Chapter 10

Diagnosis

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Key Points

- TBE appears with non-characteristic clinical symptoms, which cannot be distinguished from other forms of viral encephalitis or other diseases.
- Cerebrospinal fluid and neuro-imaging may give some evidence of TBE, but ultimately cannot confirm the diagnosis.
- Thus, proving the diagnosis "TBE" necessarily requires confirmation of TBEV-infection by detection of the virus or by demonstration of specific antibodies from serum and/or cerebrospinal fluid.
- During the phase of clinic symptoms from the CNS, the TBEV can only rarely be detected in the cerebrospinal fluid of patients.
- Most routinely used serological tests for diagnosing TBE (ELISA, HI, IFA) show cross reactions resulting from either infection with other flaviviruses or with other flavivirus vaccines.

Clinical confirmation of suspected TBEV infection

Tick-borne encephalitis (TBE) manifests as a non-specific disease with symptoms of a febrile, influenza-like illness and, in some cases, an inflammatory infection of the central nervous system (CNS) that follows a few days later. Due to the lack of specific symptoms, a definitive confirmation of the diagnosis requires taking the history of the patient with regard to a possible tick bite or ingestion of unpasteurized milk in a known or suspected endemic area, plus a positive result from a classical virological test that confirms TBEV-infection either directly by the detection of virus or indirectly via detection of specific anti-virus antibodies.[1] Prior to the introduction of molecular detection technologies such as polymerase chain reaction (PCR), the only technique available to detect TBEV infection was virus isolation, but this is rarely used today.

The most common method of detecting TBEV infection nowadays is via serological assays, which have developed from complement fixation or hemagglutination inhibition tests through to modern immunoglobulin (Ig)-specific tests such as ELISAs and immunofluorescence (IF) assays.

Understanding of the pathogenesis and immunology of TBEV infection is essential for the selection and interpretation of appropriate diagnostic tests (Fig. 1). For example, the European subtype of TBEV often induces a biphasic clinical course, whereas a monophasic course may be more prominent in those infected with the Far Eastern subtype or Siberian subtype.[2] Following a bite from an infected tick, the virus is assumed to replicate locally within antigen-presenting cells and then subsequently within nearby lymph nodes. After replicating within the lymph nodes, the virus then spreads to the internal organs via the lymph and blood (causing viremia) and begins to replicate within the reticuloendothelial system.[3] It is during this phase of the disease that the infected individual will often show non-specific, influenza-like symptoms. These symptoms will then begin to improve for several days before a second phase appears in up to 30% of infected individuals, and which includes CNS involvement varying in severity from meningeal irritation to meningoencephalo-myelitis and even death. The choice of whether a specific patient should be tested using an assay that directly or indirectly detects TBEV infection therefore depends on the phase of the infection of a given patient.

Direct detection of TBEV infection

Virus isolation

The isolation of TBEV was the first diagnostic technique established for the confirmation of clinically suspicious CNS infections such as TBE. In the past, virus isolation from blood and brain samples was performed in newborn mice, with many of the 'old' TBEV strains (e.g., Scharl, Absettarov, Sofjin, KEM II, Alsace, Schaffhausen, etc.) isolated by intracerebral inoculation of patient material or tick suspensions. Cell culture was subsequently introduced and there are now a number of immortalized cell lines that can be used to isolate TBEV from patient material. The most frequently used cell lines are currently PS cells (porcine fetal kidney cells), Vero cells (green monkey fetal kidney cells), BHK-21 (baby hamster kidney cells), and A549 cells (human lung adenocarcinoma cells), although other lineages such as human neuroblastoma cells may also be used.

Figure 1: Natural course of TBE with clinical symptoms, virus replication, and evolution of specific anti-TBE antibodies

