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Original Article

Outbreak of influenza A and B among military recruits: evidence from viral culture and polymerase chain reaction

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Background and purpose: Influenza is an important cause of acute respiratory illness among military recruits, and pneumonia is the most frequent complication. This study was performed to categorize the clinical manifestations of influenza infections among military recruits.

Methods: In this retrospective chart review, epidemiologic investigation of patients who met the definitions of acute respiratory illness, influenza-like illness, and pneumonia was conducted. Surveillance of influenza by viral culture and polymerase chain reaction was performed weekly from a random selection of 4 patients with influenza-like illness of less than 3 days duration.

Results: 2074 and 2046 men recruited to the Substitute Service Training Center in Taiwan, from November 30 to December 31, 2006 (outbreak 1), and January 11 to February 12, 2007 (outbreak 2), respectively were enrolled. During outbreak 2, 1182 men (57.7%) were identified to have acute respiratory illness, including 607 (29.6%) with influenza-like illness and 19 (0.9%) with pneumonia. During outbreaks 1 and 2, sixty two nasal and throat swabs were obtained, 15 of which were influenza A and 6 were influenza B. All the influenza A isolates were A/Wisconsin/67(H3N2) viruses. *Haemophilus influenzae* was isolated from 9 of 19 patients with pneumonia (47.3%); 8 from sputum specimens and 1 from a blood specimen. *H. influenzae* was the primary identifiable bacterium.

Conclusions: The 2 outbreaks consisted of concurrent infection of influenza A and B, with subsequent pneumonia. These results have implications for outbreak management and treatment of influenza among military recruits. Surveillance of influenza-like illness enables early detection of an outbreak and better understanding of the epidemiology of influenza in this setting.

Key words: Disease outbreaks; Influenza, human; Pneumonia

Introduction

Influenza causes significant morbidity and mortality worldwide. In the United States, influenza results in an annual average of approximately 95,000 hospital admissions [1]. The Centers for Disease Control and Prevention estimated that the annual medical costs are US$10.4 billion and the number of days of productivity lost due to illness was 44.0 million [2]. It has been estimated that 62 million people will die if a strain of influenza similar to that of the 1918 to 1920 pandemic was to emerge [3]. The first documented influenza outbreak in this pandemic was among military recruits at Fort Riley, Kansas, United States. Influenza viruses are transmitted from person to person via respiratory droplets expelled through coughing and sneezing. In a recent study, the attack rate for adults of working age was 1.5% to 2.6% [2]. In confined environments such as nursing homes, prisons, cruise ships, and military camps, the attack rate can be as high as 45% [4]. The recommended action to control influenza outbreaks [5]

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may not be sufficient in confined environments where influenza may rapidly disseminate [6].

The Substitute Service Training Center in Taiwan is the largest substitute service recruitment processing facility in Taiwan. Recruits are grouped into 15 squadrons of 100 to 150 members, and each of the 15 squadrons is organized into 4 companies. The duration of recruit training is 32 days. Squadron members have close contact with one another, as they train and live together under crowded conditions. This study was performed to categorize the clinical manifestations of influenza infections among military recruits into 3 classes and compared the diagnostic methods by polymerase chain reaction (PCR) and viral culture.

Methods

Patients

This retrospective chart review was performed at the military base, which has a 16-bed clinic; medical care was provided by Taichung Veterans General Hospital, Taichung, Taiwan. All clinic visits were recorded and clinical and laboratory data were collected systematically. Patients with pneumonia were interviewed about clinical symptoms using a standardized questionnaire.

Definitions

The definitions used in this study were adopted from previous studies [6-9] to enable comparison of the results. Acute respiratory illness (ARI) was defined as the presence of 2 or more of the following symptoms: cough, sore throat, runny nose, or sneezing. Influenza-like illness (ILI) was considered a subcategory of ARI and was defined as ARI with temperature >37.8°C. Pneumonia was defined as symptoms of ARI and infiltrates compatible with pneumonia on chest radiograph. The epidemic curves were plotted by time (X-axis) and number of patients (Y-axis).

