Does Virus–Bacteria Coinfection Increase the Clinical Severity of Acute Respiratory Infection?

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This retrospective cohort study investigated the presence of bacteria in respiratory secretions of patients hospitalized with acute respiratory infections and analyzed the impact of viral and bacterial coinfection on severity and the mortality rate. A total of 169 patients with acute respiratory infections were included, viruses and bacteria in respiratory samples were detected using molecular methods. Among all samples, 73.3% and 59.7% were positive for viruses and bacteria, respectively; 45% contained both virus and bacteria. Bacterial coinfection was more frequent in patients infected by community respiratory viruses than influenza A H1N1pdm (83.3% vs. 40.6%). The most frequently bacteria detected were *Streptococcus pneumoniae* and *Haemophilus influenzae*. Both species were co-detected in 54 patients and identified alone in 22 and 21 patients, respectively. Overall, there were no significant differences in the period of hospitalization, severity, or mortality rate between patients infected with respiratory viruses alone and those coinfected by viruses and bacteria. The detection of mixed respiratory pathogens is frequent in hospitalized patients with acute respiratory infections, but its impact on the clinical outcome does not appear substantial. However, it should be noted that most of the patients received broad-spectrum antibiotic therapy, which may have contributed to this favorable outcome. ***J. Med. Virol.***

KEY WORDS: acute respiratory infections; respiratory viruses; influenza A H1N1pdm; bacteria

INTRODUCTION

Viral and bacterial respiratory tract infections are major causes of morbidity and mortality worldwide despite the development of vaccines and powerful antibiotics [Deng, 2013]. An estimated 3–5 million people die of these infections annually, which is greater than the number of deaths caused by HIV/AIDS and tuberculosis combined [WHO, 2011]. During the 2009 pandemic influenza (H1N1pdm), bacterial pneumonia was registered in 25–30% of cases requiring hospitalization. Furthermore, almost 50% of small autopsy series highlight the importance of viral–bacterial coinfections, suggesting that several lower respiratory tract infections are caused by multiple pathogens acting in synergy [Deng, 2013].

The outcomes of this pandemic influenza were especially severe in the southern region of Brazil, with a sample positivity rate of 39% and a mortality rate of 35% [Raboni et al., 2011]. The high mortality rate observed in these patients led to questions about the presence of other factors that could be related to the severity of these infections, including associations with bacterial infection.

Therefore, this study evaluated the detection frequency of common community bacteria in respiratory tract samples from hospitalized patients with acute

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respiratory infections as well as the impacts of bacteria–virus coinfection in these patients during the H1N1 influenza A pandemic.

MATERIALS AND METHODS

Patients

Specimens collected from the respiratory tract, nasopharyngeal aspirates (n = 164, 97%), or bronchoalveolar lavage fluid ($n = 5$, 3%) from patients presenting with acute respiratory infection hospitalized from April to December 2009 were included in this study. Samples were analyzed for the detection of community respiratory viruses (CRVs) and to investigate H1N1pdm. All patient records were reviewed. Cases were reported by completing a specific notification form that included medical history, epidemiological information, laboratory findings, and clinical outcome. The Hospital de Clínicas/UFPR Institutional Ethics Review Board approved this study (IRB#: 2160.055/2010-03).

Pandemic H1N1 Influenza A Virus Detection

H1N1pdm was detected and characterized by real-time reverse transcription-PCR (rtRT-PCR) according to the Centers for Disease Control and Prevention (CDC) protocol [WHO, 2011], which detects seasonal influenza A and H1N1pdm viruses. Viral RNA was extracted using a nucliSENS easyMAG kit (bioMérieux, Marcy l'Etoile, France) according to the manufacturer's instructions.

CRV and Bacteria Detection

CRVs were detected using a multiplex RT-PCR technique. In brief, the viral genome was extracted using a High Pure viral RNA kit (Roche, Inc., Mannheim, Germany) according to the manufacturer's instructions. First-strand cDNA was synthesized by using random primers and an Improm-II reverse transcription system (Promega, Inc., Madison, WI). The resultant cDNA was subsequently subjected to PCR by using a Seeplex RV12ACE detection kit (Seegene, Inc., Seoul, South Korea) according to the manufacturer's protocol. This multiplex PCR technology enables the simultaneous detection of multiple viruses including adenovirus (AdV); coronavirus types (CoV) 229E/NL63, and OC43/HKU1; human metapneumovirus (HMPV); parainfluenza virus types 1, 2, and 3; seasonal influenza A (FLUA) and B (FLUB); respiratory syncytial virus types (RSV) A and B; and human rhinovirus (HRV) types A and B.

