Laboratory techniques in the diagnosis of syphilis: A review

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Introduction
At present, the laboratory diagnosis of syphilis is based on the visualisation of the pathogen, Treponema pallidum and/or the demonstration of specific antibodies in body fluids by means of serological tests. In this article the current methods, the test procedures that have only recently been introduced to diagnose syphilis and the diagnosis based on molecular biology techniques are reviewed.

Visualisation of the pathogen
The diagnosis of syphilis by direct visualisation of the causative organism is complicated, since at present T. pallidum cannot be routinely cultivated in vitro on artificial media. The treponemes can be observed in exudates of primary and secondary lesions and in fluid obtained from lymph node puncture upon dark-field microscopy. Recently, darkfield examination of amniotic fluid obtained by amniocentesis has been shown to be valuable in the diagnosis of congenital syphilis. The treponemes are recognised by their characteristic morphology and motility. The preparations should be examined with minimal delay because of the poor retention of motility of the treponemes in vitro. The direct or indirect fluorescent method is a suitable alternative if direct dark-field microscopy is not possible. The visualisation of T. pallidum by either method is diagnostic, provided that non-venereal treponematoses are excluded.

Rabbit infectivity testing (RIT) for routine diagnostic purposes with materials from suspected patients is impractical since the development of orchitis and/or seroconverstion in the rabbit takes rather long and appropriate facilities to house and handle the rabbits are necessary. However, RIT is the only test available to demonstrate the presence of virulent treponemes.

Histochemistry and Immunohistochemistry
Treponemes can be observed in formalin-fixed and paraffin embedded biopsy material using silver impregnation methods such as the Whartin-Starry or the Steiner method. Immunological recognition of T. pallidum in paraffin embedded tissues has been described. An immunogold staining technique for this purpose has recently been published.

Demonstration of antibodies
An ideal serological test for syphilis should have high sensitivity and specificity. In addition, such a test should be suitable for treatment-monitoring and give negative results after adequate therapy to allow a clear-cut diagnosis of reinfection. Such an ideal test is not yet available and serological testing relies on testing individuals with screening tests, followed by confirmation of the results by confirmatory tests. In screening, it is important not to miss individuals who are infected. Therefore, high emphasis is placed on the sensitivity of the test and on a high negative predictive value (NPV) of a test result. Confirmatory tests are used to distinguish true positives and true negatives among the individuals traced by the screening tests. High emphasis, therefore, is placed on the specificity and on a high positive predictive value (PPV) of test results. (For calculating NPV and PPV, see ref 12). In addition to these theoretical considerations, technical and economic aspects also play a role. These include technical simplicity, rapidity, the ease of performing IgM-based diagnostic tests, suitability for automation and financial costs.

Currently, two types of tests, namely the nontreponemal and the treponemal tests are used to determine the presence of antibodies for the serodiagnosis of syphilis. The nontreponemal tests use cardiolipin as the active ingredient in a mixture with cholesterol and lecithin to detect anti-cardiolipin antibodies. The Centers for Disease Control (CDC, Atlanta, Georgia, U.S.A.) recognise five nontreponemal tests as Standard Tests for Syphilis. These five tests have the same standardised antigen and consequently have a similar sensitivity, which varies from approximately 70% in primary syphilis to 100% in secondary syphilis. The specificity varies considerably depending on the occurrence of acute and chronic biological false-positive (BFP) reactions in the population under investigation. False-negative results may occur owing to the prozone phenomenon, which is caused by high levels of anti-cardiolipin antibodies. Upon dilution of these sera positive results are obtained, usually at high titre.

Among these five nontreponemal tests two categories can be distinguished. One category comprises those tests which can be read visually, because of the presence of a coloured substance in the antigen preparation. These tests comprise the Reagin Screen Test (RST), the Toluidin Red Unheated Serum Test (TRUST) and the Rapid Plasma Reagin (RPR) card test. The latter test, which was initially developed for field use, is the most widely used nontreponemal test. It is performed on plastic coated cards onto which circles have been imprinted. When performed...
qualitatively, standardised amounts of undiluted serum and antigen suspension containing carbon particles are mixed within the circle and spread over it. When performed quantitatively, serially diluted serum is mixed with the antigen. The card is then mechanically rotated at 100 rpm for 8 min under humid conditions. When a serum contains anticardiolipin antibodies, flocculation occurs and the carbon particles coagglutinate. The test results are read under high intensity light source in the humid state immediately after rotation and scored as reactive or nonreactive. The quantitative results are expressed as the reciprocal of the highest dilution giving a reactive result.

