Jpn. J. Infect. Dis., 62, 260-264, 2009

Original Article

Assay of *Chlamydia pneumoniae*-Specific IgM Antibodies by ELISA Method—Reduction of Non-Specific Reaction and Resetting of Serological Criteria by Measuring IgM Antibodies—

Toshio Kishimoto*, Shuji Ando, Kei Numazaki[1], Kazunobu Ouchi[2], Tsutomu Yamazaki[3], and Chikara Nakahama[4]

Department of Virology I, National Institute of Infectious Diseases, Tokyo 162-8640; [1]Department of Virology III, National Institute of Infectious Diseases, Tokyo 208-0011; [2]Department of Pediatrics II, Kawasaki Medical School, Okayama 701-0192; [3]Department of Pediatrics, Saitama Medical School, Saitama 350-0495; and [4]Nakahama Clinic, Osaka 535-0003, Japan

(Received December 24, 2008. Accepted April 21, 2009)

SUMMARY: In the present study, we tried to reduce the non-specific reactions for measuring anti-*Chlamydia pneumoniae* IgM antibodies by the ELISA kit of HITAZYME C. pneumoniae Ab-IgM (HITAZYME IgM) by using a new absorbent. We also tried to reset the IgM cut-off index (ID) of HITAZYME IgM by testing serum samples from healthy children and healthy adults with no respiratory symptoms. The results suggest that the use of the new absorbent (anti-human IgG antibodies) may reduce the non-specific reactions by rheumatoid factor and anti-nuclear antibodies, and that the setting of the higher cut-off ID (2.00), calculated as the mean ID + 3SD of the serum samples from healthy children and healthy adults, respectively, would improve the specificity of IgM during the measurement by HITAZYME IgM.

INTRODUCTION

For the diagnosis of *Chlamydia pneumoniae* infection, "HITAZYME C. pneumoniae Ab-IgG, Ab-IgA and Ab-IgM (HITAZYME IgG, HITAZYME IgA and HITAZYME IgM, respectively)" (1-5), based on enzyme-linked immunosorbent assay (ELISA), have generally been used at clinical inspection laboratories to provide inspection services under the public health insurance system in Japan.

Because the level of IgM antibodies usually increases at the acute stage of infection, the measurement of IgM has been considered to provide helpful information for the early diagnosis of *C. pneumoniae* infection. The IgM diagnostic criteria for single serum samples has been established for Wang's micro immunofluorescence test (micro-IF) (6), which has been recognized as a world standard method, and also for the authors' microplate immunofluorescence antibody technique (MFA) (7,8). As the IgM diagnostic criteria for this ELISA method (HITAZYME IgM), the cut-off index (ID) was originally set at 1.10 by calculating the mean ID + 2SD each for the IgG, IgA and IgM antibodies in healthy children (0 - 5 years of age), who had tested negative for the IgG, IgA and IgM antibodies by the micro-IF method (6). Later, an additional cut-off of ID 1.60 was set for adults by calculating the mean ID + 2SD for IgM antibodies in healthy adults with no respiratory symptoms (9,10). Since then, the cut-off of ID 1.10 for children and ID 1.60 for adults has been provisionally applied.

In recent years, with the accumulation of data, it has been noted that the IgM positive rate was higher than expected. In some positive cases, no increase of IgG or IgA was observed after the increase of IgM (11). The results of false positives related to rheumatoid factor (RF) and other factors have also been reported for adults. The need to resolve the problem of non-specific reactions and to review the serological diagnostic criteria has been pointed out (12).

Thus, in this study, we attempted to reduce non-specific reactions by using the new absorbent instead of the current latex solution for serum samples highly positive for RF and to reset the IgM cut-off ID for HITAZYME IgM by using serum samples from healthy children and healthy adults. The validity of the new IgM cut-off ID was evaluated by testing serum samples from prospective patients with pneumonia or acute bronchitis, and from retrospective patients who had already been diagnosed with acute *C. pneumoniae* infection. Based on the results of this study, we revised the past serological criteria of the authors' "Standard for the diagnosis of *C. pneumoniae* acute infection (proposed)" (13-15).

