Original Article

Acute Respiratory Disease at a Chinese Military Recruitment Training Center: Three-Year Consecutive Investigation

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Background Military recruits are at a higher risk of acute respiratory disease (ARD) and the causative agents might change over time, which needs to be investigated.

Methods The nasopharyngeal swabs and blood samples were consecutively collected from conscripts for three years in a military training center. The real-time fluorescent quantitative PCR assays were conducted for 15 species of common respiratory pathogens; the serum anti-*Legionella pneumophila* antibodies were detected by indirect immunofluorescence (IIF) assay, and serum anti-*Mycoplasma pneumoniae* antibodies, serum anti-influenza B virus and anti-influenza A virus-IgM and IgG were detected by ELISA.

Results The prevalences of ARD were 59.3% (108/182) in 2008, 23.3% (50/215) in 2009, and 19.6% (40/204) in 2010. Among the patients with ARD from 2008 to 2010, the influenza B virus infection accounted for 45.4%, 30.0% and 55.0%, and seasonal influenza A virus infection for 8.3%, 8.0% and 5.0%, respectively; the positive rates of serum anti-*Legionella pneumophila* and anti-*Mycoplasma pneumoniae* antibodies in recruits was lower than 10% each year respectively in the three years without diagnostic significance.

Conclusion The early appropriate diagnosis and treatment of ARD in military personnel will ensure the power strength of armed forces.

Key words: Military personnel; Acute respiratory disease; Polymerase chain reaction; Influenza B virus

The respiratory infections in military populations are very common throughout history.¹ Military conscripts are prone to respiratory infection than their civilian counterparts.² The outbreaks of adenovirus (Ad)-associated acute respiratory disease (ARD), or febrile respiratory illness (FRI), were repeatedly reported among US military camps, which led to significant morbidity and lost training times.^{2,3} Adenoviruses,^{4,5} influenza virus,^{6,7} respiratory syncytial virus (RSV),⁸ enterovirus,⁹ rhinovirus,⁹ *Streptococcus pneumoniae*,⁹

*Mycoplasma pneumoniae*¹⁰ and *Neisseria meningitidis*⁵ are implicated in over half of the FRI cases reported at recruit training centers, while the etiology of the remaining cases is unclear. Influenza is an important cause of acute respiratory illness among military recruits, and pneumonia is the most frequent complication.⁶

An epidemic of severe acute respiratory syndrome (SARS) occurred in China, in Spring of 2003,¹¹ followed by the outbreak of pandemic influenza A (H1N1) in 2009.^{12,13} During this period, a small-scale outbreak of FRI took place in different provinces, which drew public attention and imposed some threat to society, which urged that rapid diagnostic tools should be made available. Early etiologic diagnosis would guide accurate and efficient management of a given epidemic and alleviate social panic. We

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established a real-time fluorescent quantitative PCR assay for rapid detection of 15 species of common respiratory pathogens and investigated the prevalence and common etiological causes of ARD in recruits of a military camp in Anhui Province consecutively for three years and found that the prevalence of ARD ranged from 19.6% to 60.4% each year, and influenza B virus infection accounted for 30.0% to 55.0%.

MATERIALS AND METHODS

Ethics statement

Our research involving human participants has been approved by the Institutional Review Boards at 105th Hospital of Chinese PLA and Anhui Medical University at Hefei (IRB number: ST 105080001). We carefully explained the purposes, protocols and implications of the research to all the participants before we collected any swabs or blood, so they all gave the written informed consent. The clinical investigation has been conducted according to the principles expressed in the Declaration of Helsinki.

Sample collection

Total of 601 conscripts (559 men and 42 women with mean age of 18.5 years old) in all were recruited in a military training center from December 2007 to February 2010. The nasopharyngeal swabs (washed with 3 ml sterile normal salts and stored at -40°C) and paired sera from 200 persons with ARD out of the 601 conscripts were collected for three consecutive years. The ARD was defined as fever $\geq 37.5^{\circ}\text{C}$, with cough or sore throat and/or radiological pulmonary infiltrates.³ Recruits with ARD were eligible for enrollment.

