Arch Public Health
1999, 57, 263-274

Role of the virology laboratory in the diagnosis of central nervous system infections

by

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Abstract

Viral infections of the central nervous system (CNS) have two major clinical presentations: 'aseptic' meningitis and meningoencephalitis. Other manifestations like cerebellar ataxia, myelitis, cranial and peripheral nerve palsies, post-infectious syndromes are far less frequent. Enteroviruses are the leading cause of viral CNS infections, with mumps, herpesviruses, HIV, measles, rubella, ... being responsible for a minor part of these infections. As antiviral therapy is available and efficient on some of these infections, rapid diagnosis of viral CNS involvement has become an important goal of the virology laboratory. Conventional techniques have been disappointing, lacking sensitivity and/or rapidity. The most promising recent development in rapid detection of viruses in the CNS has been the application of molecular amplification methods on cerebrospinal fluid (CSF). Among these, polymerase chain reaction (PCR) is the most popular assay. Experience with PCR in the diagnosis of viral CNS is now growing. Various studies have demonstrated the good performances of PCR in terms of sensitivity and speci-

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ficity and its superiority compared to conventional techniques in the diagnosis of herpes simplex encephalitis, enterovirus meningitis, cytomegalovirus-related neurological diseases in immunosuppressed patients, ... Application of PCR in CNS syndrome without clear etiology like Mollaret's meningitis or in pathology usually diagnosed by brain biopsy like progressive multifocal leucoencephalitis has allowed the detection of implicated viruses (H.S.V. and J.C. virus) directly in CSF samples. Although application of PCR in the diagnosis of viral infections of the CNS appears very promising, some important problems remain unresolved. Assessment of the accuracy of PCR in CSF should compare PCR results with a gold-standard test. However, standard techniques are insensitive (culture), late (intrathecal specific antibody production) or invasive (brain biopsy). Clinical criteria alone are not able to discriminate between CNS infections of various etiologies. Accuracy of PCR in the diagnosis of viral CNS infections must be established by comparison with a body of clinical, neurological, biological investigations. The virology laboratory can play an important role not only in the diagnosis but also in the management of CNS infections, provided that information exchanges and communication between physicians and the laboratory are established.

Key words

Aseptic meningitis, meningoencephalitis, enterovirus, PCR.

Introduction

Rapid diagnosis of central nervous system viral infections has become an important goal of the virology laboratory for two main reasons. Antiviral therapy for viral infections is now available and efficient but should be administrated early in the disease to improve the prognosis of the patient. Rapid viral diagnosis could reduce costs due to unnecessary hospitalization and antibiotic treatment.

The most promising recent development in rapid detection of viruses in the cerebrospinal fluid (CSF) has been the application of molecular amplification methods. Contribution of these methods to the diagnosis of enteroviral infections which are the most common cause of central nervous system (CNS) viral infections in humans, is a highly representative

example of the progress and problems that can be encountered in these fields.

Enteroviruses (EV) are members of the picornaviridae family which includes also the genus rhinovirus responsible for the common cold. In the United States, non-polio EVs cause an estimated 10 to 15 million symptomatic infections annually. These infections occurs principally during the summer and fall. Young children are the most common victims as both the incidence and severity of enterovirus infections vary inversely in relation to patients' age (1). There are 66 distinct human EVs: 3 polioviruses, 23 coxsackies A, 6 coxsackies B, 30 echoviruses and four enteroviruses. EV infections can occur in epidemic form or in sporadic cases but many infections are asymptomatic. EVs are responsible for clinical diseases affecting many organ systems. Each disease can be caused by several different EVs and each EV can cause several different syndromes even during the same epidemic. EVs are the most common cause of aseptic meningitis and a frequent cause of encephalitis. Paralytic myelitis is becoming rare due to polio vaccination but can be observed with non-polio EVs. Cerebellar ataxia, Guillain-Barré syndrome and transverse myelitis have occasionally been associated with EV infection, but these associations are weakened by the difficulty in distinguishing pathogenecity of throat or stool isolates from coincidental viral shedding which may occur for weeks to months after EV infection (2).