MATERIALS AND METHODS

Target patients: Prospective patients suffering from pneumonia or acute bronchitis were targeted. A total of 132 patients, including 63 pediatric patients (0 - 15 years of age) and 69 adults patients (≧16 years of age), who visited 19 facilities (such as university hospitals, general hospitals and the offices of pediatricians, general physicians and respiratory physicians) from May 2005 until March 2007, became the subjects of this study. Among them, 85 cases had pneumonia (16 serious cases and 69 moderate cases) and 47 had acute bronchitis. Informed consent was obtained from all patients. Prior approval was also obtained from the Ethical Review Board at each of the facilities.

To study the retrospective cases, we also performed additional testing by using serum samples from 126 patients (including 16 children and 110 adults) with respiratory diseases who had already been diagnosed with *C. pneumoniae* acute infections by the authors' MFA method (7,8) from 1989 until

*Corresponding author: Present address: Okayama Prefectural Institute for Environmental Science and Public Health, 739-1 Uchino, Minami-ku, Okayama 701-0298, Japan. Tel: +81-86-298-2681, Fax: +81-86-298-2088, E-mail: toshio_kishimoto@pref.okayama.lg.jp

2000.

Test materials: As the test materials for our study, we collected 207 serum samples (78 pediatric samples and 129 adult samples) by drawing blood from all of the 132 prospective patients (63 children and 69 adults) mentioned above. For the testing of retrospective cases, we used a total of 224 stored serum samples (20 children and 204 adults). These serum samples were tested by both the ELISA method and micro-IF method for the measurement of antibodies.

The RFs of all the above serum samples were measured using the RF measurement kit "IATRO-RF II" (Mitsubishi Kagaku Iatron, Inc., Tokyo, Japan), and anti-nuclear antibodies (ANA) of part of the above serum samples were measured by the fluorescent antibody method (outside order to SRL, Inc., Tokyo, Japan).

To reset the IgM cut-off ID, 232 serum samples from healthy children (0 - 15 years old) and 417 serum samples from healthy adults (≧16 years old) with no respiratory symptoms were used after informed consent was obtained.

Methods for measuring of antibodies: (i) ELISA method: The levels of anti-*C. pneumoniae* IgG, IgA and IgM antibodies were measured using "HITAZYME C. pneumoniae Ab-IgG, IgA and IgM" (Hitachi Chemical Co. Ltd., Tokyo, Japan), respectively. The ID values for IgG, IgA and IgM were calculated for each. When the ID was 1.10 or greater, the case was considered to be positive for IgG and IgA. For IgM, we compared the cases by applying the new cut-off ID and the current cut-off ID of 1.10 for children and 1.60 for adults. Furthermore, a new absorbent (anti-human IgG antibodies) (16) was used instead of the latex solution (denatured human γ globulin reacted latex solution). This new absorbent has proven effective in reducing non-specific reactions caused by RF (16).

(ii) Micro-IF method (6): After the 8-fold dilution of each serum sample by using the above new absorbent, 2-fold dilution series were prepared to add to the purified *C. pneumoniae* elementary bodies (EBs) fixed on the slide glasses for reaction. After the reaction of fluorescence-labeled secondary antibodies, *C. pneumoniae* EBs were observed through the fluorescent microscope (magnification ×400). If the fluorescence of EBs could be observed under the fluorescent microscope, the number of fold times for the dilution rate was recorded as the titer of antibodies. If the fluorescence of EBs was not visible with the 8-fold serum dilution, a result was recorded as <×8. According to the criteria at the National Institute for Infectious Diseases, which was established based on our experiences, when the titer of IgM antibodies was ≧×32, the result was considered positive.

RESULTS

Resetting of IgM cut-off ID: We tested 232 serum samples from healthy children with no respiratory symptoms as well as 417 serum samples from healthy adults with no respiratory symptoms by HITAZYME IgM using the current latex solution. To improve the specificity, we calculated the mean ID + 3SD. As a result, ID 2.11 and ID 2.06 were obtained from the healthy children and healthy adults, respectively (data not shown). In addition, above 232 serum samples from healthy children and 142 serum samples, having good conditions for measuring, out of 417 from healthy adults were tested by HITAZYME IgM using the new absorbent. From the data obtained, the mean ID + 3SD were calculated, respectively. In comparison, the values of the mean ID + 3SD were almost equivalent between the new absorbent and the current latex solution (data not shown). Thus, as a provisional standard, we set the new cut-off ID at 2.00.

