Research and Reports in Tropical Medicine

Dovepress
open access to scientific and medical research

Open Access Full Text Article

ORIGINAL RESEARCH

Molecular diagnosis of microbial copathogens with influenza A(H1N1)pdm09 in Oaxaca, Mexico

Luis Román Ramírez-Palacios[1]
Diana Reséndez-Pérez[2]
Maria Cristina Rodríguez-Padilla[2]
Santiago Saavedra-Alonso[2]
Olga Real-Najarro[3]
Nadia A Fernández-Santos[4]
Mario A Rodriguez Perez[4]

[1]Laboratorio Estatal de Salud Pública de Oaxaca, Oaxaca, [2]Departamento de Inmunología y Virología, Facultad de Ciencias Biológicas, Universidad Autónoma de Nuevo León, San Nicolás de los Garza, Mexico; [3]Consejería de Educación, Madrid, Spain; [4]Instituto Politécnico Nacional (IPN), Centro de Biotecnología Genómica, Reynosa, Mexico

Background: Multiple factors have been associated with the severity of infection by influenza A(H1N1)pdm09. These include H1N1 cases with proven coinfections showing clinical association with bacterial contagions.

Purpose: The objective was to identify H1N1 and copathogens in the Oaxaca (Mexico) population. A cross-sectional survey was conducted from 2009 to 2012. A total of 88 study patients with confirmed H1N1 by quantitative RT-PCR were recruited.

Methods: Total nucleic acid from clinical samples of study patients was analyzed using a TessArray RPM-Flu microarray assay to identify other respiratory pathogens.

Results: High prevalence of copathogens (77.3%; 68 patients harbored one to three pathogens), predominantly from *Streptococcus*, *Haemophilus*, *Neisseria*, and *Pseudomonas*, were detected. Three patients (3.4%) had four or five respiratory copathogens, whereas others (19.3%) had no copathogens. Copathogenic occurrence with *Staphylococcus aureus* was 5.7%, Coxsackie virus 2.3%, *Moraxella catarrhalis* 1.1%, *Klebsiella pneumoniae* 1.1%, and parainfluenza virus 3 1.1%. The number of patients with copathogens was four times higher to those with H1N1 alone (80.68% and 19.32%, respectively). Four individuals (4.5%; two males, one female, and one infant) who died due to H1N1 were observed to have harbored such copathogens as *Streptococcus*, *Staphylococcus*, *Haemophilus*, and *Neisseria*.

Conclusion: In summary, copathogens were found in a significant number (>50%) of cases of influenza in Oaxaca. Timely detection of coinfections producing increased acuity or severity of disease and treatment of affected patients is urgently needed.

Keywords: bacteria, copathogens, microarray assay, H1N1

Introduction

Influenza viruses A and B are the main pathogens responsible for the onset of epidemics because of their evolving nature. They are RNA viruses that have a high mutation rate and ability to make "drift" changes; however, only influenza A viruses are responsible for pandemics. Worldwide, influenza A viruses are the cause of severe infections in 3–5 million people annually, and these viral infections kills 0.25–0.5 million people annually.[44] As such, influenza outbreaks produce high morbidity and mortality rates with great economic and social impact.[44]

Early findings in relation to the most recent influenza pandemic occurred in April 2009 in Mexico and soon spread to other countries. The pandemic was caused by an H1N1 variant, which came from two genetic recombination events. The first occurred in 1998, when an avian virus, an American pig virus, and virus fragments of humans had exchanged genetic materials. The following recombination with a European swine

Correspondence: Mario A Rodriguez Perez
Instituto Politécnico Nacional (IPN), Centro de Biotecnología Genómica, Boulevard del Maestro Esquina Elías Piña, Colonia Narciso Mendoza, Reynosa, Tamaulipas 88710, Mexico
Email drmarodriguez@hotmail.com

submit your manuscript | www.dovepress.com
Dovepress
http://dx.doi.org/10.2147/RRTM.S144075

© 2018 Ramirez-Palacios et al. This work is published and licensed by Dove Medical Press Limited. The full terms of this license are available at https://www.dovepress.com/terms.php and incorporate the Creative Commons Attribution – Non Commercial (unported, v3.0) License (http://creativecommons.org/licenses/by-nc/3.0/). By accessing the work you hereby accept the Terms. Non-commercial uses of the work are permitted without any further permission from Dove Medical Press Limited, provided the work is properly attributed. For permission for commercial use of this work, please see paragraphs 4.2 and 5 of our Terms (https://www.dovepress.com/terms.php).

virus strain resulted in the pandemic swine origin influenza virus.[2,30] In Mexico in recent years, this has caused at least four outbreaks with high mortality rates compared with that presenting in other countries.[6] During the winter of 2017-2018, influenza activity increased in Mexico, and 2,855 cases of influenza and 73 deaths were confirmed by March 02, 2018, of which 46 cases were A(H3N2), 11 cases A(H1N1) pdm09, 10 cases B, and the remaining six cases were not subtyped.[39]

Results emanating from different studies have shown that influenza outbreaks are characterized by high severity of symptoms with increased mortality,[6,8,13,25,32] Several factors have been associated with H1N1 disease severity, such as factors due to the virus (ie, viral pathogenic mutations, resistance to antivirals), factors inherent to host susceptibility (eg, age, sex, race), including physiological immunosuppression or acquired diseases (ie, diabetes, hypertension, obesity, asthma), factors associated with available medical services and public health facilities, and factors arising from the presence of bacterial coinfections.[1,3,6,8,9,16,18,25,32,34,35,41]

Seasonal and pandemic influenza often have complications arising from bacterial coinfections. Cillóniz et al[12] documented that in H1N1 patients with community-acquired pneumonia, the most frequently isolated bacterial pathogens were *Streptococcus pneumoniae* (26, 62%) and *Pseudomonas aeruginosa* (6, 14%). *Staphylococcus aureus* was rarely found, and *Haemophilus influenzae* was not found.[12] During the 1918 pandemic, most deaths had bacterial coinfections. Globally, more than 34% of influenza virus infections needed intensive care among hospitalized patients, from which 0.5% of all cases of influenza corresponded to healthy young individuals and at least 2.5% of total cases the elderly group and those with coinfections harbored the bacteria.[27] Symptoms of influenza cases with bacterial coinfections are similar to those with severe influenza, but the former may have a higher risk of death. Identification of coinfections should be considered in patients with influenza-like illness (ILI) presenting symptoms suggestive of pneumonia, such as dyspnea, tachypnea, and hypoxia, or with evidence of septicemia.[27] Many copathogens are known to be colonizers of the respiratory mucosa, ie, *S. pneumoniae*, *H. influenzae*, and *Neisseria meningitidis*, including the upper and lower respiratory tracts. Distinction between copathogenic colonization and coinfection is critical, because a proven coinfection is clinically correlated with signs of pneumonia and bacterial contagion producing increased acuity or severity of disease.[27]

Empirical antiviral treatment should be considered and managed in such critically ill patients. The most commonly isolated bacterial pathogens are those that colonize the nasopharynx, and this complex of virus–bacteria contributes significantly to the pathogenesis of the disease, mainly in periods of endemic influenza.[26,34] There have been studies reporting copathogens between influenza and other viruses, but few cases have observed that this produced severe complications because of coinfection.[15,33,42] Two studies have hypothesized a "viral interference", suggesting that a rhinovirus infection may interfere with the A(H1N1pdm09) influenza, but this is still not fully understood.[24,33]

The precise identification of infectious pathogens responsible for acute respiratory infections, primarily influenza, is a critical factor for proper treatment of the disease and control during outbreaks and for the appropriate use of antibiotics and antivirals. For these reasons, continuation of investigations into the pathogens commonly associated with influenza cases is urgently needed. Here, the presence of bacterial and viral copathogens are identified using clinical samples for the molecular diagnosis by resequencing microarray in study patients with confirmed influenza A(H1N1)pdm09 in Oaxaca, Mexico. We also document an association between influenza A(H1N1pdm 09) and symptoms of disease severity in dead patients with multiple-microbial infection.

