RESEARCH PAPER

Influenza and Secondary Streptococcus pneumonia Co-infections in Egypt: A Molecular and Clinical Outcome Study

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Abstract

Background: Influenza remains a global public health threat, with altered post-COVID-19 epidemiology. Secondary bacterial infections, notably Streptococcus pneumoniae (SP), drive severe complications and mortality, but contemporary data from Egypt are lacking.

Objectives: To determine the prevalence, seasonality, and subtypes of influenza among Egyptian patients with influenza-like illness (ILI), assess SP co-infection rates, and evaluate clinical outcomes.

Methods: Nasopharyngeal swabs from 240 ILI patients at Ain Shams University Hospitals were analyzed using quantitative real-time RT-PCR for influenza and conventional PCR targeting the 355-bp pneumolysin gene for SP.

Results: Influenza positivity was 20.83% (50/240): 12.92% Influenza A and 7.92% Influenza B. Influenza A peaked in winter, while Influenza B peaked in March–April. Among Influenza A cases, 51.6% were seasonal H3 and 48.4% were pandemic H1N1. Age was strongly linked to bacterial co-infection ($p = 0.003$), with infants bearing the highest burden (39.81%). SP co-infection was a significant risk factor for influenza patients ($p = 0.022$). Crucially, mortality was highly significant among co-infected patients ($p < 0.001$) compared to influenza mono-infection ($p = 0.001$), claiming nearly all co-infected individuals.

Conclusion: The convergence of influenza and SP results in a lethal synergy that drastically escalates mortality, especially in infants. Implementing dual molecular surveillance is vital to optimize antimicrobial stewardship and guide vaccination policies in Egypt.

Keywords: Influenza; Streptococcus pneumoniae; Bacterial Co-infection; Real-Time RT-PCR; Pneumolysin; Egypt.

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Introduction

Flu vaccinations and antiviral therapies may decrease flu symptoms. However, there are still significant public health concerns due to yearly flu outbreaks. It's predicted that a minimum of three to five million people will develop severe illness, and approximately 650,000 will die due to flu-related respiratory illnesses. Additionally, the influenza A and B viruses

have varying levels of pathogenicity. Influenza A viruses can cause large epidemics and even pandemics. Due to continuous antigenic shifts, influenza A viruses will continue to cause illnesses and infections to the public. Influenza A viruses also pose a large threat to the population. [1-3]

Following the COVID-19 pandemic, the flu seems to have a new distribution of infection. Flu cases were almost non-existent due to the COVID-19 public health directives, and then reappeared for the following flu season. Now, more than one type of respiratory virus will circulate during the same season. Symptoms will present differently based on age, and as a result, the classic signs and symptoms of the flu that most people expect will be different. Due to the new flu patterns, the public will benefit from continued flu surveillance to understand the types of flu that are circulating. [4-6]

Because of the difficulty in diagnosing influenza infections as distinct from other respiratory viral infections, laboratory tests are essential. "Influenza-like illness" (ILI) consisting of fever, cough, pharyngitis, rhinorrhea, malaise, and myalgia, are not unique to influenza and therefore do not assist in diagnosis. Due to the previously mentioned reasons, many clinicians rely on real-time, rapid RT-PCR tests for influenza diagnosis [7,8].

Influenza infection leads to increased morbidity and mortality, primarily due to the secondary bacterial infection. It disrupts the epithelial integrity and the mucociliary defense, and augments the adhesion of bacteria to the epithelial lining. S. pneumoniae remains the predominant infection, but other pathogenic respiratory bacteria also contribute to disease severity, including S. aureus, H. influenzae, Mycoplasma pneumoniae, and others [9-11].

It is reported in more recent literature that 20% of all influenza cases requiring hospitalization are due to secondary bacterial infections. Compared to influenza mono-infection, co-infection with secondary pathogens increases the risk of mortality, need for invasive mechanical ventilation, and ICU admission. A rapid, but incorrect, diagnostic testing of influenza infections combined with a bacterial co-pathogen leads to unnecessary, severe illness and death due to extremely poor healthcare [12].

Contemporary molecular diagnostics allow more precise characterisation of pathogens in respiratory samples. Certain real-time multiplex polymerase chain reaction (PCR) technologies allow simultaneous detection of both influenza virus and co-infecting bacteria. These newer technologies also outperform most existing technologies in terms of speed of analysis. This allows targeted antibiotic administration and regulation of the spread of infection. This is beneficial in low and middle income countries where respiratory infections continue to be a major cause of the disease burden and mortality.

