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Original Article

Higher mortality of severe influenza patients with probable aspergillosis than those with and without other coinfections

Yee-Huang Ku [a], Khee-Siang Chan [b], Chun-Chieh Yang [b], Che-Kim Tan [b], Yin-Ching Chuang [a], Wen-Liang Yu [b,c,*]

[a] *Division of Infectious Diseases, Department of Internal Medicine, Chi Mei Medical Center-Liouying, Tainan City, Taiwan*
[b] *Department of Intensive Care Medicine, Chi-Mei Medical Center, Tainan, Taiwan*
[c] *Department of Medicine, School of Medicine, College of Medicine, Taipei Medical University, Taipei, Taiwan*

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Background/Purpose: *Aspergillus*-associated infection might comprise up to 23–29% of severe influenza patients from the community throughout stay in an intensive care unit (ICU). In Taiwan, cases of severe influenza with aspergillosis are increasingly reported. Therefore, we describe the relative risk of mortality among severe influenza patients with aspergillosis and other coinfections compared to severe influenza patients without *Aspergillus* coinfections.
Methods: We retrospectively reviewed 124 adult patients with severe influenza in a tertiary medical center in southern Taiwan from January 2015 through March 2016. The definition of probable aspergillosis required abnormal radiological findings and positive *Aspergillus* galactomannan (GM) antigen and/or *Aspergillus* isolation.
Results: Probable aspergillosis (detected throughout the whole course) and other coinfections (only community-acquired) were diagnosed in 21 (17%) and 38 (31%) of all patients respectively. *Klebsiella pneumoniae* (36.8%), *Pseudomonas aeruginosa* (31.6%) and *Staphylococcus aureus* (31.6%) were the most frequent isolates of other coinfections. In-ICU mortality of *Aspergillus* group (66.7%) was significantly higher than other coinfections (23.7%, $p = 0.001$) or control group without coinfections (15.4%, $p < 0.001$), with significant odds ratios after adjusting for important variables. The factor of GM index $\geq$0.6 had a 19.82 (95% CI, 4.91 to 80.07, $p < 0.0001$) odds of expiring in an ICU among the *Aspergillus* group.

* Corresponding author. Department of Medical Research, Chi Mei Medical Center, No. 901 Zhonghua Road, Yongkang District, 710 Tainan City, Taiwan. Fax: +886 6 283 3351.
E-mail address: yuleon_md@yahoo.com.tw (W.-L. Yu).

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Conclusion: Dual *Aspergillus* and influenza infection is emerging in southern Taiwan. Meanwhile, community-acquired *P. aeruginosa* should be listed in the common copathogens with severe influenza. The 67% mortality linked to aspergillosis highlights the need for physicians to focus attention on patients with GM $\geq$ 0.6.

Introduction

Severe influenza infections are usually defined as cases requiring medical intensive care unit (ICU) admission. Invasive pulmonary aspergillosis (IPA) may occur in the setting of severe influenza even among immunocompetent hosts.[1,2] Such cases of a dual influenza and IPA coinfection have increasingly been reported since 2010. Among them, 65% of cases lacked classic immunosuppressive conditions at diagnosis,[1] which is essentially required as host factors (neutropenia, hematologic cancer and stem cell or bone marrow transplantation) according to the European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group and the National Institute of Allergy and Infectious Diseases Mycoses Study Group (EORTC/MSG) criteria.[3] Therefore, there is a need for newly revised diagnostic criteria of IPA, especially for severe influenza patients without host criteria of immunosuppression, or directly regarding severe influenza as a status of immunosuppression to fulfill the revised host criteria of EORTC/MSG for diagnosing "probable" IPA in the absence of histological confirmation.

According to EORTC/MSG criteria, the diagnosis of probable IPA requires a host factor, a typical radiological feature, and a mycological criterion like either *Aspergillus* culture or galactomannan (GM) antigen detected in serum or bronchoalveolar (BAL) fluid. Possible IPA indicates presence of host factors and clinical features but in the absence of or negative mycological criteria.[3] The second issue frequently raised for difficulty in diagnosis of IPA for the critically ill patients in ICU would be non-specific consolidation in mechanically ventilated patients and lack of radiological features of EORTC/MSG criteria in chest-computed tomographic (CT) findings. Therefore, for critically ill patients in ICU, a diagnostic algorithm was proposed for "IPA", which was less strict than EORTC/MSG criteria in a Belgium study.[4] For example, abnormal but non-specific chest X-ray imaging could be a radiological criteria and steroid treatment with a prednisone equivalent of >20 mg/d could be a host factor; whereas EORTC/MSG criteria require typical CT imaging and prolonged use of steroids with a prednisone equivalent of >0.3 mg/kg/d for >3 week respectively.[3,4] According to the ICU algorithm, the typical findings of cavity, halo sign or an air-crescent sign occurred in only 5% of critically ill patients with "IPA" (17 definite, 68 probable).[4]

In a prospective, multicenter cohort research of 220 patients hospitalized with severe presentation of pandemic (H1N1)v influenza A infection in the ICUs from the European Society of Intensive Care Medicine (ESICM) H1N1 registry before 11 February 2010,[5] hospital-acquired pneumonia (HAP) was clinically suspected in 79 patients (35.9%). Among them, *Aspergillus* spp. was the 5th top common pathogen and accounted for 4 (8.7%) of the 46 microbiologically documented patients. However, the study did not define IPA by the EORTC/MSG criteria. In the study, patients who received early corticosteroid therapy had HAP more frequently than patients who did not (26.2% versus 13.8%, $p < 0.05$). Adjusted Cox regression analysis identified that early use of corticosteroids was not significantly associated with mortality, but was still associated with an increased rate of HAP.[4]

Later, a large prospective, observational study was conducted from 2009 to 2015 in a large cohort of ICUs patients with influenza in Spain.[6] Community-acquired respiratory coinfection was defined as diagnosis within first 2 days of hospital admission. A total of 2901 ICU patients with influenza were enrolled. Overall, coinfection was diagnosed in 482 (16.6%) of patients. *Aspergillus* spp. was the 4th top common pathogen, accounting for 35 (7.3%) of patients with the community-acquired coinfection, comprising 2 definitive IPA, 25 probable IPA and 8 possible IPA, by applying "modified" EORTC/MSG criteria. In addition, *Aspergillus* spp. was an independent risk factor for ICU mortality ($p = 0.001$).[6] The study might highlight an important role of diagnosing IPA coinfection earlier in the course within 2 days among critically ill influenza patients.