The second category comprises the Venereal Disease Research Laboratory (VDRL) slide test and the Unheated Serum Reagin test which have to be read using a microscope. In the VDRL slide test freshly prepared antigen and serum which had been heated at 56°C for 30 min are used. The test is performed in a very similar manner to that of the RPR: standard amounts of serum and antigen are mixed within a ring on a glass slide by rotating of the slide mechanically. If anti-cardiolipin antibodies are present, the short antigen rods aggregate to form clumps which can be observed microscopically. A quantitative VDRL is performed with serial dilutions, usually up to 1:64, of the test serum. The VDRL test is the only test recommended for investigating cerebrospinal fluid (CSF). There is no need to heat-inactivate CSF before use.

Tests for detecting anti-lipoidal antibodies are used for screening. Their titres expressed as the reciprocal of the highest dilution giving a positive result, usually decrease spontaneously in time. The decline after treatment is used for treatment-monitoring.

The treponemal tests measure specific antibodies formed by the host in response to infection with T pallidum. In the haemagglutination tests (MHA-TP, HATTS and TPHA) erythrocytes coated with treponema antigens derived from the Nichols strain of T pallidum are used. The suspension of these erythrocytes in diluted serum containing anti-treponemal antibodies results in agglutination of the red cells, which can be read visually. Despite fairly complicated absorbing diluents provided with the kits, non-specific reactions do occur. The haemagglutination tests are suitable for testing large numbers of samples since they are simple to set up and easy to read. 13

The most widely used treponemal test is the fluorescent treponema antibody absorption (FTA-ABS) test. 14 Whole micro-organisms are fixed to glass slides and are overlaid with previously absorbed test serum. Antibodies bound to the treponemes are preferably detected with an anti-IgG conjugate. A serum dilution of 1:5 in sorphons prepared from the culture medium of Reiter treponemes or from a sonicate of the latter 15,16 results in an extremely sensitive test, especially in early syphilis. However, false-positive results due to high immunoglobulin levels 17 or anti-nuclear antibodies 18 in the test sera do occur. The FTA-ABS test is at present the confirmatory reaction of choice and is used as the gold standard. Varieties of the test are used to diagnose congenital syphilis and to determine the activity of the disease. A category is the (IgM)FTA-ABS test in which an anti-IgM conjugate is used for the detection of antitreponemal IgM antibodies. 19 In the IgS(IgM)FTA-ABS test, the IgS (=IgM containing) fraction from the test sera is used to avoid false-positive reactions caused by rheumatoid factor (RF). 20 However, the FTA-ABS test is technically complicated and requires continuous quality control checks in order to obtain reliable and reproducible results. Moreover, each test result has to be read individually by an experienced observer. This excludes automation and processing of large numbers of sera and may introduce subjectivity in interpreting the results.

Treponemal tests are performed qualitatively. Once positive, the test results usually remain so for many years and often lifelong. Hence these tests are not suitable for monitoring the effect of treatment.

Shortcomings of the present tests are their insufficient sensitivity and/or specificity in the diagnosis of incubating primary syphilis, congenital syphilis and neurosyphilis and their inability to distinguish between venereal and nonvenereal treponematoses. Moreover, since the latter tests are not specific for human immunodeficiency virus (HIV) can change the outcome of serological test for syphilis, methods that can detect (parts of) the syphilis pathogens are desirable. From a technical and economic point of view, automated tests which would be suitable for screening, confirmation purposes and treatment monitoring would be welcome.

Application of ELISA

The enzyme-linked immunosorbent assays (ELISA) are widely used in the field of infectious diseases owing to the possibility of automation and the broad potential to detect antibodies and other antigens (for a recent review see ref 21). After the first use of the indirect ELISA in the serodiagnosis of syphilis, 22 several related tests in which detergent extracted or sonicated T pallidum (Nichols) were used as an antigen have been reported. 23-27 Indirect ELISA in which components from non-pathogenic treponemes 28 or modified VDRL antigen 29 were used as an antigen have been developed. Besides use in (congenital) syphilis, 30 the latter tests have also been used to distinguish the anti-cardiolipin antibodies in syphilis from the anti-phospholipid antibodies in other diseases. 31-34 In the figure a schematic representation of commercial ELISA test principles is depicted.

