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Original Article

Investigation of Atypical Bacteria and Virus Antigens in Respiratory Tract Infections by Use of an Immunofluorescence Method

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SUMMARY: In this study an immunofluorescence (IF) method was used to investigate the antigens of viruses and atypical bacteria in respiratory tract infections (RTI) in pediatric and adult age groups. In this prospective study of 2 years (1998 - 2000), IF was used to investigate the antigens of 7 viral and 3 atypical bacteria to be used for the etiological diagnosis of RTI. Sputum (33.6%) and nasopharyngeal aspirate specimens were obtained from pediatric patients (Group I, 76 cases) and adults (Group II, 135 cases) with RTI symptoms. Antigen detection rates were found to be 44.7% in Group I and 67.4% in Group II ($P < 0.05$). The following rates for specific antigens in Groups I and II, respectively, were as follows: *Chlamydia pneumoniae*, 17.1 and 13.3% ($P > 0.05$); *Mycoplasma pneumoniae*, 0 and 9.6% ($P < 0.05$); influenza A virus, 3.9 and 16.3% ($P < 0.05$); adenovirus, 3.9 and 14.8% ($P < 0.05$); parainfluenza virus type 1, 5.3 and 7.4% ($P > 0.05$); respiratory syncytial virus, 9.2 and 1.5% ($P < 0.05$); parainfluenza virus type 2, 3.9 and 3% ($P > 0.05$); and influenza B virus, 1.3 and 1.5% ($P > 0.05$). Mixed agents were found at a rate of 2.6 and 3.7% ($P > 0.05$) in Groups I and II, respectively. Parainfluenza virus type 3 and *Legionella pneumophila* antigens were not found. Since detecting etiological agents provides an important guide for determining the most appropriate antibiotic therapy, this IF method could be applied in clinical practice for arriving at a correct diagnosis and administration of effective treatment.

INTRODUCTION

Respiratory tract infections (RTI) are one of the most common diseases of patients seen at primary health care facilities. Many drugs, especially antibiotics, are prescribed for this group of infections (1,2). Due to the non-malignant pattern of development of the disease, physicians do not require routine etiologic diagnostic procedures; instead, they rely primarily on the results of previous epidemiological studies as a basis for diagnosis and they initiate the preferred therapeutic approach accordingly. The majority of diagnostic studies are based on serological methods, and therefore they have limited value for an early diagnosis of RTI (2,3). Wide and unreasonable use of antibiotics is facilitating infections with resistant strains, which results in a significant increase in morbidity and mortality rates. Early determination of etiological agents is the best means of preventing the unreasonable use of antibiotics (4,5).

Viruses and atypical bacteria can lead to a significant rate of morbidity. For the detection of these agents, various culture methods serve as the gold standard. However, microbial cultures require substantial effort, and it is difficult to work with a great number of samples at once; moreover, the results are first obtained within 3 - 7 days (1,6). The cost of molecular techniques is high, and therefore they are also not widely used in routine situations (7). Immunofluorescence (IF) and enzyme immunoassay (EIA) methods are becoming more widely used in the diagnosis of infectious diseases. IF is easy to apply and it can also be used to determine viral antigens, in which case it is a more reliable method than culture techniques and it has high sensitivity (70 - 95%) as compared to culture; in addition, the results can be obtained within 1 - 4 h after taking samples (8,9).

In both children and adults, the distribution of etiologic agents in cases of RTI is based upon numerous factors such as age, sex, geography, and underlying disease. In this study, IF was used to study RTI in both age groups, namely, the viral antigens and atypical bacteria in RTI were investigated for a period of 2 years.

MATERIALS AND METHODS

This prospective study was conducted in the outpatient clinics of the Faculty of Medicine, Karadeniz Technical University, Trabzon, Turkey. Patients were seen at the outpatient clinics between September 1998 and August 2000. The patients included in the study were classified as either children (Group I, $n = 76$) or adults (Group II, $n = 135$).