Laboratory methods

Throat or nasal swabs were collected from all recruits with pneumonia. When there was an outbreak of ARI on the military base, throat swabs were collected weekly by random sampling of 4 patients who visited the base clinic with ILI of <3 days' duration. The specimens from throat and nasal swabs were placed in viral transport medium and stored at 4°C until delivered to the laboratory for viral culture and reverse transcriptase-PCR (RT-PCR).

Influenza virus was isolated by a cell culture method with immunofluorescence assay. The samples were inoculated onto Madin-Darby Canine Kidney cells and stained by indirect immunofluorescence assay with commercial monoclonal antibodies (Chemicon International, Temecula, CA, USA). Nucleic acid testing was performed by extracting RNA from the clinical sample using a QIAamp Viral RNA Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's recommendations. The resultant RNA was eluted with AVE elution buffer 60 μL (Qiagen). A negative control consisting of molecular grade water was included in each extraction and PCR assay. Positive virus controls from known isolated cultures in the laboratory were used.

Standard RT-PCR was performed by using a Reverse Transcriptase Kit (Promega, Madison, WI, USA). Viral RNA was reverse transcribed at 37°C for 1 h. PCR was performed using the PCR Core Kit (Qiagen). Thermocycling conditions for PCR consisted of 40 cycles of 94°C for 1 min, 52°C for 2 min, and 72°C for 3 min. The sequences of the primers used for detection of influenza A were as follows: H1 5'-GATGCAGACACAATATGTAGAGG-3' and 5'-CNCTACAGAGACATAAGCATTT-3'; H3 5'-TCAGATTGAAGTGACTAATGCT-3' and 5'-AATTTTGATGCCTGAAACCGT-3'. Amplicons were visualized by ethidium bromide staining following electrophoresis on 1.5% agarose. Specimens were sent to the Centers for Disease Control laboratories for further serologic typing.

For patients with pneumonia admitted to the clinic, culture from blood and sputum specimens were obtained. Expectorated sputum samples were collected for Gram staining. Urine samples were collected for detecting *Streptococcus pneumoniae* antigen by immunochromatographic assay (Binax-Now; Binax Inc, Portland, Maine, USA). Serology tests for *Chlamydophila pneumoniae*, *Legionella pneumophila*, and *Mycoplasma pneumoniae* were performed.

Radiograph evaluation

The chest radiographs were reviewed and classified as alveolar infiltrates, interstitial infiltrates, atelectasis, or pleural effusion.

Statistical analysis

Statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS) for Windows (Version 11.5; SPSS Inc, Chicago, IL, USA)