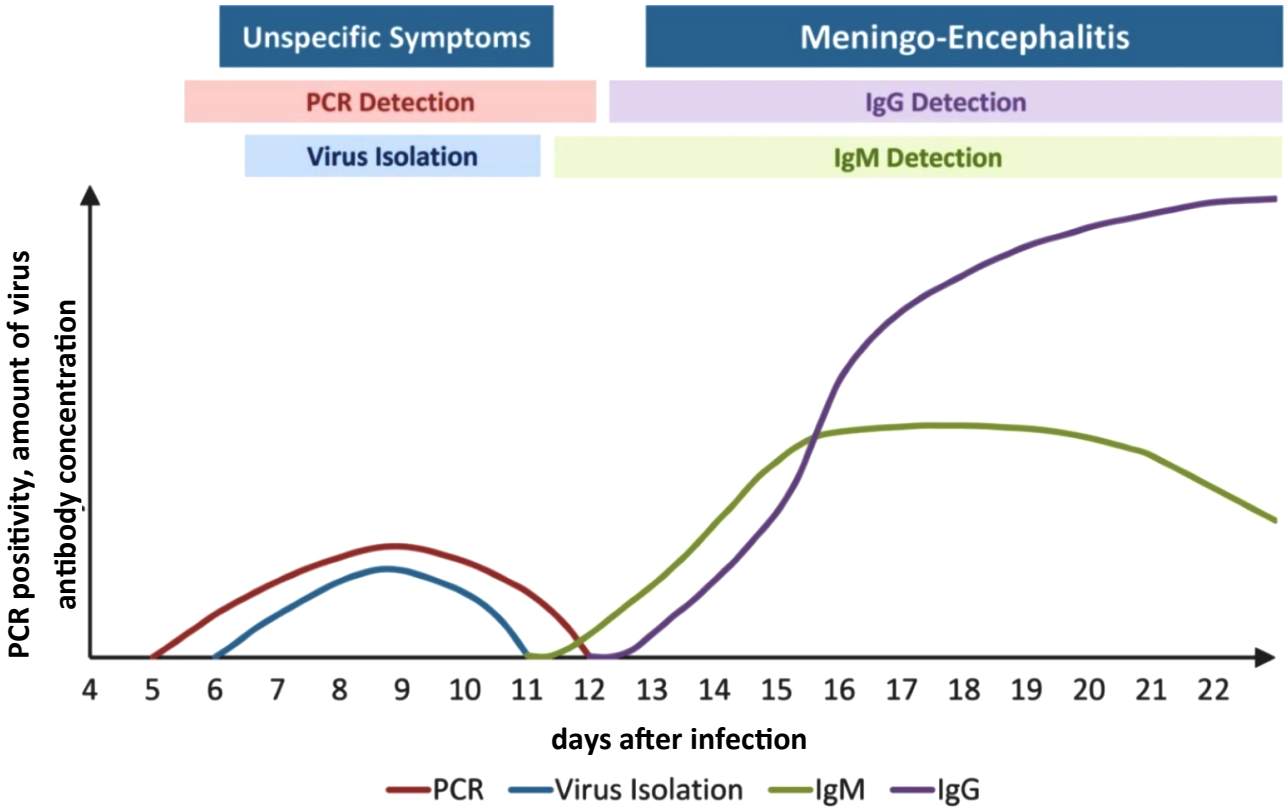

Table 1: Detection of TBEV by RT-PCR in patient samples according to stage of infection[4]

Antibody status	Serum	Blood	CSF	Brain tissue
IgM-/IgG-	30/30 (100%)	19/19 (100%)	1/10 (10%)	-
IgM+/IgG-	3/13 (23%)	3/5 (60%)	0/2 (0%)	-
IgM+/IgG+	1/34 (3%)	1/6 (16%)	0/19 (0%)	1/1 (100%)

Virus can be detected in an infected individual's blood during the first febrile phase of the disease and can be detected predominantly in brain tissue during the second phase involving neurologic symptoms.[4] The cerebrospinal fluid (CSF) does not usually contain viable virus and should therefore only be used for virus isolation under special circumstances. No systematic studies on the discharge of viable TBEV in the urine of patients infected with TBEV are available to date, but discharging in an immuno-compromised patient was observed to last for at least 56 days[5] and intermittent discharging in urine was observed for a period of more than 700 days in experimentally infected monkeys.[6]

Virus isolation is no longer routinely used for diagnosis of a TBE infection but is still needed to identify the subtype of TBEV present in brain tissue samples from fatal cases or in blood samples taken during the febrile phase of the disease. Virus isolation is also used to isolate TBEV strains from other biological material (e.g., ticks, rodents, etc.) for use in subsequent genetic and phenotypic characterization.