Materials and methods

Ethics statement

The present study involved the collaboration of one government health institution in Mexico that performed the sample collection. Before each examination, each adult who had voluntarily come to the examination point and agreed to participate was informed about the microbiological process of his/her sample, and oral consent was obtained. Parents or guardians provided oral consent on behalf of all under age child participants. The ethical committee of the health secretariat of Mexico approved the use of oral consent, given that the studies were conducted as part of the national H1N1-surveillance program and thus part of a routine public health-monitoring program conducted by the Mexican government.

Cross-sectional survey

A total of 88 study patients with confirmed H1N1 by quantitative qRT-PCR from six health districts of Oaxaca in Mexico were examined for other microbial infections. These patients were recruited from April 2009 throughout December 2012. The present study meets the operational definition criteria for ILI cases recommended by the World Health Organization.[45]

Inclusion

Only participants with confirmed H1N1 by qRT-PCR and ILI of any age who had a fever ≥38°C, cough, and headache accompanied by one or more of rhinorrhea, rhinitis, arthralgia, myalgia, prostration, sore throat, chest pain, abdominal pain, or nasal congestion were included in the study. For patients <5 years of age, irritability was substituted for headache. In those >65 years of age, fever was not required as a cardinal symptom.

Clinical specimens from ILI patients with H1N1

Individual respiratory clinical samples were collected. These included throat swab, nasopharyngeal swab, bronchoalveolar lavage, and lung biopsy according to each patient's condition. Throat and nasopharyngeal swabs were collected using a rayon or Dacron hyssop in a plastic tube containing 2–3 mL viral transport medium. Bronchoalveolar wash and lung biopsy samples were collected by trained medical staff and placed in plastic bottles containing 15–20 mL viral transport medium. All samples were kept at 2°C–8°C after sampling and during transport to a local molecular biology laboratory and stored at –80°C until testing.[17]

Detection of influenza A(H1N1)pdm09 using qRT-PCR

RNA extraction

Viral RNA was extracted using 140 µL of each clinical sample and of a positive control of influenza A(H1N1) pdm09 (donated by the Laboratory of Molecular Validation and Testing of the Institute for Epidemiological Diagnosis and Reference, Mexico) following the manufacturer's instructions from the QIAamp viral RNA minikit (Qiagen, Venlo, Netherlands). RNA extraction was completed using an automated protocol (QIAcube; Qiagen) and an elution volume of 60 µL. The RNA to be used as template was stored at –80°C until testing.

Oligonucleotides (probes and primers)

The protocol included four sets of primers and probes (universal influenza A [InfA], swine flu [swInfA], swine H1 [swH1], and RNase P [RP] primers). TaqMan primers and probes were synthesized by Biosearch Technologies (Novato, CA, USA) (Table 1).

qRT-PCR assays

We followed a US Centers for Disease Control and Prevention protocol (http://www.who.int/csr/resources/publications/swineflu/CDCRealtimeRTPCR_SwineH1Assay-2009_20090430.pdf) and used a Fast ABI 7500 thermocycler (Thermo Fisher Scientific, Waltham, MA, USA). The total reaction volume was 25 µL, including 5 µL template DNA (4–140 ng/µL), 0.5 µL primer forward (0.4 µM), 0.5 µL reverse primer (0.4 µM), 0.5 µL probe (0.1 µM), 12.5 µL 2× PCR Master Mix, 0.5 µL SuperScript III RT/Platinum Taq Mix, and 5.5 µL sterile molecular biology grade water. The PCR procedure started with one cycle of 50°C for 30 minutes (RT), one cycle of 95°C for 2 minutes (Taq inhibitor activation), followed by 45 cycles of 95°C for 15 seconds and 55°C for 30 seconds, as fluorescence data needed to be collected during the 55°C incubation step. A positive control (10 ng/µL) and a negative control of molecular biology grade water were run in parallel during all experiments. Background fluorescence was considered the correct value of the cycle threshold or cut (C_t). It was thus considered the threshold that went over the background fluorescence for each run in the

Table 1 Primers and probes used to detect influenza A(H1N1)pdm09 virus in study patients of Oaxaca, Mexico

Gen	Sequence (5′–3′)	Final concentration (µM)
InfA	InfA F: GAC CRA TCC TGT CAC CTC TGA C	0.4
	InfA R: AGG GCA TTY TGG ACA AAK CGT CTA	0.4
	InfA P: TGC AGT CCT CGC TCA CTG GGC ACG	0.1
SwInfA	SwInfA F: GCA CGG TCA GCA CTT ATY CTR AG	0.4
	SwInfA R: GTG RGC TGG GTT TTC ATT TGG TC	0.4
	SwInfA P: CYA CTG CAA GCC CAT ACA CAC AAG CAG GCA	0.1
SwH1	SwH1 F: GTG CTA TAA ACA CCA GCC TYC CA	0.4
	SwH1 R: CGG GAT ATT CCT TAA TCC TGT RGC	0.4
	SwH1 P: CA GAA TAT ACA TCC RGT CAC AAT TGG ARA A	0.1
RP	RNase PF: AGA TTT GGA CCT GCG AGC G	0.4
	RNase PR: GAG CGG CTG TCT CCA CAA GT	0.4
	RNase PP: TTC TGA CCT GAA GGC TCT GCG CG	0.1

Notes: Primers and probes synthesized by Biosearch Technologies (Novato, CA, USA). The probe was labeled with FAM at 5' and BHQ1 at 3'. A US Centers for Disease Control and Prevention protocol was used. Reprinted from World Health Organization, The WHO Collaborating Centre for influenza at CDC, *DC protocol of realtime RTPCR for influenza A(H1N1)*, 2009. Available from: http://www.who.int/csr/resources/publications/swineflu/CDCRealtimeRTPCR_SwineH1Assay-2009_20090430.pdf. Accessed March 8, 2018.[46]
Abbreviations: InfA, universal influenza A; swInfA, swine flu; swH1, swine H1; RP, RNase P.

exponential phase of the amplification curve. A cutoff value of C_t=37 was corrected accordingly.

Identification of coinfections using microarrays (TessArray RPM-Flu 3.1)

Total nucleic acid isolation

With 450 μL of the primary sample, total nucleic acids were recovered following the manufacturer's instructions from the MasterPure DNA- and RNA-isolation kit (Epicentre Biotechnologies, Madison, WI, USA). A final elution volume of 35 μL was stored at –20°C until testing.

Quantification of total nucleic acids

Amounts of total nucleic acids were estimated according to the ratio A 260:280 nm using a NanoDrop 2000c (Thermo Fisher Scientific).

Synthesis of complementary DNA

With an RPM-Flu 3.1 RT tube containing 4 μL total nucleic acids, RT was performed in order to obtain cDNA. The RT master mix comprised 4 μL 5× buffer for a single chain, 2 μL 0.1 M DTT, 1 μL 40 U/μL RNaseOut, and 1 μL 200 U/μL SuperScript III RT/Platinum Taq Mix. RT-PCR cycling conditions were 25°C for 10 minutes, 50°C for 50 minutes, and 85°C for 5 minutes. The resulting product was stored at –20°C.

Multiplex PCR amplification

With four 3.1 RPM-Flu 3.1 multiplex PCR tubes (A, B, C, and D), multiplex PCR amplification was conducted. The reaction mixture contained 5 μL cDNA, 11 μL Flexi buffer 5× GoTaq, 8.8 μL 25 mM $MgCl_2$, 0.8 μL DNA polymerase GoTaq 5 UI/μL, and 0.4 μL UDG 0.22 KU/220 μL. PCR cycling conditions were initiated with two cycles of 24°C for 10 minutes and 94°C for 2 minutes, followed by 16 cycles of 94°C for 30 seconds, 45°C for 30 seconds (increasing 1°C every cycle until 60°C), and 72°C for 90 seconds. The reaction was ended using 24 cycles of 94°C for 30 seconds and 60°C for 2 minutes. The PCR product was stored at 4°C.