With regard to the prevalence of IPA among the influenza patients in the ICUs, community-acquired and nosocomial IPA accounted for 1.2% (35/2901) and 1.8% (4/220) respectively.[5,6] In Belgium, Wauters and colleagues reported 9 (23%) IPA in 40 critically ill H1N1 patients, including 5 patients with proven disease and 4 ''probable'' infections, based on ''modified" EORTC criteria with broader definitions of risk factors.[7] For example, they enrolled Child C cirrhosis, human immunodeficiency virus (HIV) infection, cancer on therapy within recent 3 months and relatively shorter courses of steroid use as host factors.[8] In France, Guervilly and colleagues reported 5 (29.4%) patients with proven or probable IPA in a prospective cohort study of 17 H1N1 patients, based on the EORTC/MSG criteria. The mycological evidence included fungal culture and a novel diagnostic strategy of serum galactomannan and PCR for *Aspergillus* sp.[9] Therefore, the prevalence of IPA in critically ill influenza patients appears to be 15 times higher by applying broader definitions of host factors and/or mycological criteria.

In Taiwan, cases of IPA in association with severe influenza have been increasingly reported.[10–15] Yet, the relative risk of mortality associated with IPA in the influenza patients has

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not been investigated; therefore, we aimed to retrospectively review the 124 adult patients with severe influenza admitted to the 6 ICUs with a total of 96 ICU beds in a tertiary medical center in southern Taiwan, from January 1, 2015 through March 31, 2016. We hypothesized that severe influenza patients with IPA diagnosed during the whole course of episode had worse outcome than those with and without other community-acquired respiratory coinfections.

Methods

Diagnosis of influenza and definition of severe influenza

A confirmed influenza case was defined at least one positive assay for testing influenza, including rapid influenza diagnostic tests (RIDTs), real time polymerase chain reaction (PCR), viral isolation for specimens of nasopharyngeal swab and/or lower respiratory tract aspirates. Severe influenza was regarded as those influenza patients who were admitted to ICUs.

Galactomannan (GM) antigen assay

Aspergillus GM antigen was detected by Platelia *Aspergillus* Ag assay (Bio-Rad Laboratories, Marnes-La-Coquette, France) in serum or BAL fluid with a positive cut-off value of an optical density index $\geq$ 0.5. In a reference of diagnostic meta-analysis for thirteen studies with 1670 patients, a single positive test result of GM $\geq$0.5 had an estimated mean sensitivity, specificity, positive predictive value, and negative predictive value of 92%, 90%, 61%, and 98% respectively.[16]

Case definitions of IPA

Diagnosis of proven IPA was based on histological evidence on lung biopsy. IPA was considered a "probable" diagnosis for patients with severe influenza on based of the presence of acute pulmonary infiltrates of the lungs with positive determination of GM antigen in the serum or BAL fluid and/or *Aspergillus* isolates in the respiratory samples, but not necessarily needing the presence of immunocompromised disorder.[1,6–8] Only patients in whom IPA was proven or probable were enrolled in the study.

Definitions of community-acquired, nosocomial and late-onset diagnosis of IPA

Community-acquired IPA was considered when the serum or respiratory specimen collected within 48 h of hospitalization was positive for GM test or growth of *Aspergillus* sp. Nosocomial IPA was considered if initial GM test and *Aspergillus* culture were negative at initial examination, but became positive after 2 days of hospitalization. Other IPA cases without initial GM testing and fungal culture were regarded as "late-onset diagnosis", but not known as hospital-acquired cases. In that scenario, we could not exclude the possibility of delayed diagnosis of IPA, which might be acquired in the community.

Case definitions of non-*Aspergillus* other coinfection

Coinfection was defined by community-acquired respiratory copathogens identified within 2 days of hospital admission.[6] Nosocomial pneumonia would complicate the late course of influenza. We did not enroll nosocomial infection by non-*Aspergillus* other pathogens, because it would involve complicated issues of multiple resistant organisms and limited option of antimicrobial therapy. In this study, the second hypothesis was that classic respiratory coinfections in the community might predict worse outcome than for patients with influenza alone.

Data collection

The following data relevant to patient characteristics were collected: gender, age, comorbidities with immunodeficiency (as defined by EORTC/MSG criteria)[3] and with immunomodulatory disorders (such as chronic obstructive pulmonary disease, diabetes mellitus, solitary cancer, chemotherapy therapy within recent 3 months, end-stage renal disease, liver cirrhosis, autoimmune disorders, and HIV infection), types of influenza and oseltamivir treatment, results of chest X-ray and thoracic CT scan, antifungal therapy, treatment with antibiotics and corticosteroids (duration, daily and cumulative doses prior to GM testing or fungal culture). Acute Physiology and Chronic Health Evaluation II (APACHE II) score at admission, acute respiratory distress syndrome (ARDS) and need for invasive mechanical ventilation were assessed as the disease severity. Outcome was described as in-ICU mortality within the same hospital episode.

Statistical analysis

Firstly, the proportion of cases of IPA, non-*Aspergillus* other coinfection and controls (no coinfection) were determined. Proportions of patient characteristics were compared between each coinfection category and controls using the χ^2 test or Fisher exact test for categorical variables and Student's *t*-test for continuous variables, where appropriate. A two-tailed p value < 0.05 was considered statistically significant.

Odds ratio was used as a measure of relative risk of in-ICU mortality between each coinfection category and controls, with 95% confidence interval (CI) and *p*-values. Several important variables, including age, influenza (H1N1), comorbidity and disease severity (ARDS, APACHE II and ventilator use), were considered in the multivariable logistic regression model to estimate the adjusted odds ratio.

