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Prospective Evaluation of Rapid Antigen Tests for Diagnosis of Respiratory Viral Pathogens

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ABSTRACT

Acute respiratory infection is a frequently transmitted illness of concern to doctors and patients. Considering its airborne transmission, early diagnosis of such disease is particularly important. This study explored respiratory viral infections with influenza virus, parainfluenza virus, respiratory syncytial virus, human metapneumovirus, human bocavirus, coronavirus, and other early diagnostic substances as confirmed by literature resources. This study also used the corresponding monoclonal antibodies that were produced with the use of hybridoma technology, which were fixed on the chip after purification, for further serum detection. Using this method, a new technique to simultaneously detect 6 kinds of febrile respiratory viruses in a protein chip was developed. The accuracy rate of this method can be >99.65%. This product is inexpensive and capable of high-precision and high-throughput screening, which are prominent advantages.

ACUTE RESPIRATORY INFECTION (ARI) accounts for a large proportion of all acute morbidities in developed countries, and the majority of these infections (~80%) have a viral etiology [1–3]. Acute viral respiratory tract infection is the leading cause of hospitalization for infants and young children in developed countries and is a major cause of death in developing countries [4,5]. In developed countries, the number of viral respiratory episodes per year has been estimated to be 6–10 in children before school age versus 3–5 in those after this age, and ARI represents the cause of 30%–40% of hospital admissions in this category of patients [6,7]. Respiratory tract infection can lead to acute asthma exacerbations, acute otitis media, or other lower respiratory tract presentations, including bronchitis, bronchiolitis, and pneumonia [8]. A wide range of pathogens are involved in ARI, including bacteria and viruses [9]. In clinical practice a specific virus is often not identified, owing to the lack of available sensitive tests, the presence of as yet unidentified pathogens, or the failure to use appropriate tests.

The major causes of ARI in children and adults are influenza viruses A, B, and C [10,11]; parainfluenza virus (PIV) types 1, 2, and 3; adenovirus [12,13]; respiratory syncytial virus (RSV) [14]; and rhinovirus [15]. In the past decade, several new respiratory viruses, including avian influenza viruses (H5N1, H7N7, H7N3), human metapneumovirus (hMPV) [16], human

bocavirus (hBoV); severe acute respiratory syndrome coronavirus (SARS CoV), human coronaviruses (hCoV) NL63 and HKU1, polyomavirus WU/KI, parvovirus 4 and 5, mimivirus, and the swine-origin pandemic A/H1N1 influenza (H1, hemagglutinin type 1 protein; N1, neuraminidase type 1), have emerged and been recognized as causing upper respiratory tract infections and lower respiratory tract infection. The clinical importance of some of these newly discovered viruses is currently under investigation [17,18]. It is now clear that rhinoviruses and coronaviruses, once thought to cause only a common cold, can infect the respiratory tract [19] and in some cases may cause fatalities. All of the viruses mentioned above can cause both upper and lower tract infections [20] and have overlapping clinical presentations, and physicians usually can not distinguish the causative agent without a laboratory diagnosis.

Conventional methods of viral diagnosis, including cytologic, immunologic, and molecular biologic detection, are limited both by the ability to isolate only a limited range of

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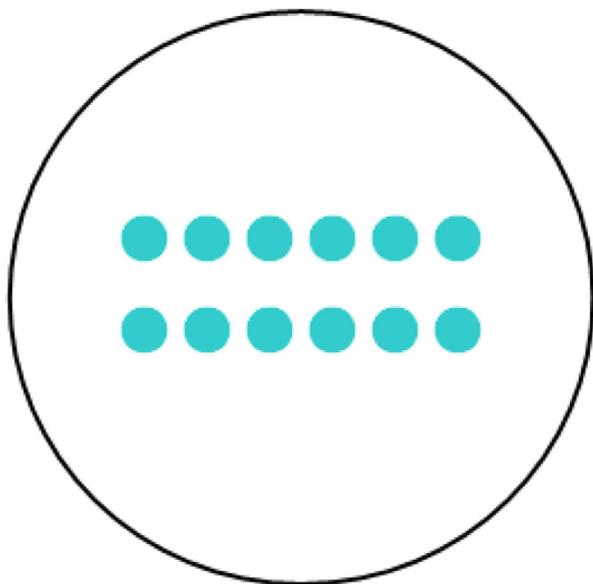


