Efficacy of clarithromycin-naproxen-oseltamivir combination in the treatment of patients hospitalized for influenza A(H3N2) infection: an open-label, randomized controlled, phase 2b/3 trial

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Summary

Background

Influenza causes excessive hospitalizations and deaths. Single agent treatment with oseltamivir in severe influenza might be insufficient. The study assessed the efficacy and safety of oseltamivir-clarithromycin-naproxen combination for treatment of serious influenza.

Methods

From February to April 2015, we conducted a prospective open-label randomized-controlled trial. Adult patients hospitalized for A(H3N2) influenza were randomly assigned to a 2-day combination of clarithromycin 500mg, naproxen 200mg and oseltamivir 75mg twice daily, followed by 3 days of oseltamivir; or oseltamivir 75mg twice daily without placebos for 5 days as control (1:1). The primary end-point was 30-day mortality. The secondary end-points were 90-day mortality, serial nasopharyngeal-aspirate (NPA) virus titer, percentage of neuraminidase inhibitor resistant A(H3N2) virus (NIRV) quasispecies by pyrosequencing, pneumonia-severity-index (PSI), and duration of hospital-stay.

Results

Among the 217 influenza A(H3N2) patients enrolled, 107 were randomly assigned to the combination treatment. The median age was 80 years and 56% were men. Adverse events were uncommon. Ten patients succumbed during the 30-day follow-up. The combination treatment was associated with lower 30-day mortality (P=0.01), less frequent ICU/HDU admission (P<0.001), and shorter hospital-stay (P<0.0001). The virus titer, PSI (day 1-3;P<0.01), and NPA specimens with NIRV quasispecies ≥5%

(day 1-2;*P*<0.01) were significantly lower in the combination treatment group. Multivariate analysis showed that combination treatment was the only independent factor associated with lower 30-day mortality (odds-ratio:0.06; 95%,confidence-interval, 0.004-0.94;*P*=0.04).

Conclusions

Combination treatment reduced both 30- and 90-day mortality and length of hospital-stay. Further study on the antiviral and immunomodulatory effects of this combination treatment for severe influenza is warranted.

Clinical trial registered with www.isrctn.com/ISRCTN11273879

Introduction

Seasonal influenza is the most important respiratory virus infection leading to hospitalization and death especially amongst elderly or individuals with chronic illness. The World Health Organization estimates that seasonal influenza causes 250,000-500,000 deaths worldwide each year[1]. During the 2014-2015 season, the antigenically drifted A/Switzerland/9715293/2013-like-H3N2 virus caused major outbreaks globally, resulting in increased morbidity and mortality.[2] Apart from the seasonal influenza, sporadic avian influenza can cause even higher mortality.[3-5] Treatment and prophylaxis of influenza is currently limited to the neuraminidase inhibitors. Nevertheless, patients with severe influenza often present beyond 48 hours from symptom onset, rendering neuraminidase treatment less effective.

Antiviral combinations with different modes of action are used for treating severe influenza infection.[6] *In-vitro* and animal studies have shown that two clinically available drugs, clarithromycin and naproxen, both possess antiviral activity.[7-9] In addition, macrolides have effects on the host response to influenza virus infection.[8,9] Clarithromycin treatment can hasten the resolution of symptoms in paediatric influenza patients.[10] Naproxen has been shown to reduce weight loss and pulmonary hemorrhage in mice infected with influenza A virus.[7] Here, we aim to assess the safety and clinical efficacy of a triple combination of clarithromycin-naproxen-oseltamivir by comparing it with oseltamivir treatment alone for severe influenza infection.

Methods

Study Design

This was a phase 2b/3, single-center open-label clinical trial with randomized treatment group assignments. Clarithromycin-naproxen-oseltamivir combination, was compared to oseltamivir alone as control in patients hospitalized for A(H3N2) influenza with pneumonia. The study protocol was approved by the institutional review board of the University of Hong Kong (ISRCTN:11273879). All patients provided written informed consent before randomization.

The study was conducted in the Queen Mary Hospital, Hong Kong between February and April 2015. The inclusion criteria were patients ≥18 years of age, auditory temperature ≥38°C plus one of the following symptoms (cough, sputum production, sore-throat, rhinorrhea, myalgia, headache or fatigue) upon admission, symptom duration ≤72 hours, laboratory-confirmed A(H3N2) influenza, radiological changes of pulmonary infiltrate by chest-radiography (CXR) or computerized-tomography (CT), and clinically required hospitalization. Initiation of antiviral treatment had to be commenced within 24 hours after admission. Patients with history of allergy to study medications, or creatinine clearance <30mL/min were excluded. All enrolled patients were given ventilatory support in case of respiratory failure.

Randomization and Intervention

Recruited patients were assigned into one of two groups by simple randomization without stratification: the study group, a triple combination of clarithromycin 500mg, naproxen 200mg and oseltamivir 75mg twice daily for 2 days, followed by 3 days of oseltamivir 75mg twice daily; or the control group, oseltamivir 75mg twice daily for 5

days (ratio 1:1). All patients received 5 days of oral amoxicillin-clavulanate 1g twice daily for empirical treatment of acute community-acquired pneumonia, and esomeprazole 20mg daily for prevention of stress- or nonsteroidal-anti-inflammatory drug (NSAID)-induced gastropathy. Patients were assigned to serial number by the study-coordinator. Each serial number was linked to a computer-generated randomization list assigning the antiviral treatment regimens. The study medications were dispensed by the hospital pharmacy and then to the patients by nurses who did not know the treatment assignment of the study patients. Enrolled patients could not differentiate their regimen from the appearance of tablets as all packings were removed. All patients were followed-up for 90 days after antiviral treatment.

Outcome Measures

The primary outcome was 30-day mortality and the secondary outcomes were 90-day mortality, serial changes in the nasopharyngeal aspirate (NPA) viral titer, percentage change of neuraminidase inhibitor-resistant A(H3N2) virus (NIRV) quasispecies, the pneumonia-severity-index (PSI) from day 1-4 after antiviral treatment, and length of hospitalization. The PSI was determined daily for all recruited patients from admission till discharge, transfer to a convalescent hospital or death (Supplement). Arterial blood-gas was measured in patients who required respiratory support.

Clinical, Virological and Radiological Assessment

Clinical findings, laboratory investigation results, admission to intensive-care-unit (ICU)/ high-dependency-unit (HDU) and requirement of ventilator support, were prospectively entered into a predesigned database. The initial diagnosis of A(H3N2) influenza virus infection was confirmed when the M and H3 genes were tested

positive by RT-PCR, and NxTAG™ Respiratory Pathogen Panel was also used to detect atypical agents including *Mycoplasma pneumoniae*, *Chlamydophilia pneumoniae* and *Legionella pneumophila* in the NPA specimens taken on admission. Subsequent virus titer was determined using quantitative RT-PCR and all laboratory procedures were performed as previously described (Supplement).[11] NPA specimens were collected daily if possible from admission till discharge, transfer to convalescent hospitals or death. Percentage of NIRV quasispecies including E119V, R292K and N294S mutants were determined by pyrosequencing analysis in any NPA specimens containing a viral titer of >1000 copies/mL, a level sufficient for accurate pyrosequencing analysis (Supplement). We performed bacteriological studies on blood, urine and sputum or endotracheal aspirates when clinically indicated.

