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ORIGINAL ARTICLE

COMPARATIVE STUDY OF ELISA AND IIFT IN DETECTING CHLAMYDIA PNEUMONIAE SPECIFIC IGG ANTIBODIES IN PATIENTS OF ACUTE CORONARY SYNDROME (ACS)

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ABSTRACT

Background: Chlamydia pnaumoniae is reported to be associated with coronary heart disease (CHD). Efficacy of available serologic tests for detecting C.pneumoniae antibodies has been debated. The aim of present study was to compare Enzyme Linked Immunosorbant Assay (ELISA) and Indirect Immunofluorescent Test (IIFT) for detecting specific antichlamydial antibodies in patients of Acute Coronary Syndrome (ACS).

Materials and Methods: Serum samples from 100 patients of ACS and 90 healthy controls were tested for the presence of Chlamydial IgG antibodies using ELISA and IIFT. To assess agreement between ELISA and IIFT, we used 'nominal scale variables". Agreement analysis was done using sensitivity, specificity, positive and negative predictive values (PPV and NPV) and diagnostic accuracy of the test.

Results: The ELISA and IIFT detected C.pneumoniae IgG antibodies in 66% and 48% respectively in patients of ACS, and 29% and 20% respectively of healthy controls. In patients of ACS, sensitivity and specificity of ELISA as compared to IIFT were 70.8% and 38.4% respectively. The PPV of ELISA for C.pneumoniae was 51.5% and NPV was 58.8%. The diagnostic accuracy of ELISA for C.pneumoniae was 54.6%. The two tests correlated in 54% of samples with a moderate agreement of =0.51.

Conclusions: The results of present study indicate that ELISA test was inferior to IIFT in detecting C.pneumoniae antibodies in patients of ACS.

Keywords: Enzyme Linked Immunosorbant Assay, Indirect Immunofluorescent Test, Chlamydia pneumonia, Acute Coronary Syndrome

INTRODUCTION

Chronic infections may initiate and perpetuate vascular endothelial damage. Chlamydia pneumoniae, an obligatory intracellular pathogen has been recently implicated in atherosclerosis. Epidemiological studies demonstrate a consistent association between elevated C.pneumoniae antibody titres and myocardial infarction or chronic coronary heart disease (CHD). Negative seroepidemiological studies have also emerged over the last few years.[1]

As majority of patients with CHD do not undergo surgery, serology for detection of raised level of C.pneumoniae antibodies has been used as indirect measure to assess association of C.pneumoniae with CHD.[2, 3] Persistently raised IgG and IgA titres, determined by microimmunofluorescence (MIF) or a variety of enzyme immunoassay (EIA) or enzyme linked immunosorbant assay (ELISA)

have been considered as one of the criteria for chronic infection. Seroepidemiological studies expose wide variations in diagnostic criteria, cut-off titres, adjustment for confounders, sample size, temporal variations in antibody and antigen titres etc. [2-6]

All immunofluorescent tests i.e. direct, indirect (IIFT) and MIF work on the same principle. Though MIF test is considered gold standard for detection of C.pneumoniae antibody, it requires a high level of expertise for correct interpretation and also an expensive fluorescent microscope. Further, extents of laboratory to laboratory variations as well as interpretation of results are not yet specifically addressed. Due to these drawbacks, several partially automated commercial ELISAs have been developed.[2,3,4]

In contrast to MIF, ELISA is easier to perform, less time consuming, objective end point, and technical accessibility and has an electronic record of results. However, these commercial ELISAs have not been fully validated. They seem to be less specific but more sensitive than the MIF test. [7]

To-date, no totally satisfactory serological method has been developed. Use of several different methods would be no problem, provided there is high grade agreement between the tests.[5]

The number of published reports investigating the role of C.pneumoniae in atherosclerosis has more than tripled in last 5 years compared to the preceding decade. Except for a few reports,[8-12] there is tremendous paucity of literature on this subject from our country. At least no work has been carried out from this part of our country.

As efficacy of available serologic tests for detection of C.pneumoniae antibody is debated, we evaluated two serological tests, ELISA and IIFT to detect C.pneumoniae specific IgG antibody in patients of Acute Coronary Syndrome (ACS).