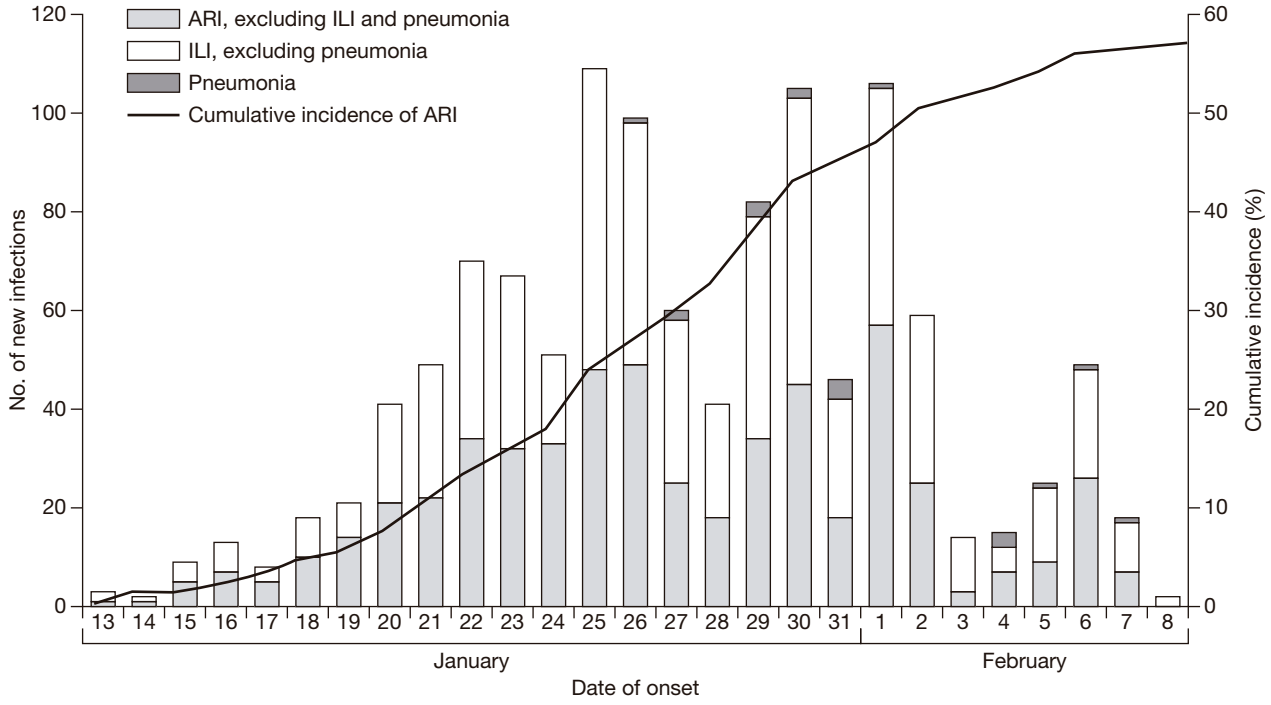

Fig. 1. Cumulative incidence of influenza during outbreak 2.
Abbreviations: ARI = acute respiratory illness; ILI = influenza-like illness.

and Microsoft Excel 2002 (Microsoft Corp, Redmond, WA, USA). Categoric variables were compared using the chi-squared test or Fisher's exact test, when appropriate. The level of significance was $p < 0.05$. The results are shown in mean ± standard deviation (SD).

Results

2074 and 2046 men were recruited to the Substitute Service Training Center from November 30 to December 31, 2006, (outbreak 1) and January 11 to February 12, 2007 (outbreak 2), respectively. During outbreak 2, the ARI attack rate was 57.7% (n = 1182), including 607 patients (29.6%) with ILI and 19 (0.9%) with pneumonia. Fig. 1 shows the epidemic curves for ARI for outbreak 2. At the weekends the clinic treated emergency patients only, which explains the artifactual cyclic lacunae visible on the curve. Fifty four percent of patients were reported during an 8-day period (25 January to 1 February) [Fig. 1]. The age of the recruits was 22.30 ± 2.04 years (range, 19-34 years). Only 2 recruits were aged 29 or older. There were no age differences between the 3 illness groups.

For outbreak 1, notification was only given during the last few days of the training course, which made surveillance and investigation difficult. Ten recruits with pneumonia were identified during this period, and specimens from 4 recruits with ILI were sent for laboratory surveillance.

The patients were distributed across all squadrons. No deaths were reported and all patients recovered without major complications.

Throat or nasal swabs were collected from 62 recruits. Specimens were collected from 10 patients with pneumonia who were admitted to hospital during outbreak 1 and from 19 patients during outbreak 2. Throat swabs were collected weekly from 4 patients with ILI for <3 days who visited the clinic during outbreak 1 (n = 4) and from 29 patients during outbreak 2.

Influenza A virus was isolated from 13 patients during outbreak 2. Influenza B virus was isolated from 1 patient during outbreak 1 and from 5 patients during outbreak 2. PCR for influenza A was positive for 2 patients during outbreak 1 (Table 1). Fifteen patients had influenza A and 6 had influenza B. All influenza A isolates were A/Wisconsin/67(H3N2) viruses.

The symptoms of the 29 patients with pneumonia who were interviewed using a standard questionnaire were compared with those of the 1163 patients with ILI and ARI during outbreak 2. Cough, sore throat, and nasal congestion were the most commonly recorded symptoms in all groups (Table 2). Several clinical

Table 1. Results of viral cultures and reverse transcriptase-polymerase chain reaction from military recruits during 2 influenza outbreaks.

	No. of positive results/specimens tested			
	Viral culture		Polymerase chain reaction	
	Influenza A	Influenza B	Influenza A	Influenza B
Outbreak 1				
Pneumonia (n = 10)	0/10	0/10	0/10	0/10
Surveillance (n = 4)	0/4	1/4	2/4	0/4
Outbreak 2				
Pneumonia (n = 19)	1/19	0/19	0/0	0/0
Surveillance (n = 29)	12/29	5/29	0/0	0/0

Table 2. Clinical symptoms of patients with pneumonia, influenza-like illness (ILI), and acute respiratory illness (ARI).