Bacterial infections were investigated using Seeplex® PneumoBacter ACE Detection kit (Seegene, Inc., Seoul, South Korea) according to the manufacturer's protocol to detect the following pathogens: *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Chlamydophila pneumoniae*, Mycoplasma pneumoniae, Legionella pneumophila, and Bordetella pertussis.

Statistical Analysis

Data were compiled using JMP version 5.2.1 (SAS Institute, Inc., Cary, NC) and analyzed using GraphPad Prism version 5.03 (GraphPad Software, Inc., La Jolla, CA). Fisher's exact test or the χ^2 test was used to assess differences between groups, and the Mann–Whitney *U*-test was used to analyze continuous variables where appropriate. Continuous data are expressed as median (interquartile range). The difference between the medians of onset of illness was calculated by the nonparametric Kruskal–Wallis test.

Univariate analysis was performed to identify independent predictors of mortality. Furthermore, multiple logistic regression analysis including all statistically significant variables identified in the univariate analysis ($P < 0.05$) was performed to determine the variables that were risk factors for death. All *P*-values are two-tailed, and the level of significance was set at $P < 0.05$.

RESULTS

A total of 169 hospitalized patients were investigated for viruses and bacteria in respiratory secretions; 51% were male, the median age was 24 years (interquartile range: 1–44 years), and 20% progressed to death. Patients were divided into six groups according to the laboratory results: (1) samples positive only for CRVs (n = 10, 6%); (2) samples positive for CRVs plus respiratory bacteria (n = 50, 29.5%); (3) samples positive only for H1N1pdm (n = 38, 22.5%); (4) samples positive for H1N1pdm plus respiratory bacteria (n = 26, 15%); (5) samples positive only for respiratory bacteria (n = 25, 15%); and (6) samples negative for all investigated pathogens (n = 20, 12%). Demographic data, clinical presentation, radiographic findings, and outcomes are shown in Table I.

A predominance of younger patients was observed in group 2. Meanwhile, hospitalization duration was longer among patients who had only bacterial infection (group 5). The frequency of myalgia was higher among patients with H1N1pdm infection (groups 3 and 4) and in those with negative results (group 6). A higher frequency of comorbidities was observed in patients with CRV and bacterial infections (groups 1 and 5). Furthermore, a higher rate of hospitalization in intensive care units (ICUs) and greater mortality rate were observed in patients with H1N1pdm infection (groups 3 and 4) than in those infected by CRVs.

The predominant radiologic finding in the investigated patients was interstitial infiltrate; this was observed in all groups, regardless of bacteria detection, including the group positive only for bacteria.

Among CRV-positive samples, 83.3% (50/60) were coinfected with bacteria. However, among FLUA H1N1pdm-positive patients, only 40.6% (26/64) were also positive for bacteria (odds ratio [OR] = 7.3, 95% confidence interval [CI]: 3.1–16.9, $P < 0.0001$).

Respiratory viruses were detected in 129 (75%) cases, 67 (39%) were diagnosed with FLUA H1N1pdm,

TABLE I. Epidemiologic and Clinical Data, and Outcomes of Hospitalized Patients with Acute Respiratory Infection Due to Pneumonia