MATERIALS AND METHODS

A total of 100 patients of ACS admitted in Intensive Coronary Care Unit of a tertiary care teaching hospital, were taken up for the study. Recruitment of patients began in January 2005 and the study was completed in June 2006.

Additionally, 90 healthy relatives of these admitted patients served as control group. These subjects were age and sex matched to the patients of ACS. None of them had any symptoms or signs of CHD as judged by negative history and normal resting ECG. All subjects were free of any chronic disease.

All 100 patients of ACS and 90 controls were screened for serum IgG antibodies against C.pneumoniae by ELISA and IIFT. Venous blood samples were drawn from all patients within 24 hours of admission. Sera were kept frozen at -20°C until analysis.

The ELISA and IFT kits manufactured by M/s EUROIMMUN Medizinische Labordiagnostika Ag, Germany, were used and the tests were carried out as per details and instructions supplied by manufacturers.

ELISA test:

Briefly, basis of antigen preparation were HEP-2 cells infected with the CDC/CWL 029 strain of C.pnuemoniae. Elementary bodies purified from cell lysates were treated with sodium dodecylsulfate. The used solutioncontain all relevant antigens localized in the outer membrane of the elementary bodies. The outer membrane is composed of lipopolysaccharide (LPS) and numerous proteins (outer membrane proteins, OMPs). The main portion is provided by the MOMP antigen (major outer membrane protein).

The test kit contains microtitre strips, each with eight break-off reagent wells coated with C.pneumoniae antigens. In the first reaction step, diluted patient samples were incubated with the wells. In case of positive samples specific IgG antibodies will bind to the antigens. To detect the bound antibodies, a second incubation is carried out using an enzyme – labelled anti-human IgG (enzyme conjugate) which is capable of promoting a colour reaction. Photometric measurement of colour intensity was made at a wavelength of between 620 nm and 650 nm within 30 minutes of adding the stop solution. The intensity of the formed colour is proportional to the concentration of antibodies against C.pneumoniae antigens.

For this quantitative assay, the upper limit of the reference range of non-infected persons (cut-off value) recommended by EUROIMMUN is 20 RU/ml. Readings were taken in automated ELISA reader.

IIFT test:

Cells (EU 38) infected with C.pneumoniae are the standard substrate used for the determination of specific antibodies to C.pneumoniae. the infected cells are mixed with non-infected cells to enable a direct comparison between positive and negative reactions. Cells infected with the bacterium are fixed as BIOCHIPs into the reaction fields of a microscope slide. EUROIMMUNE BIOCHIP slides are incubated using the proprietary TITERPLANE technique, which enables multiple samples to be incubated next to each other and simultaneously, under identical conditions. The slides were incubated with a primary antibody for 30 minutes at room temperature (+18°C to +25°C), washed twice for 5 minutes each with PBS-Tween and incubated with 20 µl of fluorescein labelled conjugated antihuman immunoglobulin for 30 minutes. Results are evaluated by fluorescence microscopy (Zeiss Axiostar Plus) with 40X objective.

Interpretation of result:

Positive reaction: In the positive specimens, evenly distributed Chlamydia pneumoniae Elementary Bodies were observed as bright fluorescent, regular, round, apple green particles (Figure-1).

Negative reaction: No specific fluorescence was observed in the cells (Figure-2).

If all cells in view were clearly fluorescing, it indicated presence of auto-antibodies against component of the cell nucleus or cytoplasm. Such pattern was differentiated from the fluorescence pattern observed in positive reaction. e.g. the cell nuclei of all infected and non-infected cell fluoresce, producing a homogenous pattern, whenever auto-antibodies are present against the cell nuclei.

Auto-antibodies against auto-antigens: If auto-antibodies are present against mitochondria, a granular fluorescent in the cytoplasm of all infected and non-infected cell is observed.

Incubation of the substrate with the positive and negative control sera provided in each kit verified correct performance of the test and aids evaluation. If a patient's sample contains antibodies against C.pneumoniae, the same pattern is essentially observed as for positive control serum. But if the positive control shows no specific fluorescence pattern or the negative control shows a clear specific fluorescence, the results were discarded and the test was repeated.