Patients included in the study were those who were not diagnosed with an RTI during in the previous month and presented with at least one of the following symptoms and findings: acute fever for less than 1 week (at least once, 37.8°C and above, temperature taken orally), coughing, sputum production, side pains, runny nose, throat pain, and/or aphonia (lowered voice). The following criteria were used to exclude patients: an immunosuppressed state, pregnancy, neoplasm, HIV (+), and discharged from the hospital within 10 days preceding the presentation of symptom.

Either sputum or nasopharyngeal aspirate (NFA) specimens were collected from the patients, depending on their sputum production at the time; specimens were produced once within

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7 days of the onset of symptoms. All subjects or parents of subjects consented to this procedure. By considering the clinical progress of the patients, decisions were made regarding the need for hospitalization. Respiratory syncytial virus (RSV); influenza A and B virus; parainfluenza virus (PIV) types 1, 2, and 3; adenovirus; and atypical bacteria including *Mycoplasma pneumoniae*, *Chlamydia pneumoniae*, and *Legionella pneumophila* were investigated in this study by using IF, as these agents are frequently responsible for RTI. The samples rapidly supplied to the laboratory that contained mucous were treated with N-acetyl-L-cysteine at a 1:1 ratio. The respiratory tract secretions (NFA or sputum) were buffered with phosphate buffered solution and were processed in a centrifuge and poured on to a special lam with small pockets; samples were then fixed with cold acetone for 5 min. IF antigen kits (Argeno Biosoft, Varilhes, France) containing *C. pneumoniae* (Anti-Chlamydia pneumoniae purified), *M. pneumoniae* (Anti-Mycoplasma pneumoniae purified), *L. pneumophila* (Anti-Legionella pneumophila purified), RSV (Anti RSV Group & Secondary FITC), adenovirus (Anti Adenovirus Group FITC), influenza A (Anti Influenza A Group FITC), influenza B (Anti Influenza B Group FITC), and parainfluenza types 1, 2, and 3 (Anti Parainfluenza Group FITC) were used, and the samples were stained by the present IF method. *C. pneumoniae* and the PIV group were stained using a direct IF method with higher sensitivity. *M. pneumoniae*, *L. pneumophila*, RSV, adenovirus, influenza A, and influenza B were investigated by using an indirect IF method (10,11).

As regard the direct IF method, the monoclonal antibody specific for all species was placed into the pockets of the lams, and the samples were incubated at 37°C. Following washing and drying, they were stained with Evans blue, which is a contrast material. With the IF method, the samples were subjected to incubation a second time after the monoclonal antibody incubation; for the second incubation, fluorescent conjugant goat anti-mouse (IgM and/or IgG) was used. Afterward, the samples were washed and dried. Glycerol drops were applied to the samples, which were then dried and covered with lamel and subjected to examination with an IF microscope, under 40x magnification. Infected cells (nuclear, cytoplasmic, or both) appeared greenish, and elementary bodies belonging to *Clamydia* were assessed as positive (10,11).

For the statistical analysis of the data, the qui-square test was carried out using SPSS 8.0.

RESULTS

While the average age in Group I was 2.71 ± 3.9 (1 month-14 years of age), the average age in Group II was 34.89 ± 15.2 (18-78 years of age). The number of cases was the lowest during the summer and highest during winter and spring (Table 1).

Monitoring of 41 pediatric patients (53.9%) and of 61 adult patients (45.2%) was carried out on inpatient basis. From 140 of the patients, NFA was used, and from 71 of the patients, sputum was taken for the study.

The viral antigen positivity in Group I was determined to be 27.6%, and the positivity of atypical bacterial antigens was 17.1%; the values for Group II were 44.4 and 23%, respectively. The positivity that was determined for all of the cases (virus + atypical bacteria) was 59.2%. The total positivity rate in Group II was higher than that of Group I ($P = 0.00699$). The highest rate of antigenic determination occurred during winter and spring. Mixed agents such as adenovirus and influenza A virus were idetified in two cases in Group I, and in five cases in Group II (three adenovirus + PIV type 1; one *C. pneumoniae* + influenza A; one *M. pneumoniae* + adenovirus) (Table 2). In the present study, no antigens belonging to either *L. pneumophila* or PIV type 3 were identified. In addition, no cross- reactions were observed.