Purification and elution of PCR products

Purification of PCR products was performed following the instructions of the QIAquick PCR purification kit (Qiagen). PCR products of tubes A–D were mixed and purified according to the manufacturer's instructions. A final elution volume of 25 μL was stored at 4°C.

Fragmentation and labeling

Reagents were used for the fragmentation and labeling of the PCR products for the resequencing GeneChip assay (Affymetrix; Thermo Fisher Scientific). Briefly, in a PCR tube, fragmentation was conducted using 23 μL purified PCR products and 2.6 μL master mix (2.5 μL fragmentation buffer 10× and 0.1 μL fragmentation reagent). PCR cycling conditions were 37°C for 5 minutes and 95°C for 10 minutes. The PCR product was stored at 0°C. Subsequently, a new PCR tube was used to perform the DNA labeling, which contained 25.6 μL fragmented PCR products and 10.4 μL master mix (7.2 μL of TdT buffer 5×, 1.2 μL labeling reagent, and 2 μL TdT 30 UI/μL). PCR cycling conditions were 37°C for 30 minutes and 95°C for 5 minutes. Then, the labeled PCR product was placed for at least 5 minutes in 0°C for hybridization.

Hybridization, washing, and sample scanning

The hybridization procedure was carried out for 16 hours at 56°C using a hybridization oven (model 640, Affymetrix). Microarrays were then washed and stained in an Affymetrix Fluidics Station. A computer CEL file containing microarray images was produced by an Affymetrix G7 scanner.

Analysis, interpretation, and reanalysis

The scanned image of the microarray was analyzed using GSEQ 4.0 software, which produced a CHP file containing the names and the sequences identified in each sample. Sequences were stored in a FASTA file, which was submitted online to retest the sequences on the manufacturer's website (http://www2.gsu.edu/~psyrab/gseq/index.html). Sequences of the pathogens identified in the FASTA file contained the results of the positive and negative controls and an overview of the sequences, including their name, C3 score, homology percentage, and length of the longest continuous sequence.

Statistical analysis

Patient data – age, sex, date, flu symptoms, clinical sampling date, and place of residence – were captured on an Excel spreadsheet. The proportion of H1N1 patients with microbial infection was calculated as the number of positive patients divided by the total (n=88) number examined and expressed as a percentage (prevalence). The associated 95% CIs of the proportion of patients harboring the pathogens were also determined. Prevalence of patients with influenza A(H1N1)pdm09 and copathogens (additional one to five pathogens) were examined for significant differences at α=0.05, indicated by no overlapping of CIs. The prevalence

of each pathogen and symptoms in the 88 patients were also examined per age-group.

Results

The influenza A(H1N1)pdm09 virus was detected and confirmed by qRT-PCR in a total of 88 clinical samples from study patients showing ILI in Oaxaca, Mexico (Table S1). To identify associated pathogens in the 88 samples, we used the microarray RPM-Flu 3.1.TessArray. A total of 71 patients presented copathogens with bacteria and/or viruses other than influenza A(H1N1)pdm09 (Table S1). Of the 71 patients, 27 (30.7%) patients harbored a single pathogen (other than influenza A[H1N1]pdm09), 28 (31.8%) harbored two pathogens, 13 (14.8%) harbored three pathogens, two (2.3%) harbored four pathogens, and one (1.1%) harbored five pathogens. The prevalence of copathogens did not vary among the patients coinfected with one to three pathogens; however, a significantly ($P<0.05$) low number of patients coharbored four to five pathogens (Table 2).

Table 2 Pathogens associated with influenza A(H1N1)pdm09 virus and microbial prevalence in 88 patients of Oaxaca, Mexico

Associated pathogens, n	Samples, n	Prevalence* (95% CI)
0	17	19% (12%–28%)
1	27	31% (22%–41%)
2	28	32% (23%–42%)
3	13	15% (8%–22%)
4	2	3% (0.1%–6%)
5	1	1% (0.002%–4%)

Note: *Point estimates.

Genera of associated bacterial pathogens identified were *Streptococcus*, *Haemophilus*, *Neisseria*, *Pseudomonas*, *Staphylococcus*, *Klebsiella*, and *Moraxella*, as well as parainfluenza coinfections and Coxsackie viruses. The most prevalent species of bacteria found ($P<0.05$) were *S. pneumoniae* (39.8%), *H. influenzae* (32.9%), *N. meningitidis* (25%), and *S. mitis* (23.9%) (Figure 1). A medium number (14.7%) of prevalent bacteria were *P. aeruginosa*. A few patients (range of prevalence 2.2%–7.9%) harbored other species of bacteria, such as *H. parainfluenzae*, *S. aureus*, *P. putida*, *P. mendocina*, and *N. gonorrhoeae*. Less prevalent species of bacteria and viruses (1.1%) found are also summarized in Figure 1. Table 3 highlights the copathogens identified as colonizers of the respiratory tract and coinfection with pathogens found in the 88 H1N1 patients of Oaxaca.

Of the 22 symptoms identified, the most common were fever in 85 patients (96%), impaired general health in 85 (96%), cough in 84 (95%), headache and myalgia in 75 (85%), rhinorrhea in 72 (82%), odynophagia in 57 (65%), cold in 56 (64%), and nasal congestion in 52 (59%). Most signs and/or symptoms were found among study participants 0.4–30 years old infected with influenza A(H1N1)pdm09 and microbial infection (Figure 2). Six of the 88 patients experienced disease severity and were admitted to the intensive care unit (Table S1).

Two of four dead patients harbored three bacteria. One male aged 30 years had *S. pneumoniae*, *H. influenzae*, and *N. meningitidis*. He was treated on an outpatient basis, and

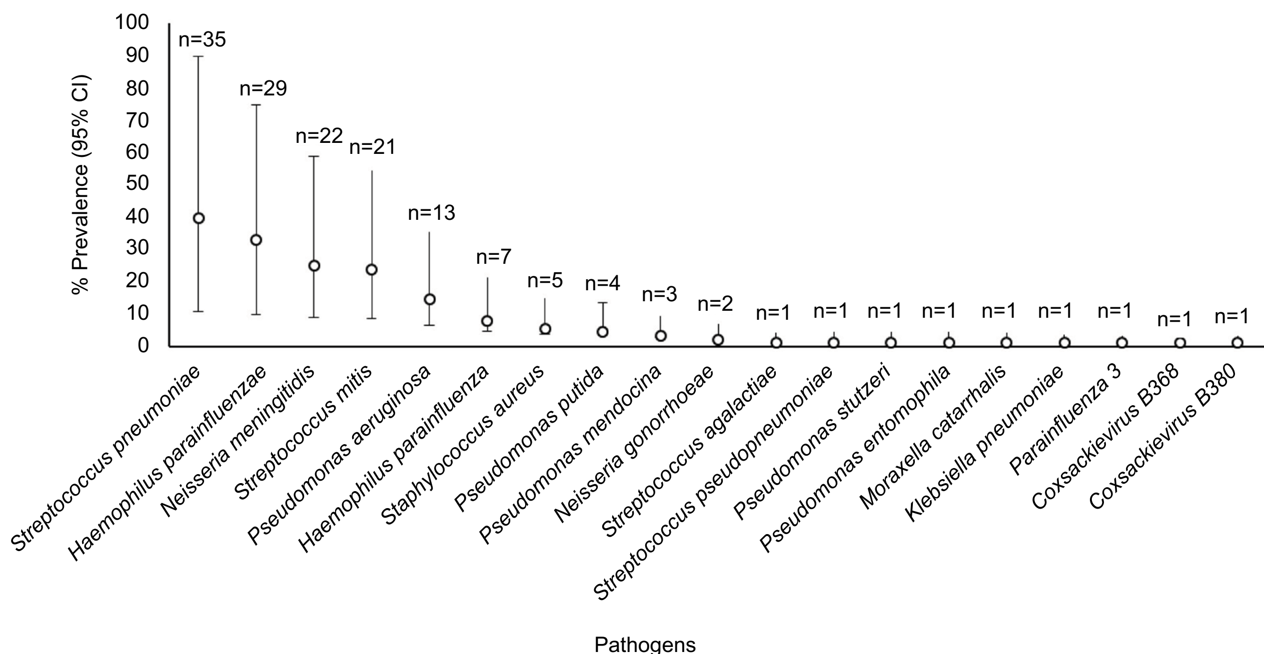

Figure 1 Number of copathogens positively associated with influenza A(H1N1)pdm09 virus and percentage of prevalence for each pathogen in patients of Oaxaca, Mexico.