Statistical analysis:

All data were collected using standardized forms and analysed by SPSS software version (10.0). To assess the agreement between ELISA and IIFT, we used "nominal scale variables"[13] < 0.20 was defined as poor agreement, =0.21 to 0.40 as fair agreement, =0.41 to 0.60 as moderate agreement, =0.61 to 0.81 as very good agreement and = 0.81 to 1.00 as very good agreement. Agreement analysis was done using sensitivity, specificity, positive and negative predictive values (PPV and NPV) and diagnostic accuracy of the test. The value of P< 0.05 was considered significant.

RESULTS

Among 100 patients of ACS, there were 61 males and 39 females. Their mean age was 55 years (range 30-86 years). Out of 61 males, 30 patients (49.1%) were current smokers. None of the females was smoker. 62 patients had acute ST elevation myocardial infarction, 20 had non-ST elevation myocardial infarction and 18 had unstable angina.

As seen in Table, with ELISA test, seropositivty to C.pneumonia was detected in 66 (66%) out of 100 patients of ACS as compared to only 29 (29%) control subjects, the difference being statistically significant (X^2=13.34, P<0.001).

Table 1: Showing results of C.pneumoniae antibody detection by Indirect Immunofluorescence and ELISA tests

ELISA test	Indirect Immuno-fluorescence test (IIFT)		Total
	IIFT +ve	IIFT -ve	
ELISA +ve	34	32	66
ELISA -ve	14	20	34
Total	48	52	100

Sensitivity = 70.8%; Specificity = 38.4%, Positive Predictive value: 51.5 %, Negative Predictive value: 58.8 %, Diagnostic accuracy of test: 54.6%, Aggreement: 0.51 (moderate)

With IIFT, seropositivity to C.pneumoniae was detected in 48 (48%) out of 100 patients of ACS as compared to only 20 (20%) control subjects,

the difference being statistically significant(X^2=13.84, P<0.001).

Though seropositivity was observed in 66% of patients of ACS with ELISA as compared to 48% with IIFT, the difference was statistically not significant (P>0.05).

Comparison of IIFT and ELISA in patients of ACS reveals that 34 (34%) patients tested positive and 20 (20%) patients tested negative by both methods. We observed a concordance of 54% and a moderate agreement (=0.51) between the two methods. Sensitivity and specificity of the ELISA as compared to IIFT were 70.8% and 38.4% respectively. PPV of ELISA for C.pneumoniae was 51.5% and NPV was 58.8%. The diagnostic accuracy of ELISA test for C.pneumonia was 54.6%. (Table-1)