DISCUSSION

Both lower and upper RTIs are seen, and the differential diagnosis of these two types is difficult when only the clinical findings are available (1,12); therefore, we did not separate these two types into two groups in the present study.

The isolation or identification of viruses and atypical bacteria that are frequently responsible for RTI is more difficult than that of other common pathogens. The majority of etiologic agents in cases of RTI are viruses (1,13). The application of a particular treatment against an atypical agent is generally not based on etiological grounds; as a result, the morbidity, the mortality, and the cost of treatment increase. Therefore, early and correct diagnosis according to reliable methods such IF or PCR is essential for the administration of appropriate antibiotic treatment (4, 12-15).

The distribution of etiologic agents responsible for RTI changes according to geography, season, age, race, and associated disease (2,4,5,10,13). Despite the yearly changes in the incidence of RTI, the incidence tends to reach a peak rate in

Table 1. Distribution of gender and seasons in the groups

Season	Group I		Group II		Total	
	men	women	men	women	men	women
Winter (Dec, Jan, Feb)	16	13	36	29	52	42
Spring (Mar, Apr, May)	14	12	22	13	36	25
Summer (Jun, Jul, Aug)	4	1	5	4	9	5
Autumn (Sep, Oct, Nov)	10	6	15	11	25	17
Total (%)	44 (58)	32 (42)	78 (58)	57 (42)	122 (58)	89 (42)

Table 2. Viruses and atypical bacteria defined in the groups

	Group I (76)		Group II (135)		Total (211)		$P^{1)}$
	n	%	*n*	%	*n*	%	
Viruses	21	27.6	60	44.4	81	38.4	< 0.05
RSV	7[2)]	9.2	2	1.5	9	4.3	< 0.05
Adenovirus	3	3.9	20	14.8	23	10.9	< 0.05
Influenza A virus	3	3.9	22	16.3	25	11.8	< 0.05
Influenza B virus	1	1.3	2	1.5	3	1.4	> 0.05
PIV type 1	4	5.3	10	7.4	14	6.6	> 0.05
PIV type 2	3	3.9	4	3.0	7	3.3	> 0.05
PIV type 3	0		0		0		
Atypical bacteria	13	17.1	31	23	44	21.1	> 0.05
C. pneumoniae	13	17.1	18	13.3	31	14.7	> 0.05
M. pneumoniae	0	0	13	9.6	13	6.2	< 0.05
L. pneunophila	0		0		0		
Mix agents	2	2.6	5	3.7	7	3.3	> 0.05
Non-identified	42	55	44	33	86	41	< 0.05
Total	34	45	91	67	125	60	< 0.05

[1)] Between Group I and Group II.

[2)] All cases were under 2 years-old.

winter. During winter, the RSV and influenza viruses are generally more prevalent. During spring, PIV (types 1 and 2) is prevalent, and during summer and fall, *L. pneumophila*, PIV type 3, and *M. pneumoniae* are more prevalent. Adenoviruses can be prevalent in any season (1,13,16-18). Our study covered a 2-year period in which the incidence rate peaked in both winter and spring.

Since the NFA contained infected epithelial tissue, it served as an ideal sample. IF was found to be a rather valuable means of diagnosing *M. pneumoniae* and *C. pneumoniae* (17,18), as well as viral agents. *Legionella* was identified in NFA sample, but a rather high- quality lower respiratory tract secretion was needed. Sputum was important for diagnosis as well (1,7). The majority of samples used in this study were NFA samples (66.4%).

As regards the distribution of etiological agents of RTI, in children who were below 2 years of age, RSV was prevalent; in elder children, other viruses (influenza, PIV, adenovirus, rhinovirus, etc.) and *M. pneumoniae* are usually more frequently seen (7, 8, 13, 15). In studies of RTI etiology, RSV has been reported at a rate of 15.8 - 69%, influenza virus at 2.8 - 23.8%, PIV at 2.3 - 5.3%, adenovirus at 5.7 - 27.3%, and *M. pneumoniae* at 2.4 - 27.4% (7, 18, 19). The two least common agents of RTI in children above 5 years of age are known to be *M. pneumoniae* and influenza. *C. pneumoniae* (0.8 - 31%), the incidence of which increases with age (18).