Effects of new absorbent in reduction of non-specific reactions: After RF quantification, 207 serum samples from prospective cases were tested by HITAZYME IgM for comparison of the effect of the new absorbent with the current latex solution. Table 1 indicates the results of 18 serum samples showing positive RF (≧30 IU/mL). As shown in Table 1, for each of the 7 serum samples (≧500 IU/mL RF), the level of ID decreased when using the new absorbent compared with the current latex solution. For 11 serum samples (<500 IU/mL RF), there was no difference in judgment of IDs between the current latex solution and the new absorbent when the new cut-off of ID 2.00 was used.

Table 2 indicates the results of the ANA measurement for some of the low RF serum samples that showed lower IDs using the new absorbent. Four out of 5 serum samples from prospective cases (A) and 5 out of 7 serum samples from retrospective cases (B) were ANA positive.

IgM-ID distribution of patients with respiratory infections: Figures 1A and B indicate the distribution histograms of IgM-ID obtained for the first serum samples from prospective pediatric patients (63 cases) when using the new absorbent and current latex solution, respectively. The rates of ID ≧ 1.10 and ID ≧ 2.00 were 42.9% (27/63) and 20.6% (13/63), respectively, when using the new absorbent and 44.4% (28/63) and 12.7% (8/63), respectively, when using the current latex solution.

Figures 2A and B indicate the distribution histograms of IgM-ID obtained for the first serum samples of prospective adult patients (69 cases) when using the new absorbent and current latex solution, respectively. In these cases, the rates of ID ≧ 1.60 and ID ≧ 2.00 were 14.5% (10/69) and 5.8% (4/69), respectively, when using the new absorbent and 15.9% (11/69) and 10.1% (7/69), respectively, when using the current latex solution.

From the results of measuring IgG and IgA by HITAZYME IgG and HITAZYME IgA, respectively, of the prospective cases (data not shown), the IgG positive rates were 19.0% (12/63) in pediatric cases and 50.7% (35/69) in adult cases,

Table 1. Comparison of IgM-ID values of prospective patients sera from results by using new absorbent compared with latex solution

Sample no.	Sex	Age	RF (IU/mL)	IgM-ID	
				Latex solution	New absorbent
1-①	M	34	61	1.22	1.07
1-②	M	34	63	1.49	1.12
2-②	F	61	75	0.95	0.44
3-①	F	61	80	1.36	1.05
3-②	F	61	88	1.13	1.22
4-②	F	37	90	0.71	0.27
2-①	F	61	101	1.08	0.48
5-②	M	66	254	0.46	0.12
4-①	F	37	308	0.43	0.25
6-①	F	33	359	1.03	0.20
7-①	F	19	384	1.34	1.60
6-②	F	33	645	1.06	0.22
8-②	M	70	753	7.17	1.80
9-②	M	80	756	6.82	0.85
9-①	M	80	1,060	4.01	0.63
8-①	M	70	1,190	6.71	1.65
10-①	F	59	1,650	2.92	0.92
9-③	M	80	3,490	8.52	0.65

RF, rheumatoid factor.

Table 2. ANA titers of samples with low RF values and decreased IgM-ID from results by using new absorbent compared with latex solution

A. Prospective patient samples

Sample no.	Sex	Age	RF (IU/mL)	IgM-ID		ANA	
				Latex solution	New absorbent		
11-①	M	76	3	2.26	0.66	×80	+
12-①	M	80	0	1.28	0.84	<×40	−
13-②	F	77	1	1.34	0.84	×40	+
14-①	M	76	15	2.42	0.44	×40	+
15-②	M	74	3	1.52	1.08	×40	+

B. Retrospective patient samples

Sample no.	Sex	Age	RF (IU/mL)	IgM-ID		ANA	
				Latex solution	New absorbent		
80	M	75	0	3.03	1.04	<×40	−
36	M	73	1	3.76	1.18	×40	+
42	M	34	9	2.43	0.96	×40	+
93	M	72	13	1.94	0.70	×40	+
102	M	82	0	2.25	0.80	×40	+
109	M	79	20	3.19	0.37	×40	+
137	M	83	10	3.68	0.20	<×40	−

ANA, anti-nuclear antibody.

