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Detection of bacterial co-infection in patients with severe influenza

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Abstract

Viral infections engender bacterial colonization in the host by causing alteration in the innate immune response to common bacterial pathogens. These alterations may lead to severe bacterial pneumonia during influenza. Bacterial co-infection is common in influenza pneumonia and increases morbidity and mortality. With the onset of the COVID-19 pandemic worldwide, research in viral diseases has intensified. With this study, it can be thought that bacterial colonization may also support viral activity in the human body. This study sought to investigate bacterial co-infection in respiratory specimens using samples from the Australian influenza epidemic of 2009 as a model for analysis. Furthermore, it was thought that this study could shed light on bacterial co-infection studies in patients with COVID-19, which caused the current pandemic. 200 clinical specimens were extracted for isolation of genomic DNA and directly tested in a multiplexed quantitative PCR. Multiplex-tandem PCR (MT-PCR) assay based on 16S rRNA consensus sequence of a family (*Enterobacteriaceae* species) and four genera (*Staphylococcus* spp., *Streptococcus* spp., *Enterococcus* spp., and *Pseudomonas* spp.) was performed. According to MT-PCR results, respiratory tract samples showed a very high rate of bacterial identification (163/200-81.5%), and Enterococci were commonly found as the dominant genus identified in influenza patients. In a total of 200 putative co-infection samples, *Enterococcus* spp. was apparently present in 139 samples (69.5%), being the dominant signal (any other signal was ≤10%) from 106 samples. *Streptococcus* spp. was next most commonly indicated with 137 positive results (68.5%), but was the dominant signal in 19 of 137. It was determined that bacterial co-infection was high in the samples taken from patients with severe influenza, and *Enterococcus* was the dominant bacteria in the upper respiratory tract of patients treated for severe pneumonia, while *Streptococcus* was more dominant in the lower respiratory tracts than the other species.

Keywords: Bacterial co-infection, secondary bacterial pneumonia, australian influenza epidemic, respiratory tract infections

Introduction

Common cold and influenza (flu) are familiar syndromes resulting from respiratory tract infections that can be both viral and bacterial. More than 200 different viruses have been discovered to cause the common 'cold'. Respiratory tract infections are caused by rhinoviruses, coronaviruses, and influenza viruses [1]. Viral infections cause alterations in the innate immune response to common bacterial pathogens and ultimately cause bacterial colonization of the host. These changes can lead to severe bacterial pneumonia during influenza [2]. Bacterial co-infection is common in influenza pneumonia, and one of the main concerns in viral infections is that bacterial co-infections increase the morbidity and mortality rate. Lower respiratory infections are frequent complications of coronavirus, influenza A and influenza B infections. Furthermore, acute bronchitis, which can result from lower respiratory infections, is more common in those patients with influenza [3,4]. In addition, *S. pneumoniae* and some other bacterial pathogens are common causes of lethal CAP (Community-acquired pneumoniae) and the most important cause of secondary bacterial pneumonia during influenza [5]. Viral pneumonia, acute respiratory distress syndrome (ARDS),

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and bacterial co-infections have also been identified as common causes of death in several studies [6,7].

It is known that the coronavirus, which causes severe pneumonia, affected almost 460 million people worldwide with the 2019 pandemic, and 6 million people died because of this [8]. In some studies, bacterial co-infection was found in 3.5-7% of COVID-19 patients, and secondary bacterial infection was detected in 14.3% of COVID-19 patients. Therefore, the overall rate of COVID-19 patients with bacterial infection was 6.9%. Moreover, it has been observed that bacterial co-infection is more common in patients in mixed ward/ICU (Intensive care unit) compared to other hospitalized patients [9,10]. The dominant bacteria were *Mycoplasma pneumonia, Pseudomonas aeruginosa, Haemophilus influenza*, and *Staphylococcus aureus* in COVID-19 patients [10,11].