PCR

The current technology of choice for the detection of TBEV is PCR, and there are several formats available. The earliest PCR-based method for detecting TBEV infection was nested RT-PCR,[7–9] but a number of real-time RT-PCR assays for the detection of viral ribonucleic acid (RNA) in various clinical and biological samples have also been described.[10] PCR-based methods have no clear role in the diagnosis of TBEV infection during the phase involving CNS symptoms because viral RNA cannot usually be detected in blood or CSF samples during this phase of the disease.[4,8] However, TBEV

can be detected in blood samples during the first febrile phase of TBE as well as in brain tissue (if available) during the phase involving CNS symptoms. The RT-PCR format is therefore a valuable diagnostic tool when there is a need to confirm an infection with TBEV as the cause of a febrile illness following a tick bite, or when confirmation of a TBEV infection is sought in fatal cases. A recent Swedish study reported that TBEV RNA could also be detected by RT-PCR in urine samples from patients for up to 19 days after the start of neurologic symptoms.[11] Another application of RT-PCR in this setting is the diagnosis of potential TBEV infections in immunosuppressed patients unable to develop antibodies to the virus. In these cases, TBEV RNA may be detectable within blood and CSF samples over a longer period of time compared with immunocompetent patients. Detectable TBEV was reported to be shed over a period of at least 56 days in 1 immunocompromised patient.[5]

Indirect detection of TBEV infection

Purified antigenic components of the TBEV particle are essential in order to be able to detect antibodies produced by a potential host. The main immunodominant structure of a TBEV particle is the dimeric envelope (E) protein, which induces hemagglutinating, neutralizing, and protective antibodies following infection or immunization. The capsid (C) protein and nonstructural protein 1 (NS1) are antigens against which the host generates complement-fixing antibodies. A more detailed description of the proteins encoded by the TBEV genome can be found in Chapter 2b.

Complement fixation assay

The complement fixation assay (CFA) is one of the oldest tests for detecting antibodies against TBEV and other flaviviruses,[12] and was used to detect anti-virus antibodies in the early phase of a potential infection. The CFA cannot differentiate between different antibody isotypes, however, because IgM and IgG (IgG1, IgG2, and IgG3 subclasses) can all bind complement. Early data showed that infected individuals display a marked increase in the generation of complement-fixing antibodies during the second phase of the infection involving CNS symptoms, about 10-14 days after being infected.[13] The titer of complement-fixing antibodies reaches a peak after 5-10 weeks and then decreases to a lower level or disappears completely following a period of up to 1 year. The detection of complement-fixing antibodies is therefore an indicator of an acute or recent TBEV infection. The test usually involves demonstrating a significant increase in antibody titer in 2 serum samples taken 10-14 days apart. During the acute phase of the disease, a 3- to 4-fold increase in titer may be expected. The CFA is cross-reactive with antibodies against other flaviviruses and can also give positive results for some time after a TBE vaccination. The CFA relies on the quality of the reagents used being excellent, especially the TBEV antigen (which was formerly mouse brain extract but extracts from infected cell cultures were subsequently used). The introduction of modern, standardized, less time-consuming assays and the lack of antigen of appropriate quality means that the CFA is now obsolete.

Hemagglutination inhibition test

The hemagglutination inhibition (HI) test exploits the ability of the E protein of TBEV and other flaviviruses to agglutinate erythrocytes isolated from male geese.[14] The agglutinating phenotype of the TBEV is lost in the presence of host antibodies against the E protein and only a small pellet of erythrocytes forms at the bottom of the test tube, whereas a larger layer of erythrocytes can be seen to form at the bottom of the tube in the absence of host anti-virus antibodies. The test can be standardized using a defined quantity/activity of antigen (usually 4 hemagglutination units), a defined concentration of erythrocytes, and serial dilutions of the serum being tested. The test can therefore be quantitated and the level of dilution at which the serum inhibits agglutination is referred to as the HI titer. It should be noted that serum contains many substances that inhibit hemagglutination and these must be removed by acetone extraction or kaolin absorption before the serum can be used in the HI test. Usually the viral antigen used in the test is isolated from infected mouse brain, although cell culture supernatant can also be used as a source of antigen when testing for other viruses.

The hemagglutination reaction detects both IgM and IgG antibody isotypes. Historically, the HI test was used to demonstrate a significant (usually 4-fold) increase in the end titer that would be indicative of an acute infection. The test was also used in seroprevalence studies because hemagglutinating antibodies usually persist for many years.