Statistics

We calculated that we would require 93 patients per group in order to detect a statistically significant 30-day mortality difference between the two treatment groups (two-sided alpha p=0.05, 80%-power). We based our calculation on a previous study in ICU influenza patients, which showed an 18% difference in 14-day mortality in patients treated with combination-therapy (17%) vs. oseltamivir alone (35%).[12] The protocol proposed recruiting at least 100 subjects per group to allow for a 7% drop-out rate due to adverse effects or premature termination.

Clinical and virological characteristics were compared. Fisher's-exact-test and χ^2-test were used for categorical variables where appropriate, whereas Mann-Whitney-*U*-test was used for continuous variables, including comparison for the percentage of NIRV quasispecies. Significant factors by univariate analysis (p<0.10) were further assessed

by the multivariate analysis by Cox-regression to identify the independent risk factors for the 30-day mortality. Sensitivity analysis of the results of the multivariate analysis were assessed by excluding patients with multiple comorbidities and Charlson-comorbidity-index >3. Log-rank test was used to compare the survival between the two groups. The viral titer and PSI was compared by linear regression. SPSS 21.0 for Windows (SPSS Inc.,IBM) was used for statistical computation. *P*-value <0.05 represents significant difference.

Results

Between February and April 2015, 334 hospitalized patients with A(H3N2) infection were initially screened (Figure-1). Two hundred and seventeen patients were enrolled after virological confirmation by RT-PCR and radiological confirmation of pulmonary infiltrate. All patients had CXR-confirmed pulmonary infiltrates or consolidation, and 14 patients also had CT-confirmed pulmonary infiltrates. Of these, 107 (49.3%) were randomly assigned to receive the triple combination treatment. All recruited patients completed the study and their outcomes were analyzed by intention-to-treat. There was no difference in patients' baseline characteristics between the two groups (Table-1). Two patients from the combination treatment developed a rise in serum creatinine to 120μmol/L and 132μmol/L respectively from baseline, three days after treatment. Creatinine level returned to the admission levels of 102μmol/L and 106μmol/L (within the normal-range), respectively, two days after completing naproxen and clarithromycin. No patients developed gastrointestinal side effects during the study period.

The combination treatment was associated with lower 30-day (*P*=0.01) (Figure-2) and 90-day mortality (*P*=0.01), less frequent ICU/HDU admission after hospitalization (*P*=0.009), and shorter acute hospital stay (*P*<0.0001) (Table-1). During the 30-day follow-up period, ten patients died, of whom one (0.9%) was from the combination treatment group and nine (8.2%) were from the control group. The results of the univariate and multivariate analyses (Table-2) demonstrated that the combination treatment was the only independent factor associated with lower 30-day mortality (odds-ratio:0.06; 95% confidence-interval, 0.004-0.94;*P*=0.04). A sensitivity analysis that excluded patients with Charlson-comorbidity-index >3 supported the multivariate

analysis finding (odds-ratio:0.01; 95% confidence-interval, 0.001-0.94;*P*=0.04). Similarly, separate sensitivity analyses that excluded patients with cerebrovascular disease (p=0.042), malignancy (p=0.022) or impaired admission renal function (creatinine >106μmol/L for male and >97μmol/L for female) (p=0.048) supported the multivariate analysis results.

There was no difference in the baseline virus titer between the two groups. (Table-1, Figure-3). The reduction in virus titer was significantly higher in the combination treatment group when compared with the oseltamivir group between day 0 and 1 (-1.45 vs. -0.27 $\log_{10}$ M-gene RNA copies/ml;*P*<0.0001). Similarly, the reduction in PSI was significantly higher in the combination treatment group when compared with the oseltamivir group between day 0 and 1 (-14.52 vs. -5.26;*P*<0.0001) (Figure-4).

Pyrosequencing analysis of 72 patients with serial NPA samples containing sufficient viral titer demonstrated a detectable percentage of NIRV quasispecies at baseline (median;IQR), E119V: 3% (2-4%) and N294S: 3% (2-3%), with no difference between the two groups (Figure-5). The percentages of patients with ≥5% NIRV quasispecies detected were significantly lower on day 1 (*P*=0.003) and day 2 (*P*=0.007) in the combination treatment group and 75% of the patients who died had ≥5% NIRV quasispecies detected on day 3.

Bacterial co-infections upon admission were uncommon with no difference between the two groups (Table-2). None of the patient was diagnosed to have an atypical bacterial infection. Six patients from each group developed nosocomial infections.

Discussion

Here we report the first prospective open-label randomized-controlled trial using a triple combination of clarithromycin-naproxen-oseltamivir as antiviral treatment of influenza A(H3N2) in hospitalized elderly patients with pulmonary infiltrates on CXR. Multivariate analysis showed that the combination treatment was the only independent factor associated with lower 30-day mortality. These findings were associated with the significantly faster reductions in virus load and PSI, and fewer patients with ≥5% NIRV quasispecies detected.

Previous experimental studies showed that macrolides and NSAIDs might lower host proinflammatory cytokine production and modulate leukocyte recruitment to tissue sites.[13] Treatment with erythromycin resulted in increased survival of H2N2-infected mice from 14 to 57%.[14] Celecoxib, a COX-2 inhibitor when combined with zanamivir and mesalazine improved survival of H5N1-infected mice from 13.3%-53.3% compared to zanamivir alone.[15] In our study, combination treatment suppressed virus replication effectively within 24 hours. Each of these three drugs acts on different stages of the virus life cycle. Clarithromycin may reduce virus attachment to host cell surface by downregulating the host cell expression of α2,6-linked sialic acid receptor on the cell surface via inhibition of NF-κB.[16] Clarithromycin also inhibit the acidification of endosomes, interfering the uncoating and fusion to host cell membrane.[16] Naproxen inhibits the virus replication by interfering with the binding of virus RNA to nucleoprotein.[7] Oseltamivir acts at the last step by inhibiting the release of progeny virus from the host cell surface.[17] Outside the host cell, the antiviral combination can inhibit virus entry into cells. Oseltamivir prevents the virus from reaching the host cell by affecting its trafficking through the sialic acid rich mucus

layer in the airway by inhibiting the virus neuraminidase activity.[18] Clarithromycin enhances the secretion of specific mucosal IgA against influenza virus.[8,9] The concentration of clarithromycin in the epithelial lining fluid exceeds that of the concentration required for the inhibition of influenza virus in human airway epithelial cells.[16,19] Furthermore, the peak serum concentration of naproxen exceeds that of the EC_{50} in MDCK cells.[7] Therefore, the lower virus titer and the better outcome in the triple combination may be related to the direct antiviral activity of clarithromycin and naproxen, in addition to their known anti-inflammatory and immunomodulatory effects.

We limited the combination treatment period to two days to minimize the potential side effects associated with NSAID and macrolide. Systematic review has demonstrated that even short-term use of NSAIDs for 72 hours can impair renal function and cause bleeding peptic ulcers, which was minimized in our study by concurrent treatment with esomeprazole.[20] Furthermore, we previously showed that the influenza virus titer is highest on presentation and the reduction in virus titer is very slow despite oseltamivir treatment.[11,21,22] Therefore early reduction of virus titer by using three drugs in the first two days would be most beneficial. Clarithromycin reduces the metabolism rate of esomeprazole by affecting the CYP2C pathway.[23] Nevertheless, no patient in our study reported adverse events due to drug-drug interaction.