In Egypt, each flu season is associated with a circulation of flu viruses. Information on flu subtypes, bacterial co-infections and outcomes of patients post COVID-19 is lacking. The existing studies from Egypt are either pre-COVID-19 or early COVID-19. These studies are not likely to reflect recent influenza viruses and bacterial co-infections. Thus, new studies are important to guide influencing testing, use of antibiotics, vaccinations and to prepare for potential new influenza outbreaks.

Due to the above reasons, the current study aimed to assess the state of influenza infections in Egypt from September 2022 to May 2025. This involved the identification of influenza viruses in patients with Influenza-Like Illness (ILI) and assessment of bacterial co-infections. The study also aimed to identify influenza viruses that were currently circulating, and assess the impact of bacterial co-infections and negative clinical outcomes, including death.

2. Subjects and methods

2.1 Study design

A cross-sectional pilot observational study was conducted at Ain Shams University hospitals to define Influenza-like Illness (temperature >37.5°C with one or more of the following: sore throat, cough, and/or nasal discharge). Participants were categorized into four groups: infants (<2 years), children (2-12 years), adults (18-65 years), and the elderly (>65 years old).

2.2. Sample collection and preparation

Sampling was restricted to swabs with a synthetic tip (e.g., polyester and Dacron) and an aluminum or a plastic support. Swab samples were collected and transported in viral transport media (VTM).

2.3. Data collection

Data were collected on age, gender, bacterial culture, and chest X-ray for each participant, from their medical records.

2.4 Viral RNA extraction and RT-PCR (Real Time Polymerase Chain Reaction)

Using the QIAamp® Viral RNA Mini Kit, we extracted RNA in a biosafety level II cabinet. For real-time PCR, we used the 7500 Fast Real-Time PCR System with the 96-well thermocycler reaction block. Using Taqman®, we performed a one step quantitative real time RT-PCR. For this experiment's reagents and probes, we used Invitrogen's SuperScript™III

Platinum® One-Step Quantitative Kit (cat# 11732-020 or 11745-100). The CDC real time RT-PCR for influenza offers primers and comprehensive probes that help us to detect the various types and subtypes of Influenza viruses. Each sample's RNA is extracted, and real time RT-PCR is performed using separate primers and probes. We used RNaseP (RP) as a positive control in this assay to determine if the samples contain human nucleic acid. In this assay, RNaseP also serves as an internal control. We used no template controls (NTC) and positive template controls (PTC) for every primer/probe set. In addition, we used Human Specimen Control (HSC), which serves as a negative control for the extraction reagents.

2.5 Bacterial DNA extraction and conventional PCR

Sample preparation: one ml of NPS sample was centrifuged at 12,000 g for 10 min. The pellet was suspended in 200 microliter digestion buffer (50 mM Tris-HCl [pH 8.5], 1 mM EDTA, 0.5% SDS). DNA extraction was performed using QIAGEN amp DNA Mini Kit (QIAGEN diagnostic, USA). DNA concentrations were measured by using Du series 650, INC, USA. PCR amplification assay: Based on the published sequence of the pneumolysin gene [19], we selected a pair of primers (59-GTGATATTTCTGTAACAGCTAC, 59-GAGAATTCCCTGTCTTTTCAAAG). The primers amplify a 355-bp region of the pneumolysin gene sequence. The DNA amplification was performed in a microprocessor-controlled incubation system (Gene Amp, PCR system 9700, Applied Biosystem, Singapore). The reaction mixture (volume, 50 ml) constituted of 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl2, 5 mM deoxynucleotides, 50 pmol of primers (BIONEER, Accuoligo) and 25 ml Taq pcr Master Mix (Qiagen, catalog number:1007544), The DNA sample was 200 ng). The PCR temperature profile was initial denaturation at 94□C for 3 min then 30 cycles of denaturation at 94□C for 1 min, Annealing at 60□C for 1 min, synthesis at 72□C for 1 min then final extension at 72□C for 5 min. The positive control for the assay was prepared from pneumococcal DNA

The PCR products were visualized by agarose gel electrophoresis using DNA molecular weight markers of 100-1000 bp. The PCR products were observed to have a size of 355 bp (base pairs).

