Review Article

Immunological tests and their interpretation in uveitis

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Uveitis is a complex disorder including both infectious and non-infectious etiologies. Clinical diagnosis is a challenge because many diseases share common clinical signs. Laboratory support is crucial for confirming the clinical diagnosis. Laboratory diagnosis includes direct tests and indirect tests. For example smear, culture, and molecular diagnostics demonstrate the pathogens, hence they are direct tests. Immunologic tests employ an antigen to detect presence of antibodies to a pathogen, or an antibody to detect the presence of an antigen, of the pathogen in the specimens. The immunological tests used in laboratories are made by producing artificial antibodies that exactly "match" the pathogen in question. When these antibodies come into contact with a sample they bind to the matching pathogen if found in the sample. Hence they are grouped under indirect evidence. There are several investigations in uveitis to reach the confirmed diagnosis including microbiological, immunological, imaging and molecular diagnostic testing. In this section we will discuss immunological investigations of infectious and non-infectious uveitis.

Key words: Infectious, investigations, non-infectious, novel infections, uveitis

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Uveitis is an ocular condition wherein a single disease can present with myriad presentations, while a single presentation can be seen in varied diseases. Example: scleritis can be seen in an infectious disease like tuberculosis while can be part of an autoimmune disease too. In 70% of the uveitic conditions, a diagnosis can be reached with proper systemic/ocular history and the ophthalmologist's clinical acumen. A tailored laboratorial approach is needed for identification in rest of the cases [Table 1]. These investigations are also needed to confirm the diagnosis and to start the patient on treatment which may be poles apart.

Infectious Uveitis

Tuberculin skin test (TST)/Mantoux test

It was first described by Koch in 1890 but was modified to intradermal use by Charles Mantoux in 1912.[1] Its a delayed type IV hypersensitivity reaction to purified protein derivative (PPD) which is prepared by precipitation of proteins from heat killed cultures of *Mycobacterium tuberculosis*. The sensitized T-cells are recruited to the site of injection where they release lymphokines leading to erythema and induration. A standard dose of 5 tuberculin units (0.1 ml) is injected intradermally on the volar surface of the forearm and the result is interpreted after 48–72 h in terms of erythema and induration [Fig. 1]. Diameter of the induration is measured perpendicular to the long axis in millimeters. An induration of 10 mm or more is considered positive, 5–9 mm doubtful and less than 5 mm negative.[2] However, this also depends on the endemicity of tuberculosis in the region. An induration of more than 5 mm is considered positive in cases of recent TB contact, HIV-positive patients, or immunosuppressed patients like post-organ transplant. On the other hand, patients without any risk for TB are considered to have a positive reaction if it exceeds 15 mm. TST has limited sensitivity and specificity of 71% and 66%, respectively.[3] It fails to distinguish latent infection from active disease.

False positivity is seen post *Bacillus–Calmette–Guérin* (BCG) vaccination, atypical mycobacterial infection, and also faulty administration. False negative, on the other hand, is due to inability or reduced ability to mount a response to the tuberculin antigen.[4-6]

This is usually seen in recent TB infection (less than 8–10 weeks), miliary tuberculosis, sarcoidosis, recent live-virus vaccination, malnutrition, immunocompromised states like HIV infection, post-organ transplants or patients on chronic corticosteroid and immunosuppressive medications, diabetes, and renal disease.

TST is a subjective test that requires technical skill for proper administration and interpretation. It is also a two-step procedure requiring multiple visits of the patient.

Interferon-gamma release assay (IGRA)

It measures the interferon gamma release after *in vitro* stimulation of T-lymphocytes with MTB antigens (ESAT-6, CFP-10, TB7-7) which are specifically absent in BCG strains and non-tuberculous mycobacteria.[6,7] There are two commercially available IGRAs.

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Table 1: Immunological tests in uveitis

Uveitic entity	Disease type	Immunological tests
Infectious Uveitis		
Bacterial disease	Tuberculosis	Mantoux Test,
		Interferon-gamma release Assay (IGRA) 6
		QuantiFeron-Gold In-Tube & T-SPOT TB
	Leprosy	Lepromin test
	Syphilis	Treponemal tests
		Non Treponemal tests
	Leptospirosis	Microagglutination test, ELISA
	Lyme disease	ELISA
	Rickettsiae	ELISA Weil Felix test,
Viral diseases	HIV	ELISA. Westren Blot
	Dengue	ELISA, Plaque Reduction Neutralization test (PRNT)
	Chikungunya	ELISA, (PRNT)
	West Nile virus	ELISA, RT PCR
Parasitic diseases	Toxoplasmosis	ELISA
	Toxocariasis	ELISA
Non-infectious Uveitis		
Collagen vascular disease	Rheumatological disorders	
	Juvenile idiopathic arthritis Systemic lupus erythematosus	Antinuclear Antibody:
	Systemic lupus erythematosus	dsDNA, ssDNA (double- and single-stranded deoxyribonucleic acid,)
	Scleroderma	Anticentromere antibody, Sm
	Rheumatoid arthritis	Rheumatoid Factor:
		Anti-Cyclic Citrullinated Peptide:
	Wegener's granulomatosis Polyarteritis nodosa (PAN) group	Antineutrophil Cytoplasmic Antibody: C ANCA, P ANCA,

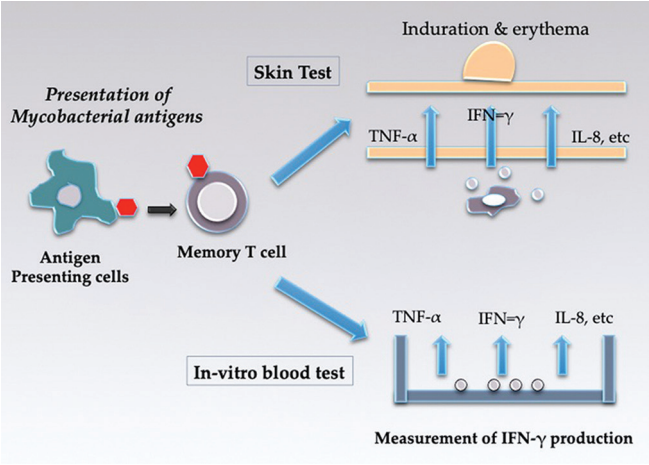

Figure 1: Figure shows both *in vivo* (TST test) and *in vitro* (IGRAs) release of inflammatory cytokines by T-cells sensitized to mycobacterial antigens. In the skin test, antigens are injected intra dermally which bring specific lymphocytes to the site causing release of cytokines resulting in induration. In the blood test, mononuclear cells from peripheral blood produce IFN-γ from sensitized T-cells which is measured by ELISA.[6] Adapted from: Andersen P, Munk ME, Pollock JM, *et al*. Specific immune- based diagnosis of tuberculosis. Lancet 2000;356:1099-04