The most common central nervous system infection caused by EVs is meningitis. Neonates are at risk for severe systemic illness especially with coxsackies B and echoviruses (3, 4). In a prospective study of neonates of less than two weeks of age with proved EV infections, 75% had clinical and/or laboratory evidence of meningitis (5). In retrospective studies, substantial mortality rates have been reported, going from 3 to 75% and were associated with the presence of myocarditis or hepatic disease (3). EVs meningitis after two weeks of age is rarely associated with severe disease or poor outcome. Onset of meningitis is sudden, fever and headache are the most consistent pattern of presentation. Other non specific signs can be associated like vomiting, rash, cough and pharyngitis, ... Transient neurological abnormalities have been reported in 5 to 10% of the cases (6, 7, 8). More than 10 000 cases of aseptic meningitis were reported in the United States annually until 1994 when aseptic meningitis has became non longer nationaly notifiable, and the actual incidence is probably ten-fold higher (9). The vast majority of cases are due to viral infections and 80 to 90% of identified etiologic agents in CSF are EVs (2). In England, the most common EVs isolated

from CSF between 1975 and 1994 were echoviruses 11, 9, 19, 30, 7 and coxsackie A9 (10).

Laboratory diagnosis

Viral culture

Isolation of an EV in CSF using cell culture remains the "gold standard" for diagnosis. However detection of CNS enteroviral infection remains technically difficult (reviewed in 11). Presence of neutralizing antibodies and low virus titers in the CSF, not exceeding 10 to 10^3 ID_{50}/ml, can hamper the recovery of the virus in cell culture. Although EVs are stable in liquid environments, inadequate collection handling and processing of specimens may reduce diagnostic sensitivity. There is a loss of infectivity when specimens are kept at room temperature for hours or are allowed to dry. No single cell type is suitable for recovery of all common EVs. For practical purposes, most laboratories use a combination of two cell lines for diagnosis. The growth requirements of different groups of EVs vary considerably. For example, inoculation into newborn mice is the method of choice for isolation of most coxsackie A viruses, but this method is not used anymore because of problems of animal maintenance. During meningitis, EV can be recovered from throat or feces. However, feces are the most sensitive and least specific site for detecting EV associated illness, particularly in children, as the virus may remain detectable in feces for weeks to months after an EV infection and may have nothing to do with a current meningitis. Last, viral culture of the CSF typically requires 3 to 7 days for a cytopathic effect to develop and is rarely of immediate help in the diagnosis. Using viral culture 25% to 35% of patients with aseptic meningitis have EV isolated from their CSF. Cultures of other sites can increases EVs recovery (table 1).

TABLE 1
Results of EV culture in aseptic meningitis

	CSF	All sites
Singer et al (7)	25%	35%
Chonmaitree et al (12)	32%	41%
Berlin et al (13)	35%	60%
Wildin et al (14)*	77%	36% (throat swabs) 85% (rectal swabs)

* EV isolated in all patients of the series

Serology

Serologic assays have a limited role in EV diagnosis, because of the great diversity of EV, the lack of a common antigen and the high percentage of cross-reactivity. Antibody derterminations can be performed by neutralization, complement fixation, hemagglutination inhibition or immunoassays. Neutralizing antibody determination are cumbersome and generally reserve to reference laboratories. Complement fixation antibodies are broadly cross-reactive among non-polio EV serotypes but have had some utilities in the diagnosis of poliovirus and coxsackies B infections. As only a part of all EV serotypes agglutinate erythrocytes, hemagglutination inhibition is an unsensitive technique. IgM assays have been developed in the eighties but clinical experience with these tests is limited (15).

Molecular methods

The most promising development in direct detection of viruses in CSF has been the application of polymerase chain reaction (PCR). Various sets of primers directed at highly conserved sequences of the 5' non-coding (NC) region of the viral genome have been designed for reverse transcription combined with PCR (RT-PCR) as EVs are RNA viruses. Various methods of extraction, of PCR products identification have been used (16, 17, 18, 19, 20). Recently, a commercial assay, the Amplicor Enterovirus test (Roche Diagnostics System – Branchburg-NJ-USA) has become available allowing the use of the same enzyme for the reverse transcription step and the PCR step and using the nucleotide dUTP and the enzyme uracyl N-glycosylase to reduce carry over contaminations (21). A lot of studies have been published that evaluate the sensitivity of RT-PCR to detect EVs in case of aseptic meningitis (22, 23, 24, 25). However, as the gold standard is not a real one because of the low sensivity of viral culture and as symptoms of CNS EV infection are not specific, a problem of case definition is encountered in all these studies. For example, in some studies a result of RT-PCR is confirmed with another RT-PCR (25, 26). Moreover as many different protocols of RT-PCR have been proposed, comparison between studies is difficult. Table 2 compiles the results of studies that have used the Amplicor test in the CSF of patients with aseptic meningitis. Sensivity of the Amplicor assay compared to culture is between 78 and 100%. Some studies have also compared Amplicor with their own in-house protocol of RT-PCR and generally these tests seems to perform a little better (25, 26, 27).