	GROUP 1 CRV n = 10 (6%)	GROUP 2 CRV + bacteria n = 50 (29.5%)	GROUP 3 FLUA H1NIp n = 38 (22.5%)	GROUP 4 FLUA H1NIp + bacteria n = 26 (15%)	GROUP 5 bacteria n = 25 (15%)	GROUP 6 negative n = 20 (12%)	*P*-value
Age y (Median, IQR)	24.5 (8–62.25)	1 (0.48–4)	35 (22.75–44.25)	26 (3.25–43.25)	22 (2–46)	45.5 (33.25–56.75)	<0.0001
Sex (%)							0.2566
Male	50	60	39	46	64	40	
Female	50	40	61	54	36	60	
Pregnant or puerperal (%)							NA
Yes	30	–	13.2	11.5	8	5	
No	70	100	86.8	88.5	92	95	
Setting (%) ICU	20	14	31.5	38.5	16	15	0.0336
Emergency room	30	54	8	19	20	20	
Nursery	50	32	60.5	42.5	64	65	
Symptom duration (days)	3.5 (1.75–6.5)	3 (2–4.25)	4.5 (3–7)	4 (2–5.25)	3 (1.5–4)	4 (2–6)	0.0891
Hospitalization duration (days)	4 (2.75–8.5)	4 (2–7)	6 (2–11.5)	4 (2–6.5)	8.5 (4.5–15.5)	6 (4–8)	0.0129
Comorbidity (%)	70	38	39	35	64	60	0.0387
Most frequent comorbidity	Cardiopathy	Chronic pneumopathy	Cardiopathy; chronic pneumopathy	Chronic pneumopathy	Cardiopathy; chronic pneumopathy	Cardiopathy; chronic pneumopathy; smokers and alcoholics	NA
Time between onset of disease and sampling, days (Median, IQR)	3 (1–7)	3 (2–5)	5 (2.75–7)	4 (3–6.5)	3 (2–4)	4 (2.25–5.75)	0.0268
Cough (%)	100	96	89	100	92	95	0.7155
Myalgia (%)	20	12	47	38	28	45	0.0057
Fever (%)	80	92	95	88	88	90	0.6674
Sore throat (%)	20	4	16	19	16	5	0.2340
Headache (%)	10	2	11	3.8	8	5	0.7098
Dyspnea (%)	70	58	63	62	68	75	0.8157
Diarrhea (%)	0	16	5	12	16	5	NA
Chest pain (%)	0	4	0	8	0	0	NA
Antibiotics (%)	70	70	92	73	80	90	0.1103
Corticoids (%)	0	17.1	12	8	12	25	NA
Death (%)	20	8	37	34	12	10	0.0047

Data are presented as numbers, percentages, or median (IQR—interquartile range). CRV, community respiratory virus; NA, not applicable; NI, not informed.

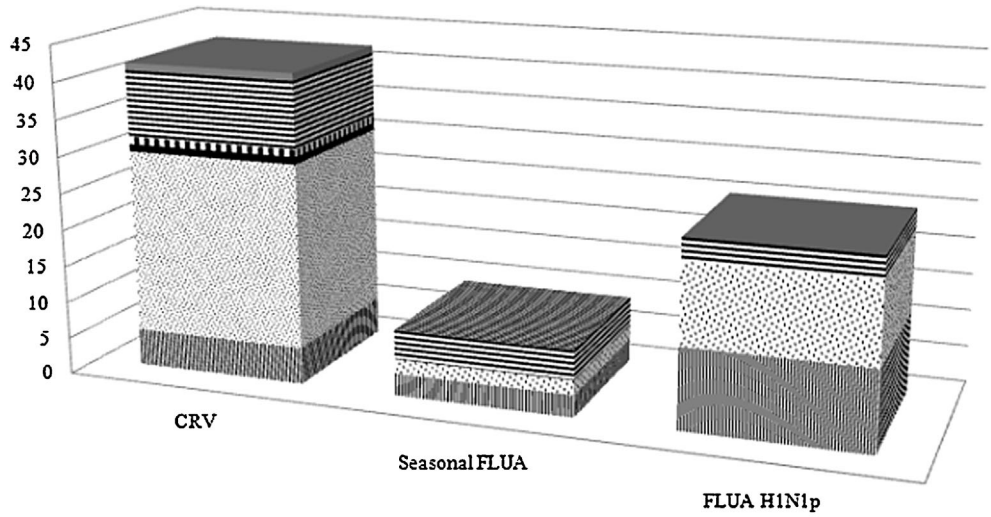
and 62 (36%) were diagnosed with CRVs, 9% of which were seasonal FLUA. In addition to seasonal FLUA, the most frequent CRVs were RSV A/B (8%) and HRV A/B (8%). Viral coinfections were more common among CRV cases (8%) than FLUA H1N1pdm cases (1%) [Raboni et al., 2011].