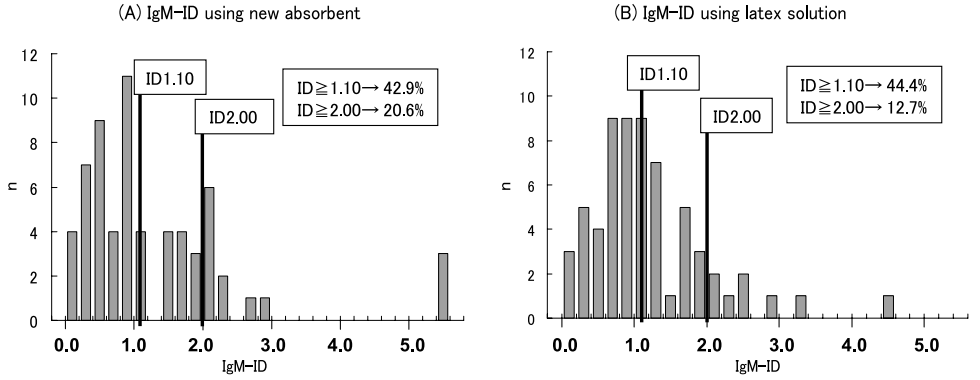

Fig. 1. IgM-ID distribution of prospective pediatric patients (n = 63) with respiratory infections from results by using new absorbent (A) compared with latex solution (B).

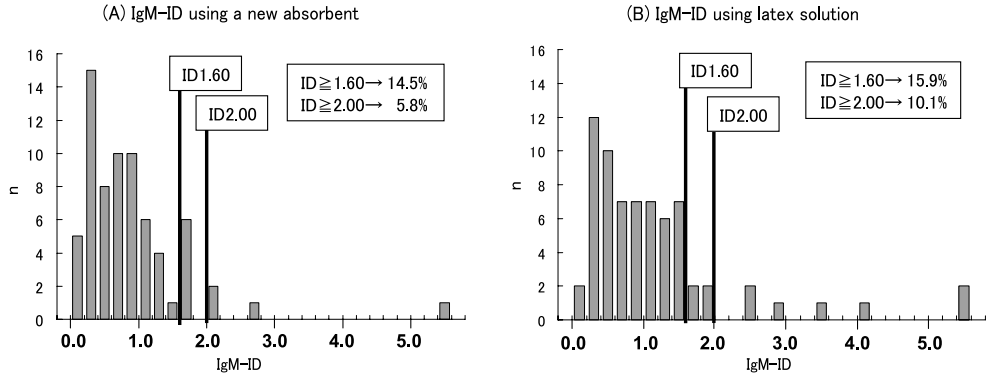

Fig. 2. IgM-ID distribution of prospective adult patients (n = 69) with respiratory infections from results by using new absorbent (A) compared with latex solution (B).

and the IgA positive rates were 6.3% (4/63) in pediatric cases and 50.7% (35/69) in adult cases. The IgG positive and/or IgA positive rates were 20.6% (13/63) in pediatric cases and 62.3% (43/69) in adult cases. In pediatric IgG and/or IgA positive cases, the rate of IgM-ID ≧ 2.00 was 38.5% (5/13) when using the new absorbent, and 15.4% (2/13) when using the latex solution. On the other hand, in adult IgG and/or IgA positive cases, the rate of IgM-ID ≧ 2.00 was 4.7% (2/43) when using the new absorbent and 11.6% (5/43) when using the latex solution.

Comparison with the results of the micro-IF method: When the 132 serum samples from prospective patients were tested by the micro-IF method for IgM antibodies, 15 samples (11.4%) showed ≧×32. In a comparison of these cases with the above positive cases of ID ≧ 2.00, the following data was obtained, 33.3% for sensitivity, 89.7% for specificity,

Table 3. New serological criteria for diagnosis of *C. pneumoniae* infections by ELISA method (HITAZYME C. pneumoniae Ab)

Acute infection	Sample	Antibody	HITAZYME	Micro-IF
Positive[1)]	Single serum	IgM	ID ≧ 2.00	≧×32
	Paired sera	IgG	ID rise ≧ 1.35	rise ≧×4
		IgA	ID rise ≧ 1.00	−
Equivocal[2)]	Single serum	IgM	1.10 ≦ ID < 2.00	−
		IgG	ID ≧ 3.00	≧×512
		IgA	ID ≧ 3.00	−

[1)]: Finally diagnosed as having acute infection.
[2)]: Might have acute infection. Acute infection was doubted.