In Group I, the total antigen positivity rate was found to be 44.7%. The viral antigen positivity rate was 27.6% and the rate for atypical bacteria was 17.1%. The most frequently found virus was RSV. By taking into consideration that the average age was 2.7 ± 3.9 in the children studied, and the majority of clinical outcomes for those below 2 years of age were related to the RTI infection, especially to bronchiolar infection; here, the RSV rate was found to be significant ($P < 0.05$). Other viruses were observed at similar rates as those reported in previous studies (7-9, 13). The rate of *C. pneumoniae* was found to be 17.8%; despite its generally lower incidence rates in the pediatric population, its incidence increases with age. Different distribution rates can also occur among children, even among those living in the same region (18).

In the present etiological study of adult cases of lower and upper RTI, the *C. pneumoniae* was found at a rate of 0 - 18%, *L. pneumophila* at 0 - 12%, and *M. pneumoniae* at 1 - 22.4%. However, it has been reported that the rate of influenza A is 9 - 9.5%, the rate of influenza B is 2 - 22%, the rate of adenovirus is 1 - 5%, the rate of RSV is 2 - 7%, the rate of PIV type 1 is 1 - 2.5%, and the rate of PIV type 2 is 1.3 - 3% (3, 7, 16, 20, 21).

In Group II, the viral antigens were found at a rate of 44.4%, and the rate of atypical bacteria antigens was 23%. Antigens belonging to influenza A virus (16.3%) and to adenovirus (14.8%) were most frequently seen. The rate of *C. pneumoniae* was 13.3% and that of *M. pneumoniae* was 9.6%. Influenza- and adenovirus-dependent RTI were more prevalent than other types, especially during winter. The rates determined here were similar to the values previously reported (5,16,17,20,22). PIV type 3 and *L. pneumophila* were seen mainly during the summer (1,2). Both culture and IF methods of identifying *L. pneumophila* antigen are known to be effective diagnostic approaches. However, high- quality lower respiratory tract samples are necessary for such studies. In this study, patients were generally seen in seasons other than summer, and the majority of samples were prepared from NFA specimens, so the reason for not being successful for determining the agent was attributed for these.

In Group I, mixed agents were found in two cases (2.6%). In Group II, mixed factors were found in five cases (3.7%). Five of the mixed cases were in form of a virus plus a virus and two were in the form of a bacteria plus a virus. In studies in which mixed RTI is the subject, determinations have been made at rates of 0 - 38% (2,22,23).

When cases are compared by age, the total positivity rate in Group II was found to be significantly higher ($P = 0.00699$). While influenza A, adenovirus, and *M. pneumoniae* antigen positivity were more frequently seen in adults, in pediatric cases, RSV was more often seen ($P < 0.05$) (Table 2). The distribution of our results was affected by different sampling sites, different groups, and different time periods of sampling.

In other studies, the rate of diagnosis, in cases having lower and upper RTI, can range between 20 - 66% (2,3,16). In such studies, serological methods are generally used to show seroconversion in paired serum samples; less frequently, antigenic definitions were used.

The rate of etiological diagnosis using IF method was determined to be 44.7% in Group I and 67.4% in Group II. When all of the present cases were taken into consideration, diagnosis of 59.2% of the cases was possible with the IF method. In lower RTI cases, the typical agents were *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Moraxella catarrhalis* (1,5,16). Other bacterial agents were not considered in this study.

The etiological diagnosis of RTIs is becoming the most important issue in the treatment of this disease. Using IF, a high positivity rate is possible to achieve, and this aspect is increasing the importance of this method, in spite of the need to search for antigens of a limited number of agents. Arriving at an etiological diagnosis is an important tool for the appropriate antibiotic treatment of RTI. We believe that this diagnostic method should be incorporated into common practice in the clinical setting to facilitate the establishment of correct diagnosis and effective treatment.

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