Pulmonary complications of influenza include primary influenza pneumonia, secondary bacterial pneumonia, pneumonia due to unusual pathogens in immunocompromised hosts, and exacerbations of chronic pulmonary disease [12]. Bacterial co-infection is common in influenza pneumonia [13,14]. The most common pathogens that cause secondary bacterial pneumonia are *S. pneumoniae, S. aureus*, and *H. influenza* [15]. It is difficult to distinguish whether pneumonia is viral or bacterial based on clinical findings. In some CAP studies, the results showed that the frequency of different types of microorganisms varies between populations. According to Johnstone et al., overall, only 39% of patients with CAP had their pathogens identified. 20% of these pathogens were bacteria followed by viruses (15%), and 4% of the patients have both. According to Jennings et al., overall, 58% of patients with CAP have their pathogens identified. 48 % of these pathogens were bacteria followed by viruses (29%), and 15% have both [16,17].

In 2009, more than 370,000 cases of pandemic influenza H1N1 (swine-origin influenza A) infection were identified worldwide, with more than 4500 deaths reported to the World Health Organization (WHO) [18]. During winter in 2009, 32.7% of H1N1 influenza cases admitted to ICU in Australia and New Zealand had respiratory risk factors, including asthma and other chronic pulmonary diseases. 48.8% of cases had acute respiratory distress syndrome or viral pneumonia, and 20.3% of cases had secondary bacterial pneumonia with 2009 H1N1 influenza infection in Australia and New Zealand ICU [19]. This study aimed to investigate the frequency of co-infection caused by Gram-negative and Gram-positive bacteria in patients with severe influenza during the 2009 Australian influenza epidemic using a multiplexed quantitative PCR assay, and it was thought that this study could shed light on bacterial co-infection studies in patients with COVID 19, which caused the current pandemic.

Material and Methods

Testing of Assay Validation

S. aureus (ATCC 25923), *E. facecalis* (ATCC 29212), *E. coli* (ATCC 25922), *S. pneumoniae* (ATCC 49619) and *P. aeruginosa* (ATCC 27853) strains (obtained from Infectious Diseases& Microbiology Department, Westmead Hospital, Sydney, Australia) used for DNA extraction of positive controls for Bacterial load assay. Bacterial cultures on blood agar plates were suspended in PBS (phosphate buffered saline) to achieve an optical density of 0.5 McFarland (~$1.5x10^8$cfu/mL) using a DensiChek densitometer (bioMérieux, Durham NC, USA). Serial dilutions were prepared in NB (Nutrient broth) (ten-fold dilutions from $1.0x10^8$cfu/mL to $1.0x10^4$cfu/mL). According to the manufacturer's instructions, each diluted sample was extracted using the NucliSENS® easyMAG® extractor (bioMérieux, Durham NC, USA). Then all extracted samples were tested in the MT-PCR Bacterial load assay. Standard curves were prepared for quantitative analysis (Appendix A).

Clinical Specimens and Performing PCR Assay

A total of 200 clinical specimens from intensive care patients with influenza from the 2009 H1N1 epidemic were then evaluated [18]. These were from sputum, endotracheal tube (ETT) aspirate, bronchial washing, bronchoalveolar lavage (BAL), nose/throat swab and nasopharyngeal aspirates (NPA) and were extracted (NucliSENS® easyMAG®, Durham, USA) for isolation of genomic DNA, and directly tested in the MT-PCR (Gene-Plex Robot & Rotor-Gene RG6000, Corbett Research, Sydney, Australia) Bacterial load assay. The Bacterial load assay (AusDiagnostics Pty. Ltd., Sydney, NSW) was used for detection and quantification of pathogens from a family *Enterobacteriaceae* species and the genera *Staphylococcus* spp., *Streptococcus* spp., *Enterococcus* spp., and *Pseudomonas* spp. using 16S rRNA consensus targets for each genus (Table 1).

Table 1. Target genes and their descriptions for bacterial load assay

Gene symbol	Description
pan-*Staphylococcus*	*Staphylococcus* spp. 16S rRNA consensus sequence
pan-*Streptococcus*	*Streptococcus* spp. 16S rRNA consensus sequence
pan-*Enterococcus*	*Enterococcus* spp. 16S rRNA consensus sequence
pan-*Enterobacteriaceae*	*Enterobacteriaceae* species 16S rRNA consensus sequences
pan-*Pseudomonas*	*Pseudomonas* spp. 16S rRNA consensus sequence
SPIKE	Artificial sequence for assay control

Results

Of these, 37 (18.5%) of 200 specimens had no detectable bacterial signal, but 163 (81.5%) specimens had evidence of bacterial colonization or co-infection, with 138 evidently polymicrobial and 25 evidently monobacterial in nature (9 streptococcal co-infection and 16 enterococcal co-infection) (Table 2, Appendix B).