There was a high frequency of bacterial co-detection among all analyzed cases: 1, 2, and 3 bacteria were found in 45%, 54%, and 1% of the positive samples, respectively. The bacteria most commonly detected were *S. pneumoniae* and *H. influenzae* (22 and 21 patients, respectively); both bacteria were co-detected in 54 patients. Rare combinations were *H. influenzae* + *S. pneumoniae* + *Mycoplasma pneumoniae* ($n = 1$), *S. pneumoniae* + *M. pneumoniae* ($n = 1$), and *M. pneumoniae* (n = 2) (Fig. 1).

The presence of bacteria in the respiratory tract was not associated with increased mortality ($P = 0.135$) or longer hospitalization ($P = 0.36$). The interval between symptom onset and sample collection was significantly shorter in patients positive for bacteria than those negative for bacteria ($P = 0.0076$).

Comorbidities were frequent and associated with severity in all groups. Among all female patients, 15% (13/83) were pregnant; however, the presence of bacteria infection was not associated with increased mortality among them ($P = 0.28$).

Logistic regression analysis was subsequently performed to determine the factors that were independent predictors of mortality. In univariate analysis, ICU admission, mechanical ventilation, and radiological pattern were significantly associated with the mortality. However, in multivariate analysis, only ICU admission (OR = 54.4, 95%CI: 12.1–310.8) and mechanical ventilation (OR = 12.4, 95%CI: 2.4–65.9) were significant independent predictors of mortality.



	CRV	Seasonal FLUA	FLUA H1N1p
S pneumoniae/M pneumoniae	1	0	0
S pneumoniae	9	4	3
M pneumoniae	1	0	0
H influenzae/S pneumoniae/M pneumoniae	1	0	0
H influenzae/S pneumoniae	25	2	11
H influenzae	5	3	11

Fig. 1. Distribution of respiratory viral and bacterial coinfections.

DISCUSSION

Overall, bacterial–viral coinfection was detected in 45% of patients hospitalized with severe respiratory infection. H1N1pdm-infected patients exhibited a significantly higher mortality rate than other groups. However, the presence of bacterial infection was not associated with higher mortality. Coinfection was more frequently found in hospitalized patients with CRV than in those with H1N1pdm. Regarding the pandemic virus, the severity of the infection appears to be related to the virulence of the pathogen because bacteria associated with community-acquired pneumonia were significantly less frequent in this group.

Acute respiratory infections are a critical disease of the 21st century, incurring high mortality rates and significantly impacting the frequency and duration of hospitalization as well as severity. Therefore, their etiology should be thoroughly investigated in order to offer patients appropriate treatment and provide community safety surveillance to detect emerging pathogens. The association between influenza and bacterial pneumonia has been recognized for a long time [Morens et al., 2014]. Epidemiologic studies in the 20th century demonstrate that the incidence of pneumonia peaks with influenza activity [Stuart-Harrisa et al., 1949; Tyrrell, 1952; Oswald et al., 1958]. The present findings of high rates of bacterial coinfection are corroborated by these previous results.

Studies comparing the impact of viral–bacterial coinfection on patient outcome report conflicting results. Viral–bacterial coinfection is an important trigger for the exacerbation of asthma and chronic obstructive pulmonary disease. In vitro analyses show that respiratory viruses can promote bacterial infection through epithelial disruption and that chronic *H. influenzae* infection potentiates the inflammatory response to respiratory viruses [Deng, 2013; Wark et al., 2013].

Although our understanding of the epidemiology of influenza viruses is changing rapidly, bacterial pneumonia remains an important contributor to the severity and lethality of influenza infections [Deng, 2013]. Bacterial coinfection was probably associated with the majority of pneumonia deaths in the 1918 influenza pandemic and has been reported in up to 34% of 2009 influenza A H1N1 pandemic cases managed in ICUs; the most common pathogens in these cases are *Staphylococcus aureus*, *Streptococcus pneumoniae*, and *Streptococcus pyogenes* [Chertow and Memoli, 2013]. However, even though bacterial pneumonia is a common contributor to poor outcomes, various epidemiological data suggest the incidence, severity, and the identified pathogens differ among epidemics and geographical locations. Few studies in Brazil have identified the bacteria species in patients hospitalized owing to community respiratory infections. Mauad et al. [2010] reported

21 necropsies of Brazilian patients infected by influenza A H1N1pdm; the only bacterial species isolated in three samples from nine investigated patients was *S. pneumoniae*. It has been suggested that there is a geographic variation of the bacterial expression of virulence factors, which could explain the observed regional differences in severity [McCullers, 2013].