Two tests, based on the indirect ELISA principle and in which antigens from T pallidum (Nichols) are used are commercially available. The Syphilis Bio-EnzaBead assay (Organon Teknika Corp.) has ferrous metal beads coated with antigens from T pallidum as the solid phase. The steps in the ELISA procedure are performed by the subsequent transfer of the beads into separate microtiter plates containing dilutions of the test sera and
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Control sera, washing fluids, conjugate solution and finally the substrate solution by means of a magnetic transfer device. This test, which has provisional status as Standard Test for Syphilis (C.D.C.) was evaluated by four groups. The results are difficult to compare owing to a lack of a final reading procedure and only one reporting group separated the results on the basis of treatment. In this latter study the specificity of the test was 94-7% and that of the FTA-ABS test 98-1%. The sensitivity in FTA-ABS positive primary and secondary syphilis was 100% and in early latent and late latent syphilis it was 96-4% and 84-6% respectively. The second test is the Captia Syphilis-G test (Mercia Diagnostics, Guildford, Surrey). The antigen is coated onto the wells of a microtitre plate. After incubation with 20-fold diluted sera and rinsing, bound anti-treponemal IgG is detected with a tracer complex consisting of biotinylated monoclonal antibody to human IgG and streptavidin conjugated to horseradish peroxidase. This detection system provides an amplification of the final signal. In their evaluation of the test, Young et al. observed an overall sensitivity of 98-4% and a specificity of 99-3%. In the group of patients investigated by Lefevre et al., the Captia Syphilis-G test showed an overall sensitivity of 98-3%. In the patients with secondary, early and late latent, neuro- and congenital syphilis and in reinjected patients, the test reached a sensitivity of 100%. The same sensitivity was observed in VDRL-reactive and VDRL-nonreactive treated patients. The sensitivity in patients with untreated primary syphilis was 82%. False-positive reactions occurred in 1-5% of the patients. All 92 sera, shown to contain Rf were negative in the Captia Syphilis-G assay. The sensitivity of 82% of the Captia Syphilis-G assay in primary syphilis did not differ from the sensitivity of 84% in the combined screening with the TPHA and the VDRL tests. The authors concluded that the Captia Syphilis-G assay can be used as a single screening test for syphilis.

A third commercially available test is the Captia Syphilis-M test (Mercia Diagnostics, Guildford, Surrey), which is based on the capture ELISA principle. In this test a separation between IgG and IgM is accomplished by the capture of IgM from the test sera by antibodies to human IgM that have been coated onto the wells of microtitre plates. This separation is essential in IgM-based diagnoses to circumvent false-positive reactions caused by Rf in the test sera. The captured IgM is detected with treponemal antigens labelled with an enzyme. This test was evaluated by Lefevre et al. and Ijssel-Muden et al. The results of both groups agreed reasonably well considering the small numbers of patients tested. The sensitivity was highest in patients with untreated primary syphilis (94% and 82% respectively) and it gradually decreased when the stage of the disease progressed. Lefevre et al. noted that patients with late latent syphilis were unreactive in this test. There was a remarkable difference in sensitivity between the groups of patients with VDRL-reactive and
VDRL-nonreactive treated latent syphilis. In the former group, the sensitivity was 30-4% and in the latter group it was 3-8%. Both groups of investigators observed positive results in all cases of congenital syphilis. False-positive reactions in newborns from mothers who had been adequately treated for syphilis were not observed. The test was also negative in individuals in whom false-positive reactions in the VDRL test were observed. Two of the six individuals with RF in their serum reacted positively in the Capita Syphilis-M showing that the specificity of this test was not absolute.

Application of molecular biology techniques
The application of molecular biology techniques to the diagnosis of syphilis thus far has served two aims:

1. The production of large quantities of treponemal proteins which can serve as antigens in serodiagnostic tests.
2. Amplification of selected treponemal DNA sequences from selected material using the polymerase chain reaction (PCR). The number of copies of this selected treponemal DNA are thus increased to enable detection using specific probes.