Neuraminidase inhibitor treatment may fail for late presenters, immunocompromised hosts or complicated with pneumonia.[6] Thus single agent treatment is unlikely to work in severe cases during outbreaks of antigenically drifted or shifted influenza, and

avian influenza infection associated with the emergence of antiviral resistant quasispecies.[6] Combination of antivirals with different modes of action might offer additive/synergistic actions that limit the development of resistant mutants.[6] Previously, we demonstrated that the combination of oseltamivir with A(H1N1)pdm09 convalescent plasma or hyperimmune-globulin, containing neutralizing antibody reduced respiratory tract virus replication, serum cytokine/chemokine responses and mortality in severe A(H1N1)pdm09 infection within the first week of treatment.[24,25] However, such treatment is limited by the practical limitations of obtaining convalescent plasma, preparation time and cost.[26] A multicenter retrospective study demonstrated that amantadine-ribavirin-oseltamivir might reduce mortality in ICU patients with severe A(H1N1)pdm09 infection when compared with oseltamivir monotherapy.[12] In outpatients, results with clarithromycin added to neuraminidase inhibitors have been inconsistent. One Japanese study did not find significant effect of adding clarithromycin to neuraminidase inhibitor,[27] whereas another non-randomized study on oseltamivir-azithromycin combination showed earlier fever resolution in non-pneumonic patients.[28]

This is the first large study to analyze the serial changes in NIRV quasispecies by pyrosequencing before and after antiviral treatment. These NIRV mutants including E119V, R292K and N294S are responsible for increasing the 50% inhibitory concentrations (IC_{50}) of oseltamivir by >100-fold.[29,30] We previously reported oseltamivir resistant quasispecies H274Y in an A(H1N1)pdm09 patient with no underlying disease even before any antiviral treatment was given.[31] Similarly, in severe or fatal A(H1N1)pdm09 influenza, D225G quasispecies of the haemagglutinin gene was more frequently detected in the lower respiratory tract and blood of infected

patients.[32,33] Another study reported that 5% of A(H1N1)pdm09 virus positive clinical samples contained H275Y mutant during oseltamivir treatment.[34] Therefore, our finding of a high percentage of low level NIRV mutant quasispecies at baseline is not surprising because A(H3N2) viruses are generally genetically unstable and intrinsically associated with high frequency of mutation.[35] Nevertheless, we found that triple antiviral combination successfully suppressed the percentage of NIRV quasispecies 24 hours after treatment, and this was associated with rapid reduction of virus load and clinical recovery.

Our study had several limitations. We could not entirely exclude the added antibacterial benefits of beta-lactam treatment; combined with a macrolide it might have minimized the effect bacterial co-infection has on virus replication. In addition, the relative therapeutic contribution of clarithromycin and naproxen remains unknown, and the two agents might have added to the risk of side-effects. Though PSI has been used and validated as a tool to identify patients at risk of complications and death from community-acquired pneumonia, we adopted the PSI and its components as markers for response to antiviral therapy, and this needs further validation. Patients enrolled in the combination group presented 1-day later than the monotherapy group, which might have put this group at some disadvantage. We only enrolled patients who presented within 72 hours from symptom onset, so the validity and generalizability of this study need to be further tested in larger studies that include immunocompromised hosts and late presenters. Additional monitoring of patients' cytokine/chemokine profiles would allow investigators to further define the immunomodulatory effects of these agents. In our study, patients refused to provide nasopharyngeal samples once they became asymptomatic, hence the rapid decrease in the number of samples after

the first day. Moreover, pyrosequencing may not be accurate when the quasispecies represent <5% of the total virus population.[36]

Conclusions

The triple combination clarithromycin-naproxen-oseltamivir treatment reduced mortality, hospital stay, subsequent ICU/HDU admission, PSI, serial virus titer and NIRV quasispecies without increased adverse effects.

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Table 1. Demographics, Clinical features, Laboratory, Chest radiographic findings, Progress, Complications and Outcome of the 217 Patients*

	Oseltamivir/ Clarithromycin/ Naproxen (n=107)	Oseltamivir (n=110)	*P* value
Demographics			
Age in years, median (IQR)	80 (72-85)	81.5 (71-87.3)	0.27
Male sex (%)	63 (58.9)	53 (48.2)	0.11
Smoker (current or ex-smoker) – no.(%)	30 (28)	24 (21.8)	0.29
Elderly home resident - no. (%)	24 (22.4)	34 (30.9)	0.18
Previous neuraminidase inhibitors - no. (%)	0 (0)	0 (0)	na
Influenza vaccination (2014/5 season)	15 (14)	10 (9.1)	0.26
PPV-23 vaccination (within 5 years)	16 (15)	23 (20.9)	0.25
Past Medical History - no. (%)			
Good past health	49 (45.8)	49 (44.5)	0.85
Charlson comorbidity index >3	12 (11.2)	16 (14.5)	0.46
Cardiovascular disease	25 (23.4)	25 (22.7)	0.91
Pulmonary disease	19 (17.8)	14 (12.7)	0.30
Cerebrovascular disease	18 (16.8)	23 (20.9)	0.44
Hepatitis B carrier	3 (2.8)	1 (0.9)	0.30
Renal disease	3 (2.8)	4 (3.6)	0.73
Malignancy	12 (11.2)	15 (13.6)	0.59

Presenting symptoms - no· (%)			
Fever	111 (100)	110 (100)	na
Cough	84 (78.5)	81 (73.6)	0.40
Sputum	69 (64.5)	65 (59.1)	0.41
Rhinorrhea	32 (29.9)	25 (22.7)	0.23
Sore throat	9 (8.4)	9 (8.2)	0.95
Chills	10 (9.3)	9 (8.2)	0.76
Wheezing	10 (9.3)	11 (10)	0.87
Headache	5 (4.7)	3 (2.7)	0.44
Dizziness	12 (11.2)	9 (8.2)	0.45
Dyspnea	48 (44.9)	57 (51.8)	0.31
Pleuritic chest pain	13 (12.1)	11 (10)	0.63
Vomiting	11 (10.3)	13 (11.8)	0.74
Diarrhea	5 (4.7)	5 (4.5)	0.96
Initial physical examination findings			
Altered mental status – no· (%)	18 (16.8)	19 (17.3)	0.93
Oxygen saturation in room air (%)	96 (94-99)	97 (95-99)	0.11
Systolic blood pressure, median (IQR), mmHg	151 (135-169)	149.5 (133-167)	0.65
Pulse rate, median (IQR), /min	94 (81-108)	93 (80-109)	0.97
Respiratory rate, median (IQR), /min	18 (16-20)	18 (16-20)	0.78
Temperature, median (IQR), °C	38.3(37.6-38.9)	38.0(37.2-38.7)	0.06
Initial laboratory findings – median (IQR)			
Total white blood cell (x 10^9/L)	7.9 (5.8-10.2)	7 (6.0-10.7)	0.64
Neutrophil (x 10^9/L)	6.0 (4.1-8.3)	5.8 (4.5-8.6)	0.95
Lymphocyte (x 10^9/L),	0.9 (0.6-1.3)	0.9 (0.5-1.3)	0.76