29.4% for positive predictive value (PPV) and 91.3% for negative predictive value (NPV). Additionally, 224 serum samples from retrospective patients were tested by the HITAZYME IgM and micro-IF method for IgM antibodies. As a result, 52 serum samples (23.2%) showed ID ≧ 2.00, while 97 serum samples (43.3%) showed ≧×32 by the micro-IF method. When the results were compared between the two methods, 36.1% for sensitivity, 86.6% for specificity, 67.3% for PPV and 64.0% for NPV were obtained for HITAZYME IgM.

Review of diagnostic criteria for HITAZYME C. pneumoniae Ab-IgM: Table 3 shows the newly revised serological diagnostic criteria. For HITAZYME IgM, if a result is ID ≧ 2.00, the case will be judged as "acute infection is positive". If a result is 1.10 ≦ ID < 2.00, the case will be judged as "acute infection is equivocal, or may be positive," because it may be judged as antibody positive under the current criteria (ID ≧ 1.10).

DISCUSSION

In this study, we first evaluated the effects of the new absorbent in comparison with the current latex solution used for pretreatment of serum samples during the process of IgM measurement by HITAZYME IgM, for the purpose of reducing the non-specific reactions that can cause a false positive (13).

In the case of patients who are positive for anti-*C. pneumoniae* IgG antibodies and positive for IgM-class RF, it is known that a "false positive" occurs when IgM-class RF non-specifically reacts with specific IgG antibodies bound to antigens. However, if the new absorbent reacts with specific IgG antibodies in advance to inhibit the binding of specific IgG antibodies to antigens, it is thought that the non-specific reaction of IgM-class RF may be reduced and, therefore, "false positives" may be decreased. Furthermore, when patients are strongly positive for specific IgG antibodies and positive for specific IgM antibodies, excessive amounts of specific IgG antibodies may bind to antigens and hamper the binding of specific IgM antibodies to antigens ("false negative"). In these cases, it is expected that the new absorbent may react with excessive specific IgG antibodies and enable specific IgM antibodies to react with antigens, thereby decreasing the "false negatives" (13).

As our basic data, when using the latex solution for pretreatment, no effects existed from co-existent substances on the measurement of IgM antibodies by HITAZYME IgM, if the RF was <500 IU/mL. Among the serum samples collected from prospective patients at each of the facilities, several serum samples showed high RF (≧500 IU/mL). As these high RF serum samples also showed high ID non-specifically for IgM (Table 1), it was believed that a high RF might have been one cause of the "false positives". In order to resolve this "false positive" problem, we used anti-IgG antibodies solution as the new absorbent, and succeeded in lowering the high IDs. It was noted that in some cases where the RF was low, the serum samples showed lower IDs. We also investigated these cases, and found that many were ANA positive (Table 2). It was suggested that a positive ANA might also have been one of the causes of the "false positives".

In this study, we also revised the IgM cut-off ID, since we considered the high IgM positive rate to be due to the too low cut-off ID obtained from using serum samples from healthy children (0 - 5 years of age) who had tested negative for IgG, IgA and IgM by the micro-IF method. At this time, we prioritized the specificity in setting the new cut-off ID, calculated the mean ID + 3SD (in which range 99.8% of healthy children and adults might be statistically included) and, consequently, set ID 2.00 as a new provisional cut-off ID. The rates of serum samples showing ID ≧ 2.00 were 1.7% (4/232) of healthy children and 2.4% (10/417) of healthy adults. We presumed that these cases might have been infected within a period of several months, but were either cured naturally or showed no symptoms at the time of measurement. However, since the use of the new absorbent produced almost the same results as the use of the current latex solution in calculation of the mean ID + 3SD of serum samples from healthy people, it was assumed that the use of the new absorbent could specifically reduce the non-specific reaction by high RF and positive ANA in the case of *C. pneumoniae* infected patients.