TABLE 2
Sensitivity of the Amplicor EV RT-PCR assay

	N positive Amplicor/ N positive culture (%)	N positive Amplicor/ N positive inhouse RT-PCR (%)
Tanel et al (22)	7/9 (78)	–
Ahmed et al (24)	13/13 (100)	–
Kessler et al (26)	26/27 (96)	34/35 (97)
Pozo et al (25)	13/13 (100)	43/46 (93)
Yerly et al (27)	12/13 (92)	25/29 (86)

Some authors have evaluated performances of Amplicor and in-house RT-PCR applied on CSF of meningitis cases occurring during an outbreak of EVs infections (table 3).

TABLE 3
Sensitivities of EV detection methods in outbreaks of aseptic meningitis

	Yerly et al. (27)	Pozo et al. (25)	Gorgievski-Hrisoho et al. (28)
EV culture	34%	26%	24%
Amplicor assay	66%	86%	85%
in house RT-PCR	76%	92%	–

The assumption is that all the patients with an aseptic meningitis during an outbreak are true positive cases of EV meningitis. Again sensitivity of in-house RT-PCR in these studies seems to be better than that of Amplicor.

According to different studies, 3 to 60% (24, 25, 26, 27, 28) more CSF are found positive by the Amplicor assay compared to culture. However, in a multicenter study which compares the Amplicor assay conducted in one reference laboratory with viral culture done in 9 different laboratories, only 4% of CSF which were culture negative were found positive by Amplicor (29).

A multicenter evaluation of EV RT-PCR has been conducted in 13 french laboratories, on a panel of 20 CSF (30). Seven of the samples were not infected, 3 were infected with a non EV virus and 10 samples were infected with 10, 1 and 0.1 ID_{50}/ml of 3 different types of EV. All the laboratories used the Amplicor test and 5 tested also the panel with their in-house RT-PCR. Eight laboratories analyzed the panel by cell culture. Sensitivities of Amplicor, in-house RT-PCR and culture were 61, 68, 30% respectively. The sensitivity varies greatly according to the viral load:

sensitivity was 96% for 10 ID_{50} and only 16% for 0.1 ID_{50}. False positive results were obtained in negative CSF and in CSF infected with another virus. False positives were more frequent with replicates suggesting that, even with the Amplicor kit, the risk of carry over contaminations is not totally eliminated. The authors concluded that differences between sensitivities obtained in the different laboratories were small, although one lab obtained a low sensitivity of 37% compared to a mean sensitivity of 61%. The main factor influencing the sensitivity of PCR is the viral load. Although a sensitivity of 0.1 ID_{50} could be obtained on dilutions of EV in culture medium (21, 31), this is difficult to reach with clinical specimens probably because of the presence of inhibitors and the degradation of nucleic acids responsible for false negative results (30, 31). Anyway, the Amplicor assay and in-house RT-PCR are much more sensitive than culture whatever the viral load. Despite the need to improve the performances of the kit, these authors concluded that the Amplicor assay appears a suitable tool for the clinical laboratory setting (30).

TABLE 4
Results of EV culture and RT-PCR in CSF and by patient

	CSF **N positive/Total (%)**	**Patients** **N positive/Total (%)**
Culture	13/75 (17%)	13/72 (18%)
RT-PCR	42/131 (32%)	40/124 (32%)

Table 4 exposed our prospective experience with RT-PCR on CSF sent to the laboratory for evaluation of aseptic meningitis. We used a nested RT-PCR directed to a sequence of the 5' NC region described for picornaviruses by Kämmerer (19). Each sample was extracted twice and 2 amplifications were conducted for each extraction. Eighteen percent of our patients had a positive culture of their CSF. Most common isolates were Echoviruses 6, 11, 30, and 9. In 32% of the patients, PCR was positive in the CSF. All culture positive CSF were found positive by RT-PCR. Patients with positive RT-PCR were young: almost half of them had less than 3 years of age. For each positive case we have had a contact with the clinician in charge of the patient and in almost all cases, biological results and clinical symptoms were compatible with an EV meningitis. In one ill-treated child with a drained intracranial hyperpressure the diagnosis of an EV infection was not confirmed. Another child presented with a rash typical of coxsackie A virus infection. A lumbar puncture was done because of high fever. There was no pleocytosis in the CSF but the RT-PCR was positive. An EV grew in a stool culture which could not be

typed. It is known that EV can be recovered from CSF even in the absence of abnormal cell count (14, 23, 32).

