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Measurement of [IgG] antibody to Mycoplasma hominis and T-mycoplasma by an enzyme-linked immunosorbent assay in cervical factor infertility.

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Summary

Sera [200] from patients attending the out patient clinic Dept. Of obstetrics and Gynaecology, LLRM medical college, Meerut, were screened for the presence of IgG class of immunoglobulins against M. hominis & T-mycoplasma due to cervical factor infertility using the ELISA technique with the use of standard PG-21 strain and serotype (CS_1) for M hominis & serotype 1 and 4 for T-mycoplasma. The test was standardized using penicillinase as an enzyme. ELISA is a rapid and reliable test and requires smaller quantity of the serum as compared to that needed for culture.

Introduction

Depending on a number of factors, including age, sexual experience & socio economic background, Mycoplasma can be isolated from the lower genito-urinary tract in, up to 53.6% of healthy adults. However, carefully controlled cultural and serological studies suggest that under certain circumstances this opportunistic micro-organism is capable of invading the upper genito-urinary tract and is of etiologic significance in postpartum fever, salpingitis and pyelonephritis. M. Hominis can also cause amniotic fluid infection, meningitis, submandibular adenitis and subcutaneous abscesses in neonates and has been implicated in congenital pneumonia. T-Mycoplasma is a cause of non-gonococcal urethritis (NGU) in men and it has been implicated in other diseases of the genito-urinary tract, such as infertility, spontaneous abortion and also perinatal morbidity and mortality.

A number of serological tests have been developed for the detection of specific antibodies to Mycoplasma hominis & T-mycoplasma but the value of many of these are limited by technical difficulties. Enzyme linked immuno-sorbent assay (ELISA) have been developed for a variety of mycoplasma species and have been found to be a rapid, reliable & sensitive means of diagnosis. The objective of the present study was to establish an ELISA for the detection of antibodies to many different micro-organisms including Mycoplasma species.

Material and Methods

Mycoplasma strain CS_1 locally isolated and confirmed by Immuno-fluorescence for M. hominis and for T-mycoplasma, serotype1, 4 and reference antisera were used for the preparation of antigen.

Antigen Preparation for M. hominis

The serotype (CS_1) was grown in PPLO broth (pH-7) supplemented with horse serum, yeast extract and

0.5 percent arginine and phenol red as an indicator. This was repeatedly subcultured (4-5 times) in broth. Five ml of broth culture was inoculated in 100 ml of the medium and incubated at 37° C for 12 to 20 h. It was harvested by centrifugation at 12,000 rpm for 1 hr at 4° C. The pellet was resuspended and washed three times using sterile carbonate-bicarbonate buffer (pH-9.6). In the end, the pellet was resuspended in 15 ml carbonate buffer. This was sonicated at 26,000 cycles/sec for 1 minute and the sonicate as such was used as an antigen. Bacterial contamination was monitored by standard laboratory methods. The protein content of the lysate was determined by Lowery's method. Antigen was preserved in aliquotes at –20°C, M hominis PG-21 antigen supplied by Dr. Gail Cassell, Alabama, USA was also used along with our antigen as a reference antigen.

Antigen Preparation for T-mycoplasma

Serotype 1 & 4 were grown in U-9B modified medium. 0.5ml of primary culture was added to 30ml of U-9B medium and incubated at 37° C for 18-20 h. This was centrifuged at 15,000 rpm for 1 hr. The pellet was resuspended in 15 ml of Carbonate-bicarbonate buffer at pH-9.6, sonicated for 1 minute at 26,000 vibrations/sec. and used as an antigen. The protein content of the antigen was determined by Lowery's method. Antigen was preserved in aliquots at –20° C and used in concentrations of 6 μg protein/well.

The antigen of T-mycoplasma serotype 1 and 4, supplied by Dr. Gail Cassell. Alabama U.S was also used as a reference antigen.

Enzyme conjugated anti-immunoglobulin

Goat antihuman IgG class immunoglobulin (Behring) was conjugated with enzyme penicillinase (E.C. 3.5, 2.6 from Hindustan Antibiotics Ltd., Pimpri, Pune) by one step glutaraldehyde method. Conjugates were stored at 4° C and diluted just before use.

Control sera:

a. Positive control
   Pooled sera from three patients where M. hominis and T-mycoplasma were isolated from genital tract were considered as positive control sera. Sera were serologically positive by immunofluorescence. Titres of ≥ 1:64 for M. hominis and ≥ 1:32 for T-mycoplasma were considered positive.
b. Negative control
   Sera from 10 healthy controls who were culture negative & immunofluorescence negative served as negative controls.