Fig 1. Cellular porin chip spotting hole arrangement diagram. From left to right: influenza virus, parainfluenza virus, respiratory syncytial virus, human metapneumovirus, bocavirus, and coronavirus.

the viruses causing respiratory diseases and by the restricted range of viruses that can be diagnosed rapidly with the use of antigen detection [21]. However, given the high cost of testing, complex operation, long cycle, high requirements of equipment and operators, and other factors, this technology has significantly limited applications in rapid diagnosis of respiratory viral infections, particularly early diagnosis, which has a decisive role in developing rapid and accurate treatment programs. Nucleic acid amplification tests (NATs) have been recognized to play an important role in diagnosing respiratory virus infection since the mid-1990s, but it was the emergence of CoV in 2003 that showcased the importance of these tests for diagnosing respiratory virus infection [22,23]. NATs have been developed for all respiratory viruses, including both conventional and emerging viruses [22].

Protein microarrays were developed because of the limitations of using DNA microarrays for determining gene expression levels in proteomics. The quantity of mRNA in the cell often does not reflect the expression levels of the proteins they correspond to. Because it is usually the protein, rather than the mRNA, that has the functional role in cell response, a novel approach was needed. Additionally post-translational modifications, which are often critical for determining protein function, are not visible on DNA microarrays. Protein microarrays replace traditional proteomics techniques such as 2-dimensional gel electrophoresis and chromatography, which were time consuming, labor-intensive, and ill-suited for the analysis of low-abundant proteins.

Among the respiratory viral infections, influenza virus, PIV, RSV, hMPV, hBoV, and CoV, which cause severe acute

respiratory syndrome, have become exceedingly common. The objective of the present study was to identify the early diagnostic substances of these 6 kinds of respiratory viruses and prepare monoclonal antibodies to develop a corresponding protein chip for rapid diagnostic kits.

MATERIALS AND METHODS

Identification and Sources of Early Diagnosis Substances

Given the simple virus structure, virus antigen determination is generally focused on the genetic material and proteins, particularly the nucleocapsid proteins, which have strong species-specific patterns. Therefore, nucleocapsid proteins can be good markers for early diagnosis. Based on the literature, the present study identified the early diagnostic markers of these 6 viruses: influenza virus (A and B) and nucleocapsid proteins of CoV (all kept in our laboratory), nucleocapsid proteins of PIV (Guangzhou Huayin Medical Technology Co), PV2 proteins of BoV (State Key Laboratory of Virology), fusion protein of hMPV F (Capital Institute of Pediatrics), and fusion protein of RSV F (Wuhan Institute of Biologic Products).

Laboratory Animals

Six- to 8-week-old female BALB/c mice were used in this experiment.

Preparation of Monoclonal Antibody

Animal Vaccination and Screening of Hybridoma. According to conventional methods, the BALB/c mice were immunized. Antigenic proteins were collected using Freund complete adjuvant at 100:100 $\mu\text{g}/\text{mice}$. The fusion of spleen cells and SP2/0 cells in mice was detected, and indirect enzyme-linked immunosorbent assay (ELISA) was used to screen hybridoma cells that secrete positive antibodies. Phalanx titration method was used to determine the most viable antigen protein concentration and to clone positively screened hybridoma cells. Cloning and subcloning of positive holes were repeated. Finally, a high A450 value was selected, as well as good-cell viability and single-hole cell lines for culture proliferation.