In a total of 200 putative co-infection samples, *Enterococcus* spp. was apparently present in 139 samples (69.5%), being the dominant signal (any other signal was ≤10%) from 106 samples. *Streptococcus* spp. was next most commonly indicated with 137 positive results (68.5%), but was the dominant signal in 19 of 137. *Pseudomonas* spp. was not commonly identified with 17 positive results (9.7%), and being the dominant signal in only 1 of 17 (Table 3; Appendix B).

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Table 2. Bacterial colonization or co-infection in patients with severe influenza

No bacteria detected	Bacteria detected		TOTAL
37 (18.5%)	163 (81.5%)		200 (100%)
	Monobacterial	More than one bacterial type indicated	
	25 (12.5%)	138 (69%)	
	Streptococcus spp.	*Enterococcus* spp.	
	9 (4.5%)	16 (8%)	

Comparation of specimens from upper and lower respiratory tract

45 out of 200 clinical specimens were not clearly identified by their origin, but 34 were identified lower respiratory tract specimens (eg. ETT aspirate, tracheal aspirate, bronchial washing). *Streptococcus* spp. is the leading cause of co-infection in the lower respiratory tract (19 positive results-19/44:55.9%). However, one of 19 positive signals for *Streptococcus* spp. appeared as the dominant signal and *Enterococcus* spp. (18 positive results- 18/34:52.9%) gave the highest number of dominant signals (12/34) (Appendix B). *Enterococcus* spp. was most commonly detected in upper respiratory tract specimens (92 positive results- 76%) and it was detected as the strongest signal in 73 samples of these 92 (Table 4 and Appendix B). In all cases described as the 'dominant' signal, there was no other that was >10% in intensity normalized.

In total, 85 of 110 positive signals for Enterococci appeared as the dominant signal. Of the 110 samples in which Enterococci were detected, 81 also yielded a Streptococcal signal (73.6%) which was much weaker, and was in all cases <10% of that for *Enterococcus* spp., suggesting some cross-reactivity. There were only 3 samples in which Streptococci was the dominant signal. There were 9 samples which the *Streptococcus* signal was strong and unique (Appendix B).

Table 3. The number and percentage of bacterial types identified in patients with influenza (n=200) and the number and percentage in which it was the dominant signal

Signals*	*Staphylococcus* spp.	*Streptococcus* spp.	*Enterococcus* spp.	*Enterobacteriaceae* species	*Pseudomonas* spp.
any	66/200 (33%)	137/200 (68.5%)	139/200 (69.5%)	45/200 (22.5%)	17/200 (8.5%)
dominant	31/66 (46.9%)	19/137 (13.8%)	106/139 (76.2%)	6/45 (13.3%)	1/17 (5.8%)

*37/200 (18.5%) had no bacterial signal detected

Table 4. The number and percentage of bacterial types identified in patients with influenza (n=200) and the number and percentage in which it was the dominant signal

	No	*Staphylococcus* spp.	*Streptococcus* spp.	*Enterococcus* spp.	*Enterobacteriaceae* species	*Pseudomonas* spp.
LRT						
any	**34**[a]	15/34 (44.1%)	19/34 (55.9%)	18/34 (52.9%)	10/34 (29.4%)	4/34 (11.7%)
dominant		10/15 (66.6%)	1/19 (5.2%)	12/18 (66.6%)	1/10 (10%)	0/4 (0%)
URT						
any	**121**[b]	40/121 (33.1%)	88/121 (72.7%)	92/121 (76%)	26/121 (21.5%)	9/121 (7.4%)
dominant		15/40 (37.5%)	12/88 (13.6%)	73/92 (79.3%)	6/26 (23.1%)	0/9 (0%)
Total	**155**	55	110	110	36	13