QuantiFeron-Gold In-Tube (QFT, Cellestis Inc, Carnegie, Australia) – It is a whole blood enzyme-linked immunosorbent assay (ELISA) which measures IFN-γ secreted in response to stimulation with ESAT-6, CFP-10, and TB-77 antigens.

T-SPOT TB (Oxford Immunotec, Abingdon, UK) – It is a T cell-based enzyme-linked immunospot assay (ELISPOT) which measures T-cell spot formation after exposure to ESAT-6 and CFP-10 antigens.

Not only IGRAs indicate infection but also help quantify the severity. Higher the mycobacterial load, higher is the T-cell sensitization producing high IFN-γ levels. It is a more specific test of *M. tuberculosis* indicating infection or previous exposure. Also it is not influenced by prior BCG vaccination or exposure to atypical mycobacteria. It requires a single patient visit and gives an *ex vivo* objective measure of IFN-γ release. The main disadvantages of IGRAs are higher cost, need for trained technician, specialized instruments, and logistic issues as the blood samples are time and temperature sensitive. Also, IGRA has reduced sensitivity in immunocompromised patients and children.[8] Recently, QuantiFeron-TB Gold PLUS (QFT-PLUS) has been introduced as a new generation QFT-GIT. The QFT-PLUS contains two antigen tubes, TB1 and TB2: the TB1 tube contains ESAT-6 and CFP-10 derived peptides (TB 7.7 was removed) and is designed to induce CD4+ response; the TB2 contains both the same long peptides of TB1 and newly designed peptides which stimulate IFN-γ production by both CD4+ and CD8+ T cells. In a recent meta-analysis concerning the diagnostic test accuracy of QFT-PLUS in detection of TB, the authors conclude that QFT-PLUS performs equivalently

to the QFT-GIT for detection of patients at risk for latent TB. Further studies are still needed to prove their efficacy in immunocompromised patients and children.[9]

The role of TST and IGRAs in the diagnosis of intraocular tuberculosis depends significantly on whether the patient is from low or high TB endemic areas.[10] IGRAs can be used in diagnosing latent TB in low endemic areas where its specificity is 92–97%.[10] In a study by Chee *et al*., the authors reported that QFT is only slightly superior to TST in the diagnosis of TB-related uveitis. It is helpful to perform both tests simultaneously to improve accuracy and avoid negative or indeterminate test results.[11] Most of the referral uveitis centers in India now prefer a combination of Mantoux test and IGRA to diagnose ocular tuberculosis.[12,13] The pros and cons of TST and IGRA is discussed in the table [Table 2].

Serological tests for leprosy

Lepromin test

As the incidence of leprosy is coming down, this test is rarely used. The lepromin skin test is used to determine whether the patient has leprosy and if so the type of leprosy he or she has. Inactivated leprosy bacteria is injected just under the skin, on the forearm and examined after 28 days to see if there is a reaction. People who do not have leprosy will have little or no skin reaction to the antigen. People with lepromatous leprosy, will also have no skin reaction to the antigen because they are anergic to the antigen even though their body is loaded with leprosy bacilli. A positive skin reaction will be seen in people with tuberculoid and borderline tuberculoid leprosy.[14]

Serological tests for syphilis

Syphilis, a great mimicker, is caused by *Treponema pallidum*. It has diverse ocular manifestations and can present as anterior, intermediate, posterior, or panuveitis usually in the late secondary stage. It also shares clinical features with other treponemal and non-treponemal diseases. Hence, it is important that the clinical diagnosis is always supported by appropriate laboratory investigations. It can be diagnosed either by direct or indirect methods. Direct method includes detection of *T. pallidum* by dark field microscopy, direct fluorescent antibody stains, polymerase chain reaction (PCR), and immunohistochemistry. The sensitivity and specificity of dark field microscopy in the diagnosis of syphilis is 90% and 100%, respectively.[15,16] But this method is seldom applicable as it not commonly available, is technically challenging, and requires an experienced microbiologist. Indirect methods include the serological tests which remain the standard detection method.[17] Serological tests are categorized into two groups: non-treponemal and treponemal.

Non-treponemal tests

The term "non treponemal" refers to the antigens cardiolipin and lecithin which are extracts of normal mammalian tissues. The test detects both IgG and IgM antiphospholipid antibodies against these antigens formed by the host in response to the lipoidal material released from the damaged host cells as well as to lipids on the surface of *T. pallidum*. Seroconversion usually occurs from 21 days of exposure till 6 weeks after infection. Two tests commonly used are the VDRL (Venereal Disease Research Lab) & the RPR (rapid plasma reagin). These are rapid, simple, and inexpensive tests used as screening tools. But they have their limitations such as reduced sensitivity in primary, late latent and tertiary syphilis. False-positive results due to cross-reactivity are seen in bacterial (leprosy, chancroid, endocarditis), viral (HIV, chickenpox) and parasitic (malaria, trypanosomiasis) infections besides connective tissue diseases (systemic lupus erythematosus, rheumatoid arthritis), advanced age, drug addiction, and pregnancy.[18] False-negative results can be seen in patients with concomitant ocular syphilis and HIV infection due to prozone phenomenon.[19]

The tests are interpreted as reactive, weakly reactive, and non-reactive. A four-fold increase in antibody titer indicates infection, reinfection, or treatment failure. A four-fold reduction in antibody titer suggests response to treatment. These tests are recommended to monitor the course of disease during and after treatment. Patients are evaluated after 3, 6, and 12 months post-treatment to assess the response of therapy and detect any reinfection. If the non-treponemal test shows persistent reactivity at 6 months despite treatment or fails to show a four-fold decrease in antibodies titers within a year, it is reported as sero-resistance or serofast state.[20]