Test with antigens derived via the recombinant DNA technology
An assay based on the indirect ELISA in which the 4D membrane protein with a molecular mass of 190 kDa derived via the recombinant DNA technique was published by Rudolf et al. In this assay anti-treponemal IgG in sera was measured. All 172 VDRL-negative control sera and the sera from 20 individuals with VDRL-positive BFP reactions were negative, indicating a very high specificity. The authors noted an extremely low optical density with these sera which they assumed to be due to the use of a purified recombinant antigen rather than whole T. pallidum sonicate. With stored sera from syphilis patients, all treated less than 3 months before, the 4D ELISA reached a sensitivity of 81% in primary syphilis and 100% in secondary and early latent syphilis. In patients with late latent syphilis the sensitivity was 86%. However, in patients with cardiovascular and neurosyphilis the test showed a sensitivity of 57%. Using fresh sera from a group of patients with dark-field positive primary syphilis, the 4D ELISA showed a sensitivity of 83% and that of VDRL and the FTA-ABS test both reached 88%. Eighty percent of the sera from yaws patients and 73% of the sera from patients with pinta were positive in the 4Da ELISA. This suggested the presence of a molecule similar to the 4D molecule in T. pertenue and T. carateum, but also showed that a test based on the 4D antigen is unable to distinguish serologically between infections caused by the three treponeme species. The authors suggested that the sensitivity of recombinant antigen based ELISA can be improved further by including additional cloned T. pallidum antigens which are immunogenic in all stages of syphilis.

A second study assessed a 37 kDa treponemal protein expressed in E. coli for its diagnostic value in a radioimmunoassay. In a group of 81 treated and untreated patients with various stages of syphilis, 71 sera reacted positively with the 37 kDa protein resulting in a sensitivity of 87-7%, while the FTA-ABS test showed a sensitivity of 100%. The specificity of the test was 95-4%. In a group of 50 sera from patients with untreated primary syphilis, 38 were reactive with the 37 kDa protein resulting in a sensitivity in primary syphilis of 76%. The sensitivity of the FTA-ABS test was 86% in this group. Four out of 42 sera from BFP reactors reacted positively with the 37 kDa protein, while all sera were negative in the FTA-ABS test.

Schouls et al. evaluated two recombinant DNA products, namely the 42 kDa treponemal membrane protein A (TmpA) and the 34 kDa treponemal membrane protein B (TmpB) for use in the serodiagnosis of syphilis. TmpB was found to be unsuitable as a single antigen because a considerable portion of the sera from patients with syphilis did not react with TmpB. In the TmpA-ELISA, 21 out of 24 (87-5%) sera of patients with untreated primary syphilis, all FTA-ABS positive, were also positive in the TmpA-ELISA. The sera from patients with untreated secondary syphilis (n = 25) and latent syphilis (n = 19) reacted positively in the Tmpa and were also positive in FTA-ABS, TPHA and VDRL tests. Sera from patients with treated syphilis were divided into groups with a positive VDRL (n = 30) and a negative VDRL (n = 30). In the VDRL-reactive group, 25 sera were positive in the TmpA-ELISA, while in the VDRL-nonreactive group, 10 sera were positive. The authors observed a correlation between the VDRL-titre and the presence of anti-TmpA antibodies and suggested that in addition to screening, the TmpA-ELISA would also be useful for monitoring the effect of therapy. This suggestion was investigated by Jisselmaiden et al. In this study the TmpA-ELISA had a sensitivity of 76% (42/55) in untreated primary syphilis, a sensitivity of 100% in secondary and of 98% in early latent syphilis. The lower sensitivity in primary syphilis in this study was due to the incorporation of dark-field positive but FTA-ABS negative patients. In treated VDRL-reactive syphilis patients, the TmpA-ELISA had a sensitivity of 85% and in VDRL-nonreactive syphilis patients a sensitivity of 37%. In a group of 27 patients with VDRL-negative syphilis, it was shown that in the sera from 25 patients with early syphilis, the kinetics of the decline in anti-TmpA antibodies paralleled the decline in anti-cardiolipin antibodies in response to therapy. The two exceptions were the sera from a patient with late latent syphilis and from a patient with asymptomatic neurosyphilis. The authors recommended the TmpA-ELISA assay (commercially available from Euro-Diagnostics, Apeldoorn, The Netherlands) as a screening test which would also be suitable for treatment-monitoring.