Conclusion

EV RT-PCR allows a better diagnosis of aseptic meningitis due to EVs. Sensitivity is affected by the viral load in the CSF. Clinical sensitivity is lowered by the presence of inhibitors in clinical samples, by degradation of nucleic acids and by storage conditions. One of the studies previously mentioned had shown a decrease sensitivity of the Amplicor assay when CSF have been stored at -20°C (25). Thus retrospective studies are probably not recommended to evaluate the Amplicor assay. Contamination remains a problem even with an assay using the uracyl-N-glycosylase protocol. All EVs are not detected with the same efficiency. For example, the Amplicor assay is known to be insensitive to Echovirus 1, 5, 22 and 23 (33). Another study (29) have found negative Amplicor EV test result on EVs that grew in culture. However, as the Amplicor EV test does not included an internal control of amplification in its current design, it was impossible to determine if these EVs were not recognized by the Amplicor primers or if there was inhibitors in the CSF samples. The ability of in-house RT-PCR to detect various serotypes of EVs have also been evaluated, mostly on EV strains with different efficiency depending of the PCR protocol (17, 18, 19). Experience with clinical specimens is limited, although, generally, in-house RT-PCR seems to be more sensitive than the Amplicor test (25, 26, 27). Despite these limitations, PCR is a major advance in the diagnosis of CNS infections. The technique provides a diagnosis in a few hours, a sufficient time to influence clinical decisions. A study of a large outbreak of EV meningitis has shown that 90% of the patients are hospitalized for one or two days, 74% receives at least one doses of intravenous antibiotics, 32% undergo a head computer tomography (34). In another study authors have calculated that a 17% to 35% of cost reduction can be obtained by the use of routine EV RT-PCR, allowing early discharge from the hospital, reducing unnecessary investigations and empiric therapy (35). These studies suggested that patients with aseptic meningitis could benefit from a rapid test like RT-PCR for EV detection.

RT-PCR for detection of EV infections of the CNS is promising, although sensitivity should be precised using controls of amplification, evaluation of clinical samples infected by the most common serotypes encountered in CNS diseases, quality controls, ... Beside classical precautions in the PCR laboratory, a control of sensitivity and specificity

could also be evaluated by continuous communication between physicians in charge of the patients and the laboratory. This communication would allow to order appropriate tests, to carry out appropriate specimen collection, transport, storage, to identify rapidly possible problems by confronting laboratory results with clinical data.

Résumé

Les infections virales du système nerveux central (SNC) sont essentiellement responsables de deux tableaux cliniques: la méningite 'aseptique' et la méningo-encéphalite. De façon moins fréquente, des infections virales peuvent également causer d'autres atteintes neurologiques comme des ataxies cérébelleuses, des myélites, des paralysies des nerfs crâniens ou périphériques, des syndromes post-infectieux. Les entérovirus sont les agents principaux des infections virales du SNC. Une part mineure de ces infections est due aux oreillons, au virus du groupe herpès, au VIH, à la rougeole, à la rubéole, ... Des traitements antiviraux ayant montré une efficacité dans certaines de ces infections, le diagnostic rapide d'une atteinte virale du SNC est devenu un objectif important du laboratoire de virologie. Les techniques conventionnelles sont décevantes; elles manquent de sensibilité et/ou de rapidité. Actuellement, le développement le plus prometteur dans le domaine de la détection rapide des virus au niveau du SNC est l'application des méthodes d'amplification moléculaire au prélèvement de liquide céphalo-rachidien (LCR). Parmi ces méthodes, la réaction de polymérase en chaîne (PCR) est devenue la plus populaire et l'expérience en ce domaine ne cesse de croître. Diverses études ont montré les bonnes performances de la PCR en terme de sensibilité et spécificité et sa supériorité sur les techniques conventionnelles, en particulier dans le diagnostic de l'encéphalite herpétique, des méningites à entérovirus, des atteintes neurologiques causées par le cytomégalovirus chez les patients immunocompromis, ... L'utilisation de la PCR dans des syndromes sans étiologie claire jusqu'alors, comme la méningite de Mollaret ou dans les pathologies habituellement diagnostiquées par biopsie cérébrale, comme la leucoencéphalite multifocale progressive, a permis la mise en évidence des virus impliqués (virus de l'herpès simplex et virus JC) directement dans des échantillons de LCR. Malgré toutes les avancées dans ce domaine, certains problèmes doivent être résolus à l'avenir. Afin de déterminer la précision de la technique PCR appliquée au LCR, il faut pouvoir comparer les résultats de PCR avec une technique 'gold standard'. Malheureusement les techniques considérées comme des standards sont soit insensibles (culture), soit tardives (recherche d'une production intrathécale d'anticorps spécifiques), soit invasives (basées sur une biopsie cérébrale). Les critères cliniques, seuls, ne permettent pas de discriminer les infections du SNC d'étiologies variées. La précision de la PCR pour le diagnostic des infections virales du SNC doit être établie en se basant sur un ensemble de données cliniques, neurologiques et biologiques. Le laboratoire de virologie peut être amené à jouer un rôle important non seulement dans le diagnostic, mais également dans la prise en charge des infections du SNC à condition de développer l'échange d'informations et la communication entre les cliniciens et le laboratoire.