Treponemal tests

These tests detect specific treponemal antibodies. Various tests used more commonly are:

1. T pallidum *hemagglutination assay (TPHA):*
   It is a microhemagglutination assay for Ig G & IgM antibodies where sensitized sheep RBCs are coated with T. pallidum (Nichol's strain). The test is reported as reactive if agglutination occurs in a dilution of 1:80 or more. It is usually seen at 4th to 5th week of infection.
2. Fluorescent treponemal antibody absorption (FTA-ABS)
   It is an indirect immunofluorescence antibody test where the reactivity shows by 3rd week of infection.
3. Chemo luminescence immunoassays (CLIA)
4. Enzyme immunoassays (EIA)
5. Rapid tests.

These are used either as agglutination tests or as immunochromatographic strips at the point of care sites. They are easy to perform, require minimal training, and results are available in less than 30 min. However, due to poor quality control, laboratory based tests are recommended.

Treponemal tests remain reactive for years with or without treatment. They are mainly used to confirm the reactivity in

Table 2 : Pros and Cons of TST and IGRA

TST	IGRA
Measures skin induration after PPD injection	Detection of IFN-γ
Affected by BCG vaccination and other mycobacteria	Unaffected by BCG vaccination and other mycobacteria
Less sensitive and specific	More sensitive and specific
Cheaper/easy availability	costlier
Need for review within 48-72 h	Sample handling difficult

non-treponemal tests. Also, they are not useful to monitor the response to treatment, relapse or re-infection and correlate poorly with disease activity. These are more expensive and difficult to perform.[21]

Three testing algorithms are recommended now-a-days, which includes traditional algorithm, reverse sequence algorithm, and the European Centre for Disease Prevention and Control (ECDC) algorithm.[22,23] The traditional algorithm uses a non-treponemal test for screening and the reactive samples are then tested by a treponemal assay for confirmation. The reverse sequence algorithm recommended by the Centers for Disease Control and Prevention (CDC) uses an automated, treponemal test such as EIA or CIA as a screening test followed by a non-treponemal test like RPR for reactive samples. This technique allows rapid analysis. However, the results of EIA screening and RPR are often reported to be discordant. In such situations, CDC recommends a different treponemal test to identify active syphilis.[24,25]

The European Centre for Disease Prevention and Control algorithm uses a treponemal immunoassay that is followed by a second, different treponemal assay as a confirmatory test in high-prevalence populations.[26] All the algorithms have their own pros and cons, hence it is important that the clinician decides about these tests based upon local syphilis prevalence, careful history of the patient including sexual history, medical history or previous treatment history of syphilis and the clinical presentation.

In India, the traditional algorithms including both non-treponemal and treponemal tests is commonly used by uveitis experts. If the clinical assessment suggests a syphilis etiology but the screening tests are non-reactive, one can repeat the tests after 2–3 weeks to confirm the diagnosis. As syphilitic ocular manifestations can mimic other uveitic conditions, routine use of serological tests in all patients with intraocular inflammation of unknown origin is essential. CDC also recommends lumbar puncture for cerebrospinal fluid (CSF) analysis in all patients with ocular syphilis to detect neurosyphilis.[24] The diagnosis is based upon positive VDRL, elevated total protein, and CSF pleocytosis. The CSF VDRL has a very high (99.8%) specificity but sensitivity is only 50%. Consequently, a negative CSF–VDRL does not rule out neurosyphilis. Specific treponemal tests are not useful due to false-positive reaction. The diagnosis is then confirmed with co-relation with serological tests. Besides serological tests in ocular syphilis, intraocular fluid analysis of non-treponemal and treponemal antibodies may also play an important role in diagnostic dilemma.[26,27] In a recent study by Silpa-Archa *et al*., authors have concluded that vitreous treponemal antibodies can act as a supplementary test to serology for confirmation of syphilitic chorio-retinitis.[27]

Leptospirosis

Leptospirosis is a waterborne spirochete, dark field microscopy can visualize the organisms from body fluids, but the test is less specific and false positives are more common. Microagglutination test (MAT) is the gold standard test where the alive motile bacteria is added to titrated amounts of patients serum.[28] If the serum has antibodies agglutination happens which can be visualized by dark field microscopy. The sensitivity of the test is low, however it is highly specific.

The MAT is a complex test to control, perform, and interpret. Live cultures of all serovars required for use as antigens have to be sub-cultured every week. The repeated weekly subculture of large numbers of strains presents hazards for laboratory workers and laboratory-acquired infections have been reported. Other drawbacks include the continuous risk of cross-contamination of the cultures, necessitating periodic verification of each serovar.

The IgM-ELISA is more readily commercially available and is less labor-intensive than MAT. The result of IgM-ELISA should be considered preliminary and further confirmation by MAT is recommended. Other serological tests that are available include macroscopic agglutination, indirect haemagglutination, lepto dipstick, microcapsule agglutination tests, and lateral flow assay. In addition to the above serologic assays, polymerase chain reaction and immunohistochemical (IHC) assays are sensitive methods of diagnosis.[29]

A short description on the methodology of ommunological test used in these diseases. Enzyme immunoassays, Western blot test are commonly used immunological tests. These tests use an antigen to identify the presence of antibodies to a microbe, or an antibody to identify the presence of an antigen or of the pathogen in the samples.

ELISA

It is a plate based assay technique to detect and quantify viruses, proteins, antibodies and hormones. We can use various antigen–antibody combinations. The procedure includes either an enzyme labelled antigen or antibody. From the final enzymatic activity the result can be interpreted qualitatively [Fig. 2].

Western blot

This is a sensitive assay for identification of proteins. It is based on the immunochromotography principle. In this test, the proteins are first separated into polyacrylamide gel according to their molecular weight and then transferred to nitrocellulose membrane. Then proteins are identified using initial specific primary antibody followed by secondary enzyme labelled antibody and substrate [Fig. 3].