2.6 Statistical analysis:

All the data were recorded and transferred to given tables. The data were entered in Statistical Package for Social Sciences (SPSS) (Version 16.0.1, Chicago, Illinois, USA) on a personal computer. Descriptive statistics and the results of the tests were shown by p-values.

A p-value of <0.05 was interpreted as statistically significant whereas <0.01 was interpreted as highly significant.

Interpretation of the results:

1. NTC reactions for each of the probe/primer sets must not show any fluorescence growth curve crossing the threshold line. If any NTC reaction for primer and probe show a positive signal, the sample is possibly contaminated. The entire reaction must be repeated strictly following standard operating procedures.

2. All the clinical samples must cross the threshold line at or before 37 cycles, thus indicating the presence of amplifiable RNA from human RNase P gene indicating the specimen is of good quality. However, it is also possible that some samples may fail to give positive reactions due to low cell numbers in the original clinical sample.

A. Samples taken from animal/avian species or cell culture show a weak RP reaction or even no RP reaction. The inability to detect RNase P in any of the clinical samples may be attributed to:

(a) Lack of RNA or transport of RT-PCR inhibitors during extraction of nucleic acid from clinical samples.

(b) Lack of sufficient human cellular material in the sample.

(c) Errors during the execution of the assay

(d) Faulty reagents or equipment.

3. The HSCs should NOT display fluorescence growth curves for the InfA, swFluA, and swH1 primer/probe sets, which should remain below the threshold in 40 cycles. If any of the influenza specific primer/probes display a fluorescence growth curve that crosses the threshold, the following should be attributed:

(a) RNA Extraction Reagents have likely been contaminated. Validate the run and repeat RNA extraction reagents integrity if necessary.

(b) RNA Extraction contamination and/or assay setup contamination have occurred. Validate the run and strictly adhere to procedure when repeating the assay.

4. PTC reactions should produce a positive result for InfA, swInfA, swH1, and RP reactions before the 40 cycle threshold. If expected positive reactivity is not achieved, invalidate the run and repeat the assay with strict adherence to procedure guidelines. Failure to produce PTC reactivity should be documented along with the corrective actions. PTC reagents should not be used if they do not produce the expected results.

5. A specimen is considered presumptive positive for influenza A if InfA reaction growth curves cross the threshold within 40 cycles. The presence of influenza A can indicate the presence of both Univ SW and SW

H1. A specimen is presumptive positive for swine influenza A/H1 if BOTH InfA and the respective subtype (swine influenza A or swine influenza H1) reaction growth curves cross the threshold within 40 cycles. The guidance of CDC should be obtained if a specimen is positive for InfA and one of the subtype reactions or InfA only.

6. If growth curves for InfA do not cross the threshold after 40 cycles with the other controls meeting stated requirements, then the specimen is considered negative for influenza virus.

Limitations:

1. Analysts should be trained and familiar with testing procedures and interpretation of results prior to performing the assay.
2. A false negative result may occur if inadequate numbers of organisms are present in the specimen due to improper collection, transport or handling.
3. A false negative result may occur if an excess of DNA/RNA template is present in the in the reaction. If inhibition of the RP control reaction is noted for a particular sample, extracted RNA can be tested at 2 or more dilutions (e.g., 1:10 and 1:100) to verify the result. The gel was examined under ultraviolet light as ethidium bromide intercalated between the bases of the DNA and fluoresced. Photographs were taken using Polaroid camera.Marker gave different bands ranging between (100-1000)bp.The negative control was examined to exclude any source of contamination. Samples were compared with controls.

Results

The present study detected an influenza positivity rate of 50/240 (20.83%). Of the positive samples, 19 (7.92%) were influenza B virus and 31 (12.92%) were influenza A virus. Among the A subtype samples, 15 (48.4%) were H1N1 virus and 16 (51.6%) were seasonal H3N1 virus. In this study, the greatest number of A cases were recorded and the highest number of B cases were recorded in the months of March and April. The highest rates of pandemic H1N1 influenza infection were recorded among the females and the highest rates of seasonal H3N1 influenza were recorded among the males. There was a statistically significant association (p value: 0.022) with co-infection of bacteria with influenza A patients. Among influenza A (11.11%), influenza B (6.17%), and influenza H3N1 (11.11%), samples were reported and are shown in Table (1). In the present study, co-infections were considered risk factors for flu infection. Based on age, a statistically significant effect (p value: 0.003) was observed with co-infections. Of the total study population, 43 (39.81%) infants, 34 (31.48%) children, 14 (31.48%) adults, 17 (15.74%) elderly, co-