Table 3 The 18 bacteria found in the 88 H1N1 patients of Oaxaca, Mexico: differences between pathogen cocolonizer and coinfection

Type of pathogen	Colonizer	Pathogen
Streptococcus pneumoniae		X
Haemophilus influenzae		X
Neisseria meningitidis		X
Streptococcus mitis	X	X
Pseudomonas aeruginosa	X	X
Haemophilus parainfluenzae	X	X
Staphylococcus aureus	X	X
Pseudomonas putida	X	
Pseudomonas mendocina	X	
Neisseria gonorrhoeae		X
Coxsackievirus	X	
Streptococcus agalactiae	X	
Streptococcus pseudopneumoniae	X	X
Pseudomonas stutzeri	X	
Pseudomonas entomophila	X	
Moraxella catarrhalis		X
Parainfluenza 3	X	X
Klebsiella pneumoniae		X

Note: Bacteria marked in yellow can cause sepsis. *N. meningitides* is highly pathogenic.

presented such symptoms as fever, impaired general health, odynophagia, cough, and headache. A 29-year-old female had *S. aureus*, *S. mitis*, and *S. pneumoniae*. She had multiple symptoms (Table S1) and died during the period of confinement (puerperium) just after childbirth. The remaining two dead patients harbored two bacteria. One male aged 22 years had *S. pneumoniae* and *N. gonorrhoeae.* He was treated on an outpatient basis, presented multiple symptoms, and was asthmatic. One 8-month-old male had *H. influenza* and *S. mitis*. The baby presented multiple symptoms, and was also asthmatic.

Discussion

Mexico has suffered at least four outbreaks produced by the influenza A(H1N1)pdm09 virus, with higher mortality rates than those reported by other countries.[4,10] As mentioned, there are viral and host factors,[18,7,22] timeliness of medical treatment of the disease,[11,20,37] and presence of copathogens[8,25,34,40] associated with increased severity of disease caused by this virus, among other factors, which may explain the epidemiological scenario in Mexico. During the first outbreaks of the virus in Mexico, increased mortality was noted because of the presence of coinfections with other pathogens.[20,31] Therefore, it is of paramount importance to investigate which organisms are causing coinfections to implement a monitoring and surveillance system for the disease in Mexico.

Here, we used the standard qRT-PCR protocol to identify the influenza A(H1N1)pdm09 virus in conjunction with a microarray assay for detecting other associated pathogens in positive patients to influenza A(H1N1)pdm09. The microarray assay has been shown to be capable of detecting in a single sample up to 30 viruses and bacteria that may produce ILI.[28] The identification of such pathogens is based on sequence analysis and subsequent search in the GenBank database, which helps to identify specific viral types and species of bacteria present in the sample.[28,43]

There was a significantly high prevalence rate of influenza patients with one and three associated pathogens (Table 2). The bacteria species most frequently identified was *S. pneumoniae* (39.8% of total individuals with copathogens), which differs somewhat with percentages (7.5%–18.6%) reported elsewhere.[3,8,13,29] *H. influenzae* (32.9%) and *N. meningitidis* (25%) also showed high prevalence rates. *N. meningitidis* is associated with greater severity of illness but requires a process of immunosuppression by patients.[5,14,23] Other associated pathogens that presented elevated prevalence rates were *S. mitis* (23.9%) and *P. aeruginosa* (14.7%), which coincide with other studies that reported (1%–13.9%) coinfections with those pathogens.[3,29] However, when comparing the results of this study with previous studies, prudence should be exercised, given that anatomic sites sampled and techniques of detection (and sensitivity and specificity) were somewhat different.

It is noteworthy that the presence of a high number of patients harboring several pathogens producing respiratory infections can be explained by considering the sanitary conditions of a particular country. For example, the prevalence of meningococcal disease varies among countries: 0.3–4 cases per 100,000 people.[36] In addition, there were only three cases where the pandemic virus was found in coinfection with parainfluenza 3 and Coxsackie virus. The results of our study are consistent with other studies that have reported lower rates of viral coinfections with respiratory syncytial virus, coronavirus, influenza B virus, adenovirus, and parainfluenza virus without significant complications or increase in the severity of the disease.[3,15,40] Similarly, some associations were found in most patients with certain signs and/or symptoms with influenza A(H1N1)pdm09 alone and with other pathogens (Table 3). Although this table does not address severity of illness or indicate that treatment of the bacterial infection was necessary, the evolution in the clinical status of the patients was more severe in some patients who interestingly

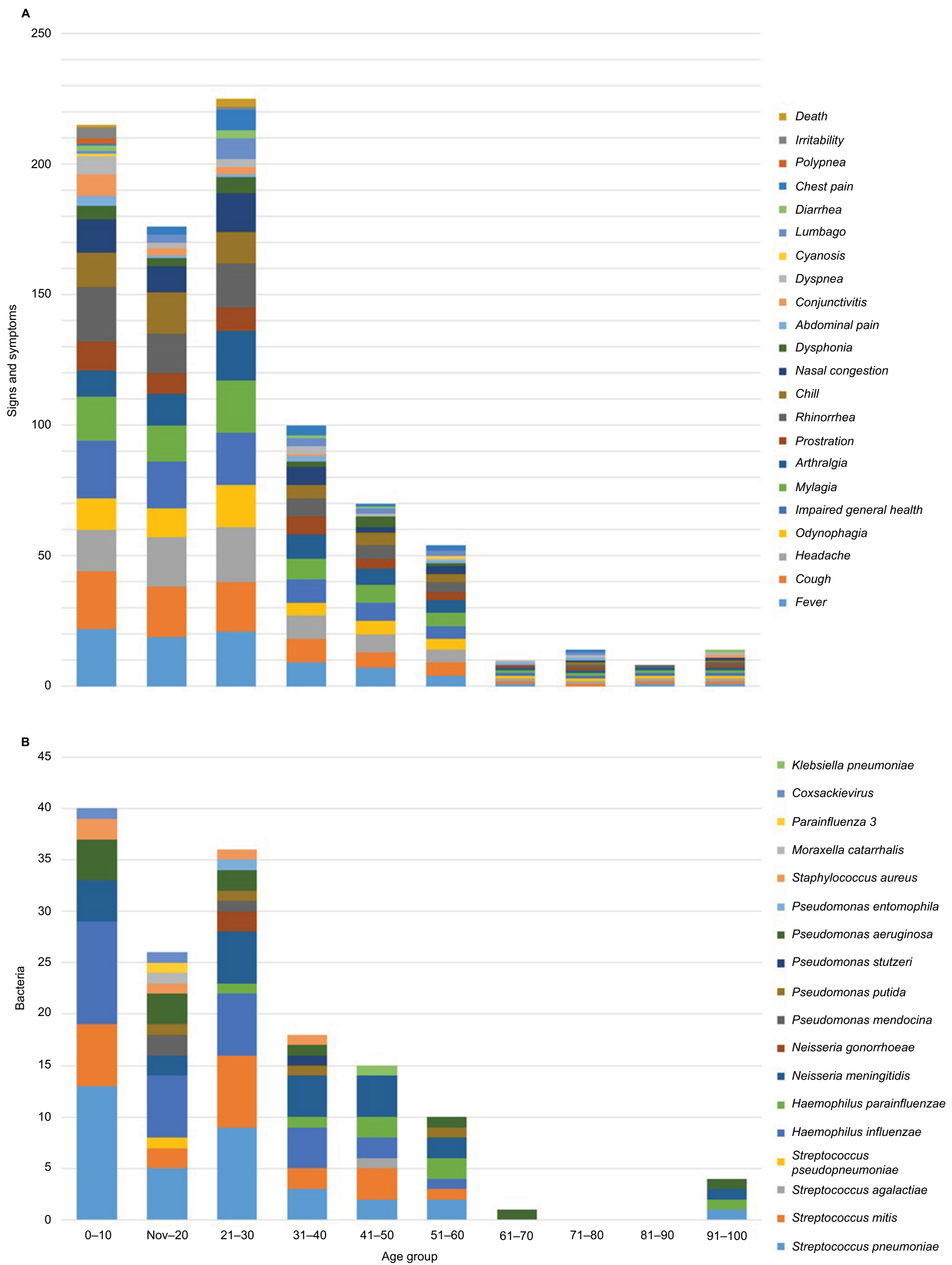

Figure 2 Signs and/or symptoms of study participants per age group infected with influenza A(H1N1)pdm09 and microbial infection.
Notes: (**A**) Signs/symptoms; (**B**) bacteria harbored by patients.

