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Performance of Microimmunofluorescence Test in Detection of anti-*ChlamydiaTrachomatis* Immunoglobulinsin Iraqi Infertile Males with Special Refer to Age Group Distribution

Ali Ibrahim Ali Al-Ezzy

Department of pathology, College of Veterinary medicine, Diyala University

Abstract: ***Background: The incidence of anti-chlamydia trachomatis immunoglobulins in serninal plasma and serum of infertile men was detected by microimmuno fluorscence (MIF) test .The main objective was to determine and compare the performance of seminal plasma and serum in the diagnosis of chlamydia trachomatis genital infection in Iraqi infertile men. Patients and Method: Seminal fluid and blood samples were collected from 178 infertile men and35 normalvolunteers .Serum and seminal plasma were examined by MIF technique. Results: Anti-chlamydia trachomatis immunoglobulins were detected by MIF in 68(49.27%) of serum. 108(75%)in seminal plasma and 39(37.5%)in bothserum and seminal plasma. Conclusion: Detection of anti-chlamydia trachomatis immunoglobulins by MIF in seminal plasma of infertile male more sensitive than serum and applicable for infertility centers.***

Keywords: *Chlamydia trachomatis*, infertility, immunoglobulines, semen parameters, microimmunofluorescence test, age,Iraq

1. Introduction

The significance of *C.trachomatis* as a cause of male infertility is debated[1]. There is epidemiological evidence that a symptomatic infection with *C.trachomatis*in men is associated with unexplained infertility[2].*C.trachomatis*regard a relevant for male infertility as infection may lead to stenosis in infected organs may also induce autoantibodies and /or inflammation and may impaired semen quality[3] . The difficulty of definitive diagnosis of *C.trachomatis* is the major reason for the debateregarding its significance [4].the classical cell culture technique cannot be used for semen samples due to cytotoxicity of semen [5].Consequently serological diagnosis of chlamydial infection was attempted but neither IgA, nor IgG in serum was reliable marker for acute infection [6].Inaddition, a further chlamydia species ,*C.pnumoniae*was detected. Antibodies against this species accounted for half of all Chlamydia IgGpositive cases attending genitourinary clinic[7].

This study was designed to determine the incidence of antichlamydia trachomatis immunoglobulins in seminal plasma and serum of infertile males using MIF test and compare the usefulness of seminal plasma and serum in the diagnosis of chlamydial genital infection in infertile men.

2. Patients and Methods

One hundred seventy eight infertile patients were examined in Kamal Al-Samarae Hospital for infertility and In vitro Fertilization from November 2000 to November 2001. Ethics committee of Baghdad University, college of medicine, approved the present research. At first the aim of study was explained for all participants and after obtaining their oral and signed consent they have been studied.The age of the patient ranged between 22-55 years and the duration of infertility problem ranged from 1-18 years. Serum and Seminal plasma were collected from 104 male, serums was collected from 34 male and seminal plasma was collected from 40 infertile male, the total serum samples were 138 and the total samples of seminal plasma were 144. The criteria for patients' selection were those having abnormal picture of seminal fluid during examination with history of primary and secondary infertility. Thirty five normal fertile volunteers were examined as a control group.

Patients were provided with clearly written or oral instructions as appropriate concerning the collection of semen sample. Each sample was collected after a minimum of 48 hours but not longer than seven days of sexual abstinence[8]. The sample was obtained by masturbation and ejaculated into clean, wide mouth container. The name of the man, the period of abstinence, the date and time of collection and the interval between collection and analysis were recorded.Seminal plasma was separated by centrifugation of semen samples at 300-500 g which is yet enough to remove the seminal plasma from cellular components. Seminal plasma was dispensed in aliquots and was kept at – 20^{o}C until used [8].

Blood samples

Ten ml of blood sample was collected from each infertile and normal fertile volunteer. Freshly isolated blood sample was incubated at 37^{o}C in water bath or incubator and centrifuged at 2000g for 30 minute and then after that serum was very carefully aspirated from the cells, blood clot, and aliguoted as required then kept at-20^{o}C until used[9].

Microimmunofluorescence test (MIF)

MIF test was used to examine the sera and seminal plasma of infertile and fertile males. Preparation of antigen smears. Tenμl of *C. trachomatis* antigen was applied to each slide circle, slide was air dried for at least 30 minute, it was important that all antigen dots were completely dried and fixed

in acetone for 10-15 minute at room temperature. Then the fixed slides were wrapped with clean papers and kept in airtight container at -20°C until used[10].

Fluorescein labeled conjugate dilution:

Two folds dilution of immunoglobulins conjugate were made in PBS, starting with 1:5 to 1:80. These were tested by MIF to demonstrate a dilution, which gives best fluorescence. Thus a dilution 1:10 was chosen for use in the test. Each immunoglobulin conjugate was reconstituted according to labeled directions and dispensed in to 50µl aliquots and were kept at -20°C until used.