Application of PCR technique to the diagnosis of syphilis
The polymerase chain reaction has been used
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by Hay et al.50 to detect DNA sequences specific for pathogenic treponemes in cerebrospinal fluid (CSF). Sequences of 519 and 428 base pairs (bp) from theTmpA and 4D genes respectively were amplified. Using the method, it was possible to detect 65 organisms in 0.5 ml CSF as determined by diluting suspensions of Nichols treponemes. The specificity using 30 CSF specimens from individuals not suspected of HIV infection or syphilis was at least 96.7%. From the 19 CSF specimens examined for latent or tertiary syphilis, 10 (53%) were positive. Twenty-eight specimens obtained from HIV-positive patients, of whom 14 had "CNS disease" were tested for the presence of treponemal DNA. Seven patients from the CNS disease group were positive. From the remaining seven patients in the CNS disease group and from the 14 patients without CNS disease, three and five patients respectively had a history of syphilis. None of these 21 patients were positive using the PCR technique. Noordhoek et al54 used PCR to detect treponemal DNA in CSFs from 27 patients with untreated symptomatic and asymptomatic neurosyphilis. The gene for the 39 kDa basic membrane protein was chosen for amplification. This was performed in two parts. In the first part the primers allowed the amplification of a 617 bp fragment, in the second part a second primer set (nested primers) allowed amplification of a 500 bp fragment from the previously amplified 617 bp fragment. With this PCR method, it was possible to detect that amount of isolated treponemal DNA which was equivalent to a single treponeme and a minimum of 100 treponemes per ml when suspended in CSF. Specificity was assessed using a large variety of microorganisms including Borrelia burgdorferi. Specific amplification was only noted with DNA from T. pallidum ssp pallidum and pertenue. Positive results were obtained with CSFs from five out of seven patients with acute neurosyphilis. None of the four patients with chronic neurosyphilis but two out of 16 patients with asymptomatic neurosyphilis were positive. However, CSFs obtained up to three years after treatment were frequently positive using PCR. This was rather puzzling since none of the patients showed evidence of active disease following treatment and the titres in the serological tests declined slowly.

Burstein et al53 amplified a 658 bp portion of the gene coding for the 47 kDa membrane protein. The probe used to detect the amplified DNA by hybridisation consisted of a large 496 bp fragment included in the amplified DNA. Using this method, the authors were able to detect treponemal DNA in some samples calculated to contain a single treponeme. Special attention was paid to DNA that could possibly contaminate clinical isolates. No specific amplification products were obtained from any of the tested skin microbes, central nervous system pathogens and microbes causing STD. Specific amplification occurred only with the DNA from T. pallidum ssp pertenue. Positive PCR results, confirmed by RIT were obtained with the amniotic fluid from the two pregnant women with untreated syphilis, with the serum and the CSF from the patient with untreated early latent syphilis, but whose CSF showed normal clinical laboratory tests and with the CSF from the patient with syphilitic meningitis. The CSFs from two patients with general paresis were negative.

The study by Grimprel et al54 focused on the inhibition of PCR by contaminants in clinical samples to optimise the method for the diagnosis of congenital syphilis. This inhibition was noted in a previous report, especially when larger volumes were used for amplification.53 Four different methods were used to prepare DNA prior to amplification: a boiling method, a low spin centrifugation method, the alkaline lysis extraction method and a spin extraction method. The authors suggested that preparation of DNA from amniotic fluids should be performed by the boiling and the low spin centrifugation method. For neonatal CSF and sera the simple boiling method should be initially used with 10 and 20 μl volumes and if necessary, followed by the low spin centrifugation method. The method showed a specificity of 100% indicating that false-positive results due to contamination of the samples during collection and processing had not occurred. The sensitivity of the PCR, as compared with RIT was 100% for amniotic fluids, 71% for neonatal CSFs and 68% for sera. An interesting aspect was the detection of an in utero treatment failure. Used in this manner, the PCR technique would be useful to establish optimal treatment schedules for congenital syphilis.