Determination of risk factors for false-positive GM testing and risk for IPA coinfection as well as comparison of difference in the chest X-ray patterns were assessed between IPA cases ($n = 21$) and subgroup of controls ($n = 29$), 24 of whom were time-matched by GM testing or fungal cultures within one month to those of IPA cases. The case—control matching ratios were 3:1 ($n = 1$), 1:1 ($n = 16$), 1:3 ($n = 3$), 1:4 ($n = 4$) and 5 controls with the timing of GM testing beyond one month to the cases. Risk factors for in-ICU mortality were evaluated within the IPA group.

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All statistics were performed using Stata version 12.1 (Stata Press, College Station, TX, USA).

Results

Patients

A total of 124 adult patients (>18 years old) with severe influenza were identified (Table 1). Positive RITD was found in 99 patients, PCR-H1N1 in 41 patients, PCR-H3N2 in 18 patients, and other influenza A in 28 patients. All patients received oseltamivir therapy for influenza. 77 patients (62%) were male and 81 patients (65%) had comorbidities with immunodeficiency or immunomodulatory diseases, including renal transplant recipient ($n = 2$), diabetes mellitus ($n = 51$), chronic obstructive pulmonary disease ($n = 22$), end-stage renal disease ($n = 14$), solitary cancers ($n = 13$), autoimmune disorders ($n = 5$), liver cirrhosis ($n = 4$), and HIV infection ($n = 2$). Multiple comorbidities could be found in a single patient. There was no evidence of neutropenia, hematological malignancy, stem cell transplant recipient and inherited severe immunodeficiency. Evaluation of disease severity included APACHE II (mean, 20.8), development of ARDS (72 patients, 58%) and use of invasive respiratory ventilator (93 patients, 75%).

Table 1 Demographic data, comorbidity, and disease severity for 124 influenza patients in the intensive care units with copathogens diagnosed or isolated in 59 patients including probable *Aspergillus* infection ($n = 21$) and other community-acquired coinfections ($n = 38$) compared to controls without coinfections ($n = 65$).

Co-pathogens	*n*	Man (*n*), p^a	Age (mean), p^b	Comorbidity (*n*), p^a	H1N1 (*n*), p^a	ARDS (*n*), p^a	APACHE II, p^b	MV (*n*), p^a
Total population	124	77 (62%)	65	81 (65%)	41 (33%)	72 (58%)	20.8	93 (75%)
No coinfection (controls as reference)	65	42	66	42	22	34	19.5	45
Probable *Aspergillus* infection	21	11, 0.316	63, 0.194	13, 0.822	8, 0.722	19, **0.002**	25.2, **0.008**	21, **0.002**
1. Mono *Aspergillus*	14	7, 0.307	61, 0.152	8, 0.599	7, 0.255	14, **<0.001**	26.1, **0.005**	14, **0.016**
Mixed with other pathogens	7	4, 0.698	65, 0.472	5, 0.719	1, 0.418	5, 0.442	23.3, 0.259	7, 0.179
2. *Aspergillus* diagnosed >48 h	16	8, 0.281	64, 0.632	10, 0.874	5, 0.844	**14**, **0.011**	22.8, 0.150	**16**, **0.009**
Community-acquired *Aspergillus*	5	3, 1.000	59, 0.312	3, 1.000	3, 0.341	5, 0.062	**32.8**, **<0.001**	5, 0.312
Non-*Aspergillus* other coinfections	38	24, 0.882	67, 0.351	26, 0.694	11, 0.607	19, 0.821	21.6, 0225	27, 1.000
1. *Klebsiella pneumoniae*	14	10, 0.761	67, 0.403	11, 0.367	5, 0.894	4, 0.122	20.7, 0.601	10, 1.000
Mono *K. pneumoniae*	9	7, 0.709	71, 0.180	7, 0.709	3, 1.000	3, 0.479	20.1, 0.826	5, 0.460
Mixed with other pathogen(s)	5	3, 1.000	60, 0.197	4, 0.654	2, 1.000	1, 0.357	21.8, 0.540	5, 0.312
2. *Pseudomonas aeruginosa*	12	7, 0.678	68, 0.330	7, 0.678	4, 1.000	7, 0.701	22.1, 0.305	9, 1.000
Mono *P. aeruginosa*	6	4, 0.920	70, 0.229	4, 1.000	2, 1.000	2, 0.429	20.0, 0.886	4, 1.000
Mixed with other pathogen(s)	6	3, 0.662	65, 0.456	3, 0.662	2, 1.000	5, 0.213	24.2, 0.168	5, 0.662
3. *Staphylococcus aureus*	12	7, 0.678	62, 0.229	9, 0.741	5, 0.602	6, 0.883	23.7, 0.125	10, 0.491
Mono *S. aureus* (MRSA, $n = 2$)	2	0, N/A	64, 0.437	2, N/A	2, N/A	1, 1.000	29.0, 0.098	2, N/A
Mixed with others (MRSA, $n = 7$)	10	7, 1.000	62, 0.228	7, 1.000	3, 1.000	5, 0.892	22.6, 0.282	8, 0.714
4. *Haemophilus influenzae*[c]	5	3, 1.000	61, 0.261	2, 0.353	3, 0.341	2, 0.669	21.1, 0.652	4, 1.000
5. *Streptococcus pneumoniae*[c]	4	4, N/A	60, 0.210	4, N/A	0, N/A	3, 0.618	33.3, **0.001**	4, N/A
6. *Escherichia coli*[c]	2	1, 1.0000	68, 0.415	2, N/A	0, N/A	1, 1.000	20.0, 0.469	2, N/A
7. *Legionella pneumophila*[c]	2	2, N/A	53, 0.112	1, 1.000	0, N/A	2, 0.495	24.5, 0.398	1, 0.532
8. Non-tuberculous mycobacteria[c]	2	0, N/A	82, 0.071	0, N/A	0, N/A	0, N/A	28.0, 0.127	2, N/A
9. *Mycoplasma pneumoniae*	1	0, N/A	57, N/A	0, N/A	0, N/A	1, N/A	16.0, N/A	1, N/A
10. *Cryptococcus* spp.[c]	1	1, N/A	58, N/A	1, N/A	0, N/A	1, N/A	7.0, N/A	1, N/A