Microimmunofluorescence test procedure :

The thawed slides were incubated for 30 min. at 37°C with the appropriate antiserum or seminal plasma dilution (two-fold dilution from 1:2 to 1:256) diluted in PBS, pH7.2. Three circles of each slide were used for positive serum, negative serum and antigen controls. Before being washed by dipping in two PBS Jar each for 5min. and in distilled water for 5min.; 10µl fluorescein isothiocyanate conjugate at a predetermined working dilution was added to each well which was freshly diluted 1:10 and stained with 1% Evan's blue (50µl conjugate + 50µl of Evan's blue +400µl PBS). The slides were incubated for 30min. at 37oC in moist chamber and dark place. Then the slides were washed with PBS and distilled water as previously described. Glycerin buffer was used in mounting step by adding small drop on each circle of the slide and then a cover slip was applied over the slide for examination by fluorescent microscope with a 40X Lens and exciter filter No.3 and barrier filter No. 3[11]. The highest dilution giving specific fluorescence associated with elementary bodies was regarded as end point[10].

3. Results

The present work revealed thatthe incidence of *C.trachomatis* (genital serotypes) positive microimmunofluorescence in the serum of infertile males represents 68(49.27%) out of 138 serum samples (Table 1). The highest incidence (36.76%) was at the age group (27-31) years. The least incidence (1.47%) was at the age group (52-56) years. IgA was the highest immunoglobulin class that detected (21.73%), IgA and IgG were (14.49%), IgG was (7.24%) and IgM was (5.79%) among total positive. The highly incidence of IgA (22.05%) was at the age group (27-31) years. The least immunoglobulin class was IgM, (5.79%). The highest incidence of positive IgM was (5.88%) at the age group (27-31) years and detected less frequently (1.47%) at the age group (52-56) years.The incidence of *C.trachomatis* (genital serotypes) positive MIF in seminal plasma of infertile males represents 108(75%) out of 144 which gave specific fluorescence as shown in table (2) and figure (1). The highest incidence of positive seminal plasma were (41.66%) in the age group (27-31) years and least incidence was (0.92%) in the age group (52-56) years. IgA was the highest immunoglobulin class that frequently detected (44.44%), IgA and IgG was (27.77%), IgG was (2.08%) and IgM was the least frequent class that detected only in (0.69%) among total positive.Highly incidence of IgA (24.07%) was in the age group (27-31) years. IgA was detected least frequently (0.92%) in the age group (52-56) years. IgM was the least immunoglobulin classes which was detected in (0.92%) at the age group (22-26) years.

The incidence of *C. trachomatis* (genital serotypes) positive MIF in seminal plasma and serum represents 39(37.5%) out of 104 infertile males as shown in table (3). The highest incidence of positive cases were (41.02%) in the age group (27-31) years while the least one (2.56%) in the age group (42-46) and (47-51) years. IgA was detected in (34.6%) while IgA and IgG were detected only in (2.88%). The majority of IgA (38.46%) was detected in the age group (27-31) years.

Table 1: Distribution of (68) positive serum samples of infertile males according to the age groups

Age group (year)	Total cases	IgM positive serum	IgG positive serum	IgA positive serum	IgA,IgG positive serum	Total positive
	No. %	No. %	No. %	No. %	No. %	No. %
22-26	12(6%)	0(0%)	1(1.47%)	3(4.41%)	2(2.94%)	6(8.82%)
27-31	48(25%)	4(5.88%)	0(0%)	15(22.05%)	6(8.82%)	25(36.76%)
32-36	23(15%)	2(2.94%)	2(2.94%)	4(5.88%)	7(10.29%)	15(22.05%)
37-41	37(16%)	0(0%)	7(10.29%)	8(11.76%)	1(1.47%)	16(23.52%)
42-46	13(3%)	1(1.47%)	0(0%)	0(0%)	2(2.94%)	3(4.41%)
47-51	3(2%)	0(0%)	0(0%)	0(0%)	2(2.94%)	2(2.94%)
52-56	2(1%)	1(1.47)	0(0%)	0(0%)	0(0%)	1(1.47%)
Total	138(68%)	8(5.79%)	10(21.73%)	30(21.73%)	20(14.49%)	68(49.27%)

Table 2: Distribution of (108) positive seminal plasma of infertile males according to the age groups