Between (2022-2025), we identified SP (Staphylococcus Pseudintermedius) as a significant contributor to the increase in mortality rates of patients infected by the virus (P-value<0.001). However, SP had less of an effect on the mortality rates of patients infected by influenza (P-value=0.001).. Table (1), shows most of the SP-infect

Analysis of RT-PCR in Fig 2 and Fig 3 shows the presence of 29 samples of positive RNP along with positive and negative controls for each of the genes. A straight line represents a negative control and a positive control is represented by a curve. This can be determined by CT (Threshold Cycle). CT is the average value of copies of a given gene, which increases.

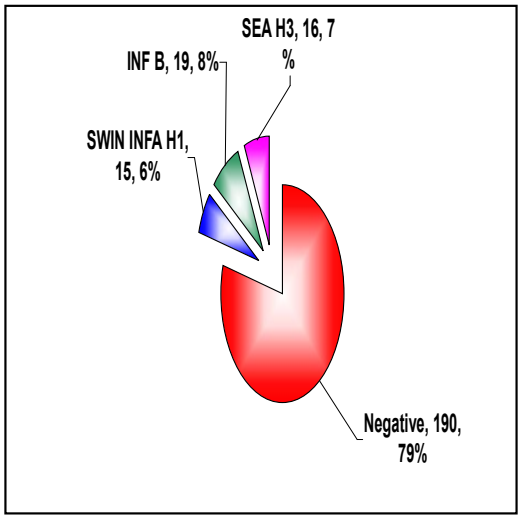

Fig (1) :Percentages of Influenza types and subtypes in the studied patients.

Our results were illustrated in table (1)

SP patients detected by PCR	Influnza patients	Mortality						Chi-Square	
		Alive		Died		Total			
		N	%	N	%	N	%	X^2	P-value
Negative	Negative	122	86.52	10	55.56	132	83.02	11.211	0.011*
	SWIN INFA H1	5	3.55	1	5.56	6	3.77		
	INF B	8	5.67	6	33.33	14	8.81		
	SEA H3	6	4.26	1	5.56	7	4.40		
	Total	141	100.00	18	100.00	159	100.00		

Positive	**Negative**	23	82.14	35	66.04	58	71.60	7.898	0.048*
	SWININFAH1	4	14.29	5	9.43	9	11.11		
	INF B	0	0.00	5	9.43	5	6.17		
	SEAH3	1	3.57	8	15.09	9	11.11		
	Total	28	100.00	53	100.00	81	100.00		

Table (1): Rate of Mortality in patients with Influnza virus and patients with SP infection . *significant

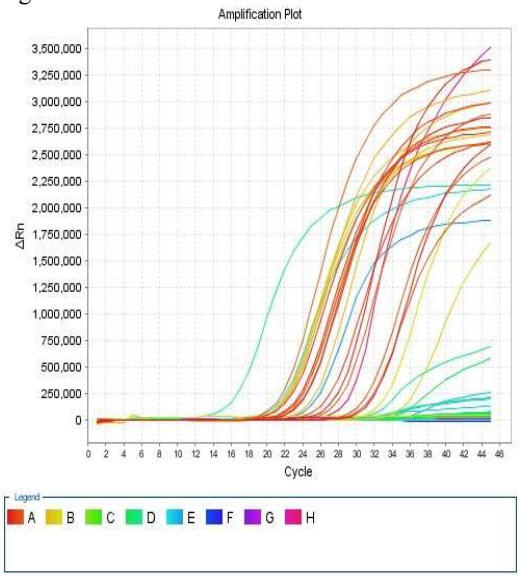

Fig (2): The amplification plot of (RNP, INF A, INF B) Amplification plot for 29 samples with positive and negative controls illustrating the relation between number of cycles with average number of gene copies. Note: letters (A-H) showed position of samples in plate by different colors.