A total of 207 serum samples (78 pediatric samples and 129 adult samples) were collected from 132 prospective patients (63 children and 69 adults) and the paired or plural serum samples obtained from the same patients were studied for the difference among the IDs. However, almost no differences were observed among the IDs of paired or plural serum samples (data not shown). Therefore, in order to exclude the effects of IDs from the plural serum samples from one patient, the data of the first serum samples from the prospective patients were used, as shown, for example, in Figures 1 and 2.

As shown in Figures 1 and 2, the distribution of IgM-ID histograms had a peak at ID < 2.00. The number of cases began to decrease for ID ≧ 2.00. That is 20.6% (13/63) of prospective pediatric patients and 5.8% (4/69) of prospective adult patients showed ID ≧ 2.00. On the other hand, 23.3% of the 224 retrospective patients showed ID ≧ 2.00 for IgM when measured by HITAZYME IgM. It is thought that, for the retrospective patients, since many of the cases of IgM-ID < 2.00 showed IgG and/or IgA positive, there is no problem in the judgment of *C. pneumoniae* infection.

The comparison of the results between the HITAZYME IgM and micro-IF methods showed that under the new diagnostic criteria, because of the low sensitivity and PPV, some positive cases may be overlooked if antibodies do not increase sufficiently at the time of measurement. Therefore, if the specificity and NPV are high and the case shows ID ≧ 2.00 for IgM, there is greater certainty that the sample is positive, and the problem of a high positive rate due to the low cut-off ID can be resolved. Taking into account the low sensitivity, it is proposed that a case of 1.10 ≦ ID < 2.00 will be judged equivocal (in some cases judged as equivocal, however, the titer of antibodies may increase sometime after the measurement), and final judgment will be given when findings of the IgG and IgA are obtained.

To date, it has been reported that in the case of reinfection, the titer of IgM antibodies to *C. pneumoniae* do not increase

at all or do not increase enough for measuring. Even if the IgM antibodies are measurable, we believe that improving the specificity is important for providing more accurate and helpful information for the diagnosis of *C. pneumoniae* infection at clinical sites.

Therefore, we concluded that the value of ID 2.00 is a practical, valid new cut-off ID value of IgM for increasing the specificity in diagnosing by HITAZYME IgM, and propose that the new IgM cut-off ID be set at 2.00 for both children and adults as a new IgM diagnostic criteria for acute infection (Table 3) (14,15).

Where small children are concerned, however, it is known that the titers of IgG and IgA may not increase sharply soon after the infection begins. Even if the result is ID $\geqq$ 1.10, it is still possible that the case may be positive. This is why we have proposed to include an "Equivocal" (1.10 $\leqq$ ID < 2.00) range in the new criteria.

ACKNOWLEDGMENTS

This was a joint study conducted with the participants of the "Meetings for the Study of Serological Diagnosis of *C. pneumoniae* Infection". We express thanks to Dr. Shuusaku Haranaga of Ryukyu University School of Medicine, Dr. Yasuhito Higashiyama of Nagasaki University of Medicine, Dr. Kiyoyasu Fukushima of The Japanese Red Cross Nagasaki Genbaku Isahaya Hospital, Dr. Naoki Tsumura, Dr. Tomotaka Kawayama and Dr. Junichi Honda of Kyurume University School or Medicine, Dr. Tadashi Ishida of Kurashiki Central Hospital, Dr. Naoyuki Miyashita of Kawasaki Medical School, Dr. Takaya Maruyama of Mie University School of Medicine, Dr. Satoshi Iwata of National Hospital Organization Tokyo Medical Center, Dr. Hisaichi Bannai and Dr. Teruko Komoda of Kyorin University, Dr. Toshihiro Tanaka of Mito Kyodo General Hospital, Dr. Shuichiro Inoue of Inoue Shiro Pedriatrics Clinic, Dr. Tomohiro Oishi of Niigata Prefectural Shibata Hospital, and Dr. Katsushi Kaji and Dr. Urara Koudera of Nakano Children's Hospital.