Age group (Years)	Total No. of cases	IgM positive seminal plasma	IgG positive seminal plasma	IgA positive seminal plasma	IgA,IgG positive seminal plasma	Total Positive
	No. %	No. %	No. %	No. %	No. %	No. %
22-26	19(13%)	1(0.92%)	2(1.85%)	7(6.48%)	3(2.77%)	13(12.03%)
27-31	57(40%)	0(0%)	0(0%)	26(24.07%)	19(17.59%)	45(41.66%)
32-36	19(13%)	0(0%)	0(0%)	11(10.18%)	4(3.7%)	15(13.88%)
37-41	33(23%)	0(0%)	0(0%)	13(12.03%)	10(9.25%)	23(21.29%)
42-46	13(9.02%)	0(0%)	0(0%)	6(5.55%)	3(2.77%)	9(8.33%)
47-51	2(1.38%)	0(0%)	1(0.92%)	0(0%)	1(0.92%)	2(1.85%)
52-56	1(0.69%)	0(0%)	0(0%)	1(0.92%)	0(0%)	1(0.92%)
Total	144(100%)	1(0.69%)	3(2.08%)	64(44.44%)	40(27.77%)	108(75%)

Table 3: Distribution of (39) positive seminal plasma and serum of infertile males according to age groups

Age group (Years)	Total cases	IgA positive seminal plasma And serum	IgA,IgG positive seminal plasma and serum	Total Positive
	No. %	No. %	No. %	No. %
22-26	10(2%)	2(5.12)	0(0%)	2(5.12%)
27-31	41(16%)	15(38-46%)	1(2.56%)	16(41.02%)
32-36	13(10%)	9(23.07%)	1(2.56%)	10(25.64%)
37-41	26(9%)	9(23.07%)	0(0%)	9(23.07%)
42-46	11(1%)	1(2.56%)	0(0%)	1(2.56%)
47-51	2(1%)	0(0%)	1(2.56%)	1(2.56%)
52-56	1(0%)	0(0%)	0(0%)	0(0%)
Total	104	36(34.6%)	3(2.88%)	39(37.5%)

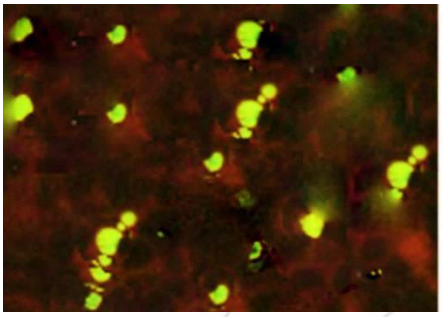

Figure 1: Specific fluorescence of *C.trachomatis* (100X) by microimmunofluorescence test in seminal plasma of infertile male

4. Discussion

The incidence of antibodies to genital *C. trachomatis* was detected in (75%) seminal plasma, (49.27%) serum and in (37.5%) both serum and seminal plasma of infertile males using MIFtest . The specificity of MIF is higher in detection of antibodies in seminal plasma than serum. This result come in contrary with others [12],they reported that antichlamydial antibodies were detected in (87.5%) of serum and only in (29%) of semen samples of infertile malesusing genus-specific ELISA. Genus specific ELISA can detect antibodies in serum that have no relation with the genital infection with *C.trachomatis*, like in case of *C.pneumoniae* infection which is very common in adult population and antibodies to this species accounted for up to half of all positive cases attending genitourinary clinics. [13]

In this study antichlamydial immunoglobulins were detected in (49.27%) serum samples and in (37.5%) both in seminal plasma and serum. This variation inmay attributed to the fact that in MIF the immunodominant antigen is major outer membrane protein which is an important surface antigen of both elementary bodies and reticulate bodies , display genus, species, subspecies and serovar-specific antigen determinants that are recognized during human infection and is known to cross-react with sera prepared against either of the three species of chlamydiae [14, 15].This opinion come in agree with [16] they reported that cross-reactive IgG against *C.trachomatis* D to K strains was present in 32.6% to 55%, and they found that serum antibodies to *C.pneumoniae* and *C.psittaci* account for up to half of all chlamydia IgG positive cases in MIF of males attending genitourinary clinics. This supported by [17], they reported that genital infection with *C.trachomatis* in male in contrast to the female produces only weak antigenic stimulus resulting in modest antibody responses and about 20% of infected males does not even mount an immunological response at all, for this reason reaction by the host fade away and the determination of chlamydial antibodies in serum are unsuitable for diagnosing infertility in men.For all previously mentioned reasons, the detection of chlamydial antibodies in seminal plasma by MIF are more specific as it is reflect local immune response to infection and the probability of cross-reactivity with other chlamydial species was unlikely [2]

The incidence of positive cases by MIF was higher among (27-31) years in which antichlamydial antibodies were detected in (36.76%) serum, (41.66%) seminal plasma, and in (41.02%) both serum and seminal plasma. This result come in agreement with other studies ,found that the highest incidence of antichlamydial immunoglobulins was among the age group (20-30) years in which antibodies were detected in (29.2%) of seminal plasma and in (8.33%) of serum[18]. While [19]found that (68.7%) of serum and (25.2%) of seminal plasma of infertile males at the age group (20-57) years have positive titer for C. trachomatis using genus-specific ELISA test.