A further development in the HI test was the treatment of serum samples with 2-mercaptoethanol in order to reduce the disulfide bonds present in native IgM pentamers to leave inactive IgM monomers.[15] This additional treatment step will cause HI titers to decrease in the presence of IgM antibodies, with a significant (at least 4-fold) decrease in HI titer indicating acute TBEV infection.

One disadvantage of the HI test is that there is a broad cross-reactivity with all flaviviruses[14] and therefore samples from patients infected with more than 1 flavivirus, or from those recently vaccinated, may lead to non-specific cross-reaction and inaccurate determinations of titer. The HI test is still used in several countries and is recommended by the World Health Organization for distinguishing between primary and secondary flavivirus infection.

Immunofluorescence assay

The use of IF to detect antibodies against TBEV usually involves indirect assays that require cells infected with TBEV to be spotted, fixed, and permeabilized on slides.[16] A characteristic, fluorescent, cytoplasmic staining pattern can be seen and quantified using serial dilutions of the serum being tested; antibody isotypes can be distinguished using fluorescent conjugates specific to IgM or IgG. For IgM testing, the higher-affinity IgG antibodies must be removed in order to avoid false-negative results. The sensitivity of IF assays appears to be like the HI test (the author's personal observation). IF assays that detect IgM antibodies against TBEV are moderately specific and occasionally show low levels of cross-reactivity to other anti-flavivirus antibodies following a recent infection or vaccination in the patient's history (the author's personal observation). According to our laboratory's experience, IF assays that detect IgG antibodies against TBEV perform specifically if there is only a TBEV infection or vaccination in the medical history. In contrast, diagnosis of patients with a history of infection or vaccination by a flavivirus other than TBEV can be difficult due to cross-reacting antibodies.

Low antibody titers that subsequently become undetectable occur following TBE vaccination and therefore IF assays are not recommended to test for immunity against TBE. After 2 flavivirus infections or vaccinations, a secondary response similar to the one seen in the HI test can often be detected as a high and broadly cross-reactive titer (the author's personal observation).

Neutralization test

The neutralization test (NT) exploits the capacity of antibodies to neutralize infectious viruses,[17] with several different formats available. One type of NT uses a standardized virus preparation and varying serum dilutions, while another format uses a standardized serum dilution and varying virus concentrations. Other examples are the plaque reduction NT (PRNT), which is used to evaluate the neutralization titer by analyzing the serum dilution at which the number of viral plaque-forming units is reduced by 50% or 90%, and the 'tissue culture infection dose 50%' (TCID50) test. The TCID50 test involves a defined number of infectious or lethal doses undergoing neutralization by varying concentrations of the serum being tested. The dilution at which 50% of the original quantity of virus is neutralized is termed the TCID50 titer and is usually calculated using the formula of Reed and Muench.[18]

Neutralizing antibodies usually occur about 2 weeks after vaccination or infection. They are thought to be the most specific antibodies produced by the host, and with the lowest cross-reactivity to other flaviviruses. Therefore, one scenario that indicates the use of an NT is when it is necessary to distinguish between specific anti-TBEV antibodies and antibodies against other flavivirus types. A second scenario in which an NT is useful is when there needs to be a reliable demonstration of immunity: only the detection of neutralizing antibodies is thought to be a reliable surrogate marker for an existing immunity against TBE.

ELISA

The ELISA format is the most commonly used test for detecting antibodies against TBEV.[19,20] The ELISA is usually conducted in a standardized format and can be automated. The various formats of anti-TBEV ELISAs on the market use different antigens, such as European subtype strains (e.g., Hypr, K23, Neudoerfl, K 1074) or Far Eastern subtype strains (e.g., Moscow B-4). The antigens used in the assays are whole-cell lysates or purified extracts derived from whole-cell lysates.[21] The results obtained from different ELISAs are not comparable due to the different amounts of antigen used. In general, ELISAs exhibit high levels of sensitivity but only moderate specificity due to cross-reactivity with antibodies against dengue virus (caused by infections) or yellow fever virus (caused by vaccinations) and other flaviviruses.