REFERENCES

1. Kishimoto, T., Kubota, Y., Matsushima, T., et al. (1996): Assay of specific anti-*Chlamydia pneumoniae* antibodies by ELISA method: 1. Evaluation of ELISA kit using outer membrane complex. J. Jpn. Assoc. Infect. Dis., 70, 821-829 (in Japanese).
2. Morikawa, T., Kishimoto, T., Izutsu, H., et al. (2000): HITAZYME C. pneumoniae; a new ELISA for measuring specific anti-*Chlamydia pneumoniae* antibodies. Proceedings of the 4th Meeting of the European Society for Chlamydia Research, 4, 122.
3. Kishimoto, T., Kubota, Y., Matsushima, T., et al. (1996): Assay of specific anti-*C. pneumoniae* antibodies by ELISA method: 2. Studies on clinical usefulness and serological diagnostic standards. J. Jpn. Assoc. Infect. Dis., 70, 830-839 (in Japanese).
4. Kishimoto, T., Matsushima, T., Morikawa, T., et al. (1999): Assay of specific anti-*Chlamydia pneumoniae* antibodies by ELISA method: 3. Setting of serological criteria. J. Jpn. Assoc. Infect. Dis., 73, 457-466 (in Japanese).
5. Kishimoto, T., Morikawa, T., Izutsu, H., et al. (2000): Study of diagnostic criteria by HITAZYME C. pneumoniae for detection of acute *Chlamydia pneumoniae* infection. Proceedings of the 4th Meeting of the European Society for Chlamydia Research, 4, 108.
6. Wang, S.-P. and Grayston, J.-T. (1970): Immunological relationship between genital TRIC, lymphogranuloma venereum and related organisms in a new microtiter indirect immunofluorescent test. Am. J. Ophthalmol., 76, 367-374.
7. Bessho, S. and Matsumoto, A. (1984): A simple antibody assay method using inclusion bodies of *Chlamydia psittaci* as antigen–microplate immunofluorescence technique. Igakunoayumi, 128, 571-572 (in Japanese).
8. Kishimoto, T. (1990): Study of *Chlamydia pneumoniae* TWAR infectious diseases (1) –Examination of infection to mice and detection of anti-TWAR antibodies in serum by MFA method. J. Jpn. Assoc. Infect. Dis., 64, 124-131 (in Japanese).
9. Kishimoto, T., Ogawa, M., Shiga, S., et al. (2001): Setting of diagnostic criteria on adult human by *C. pneumoniae*–specific IgM antibodies using HITAZYME C. pneumoniae. J. Jpn. Assoc. Infect. Dis., 75, 194 (in Japanese).
10. Kishimoto, T. (2001): Emerging respiratory infectious diseases, *Chlamydia pneumoniae* infections. J. Respir. Mol. Med., 5, 215-223 (in Japanese).
11. Nakata, S., Omata, T., Takahashi, Y., et al. (2002): *Chlamydia pneumoniae* specific IgG, IgA and IgM responses were dissociated in children with acute lower respiratory tract infection. J. Jpn. Pediatr. Pulmo., 13, 136-140 (in Japanese).
12. Miyashita, N., Obase, Y., Fukuda, M., et al. (2006): Evaluation of serological tests detecting *Chlamydophila pneumoniae*-specific immunoglobulin M antibody. Intern. Med., 45, 1127-1131.
13. Sunagawa, K. and Iwai, S. (1998): Diagnosis and treatment of *Chlamydia pneumoniae* infection. The Records of Luncheon Seminar I at the Combined Meeting of 47th Meeting of the Japanese Society of Infectious Diseases of East Japan and 45th Meeting of the Japanese Society of Infection and Chemotherapy of East Japan (in Japanese).
14. Kishimoto, T., Ando, S., Yamazaki, T., et al. (2007): Resetting of diagnostic criteria of "HITAZYME C. pneumoniae Ab-IgM" in serological diagnosis of *Chlamydia pneumoniae* infectious diseases. J. Infect. Chemother., 55 Suppl., 196 (in Japanese).
15. Uehara, S. and Sunagawa, K. (ed.) (2007): The method of detection of pathogenic microbes for respiratory infectious diseases in children. 2. Micoplasma and Chlamydia: Guidelines for the management of respiratory infectious diseases in children in Japan 2007. p. 16-20 (in Japanese).
16. Martins, T.B., Jaskowski, T.D., Mouritsen, C.L., et al. (1995): An evaluation of effectiveness of three immunoglobulin G (IgG) removal procedures for routine IgM serological testing. Clin. Diagn. Lab. Immunol., 2, 98-103.