Test Sera

Serum samples were collected from 29 infertile women due to cervical factor infertility [Positive Post coital test]

Samples from 40 healthy controls with no known health problem served as the control. All the sera were stored at –20° C before use.

Standardization & determination of cut off limit

Negative and Positive controls were doubly diluted from 1:50 to 1:800 in PBS (pH-7.2) before use. Incubation time, concentration of antigen and conjugate were determined by checkerboard titration. Antigen lysate (20, 30, 40 μg protein/ml) was diluted in sodium bicarbonate buffer (pH-9.6). To each well 200 μl of antigen was added. Flow laboratory EIA microtitration plates with flat bottom wells were used. Plates were kept in the refrigerator overnight (16-18h). The plates were then washed 5-6 times using saline-Tween 20 (0.05%. Tween 20 in 0.85% NaCl). Two percent bovine serum albumin (BSA) in PBS (pH-7.2) was added to each well (200μl well) and the plate was incubated at room temperature for 30 minutes. The plate was again washed 5-6 times with washing buffer. Serum (200 μl) in various dilutions (1:50 to 1:800) was added to each well, and allowed to incubate at 37°C for 4-5 hours in a moist chamber. The plate was again washed 5-6 times with washing buffer and then 1:1200 dilution of anti-human IgG penicillinase conjugate (1:1000, 1:1200, 1:4000) was added to each well (200 μl). The plate was incubated at 37° C overnight in a moist chamber. Next day, the plate was washed 5-6 times and then the indicator, starch-iodine penicillin V was added. This was incubated at room temperature for 30 minutes. The Reaction was stopped by 5 N HCl; readings were recorded with air blank using ELISA reader.

The discrimination between the negative samples and positive samples was clearly evident at sample dilution 1:200 both visually as well as by ELISA reader. An optical density of negative value minus SD was considered as the cut-off limit.

Standard Conditions for ELISA for detection of IgG to M. hominis & T-mycoplasma
Table-I

ELISA reagent	Concentration	Incubation time	Incubation temp.
Antigen	30 μg Protein/ml	18 hours	4° C
Serum	1:200	4-8 hours	37° C
Conjugate	1:1200	Overnight	37° C
Substrate	Starch/Iodine	30 min.	Room temp.

Results & Discussion

Of the 29 women with positive post coital test, 7 were culture positive and 5 were seropositive (Mycoplasma hominis) whereas in two cases ELISA was positive but culture was negative.

In case of T-mycoplasma antibodies 18 cases were culture positive while 16 were seropositive for ELISA, whereas 4 were positive by ELISA but cultured as negative.

In control cases, out of 40, 2 were culture positive [M. hominis] whereas 2 were positive by ELISA but culture negative. Positivity of M. hominis was not significant as compared to control group.

In case of T-mycoplasma, out of 40 control samples, 10 samples were culture positive as well as seropositive, whereas 3 were positive by ELISA but cultured as negative. Culture isolation as well as ELISA positivity were significantly higher in our study group as compared to control group. ($P<0.001$)

The observed discrepancies between cultural status and presence of antibody are predictable. A percentage of culturally negative and serologically positive individuals is not uncommon, whether this represents failure in culture technique, previous clearance of the organism, or nonspecificity is not known. Culturally negative individuals were most frequently positive in the IgG class, suggesting that these individuals may have had previous exposure to and subsequent clearance of Mycoplasma hominis.

The assays currently used to determine the presence of antibody require careful regulation of mycoplasma cell numbers and multiple strains of M. hominis. Few clinical laboratories possess the facilities or expertise to perform such assays. ELISA technology, however, has been readily adopted by commercial sources for routine use in the clinical setting.

The ELISA makes it possible to study the class specific antibody response. The assay is highly reproducible as proved by the repeat assay of 20 samples which gave equivocal results at an interval of 4 weeks. ELISA is rapid, reliable and has the ability to differenciate quantitatively among immunoglobulin classes. ELISA is easier to perform than other commonly used serological tests.

Table-II

Group	Mycoplasma hominis Culture				T-mycoplasma Culture			
	+ve ELISA		-ve ELISA		+ve ELISA		-ve ELISA	
	+ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve
Study group (29)	5 (17.24)*	2 (6.89)*	2 (6.89)*	20 (68.96)*	16 (55.17)*	2 (6.89)*	4 (13.79)*	7 (37.93)*
Control Group (40)	2 (5)*	-	2 (5)*	36 (90)*	10 (34.48)*	Nil (-)	3 (7.5)*	27 (67.5)*

* Percentage value