Note. *n*: number of patients; H1N1: influenza A (H1N1)pdm virus infection; ARDS: Acute respiratory distress syndrome; APACHE II: Acute Physiology and Chronic Health Evaluation II score (mean); MV: intubation with mechanical ventilation; MRSA: methicillin-resistant *Staphylococcus aureus*; N/A: not applicable for data such as zero appeared in a variable.
Bold highlights a significant *p* value of less than 0.05.
Superscript: [a]Comparison for proportion of man, comorbidity, H1N1, ARDS or ventilator use in patients of each coinfection category to controls (no coinfection); [b]Comparison for distribution of age or APACHE II score in patients of each coinfection category to controls (no coinfection); [c]Might coexist with other pathogen(s).

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IPA-influenza dual infection

Aspergillus isolation was found in eight patients, and determination of GM was positive in 20 patients (including 7 patients with positive *Aspergillus* isolation). One patient with *Aspergillus* isolation was not tested for GM assay. Only one patient undertook BAL GM assay due to poor response to voriconazole therapy. The initial serum GM index was 0.81, but the follow-up BAL GM index 8 days later revealed more than 9.02. No cases received lung biopsy. In total, "probable" IPA was diagnosed in 21 patients throughout the ICU course, accounting for 16.9% of severe influenza patients. However, only one case of renal transplant recipient receiving T-cell immunosuppressants and prolonged steroid use could be regarded as probable IPA, based on EORTC/MSG criteria.[3] There were 5 cases defined as community-acquired IPA and 16 patients were "late-onset diagnosis" of IPA. The former had significantly higher APACHE II score, and the latter had significantly higher ARDS and ventilator use than controls without coinfections. There were no cases classified as nosocomial IPA. Overall, patients with IPA did not have significantly different demographics and comorbidities, but had higher disease severity (APACHE II, ARDS, and ventilator use) than did the controls (Table 1).

Rates of non-*Aspergillus* other coinfections

Coinfection, defined as being community-acquired, occurred in 38 (30.6%) patients (Table 1). *Klebsiella pneumoniae* (36.8%) was the bacterium most often identified, followed by *Staphylococcus aureus* (31.6%) and *Pseudomonas aeruginosa* (31.6%). Methicillin-resistant *S. aureus* (MRSA, $n = 9$) was more prevalent than methicillin-sensitive *S. aureus* ($n = 3$). Patients with other coinfections did not have significantly different demographics, comorbidities and disease severity than did the controls, except for *Streptococcus pneumoniae* with a higher mean APACHE II score than for the controls (Table 1).

Clinical outcomes

Overall ICU mortality occurred in 33 (26.6%) of 124 patients with severe influenza (Table 2). The in-ICU mortality of controls (10/65, 15.4%) was significantly lower than that of all patients with coinfection (23/59, 39.0%, $p = 0.003$) and those with IPA (14/21, 66.7%, $p < 0.0001$), even adjusted by age, influenza (H1N1), comorbidity and disease severity (APACHE II scores, ARDS and ventilator use), but was not significantly different to patients with other coinfection (9/38, 23.7%, $p = 0.295$). Specifically, *P. aeruginosa* reached significant contribution to fatal outcome in the ICUs in comparison to non-coinfected controls ($p = 0.035$) with an odds ratio of 3.93 (CI 95% 1.04–14.87, $p = 0.044$), which became statistically insignificant when adjusted for comorbidity and disease severity. Meanwhile, patients with *K. pneumoniae* mixed with other pathogens had a 44.48 (CI 95% 1.06–1866.40, $p = 0.047$) odds of dying while adjusting for all other variables in comparison to patients without coinfections (Table 2). Therefore, odds of in-ICU mortality by *P. aeruginosa* and *K. pneumoniae* coinfections were confounded by comorbidity factors and disease severity. Furthermore, influenza patients with IPA had a significantly higher in-ICU mortality than those with other coinfections (66.7% vs. 23.7%, $p = 0.001$), except for *P. aeruginosa* coinfection (66.7% vs. 41.2%, $p = 0.162$).

Risk factors for false-positive GM

Detailed analysis of antibiotic use prior to the day of GM testing was evaluated for difference in proportion between GM-positive and GM-negative patients with time-matched controls (Table 3). However, no factors with statistical significance could be detected, including piperacillin-tazobactam use and for different kinds of duration (>3 days, >5 days or >7 days), use of cephalosporins, and/or use of carbapenems.

Risk factors for acquisition of IPA-influenza dual infection

The use of steroids prior to ICU admission, prior to GM testing, >3 days duration before GM testing, and use of prednisolone equivalent cumulative doses of mean value, >300 mg, >200 mg, >0.3 mg/kg/day for more than 3 weeks or >20 mg/day did not significantly differ between influenza patients with and without IPA (time-matched controls). In addition, immunomodulatory comorbidities in severe influenza patients with IPA and controls are shown in Table 4. Overall, only one factor of cancer disorder was significantly associated with IPA ($p = 0.029$).

Risk factors for ICU mortality in influenza patients with IPA

The in-ICU mortality of severe influenza patients with IPA was 66.7% (14/21). The mortality did not significantly differ between 8 patients with *Aspergillus* isolation and 13 patients with GM-positive only (Table 5). The influenza H1N1, other coinfections, comorbidities, use of steroids with variable cumulative dose, disease severity and voriconazole therapy were not significantly associated with in-ICU mortality. Nonetheless, two risk factors of GM index ≥0.6 ($p = 0.025$) and age <65 years ($p = 0.025$) were significantly correlated with in-ICU mortality. The multivariable model for odds ratio further revealed that GM index ≥0.6 had a 19.82 (95% CI, 4.91 to 80.07, $p < 0.0001$) odds of dying, implying an independent risk factor for in-ICU mortality in the setting of severe influenza patients with IPA.