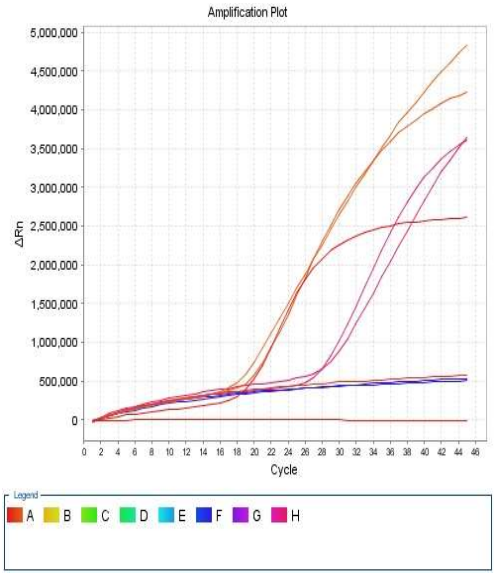

Fig (3) : Amplification plot for one positive sample (pandemic swine H1N1 & swin H3N1) with positive and negative controls illustrating the relation between number of cycles with average number of gene copies, showing (CT=10)

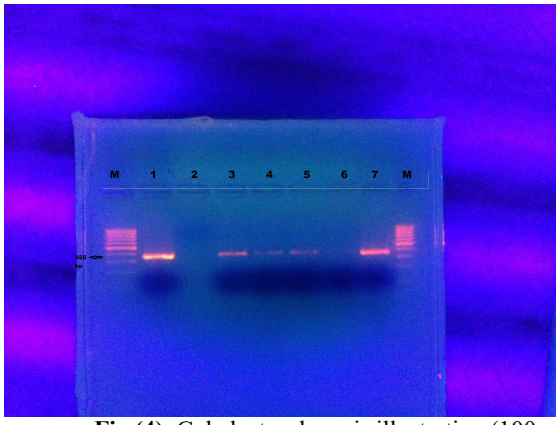

Fig (4): Gel electrophoresis illustrating (100-1000) base pair (bp) loading marker(M) (Pyron, Sigma, USA) in first and last lanes. In lane (1) SP positive control , in lane (2) SP negative control ,in lanes 3,4,5,6,7 SP positive samples at 355 bp.

Discussion

Post COVID-19, regionalized molecular surveillance will be valuable for identifying emerging respiratory pathogens and facilitating the development of focused clinical and vaccination pathways. This study provides a cross-sectional analysis of co-circulating type and subtype influenza patterns in addition to co-occurring Streptococcus pneumoniae (SP) bacterial co-infection among Egyptian patients with influenza-like illness (ILI) from September 2022 to May 2025. Our study suggests deaths are much greater among patients positive for both the virus and S. pneumoniae, indicating an extremely high risk of death among younger children.

Post-Pandemic Distribution and Seasonality of Influenza

In our sample, 20.83% of the population reported flu-like symptoms and influenza was detected in 20.83%. The predominating cause of incidence in Egypt of Acute Respiratory Infections suggests a resurgence of cases post COVID-19 related control measures, as reported in previous studies. [16]

Influenza A was reported more frequently than Influenza B (12.92% vs. 7.92%) and is consistent with previously published studies where influenza A is the key driver of winter influenza seasons. [17] Of the influenza subtypes, H3 and 2009 pandemic H1N1 were nearly identical (51.6% vs. 48.4%).

Interesting correlated data patterns related to timing of influenza and gender have been discovered. Winter peaks are evident for Influenza A, and for Influenza B, peaks occur in March and April. WHO Eastern Mediterranean Region data indicate that the majority of region hospitals continue influenza testing into spring. [18, 19] Females showed a 73.33% prevalence of pandemic H1N1 and males showed a 68.75% prevalence of seasonal H3. Although we can't currently explain these patterns, they show undeniable targeting differences among the subtypes.

Demographic Imbalances and the Pediatric Risk

A very strong correlation was found between age and risk of bacterial co-infection ($p = 0.003$). The highest risk was recorded for infants, accounting for 39.81% (43 out 108) of all co-infections, while children comprised 31.48%. This represents a serious concern for pediatric healthcare services in Egypt.

Immaturity of the immune system, in combination of the incomplete immunization of infants, increases the risk for infants to contract rapid bacterial infections following viral infections. Molecular tests should be done as soon as possible for infants with flu-like symptoms to prevent rapid onset of illness.