The various formats of ELISA can distinguish between different antibody isotypes, although only IgM and IgG are usually relevant for a diagnosis of TBEV infection (IgA does not play any role in diagnosis but may be detectable in serum and CSF). IgM antibodies are usually already present at the onset of clinical CNS disease, or at least a few days after onset of neurologic symptoms, and can be detected for about 6 weeks after the onset of CNS symptoms. A μ-capture ELISA has the highest specificity for IgM testing. When using the 2-layer ELISA format, IgG has to be removed before testing in order to avoid false-negative results. Diagnostic tests for anti-TBEV IgM are usually more specific than IgG tests with regard to cross-reactivity with other flaviviruses (the author's personal observation).

Assays evaluating IgG antibodies are usually produced in a conventional 2-layer sandwich format. Anti-TBEV IgG is broadly cross-reactive with other anti-flavivirus IgG antibodies. ELISAs for detecting IgG anti-TBEV antibodies display a high sensitivity (up to 99%), but only moderate specificity (40–80%) if sera from patients or vaccinees exposed to other flaviviruses are tested.[21] The specificity can be up to 97%, however, when samples with no history of exposure to other flaviviruses are tested. IgG antibodies against TBEV are usually present at the onset of CNS symptoms, reach a maximum titer after about 6 weeks, and persist for years. The antibody titers present after natural infections are usually much higher than those that develop after vaccination.[22]

As with diagnostic tests for other flaviviruses, different types of antigen have been investigated in ELISAs in order

to increase the sensitivity and specificity of testing. The use of NS1 protein as the antigen to be detected shows some increase in specificity but a decrease in sensitivity. ELISAs based on NS1 do not detect anti-TBEV antibodies after vaccination, and therefore this format could be capable of distinguishing between an infection-induced and vaccination-induced immune response, which might be a relevant diagnostic question when CNS symptoms occur after vaccination. In a recent development, antibodies against the non-structural protein 1 (NS1) showed a high specificity. The detection of NS1 antibodies against TBE is also the proof for an active viral replication and therefore indicates past or recent TBE virus infection. Although it could be shown in a recent publication that traces of NS1 were detectable by mass spectrometry, it could be clearly shown that this test was able to differentiate between vaccine-induced and infection-induced antibodies.[23-25]

Secondary antibody response type

Pre-existing immunity due to previous infection or vaccination with other flaviviruses could modify the immune response to TBEV infection or TBE vaccination. In such cases, a low IgM and high IgG antibody response can usually be observed (the author's personal observation). In addition, reactivity against other flaviviruses (dengue virus, West Nile virus, yellow fever virus, Japanese encephalitis virus) can be observed independent of whether these infections, or vaccinations against these viruses, have occurred or not. Therefore, broad cross-reactivity against different flaviviruses or high IgG antibody titers should raise the suspicion of a secondary immune response (Fig. 2). Patients with TBE vaccination failure can often also display a serologic pattern consistent with a secondary immune response.

Avidity testing

The avidity of an antibody is an artificial index that indicates the binding activity of an antibody to a specific antigen. The avidity of an antibody usually increases with time after infection[26] and reaches its peak after weeks to months. The avidity index may therefore help to differentiate recent and past infections. The testing of avidity is performed by testing the sera in parallel ELISAs with and without washing with 8M urea. The avidity index is calculated as a percentage using the formula: (optical density [OD] of IgG with urea / OD of IgG without urea) ×100. Sera with an avidity index <40% are of low avidity and indicate a recent infection, whereas an avidity index >80% indicates an old infection. Avidity testing is used in suspected West Nile virus infections as there is sometimes a persistent IgM that

Figure 2: Schematic diagram of the course of specific anti-TBE antibodies in primary or secondary flavivirus infection