Hemoglobin (g/dL)	11.9 (10-13.5)	12 (10·5-13.2)	0.63
Hematocrit	0.35 (0.3-0.4)	0.36 (0.32-0.4)	0.43
Alanine transaminase (IU/L)	20 (15-27)	19.5 (14-32)	0.82
Aspartate transaminase (IU/L)	29 (23-40)	31.5 (23.8-44)	0.31
Alkaline phosphatase (IU/L)	71 (54-89)	75 (57.5-93)	0.38
Sodium (mmol/L)	137 (133-140)	137(135-140)	0.07
Creatinine (μmol/L)	83 (69-99)	86 (66-112.3)	0.35
Urea (mmol/L)	5.6 (4.4-7.2)	5·6 (4·4-9.8)	0.40
Glucose (mmol/L)	6.3 (5.0-7.6)	6 (5.0-7.6)	0.86
Arterial pH (67 patients)	7.44 (7.40-7.48)	7.40 (7.40-7.45)	0.32
Arterial PO_2 (kPa) (67 patients)	10.6 (8.8-13.2)	12.2 (9.8-17.8)	0.16
Initial radiological findings – no. (%)			
Infiltrate	96 (89.7)	97 (88.2)	0.80
Right lower zone	37	41	
Right upper zone	2	1	
Left lower zone	41	39	
Left upper zone	5	4	
Diffuse	13	17	
Consolidation	9 (8.4)	8 (7.3)	0.80
Pleural effusion	2 (1.9)	5 (4.5)	0.27
Baseline viral titer, mean (SEM), log_{10} M gene RNA copies/ml	6.86 (0.19)	6.79 (0.19)	0.78
Baseline PSI, mean (SEM)	95.11 (2.33)	96.65 (2.14)	0.74
Days of symptoms before starting antiviral treatment, median (IQR)	2 (1-3)	1 (1-3)	0.17
Respiratory support upon admission –			

no. (%)			
Oxygen	42 (39.2)	36 (32.7)	0.32
Mechanical ventilation	2 (1.8)	1 (0.9)	0.55
BiPAP	7 (6.5)	8 (7.3)	0.83
CPAP	6 (5.6)	4 (3.6)	0.49
Respiratory support during hospitalization – no. (%)			
Mechanical ventilation	2 (1.9)	6 (5.5)	0.16
BiPAP	10 (9.3)	18 (16.4)	0.12
CPAP	9 (8.4)	17 (15.5)	0.11
Complications – no. (%)			
Bacterial co-infection upon presentation†	5 (4.7)	3 (2.7)	0.45
Ventilator associated pneumonia	0 (0)	1 (0.9)	0.32
Nosocomial infection‡	6 (5.6)	6 (5.5)	0.96
Admission to ICU during hospitalization no. (%)	2 (1.9)	7 (6.4)	0.10
Admission to HDU during hospitalization– no. (%)	17 (15.9)	34 (28.2)	**0.009**
Days of hospitalization, median (IQR)			
Acute hospital	2 (1-3)	3 (2-4)	**<0.0001**
Convalescent hospital	0 (0-4)	0 (0-7)	0.96
Readmission ≤30 days from discharge – no. (%)	20 (18.7)	19 (17.3)	0.79
Mortality – no. (%)			
30-day§	1 (0.9)	9 (8.2)	**0.01**
90-day	2 (1.9)	11 (10)	**0.01**

*PPV-23: 23-valent polysaccharide pneumococcal vaccine; IQR: interquartile range; SEM: standard error of mean; PSI: pneumonia severity index; BiPAP: bilevel positive airway pressure; CPAP: continuous positive airway pressure; ICU: intensive care unit; HDU: high dependency unit; †*Streptococcus pneumoniae* in blood culture (n=1 in combination-treatment-group); Methicillin sensitive *Staphylococcus aureus* in blood culture (n=1 in the control-group). *Haemophilus influenzae* in sputum culture of 5 patients (n=3 in combination-treatment-group and n=2 in control-group); Significant bacteriuria for *Streptococcus agalactiae* (n=1 in combination-treatment-group); ‡ *Pseudomonas aeruginosa* in sputum culture (n=5 in combination-treatment-group; n=4 in control-group); Endotracheal aspirate positive for mixed infection by methicillin resistant *Staphylococcus aureus*, *Klebsiella* spp, and *Pseudomonas aeruginosa* in control group (n=1); Positive urine culture was for *Enterococcus faecalis* (n=1 from each group). §One patient in the combination-treatment-group died of congestive heart failure (CHF). Nine patients in the control-group died, of which three patients died of chronic obstructive pulmonary disease exacerbation, three patients died of CHF, and three patients died of severe pneumonia. All patients succumbed received ventilatory support. Significant *P*-value <0.05 bolded.

Table 2. Univariate and Multivariate Analysis for Baseline Factors Associated With 30-day Mortality*

	Died (n=10)	Survived (n=207)	*P* value
Univariate analysis			
Demographics			
Age in years, median (IQR)	87.5(72.8-90.5)	80 (71-86)	0.15
Male sex (%)	8 (80)	108 (52.2)	**0.09**
Smoker (current or ex-smoker) – no.(%)	1 (10)	53 (25.6)	0.27
Elderly home resident - no. (%)	3 (30)	55 (26.6)	0.66
Influenza vaccination (2014/5 season)	1 (10)	24 (11.6)	0.88
PPV-23 vaccination (within 5 years)	2 (20)	37 (17.9)	0.86
Past Medical History - no. (%)			
Good past health	2 (20)	96 (46.4)	0.10
Charlson comorbidity index >3	1 (10)	27 (13)	0.78
Cardiovascular disease	3 (30)	47 (22.7)	0.59
Pulmonary disease	3 (30)	30 (14.5)	0.18
Cerebrovascular disease	5 (50)	36 (17.4)	**0.01**
Hepatitis B carrier	0 (0)	4 (1.9)	0.66
Renal disease	1 (10)	6 (2.9)	0.21
Malignancy	3 (30)	24 (11.6)	**0.09**
Presenting symptoms - no· (%)			
Fever	8 (80)	162 (78.3)	0.90
Cough	8 (80)	157 (75.8)	0.76
Sputum	7 (70)	127 (61.4)	0.58