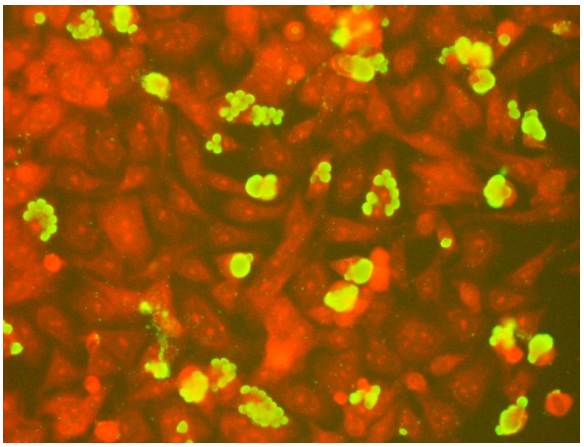

Figure 1: Positive immuno-fluorescence reaction

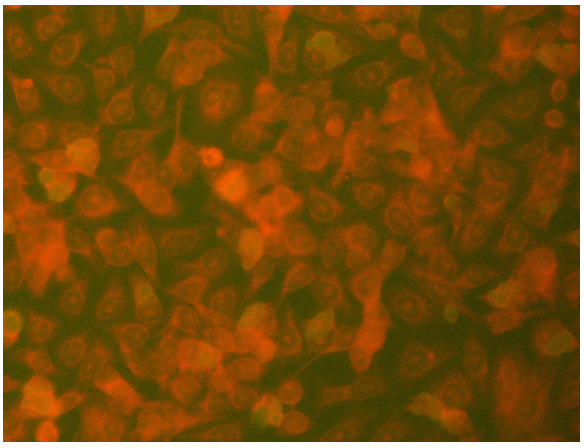

Figure 2: Negative immuno-fluorescence reaction

DISCUSSION

Saikku et al [14] first noted presence of elevated antibody levels or Chlamydial lipopolysaccharide (LPS) containing immune complexs in the sera of 50% to 68% of patients with acute myocardial infarction or chronic CHD as compared to 7% to 12% among controls. In the present study also, a significantly increased prevalence of seropositivity (66% ELISA and 48% IIFT) amongst 100 patients of ACS was noted as compared to 29% and 20% respectively among 90 healthy controls. The relation persisted virtually unchanged after adjustment for a wide range of possible confounding factors. As noted in present study, a number of published reports of different designs in Europe and the USA[1, 6, 14-16] generally support association of elevated IgG and / or IgA C.pneumoniae antibodies with acute myocardial infarction, although there are inconsistencies between reports. The findings of the present study are at variance with the reports of other research workers.[17-20]

Comparatively, very little work has been carried out from our country. Seropositivity in CHD as detected by ELISA ranged from 37.5% to 64%.[8, 9, 12]

Many factors influence selection of appropriate diagnostic test for serological detection of Chlamydial antibodies e.g. number of samples to be processed, cost of the test and expertise available. Moreover, before using a new test, sensitivity and specificity of each assay needs to be considered.[4]

In the present study, ELISA test revealed seropositivity in 66% of cases of ACS as compared to 48% by IIFT. The sensitivity and specificity of ELISA were 70.8% and 38.4% respectively as compared to IIFT. The PPV was 51.5%, the NPV was 58.8% and the diagnostic accuracy of ELISA for C.Pneumoniae was 54.8%.

These results compare favourably with observations made in the only other such study [12] carried out from our country. Satpathy et al [12] noted that using Immunocomb assay, 73.7% serum samples were positive for C.pneumoniae, while MIF assay detected 50.8% serum samples as positive. The sensitivity and specificity of the Immunocomb assay were 79.3% and 32.14% respectively in detecting C.pneumoniae antibodies. PPV of Immunocomb for C.pneumoniae was 54.8% and NPV was 60%. The diagnostic accuracy of Immunocomb test for C.pneumoniae was 56.1%.

The prevalence of antibodies to C.pneumoniae increases with age and is about 50-70% in

normal middle aged individuals as reported in Western literature.[6,7,14,21] In few studies carried out from our country, the prevalence of seropositivity ranged from 0-81%.[8-12] In the present study, 29% of controls were seropositive by ELISA and 20% by IIFT. Seropositivity observed by ELISA or IIFT was significantly more in patients of ACS as compared to controls, but the difference in seropositivity prevalence as judged by either method amongst controls was statistically not significant (P>0..05).

As such also, cross reactivity with other Chlamydial species may lead to unacceptable high rate of false positives. Therefore, while conducting seroepidemiological studies for association of C.pneumoniae with CHD, in view of high prevalence of C.pneumoniae seropositivity even in healthy individuals, only a species specific test should be preferred.

Different serological methods are being used, and the different results may be related to the choice of method. Some investigators have used methods detecting species specific MOMP antibodies. Others have used methods detecting genus specific LPS antibodies. MIF and IIFT test is an indirect fluorescent antibody test that measures specific antibodies to epitomes present in the cell walls of the elementary body particles. Even MIF methods differ from commercial methods to in-house MIF techniques. The different assays each have introduced minor variations in the materials used or in procedures to be followed e.g. different strains of C.pneumoniae as the antigen, different incubation times of sera with the antigen, and the use of fluorescein isothiocyanide – labelled anti-human immunoglobulin antibodies from different manufacturers.[21]

Some striking differences were observed with ELISA and IIFT in the present study. Some patients were seropositive by one method and seronegative by the other. This observation may be due to technical methodological differences, but it might also reflect differences in the immunological responses among individuals.