The high incidence of antichlamydial immunoglobulins among the age group (27-31) years especially in seminal plasma that reflect the local immune response against *C.trachomatis* as asexually transmitted pathogen may attributed to the fact that this age represent the age of highest sexual activities beside social, economic, moral and religious factors [20].

The incidence of antichlamydial immunoglobulins was very low among the age group (52-56) years in which only (1.47%) of serum and (0.92%) of seminal plasma were positive in MIF test. This may attributed to the fact that few number of infertile males that attended to infertility clinic belong to this age group. Also may be due to decrease sexual activities among this age group. With respect to class of immunoglobulins that detected in serum, seminal plasma and in both serum and seminal plasma among infertile males, IgA was the highest one, followed by both IgA an IgG, then IgG alone and the least class was IgM in all age groups.

The highest incidence of IgA was among the age group (27-31) years in which it was detected in (22.05%) serum, (24.07%) seminal plasma and in (38.46%) both serum and seminal plasma This result come in agreement with that reported by [18]found that antibodies of class IgA were detected in (29.2%) seminal plasma and in (8.33%) serum of infertile males in age group (20-30) years. [21],reported that antichlamydial IgA was detected in (24.7%) seminal plasma while in serum only (14.5%) had IgA among asymptomatic infertile males at second to third decade of life, using species-specific ELISA. This result come in country with [19], they reported that antichlamydial IgA was detected in (19.9%) seminal plasma and in (29.7%) serum among age group (20-57) years, using genus-specific ELISA.

The high incidence of IgA in seminal plasma (24.07%) and both in seminal plasma and serum (38.46%) support the fact that the stimulus for antibody production was with in genital tract and IgA in seminal plasma consider valuable indicator for chlamydial genital infection [22].In this study ,both IgA

and IgG detected in seminal plasma were more than that of serum or both serum and seminal plasma. The highest incidence was among age group (27-31) years. Both IgA and IgG were detected in (8.82%) serum, (17.59%) seminal plasma and in (2.56%) both in serum and seminal plasma. This result coincide with [23], reported that (15.8%) of infertile males among the age group (20-30) years, had both IgA and IgG in seminal plasma using MIF. In contrast to this result ,[24] reported that both antibodies were detected in (19%) of serum and only in (3.8%) of seminal plasma of infertile males among the age group (20-57)years, using genus-specific ELISA. The higher incidence of both IgA and IgG in seminal plasma using MIF support the fact that infertile males had previously encountered with sexually transmitted *C.trachomatis* in the form of chronic asymptomatic infection within genital tract.[25].

The incidence of IgG was (7.24%) in serum, followed by (2.08%) in seminal plasma. The highest incidence of IgG in serum was (10.29%) among age group (37-41) years, while the least one was (1.74%) among (22-26) years. This result come in agreement with that of[26], they reported that chlamydial IgG antibodies were present in (9.8%) serum of infertile males with age range (20-57) years. The incidence of IgG alone in seminal plasma was very low and detected only in (1.85%) among age group (22-26) years. This result come in agreement with that of [6], they reported that the incidence of IgG positive seminal plasma was found only in (1.6%) infertile males a second to third decade of life. others disagree with this result[19], they reported that seminal antichlamydial IgG was detected only in (5.3%) infertile males among the age group (20-57) years. This support the fact that IgG is the major antibody classes in the blood while external secretions contain small amount of IgG .[27]
IgM was detected in (5.79%) serum and in (0.69%) seminal plasma among infertile males. The highest incidence (5.88%) in serum was among the age group (27-31) years. The low incidence of serum IgM support the fact that chlamydial infection tend to by chronic beside the possibility of detection of cross- reacting IgM specific for other chlamydial species. [6, 15].There was no previous or recent studies focused on detection of antichlamydial IgM in seminal plasma depending on the fact that chlamydial infection tend to be chronic, so that search for IgM antibody in infertile males seems to have limited diagnostic value.

This study revealed that immunoglobulin classes detected less frequently in the age group (52-56) year, in which IgM was detected in (1.47%) serum and IgA was detected in (0.92%) seminal plasma. This result coincide with [28],they found that (1.33%)of male attending venereal disease clinic with specific antichlamydial serum IgM were at the age more than 50 years, using MIF test. With respect to IgA class there was only one study that carried out by[24], they found IgA in (19.9%) seminal plasma of infertile males with age (20-57) years. The low incidence of antichlamydial antibodies in patients at the age more than 50 years may be refers to the fact that at this age sexual activities will be decrease and extra martial relations will be very limited.

In conclusion, this study found that detection of antichlamydia trachomatis immunoglobulin in seminal plasma of infertile males more sensitive than serum by MIF test and applicable for infertility centers.

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