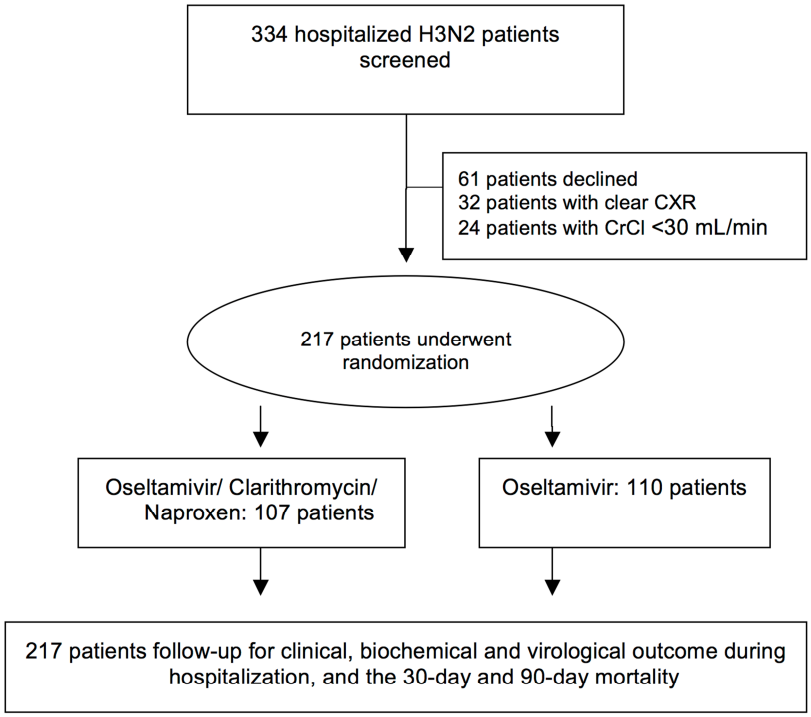
Rhinorrhea	0 (0)	57 (27.5)	**0.05**
Sore throat	0 (0)	18 (8.7)	0.33
Chills	1 (10)	18 (8.7)	0.89
Wheezing	0 (0)	21 (10.1)	0.29
Headache	0 (0)	8 (3.9)	0.53
Dizziness	1 (10)	20 (9.7)	0.97
Dyspnea	8 (80)	97 (46.9)	**0.04**
Pleuritic chest pain	2 (20)	22 (10.6)	0.36
Vomiting	2 (20)	22 (10.6)	0.36
Diarrhea	0 (0)	10 (4.8)	0.48
Initial physical examination findings			
Altered mental status – no· (%)	2 (20)	35 (16.9)	0.80
Oxygen saturation in room air (%)	95.5(91.8-98.3)	97 (95-99)	0.15
Systolic blood pressure, median (IQR), mmHg	138.5 (128.5-149.8)	151 (134-169)	**0.06**
Pulse rate, median (IQR), /min	94 (80-112.5)	94 (81-108)	0.80
Respiratory rate, median (IQR), /min	22 (16-28.5)	18 (16-20)	**0.06**
Temperature, median (IQR), °C	38.0(36.9-38.4)	38.2(37.5-38.8)	0.32
Initial laboratory findings – median (IQR)			
Total white blood cell (x 10^9/L)	8.3 (4.9-12.1)	7.3 (5.9-10.2)	0.69
Neutrophil (x 10^9/L)	7.5 (4.1-10.1)	5.8 (4.4-8.4)	0.43
Lymphocyte (x 10^9/L),	0.56(0.27-0.91)	0.9 (0.59-1.27)	**0.04**
Hemoglobin (g/dL)	12.4 (8.9-12.9)	11.9(10.1-13.4)	0.97
Hematocrit	0.37(0.33-0.39)	0.35 (0.31-0.4)	0.53
Alanine transaminase (IU/L)	18 (15.8-38.8)	20 (15-30)	0.77
Aspartate transaminase (IU/L)	36 (31-61.8)	30 (23-42)	0.12

Alkaline phosphatase (IU/L)	90.5(77.8-93.8)	73 (55-91)	**0.07**
Sodium (mmol/L)	139.5 (136.8-141.5)	137 (133-140)	**0.07**
Creatinine (μmol/L)	98 (89-111.8)	83 (68-101)	**0.03**
Urea (mmol/L)	8.6 (5-11)	5.6 (4.3-7.2)	**0.04**
Glucose (mmol/L)	6.8 (5-8.6)	6 (5-7.5)	0.75
Arterial pH (67 patients)	7.41(7.34-7.45)	7.41 (7.4-7.48)	0.55
Arterial PO_2 (kPa) (67 patients)	9.8 (7.05-10.9)	12.2 (9.5-15.8)	**0.06**
Initial radiological findings – no. (%)			
Infiltrate	10 (100)	183 (91.5)	0.34
Consolidation	0 (0)	17 (0.5)	0.34
Pleural effusion	0 (0)	7 (3.4)	0.55
Baseline viral titer, mean (SEM), log_{10} M gene RNA copies/ml	6.99 (0.57)	6.82 (0.14)	0.76
Baseline PSI, mean (SEM)	111.2 (4.26)	95.15 (1.62)	**0.02**
Days of symptoms before starting antiviral treatment, median (IQR)	2.5 (1-3)	2 (1-3)	0.40
Respiratory support upon admission – no. (%)			
Oxygen	2 (20)	12 (5.8)	**0.07**
Mechanical ventilation	0 (0)	3 (1.4)	0.70
BiPAP	3 (30)	12 (5.8)	**0.003**
CPAP	1 (10)	9 (4.3)	0.41
Admission to ICU/ HDU upon admission – no. (%)	3 (30)	22 (10.6)	**0.06**
Oseltamivir/ Clarithromycin/ Naproxen combination treatment	1 (10)	106 (51.3)	**0.01**

Multivariate analysis for independent risk factors of 30-day mortality	Odds ratio (95% confidence interval)	*P* value
Oseltamivir/ Clarithromycin/ Naproxen combination treatment	0.06 (0.004-0.94)	**0.04**

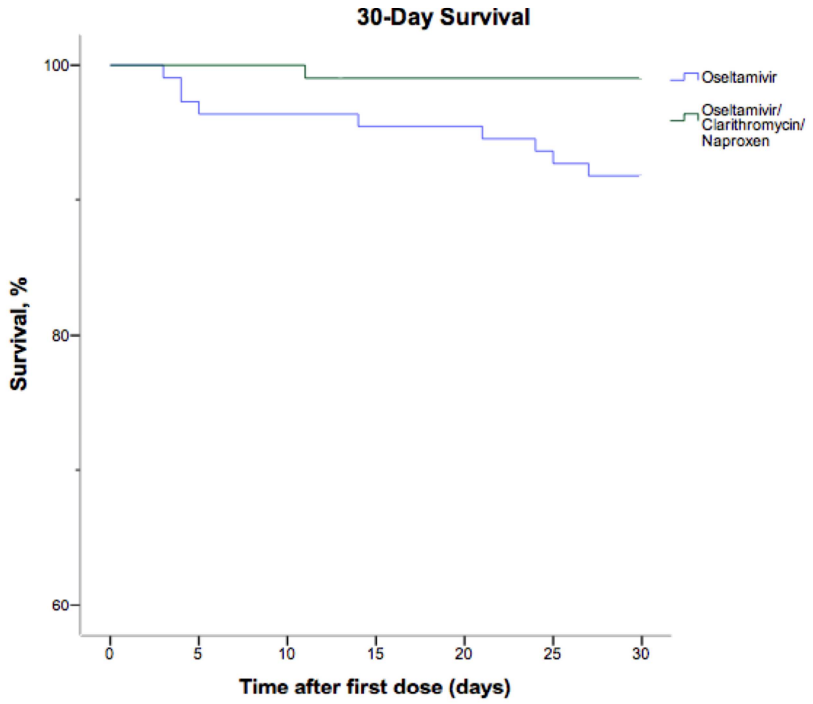
*PPV-23: 23-valent polysaccharide pneumococcal vaccine; IQR: interquartile range; CI·: confidence interval; BiPAP: bilevel positive airway pressure; CPAP: continuous positive airway pressure; SEM: standard error of mean; ICU: intensive care unit; HDU: high dependency unit; PSI: pneumonia severity index. Significant *P*-value <0.10 bolded (factors including sex, past medical history of cerebrovascular disease and malignancy, presenting symptoms of rhinorrhea and dyspnea, initial physical examination findings of systolic blood pressure and respiratory rate, initial median lymphocyte count, alkaline phosphatase, sodium, creatinine, urea, arterial PO_2, baseline PSI, respiratory support with O_2 and BiPAP, admission to ICU/HDU upon admission, oseltamivir/clarithromycin/naproxen combination treatment, from the univariate analysis were included in the multivariate analysis) .