Several authors reported a high frequency of community-acquired pneumonia during the influenza A H1N1 pandemic. However, despite these patients' higher severe pneumonia index scores, their mortality rate was similar to that of patients without bacterial coinfection [Dhanoa et al., 2011; Cillóniz et al., 2012; Blyth et al., 2013; Martín-Loeches et al., 2013]. It is likely that coinfection contributed to the need for ICU admission in patients without other risk factors for severe influenza disease [Blyth et al., 2013].

The American CDC recognizes the importance of early empirical antibiotics in H1N1pdm-infected patients who might have concurrent bacterial pneumonia [CDC, 2009; Shieh et al., 2010]. Therefore, a protocol has been established in our institution on the basis of the evidence of frequent bacterial coinfection. In this protocol, all patients admitted to the ICU with pneumonia, even those only suspected of viral infection, receive antibiotics, usually a combination of ceftriaxone and azithromycin or respiratory quinolone. This approach may be associated with the attenuated severity and consequently decreased mortality rate observed in this study.

Of note, Falsey et al. [2012] reported that only respiratory viruses were detected in 28.4% of patients for whom antibiotics were prescribed unnecessarily; this resulted in an increased risk of developing *Clostridium difficile* colitis. Therefore, early empirical antibiotic therapy for patients with an unstable condition is appropriate but is not without risk.

Despite the interesting findings, the present study has some limitations that should be kept in mind when interpreting the results. First, the diagnostic value of nasopharyngeal aspirates is questionable because pathogen detection in asymptomatic patients is relatively common [Pavia, 2013]. Nevertheless, the prevalence of coinfection differed significantly between the CRV and H1N1pdm infection groups, suggesting the bacterial positivity found in these cases could be clinically relevant. Moreover, Obasi et al. [2014] reported a significantly lower detection rate of bacteria in respiratory secretions from healthy adult than patients with acute respiratory infection. Second, only the results of molecular methods were analyzed. Thus, it is possible that bacterial and viral infections diagnosed on the basis of DNA and RNA detection overestimate the incidence of infection because it is often unfeasible to distinguish colonization from infection. Third, the presence of viral RNA could merely indicate prolonged viral detection following a previous severe respiratory tract infection. Therefore, other factors such as clinical parameters, radiographic patterns, and biomarkers should be used to predict the risk of bacterial infection [Falsey et al., 2012]. Fourth, although *S. aureus* is usually described in association with influenza infections, it was not evaluated in the present study because the kit used does not detect it and bacterial culture was not performed because of safety reasons. However, this pathogen is not usually identified in community respiratory infections in our region. A previous study of Brazilian patients who died from influenza A H1N1pdm pneumonia also failed to detect this species [Mauad et al., 2010].

In conclusion, during the 2009 influenza pandemic, bacterial–viral coinfection was frequent in hospitalized patients. However, the precise effects of this coinfection on ICU admission remain unclear. Furthermore, a higher mortality rate was observed among H1N1pdm-infected patients independent of the bacteria present. Finally, this study demonstrates that bacterial coinfection is commonly associated with viral respiratory infection, while coinfection appears not to influence the outcome.

DISCLAIMER AND AUTHOR'S CONTRIBUTIONS

All authors contributed to development of this manuscript, approved the final version of this report and declare have no competing financial interests.

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REFERENCES

Blyth CC, Webb SA, Kok J, Dwyer DE, van Hal SJ, Foo H, Ginn AN, Kesson AM, Seppelt I, Iredell JR, ANZIC Influenza Investigators, COSI Microbiological Investigators. 2013. The impact of bacterial and viral co-infection in severe influenza. Influenza Other Respir Viruses 7:168–176.

Center for Disease Control and Prevention (CDC). 2009. Bacterial coinfections in lung tissue specimens from fatal cases of 2009 pandemic influenza A (H1N1)—United States. MMWR Morbi Mortal Wkly Rep 58:1071–1074.

Chertow DS, Memoli MJ. 2013. Bacterial coinfection in influenza: A grand rounds review. J Am Med Assoc 309:275–282.

Cillóniz C, Ewig S, Menéndez R, Ferrer M, Polverino E, Reyes S, Gabarrus A, Marcos MA, Cordoba J, Mensa J, Torres A. 2012. Bacterial co-infection with H1N1 infection in patients admitted with community acquired pneumonia. J Infect 65:223–230.