Cloning and Purification of the Positive Cell Lines. The positive hybridoma cell lines were cloned and purified from positive clone holes with the use of conventional methods until all cloned cell holes were positive in hemagglutination inhibition (HI) testing. Hybridoma cell lines received a continuous passage of 10, 20, and 30 generations. With the use of indirect ELISA determination of cell supernate titer, hybridoma cell lines were screened, which stabilized antibody secretion.

Measurement of Biologic Characteristics of Monoclonal Antibodies

Titers of monoclonal antibody in ascites were measured by means of indirect ELISA and HI test. Monoclonal antibody subtypes were detected by SBA Clonotyping System/HRP antibody subtype identification kits. Antibody specificity was identified with the use of Western blot.

Preparation of Microhole Plate Protein Chips

The microhole plate was placed on a vacuum filtration apparatus. Up to 50 μL 70% methanol concentration was added into each hole. Samples were incubated at room temperature for 30 seconds, and the filtration device was set to low vacuum. Each hole was rinsed with 200 μL ultrapure water twice and then activated.

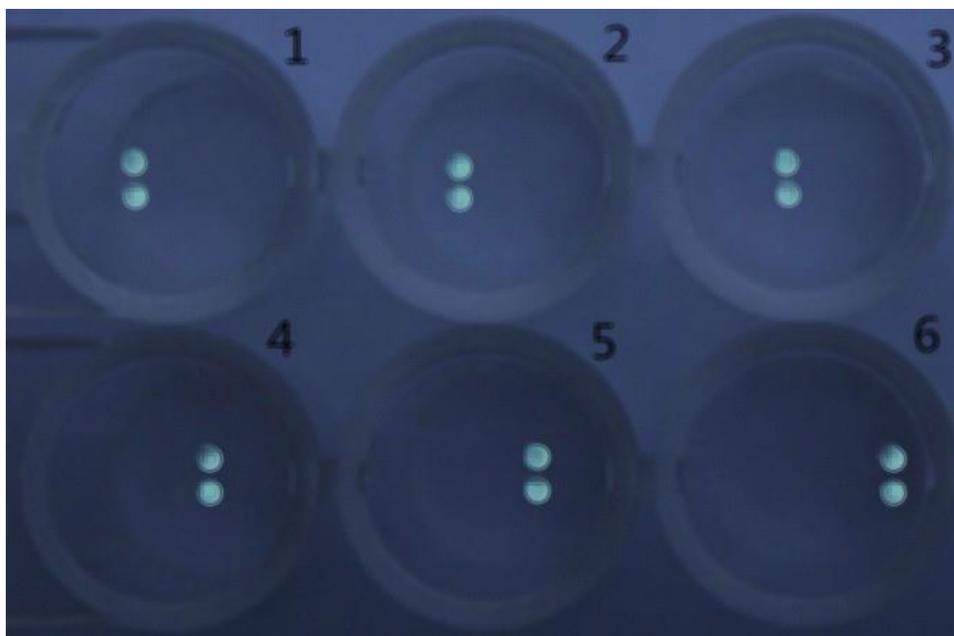


Fig 2. Six kinds of respiratory viruses in microhole plate chip serum detection. (1) influenza virus; (2) parainfluenza virus; (3) respiratory syncytial virus; (4) human metapneumovirus; (5) bocavirus; and (6) coronavirus.

The determined optimal concentration of dilution of the antibody proteins was detected in a well that raised and activated the microhole plate, 25 nL antibody proteins was sprayed using a tiny sprayer, much like that found in an inkjet printer (SmartArrayer 48, Bio-Rad, USA) in each hole with a microarrayer. Samples were quickly placed in the microhole plate into a wet box at 37°C and were fixed for 2 hours. The slide was removed after hydration, washed thrice with phosphate-buffered saline solution with Tween (PBST) for 5 minutes each time, dried at 37°C, and then placed in a sealed bag for preservation at 4°C.