Figure 1. Recruitment Flow-Chart Of The 217 Subjects



CXR: chest radiograph; CrCl: creatinine clearance

Figure 2. 30-day Survival in Patients Treated With Oseltamivir/ Clarithromycin/ Naproxen Combination and With Oseltamivir alone

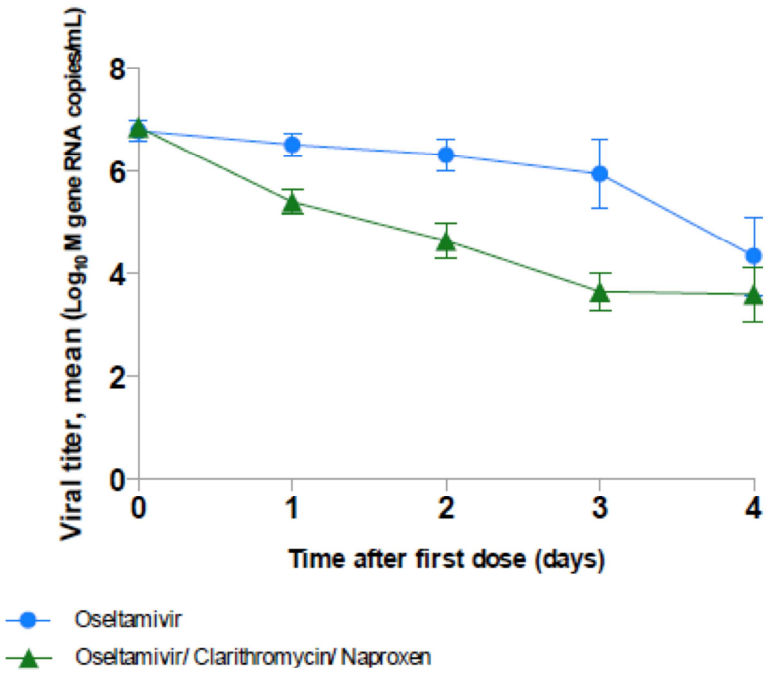


Days after first dose/ Number survived	0	5	10	15	20	25	30
Oseltamivir group (n)	110	106	106	105	105	102	101
Oseltamivir/ Clarithromycin/ Naproxen group (n)	107	107	107	106	106	106	106

Log-rank test: *P*=0.01

Figure 3. Profile Of Viral Titer After Treatment

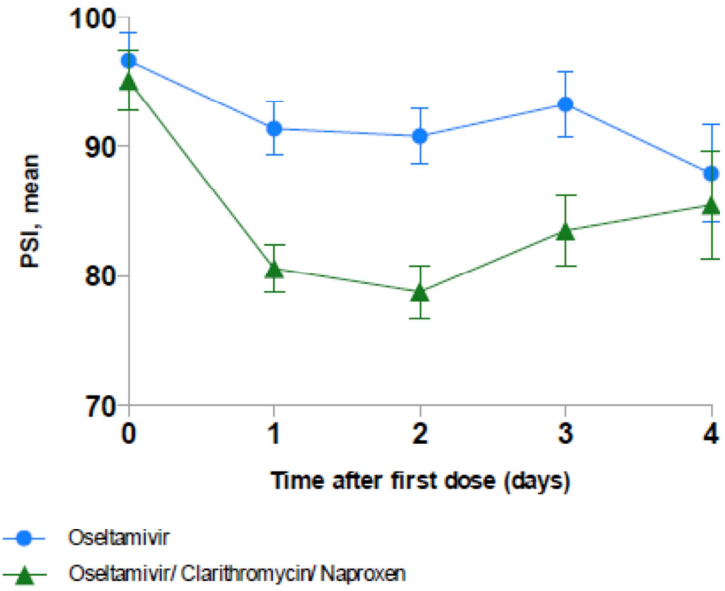
(Error-bars represent standard error of mean)



Days after first dose	0	1	2	3	4
Oseltamivir group (n)	110	80	31	9	5
Viral titer, mean (SEM), log_{10} M gene RNA copies/ml	6.79 (0.19)	6.52 (0.23)	6.32 (0.32)	5.95 (0.67)	4.34 (0.77)
Oseltamivir/ Clarithromycin/ Naproxen group (n)	107	87	42	13	10
Viral titer, mean (SEM), log_{10} M gene RNA copies/ml	6.86 (0.19)	5.41 (0.24)	4.65 (0.36)	3.65 (0.35)	3.60 (0.52)
P value	0.78	**0.001**	**0.002**	**0.006**	0.25

SEM: standard error of mean

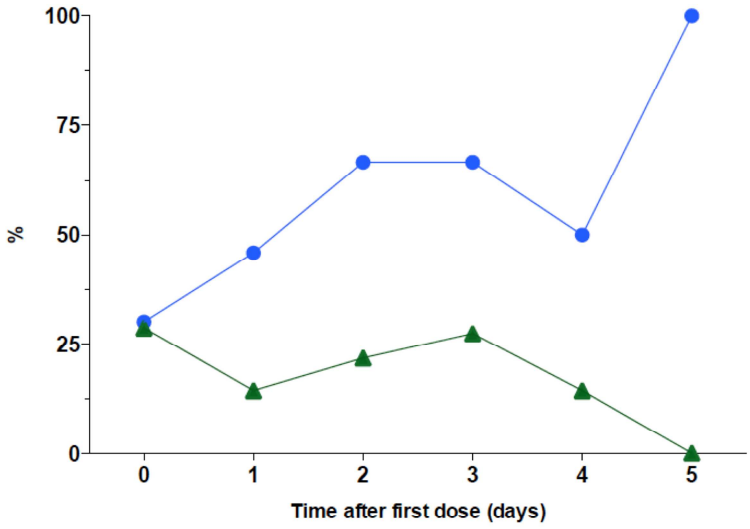
Figure 4. Profile of Pneumonia Severity Index After Treatment
(Error-bars represent standard error of mean)



Days after first dose	0	1	2	3	4
Oseltamivir group (n)	110	110	95	61	31
PSI, mean (SEM)	96.65 (2.14)	91.39 (2.05)	90.80 (2.09)	93.25 (2.57)	87.97 (3.70)
Oseltamivir/ Clarithromycin/ Naproxen group (n)	107	107	89	43	20
PSI, mean (SEM)	95.11 (2.33)	80.59 (1.82)	78.76 (2.03)	83.53 (2.70)	85.50 (4.16)
P value	0.74	**<0.0001**	**<0.0001**	**0.007**	0.58

PSI: pneumonia severity index
SEM: standard error of mean

Figure 5. Percentage Of Patients With ≥ 5% and ≥ 10% NIRV Quasispecies Detected



Days after first dose	0	1	2	3	4	5
Oseltamivir group (n)	37	37	16	6	4	1
≥5% NIRV quasispecies (%)	30	45.9	66.7	66.7	50	100
≥10% NIRV quasispecies (%)	5.4	13.5	2.7	0	0	0
Oseltamivir/ Clarithromycin/ Naproxen group (n)	35	34	21	9	8	2
≥5% NIRV quasispecies (%)	28.6	14.3	21.7	27.3	14.3	0
≥10% NIRV quasispecies (%)	5.9	0	0	0	0	0
P-value (≥5%)	0·91	**0·003**	**0·007**	0.15	0.62	0·33

Abbreviations list

WHO: world health organization

NSAID: non-steroidal anti-inflammatory drugs

NPA: nasopharyngeal aspirate

NIRV: neuraminidase inhibitor resistant A(H3N2) virus

PSI: pneumonia-severity-index

RT-PCR: reverse transcrpition polymerase chain reaction

ICU: intensive care unit

HDU: high dependency unit

BIPAP: bilevel-positive-airway-pressure

CPAP: continuous-positive-airway-pressure

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e-Appendix 1.