Lymes disease

ELISA

This test is used most often to detect Lyme disease, ELISA detects antibodies to Borrelia burgdorferi. But because it can sometimes provide false-positive results, it's not used as the sole basis for diagnosis. This test might not be positive during the early stage of Lyme disease, but the rash is distinctive enough to make the diagnosis without further testing in people who live in areas infested with ticks that transmit Lyme disease.[30]

Western blot test

If the ELISA test is positive, this test is usually done to confirm the diagnosis. In this two-step approach, the Western blot detects antibodies to several proteins of Borrelia burgdorferi.[30]

Serological tests for rickettsiae

Rickettsial infections are caused by obligate intracellular gram-negative bacteria. It is usually transmitted to humans by the bite of ticks and mites. Rickettsial agents are classified into three groups: the scrub typhus (ST), murine typhus (MT), and spotted fever (SF). Diagnosis is based on culture, nucleic acid amplification, and serological tests including Weil-Felix test (WFT), ELISA, rapid diagnostic tests (RDT), and immunofluoresceny assay (IFA).[31] WFT is a hemagglutination test which is widely used for screening due to low cost and

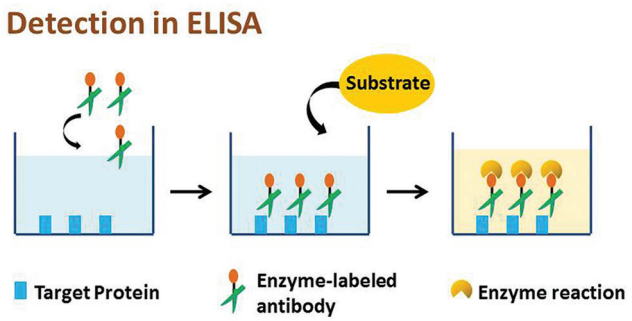

Figure 2: Depicts the steps in Enzye Immuno Assay

easy availability, but it lacks specificity. ELISA is less expensive and easy to perform. IFA is the gold standard for diagnosis of rickettsial infection but it needs skill, precision and is not routinely available. Serological diagnosis of rickettsial disease has various disadvantages due to poor sensitivity during acute infection where antibodies are not detectable within 10–14 days, cross-reactions with other rickettsial infections and being an indirect methods depending upon host responses. A recent study from India compared Weil-Felix test and IgM ELISA in the diagnosis of scrub typhus and it concluded that IgM–ELISA is more sensitive, rapid and specific in early phase of disease while Weil-Felix test specificity increases with increasing titres.[32]

Serological tests for HIV

HIV continues to be one of the major infectious causes of global public health issue. But with the availability of early diagnostic techniques and treatment for HIV and its associated opportunistic infections, patients living with HIV AIDS can still lead a long and productive life.[33]

Diagnosing HIV is the first crucial step in the treatment and prevention of AIDS. To diagnose the disease early with proper serological investigations, we should know the pattern of emergence of antigens and antibodies in the serum.

Immediately after HIV infection low levels of HIV RNA will be present intermittently in the patient's plasma. Nearly after 10 days, this RNA quantitatively increases in the plasma. Next, the p24 antigen is expressed and it quantitatively increases to levels that are microbiologically detectable. But this increase is transient. It is because of the development of antibody against P24 antigen which then forms immune complexes making their detection difficult. After 2 weeks of RNA development, IgM antibodies develop which is followed by IgG antibodies. These IgG antibodies will be present throughout the course of the disease.

Appropriate selection of the right investigation according to the timeline of the disease is mandatory to detect the disease at the earliest.

The following are the Lab investigations available for HIV:[34]

1. HIV Enzyme Immuno Assay
2. p24 antigen
3. Western Blot
4. Qualitative and quantitative PCR.

Enzyme immunoassay (EIA)[35]

Enzyme immunoassay was developed as the initial screening test for HIV infected patients before the window period. Five

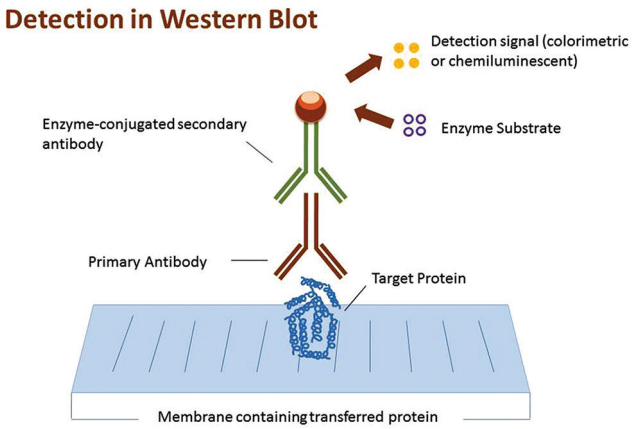

Figure 3: Depicts the steps in Western Blot

generations of assays have been developed so far based on the sensitivity and specificity of the tests to detect the HIV variants. The first generation could detect only the IgG antibodies against HIV. The recent generations can detect both IgG/IgM antibodies of both HIV I/II and also P24 antigen. The third and fourth generation assays reduce the window period to 20–25 days post-infection and the fifth generation assays reduce it to 1 week.

While the third generation immunoassay is commonly used in screening, the fourth generation is being used in facilities like Blood bank.

The disadvantage of this test is the chances of false-positive cases. So the EIA positive cases has to be re-evaluated either with a confirmatory test like Western blot or with a second EIA test using a different part of viral antigen for the detection of the antibody.

p24 antigen immuno assay

This test again uses EIA base to detect disrupted p24 antigen from the serum. This test may be positive in a recently infected patient with a HIV EIA negative test, necessitating a follow up evaluation weeks after the initial testing. p24 may not be detectable in all patients who are already positive for HIV antibodies.

Western blot[36]

This immunosorbent blot technique has been used to detect antibody to each viral protein. The test as discussed earlier contains a nitrocellulose strip having both the core and envelope protein of the HIV virus. The patient serum reacts with the strip after Gel Electrophoresis. According to the type of antibody present, the serum will bind to the antigen and produce a characteristic color band. Presence of one core band and one envelope band is mandated to declare a positive result. If the bands are indeterminate, a follow-up test has to be done after a month for acquiring a definitive band pattern. In case of failure to acquire a definitive band in a repeat test confirms that the patient is HIV negative and the band is due to nonspecific antibody. True HIV negative can be further confirmed by PCR.