Symptom	Pneumonia during outbreaks 1 and 2 (n = 29) No. (%)	ILI excluding pneumonia during outbreak 2 (n = 607) No. (%)	ARI excluding ILI and pneumonia during outbreak 2 (n = 556) No. (%)
Fever	29 (100)	607 (100)	0 (0)
Cough	28 (97.10)	586 (96.50)	530 (95.30)
Malaise	25 (86.20)[a,b]	362 (59.60)[a]	60 (10.80)
Sore throat	22 (75.90)	554 (91.30)	499 (89.70)
Nasal congestion	22 (75.90)	404 (66.60)[d]	401 (72.10)
Chills	21 (72.40)[c]	55 (9.00)[a]	0 (0)
Headache	16 (55.20)[a,b]	158 (26.00)[a]	33 (5.90)
Myalgia	15 (51.70)[a]	258 (47.00)[a]	7 (0.01)
Diarrhea	9 (31.00)[c]	15 (2.50)[d]	5 (0.01)
Vomiting	8 (27.60)[c]	33 (5.40)[a]	7 (0.01)
Dizziness	5 (17.20)[d]	83 (16.70)[a]	37 (0.07)
Arthralgia	5 (17.20)[c]	11 (1.80)	7 (0.01)
Dyspnea	2 (6.80)[c]	1 (0.20)	0 (0)
Fever >7 days	4 (13.70)	0 (0)	0 (0)
Biphasic fever pattern	19 (65.50)	0 (0)	0 (0)

[a] $p < 0.01$ versus ARI.
[b] $p < 0.05$ versus ILI.
[c] $p < 0.01$ versus ILI and ARI.
[d] $p < 0.05$ versus ARI.

characteristics were more frequent in individuals with pneumonia, in particular chills and headache. Malaise and myalgia were significantly less frequent in the patients with ARI when compared with the other patients. The mean temperature of the patients, recorded at admission to hospital, was 38.4 ± 0.6°C. Nineteen patients with pneumonia (65.5%) had a recurrence of fever after the initial influenza symptoms had disappeared. Fever continued for more than 7 days in 4 patients (13.7%).

The initial laboratory findings of 29 patients with pneumonia showed leukocytosis and elevation of muscle-associated enzymes (Table 3). The increase in aspartate aminotransferase was more prominent than that of alanine aminotransferase.

Sputum samples were obtained from all patients with pneumonia. Of the 21 samples (72.4%) that were suitable for analysis, 18 showed a predominant bacterial morphology. There were 6 Gram-positive cocci in chains, 2 Gram-positive cocci in groups, 8 Gram-negative coccobacilli, and 2 Gram-negative bacilli. The microbiology results are summarized in Table 3. Only 8 sputum specimens yielded positive bacterial growth (all were *Haemophilus influenzae*). *H. influenzae* was isolated from blood in 1 patient. Urine pneumococcal antigen assay was positive for 1 patient. Influenza A virus was isolated from a nasal swab specimen in 1 patient. The antimicrobial resistance pattern for the 9 *H. influenzae* isolates is shown in Table 4. All of the *H. influenzae* isolates were susceptible to ampicillin-

Table 3. Clinical characteristics of 29 patients with pneumonia at presentation.

Variable	Mean ± SD	Reference range	No. of positive results (%)
Laboratory values			
White cell count ($\times 10^9$/L)	12.545 ± 3.172	4.5-11.0	
Hemoglobin concentration (g/L)	130 ± 11	120-175	
Platelet count ($\times 10^9$/L)	299 ± 120	150-450	
C-reactive protein (μg/L)	130,000 ± 770,000	68-8200	
Creatine phosphokinase (U/L)	768 ± 1060	50-200	
Lactate dehydrogenase (U/L)	272 ± 115	50-200	
Aspartate aminotransferase (U/L)	95 ± 78	20-48	
Alanine aminotransferase (U/L)	55 ± 72	10-40	
Microbiology investigation			
Gram stain			
Good-quality sample[a]			21 (72.4)
Gram-positive cocci in pairs and chains			6 (28.6)
Gram-positive cocci in groups			2 (9.5)
Gram-negative coccobacilli			8 (38.1)
Gram-negative bacilli			2 (9.5)
Gram-positive cocci in chains plus Gram-negative bacilli			2 (9.5)
Gram-positive cocci in groups plus Gram-negative bacilli			1 (4.8)
Sputum culture			8 (27.5)
Blood culture			1 (3.4)
Urine pneumococcal antigen assay			1 (3.4)
Serologic assays			
Chlamydia pneumoniae			0 (0)
Legionella pneumophila			0 (0)
Mycoplasma pneumoniae			0 (0)