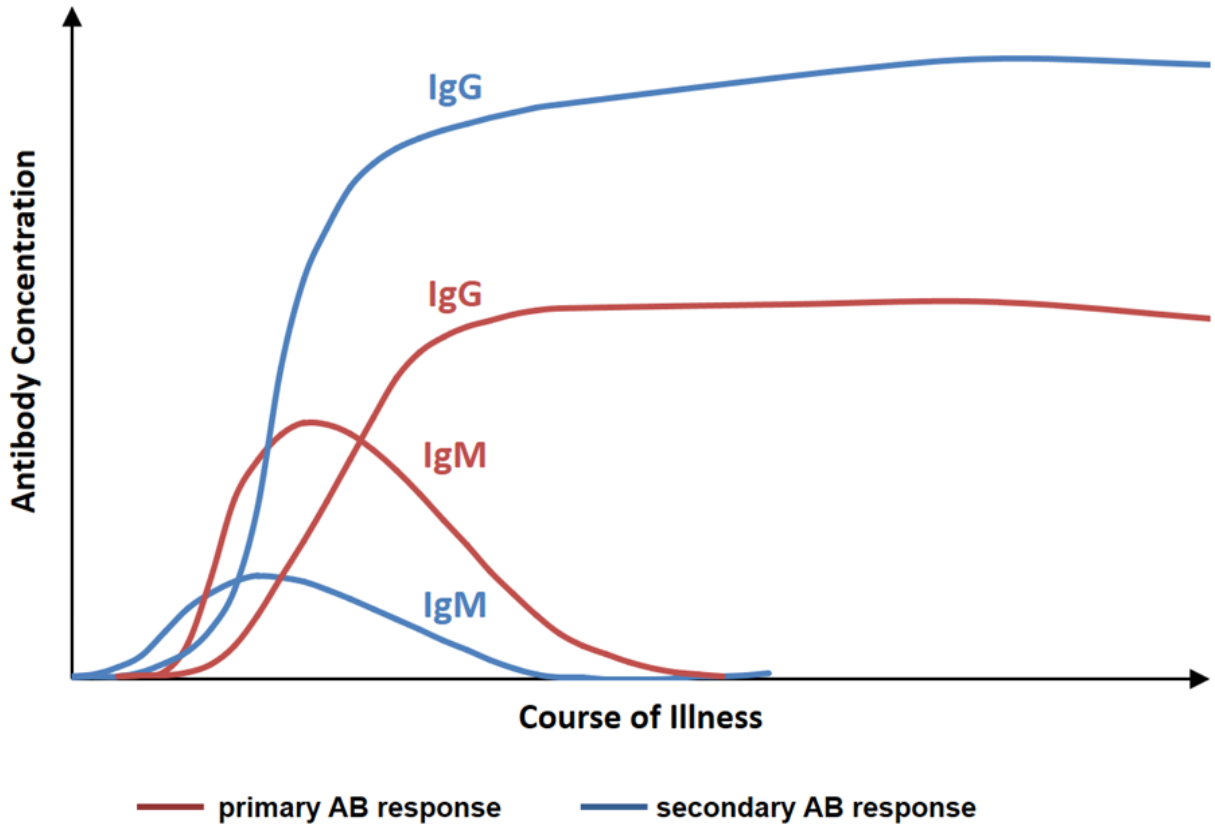

Table 2: Possible serologic constellations, their possible interpretation, and steps necessary for confirmation of TBE infection

Serologic constellation				Local CSF antibody production	Interpretation	Activity
IgM (serum)	IgG (serum)	IgM (CSF)	IgG (CSF)			
+	-	-	-	-	False-positive IgM; early phase of infection	Serologic control after 7 days; re-testing with other test format
+	+	-	-	-	Possible status after previous vaccination; very early in state of TBE infection	Serologic control after 7 days (increase in antibodies); cerebrospinal re-testing after 7 days
-	+	-	-	-	Past infection or vaccination; passive antibody transfer	Avidity testing in cases with neurologic symptoms
+	+	+	+	+	Acute or post-acute TBE infection	
-	-	-	+	Not calculable	Possibly incorrect result	Re-testing with other test format
-	-	+	-	Not calculable	Possibly incorrect positive result	Re-testing with other test format

can confound interpretation of whether an infection is recent or not. In TBEV infections, persistent IgM from a past infection is uncommon and therefore avidity testing is not routinely performed in cases of suspected TBEV infection.[19] In our laboratory, avidity testing is used to differentiate passively transferred IgG antibodies from infection-induced antibodies, e.g. to exclude Guillain-Barré syndrome in suspicious cases. Preliminary avidity testing of IgG in vaccinated persons shows that high avidity IgG is only produced after a complete basic vaccination (the author's personal observation).

Antibody testing of CSF

Both IgM and IgG anti-TBEV antibodies can be detectable in CSF at the onset of CNS symptoms, and their detection can be important in special circumstances or for supporting the diagnosis of a TBEV infection. IgM is produced locally within the CNS but is not passively transferred into the CSF to a great extent.