These nonspecific antibodies make it difficult in using this test as a screening tool. This test is also difficult to perform in a high volume due to its labor intensive nature.

Rapid diagnostic tests (RDT)[37]

RDT is fast becoming the primary methodology of HIV testing in recent years in resource limited settings. It uses both

immunochromatographic and immunofiltration techniques. It can detect HIV I/II antibodies and/or p24 antigen. It takes less than 30 min and does not need venepuncture blood. All these make it the ideal test for community based testing.

But studies have proven that it is still not as effective as ELISA in screening for HIV.[38]

Other immunuological test commonly done in these HIV patients is PCR when they develop opportunistic ocular infections. Previous studies have revealed that the sensitivity of PCR for the CMV retinitis identification can be as high as 95% and the specificity 99% in immunocompromised patients.[38] Similarly the sensitivity of the assay for the diagnosis of herpetic retinitis in HIV patients is 100% and the specificity 97%.[38] Again the sensitivity of PCR in ocular fluid in Ocular Toxoplasmosis is as high as 75% in immunocompromised patient in contrast to their immunocompetent counterpart where the sensitivity is only 30–40%.[39]

Newer rare infections

Newer viral infections such as Chikungunya, West Nile virus and rickettsial infections are tested by ELISA tests usually in reference laboratories.[40] Commercial kits have to be checked whether they are validated in different population.

Serological tests for dengue

Dengue is a mosquito-borne infection, transmitted by mosquitoes *Aedes aegypti* and *Aedes albopictus*. Four dengue virus serotypes of Flavivirus genus have been known to infect humans, which includes DEN-1, DEN-2, DEN-3, and DEN-4.[41] Primary infection with dengue virus results in serum IgM antibodies by 3–5 days post-infection and persist for 1–2 months post infection. Secondary infections show a rapid increase in serum IgG as early as 3 days of illness and late appearance of IgM antibodies. Hence, a ratio of IgM to IgG during the acute phase of the disease can help identify whether it is a primary or a secondary infection. The standard serological test for dengue virus infection is enzyme linked immunosorbent assay (ELISA) for IgM and IgG antibodies. This is a simple, easy to perform test with high sensitivity.

IgM- antibody capture ELISA (MAC-ELISA)[42,43] - is used for qualitative detection of serum IgM antibodies. The only limitation is that it lacks specificity and shows cross-reactivity with other flavi viruses. Anti-dengue IgM is a marker of recent infection and anti-dengue IgG in the serum denotes past infection. However, presence of a high titre or a four-fold increase in the serum titres of IgG confirms dengue infection.

Plaque Reduction Neutralization test (PRNT)[42,43]- It can detect specific neutralizing antibodies against dengue virus and other flavi viruses. It measures the titres of neutralizing antibodies in the serum of patients. It is usually used to distinguish dengue virus from other viruses like Zika or yellow fever. The limitations of PRNT are that it is labor intensive, expensive to perform, and requires special laboratories to perform.

Serological tests for chikungunya

Chikungunya is caused by chikungunya virus (CHIKV), an arthropod virus and a member of Alphavirus genus. This infection has a clinical similarity with other arboviruses like dengue virus and Zika virus, so it is important to have a good diagnostic evaluation. Common laboratory tests for chikungunya include serological tests for IgM and IgG antibodies and viral RNA detection by reverse transcription–polymerase chain reaction (RT-PCR).[44] Center for Disease Control and Prevention (CDC) has proposed a testing algorithm for diagnosing chikungunya based upon the characteristics of infection and the timing of collection of serum for analysis. During the first week of infection, RT–PCR, a molecular diagnostic test should be ordered to detect viremia. As IgM levels rise by day 7, IgM antibody assay is helpful. After 14–21 days, both IgM and IgG test will be positive. IgM levels wane over next few months and IgG remains for years as a marker of past infection. A four-fold rise in antibody titre in acute and convalescent sera or specific IgM antibodies are diagnostic of Chikungunya infection.[44] Various serological tests available are:

1. IgM- antibody capture ELISA (MAC-ELISA)
2. Immunofluorescent assay
3. Hemagglutination – inhibition tests
4. Plaque Reduction Neutralisation test (PRNT) – It is highly specific and gold standard for confirmation of serological tests.

Serological tests for toxoplasmosis

Ocular toxoplasmosis, caused by *Toxoplasma gondii*, is the leading cause of infectious posterior uveitis worldwide.[45] It can be congenital and acquired or can be primary or secondary due to reactivation of the previous latent infection.[46,47] Diagnosis is mainly clinical but serological assay helps in confirming the same. Seroprevalence of anti- *T. gondii* antibodies is highly variable in various population and ranges from 5 to 54% in Europe, 12 to 58% in Asia, 16 to 40% in North America, and upto 80% in South America.[45,48] In India, the seroprevalence of toxoplasmosis is 24.3%, varying from 4.7% to 51.8% in North India to 37.3% in South India.[49] Active infection stimulates both innate as well as acquired immunity leading to production of antibodies like IgM, IgA, IgE and IgG from B-cells. IgM antibodies appear at the end of first week of infection, begin to fall in 4–8 weeks and may persist in low levels upto a year. IgG antibodies appear after 2 weeks, peak by 3 months of infection and persist throughout life due to the presence of latent cysts.[49] Serological tests which are available for diagnosing ocular toxoplasmosis can be divided into two groups.[50]

Screening methods

These are simple to perform, low cost and require small amount of serum.

- ELISA (enzyme-linked immunosorbent assay)
- CLIA (Chemiluminescence immuno assay).

Confirmation methods

These are complex and expensive to perform.

- IFAT (immunofluorescent antibody test)
- ISAGA (immunosorbent agglutination assay)
- Avidity test.