Supplementary method on pneumonia severity index

The pneumonia severity index (PSI) was determined daily for all recruited patients from admission till discharge, transfer to a convalescent hospital or death by the following formula accordingly (1): Age (years) + sex (if female -10) + nursing home resident (+10) + neoplastic disease (+30) + liver disease history (+20) + congestive heart failure history (+10) + cerebrovascular disease history (+10) + renal disease history (+10) + altered mental status (+20) + respiratory rate >30 breaths/minute (+20) + pulse rate ≥125 beats/minute (+10) + systolic blood pressure <90 mmHg (+20) + temperature <35°C or ≥40°C (+15) + arterial pH <7.35 (+30) + blood urea nitrogen ≥30 mg/dL or 10.7 mmol/L (+20) + sodium <130 mmol/L (+20) + glucose ≥14 mmol/L (+10) + haematrocrit <30% (+10) + partial pressure of arterial O_2 <60 mmHg (+10) + pleural effusion (+10).

Supplementary method on influenza A virus RT-PCR

The diagnosis of influenza A virus was performed by quantitative RT-PCR targeting the M gene as we previously described with modifications (2). Total nucleic acid was extracted directly from nasopharyngeal aspirate (NPA) specimens by using the NucliSens EasyMAG instrument (bioMerieux, Marcy-l'Étoile, France), according to the manufacturer's instructions. Briefly, 250 µL of an NPA was added to 2 mL of lysis buffer and the mixture was incubated for 10 min at room temperature. This was followed by automatic magnetic separation. Nucleic acid was recovered in 55 µL elution buffer. These total nucleic acid extracts were used for all subsequent tests described below. Real-time one-step RT-PCR assays were performed by using Invitrogen SuperScript III Platinum One-Step Quantitative Kit in a StepOnePlus instrument (Applied Biosystems, Foster City, CA). Briefly, 5 µL purified RNA was amplified in a 25 µL reaction containing 0.5 µL Superscript III Reverse Transcriptase/Platinum Taq DNA polymerase (Invitrogen, Carlsbad, California, USA), 0.05 µL ROX reference dye (25 mM), 12.5 µL of 2X reaction buffer, 800 nmol/L forward primer(5'-GACCRATCCTGTCACCTCTGAC-3'), 800 nmol/L reverse primer 5'-AGGGCATTYTGGACAAAKCGTCTA-3', and probe 200 nmol/L (FAM-5'-TGCAGTCCTCGCTCACTGGGCACG-3'-BHQ1). Reactions were first incubated at 50°C for 30 min, followed by 95°C for 2 min, and were then thermal cycled for 50 cycles (95°C for 15 s, 55°C for 30 s). For quantitative assay, a reference standard was prepared using pCRII-TOPO vector (Invitrogen, San Diego, CA) containing the corresponding target viral sequences. A series of 6 log 10 dilution equivalent to 1 x 10^1 to 1 x 10^6 copies per reaction were prepared to generate calibration curves and run in parallel with the test samples.

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Supplementary method on pyrosequencing analysis of neuraminidase inhibitor-resistant A(H3N2) virus (NIRV) quasispecies

Percentages of NIRV mutant quasispecies including E119V, R292K and N294S, were determined by pyrosequencing analysis as we previously described with modifications (3). Only NPA specimens positive for influenza A M gene by RT-PCR with a viral load of >1000 copies/mL and H3 gene positive by NxTAG™ Respiratory Panel are processed. Note that a surrogate viral load level using the M gene viral load at or above 1000 copies/mL is required for accurate pyrosequencing analysis. These total nucleic acid extracts from NPAs (as above) were amplified by RT-PCR and pyrosequenced with primers designed according the A(H3N2) virus neuraminidase (NA) gene sequence of A/Switzerland/9715293/2013 using PyroMark Assay Design 2.0. One step RT-PCR amplification was performed using PyroMark™ RT-PCR Kit (QIAGEN, Hilden, Germany) with two sets of primers: forward primer F1 (5'-CTATTCATTGAGGAGGGGAAAATC-3'); reverse biotinylated primer, R1 (5'-CCATGACCACCTTCTTCATTGTTA-biotin-3') for R292K-N294S; forward primer, F2 (5'-TTTGCACCTTTCTCTAAGGACAAT-3'); reverse biotinylated primer, R2 (5'-GAACACCCAACTCATTCATCAAT-3') for E119V. The RT-PCR was performed under the following conditions: 2.5 μL of nucleic acid extract, 5 μL of RT-PCR buffer (5X), 1 μL of dNTP mix (final: 400 μM each), 5 μL Q-solution (5X), 2.5 μL CoraLoad concentrate (10X), 1 μL of primer F/Primer R set (final 0.6 μM), 1 μL enzyme and 7 μL of nuclease free water. Total reaction volume was 25 μL. The completed mix was then transferred into a thermal cycler GeneAmp® PCR System 9700 (Applied Biosystems, Foster City, California, USA). Tempcycling conditions were as follows: 30 min at 50°C for reverse transcription and 15 min at 95°C for inactivation of reverse transcriptase and activation of HotStar Tag DNA Polymerase, followed by 45 cycles of 30 s at 94°C, 30 s at 60°C and 30 s at 72°C, then final extension 10 min at 72°C.

The resulting biotinylated PCR products were immobilized onto Streptavidin Sepharose High Performance beads (GE healthcare, Chicago, Illinois, USA) with pyroMark binding buffer and then purified using the PyroMark Q24 Vacuum Prep Workstation. Finally single-stranded DNA was then transferred to 20 μL PyroMark Advanced annealing buffer containing 0.375 μM sequencing primer targeting the region of interest (E119V: GGACATCTGGGTGACA; R292K-N294S: GGTGTCAGATGTGTCTGC). The mixture was loaded into PyroMark Q24 Advanced instrument (Qiagen, Hilden, Germany). The data was analysed by PyroMark Q24 Advanced software.