Classification of radiological findings

Results of chest X-ray patterns were mainly described as peribronchial infiltrations, bilateral lung infiltrates, multiple patches with necrotizing processes, diffuse ground-glass appearance, extensive consolidation on one side of the lung fields, extensive consolidation on bilateral lung fields, diffuse air-space acute respiratory distress syndrome (ARDS) pattern, nodular lesion or patches, as well as EORTC/MSG clinical criteria, including halo sign, air-crescent sign, and cavitation. There were 8 patterns of chest X-ray film findings detected on the day of testing GM

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Table 2 Risk of mortality in the intensive care units for the pathogens isolated in 59 of 124 critically ill influenza patients with all *Aspergillus* infection ($n = 21$) and non-*Aspergillus* coinfection ($n = 38$) compared to controls ($n = 65$).

Co-pathogen	*n*	Mortality	p^a	p^b	Odds ratio (OR, 95% CI, p^c)	aOR (95% CI, p^d)	aOR (95% CI, p^e)
Controls: no coinfection	65	10 (15.4%)		(reference)[b]	1.00 (reference)	1.00 (reference)	1.00 (reference)
Aspergillus infection and other coinfections	59	23 (39.0%)		**0.003**	**3.51 (1.50−8.25, 0.004)**	**3.43 (1.25−9.40, 0.017)**	**3.02 (1.16−7.85, 0.023)**
A: *Aspergillus* infection	21	14 (66.7%)	(reference)[f]	**<0.001**	**11.00 (3.55 −34.06, <0.001)**	**13.11 (2.64 −65.12, 0.002)**	**6.29 (1.75 −22.56, 0.005)**
1. Mono *Aspergillus*	14	9 (64.3%)	1.000	**0.001**	**9.90 (2.74 −35.74, <0.001)**	**7.51 (1.32 −42.64, 0.023)**	**4.18 (1.01 −17.23, 0.048)**
Mixed with other pathogens	7	5 (71.4%)		**0.004**	**13.75 (2.34 −80.95, 0.004)**	**160.66 (5.50 −4693.39, 0.003)**	**24.88 (2.03 −304.78, 0.012)**
2. *Aspergillus* diagnosed >48 h of admission	16	9 (56.3%)	N/A	**0.001**	**7.07(2.14−23.38, 0.001)**	**4.37 (1.16 −16.47, 0.030)**	**8.89 (1.71 −46.08, 0.010)**
Community-acquired *Aspergillus*	5	5 (100%)		N/A	N/A	N/A	N/A
B: Non-*Aspergillus* coinfection (community-acquired)	38	9 (23.7%)	**0.001**[f]	0.295	1.71 (0.62−4.67, 0.298)	1.91 (0.55−6.67, 0.313)	1.71 (0.55−5.34, 0.358)
1. *Klebsiella pneumoniae*	14	2 (14.3%)	**0.005**[f]	1.000	0.92 (0.18−4.73, 0.917)	2.14 (0.21−21.47, 0.518)	1.25 (0.19−8.35, 0.815)
Mono *K. pneumoniae*	9	0 (0)	N/A	N/A	N/A	N/A	N/A
Mixed with other pathogens	5	2 (40.0%)		0.201	3.67 (0.54−24.81, 0.183)	**44.48 (1.06 −1866.40, 0.047)**	10.35(0.63 −170.70, 0.102)
2. *Pseudomonas aeruginosa*	12	5 (41.2%)	0.162[f]	**0.035**	**3.93 (1.04 −14.87, 0.044)**	3.36 (0.54−20.79, 0.193)	3.99 (0.83−19.15, 0.084)
Mono *P. aeruginosa*	6	2 (33.3%)	1.000	0.266	2.75 (0.44−17.08, 0.278)	4.00 (0.28−56.75, 0.306)	4.42 (0.52−37.79, 0.175)
Mixed with other pathogens	6	3 (50.0%)		0.070	5.50 (0.97−31.22, 0.054)	3.96 (0.42−37.67, 0.231)	3.55(0.50−25.12, 0.204)
3. *Staphylococcus aureus*	12	3 (25%)		0.416	1.83 (0.42−7.98, 0.419)	0.68 (0.04−11.25, 0.790)	0.73 (0.08−6.62, 0.781)
Mono *S. aureus* (MRSA, 2)	2	1 (50%)	0.455	0.304	5.50 (0.32−95.33, 0.241)	0.84 (0.001 −469.66, 0.955)	4.12 (0.27 −630.24, 0.074)
Mixed with other pathogens (MRSA, 7)	10	2 (20%)		0.657	1.38 (0.25−7.45, 0.712)	0.69 (0.04−12.44, 0.804)	0.61 (0.06−6.29, 0.681)
4. *Haemophilus influenzae*[g]	5	2 (40%)		0.201	3.67 (0.54−24.81, 0.183)	8.70 (0.48 −159.40, 0.145)	4.52 (0.38−53.76, 0.233)
5. *Streptococcus pneumoniae*[g]	4	2 (50.0%)		0.137	5.50 (0.69−43.70, 0.107)	2.74 (0.06 −133.57, 0.611)	0.92 (0.04−20.14, 0.957)
6. *Legionella pneumophila*[g]	2	1 (50%)		0.304	5.50 (0.38−95.33, 0.242)	N/A	4.10 (0.20−83.29, 0.359)
7. *Escherichia coli*[g]	2	0 (0)		N/A	N/A	N/A	N/A
8. Non-tuberculous mycobacteria[g]	2	0 (0)		N/A	N/A	N/A	N/A
9. *Mycoplasma pneumoniae*	1	0 (0)		N/A	N/A	N/A	N/A
10. *Cryptococcus* spp.[g]	1	1 (100%)		N/A	N/A	N/A	N/A

Note. N/A: not applicable for data such as zero appeared in a variable.
MRSA: Methicillin-resistant *Staphylococcus aureus*.
Bold highlights a significant p value of less than 0.05.
Superscript: [a]Comparison for proportion of ICU mortality between categories in the block; [b]Comparison to patients without coinfections for proportion of ICU mortality; [c]Comparison to patients without coinfections for odds ratio of ICU mortality; [d]P value for adjusted odds ratio (OR) by age, influenza (H1N1), comorbidity, ARDS, APACHE II and ventilator use; [e]P value for adjusted odds ratio (OR) by ARDS, APACHE II and ventilator use; [f]Comparison to all *Aspergillus* infections for proportion of ICU mortality; [g]Might coexist with other pathogen(s).