Clinical Sample Test

We collected 299 patients with influenza virus infection, 288 with PIV infection, 305 with RSV infection, 278 with hMPV infection, 291 hBoV-infected patients, and 28 CoV-infected patients, and 120 healthy control subjects from the Department of Clinical Laboratory, Guangzhou Center for Disease Control and Prevention, People's Republic of China, to evaluate the rapid antigen tests for diagnosis of respiratory viral pathogens. All patients were affirmed as a single virus infection with the use of conventional respiratory virus detection methods. The normal control subjects were diagnosed without any kind of respiratory infections. Written informed consent conforming to the tenets of the Declaration of Helsinki was obtained from each participant before the study. The Institutional Review Board of Guangzhou Center for Disease Control and Prevention approved this study.

Up to 50 µL was obtained from each serum sample, which was added into 500 µL sample diluent (containing goat anti-human IgG), and then incubated for 15 minutes to neutralize the IgG. About 50 µL of the diluted serum was added into the detection hole, which was incubated for 1 hour at 37°C. The chips were washed with PBST thrice for 5 minutes each time. Approximately 50 µL horseradish peroxidase (HRP)-labeled mouse anti-human

IgM monoclonal antibody was added, and the sample was incubated for 0.5 hour at 37°C. The chips were washed again with PBST thrice for 5 minutes each time. Up to 30 µL tetramethylbenzidine (TMB) chromogenic reagent was added into each hole and kept chromogenic for 5 minutes at 37°C. The results were recorded visually with the use of a digital camera or scanner.

Validation and Analysis of Results

When a signal was detected, it was considered to be positive. The receiver operating characteristic (ROC) curve is a fundamental tool for diagnostic test evaluation [24]. In an ROC curve the true positive rate (sensitivity) is plotted as a function of the false positive rate (100 – specificity) for different cutoff points of a parameter. Each point on the ROC curve represents a sensitivity-specificity pair corresponding to a particular decision threshold. The area under the ROC curve (AUC) is a measure of how well a parameter can distinguish between 2 diagnostic groups (diseased/normal). The sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of cellular porin chip kit were calculated.

RESULTS

Serum chips revealed the contents of influenza virus, PIV, RSV, hMPV, BoV, and CoV in the serum microarray. The microhole distribution of each type of virus antibody is shown in Fig 1. Part of the microhole plate test results is shown in Fig 2, which indicated no cross-influence among the results.

The ROC curves for patients with acute respiratory infection compared with patients without respiratory infection were plotted. The AUCs for protein chip detection were: influenza virus, 0.985 (95% CI, 0.97–1; $P < .001$);

Table 1. Cellular Porin Chip Serum Pathogen Test Results

Pathogen Test	Diagnosis (Gold Standard)		Sensitivity	Specificity	PPV	NPV	Accuracy
	Case	Control					
Influenza	(n = 299)	(n = 120)	98.66	98.33	99.32	98.33	98.56
Positive (+)	295	2					
Negative (-)	4	118					
PIV	(n = 305)	(n = 120)	98.36	96.66	98.68	96.66	97.88
Positive (+)	300	4					
Negative (-)	5	116					
RSV	(n = 305)	(n = 120)	98.36	97.5	99.00	97.5	98.11
Positive (+)	300	3					
Negative (-)	5	117					
hMPV	(n = 278)	(n = 120)	98.2	95	97.84	95	97.23
Positive (+)	273	6					
Negative (-)	5	114					
hBoV	(n = 291)	(n = 120)	98.97	95.83	98.29	95.83	98.053
Positive (+)	288	5					
Negative (-)	3	115					
CoV	(n = 28)	(n = 120)	100	99.16	96.55	99.16	99.32
Positive (+)	28	1					
Negative (-)	0	119					

Abbreviations: PPV, positive predictive value; NPV, negative predictive value; PIV, parainfluenza virus; RSV, respiratory syncytial virus; hMPV, human meta-pneumovirus; hBoV, human bocavirus; CoV, coronavirus.