[a]Good quality sample = >25 polymorphonuclear cells and <10 squamous cells observed under low-power field.
Abbreviation: SD = standard deviation.

sulbactam, cefotaxime, ciprofloxacin, and imipenem. Antibiotic susceptibility results from the hospital laboratory database were analyzed for comparison.

The most common chest radiographic finding in the patients with pneumonia was alveolar infiltrates in a single lobe, usually the right lower lobe (Table 5). A single alveolar infiltrate was noted in 20 patients (68.9%). Two patients (6.8%) with left lower lobe alveolar opacity also had atelectasis and 1 (3.4%) with right middle lobe alveolar infiltrates had concurrent pleural effusion.

Discussion

In this study, ARI spread rapidly through the military base, affecting approximately 57.7% of the recruits from January 11 to February 12, 2007. The primary pathogens were identified as influenza A/Wisconsin/67(H3N2) virus and influenza B. Nine patients were found to have secondary *H. influenzae* pneumonia. To the authors' knowledge, concurrent outbreaks of influenza A and influenza B in a military camp have not been described previously, although they have been reported in a nursing home [10], a cruise ship [11], and a children's rehabilitation center [12]. In this study, influenza A virus was detected in only 1 of 29 patients with pneumonia (3.4%). Of 33 specimens collected from surveillance patients with ILI, influenza virus was detected in 20 specimens (60.6%). Several studies have shown positive influenza virus culture rates of 27% to 40% [7,13]. The low rate of influenza isolation in patients with pneumonia could be due to the delayed sampling of clinical specimens. The time from symptom onset to clinic admission was more than 5 days for most patients with pneumonia in this study. The viral titers measured by serial 10-fold dilutions of viral culture decline rapidly 72 h after the onset of illness [14]. In a human influenza infection experiment, the positive culture rates were highest, approximately 86%, on days 2 and 3 after infection [14].

More than 70% of the patients had pneumonia characterized by prolonged fever for more than 7 days or a biphasic fever pattern. This fever pattern suggested a secondary bacterial infection [15-17]. Leukocytosis

Table 4. Comparison of the antimicrobial susceptibility patterns for *Haemophilus influenzae* isolates from military recruits and from the Taichung Veterans General Hospital laboratory database, 2006.

Antimicrobial	Military recruits (n = 9)[a] No. (%)	Laboratory database (n = 278) No. (%)
Ampicillin	3 (33.3)	100 (35.9)
Ampicillin-sulbactam	9 (100)	243 (87.4)
Chloramphenicol	5 (55.5)	177 (63.7)
Trimethoprim-sulfamethoxazole	4 (44.4)	105 (37.7)
Cefotaxime	9 (100)	277 (99.6)
Ciprofloxacin	9 (100)	253 (91.0)
Imipenem	9 (100)	278 (100)

[a]8 isolates from sputum and 1 from blood.

Table 5. Chest radiograph findings of 29 military recruits with pneumonia.

Chest radiograph pattern	No. (%)
Alveolar	23 (79.3)
Right lower lobe	10 (34.4)
Right middle lobe	4 (13.7)
Left lower lobe	7 (24.1)
Multiple lobes	2 (6.8)
Interstitial	6 (20.6)
Right lower lobe	4 (13.7)
Left lower lobe	1 (3.4)
Multiple lobes	1 (3.4)
Atelectasis	2 (6.8)
Pleural fluid	1 (3.4)

and elevation of serum C-reactive protein levels are considered to be indicators of systemic bacterial infection, rather than of viral infections, but both were highly variable in this study, in line with other studies [18]. Elevated creatine phosphokinase was observed in 9 patients (31%) in this study. Myositis is an uncommon self-limiting complication of influenza, mainly found in children infected by influenza B [19,20]. Although myositis has been described in case reports of elderly people, in whom it has a strong correlation with influenza A infection [21,22], it is not well documented in young healthy adults. Further study is needed to reveal the nature of influenza-associated myositis in this population.