Attempts to distinguish sub-species of T. pallidum Recombinant DNA studies showed a single base pair difference between the genes tpf-1 and tylf-1 which code for homologous sub-units of the 190 kDa proteins of T. pallidum ssp pallidum and ssp pertenue respectively.54 This difference was exploited in attempts to distinguish between DNA extracted from rabbit derived T. pallidum ssp pallidum and ssp pertenue after amplification using the PCR technique. DNA from three of the four T. pallidum ssp pallidum strains contained the "pallidum" trait. In five out of the six strains of T. pallidum ssp pertenue the "pertenue" trait was present. This indicated that the observed difference between the tpf-1 and tylf-1 genes did not constitute a definite trait for either T. pallidum sub-species.55 The sera from patients with syphilis and yaws could not be distinguished in the ELISA in which two synthetic homologous oligopeptides of 15 amino acids each reflecting the one base pair difference were used as antigens.57

Discussion
Screening is performed by a single lipoidal test, usually the VDRL (USA) or a screening combination of VDRL and TPHA (Europe). Neither test can be fully automated easily and the results are subjective and recorded manually. Fully automated tests in which these two aspects are incorporated would reduce the
work-load considerably, especially for testing large numbers of sera. Tests based on the ELISA methodology offer this possibility. The Captia Syphilis-G test was initially recommended as a confirmatory test because of its sensitivity in primary syphilis of 82%. This test was recommended as a single screening test when it was shown that this did not differ significantly from the sensitivity of the combined screening with VDRL and TPHA. It has a sensitivity of 100% in treated and untreated secondary, latent and late syphilis. Since the test has a sensitivity of 100% in VDRL-reactive and VDRL-nonreactive treated syphilis at all stages, the test would be unsuitable for treatment-monitoring. For this purpose, the VDRL can be used, but the Captia Syphilis-M test has been recommended as an alternative. This test has been recommended for the diagnosis of congenital syphilis and offers the possibility of IgM-based diagnosis outside the setting of specialised laboratories. A disadvantage is the specificity, which is not absolute in sera containing RF. The Syphilis Bio-EnzaBead test, designed as a confirmatory test was evaluated by four groups. In these studies the Bio-EnzaBead test had a somewhat lower specificity than the FTA-ABS test. This does not favour the replacement of the latter by this test. The test showed a lower sensitivity in treated syphilis as compared with the FTA-ABS and the MHA-TP. This indicated that the test may be valuable in treatment-monitoring. However, the time period between treatment and nonreactive test results requires further investigations.

From the three published tests in which a recombinant DNA protein was used as an antigen, only the Tmpa-ELISA was evaluated as a diagnostic test. This test had a high sensitivity in untreated patients. However, in treated patients the sensitivity varied. The sharp decrease in the Tmpa-TMP antibodies within a year after treatment correlated with the decline in VDRL titre. For this reason, the authors recommended this test for screening and for monitoring the effect of treatment. PCR seems pre-eminently suitable for detecting DNA from low numbers of treponemes in clinical samples. The few PCR studies published focused on the diagnosis of congenital and neurosyphilis. In two studies it was shown that the method of preparing DNA prior to amplification has to be considered in order to remove or inactivate contaminants in the clinical material which would otherwise inhibit the DNA polymerase reaction catalysed by the Taq polymerase. The results of optimised PCR method showed a good correlation with RIT using amniotic fluids. Notably, the amniotic fluid from both pregnant women with primary syphilis showed negative PCR- and RIT results. The number of positive PCR results was considerably less with neonatal CSFs and sera.

Both PCR studies with CSF from patients with untreated late syphilis showed a large difference in the overall positive rate (53% versus 26%). Unfortunately, RIT was not performed in these studies. However, the results differ from the RIT results with the CSFs from patients with late syphilis in the study by Lukehart et al. Furthermore, the presence of DNA in CSFs from intravenously treated patients stressed the importance of simultaneous RIT studies. The question as to whether the presence of treponemal DNA reflects the presence of virulent treponemes or DNA from dead treponemes is highly relevant for investigating patients in whom the serological detection of syphilis is hampered by coinfection with HIV. Further studies are necessary to establish the exact value and place of PCR technique in the armament of currently used diagnostic tests for syphilis.

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