PIV, 0.984 (95% CI, 0.921–1.062; $P < .001$); RSV, 0.979 (95% CI, 0.961–0.997; $P < .001$); hMPV, 0.966 (95% CI, 0.942–0.990; $P < .001$); BoV, 0.974 (95% CI, 0.701–0.859; $P < .001$); and CoV, 0.996 (95% CI, 0.986–1.005; $P < .001$), respectively. Sensitivity, specificity, PPV, NPV, and diagnostic accuracy of acute respiratory virus were calculated according to the ROC and listed in Table 1.

DISCUSSION

The diagnosis of ARI relies on clinical examination [25], radiologic exploration [26], and biologic nonspecific inflammatory tests (including the level of C-reactive protein or procalcitonin). The identification of the causative agent(s) is often omitted or limited to a few pathogens easy to detect by rapid antigen direct tests (influenza viruses and RSV in respiratory specimens [27–29], *Streptococcus pneumoniae* and *Legionella pneumophila* in urine specimens). However, the distinction between viral and bacterial infections is often impossible with the use of nonmicrobiologic criteria [30].

The need for precise and rapid identification of the causative agents of ARI has been recently reviewed [31–33]. The main advantages of this strategy are: (1) better use of antimicrobials, including antiviral drugs and antibiotics, thus limiting the development of bacterial resistance; (2) reduction of unnecessary paraclinical explorations and of the duration of hospitalization; (3) rapid implementation of isolation measures when necessary, thus limiting the risk of nosocomial transmission; (4) collection in real time of new epidemiologic data on the seasonal spread of pathogens; and (5) identification of simultaneous or successive infections that may justify specific intervention or explain the severity of the clinical picture.

Protein chip has been recognized to play an important role in diagnosing respiratory virus infection. Protein chip is a high-throughput method used to track the interactions and activities of proteins and to determine their function, including on a large scale [34]. Its main advantage lies in the fact that large numbers of proteins can be tracked in parallel. The chip consists of a support surface, such as a glass slide, nitrocellulose membrane, bead, or microtiter plate, to which an array of capture proteins is bound [35]. Probe molecules, typically labeled with a fluorescent dye, are added to the array. Any reaction between the probe and the immobilized protein emits a fluorescent signal that is read by a laser scanner [36]. Protein microarrays are rapid, automated, economical, and highly sensitive, consuming small quantities of samples and reagents [37]. The concept and methodology of protein microarrays was first introduced and illustrated in antibody microarrays (also referred to as antibody matrix) in 1983 in a scientific publication and a series of patents [38]. The high-throughput technology behind the protein microarray was relatively easy to develop, because it was based on the technology developed for DNA microarrays, which have become the most widely used microarrays [39].

Protein array detection methods must give a high signal with a low background. The most common and widely used method for detection is fluorescence labeling, which is highly sensitive, safe, and compatible with readily available microarray laser scanners. Other labels can be used, such as affinity, photochemical, or radioisotope tags. These labels are attached to the probe itself and can interfere with the probe-target protein reaction [40]. Therefore a number of label-free detection methods are available, such as surface plasmon resonance, carbon nanotubes, carbon nanowire sensors (where detection occurs via changes in

conductance), and microelectromechanical system cantilevers [41,42]. All of these label-free detection methods are relatively new and are not yet suitable for high-throughput protein interaction detection; however, they do offer much promise for the future [43,44].

Protein chip is widely used for rapid detection of various diseases and establishes a certain foundation for early detection of febrile respiratory diseases. In the present study, 6 fixed early markers of corresponding febrile respiratory virus antibodies on an antibody chip were detected by means of fixing the antibodies and specific recognition of early virus markers that are found in human serum. The results showed that the specificity of selected antibodies for early markers are extremely strong, and the positive rate is ~100%, in which PIV, RSV, and BoV had 1 undetected case, probably because of experimental operation and other operator factors. However, the overall positive detection rate meets the required standard for a rapid-detection chip and has consistent accuracy compared with the ELISA method. The chip can be extremely useful for rapid detection of these 6 kinds of viral infection at an early stage.

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