Based on the laboratory findings, this study identified *H. influenzae* as a major cause of secondary bacterial pneumonia after influenza. As in most studies of community-acquired pneumonia, the causative microorganisms remained unidentified in a sizable proportion of patients (69%) [13,23], but the clinical picture was consistent with bacterial pneumonia. The epidemic curves of pneumonia were approximately 10 days behind those of influenza.

During the past few decades, many studies have demonstrated the correlation between influenza and post-viral bacterial pneumonia [24-26]. Research has shown the effect that influenza virus neuraminidase has on the probability of acquiring secondary infections [26]. During the influenza pandemic of 1918 to 1919, pneumonia was a major cause of death among troops. At Fort Riley, Kansas, United States, necropsy lung cultures yielded *Bacillus influenzae* (now known as *H. influenzae*) alone or with other pathogens in 41.1% of patients [27,28]. *Pneumococcus* was found in 58.8% of the cultures. *H. influenzae* has long been recognized as a pathogen of community-acquired pneumonia and is also associated with influenza virus infection or ILI [29]. Studies have shown that *Streptococcus pneumoniae*, *Staphylococcus aureus*, and *H. influenzae* are the most common bacterial pathogens in secondary bacterial pneumonia of influenza [30,31]. A prospective multicenter study of adult community-acquired pneumonia in Taiwan showed that the most common pathogen is *S. pneumoniae* (23.8%), with *H. influenzae* accounting for only 4.8% of all cases [23]. The *H. influenzae* isolates in this study remained highly susceptible to ampicillin-sulbactam, cefotaxime, and ciprofloxacin, which were compatible to the microbiologic susceptibility results from the Taichung Veterans General Hospital laboratory database. Among the 8 sputum specimens that yielded *H. influenzae*, 6 showed characteristic Gram-negative coccobacilli by Gram stain. Hence, Gram stain is helpful for the diagnosis of the pathogen of pneumonia. A similar observation has been described previously [32].

This study has several limitations. First, the diagnosis of other important respiratory viruses in this population, such as adenovirus and respiratory syncytial virus, may be hindered by the lack of sensitive serologic testing [33]. However, this was unavoidable

because the need for acute-phase and convalescent-phase specimens limited their use for these patients. Second, chest radiograph is not likely to be done for all patients with ILI. Therefore, the number of patients with pneumonia may have been underestimated. Third, since the measurements for ILI and ARI were obtained by medical records review, they were inherently incomplete. Symptoms elicited by standardized questionnaire may be more characteristic for the outbreak [34] and the outbreak magnitude may have been underestimated since some recruits with ARI probably did not seek medical care. In an influenza outbreak aboard a cruise ship, only 22% of passengers with ARI sought medical care [35]. Finally, few influenza viruses were isolated during the first outbreak due to a delay in sampling and specimen collection in the form of throat swabs. Recent investigation has shown that throat swabs are less sensitive than nasal swabs for detecting influenza viruses. The positive culture rates of influenza virus were 46.0% to 64.6% for nasal swabs and 24.0% to 51.8% for throat swabs [14,36].

In conclusion, this study demonstrated that the 2 outbreaks in the Substitute Service Training Center consisted of a concurrent infection of influenza A and B and subsequent *H. influenzae* pneumonia. These results have implications for outbreak management and raise questions regarding the need for influenza vaccination for military recruits. The study results also highlight the need for regular influenza surveillance in this population. Control of an influenza outbreak depends on early detection. Although detection of an influenza outbreak can be made by examining a cluster of patients with similar symptoms, laboratory confirmation of influenza can be difficult due to delayed sampling and the time-consuming process of viral culture. Active surveillance by PCR and viral culture among patients with ILI is mandatory for the early identification of an influenza outbreak and will help to better define the epidemiology of influenza among military recruits receiving basic training and to develop more effective control measures.

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