A real-time fluorescent quantitative PCR assay

The reference strains of influenza A virus/Guangzhou/555/2006 (H1N1), influenza A virus /Anhui/1/2005(H5N1), influenza B virus/Jilin/20/2003, SARS coronavirus BJ01, human coronavirus OC43 ATCC VR-759, rubella virus Gos, epidemic mumps virus SP, human respiratory syncytial virus ATCC VR-26, measles virus Changchun-47, adenovirus type 2 ATCC VR-1079AS/RB, *Mycoplasma pneumoniae*

ATCC M129-B7, *Chlamydia pneumoniae* TW-183, *Legionella pneumophila* ATCC 43703, *Streptococcus pneumoniae* ATCC 700669 and *Streptococcus suis* 05ZYH33 were obtained from the Military Medical Science Academy of the People's Liberation Army and the National Institute for the Control of Pharmaceutical and Biological Products (Beijing). About 140 μl washing suspension of nasopharyngeal swabs or the supernatants of cultured reference strains were extracted for DNA (QIAGEN Plasmid Purification®, Qiagen, Germany) and RNA (QIAamp viral RNA mini kit®, Qiagen, Germany) and the obtained pipettes were stored at -80°C. The primers and probes were designed by Primer Express 3.0 (ABI) and Primer3 Input 4.0, and were compared to the reference strains for homology in the NCBI database (<http://www.ncbi.nlm.nih.gov/>, Table 1).

DNA and RNA of the pathogen genomes mentioned above were amplified by PCR. The products of the amplification were cloned to pMD19T plasmid and transformed to competent DH-5 α bacteria. The positive clones were screened by PCR. The plasmids were extracted by TIANprep mini plasmid kit® (Tiangen, China) and the positive ones were sequenced in order to verify the inserted sequence as correct. The known plasmid was added ddH₂O by 10 series as a reference by which the sensitivity, specificity and the lowest limits of detection of the primers and probes of targeted genes of the fifteen pathogens were determined. Then, the standard curve was established.

PCR (7300 Real-Time PCR System, Applied Biosystems, CA 94404, USA) was carried out by 2 $\times$ Master Mix 10 μl , sense and antisense primers (10 $\mu\text{mol/L}$) 0.5 μl each, fluorescent probe (10 $\mu\text{mol/L}$) 0.25 μl , template 2 μl , and ddH₂O added to 20 μl . The reaction was carried out at 95°C for 10 min; at 94°C for 15 s, at 58°C for 45 s, with 40 circles.

Serum detection

Serum anti-*Legionella pneumophila* antibodies were measured by indirect immunofluorescent assay (EUROIMMUN Co., Germany, serotype 4); serum anti-*Mycoplasma pneumoniae* antibodies, and serum anti-influenza antibodies (IgM and IgG) were detected by ELISA (Virion-Serion Co., Hongzhou). A four-fold

Table 1. Primers and probes of targeted genes of fifteen pathogens

Pathogens	Primers and probes (5'-3')	Target gene	Products (bp)
Influenza A virus (H ₃ N ₂)	H5-1: GCAAACAACCTCGACAGAGCA H5-2: GAGGAGCCATCCAGCTACAC H5 probe: CACAACGGGAAGCTCTGCGATCT	HA	162
Influenza A virus (H1N1)	MP-1: AGTTGCATGGGTCTCATATACAACA MP-2: TGCTGGGAATCAGCAATCTG MP probe: TTTGGCCTRGTRTGTGCCACTT	MP	101
Influenza B virus	BV-1: TGATTGGGCATTCACAGATG BV-2: ACCTCCTTTGATYGCAACAC BV probe: AAAGAGTTGGACTTGACCCTTCATTAAT	NP	149
Respiratory syncytial virus	RSV-1: CCAACGGAGCACAGGAGATA RSV-2: TCCCATTGATGTCTTGACGA RSV probe: CCATTWGCYTTWACATGATATCCHGCA	NP	145
Rubella virus	RV-1: ACGCCGCACGGACAACCT RV-2: TGTGGTTGCCGGTGAATTC RV probe: AGGTCCAGGTCCCGCCCGAC	NP	86
Measles virus	MV-1: ATGGTTGGATGTGGTGAGGAA MV-2: CCAGGATTAGAGCCACCATGA MV probe: AGGATTGCCGAGGACCTCTCTTTACGC	NP	74
Epidemic mumps virus	MSV-1: TCAGATAGAACGCTGYGAGA MSV-2: AGCAAAGCATAGGCAGCAAT MSV probe: ATTCCAAATGCACGCGCCAATCTTAC	NP	108
<i>Legionella pneumophila</i>	LP-1: AAAGGCATGCAAGACGCTATG LP-2: TGCCATCAAATCTTTCTGAAACTT LP probe: TGGCGCTCAATTGGCTTTAACCGA	mip	147
<i>Chlamydia pneumoniae</i>	CPN-1: CGTGGAGCCTTATGGGAATG CPN-2: CAACCTTAGGTTTGGACTGTGCAT CPN probe: TTGTGCAACTTTGGGAGCTGAATTCCA	ompA	76
<i>Streptococcus pneumoniae</i>	P1: GATCGTGCTCCGATGACTTA P2: AATTGCTGGGGTCTTCCACT P probe: CTGGTTTGGCAAGTAGCGATAGCTTTCTCC	ltyA	85
<i>Streptococcus suis</i>	SS-1: AAAACCGTGCAGCTGAAATC SS-2: CGGAATAACTGGTGCTTGGT SS probe: GCCGGAGGCGCTGAATGCTA	epf	71
<i>Mycoplasma pneumoniae</i>	P1-1: GCAGTTGCTGGCGCTAAGTT P1-2: AAGCGAGGTACGGTAGCGGTAT P1 probe: TGGTAGGGAACCTCGTTTTAGCGGGTACC	P1	86
SARS coronavirus	S-1: AAGCCTCGCCAAAAACGTAC S-2: AAGTCAGCCATGTTCCCGAA S probe: CGTCACTCAAGCATTTGGGAGACG	NP	229
Adenovirus type 2	AD-1: CAACAAGTCAACGGATGTGG AD-2: CCGGGCTGTAGTCATTGTTT AD probe: AACGACCACAGCAACTTTCTAACCACGGT	Penton	92
Human coronavirus	CV-1: ACAGCTGGTGCGTTTTTCTT CV-2: GCCGTTATAGCGCAATTCAT CV probe: TGGGAGTCTGACGAGCCTCAGA	NP	111