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Table 3 Assessing risk factors for false-positive galactomannan (GM) assay of severe influenza patients in intensive care units (ICU).

Variables risk for false-positive GM testing (*n*)	GM-positive ($n = 20$)[a]	GM-negative ($n = 29$)	*p*
Use of piperacillin-tazobactam before the day of GM testing	13	19	0.970
Use of piperacillin-tazobactam ≥3 days before the day of GM testing	8	9	0.517
Use of piperacillin-tazobactam ≥5 days before the day of GM testing	5	4	0.456
Use of piperacillin-tazobactam ≥7 days before the day of GM testing	2	4	1.000
Use of cephalosporins (cefuroxime, ceftazidime, ceftriaxone, cefpirome or cefoperazone-sulbactam) before the day of GM testing	4	10	0.345
Use of carbapenems (imipenem, meropenem or doripenem) before the day of GM testing	4	4	0.697
Use of β-lactams (cephalosporin and/or carbapenem) before the day of GM testing	7	14	0.394

[a] One IPA patient was identified by *Aspergillus* culture without GM testing.

Table 4 Assessing risk factors for acquiring aspergillosis among severe influenza patients in intensive care units (ICU).

Potential risk for acquiring aspergillosis (one case was defined by positive *Aspergillus* culture but not by GM testing)	Aspergillosis ($n = 21$)	No aspergillosis (n = 29)	*p*
Cases of steroid use (prednisolone, hydrocortisone and/or methylprednisolone) before the day of GM testing	16	24	0.567
Cases of steroid use (prednisolone, hydrocortisone and/or methylprednisolone) ≥3 days before the day of GM testing	12	20	0.390
Cases of steroid use (prednisolone, hydrocortisone and/or methylprednisolone) before ICU admission	1	3	0.630
Use of prednisolone equivalent doses prior to GM testing:			
- Cumulative doses (mean ± SD, mg)	482.5 ± 870.2	487.7 ± 523.1	0.981
- Cases ≥ 300 mg of cumulative doses	7	16	0.126
- Cases ≥ 200 mg of cumulative doses	9	18	0.179
- Cases ≥ 0.3 mg/kg/day for more than 3 weeks[a]	1	3	0.630
- Cases > 20 mg/day	15	19	0.658
Cases with immune modulatory comorbidities	13	21	0.432
- Cases with a cancer disorder	5[b]	1[c]	**0.029**
- Cases with diabetes mellitus	7	12	0.563
- Cases with autoimmune disorders	0	1[d]	1.000
- Cases with liver cirrhosis	2	0	0.171
- Cases with chronic obstructive pulmonary disorder	2	7	0.271
- Cases with human immunodeficiency virus infection	0	2	0.503
- Cases with chemotherapy within 3 months	1	1	1.000
Cases on treatment with other T-cell immunosuppressants[a]	1	1	1.000
Mortality of patients with and without aspergillosis	14	7	**0.003**

Bold highlights a significant *p* value of less than 0.05.
[a] The same renal transplant recipient.
[b] Choriocarcinoma ($n = 1$), prostate adenocarcinoma ($n = 1$), cervical cancer ($n = 1$), urothelial carcinoma of urinary bladder with recent local chemotherapy ($n = 1$), and hepatoma ($n = 1$).
[c] Colon adenocarcinoma.
[d] Polymyositis ($n = 1$).

or *Aspergillus* culture (Fig. 1). These patterns did not show significant difference between influenza patients with and without IPA (Table 6). The EORTC/MSG clinical criteria was not found during initial diagnosis in our time-matched control study, but one patient developed ball-in-hole appearance in the chest X-ray films and CT images during the latter course of the disease.[13]

Throughout the whole course of the 21 patients with IPA, CT of the chest was performed in 6 patients, showing diffuse ground-glass appearance ($n = 3$), diffuse consolidation ($n = 1$), lung abscess ($n = 1$), and ball-in-cavity appearance ($n = 1$). Thoracic CT was not performed for those patients without IPA.

Discussion

In our study, "probable" IPA (16.9%), either acquiring initially at the community or "late-onset diagnosed" throughout the ICU stay, significantly corresponded to higher odds of in-ICU

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Table 5 Variables predicting mortality of severe influenza patients with probable aspergillosis.

Case variables	Number ($n = 21$)	Death ($n = 14$)	Survival ($n = 7$)	*P1*	OR (95% CI, *P2*)
Galactomannan (GM) index					
≥1.0	9	7	2	0.642	
≥0.7	12	10	2	0.158	
≥0.6	16	13	3	**0.025**	19.82 (4.91–80.07), **<0.0001**
GM-positive only	13	9	4	1.000	
Aspergillus isolate (positive)	8	5	3		
GM and *Aspergillus* culture (both positive)	7	5	2	1.000	
Rapid influenza antigen (positive)	11	5	6	0.064	
Influenza A (H1N1pdm09)	8	6	2	0.656	
Coinfections (community-acquired)	7	5	2	1.000	
Host at risk for aspergillosis	13	11	2	0.056	
Cancer	5	5	0	0.124	
Renal transplant	1	1	0		
Diabetes mellitus	7	6	1	0.337	
COPD	2	1	1		
ESRD	2	1	1		
Liver cirrhosis	2	0	2		
Use of steroid	16	11	5	1.000	
Cumulative prednisolone equivalent dose (prior to GM testing or *Aspergillus* isolation)					
≥100 mg	12	7	5	0.642	
≥200 mg	9	6	3	1.000	
≥300 mg	7	4	3	0.638	
≥500 mg	3	2	1	1.000	
Risk host + steroid use	10	9	1	0.064	
Age < 65	16	13	3	**0.025**	0.90 (0.35–2.31), 0.830
APACHE II score ≥20	13	9	4	1.000	
ARDS	8	7	1	0.173	
Ventilator use	21	14	7	1.000	
Voriconazole (duration for ≥3 days)	14	11	3	0.156	