Interpretation of serological analysis plays the vital role in determining the need for anti-toxo medications in uveitis. Absence of both the IgM and IgG antibodies in the serum rules out the presence of any toxoplasmic infection. A positive IgG and negative IgM only denotes previous infection and can be misleading. High serum IgG titres are not the reliable indicators of either recent or reactivation of latent infection. However, if the level of IgG is 3–4 times higher than normal or repeat serology 3 weeks later shows increasing titres, it may be considered active infection. In congenital toxoplasmosis, presence of IgM and/or IgA antibodies confirm acute infection.

Only IgG antibodies in a neonate rules out toxoplasmosis as these are maternal antibodies which cross the placenta and are later eliminated by the neonate. In immunosuppressed patients like HIV+ or post organ transplant patients with chronic immunosuppressive therapy, positive serology suggests infection, however negative tests donot exclude concurrent infection. Besides serum, levels of antibodies can also be tested in aqueous humor. The Goldmann–Witmer or Witmer–Desmonts coefficient (GWC) is a valuable test that compares the intraocular antibody production to that of serum as measured by ELISA. GWC greater than 4 is suggestive of recent infection.[51,52] In a study by Fekkar *et al*., the authors have compared the sensitivities and specificities of three biological methods including immunoblotting, GWC and real time PCR for diagnosis of ocular toxoplasmosis. The study concluded that a combination of all the three techniques improved the sensitivity to 97%. thus increasing the diagnostic yield of ocular toxoplasmosis especially in atypical lesions.[53]

Serological tests for toxocariasis

Toxocariasis is a common zoonotic disease worldwide mainly in children. But diagnosis of ocular toxocariasis is underrated. Usually the diagnosis in ocular toxocariasis is clinical. Serological evidence may help to confirm the diagnosis. But seroprevalance is high in general population due to socio economic conditions. ELISA-based IgG antibodies may add a value to the clinical diagnosis. Still a negative result does not exclude ocular toxocariasis. In such scenario, a positive vitreous titre for toxocara antibody may help.[54-56]

Non-infectious Uveitis

Antinuclear antibody

Antinuclear antibodies (ANA) are a specific group of antibodies that recognize nuclear and cytoplasmic cell structures. ANA testing by immunofluorescence (IF) is the standard method and is used as a first-step screening test for autoimmune diseases.[57] Other laboratory tests used for ANA detection are enzyme immunoassay (EIA) and enzyme-linked immunosorbent assay (ELISA). Immunofluorescence test detects the presence of ANA in the blood of the patient which adhere to reagent test cells and forms different fluorescence patterns. Different patterns of ANA staining on IF may be grouped broadly into 3 subclasses including nuclear, cytoplasmic, and mitotic. These distinct fluorescence patterns are associated with certain autoimmune diseases. Another test parameter is ANA titer, which is directly proportional to the antibody concentration and is expressed with a quantitative scale of values.[58] Higher titers of ANA are more clinically significant.[57] Although the ANA test has a nearly 100% sensitivity for the diagnosis of systemic lupus erythematosus (SLE), it is not specific for this diagnosis and may be positive in other systemic autoimmune rheumatic diseases as well.[57] ANA may also be found in organ-specific autoimmune diseases, in viral infections and even in healthy individuals.[59] Therefore, in case of a positive ANA result, additional testing for anti-double stranded DNA (anti-dsDNA) antibodies and antibodies to specific extractable nuclear antigens (ENAs) such as anti-Ro (also called anti-SSA), anti-La (also called anti-SSB), anti-Sm, anti-RNP should be performed.[58,60]

ANA in healthy individuals is generally in low titers. Usually a titer of 1:160 is considered as significant for the diagnosis of connective tissue diseases; however, the results need interpretation within the clinical context.[61]

Routine testing of all uveitis patients for ANA has been shown to have a low positive predictive value of 0.6–2.9% in different studies; therefore, ANA testing should be included in the work up of patients with specific signs and symptoms of SLE such as a rash, symmetric polyarthritis, nephritis or pleuropericarditis and patients presenting with possible ocular manifestations of SLE including episcleritis, scleritis, and retinal/choroidal vasculitis.[62,63] Retinal vasculitis in SLE patients correlates with the systemic disease activity and may indicate inadequate control of the systemic disease. Hence, early diagnosis may aid as a prognostic factor for survival.[64] The presence of anti-dsDNA and antiphospholipid antibodies should also be tested in patients with retinal vasculitis; because anti-dsDNA which is a lupus specific antibody also correlates with the disease activity in SLE patients and there is a known association of antiphospholipid syndrome with SLE retinopathy.[65]

In addition to the entities above, all non-infectious pediatric anterior uveitis patients should be screened for ANA positivity. Anterior uveitis is the most common type of uveitis seen in pediatric population and juvenile idiopathic arthritis (JIA) is the predominant systemic disease associated with uveitis in children.[66,67] One of the main diagnostic criteria for JIA is ANA positivity and it is a known risk factor for ocular involvement in patients with JIA. Moreover, the presence of ANA is significantly associated with ocular complications at presentation.[68,69] Therefore, testing pediatric patients with anterior uveitis for ANA has a tremendous value in that it may affect the management of the systemic disease and also it identifies those patients at high risk for chronic anterior uveitis.

Rheumatoid factor

Rheumatoid factors (RFs) are a class of immunoglobulins (Igs) that have different isotypes and affinities and are defined as antibodies directed against the C-terminal domain of the constant region of the heavy chain (Fc fragment) of IgG. Different RFs recognize different parts of the IgG-Fc. IgM RFs are the most frequently observed isotype, but IgG, IgA, IgE, and IgD RFs are also detected. Nephelometry and ELISA are the methods used to detect RF in clinical practice.[70]

High levels of RF is usually associated with systemic autoimmune diseases, such as rheumatoid arthritis (RA), SLE, mixed connective tissue disease, and Sjogren's syndrome. However; RFs may also be detected in patients with nonrheumatic conditions such as infections and chronic diseases, as well as in healthy subjects.[71,72]

Rheumatoid factor titer in RA patients may be used in monitoring disease activity and treatment response since decrease in the RF levels parallels the decrease of clinical activity in patients under treatment.[73] High titers of RF in RA patients is associated with more aggressive joint disease and increased frequency of extra-articular manifestations including ocular involvement which may also be the initial manifestation of the disease.[74,75] Therefore, RF testing is usually included in the work-up of patients with episcleritis, scleritis, peripheral ulcerative keratitis (PUK), anterior uveitis, and dry eye which are known ocular manifestations of rheumatic diseases including RA that may have RF seropositivity. RF testing is also included in the workup of pediatric patients with noninfectious ocular inflammation. According to the International League of Associations for Rheumatology (ILAR) classification JIA has 7 subtypes and 1 of them is RF positive polyarticular JIA which

is genetically more similar to adult RA.[76] The risk of anterior uveitis in patients with RF positive polyarticular JIA is less than other subtypes of JIA.