or greater increase of antibody titers between acute and convalescent phase sera was considered indicative of acute infection.

than 0.05 was considered statistically significant.

RESULTS

Statistical analysis

Statistical analyses were performed through SPSS v.13 (PASW 13.0). The differences of positive rate of sera data were analyzed by a Chi-square test and *P* lower

Specificity and sensitivity of PCR assays

We successfully set up a real-time fluorescent quantitative PCR assay which simultaneously detected 15 species of common respiratory pathogens. The meth-

Figure 1. Chest roentgenography showing pulmonary spherical infiltrates in a conscript with acute respiratory disease (in 2008).

od had a linear range of detection at 1×10^2 to 1×10^9 copies per microliter and exhibited a high specificity and sensitivity of approximately 10^1 to 10^2 copies per microliter for each pathogen, as well as a high reproducibility with coefficient of variation (CV) ranging from 0.28% to 4.29%.

Incidence of ARD and clinical manifestations

The incidences of ARD in our series were 59.3% (108/182), 23.3% (50/215) and 19.6% (40/204) in 2008, 2009 and 2010, respectively. The first and second generation of febrile respiratory diseases in the military conscripts broke out in December 30, 2007 and January 6, 2008 fifteen days after the conscripts were recruited in the training camp. Their main clinical manifestations were moderate to high fever, cough in 28.7% (31/108) of them, pulmonary infiltrates (Figure 1 and Figure 2) with normal peripheral white blood cell counts. The symptoms persisted for one week and all patients recovered completely. The close contactors were given azithromycin for three days and immunoglobulin intravenously after azithromycin failure. ARD occurred sporadically in two-month

Figure 2. CT scan showing pulmonary spherical infiltrates in a conscript mentioned in Figure 1 with acute respiratory disease (in 2008).

training periods in 2009 and 2010, with less pneumonia.

Confirmation of pathogens

Our fluorescent quantitative PCR results showed positive *Legionella pneumophila*, *Microplasma pneumoniae*, human respiratory syncytial virus, measles virus or *Streptococcus pneumoniae* in some specimens, while influenza virus type B accounted for the majority of infections (Table 2).

Prevalence of positive antibodies

Some recruits had positive serum anti-*Legionella pneumophila* and anti-*Microplasma pneumoniae* antibodies in acute phase, which did not increase in convalescent-phase sera. However, serum anti-influenza B virus antibody titers increased significantly in convalescent phase, so positive detection rates of infection increased (Table 3).

DISCUSSION

Circumstances in Garrisons and other military training units make the recruits susceptible to the outbreaks of ARD.¹⁴⁻¹⁶ The continuing high incidence of ARD in the military camps underscores the establishment of rapid etiologic diagnosis as accurate as possible. Patients presenting the combination of fever, cough, tachycardia and peripheral white blood cell counts <

Table 2. The positive rates of pathogens detected with throat swab [cases (%)]

Year	n	Influenza B	Influenza A	Respiratory syncytial	Measles	<i>Legionella pneumophila</i>	<i>Microplasma pneumoniae</i>	<i>Streptococcus pneumoniae</i>
2008	108	49 (45.4)	9 (8.3)	3 (2.8)	1 (0.9)	12 (11.1)	26 (24.1)	0 (0.0)
2009	50	15 (30.0)	4 (8.0)	0 (0.0)	0 (0.0)	12 (24.0)	12 (24.0)	0 (0.0)
2010	40	22 (55.0)	2 (5.0)	0 (0.0)	0 (0.0)	6 (15.0)	8 (20.0)	3 (7.5)