IgG is transferred passively, however, especially during inflammatory processes in the CNS that disturb the blood–brain barrier. The detection of IgG in the CSF is therefore not primarily indicative of an acute TBEV infection.

IgM can be detectable within the CSF during the first days of CNS symptoms in only 50% of patients and may only become detectable in the remainder during the next 10 days.[1] Therefore, the detection of IgM in serum samples is superior to the detection of IgM in CSF for the diagnosis of TBE. The detection of IgM in CSF may help to distinguish an acute TBEV infection from the antibody response induced by a recent vaccination; an 'IgM index' can be calculated for this purpose (Fig. 3).

Figure 3: Calculation of IgM index

$$\text{IgM index} \quad \frac{\text{Titer TBE-IgM (CSF)}}{\text{Titer TBE-IgM (SER)}} > \frac{\text{Total IgG (CSF)}}{\text{Total IgG (SER)}}$$

The production of IgG antibodies within the CSF must be demonstrated in order to prove that a patient has a neurologic TBEV infection,[27] and this can be evaluated by calculating the CSF serum index according to Reiber et al.[28]

There are different options for the calculation, with the most commonly used shown in Fig. 4.

Figure 4: Calculation of intrathecal antibody production

$$\text{IgG index} = \frac{\frac{\text{OD TBE-IgG (CSF)}}{\text{OD TBE-IgG (SER)}}}{\frac{\text{Total IgG (CSF)}}{\text{Total IgG (SER)}}} > 2$$

$$\text{IgG index} = \frac{\frac{\text{OD TBE-IgG (CSF)}}{\text{OD TBE-IgG (SER)}}}{\frac{\text{Albumin (CSF)}}{\text{Albumin (SER)}}} > 2$$

Serological cross reactions with other flaviviruses

Due to the close genetic relationship between the members of the genus Flavivirus within the family Flaviviridae some cross-reactions in the available serological tests might be expected. These serological cross-reactions are mainly directed against the E protein of the flaviviruses and known for most of the available serological tests and they may cause difficulties in the serological diagnosis of flavivirus infections.

Structural test formats like ELISA are especially prone to serological cross reactions; however, also hemagglutination inhibition and indirect immunofluorescence test systems show varying degrees of cross-reactions between flavivirus infections or flavivirus vaccinations. The test with the highest specificity against other flaviviruses is the neutralization test, which is believed to be highly specific for the respective flavivirus.

But besides the test systems also the different immunoglobulin classes exhibit varying degrees of cross-reactivity. While different IgG-class antibodies show high cross-reactions among the members of the flaviviruses, antibodies of the IgM-class are highly specific and usually exhibit low or no cross-reactions.

The degree of cross-reactions between different flavivirus antibodies is also dependent on the serological status of the patient resp. vaccinee. In patients exhibiting a primary immune response due to the first contact of his immune system with a flavivirus a monospecific immune response can be mainly seen with only low and mainly short-lived cross-reactions against other flaviviruses. The titer difference, which can be usually be found is significant, which means there is a significantly higher titer to the infecting resp. vaccinating flavivirus in comparison to other related, but non-applied flaviviruses.

If a patient or a vaccinee was already infected with or vaccinated with/against another flavivirus, a second flavivirus infection or vaccination may cause a serological response of the secondary type. Here high antibodies against a different number of flaviviruses can be seen. The titers are high against all flaviviruses and the infecting resp. vaccinated flavivirus cannot be distinguished anymore. Sometimes the second flavivirus induces a strong serological answer of the IgG antibodies against the flavivirus of the first infection or vaccination, which might cause disturbance and may lead to a wrong diagnosis.

These cross-reactions are also important for defining an immunity. Cross-reacting antibodies are non-protective. If a vaccinee gets e.g. yellow fever vaccine and Japanese encephalitis vaccine, there may also be cross-reacting antibodies against TBEV. If only an ELISA test is conducted this test may become positive and lead to the suspicion of immunity, which is not the case in this situation. Therefore, the diagnosis and immunity testing of flaviviruses should always include an evaluation of immune responses against different flaviviruses like TBEV, yellow fever virus, Japanese encephalitis virus, dengue viruses and West Nile virus. Only the history of the patient or vaccinee together with the serological results against the most common flaviviruses and flavivirus vaccinations will give a realistic picture of the immune status and of a potential infection.

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