The differences in specificity level between the tests are one possible reason why some patients were seronegative by IIFT and seropositive by ELISA. The fact that some samples were seropositive by IIFT and seronegative by ELISA could be a result of higher sensitivity of the IIFT method, but there may be also other explanations for the diverging results than the sensitivity and specificity aspect of the tests. We cannot fully explain why some individuals have a dominance of persisting C.pneumoniae LPS or MOMP antibodies and others have dominance of species specific C.pneumoniae antibodies detected by IIFT. Whether this is caused by differences in the properties of the infectious agents, the clinical infection they induce, or the human immunological response, remain to be settled.

As per the claims of the manufacturer (EUROIMMUN), when ELISA and MIF were compared, 30 from 35 sera found positive in MIF and 15 from 15 sera found negative in MIF corresponding with ELISA results. Similarly, the sensitivity and specificity of IIFT were 98% and 100% respectively. Further, sensitivity and specificity of ELISA with reference to IIFT were 84.2% and 81.5% respectively.

Cross-reactivity with other Chlamydial species may also lead to unacceptably high rate of false positives in patient groups with other prevalent Chlamydial infection. Hence, only species specific test is needed to document real association of C.pneumoniae with CHD.[5]

The three Chlamydial species – C.trachomatis, C.psittasi and C.pneumoniae are very similar. Therefore, antibody against C.trachomatis and C.psittasi almost always show cross reactions with the LPS as well as the MOMP antigen of C.pneumonia. Thus, an exclusive determination of species specific antibodies against C.pneumoniae is not possible with the currently available tests systems. [5]

Cross reactivity with C.trachomatis and C.psittasi cannot be ruled out also in the IIFT with infected cells as the substrate or in MIF with elementary bodies as the substrate.

Some cross reactivity between MOMP of the different Chlamydial species might have occurred in the current estimation in the present study. Prevalence of C.psittasi antibodies is too low to have any significant influence on the result. [5] Specific methods for C.psittasi were not included in this study because no such ELISA is available in our country. Cross reactivity with C.trachomatis cannot be ruled out. However, the prevalence of C.trachomatis seropositivity is very low in our country. In one of the studies,[12] Immunocomb assay detected 74% of specimens as positive for C.pneumoniae as against only

18% for C.trachomatis antibodies in healthy blood donors.

Since ELISA, in contrast to the earlier complement fixation tests for Chlamydial LPS, is based on a Chlamydia specific small fragment of the LPS content, the probability of cross reactions to other gram negative bacteria is far lower than with earlier studies.[5]

Since we did not include serological analysis for other gram negative bacteria in our study, cross reactivities with the LPS components from non-Chlamydial organisms cannot be completely ruled out. All the same, manufacturers claim that no cross reactivity with other bodies has been noticed with either method.

A potential limitation of our study is that we only evaluated seropositivity to IgG and not to IgA. However, we do not believe this to be a major issue, because the great majority of cross-sectional and retrospective studies that suggested a positive association relied on IgG serology to determine exposure status. [4]

Schumacher et al [5] have pointed out that comparison of different assays for large-scale clinical trials may be valuable, but results should be viewed with caution. It is possible to make any assay appear sensitive by comparing it with a sub-optimal test, or by comparing the results of specimens of newer, more species specific assays. MIF is the only serological test that detects species and serovar specific responses against genus Chlamydia and thus scores over many of the commercially available ELISA tests. MIF and ELISA tests also detect reactivity to genus specific antigen of Chlamydial elementary or reticular bodies.[2,4,5]

The results of the present study indicate that ELISA test was inferior to IIFT in detecting C.pneumoniae antibodies in patients of ACS. However, ELISA has the advantages of rapidity and ease of performance. Hence, it can be very well used as a method for presumptive serology. Confirmatory diagnosis should include combination of tests, rather than deriving inference on the basis of single test results. Standardization and optimization of commercial ELISA tests, relative to MIF test, may enhance the performance of the former.[7]

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