[a]10 of 34 samples have no bacterial signal, b15 of 121 samples have no bacterial signal

Discussion

In earlier studies, bacterial co-infection was detected in between 4 and 28% of influenza cases [16,17,20-23]. *S. pneumoniae* is the major cause of bacterial co-infection in lung tissue, complicating approximately 13% of total cases of 2009 Pandemic Influenza A (H1N1) in US and 9.6% of influenza cases in the patients admitted to ICU in Spain [20,22]. According to Lee et al., *S. pneumoniae* is the leading cause of bacterial co infection, infecting 17% of H1N1/09 influenza patients who died in New York [22]. After S. pneumoniae co-infection, *S. pyogenes* has been reported as the second most important cause of co-infection, followed by *S. aureus, S. mitis* and *H.influenzae* [20,23].

In this study, 4 genera including *Staphylococcus* spp., *Streptococcus* spp., *Enterococcus* spp. and *Pseudomonas* spp. and a family, *Enterobacteriaceae* species, were investigated by using Bacterial load MT-PCR assay. The Bacterial load assay was performed on the NucliSENS® easyMAG® extractor and is expected to contain total nucleic acid. No special efforts were made to preserve RNA and it is not likely that mRNA survived other parts of the handling processes. *Enterococcus* spp. is the most commonly detected genus, being found in 79.4%. *Streptococcus* spp. was just as common (78.3%) and this was followed by *Staphylococcus* spp. (37.7%), *Enterobacteriaceae* species (25.7%) and *Pseudomonas* spp. (25.7%). The high rate of *Enterococcus spp*. might be due to its ability to survive the antibiotic treatment used for severe pneumonia (e.g. ceftriaxone), contamination, and/or cross reaction in the 16S rRNA gene between *Enterococcus* spp. and *Streptococcus* spp. [24]. *Enterococcus* spp. appear to be the most commonly encountered co-pathogen in severe influenza during the 2009 Australian influenza epidemic. Ceftriaxone is the recommended

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treatment for severe CAP [25,26]. Ceftriaxone is not active against *Enterococcus* spp. and can be expected to select for it as a colonizer of the respiratory tract. This assay does not distinguish between colonization and infection, however, it may be that surrogate markers of inflammation are crucial (e.g. cytokine assays, Gram-stain or other markers of neutrophil counts).

When comparing the number of strains that cause co-infection, there is a minor difference between the rate of strains identified in the lower respiratory tract and upper respiratory tract. The major genera and family identified were *Streptococcus* spp. (55.9%); followed by *Enterococcus* spp. (52.9%), *Staphylococcus spp*. (44.1%), *Enterobacteriaceae* species (29.4%) and *Pseudomonas* spp. (11.7%). There were dominant signals from *Enterococcus* spp. in the samples identified as BAL or other suitable deep specimens (n=34). Although *Streptococcus* spp. appeared in 19 of the lower respiratory tract samples, *Enteococcus* spp. has more primary signals (12 of 18 positive signals) than *Streptococcus* spp. (1 of 19 positive signals), and followed by *Staphylococcus* spp. (10 of 15 positive signals). This may be explained as a result of cross-reaction between the genera, and it would be important to try and obtain more specific identification (e.g. *S. pneumoniae*) in future studies, using specific targets (e.g. *lytA, ply*). It may be that true co infection (e.g. by *S. pneumoniae*) are more likely to be represented by dominant signals in the lower respiratory tract, but this is yet to be tested.

Cross reaction between *Staphylococcus* spp., *Streptococcus* spp., *Enterococcus* spp., *Enterobacteriaceae* species and *Pseudomonas* spp. might occur due to the similarity between 16S rRNA gene sequences. Levels of relatedness at nucleic acid sequence are high for *Streptococcus* spp. and *Enterococcus* spp., and this appears to be reflected to some extent in the results. In relation to this, there are several potential consensus regions for cross reactivity in the 16S rRNA gene between *Enterobacteriaceae* species and *Pseudomonas* spp. particularly including the regions between 313-370 bp., 760-824 bp., 879-965 bp., 1371-1429 bp., (Figure 1). The relatedness at nucleic acid sequence level is 100% for all these regions.