Bold highlights a significant *p* value of less than 0.05.
P1: Fisher's exact test for difference of mortality in each variable; *P2*: Multivariable model for odds ratio of mortality; GM: galactomannan; ESRD: end-stage renal disease; COPD: chronic obstructive pulmonary disease; ARDS: acute respiratory distress syndrome.

mortality than influenza patients with or without other coinfections, even adjusting for confounding factors such as comorbidities and disease severity. The main result is consistent to our initial hypothesis. However, community-acquired non-*Aspergillus* coinfections did not correlate with higher in-ICU mortality compared to those patients without coinfections, against our second hypothesis, except for *P. aeruginosa* (before multivariable adjusting) and *K. pneumoniae* being mixed with other pathogens (after multivariable adjusting). Therefore, cofactors of comorbidity and disease severity would influence odds of mortality by *P. aeruginosa* and *K. pneumoniae* but not aspergillosis. Physicians should be alert to the new emerging trend of *Aspergillus* sp. and *P. aeruginosa* as initial copathogens. The incidence of IPA in our report was similar to a small retrospective study in Belgium (16.9% vs. 22.5%, $p = 0.429$), which found 9 of 40 critically ill influenza A (H1N1) patient developed IPA.[7] Meanwhile, a large study in Spain also reported increasing trends of *P. aeruginosa* and *Aspergillus* coinfections in severe influenza patients.[6]

Our data showed some difference to previous European and US reports.[17,18] Martin-Loeches et al. reported that community-acquired coinfection, defined as any infection diagnosed within the first 2 days of hospitalization, was associated with increased mortality (26.2% vs 15.5%; OR, 1.94; 95% CI, 1.21–3.09) in ICU patients with pandemic 2009 influenza A(H1N1) virus infection.[17] However, Cox regression analysis did not confirm a significant association between coinfection and ICU mortality. Compared to other coinfections (*S. pneumoniae*, *S. aureus*, *P. aeruginosa*), *Aspergillus* spp was more frequently isolated in COPD patients ($p < 0.05$).[17] However, COPD was only found in 2 of our 21 IPA patients. On the contrary, Rice et al. reported that bacterial coinfected patients had a significantly higher hospital mortality than patients without coinfection (31% vs. 21%, $p = 0.002$). Especially *S. aureus* at admission was independently associated with increased mortality,[18] but *S. aureus* was not associated with worse outcome in our study. Vancomycin was not routinely used in empirical therapy, so the significance of MRSA coinfection remained uncertain.

Why has IPA been increasingly noticed in southern Taiwan?[10–15] We have proposed sub-chronic exposure of high-level *Aspergillus* spore-containing ambient air as the primary environmental insult in our previous reports.[14] The

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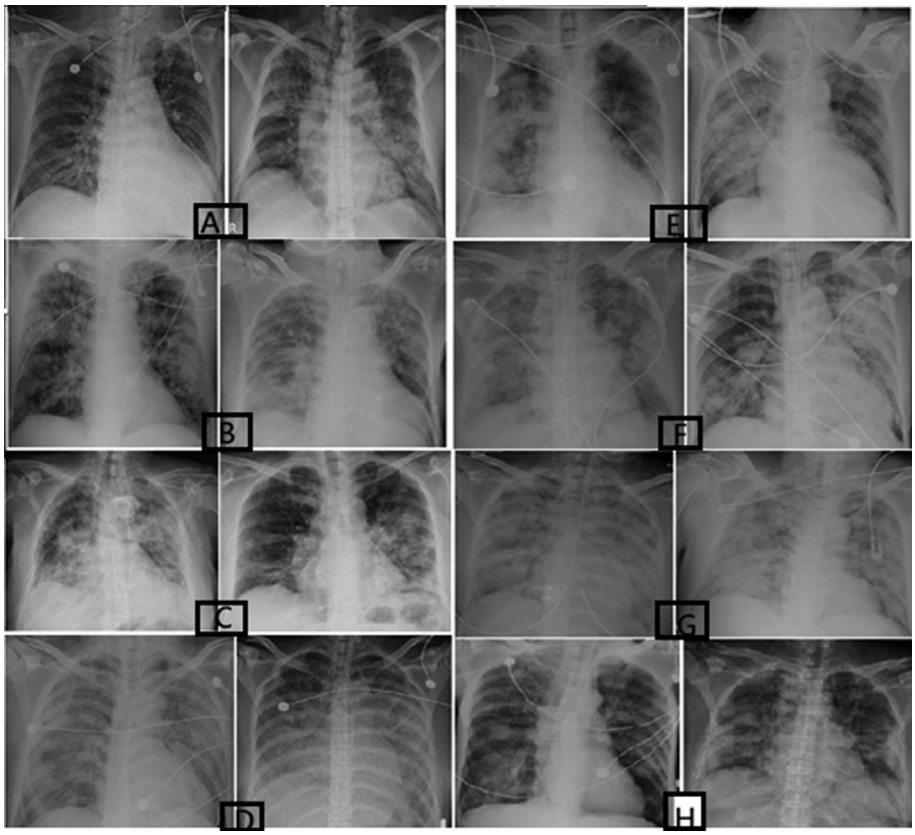

Figure 1 Eight chest radiographic patterns (A—H) of the severe influenza patients with probable invasive pulmonary aspergillosis are presented. Each pattern is representative of two different patients. A: peribronchial infiltrations; B: bilateral lung infiltrates; C: multiple patches with necrotizing processes; D: diffuse ground-glass appearance; E: extensive consolidation on one side of the lung fields; F: extensive consolidation on bilateral lung fields; G: diffuse air-space acute respiratory distress syndrome pattern; H: nodular lesions or patches.