TheCombined Effects of Influenza and Streptococcus pneuomoniae

Secondary bacterial infections are well known to complicate flu illnesses and, in many instances, are responsible for fatal outcomes. For this study, a Polymerase Chain Reaction positive result for any of the Streptococcus pneumoniae groups was associated with negative outcomes and reached statistical significance ($p=0.022$). Of the 81 subjects for whom S. pneumoniae was detected, 23 also tested positive for influenza. Of the 23, 9 were pandemic H1N1, 9 were seasonal H3, and 5 were influenza B.

The most notable and worrisome finding of this study was the sharp rise in the number of deaths from coinfections. Among patients infected with S. pneumoniae, the coinfections were highly statistically significant and likely represented serious illness and a worse outcome ($p=0.001$). Among pandemic H1N1 patients, the mortality was 55.56%; for the B influenza the mortality was 100%; and for seasonal H3 coinfections, the mortality was 88.89%.

Respiratory problems associated with the loss of ciliary function are made worse as comorbidities allow the bacteria to invade and adhere to the tissues of the respiratory system. They also impede the neutrophils from engulfing and killing the bacteria, and allow ciliated airways to be destroyed. If this happens along with the flu, especially when pneumatic bacteria invade the lung tissue, the damage of the alveoli can be extensive and cause Acute Respiratory Distress Syndrome and failure of other organ systems..

Diagnostic Utility of Combined PCR Assays

Due to the unspecific nature of the symptoms associated with a flu and common illnesses, they are often misdiagnosed. Our study underscores the importance of elevated molecular diagnostics. The use of rapid, real-time reverse transcriptase-polymerase chain reaction (rRT-PCR) assays, along with conventional PCR to amplify a 355 base pair fragment within the pneumolysin gene of Strep. pneumoniae, allows labs to identify pathogens and provide results much earlier than the traditional culture and diagnostic methods.

These protocols must be established at tertiary care hospitals such as Ain Shams University Hospitals. Early diagnosis of pathogens leads to improved antimicrobial stewardship and reduces unnecessary broad-spectrum antibiotic use.

Comparison with Previous Studies

The frequency and clinical importance of viral-bacterial co-infections reported in this work is commensurate with existing global and sub-regional literature depicting the synergies of respiratory viruses and bacterial pathogens. There are well documented global and regional literatures describing influenza associated viral bacterial co-infections as major drivers of severe illness and high case fatality rates for both seasonal and pandemic influenza outbreaks (Jia et al., 2017) [25]. Our results are in line with existing Egyptian literatures on co-infections that cause acute respiratory infections. El Seify et al. (2016) [26], working at Ain Shams University, documented pathogens in 65.5% children with community acquired pneumonia where influenza was the predominant virus and S. pneumoniae and S. aureus were the predominant pathogens. El Baroudy et al. (2018) [27] noted that 10.6% of symptomatic Egyptian children who presented with illness attributable to viral co-infections and atypical bacterial infections had more severe illness. Co-infections are generally not documented by conventional cultures while rapid molecular assays improve the detection of co-infections. From the literature, it is known that viral

infections change the host's susceptibility and affect the early protective barriers by altering the function of alveolar macrophages (Jia et al., 2017) [25].

Strengths and Limitations

One of the primary strengths of the study was the use of a sensitive prospective design to collect information. Multiplex real-time PCR was a diagnostics technique used, which improved the study's ability to identify co-infectious agents that culture techniques were unable to capture. Additionally, given the paucity of information from this region, this study provides much needed information regarding the clinical burden, surveillance of pathogens, and adherence to vaccinations.

The study has several limitations. First, the single-center and hospital-based data collection prevents this study from drawing conclusions beyond that of the asymptomatic population. Second, multiplex PCR cannot differentiate active infection from colonization or shedding, which may result in overestimation of co-infection. Lastly, some of our data were cross-sectional. Consequently, we were unable to controlling for the order of infections, which is clinically relevant to the Outcome and Mortality of the infections studied.

Conclusion

Viral-bacterial co-infections contribute to a significant clinical burden with more severe illness compared to single pathogen infections. Our results underscore the importance of using the multiplex real-time PCR assay in place of standard cultures for disease diagnosis. Given the regional burden is significant and severe illness can occur, increasing awareness of the underutilized flu and pneumococcal vaccines is important. Costs and skepticism currently limit their use. Subsequently, future multi-center studies should concentrate on the chronological sequencing of polymicrobial infections to optimize empirical antimicrobial therapies.

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