harbored copathogens: six patients of this group (6.8%) were admitted to the intensive care unit, and four (4.5%) died as a consequence of the coinfections.[3,15,40]

The 30-year-old male could have died as a consequence of infection with *N. meningitidis*, as influenza could have facilitated meningococcal colonization.[5,19] The 29-year-old female was in puerperium and presented 11 symptoms, H1N1, and three pathogenic bacteria, such as *S. aureus*. One study found that the majority of *S. aureus* isolated in both children and adults were methicillin resistant.[27] As a consequence, she was treated as inpatient in an intensive care unit.

The other two dead patients were asthmatic. They presumably died because adults and children with asthma are more likely to develop pneumonia after getting sick with the flu than people who do not have asthma (https://www.cdc.gov/flu/pdf/freeresources/updated/treating-influenza-2017.pdf). The 22-year-old male harbored *S. pneumoniae*, which appears to have a synergistic relationship with influenza.[38] This patient should have been treated on an inpatient basis and sent to the intensive care unit, as he was asthmatic and presented 10 different symptoms. However, he was treated on an outpatient basis. The 8-month-old male had eight symptoms and harbored *S. mitis*, which could have elevated the risk of and exacerbated the influenza infection.[21]

A total of 21 of 88 H1N1 patients harbored *S. mitis*. All H1N1 patients who had complications and those that were submitted to intensive care units were treated with the neuraminidase inhibitor oseltamivir (Tamiflu) within 48 hours of first symptoms of infection. An increase in the number of *S. mitis* cases could also have hampered the efficacy of the viral neuraminidase inhibitor drug.[21] Of the four H1N1 patients who died, *S. mitis* was present in three.

Copathogens were found in a significant number (>50%) of influenza cases in Oaxaca. Although some copathogens may be simply asymptomatic colonizing bacteria, other manifested proven coinfection and disease severity in some patients. Given that copathogens were found commonly, this could have an impact on the disease, but more studies need to be conducted, eg, investigating if coinfections between the pandemic virus and other pathogens can be the source of secondary infections when patients are in a status of immunosuppression. Moreover, other factors explain the increase in the severity of symptoms and mortality rate caused by H1N1 in Oaxaca. It has been noted that in areas of Oaxaca, sanitary conditions are not adequate, and this may allow proliferation of the pathogenic agents detected. A monitoring and surveillance system based on molecular diagnostics for all respiratory pathogens should be implemented, as well as strengthening of sanitary measures in the local Oaxaca population, both of which are urgently needed.

Acknowledgments

MARP holds a scholarship from Comisión de Operación y Fomento de Actividades Académicas COFAA/IPN. The present work was sponsored by the National Council of Science and Technology (CONACYT, Mexico) and the government of the state of Oaxaca (FOMIX) (grant 122497).

Disclosure

The authors report no conflicts of interest in this work.

References

1. Aktürk H, Sütçü M, Badur S, et al. Evaluation of epidemiological and clinical features of influenza and other respiratory viruses. *Turk Pediatri Ars*. 2015;50(4):217–225.
2. Mossad SB. The resurgence of swine-origin influenza A (H1N1). *Cleve Clin J Med*. 2009;76(6):337–343.
3. Blyth CC, Webb SA, Kok J, et al. The impact of bacterial and viral co-infection in severe influenza. *Influenza Other Respir Viruses*. 2013;7(2):168–176.
4. Borja-Aburto VH, Chowell G, Viboud C, et al. Epidemiological characterization of a fourth wave of pandemic A/H1N1 influenza in Mexico, winter 2011–2012: age shift and severity. *Arch Med Res*. 2012;43(7):563–570.
5. Cartwright KA, Jones DM, Smith AJ, Stuart JM, Kaczmarski EB, Palmer SR. Influenza A and meningococcal disease. *Lancet*. 1991;338(8766):554–557.
6. Castillo-Palencia JP, Laflamme L, Monárrez-Espino J. Occurrence of AH1N1 viral infection and clinical features in symptomatic patients who received medical care during the 2009 influenza pandemic in central Mexico. *BMC Infect Dis*. 2012;12:363.
7. US Centers for Disease Control and Prevention. Intensive-care patients with severe novel influenza A (H1N1) virus infection: Michigan, June 2009. *MMWR Morb Mortal Wkly Rep*. 2009;58(27):749–752.
8. Chertow DS, Memoli MJ. Bacterial coinfection in influenza: a grand rounds review. *JAMA*. 2013;309(3):275–282.
9. Chertow DS. Contribution of bacterial coinfection to severe influenza infection. *Crit Care Med*. 2012;40(5):1664–1665.
10. Chowell G, Echevarría-Zuno S, Viboud C, et al. Recrudescent wave of pandemic A/H1N1 influenza in Mexico, winter 2011–2012: age shift and severity. *PLoS Curr*. 2012;4:RRN1306.
11. Chowell G, Viboud C Simonsen L, et al. Impact of antiviral treatment and hospital admission delay on risk of death associated with 2009 A/H1N1 pandemic influenza in Mexico. *BMC Infect Dis*. 2012;12:97.
12. Cillóniz C, Ewig S, Menéndez R, et al. Bacterial coinfection with H1N1 infection in patients admitted with community acquired pneumonia. *J Infect*. 2012;65(3):223–230.
13. Domínguez-Cherit G, Lapinsky SE, Macias AE, et al. Critically Ill patients with 2009 influenza A (H1N1) in México. *JAMA*. 2009;302(17):1880–1887.
14. Feiterna-Sperling C, Edelmann A, Nickel R, et al. Pandemic influenza A (H1N1) outbreak among 15 school-aged HIV-1-infected children. *Clin Infect Dis*. 2010;51(11):e90–e94.
15. Goka E, Vallely P, Mutton K, Klapper P. Influenza A viruses dual and multiple infections with other respiratory viruses and risk of hospitalisation and mortality. *Influenza Other Respir Viruses*. 2013;7(6):1079–1087.
16. Hersh AL, Maselli JH, Cabana MD. Changes in prescribing of antiviral medications for influenza associated with new treatment guidelines. *Am J Public Health*. 2009;99(Suppl 2):S362–S364.