Table 6 Comparison of difference in the chest X-ray patterns of critically ill influenza patients with and without probable aspergillosis (time-matched controls).

Patterns of chest X-ray film findings on the day of galactomannan testing or *Aspergillus* isolation (*n*)	Aspergillosis ($n = 21$)	No aspergillosis ($n = 29$)	Overall $p = 0.402$[a]
Type 1 peribronchial infiltrations (6)	1	5	
Type 2 bilateral lung infiltrates (7)	3	4	
Type 3 multiple patches with necrotizing processes (2)	1	1	
Type 4 diffuse ground-glass appearance (2)	2	0	
Type 5 extensive consolidation on one side of the lung fields (16)	5	11	
Type 6 extensive consolidation on bilateral lung fields (6)	2	4	
Type 7 diffuse air-space acute respiratory distress syndrome pattern (8)	5	3	
Type 8 nodular lesions or patches (3)	2	1	
Type 9 cavity, halo sign, crescent formation[b] (0)	0[c]	0	

[a] Fisher's exact test.
[b] EORTC/MSG criteria for clinical data.[3]
[c] One patient developed ball-in-hole appearance in the chest X-ray films and computer tomographic images during the latter course of the disease.[13]

theory was based on the fact that air particulate matter had been highly elevated for 2 months prior to the influenza epidemic in Tainan city,[14] as well as the evidence of significant increase of *Aspergillus* spores in the ambient air when air particle matter increased in Tainan city.[19] Thus we believed that IPA was established in the severe influenza patients who had prior environmental exposure and thus high incidence of dual IPA-influenza coinfection as detected in the area.

However, over-diagnosis of dual IPA-influenza coinfection is concerning. In an editorial comment, Martin-Loeches and Valles cited the reports of low rates of IPA (<2%) in

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influenza A (H1N1) patients,[5,17,20] and expressed concern about the suboptimal diagnostic tools based on *Aspergillus*-positive cultures or GM antigen criteria that might drive physicians to overuse the antifungal drugs.[21] By similar reasoning, Wichmann and Kluge somewhat doubted that IPA was an emerging disease of influenza patients in the ICU.[22] In another editorial comment, Luyt and Rice called it a "surprisingly high rate" of *Aspergillus* coinfection (7.3%) in the severe influenza patients as it has rarely been described as a community-acquired coinfection but more as a secondary fungal infection.[6,23] Nonetheless, our data offered evidence supporting the even higher rate of dual IPA-influenza coinfection (16.9%) as reported by Wauters et al. (22.5%),[7] suggesting that physicians should confront the new trend of epidemiology.

Our data offer some new insights into the risk factors for acquisition and mortality of IPA in the severe influenza patients. We found solitary cancer a significant predisposing factor for IPA in severe influenza patients. Detailed analysis of serial variables of corticosteroid use and antibiotic therapy did not show statistically significant difference between the severe influenza patients with and without IPA. False-positive GM assay has been reported in patients receiving piperacillin-tazobactam, or even other β-lactams including cephalosporins and carbapenems.[24–26] However, recent studies rarely observe significant interaction between piperacillin/tazobactam administration and *Aspergillus* GM assays in patients with hematopoietic stem cell transplant, cancer or without known risk factors for IPA.[27–30] Meanwhile, GM $\geq$ 0.6 index is an independent risk factor for mortality of the severe influenza patients with IPA. As we know in the literature, predictors of risk for mortality within the subpopulation have not yet been reported. In addition, the radiographic patterns on the day of GM testing were similar between the severe influenza patients with and without IPA, making it difficult to decide on the ideal timing to test GM assay for the influenza patients just based on the radiographic findings.

The present study has some potential limitations. First, lung biopsy was not performed for all the enrolled patients with the severe influenza. BAL GM assay was performed for only one patient and only 6 patients underwent a thoracic CT imaging study. During the influenza epidemic, BAL and CT of thorax were rarely performed because of the high risk of generating infectious aerosols and potential risk of worsening hypoxemia during invasive procedure or transferring the ARDS patients out of ICU to the radiologic department.[6,17] Therefore, there might be an underestimation for IPA incidence of the severe influenza patients. Second, this is a retrospective study, and we could only obtain some but not all patients with GM data at initial admission. We could not exclude the possibility of community-acquired IPA for those with "late-onset diagnosis" of IPA. Third, as there is a small case number of dual influenza and IPA coinfection, the statistical power might not be enough to assess risk factors for acquisition of IPA and mortality within this subpopulation. Fourth, although we could not detect significant difference in steroid use between influenza with and without IPA, it does not mean there could not be a significant role for some patients individually. Five patients of community-acquired IPA without prior steroid use might thus reduce the impact of steroid use on the pathogenesis of IPA. However, our study may hint at an important role of environmental exposure but is not limited to steroid use on the pathogenesis of IPA in severe influenza patients. Fifth, we neglected the nosocomial pneumonia of other pathogens, which might confound the disease course. Lastly, we compared relative risk of death (odds ratio) adjusted by multiple variables between influenza patients with and without IPA coinfection. However, we did not perform multivariate analysis to identify the risk factors for mortality of all patients with severe influenza. Therefore, we could not confirm IPA as an independent predictor for mortality of all severe influenza patients.

In conclusion, patients with *Aspergillus* coinfections, whether acquiring in the community or during the ICU stay, have higher in-ICU mortality (66.7%) than those without coinfections (15.4%) and those with other community-acquired coinfections (23.7%) in the setting of severe influenza, while adjusting for all important variables. We particularly emphasize the importance of applying *Aspergillus* GM testing. Earlier diagnosis and therapy should be cautiously considered for patients with severe influenza in ICU.

Ethical approval

The Institutional Review Board (IRB) of the Chi Mei Medical Center, Tainan, Taiwan, approved the study (IRB number 10503-003).

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Conflicts of interest

The authors have no conflicts of interest relevant to this article.

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