Deng JC. 2013. Viral–bacterial interactions—Therapeutic implications. Influenza Other Respir Viruses 7(s3):24–35.

Dhanoa A, Fang NC, Hassan SS, Kaniappan P, Rajasekaram G. 2011. Epidemiology and clinical characteristics of hospitalized patients with pandemic influenza A (H1N1) infections: The effects of bacterial coinfection. J Virol 8:501.

Falsey AR, Becker KL, Swinburne AJ, Nylen ES, Formica MA, Hennessey PA, Criddle MM, Peterson DR, Baran A, Walsh EE. 2012. Bacterial complications of respiratory tract viral illness: A comprehensive evaluation. J Infect Dis 208:432–441.

Martín-Loeches I, Sanchez-Corral A, Diaz E, Granada RM, Zaragoza R, Villavicencio C, Albaya A, Cerdá E, Catalán RM, Luque P, Paredes A, Navarrete I, Rello J, Rodriguez A, H1N1 SEICYUC Working Group. 2013. Community-acquired

respiratory coinfection in critically ill patients with pandemic influenza A (H1N1) virus. Chest 139:555–562.

Mauad T, Hajjar LA, Callegari GD, da Silva LF, Schout D, Galas FR, Alves VA, Malheiros DM, Auler JO Jr., Ferreira AF, Borsato MR, Bezerra SM, Gutierrez PS, Caldini ET, Pasqualucci CA, Dolhnikoff M, Saldiva PH. 2010. Lung pathology in fatal novel human influenza A (H1N1) infection. Am J Respir Crit Care Med 181:72–79.

McCullers JA. 2013. Do specific virus-bacteria pairings drive clinical outcomes of pneumonia? Clin Microbiol Infect 19:113–118.

Morens DM, Taubenberger JK, Fauci AS. 2014. Predominant role of bacterial pneumonia as a cause of death in pandemic influenza: Implications for pandemic influenza preparedness. J Infect Dis 198:962–970.

Obasi CN, Barret B, Brown R, Vrtis R, Barlow S, Muller D, Gern J. 2014. Detection of viral and bacterial pathogens in acute respiratory infections. J Infect 68:125–130.

Oswald NC, Shooter RA, Curwen MP. 1958. Pneumonia complicating Asian influenza. BMJ 2:1305–1311.

Pavia A. 2013. What is the role of respiratory viruses in community acquired pneumonia? What is the best therapy for influenza and other viral causes of CAP? Infect Dis Clin North Am 27: 157–175.

Raboni SM, Stella V, Cruz CR, França JB, Moreira S, Gonçalves L, Nogueira MB, Vidal LR, Almeida SM, Debur MC, Carraro H Jr., dos Santos CN. 2011. Laboratory diagnosis, epidemiology, and clinical outcomes of pandemic influenza A and community respiratory viral infections in southern Brazil. J Clin Microbiol 49:1287–1293.

Shieh WJ, Blau DM, Denison AM, DeLeon-Cames M, Adem P, Bhatnagar J, Summer J, Lie L, Patel M, Batten B, Breer P, Jones T, Smith C, Bartlett J, Montague J, White E, Rollin D, Gao R, Seales C, Jost H, Metcalfe M, Goldsmith CS, Humphrey C, Schmitz A, Drew C, Paddock C, Uyeki TM, Zaki SR, 2010. Pandemic influenza A (H1N1): Pathology and pathogenesis of 100 fatal cases in the United States. Am J Pathol 177:166–175.

Stuart-Harrisa CH, Laird J, Tyrrell DA, Kelsall MH, Franks ZC, Pownall M. 1949. The relationship between influenza and pneumonia. J Hyg 47:434–448.

Tyrrell DA. 1952. The pulmonary complications of influenza as seen in Sheffield in 1949. Int J Med 21:291–306.

Wark PAB, Tooze M, Powell H, Parsons K. 2013. Viral and bacterial infection in acute asthma and chronic obstructive pulmonary disease increases the risk of readmission. Respirology 18: 996–1002.

WHO Global Influenza Surveillance network. 2011. Manual for the laboratory diagnosis and virological surveillance of influenza 2011. http://whqlibdoc.who.int/publications/2011/9789241548090_eng.pdf, accessed on 07/22/2014