17. Hubbard K, Pellar G, Emanuel P. Suitability of commercial transport media for biological pathogens under nonideal conditions. *Int J Microbiol*. 2011;2011:463096.
18. Hurt AC, Holien JK, Parker MW, Barr IG. Oseltamivir resistance and the H274Y neuraminidase mutation in seasonal, pandemic and highly pathogenic influenza viruses. *Drugs*. 2009;69(18):2523–2531.
19. Jacobs JH, Viboud C, Tchetgen ET, et al. The association of meningococcal disease with influenza in the United States, 1989–2009. *PLoS One*. 2014;9(9):e107486.
20. Jain S, Benoit SR, Skarbinski J, Bramley AM, Finelli L. Influenza-associated pneumonia among hospitalized patients with 2009 pandemic influenza A (H1N1) virus: United States, 2009. *Clin Infect Dis*. 2009;54(9):1221–1229.
21. Kamio N, Imai K, Shimizu K, et al. Neuraminidase-producing oral mitis group streptococci potentially contribute to influenza viral infection and reduction in antiviral efficacy of zanamivir. *Cell Mol Life Sci*. 2015;72(2):357–366.
22. Kumar A, Zarychanski R, Pinto R, et al. Critically ill patients with 2009 influenza A (H1N1) infection in Canada. *JAMA*. 2009;302(17):1872–1879.
23. Legriel S, Merceron S, Tattevin P, et al. Favorable outcome after life-threatening meningococcal disease complicating influenza A (H1N1) infection. *Infection*. 2011;39(5):477–480.
24. Linde A, Rotzén-Ostlund M, Zweygberg-Wirgart B, Rubinova S, Brytting M. Does viral interference affect spread of influenza? *Euro Surveill*. 2009;14(40):19354.
25. López-Cervantes M, García J, Pacheco RL, Escamilla RA, Villanueva M. La influenza A/H1N1 2009: una crónica de la primera pandemia del siglo XXl. *Rev Digit Univ*. 2010;11(4):1–19.
26. Martín-Loeches I, Sanchez-Corral A, Diaz E, et al. Community-acquired respiratory coinfection in critically ill patients with pandemic 2009 influenza A (H1N1) virus. *Chest*. 2011;139(3):555–562.
27. Metersky ML, Masterton RG, Lode H, File TM Jr, Babinchak T. Epidemiology, microbiology, and treatment considerations for bacterial pneumonia complicating influenza. *Int J Infect Dis*. 2012;16(5):e321–e331.
28. Metzgar D, Myers CA, Russell KL, et al. Single assay for simultaneous detection and differential identification of human and avian influenza virus types, subtypes, and emergent variants. *PLoS One*. 2010;5(2):e8995.
29. Muscedere J, Ofner M, Kumar A, et al. The occurrence and impact of bacterial organisms complicating critical care illness associated with influenza A (H1N1) infection. *Chest*. 2013;144(1):39–47.
30. Neumann G, Brownlee GG, Fodor E, Kawaoka Y. Orthomyxovirus replication, transcription, and polyadenylation. *Curr Top Microbiol Immunol*. 2004;283:121–143.
31. Palacios G, Hornig M, Cisterna D, et al. *Streptococcus pneumoniae* coinfection is correlated with the severity of H1N1 pandemic influenza. *PLoS One*. 2009;4(12):e8540.
32. Randolph AG, Vaughn F, Sullivan R, et al. Critically ill children during the 2009–2010 influenza pandemic in the United States. *Pediatrics*. 2011;128(6):e1450–e1458.
33. Rhedin S, Hamrin J, Naucler P, et al. Respiratory viruses in hospitalized children with influenza-like Illness during the H1N1 2009 pandemic in Sweden. *PLoS One*. 2012;7(12):e51491.
34. Rice TW, Rubinson L, Uyeki TM, et al. Critical illness from 2009 pandemic influenza A virus and bacterial coinfection in the United States. *Crit Care Med*. 2012;40(5):1487–1498.
35. Rodríguez-Valero M, Calleros HM, Escobar GA, et al. Difference between early clinical features of swine origin A H1N1 influenza confirmed and not confirmed infection in Mexico. *J Infect Dev Ctries*. 2012;6(4):302–310.
36. Sáfadi MA, Cintra OA. Epidemiology of meningococcal disease in Latin America: current situation and opportunities for prevention. *Neurol Res*. 2010;32(3):263–271.
37. Serna-Ojeda JC, Castañón-González JA, Macías AE, Mansilla-Olivares A, Domínguez-Cherit G, Polanco-González C. [Survey about responsiveness of third-level hospitals to a medical disaster: after the pandemic influenza in Mexico]. *Gac Med Mex*. 2012;148(3):227–235. Spanish.
38. Short KR, Habets MN, Hermans PW, Diavatopoulos DA. Interactions between *Streptococcus pneumoniae* and influenza virus: a mutually beneficial relationship? *Future Microbiol*. 2012;7(5):609–624.
39. Sistema Nacional de Vigilancia. Dirección General de Epidemiología. Secretaría de Salud/Sistema de Notificación Semanal de Casos Nuevos, corte al 2 de marzo del 2018. Secretaría de Salud: México; 2018. Available from: https://www.gob.mx/cms/uploads/attachment/file/304904/INFLUENZA_2018_SE09.pdf. Accessed March 8, 2018.
40. Stefanska I, Romanowska M, Donevski S, Gawryluk D, Brydak LB. Co-infections with influenza and other respiratory viruses. *Adv Exp Med Biol*. 2013;756:291–301.
41. Volkow P, y Pérez-Padilla JR. Respuesta hospitalaria a la epidemia de influenza. In: Córdova-Villalobos JA, Valdespino-Gómezc JL, de León-Rosales S, editors. *La Epidemia de Influenza A/H1N1 en México*. Madrid: Editorial Médica Panamericana; 2010:34–44.
42. Wang D, Chen LL, Ding YF, et al. Viral etiology of medically attended influenza-like illnesses in children less than five years old in Suzhou, China, 2011 to 2014. *J Med Virol*. 2016;88(8):1334–1340.
43. Wang Z, Malanoski AP, Lin B, et al. Resequencing microarray probe design for typing genetically diverse viruses: human rhinoviruses and enteroviruses. *BMC Genomics*. 2008;9:577.
44. World Health Organization. FluNet. World Health Organization: Geneva; 2018. Available from: http://www.who.int/influenza/gisrs_laboratory/flunet/en. Accessed March 8, 2018.
45. World Health Organization. *WHO Guidelines for Pharmacological Management of Pandemic Influenza A (H1N1) 2009 and Other Influenza Viruses: Part 1 – Recommendations*. World Health Organization: Geneva; 2010. Available from: http://www.who.int/csr/resources/publications/swineflu/h1n1_guidelines_pharmaceutical_mngt.pdf. Accessed March 8, 2018.
46. World Health Organization. *CDC protocol of realtime RTPCR for influenza A(H1N1)*. The WHO Collaborating Centre for influenza at CDC Atlanta, United States of America: World Health Organization, Geneva; 2009. Available from:http://www.who.int/csr/resources/publications/swineflu/CDCRealtimeRTPCR_SwineH1Assay-2009_20090430.pdf. Accessed March 8, 2018.

Supplementary material

Table S1 Symptoms, clinical evolution, and positivity to bacteria of 88 influenza A(H1N1)pdm09 patients in Oaxaca, Mexico

