus (N = 19)	10.5%	52.6%	26.3%	10.5%	21.1%

\*P < 0.05 compared to H1N1 mono-infection.



**Fig. 1** Quantitative RT-PCR of influenza in mono and coinfecting patients. Boxplots demonstrate lower and upper quartiles (light and dark shaded respectively) as well as minimum and maximum values (bars) for each group.

happened with the 2009 pandemic influenza virus can be extrapolated to seasonal influenza viruses. Also, relative humidity affects both influenza virus transmission and influenza virus survival.<sup>51</sup> Comparisons of findings when influenza is spread mainly by contact (similar to rhinovirus at times of higher absolute humidity) and when it is spread as small particle aerosol (during the peak and lower humidity) may differ. This may explain some differences in our findings compared to similar studies.<sup>38</sup> Furthermore, while we point out that disease severity likely depends on the type of the co-infecting pathogen, the numbers of certain co-detected pathogens in the H1N1 infected patients are too small for more detailed analysis. Continued investigation over multiple seasons will be required to answer these questions. Despite these limitations, we demonstrate that clinical relevance of influenza viral co-infection varies greatly and may be associated with the co-infecting pathogen involved.

The recognition of respiratory viral co-infections is increasing with advancement of sensitive multiplex screening of respiratory samples. As the sensitivity of diagnostic methods increase, the number of viral co-infections will continue to rise. Further investigation quantifying the effect of viral co-infection and identification of relevant copathogen pairings will be paramount to our understanding of viral pathogenesis.

## Ethical approval

Internal Review Board: Collection of specimens and clinical data were approved by the University Hospitals Human Investigation Committee.

## Conflicts of interest

None of the authors report conflicts of interest.

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