Three sequences of NA gene corresponding to the nucleotide position from 289 to 496 (208 bp) and 763 to 1043 (281 bp) were synthesised and then cloned into a plasmid (pIDTSMART-KAN, Integrated DNA Technologies, Iowa, USA). Such plasmids were used as controls for detection of mixed nucleotides (A or T), (G or A), (A or G) at codon 119, 292, 294 in NA respectively. Biotinylated DNA amplicons from the control plasmids were generated by using the primers for E119V, R292K and N294S. The DNA concentrations of the biotinylated PCR products were determined by Nanodrop ND-1000

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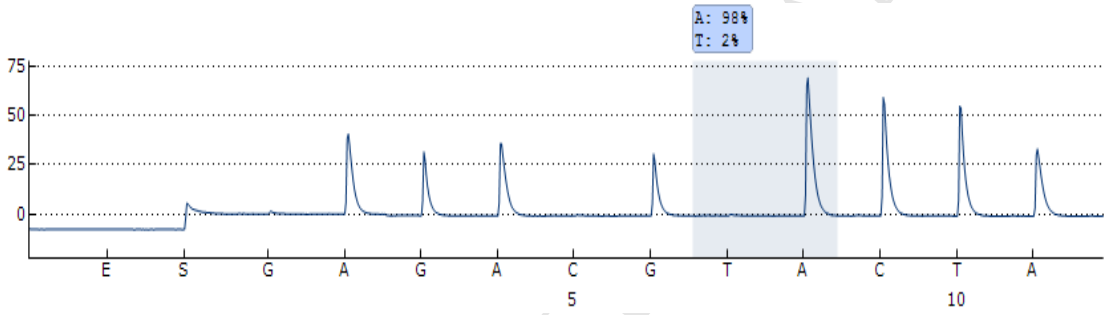
spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA) and mixed at different ratios for pyrosequencing and finally analysed by PyroMark Q24 Advanced software. All the primers for DNA amplification and sequencing primers were used as described above. Examples of the pyrosequencing read out (highlighted in blue) are shown.

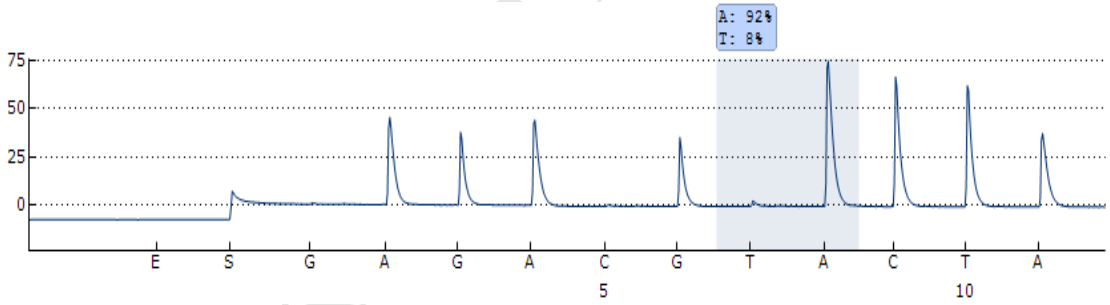
No.	Expected value	Observed value					
	Mutant (M) vs Wild (W)	E119V		R292K		N294S	
		M (GTA)	W (GAA)	M (AAA)	W (AGA)	M (AGC)	W (AAC)
1	0% vs 100%	2%	98%	0%	100%	2%	98%
2	8% vs 92%	8%	92%	8%	92%	11%	89%

E119V pyrosequencing read out AGAGA/TACCTTA

(1) M vs W = 0% vs 100%



(2) M vs W = 8% vs 92%



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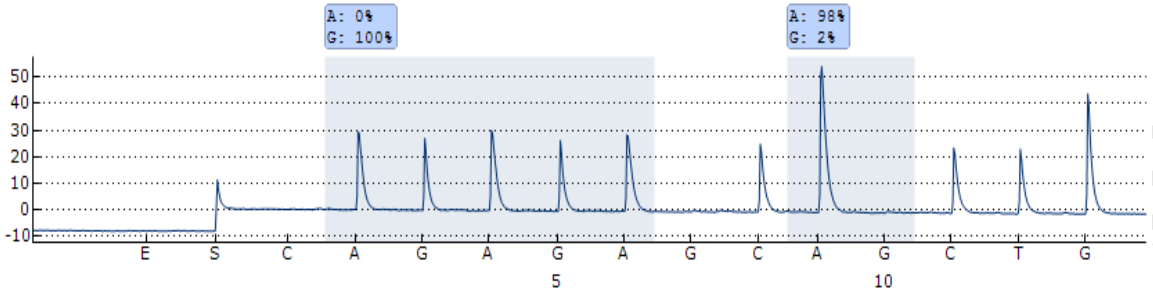
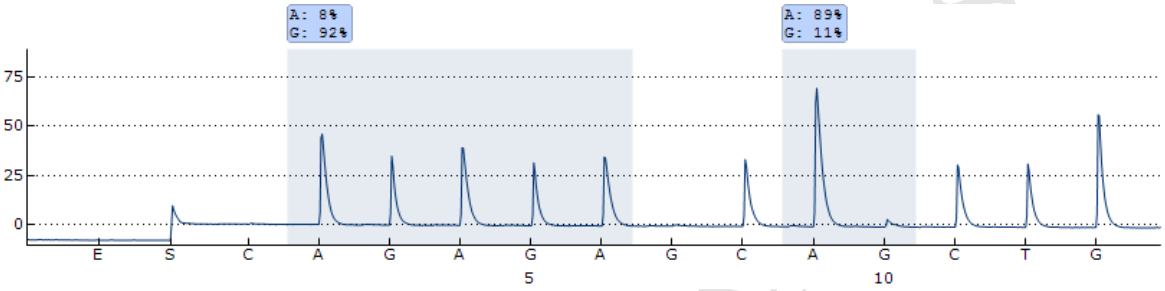
R292K_N294S pyrosequencing read out AG/AAGACAA/GCTG

(1) M vs W = 0% vs 100%



(2) M vs W = 8% vs 92%



Supplementary method on NxTAG™ Respiratory Panel

The NxTAG™ Respiratory Pathogen Panel (Luminex, Texas, USA) is a qualitative target probed based assay with internal control used for the detection of nucleic acids from multiple respiratory viruses and bacteria. A total 22 targets including Influenza A, Influenza A-H1 subtype, Influenza A-2009 H1N1 subtype, Influenza A-H3 subtype, Influenza B, Respiratory Syncytial Virus A, Respiratory Syncytial Virus B, Coronavirus 229E, Coronavirus OC43, Coronavirus NL63, Coronavirus HKU1, Human Metapneumovirus, Rhinovirus/Enterovirus, Adenovirus, Parainfluenzavirus 1, Parainfluenzavirus 2, Parainfluenzavirus 3, Parainfluenzavirus 4, Human Bocavirus, *Chlamydophila pneumoniae, Mycoplasma pneumoniae* and *Legionella pneumophila* are detected. Nucleic acid extraction was performed by using the NucliSens easyMAG instrument (bioMerieux,), according to the manufacturer's instructions. Briefly, 200µl of each NPA sample was spiked with 10 µl of MS2 (internal control) and then the mixture was added to 2 ml of lysis buffer and incubated for 10 min at room temperature. The lysed sample was then transferred to the well of a plastic vessel with 100µl of silica. This was followed by automatic magnetic separation. Nucleic acid was recovered in 110 µL elution buffer. The extract nucleic acid was stored at -70°C until testing with NxTAG RPP. Thirty-five microlitre of purified nucleic acid extract was added to strips sealed with foil, which contained lyophilised bead reagents. They were mixed to re-suspend the reaction reagents according to manufacturer instructions. The reaction was amplified via RT-PCR in a 96-well ABI 9700 thermal cycler, and the hybridised, tagged beads were then sorted and read on the MAGPIX® instrument. The signals were analysed using the SYNCT™ Software.

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