Table 3. The positive rates of serum antibodies (%)

Year	n		Anti-influenza A		Anti-influenza B		Anti- <i>Mycoplasma pneumoniae</i>	Anti- <i>Legionella pneumophila</i>
			IgM	IgG	IgM	IgG		
2008	108	acute	5 (4.6)	2 (1.9)	60 (55.6)	30 (27.8)	12 (11.1)	3 (2.8)
		convalescent	0 (0.0)	5 (4.6)	4 (3.7) ¹	65 (60.2) ¹	13 (12.0)	5 (4.6)
2009	50	acute	4 (8.0)	1 (2.0)	18 (36.0)	6 (12.0)	4 (8.0)	4 (8.0)
		convalescent	0 (0.0)	4 (8.0)	2 (4.0) ¹	19 (38.0) ¹	8 (16.0)	4 (8.0)
2010	40	acute	2 (5.0)	1 (2.5)	26 (65.0)	15 (37.5)	1 (2.5)	0 (0.0)
		convalescent	0 (0.0)	2 (5.0)	3 (7.5) ¹	28 (70.0) ¹	1 (2.5)	1 (2.5)

Note: ¹ vs acute stage, $P < 0.05$

$10 \times 10^9/\text{ml}$ are sometimes suggestive of influenza infection, H1N1 for instance. However, influenza could not reliably be distinguished from other acute respiratory illnesses in adult patients just based on clinical features.^{17,18} The pathogens of ARD are usually confirmed by polymerase chain reaction, culture and serologic detection. We had set up a real-time fluorescent quantitative PCR assay which can simultaneously detect 15 species of common respiratory pathogens, which is a potential tool for rapid screening and identification of common respiratory agents during an ARD epidemic in military centers.

A total of 102 600 cases with upper respiratory infection (URI) were recorded among young military conscripts in the Finnish Defence Forces from October 1991 to March 1994.¹⁹ While some countries have applied surveillance programs to monitor ARD in military training camps, limited data on recruits with ARD infection are available in Chinese armed forces. Our study showed that the incidences of ARD in our series were 59.3%, 23.3% and 19.6% each year from 2008 to 2010, respectively. Among the common causative pathogens, influenza virus type B was the most prevalent. Lee et al reported that 821 (28.7%) out of 2 858 cases recruited from 11 May 2009 to 25 Jun 2010 in Singapore had influenza, among which 434 (52.9%) with 2 009 influenza A (H1N1), 58 (7.1%) with seasonal influenza A (H3N2) and 269 (32.8%) with influenza B.²⁰ Evolutionary antigenic changes in haemagglutinin (HA) and neuraminidase (NA) of influenza virus surface glycoproteins, a phenomenon known as antigenic drift, always result in recurrent outbreaks in civilian and military communities.^{19,20}

Chinese scholars have established a multiplex real-time RT-PCR for simultaneous detection of influenza virus type B and some subtypes of influenza A

virus, which exhibit a high specificity, sensitivity and reproducibility.²¹ We believe that method in this article have achieved similar efficacy.

Mycoplasma pneumoniae is another common pathogens that causes community-acquired respiratory tract infection. Although outbreaks of *Mycoplasma pneumoniae* in army training units has sometimes been reported,²² data regarding medical history, symptoms, signs and laboratory detection in our series did not support such an infection. Some rapid identification of *M. pneumoniae* culture were easy to perform and provide quick results, but showed poor concordance with *M. pneumoniae* PCR and serum *M. pneumoniae* antibody detection due to high fungus contamination,²³ which suggested that the diagnosis of *M. pneumoniae* requires further investigation.

The Armed Forces Health Surveillance Center of the US embarked on prevention and management of pandemic H1N1 influenza in 2009,²⁴ and different vaccines against influenza, adenovirus and mycoplasma, immunostimulators, and other measures of urgent prophylaxis including various combinations were observed in the military.^{16,25,26} Studies suggested that asthmatic conscripts could enhance their physical fitness by training similarly to non-asthmatic ones.²⁷ Chinese doctors even found that low-dose recombinant human interferon alpha-2b nasal spray could prevent acute viral respiratory infections in military recruits.²⁸ Further studies are warranted to furtherly define the etiologic agents of ARD among recruits and the utility of prophylactic measures capable of reducing the incidence of ARD among military personnel.

FOOTNOTES

Competing interests: The authors have declared no competing interests.

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