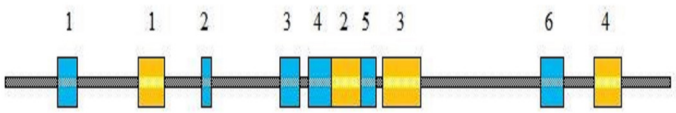

Figure 1. Potential regions for cross reaction and primer design in 16S rRNA gene aligned sequences of *Pseudomonas* spp. and *Enterobacteriaceae* species (Position 1 refers to position 1 of accession number NC_002695). *Blue boxes* (Potential regions for primer design 64.4%, 34.8%, 64.4%, 75.9 %, 50% and 50% identity, respectively)= 1/123-167 bp., 2/457-479 bp., 3/639-683 bp., 4/706-759 bp., 5/825-860 bp., 6/1245-1296 bp., respectively. *Yellow boxes* (Potential regions for cross reaction 100% identity)= 1/313-370 bp., 2/760-824 bp, 3/879-965 bp., 4/1371-1429 bp., respectively

On the other hand, there are several regions in 16 S rRNA gene sequences, with sufficient variation between *Enterobacteriaceae* species and *Pseudomonas* spp. to allow primer design and avoid cross-reaction including the regions between 123-167 bp., 457-479 bp., 639-683 bp., 706-759 bp., 825-860 bp., 1245-1296 bp. (Figure 1). Levels of relatedness at nucleic acid sequence level are 64.4%, 34.8%, 64.4%, 75.9%, 50% and 50% identity, respectively.

The art of primer design in multiplexed assays of this type is to identify regions that are sufficiently distinct between genera but highly conserved within them. The actual sequence of the primers used in the multiplex PCR-reverse line blot (mPCR-RLB) assays described are commercial-in-confidence but appear to have struck this balance reasonable well. Future work would logically be to confirm/subtype each positive signal with more specific PCR and sequencing, but that was beyond the scope of this work.

Sample contamination in the laboratory is uncommon in automated "closed" systems (MT-PCR/ BACTEC FX) and is unlikely to explain. However, antibiotic resistance in bacterial pathogens has been a growing problem in the last decade [27-31]. Moreover, antibiotics might not sterilize the respiratory tract of patients, with even relatively susceptible pathogens growing in endotracheal tubes and pooled oropharyngeal secretions into which bacterial penetration is minimal. Many such respiratory tract contaminants are routinely dismissed when cultured in the laboratory from specimens of this type and go unreported. An unbiased nucleic acid-based method such as we employed identified these readily.

Bacterial co-infection is a major determinant of the outcome of influenza patients as it is associated with increased morbidity and mortality [21,22]. In contrast to a number of previous studies, this work shows that *Enterococcus* spp. is the dominant member of the respiratory tract facultative/aerobic microflora in patients admitted to ICU with influenza. *Streptococcus* spp. and *Staphylococcus* spp. were next in importance numerically but are much more likely to include truly pathogenic species (eg. *S. pneumoniae, S. aureus*). It may be that the *Enterooccus* spp. signal is related to the colonization of airways in antibiotic-treated incubated patients, and it would be useful to examine a set of deep specimens known to have been collected on first intubation and without prior antibiotics. Although a quantitative approach is feasible, these specimens vary greatly in their original quality and subsequent handling. However, a combination of greater specificity (e.g. to species level) and quantitation would answer many of the questions about the significance of these signals. Even with all these limitations, this is a useful proof-of- principle.

Conclusion

As a result of this study, a very high rate of bacterial identification (163/200-81.5%) was observed in influenza patients. *Enterococcus* spp. was the predominant bacterial pathogen identified in patients with influenza. In contrast to Streptococci reports dominating the lower respiratory tract, Enterococci is the dominant bacteria in the upper respiratory tract of patients treated for severe pneumonia in this study. In addition, although

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there was no significant difference, parallel to the literature, Streptococci appeared to be more dominant than other genera in the lower respiratory tract.

Conflict of interests
The authors declare that there is no conflict of interest in the study.

Financial Disclosure
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Ethical approval
Ethics approval was not required for this study.

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