			Symptoms																							Bacteria																	
Age	**Sex**	**Evolution of patient clinical status**	**Fever**	**Cough**	**Headache**	**Odynophagia**	**Impaired general health**	**Myalgia**	**Arthralgia**	**Prostration**	**Rhinorrhea**	**Chill**	**Nasal congestion**	**Dysphonia**	**Abdominal pain**	**Conjunctivitis**	**Dyspnea**	**Cyanosis**	**Lumbago**	**Diarrhea**	**Chest pain**	**Polypnea**	**Irritability (headache if <5 years old)**	**Death**	**Clinical sample**	***Streptococcus pneumoniae***	***Streptococcus mitis***	***Streptococcus agalactiae***	***Streptococcus pseudopneumoniae***	***Haemophilus influenzae***	***Haemophilus parainfluenzae***	***Neisseria meningitidis***	***Neisseria gonorrhoeae***	***Pseudomonas mendocina***	***Pseudomonas putida***	***Pseudomonas stutzeri***	***Pseudomonas aeruginosa***	***Pseudomonas entomophila***	***Staphylococcus aureus***	***Moraxella catarrhalis***	***Parainfluenza 3***	**Coxsackievirus**	***Klebsiella pneumoniae***
0.4	M	ICU	+	+	−	+	+	−	−	+	+	−	+	−	−	−	+	−	−	−	−	−	−	−	NS	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
0.5	M	I	−	+	−	−	+	−	−	+	+	−	+	+	−	−	+	−	−	−	−	+	+	−	NS	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
0.6	F	O	+	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	TS	+	+	−	−	+	−	+	−	−	−	−	−	−	−	−	−	−	−
0.8	M	ICU	+	+	−	−	+	−	−	+	+	−	+	−	−	+	+	+	−	−	−	−	−	+	LB	−	+	−	−	+	ND	−	−	−	−	−	−	−	−	−	−	−	−
0.8	F	O	+	+	+	+	+	+	+	−	+	+	+	−	−	+	+	−	−	−	−	−	−	−	NS	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−
1	F	O	+	+	+	+	+	+	−	−	+	+	+	−	−	+	+	−	−	−	−	−	−	−	NS	+	+	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−
1	F	I	+	+	+	+	+	+	−	−	+	+	−	−	−	+	−	−	+	+	−	−	−	−	NS	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−
1	M	O	+	+	−	−	+	−	−	−	+	−	−	−	−	−	+	−	−	−	−	−	+	−	NS	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
2	M	I	+	+	−	−	+	−	−	−	+	+	−	+	+	−	−	−	−	+	−	−	−	−	TS	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
3	F	O	+	+	+	−	+	+	−	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	TS	+	+	−	−	+	−	−	−	−	−	−	+	−	−	−	−	−	−
4	F	O	+	+	+	−	+	+	+	−	+	−	−	−	+	−	−	−	−	−	−	−	−	−	TS	+	+	−	−	+	−	−	−	−	−	−	+	−	−	−	−	+	−
5	M	O	+	+	+	+	+	+	+	−	+	−	−	−	+	−	−	−	−	−	−	−	−	−	TS	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
5	M	O	+	+	+	+	+	−	−	+	+	+	+	+	−	+	−	−	−	−	−	−	−	−	TS	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−
6	M	O	+	−	+	−	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	TS	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−
7	M	O	+	+	+	+	+	+	+	−	+	+	+	+	−	+	−	−	−	−	−	−	−	−	TS	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
8	M	O	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	TS	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
8	M	I	+	+	+	+	+	+	+	+	+	+	+	+	−	+	−	−	−	−	+	−	−	−	TS	−	−	−	−	−	−	−	−	−	−	−	+	−	+	−	−	−	−
8	M	I	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	TS	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−
9	M	O	+	+	+	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	TS	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−
9	F	O	+	+	+	+	+	+	−	+	+	−	−	−	+	+	−	−	−	−	−	−	−	−	TS	−	−	−	−	+	−	+	−	−	−	−	−	−	−	−	−	−	−
9	F	ICU	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	BAL	−	+	−	−	+	−	+	−	−	−	−	−	−	−	−	−	−	−
9	M	I	+	+	−	−	+	+	+	−	+	−	+	−	−	−	+	−	−	−	−	+	+	−	TS	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
10	M	I	+	+	+	−	+	+	−	−	+	+	+	−	−	−	−	−	−	−	−	−	−	−	TS	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
11	M	O	+	+	+	+	+	+	+	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	TS	+	−	−	−	+	−	+	−	−	−	−	−	−	−	−	−	−	−

(*Continued*)

Table S1 (*Continued*)

Age	Sex	Evolution of patient clinical status	Symptoms																						Clinical sample	Bacteria																	
			Fever	Cough	Headache	Odynophagia	Impaired general health	Myalgia	Arthralgia	Prostration	Rhinorrhea	Chill	Nasal congestion	Dysphonia	Abdominal pain	Conjunctivitis	Dyspnea	Cyanosis	Lumbago	Diarrhea	Chest pain	Polypnea	Irritability (headache if <5 years old)	Death		*Streptococcus pneumoniae*	*Streptococcus mitis*	*Streptococcus agalactiae*	*Streptococcus pseudopneumoniae*	*Haemophilus influenzae*	*Haemophilus parainfluenzae*	*Neisseria meningitidis*	*Neisseria gonorrhoeae*	*Pseudomonas mendocina*	*Pseudomonas putida*	*Pseudomonas stutzeri*	*Pseudomonas aeruginosa*	*Pseudomonas entomophila*	*Staphylococcus aureus*	*Moraxella catarrhalis*	*Parainfluenza 3*	Coxsackievirus	*Klebsiella pneumoniae*
11	M	I	+	+	+	+	+	−	−	−	+	+	+	+	−	−	−	−	−	−	−	−	−	−	TS	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−
12	M	I	+	+	+	−	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	TS	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−
12	M	I	+	+	+	−	+	+	−	−	+	−	+	−	−	+	−	−	−	−	−	−	−	−	TS	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
13	F	O	+	+	+	+	+	+	+	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	TS	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	+	−
13	F	O	+	+	+	−	+	+	−	+	+	+	+	−	−	+	−	−	−	−	−	−	−	−	TS	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
13	F	O	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	TS	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−
14	F	O	+	+	+	+	−	+	+	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	TS	−	−	−	−	+	−	−	−	+	−	−	−	−	+	−	−	−	−
14	M	O	+	+	+	−	+	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	TS	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
14	F	O	+	+	+	−	+	+	+	+	+	+	+	−	−	−	+	−	−	−	+	−	−	−	TS	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−
14	M	O	+	+	+	−	+	+	+	+	+	+	+	−	−	−	−	−	+	−	−	−	−	−	TS	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
15	F	O	+	+	+	+	+	+	−	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	TS	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−
16	F	O	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	+	−	+	−	−	−	TS	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−
16	M	O	+	+	+	−	+	−	+	−	+	+	−	−	−	−	+	−	−	−	+	−	−	−	TS	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−
17	M	O	+	+	+	+	+	+	+	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	TS	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−
17	M	O	+	+	+	+	+	+	+	−	+	+	+	−	−	−	−	−	−	−	−	−	−	−	TS	−	−	−	−	+	−	−	−	−	−	−	+	−	−	−	−	−	−
17	M	O	+	+	+	−	+	−	−	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	TS	+	+	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−
17	M	O	+	+	+	+	+	+	+	−	+	+	+	+	+	+	−	−	+	−	−	−	−	−	TS	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
19	M	O	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	TS	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−
21	M	O	+	+	+	+	+	+	−	−	+	−	+	−	−	−	−	−	+	−	+	−	−	−	TS	+	−	−	−	+	−	−	−	−	−	−	+	−	−	−	−	−	−
21	F	O	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	+	−	−	−	−	−	TS	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−
21	F	O	+	+	+	−	+	+	+	+	+	+	+	−	−	−	−	−	−	−	+	−	−	−	TS	+	−	−	−	+	−	+	−	−	−	−	−	−	−	−	−	−	−
22	M	O	+	+	+	+	+	+	+	+	+	+	+	+	−	+	−	−	+	−	+	−	−	−	TS	+	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−
22	M	O	+	+	+	−	+	+	+	+	+	−	+	−	−	−	+	−	−	−	−	−	−	+	LB	+	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−
24	M	O	+	−	+	+	+	+	+	+	−	+	+	−	−	−	−	−	+	+	+	−	−	−	TS	−	−	−	−	+	−	−	+	−	−	−	−	−	−	−	−	−	−
24	F	I	+	+	+	+	+	+	+	−	+	+	+	+	−	−	−	−	−	−	−	−	−	−	TS	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
25	F	O	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	+	+	−	−	−	−	TS	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−

(*Continued*)

Table S1 (*Continued*)