Mots-clés

Méningite aseptique, méningo-encéphalite, entérovirus, PCR.

Samenvatting

Virale infecties ter hoogte van het centraal zenuwstelsel (CZS) komen voor onder twee klinische vormen: 'aseptische' meningitis en meningo-encefalitis. Andere verschijningsvormen, zoals cerebellaire ataxie, myelitis, craniale en perifere zenuwverlamming en postinfectieuze syndromen komen veel minder voor. Vooral het enterovirus veroorzaakt CZS-infecties. Bof, herpesvirus, HIV, mazelen, rodehond, enz. zijn verantwoordelijk voor een beperkter aandeel. Het vaststellen van de virale oorsprong, is een belangrijke doelstelling geworden van het laboratorium voor virologie, aangezien antivirale therapie beschikbaar is en efficiënt blijkt voor enkele van de bovenstaande infecties. Traditionele technieken zijn teleurstellend door hun gebrek aan gevoeligheid en/of snelheid. De meestbelovende recente ontwikkeling voor snelle virusopsporing in het CZS is de toepassing van moleculaire amplificatietechnieken op cerebrospinaal vocht (CSV). De polymerase chain reaction (PCR) blijkt de populairste techniek. Voor de diagnose van de virale oorsprong van CZS-infecties wordt steeds vaker gebruik gemaakt van de PCR. Verschillende studies hebben de superioriteit aangetoond in termen van gevoeligheid, specificiteit en kwaliteit, in vergelijking met traditionele technieken: voor de diagnose van herpes simplex encefalitis, enterovirus meningitis, cytomegalovirusgebonden neurologische aandoeningen bij immunosuppressieve patiënten, enz. Bij het Mollaretsyndroom, waarbij duidelijke etiologie ontbreekt, of bij de pathologie, die gebruikelijk wordt gediagnosticeerd door middel van hersenbiopsie zoals progressieve multifocale leuko-encefalitis, heeft de toepassing van de PCR het mogelijk gemaakt om betrokken virussen (HSV en JC-virus) rechtstreeks op te sporen in stalen van CSV. Hoewel de PCR voor de diagnose van virale CZS-infecties bijzonder veelbelovend lijkt, blijven enkele belangrijke problemen onopgelost. Om de nauwkeurigheid te bepalen, zouden PCR-resultaten moeten worden vergeleken met een 'gold standard', ongeacht het feit dat standaardtechnieken ongevoelig zijn (cultuur), laattijdig (intrathecale specifieke antistoffenproductie) of invasief (hersenbiopsie). Klinische criteria alleen kunnen geen onderscheid maken tussen de verschillende etiologieën van CZS-infecties. De nauwkeurigheid van de PCR bij de diagnose van virale CZS-infecties moet worden ontwikkeld door vergelijking met een geheel van klinische, neurologische en biologische gegevens. Op voorwaarde dat de informatie-uitwisseling en de communicatie tussen artsen en het laboratorium worden uitgewerkt, kan het laboratorium voor virologie hierbij een belangrijke rol spelen en niet alleen voor de diagnose, maar ook voor het beleid bij CZS-infecties.

Sleutelwoorden

Aseptische meningitis, meningo-encefalitis, enterovirus, PCR.

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