Although RF is known as the cheapest modality for the screening of RA especially in ophthalmology clinics, it is often not detected early in the course of the disease and it is not specific for RA. Anti-Cyclic Citrullinated Peptide (Anti-CCP) antibody testing can enable earlier and more specific diagnosis. Hence, anti-CCP should also be tested when there is high suspicion of RA, despite RF being within normal range.

Anti-Cyclic citrullinated peptide

Citrulline is an atypical amino acid that has been implicated in the pathogenesis of RA. Citrullinated proteins are present in the inflamed synovium of RA patients and this increased citrullination of peptides in an inflamed joint leads to the development of antibodies to citrullinated protein antigens. Anti-CCP in blood is detected by ELISA and is found to be a more specific serum test for RA than the RF titer and may be detected positive before the onset of clinical RA symptoms. Based on the American College of Rheumatology criteria the sensitivity and specificity of anti-CCP positivity for the diagnosis of RA were detected as 73.5% and 100%, respectively.[77] Therefore, Anti-CCP antibody testing is useful when the diagnosis of RA is still not definite, especially early in the disease course.[78]

It was shown that anti-CCP and RF positive RA patients tend to have more and worse ocular involvement.[75] Anti-CCP test is useful when attempting to confirm the diagnosis of RA in patients with typical ocular symptoms such as episcleritis, scleritis, or PUK despite the absence of systemic signs.

Antineutrophil cytoplasmic antibody

Antineutrophil cytoplasmic antibody (ANCA) reacts against the proteins located in the granules of neutrophil cytoplasm and creates 3 different characteristic appearances on IF. Two of these are cytoplasmic and perinuclear that are well-defined staining patterns and regarded as positive. The last one is a non-specific reaction as a result of an excess of various antibodies that creates a diffuse pattern. The cytoplasmic appearance called c-ANCA is almost always directed against PR3-ANCA and is usually found in granulomatosis with polyangiitis (GPA). The perinuclear pattern called p-ANCA is usually directed against MPO-ANCA and is more commonly found in microscopic polyarteritis.[79,80]

ANCA-associated vasculitides (AAV) are a group of systemic diseases that primarily effect small and medium sized vessels with multisystem involvement including the eyes.[81]

Frequency of ocular involvement in AAV in different studies goes up to 70%. Ocular inflammation is the initial manifestation leading to diagnosis to AAV in some of these studies.[81-85] Therefore, patients with scleritis, PUK, orbital inflammation, and retinal or orbital vasculitis who are being investigated for an underlying systemic disease should be screened for ANCA for the possibility of AAV including GPA, eosinophilic GPA (Churg- Strauss Syndrome), and microscopic polyarteritis which can be life threatening. Since ANCA specifity is predictive for response to treatment and long-term prognosis both p-ANCA and c-ANCA should be screened in these patients as c-ANCA positive patients are at higher risk for relapse.[86] Early diagnosis and treatment of the ocular inflammation and the underlying disease can be lifesaving. Hence, ANCA serology could be considered as a screening laboratory test in these patients.

HLA typing

Human leucocyte antigens (HLA) are cell surface molecules encoded by a highly polymorphic family of genes involved in immunity and responsible for identifying self *versus* non-self. The HLA loci are a part of the genetic region known as the major histocompatibility complex (MHC) located at 6p21.3 on the short arm of chromosome 6. MHC Class I includes the HLA-A, HLA-B, and HLA-C antigens that are expressed, to varying degrees, on the surface of all nucleated cells and present endogenously produced peptides, including native proteins as well as damaged, degraded, or misfolded proteins, and also viral proteins to CD8-positive T lymphocytes. MHC Class II includes the HLA-DP, HLA-DQ, and HLA-DR antigens that have a more limited expression on specific immune cells and present exogenously produced peptides, such as bacterial proteins, to CD4-positive T lymphocytes.[87,88]

HLA typing has been conventionally based on serologic methods.[89] Molecular typing methods used in modern medicine allows the definition of HLA alleles to different levels of resolution. The "four-digit" typing distinguishes alleles based on the sequence of peptide-binding region of the HLA molecule (e.g, *HLA-B*51:01*).[87,89]

HLA typing is essential for solid organ and bone marrow transplantation as well as in non-transplant settings such as disease association and pharmacogenomics.[87] Numerous diseases, particularly those that are immune-mediated, are associated with certain HLA alleles.[87] Table 3 shows HLA associations of selected uveitic entities.

In general, HLA typing has limited usefulness for the diagnosis of uveitis and routine screening is not recommended.[90,91] When there is a weak association, because of the low percentage of patients with the disease having the HLA type or the high prevalence of the HLA type in the normal population, HLA typing is not useful. Even when there is a strong HLA association, the positive predictive value of HLA typing, that is, the likelihood that a patient with a specific HLA antigen will have the uveitic entity in question, depends not only on the sensitivity and specificity of the HLA test, but also on the prevalence of the disease in question.[90] Although birdshot chorioretinopathy has the strongest association with an MHC Class I antigen, HLA-A29, routine testing in all patients with posterior uveitis is not recommended because birdshot chorioretinopathy is a rare entity even among posterior uveitides seen in Caucasians who have an HLA-A29 prevalence of 5%.[92-94] Zamecki and Jabs reported that the positive predictive value of HLA-A29 was 0.47 in patients with posterior uveitis.[90] Thus only patients with clinical features consistent with this entity should be tested because a positive result is diagnostic and a negative result will indicate that a diagnosis of birdshot chorioretinopathy is highly unlikely.[92-94] Birdshot chorioretinopathy has not been reported in any of the recent uveitis series from India,[95-99] which is not surprising because the HLA-A29 antigen is extremely rare or even nonexistent in the Indian populations of diverse ethnicity.[100-102]