Age	Sex	Evolution of patient clinical status	Symptoms: Fever	Cough	Headache	Odynophagia	Impaired general health	Myalgia	Arthralgia	Prostration	Rhinorrhea	Chill	Nasal congestion	Dysphonia	Abdominal pain	Conjunctivitis	Dyspnea	Cyanosis	Lumbago	Diarrhea	Chest pain	Polypnea	Irritability (headache if <5 years old)	Death	Clinical sample	Bacteria: *Streptococcus pneumoniae*	*Streptococcus mitis*	*Streptococcus agalactiae*	*Streptococcus pseudopneumoniae*	*Haemophilus influenzae*	*Haemophilus parainfluenzae*	*Neisseria meningitidis*	*Neisseria gonorrhoeae*	*Pseudomonas mendocina*	*Pseudomonas putida*	*Pseudomonas stutzeri*	*Pseudomonas aeruginosa*	*Pseudomonas entomophila*	*Staphylococcus aureus*	*Moraxella catarrhalis*	*Parainfluenza 3*	Coxsackievirus	*Klebsiella pneumoniae*
25	M	O	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	+	−	+	−	−	−	TS	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
26	F	O	+	+	+	+	+	+	+	−	+	+	+	−	−	−	−	−	+	−	+	−	−	−	TS	+	+	−	−	−	+	+	−	−	−	−	−	−	−	−	−	−	−
26	F	O	+	+	+	+	+	+	+	+	+	+	+	+	−	+	−	−	−	−	−	−	−	−	TS	+	+	−	−	+	−	+	−	−	−	−	−	−	−	−	−	−	−
26	M	O	+	+	+	−	+	+	+	−	+	−	+	−	−	−	−	−	−	+	−	−	−	−	TS	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−
26	F	O	+	+	+	+	+	+	+	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	TS	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
26	F	I	+	+	+	+	+	+	+	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	TS	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
27	F	O	+	+	+	+	+	+	+	−	+	+	−	−	−	−	−	−	−	−	−	−	+	−	TS	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
29	F	ICU	+	+	+	+	−	+	+	−	+	+	+	−	−	−	+	−	−	−	+	−	−	+	LB	+	+	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−
29	F	ICU	+	−	+	−	+	+	+	+	−	−	+	+	−	−	+	−	−	−	+	−	−	−	BAL	+	+	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−
29	F	O	+	+	+	+	+	+	+	−	+	+	+	−	−	−	−	−	+	−	−	−	−	−	TS	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
29	F	O	+	+		−	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	TS	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
30	F	I	+	+	+	+	+	+	+	−	+	+	+	+	+	+	−	−	−	−	−	−	−	−	TS	+	+	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−
30	M	O	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	LB	−	+	−	−	+	−	+	−	−	−	−	−	−	−	−	−	−	−
32	M	O	+	+	+	−	+	+	+	+	+	−	+	−	−	−	−	−	−	−	+	−	−	−	TS	+	+	−	−	+	−	+	−	−	−	−	−	−	−	−	−	−	−
34	F	O	+	+	+	−	+	+	+	+	−	+	−	−	−	−	+	−	+	−	+	−	−	−	TS	+	−	−	−	+	−	+	−	−	−	−	−	−	−	−	−	−	−
36	F	O	+	+	+	+	+	+	+	+	+	−	+	+	−	+	−	−	−	−	−	−	−	−	TS	+	+	−	−	+	−	+	−	−	−	+	−	−	−	−	−	−	−
36	M	I	+	+	+	−	+	+	+	−	−	−	+	−	−	−	+	−	−	−	+	−	−	−	TS	−	−	−	−	−	−	−	−	−	−	−	+	−	+	−	−	−	−
36	F	O	+	+	+	+	+	+	+	−	+	+	+	−	+	−	−	−	−	−	−	−	−	−	TS	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−
38	M	O	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	TS	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−
38	M	O	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	+	+	−	−	−	−	TS	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
38	M	O	+	+	+	−	+	+	+	+	+	−	+	−	+	−	+	−	+	−	+	−	−	−	TS	−	−	−	−	+	−	+	−	−	−	−	−	−	−	−	−	−	−
40	M	O	+	+	+	+	+	−	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	TS	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
41	F	I	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	TS	−	+	−	−	+	−	+	−	−	−	−	−	−	−	−	−	−	−
41	M	O	+	+	+	−	+	+	+	+	+	+	−	+	−	−	−	−	+	−	+	−	−	−	TS	−	+	−	−	−	+	+	−	−	−	−	−	−	−	−	−	−	−
43	F	I	+	−	+	+	+	+	+	−	+	−	−	−	−	−	−	−	+	−	−	−	−	−	TS	+	−	+	−	+	−	+	−	−	−	−	−	−	−	−	−	−	−
44	F	ICU	+	+	+	+	+	+	−	−	+	−	−	+	−	−	−	−	−	−	−	−	−	−	BAL	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

(*Continued*)

Table S1 (*Continued*)

			Symptoms																						Bacteria																	
Age	**Sex**	**Evolution of patient clinical status**	**Fever**	**Cough**	**Headache**	**Odynophagia**	**Impaired general health**	**Myalgia**	**Arthralgia**	**Prostration**	**Rhinorrhea**	**Chill**	**Nasal congestion**	**Dysphonia**	**Abdominal pain**	**Conjunctivitis**	**Dyspnea**	**Cyanosis**	**Lumbago**	**Diarrhea**	**Chest pain**	**Polypnea**	**Irritability (headache if <5 years old)**	**Death**	**Clinical sample**	***Streptococcus pneumoniae***	***Streptococcus mitis***	***Streptococcus agalactiae***	***Streptococcus pseudopneumoniae***	***Haemophilus influenzae***	***Haemophilus parainfluenzae***	***Neisseria meningitidis***	***Neisseria gonorrhoeae***	***Pseudomonas mendocina***	***Pseudomonas putida***	***Pseudomonas stutzeri***	***Pseudomonas aeruginosa***	***Pseudomonas entomophila***	***Staphylococcus aureus***	***Moraxella catarrhalis***	***Parainfluenza 3***	**Coxsackievirus**	***Klebsiella pneumoniae***
48	F	O	+	+	+	+	+	+	+	+	−	+	−	+	−	−	−	−	−	+	−	−	−	−	TS	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
49	F	O	+	+	+	−	+	+	+	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−	TS	+	+	−	−	−	+	+	−	−	−	−	−	−	−	−	−	−	+
49	F	I	+	+	+	+	+	+	+	+	−	+	+	+	−	−	+	−	−	−	−	−	−	−	TS	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
51	F	O	−	+	+	+	+	+	+	−	+	−	−	−	+	−	−	−	−	−	+	−	−	−	TS	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−
52	M	O	+	+	+	+	+	+	+	+	+	+	+	+	−	−	+	+	+	−	−	−	−	−	TS	+	+	−	−	ND	+	+	−	−	+	−	−	−	−	−	−	−	−
53	F	O	+	+	+	−	+	+	+	−	+	−	+	−	−	−	−	−	−	−	−	−	−	−	TS	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
55	M	O	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	+	−	−	−	TS	+	−	−	−	+	−	+	−	−	−	−	+	−	−	−	−	−	−
57	F	I	+	+	+	+	+	+	+	+	−	+	−	−	−	−	−	−	+	−	−	−	−	−	TS	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
68	F	O	+	+	+	+	+	+	+	+	−	−	−	−	+	−	+	−	−	−	−	−	−	−	TS	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−
72	M	I	−	+	+	+	+	+	+	+	+	+	+	−	+	−	+	−	+	−	+	−	−	−	TS	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
89	M	I	+	+	+	+	+	+	+	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	TS	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
92	F	I	+	+	+	+	+	+	+	+	+	+	+	−	−	+	+	−	−	+	−	−	−	−	TS	+	−	−	−	−	+	+	−	−	−	−	+	−	−	−	−	−	−

Abbreviations: M, male; F, female; O, outpatient; I, inpatient; ICU, intensive care unit; TS, throat swab; LB, lung biopsy; BAL, bronchoalveolar lavage; NS, nasopharyngeal swab; ND, no data.

Research and Reports in Tropical Medicine

Dovepress

Publish your work in this journal

Research and Reports in Tropical Medicine is an international, peer-reviewed, open access journal publishing original research, case reports, editorials, reviews and commentaries on all areas of tropical medicine, including: Diseases and medicine in tropical regions; Entomology; Epidemiology; Health economics issues; Infectious disease; Laboratory science and new technology in tropical medicine; Parasitology; Public health medicine/health care policy in tropical regions; and Microbiology. The manuscript management system is completely online and includes a very quick and fair peer-review system. Visit http://www.dovepress.com/testimonials.php to read real quotes from published authors.

Submit your manuscript here: https://www.dovepress.com/research-and-reports-in-tropical-medicine-journal