Indiscriminate testing for HLA-B27 is not recommended in all patients with uveitis. On the other hand, routine HLA-B27 testing is included in the diagnostic algorithm for patients with acute anterior uveitis as it can help to identify a distinct uveitis entity and also a previously undiagnosed systemic disease association.[90,91] Patients with HLA-B27–

Table 3: HLA associations of selected uveitis entities

Uveitis entity	HLA antigen	Frequency (%)
Birdshot chorioretinopathy	A29	96-100
Acute anterior uveitis	B27	40-82
Ankylosing spondylitis	B27	92
Behçet disease	B51	59
Vogt-Koyanagi-Harada disease	DQ4	83
	DQA1*0301	Up-100%
	DR4	93
	DRB1*0405	Up to 95%
Intermediate uveitis	DR15	47-72
Multiple sclerosis	DRB1*1501	Up-62
Tubulointerstitial nephritis and uveitis	DQA1*01/ DQB1*05/DRB1*01	72

associated uveitis typically have a recurrent, acute, unilateral or unilateral alternating, nongranulomatous anterior uveitis of limited duration.[103,104] In a meta-analysis of studies comparing HLA-B27-positive and HLA-B27-negative patients with acute anterior uveitis, specific characteristics linked to the antigen included a strong association with ankylosing spondylitis (RR = 9.9), hypopyon (RR = 5.5), and fibrinous reaction (RR = 8.7).[105] Therefore, in patients who first present with acute anterior uveitis, HLA-B27 testing is performed to confirm the specific phenotype of acute anterior uveitis, to predict its recurrent nature, and most importantly, to predict systemic disease association for an early referral to a rheumatologist.[90,103] Approximately 50–75% of patients with HLA-B27 acute anterior uveitis have an associated spondyloarthropathy.[104] Acute anterior uveitis is significantly more common in HLA-B27-positive than in HLA-B27-negative patients with ankylosing spondylitis.[106] Haroon *et al*.[107] have proposed a diagnostic algorithm (DUET; Dublin Uveitis Evaluation Tool) that would be useful to determine which patient presenting with acute anterior uveitis, should be referred to a rheumatologist. In this algorithm, HLA-B27 was checked in all patients who presented with acute anterior uveitis and back pain (onset <45 years of age and duration >3 months) or joint pains; and those who were HLA-B27-positive were referred to a rheumatologist. Patients who were HLA-B27-negative, but had psoriasis were also referred to a rheumatologist. The algorithm identified a previously undiagnosed spondyloarthropathy in around 40% of patients who presented with acute anterior uveitis.[107] The reported frequencies of HLA-B27-associated uveitis at tertiary care centers were 6% and 10.5% in South India[95,96] and 9.5% in North India.[97] This entity accounted for 12–30% of anterior uveitis in these series.[95-97] While systemic disease association of HLA-B27-positive patients was not specified in any of these series, in a recent report from a tertiary eye care center of central India, 7% of uveitis cases had spondyloartropathy.[98] Mishra and Bharucha[108] reported that the HLA-B27 frequency was 65.7% in acute anterior uveitis patients seen at a tertiary care center in Maharashtra. Only 4.5% of their HLA-B27-positive cases had systemic disease.[108] HLA-B27 is present in approximately 6–8% of the normal population in the Indian subcontinent;[109] and the frequency of HLA-B27 uveitis is similar to the figures in uveitis series reported from North America (6.7%),[110] Italy (7.7%)[111], Germany (10%),[112] and France (17.4%).[113] While more than 90% of Caucasian patients with ankylosing spondylitis have the HLA-B27 antigen,[104] only 21% of a south Indian population of ankylosing spondylitis patients tested positive for HLA-B27 antigen by the serologic method, but the HLAB*27 allele frequency was found to be 74% by the molecular method.[114] Thus the method of HLA-B27 typing should be taken into consideration when it is used as a diagnostic test in patients presenting with acute anterior uveitis.

Although HLA associations have been identified in other uveitic entities, the diagnostic value of routine HLA typing is questionable. Zamecki and Jabs[90] have shown that the positive predictive value of HLA test for uveitic entities such as Behçet's disease and Vogt-Koyanagi-Harada disease was 0.3 or lower.

The diagnosis of Behçet's disease is based on a combination of clinical manifestations, including intraocular inflammatory findings, recurrent oral ulcers, genital ulcers, and skin lesions. Although HLA-B51 antigen is associated with the disease,[115] HLA-B51-positivity has not been included as a diagnostic criterion in any of the diagnostic or classification criteria sets that are currently used.[116-118] The value of HLA-B51 testing in predicting disease severity is also controversial.[115]

Vogt-Koyanagi-Harada (VKH) disease is a clinical diagnosis and HLA-typing is not routinely performed when this diagnosis is considered. In a meta-analysis of articles relevant to the genetics of VKH disease, MHC Class II genes, specifically HLA-DQ A1*0301 and HLA-DRB1*0405 alleles showed the strongest association in various ethnic groups.[119]

Both idiopathic intermediate uveitis (pars planitis) and multiple sclerosis have been found to be associated with the HLA-DR15 subtype of HLA-DR2.[120-122] Thus HLA typing has no value in predicting the development of multiple sclerosis in patients with intermediate uveitis.

Strong associations have been found between specific HLA-DQ and HLA-DR alleles and the tubulointerstitial nephritis and uveitis (TINU) syndrome.[123] However, there is limited value of HLA typing as a routine test in this rare entity.

Conclusion

Investigations are an integral part in managing patients with infectious and non-infectious uveitis. But; it should be tailored to the patient's presentation. Tests should add value to the clinical diagnosis. Sometimes even negative test results have equally important value in diagnosing a disease. Few tests helps in diagnosing the disease, few in prognosticating the disease course and few in deciding the treatment pattern. Clinicians should never hesitate to re-evaluate/investigate the patient if the disease does not follow the anticipated course.

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