



**DETECTION OF ENTEROVIRUSES AND ADENOVIRUS BY REAL-
TIME POLYMERASE CHAIN REACTION IN CELL CULTURE
NEGATIVE STOOL SPECIMENS OF PATIENTS WITH ACUTE
FLACCID PARALYSIS IN MOROCCO**

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ABSTRACT

In the era of polio eradication, acute flaccid paralysis (AFP), a major clinical presentation of poliovirus infection, continues to pose a public health problem. Several infectious agents comprising different families of viruses have been isolated from cases of AFP. Non-polio Enteroviruses (NPEVs) are considered to be the main infectious agents in AFP cases; further investigations have shown the isolation of *Adenovirus* from the stools of these patients. However, some Enteroviruses do not grow in the conventional cell lines used for the detection of Enteroviruses which is laborious and time consuming. Thus, more sensitive and faster methods are urgently needed for accurate detection of Enteroviruses in stool samples from AFP cases.

In this study, we aimed to detect Enteroviruses and Adenoviruses in 225 culture negative stool specimens collected from AFP cases by RT-PCR. The Enteroviruses were present in 30/225 samples of AFP patients (13%), and Adenovirus were detected in 18/225 samples (8%). Of note, a number of 5/225 samples (2.2%) showed coinfection of Enteroviruses and Adenoviruses. These results show that the RT-PCR is more rapid and sensitive than the culture. It is therefore necessary to replace cell culture with molecular methods to improve the sensitivity of detecting Enteroviruses in patients with AFP and to expand the range of viruses sought to demonstrate their involvement in the onset of paralysis.

INTRODUCTION

Acute flaccid paralysis (AFP) is known as a weakness in one or more limbs or the respiratory or bulbar muscles, resulting from damaged lower motor neurons in children under 15 years old [1].

The most common viral causes are polio and non-polio Enteroviruses. In the era of polio eradication, the emergence of other neurotropic viruses, which may cause infections mimicking acute paralytic polio, is of major concern [2].

The poliomyelitis eradication program has progressed through AFP successful surveillance. The main aim of AFP surveillance is the detection of poliovirus, however neurological diseases can be also induced by either non-infectious or infectious pathogens other than poliovirus. Indeed, a number of known viral families have been frequently detected in stool specimens of AFP cases [3].

Among infectious agents, viruses from different families such as Enteroviruses, Flaviviruses, Herpes viruses, Rabies virus and some Arboviruses have been isolated frequently from stool samples of AFP [3]. However, their etiology needs to be further elucidated.

Human Enteroviruses (EVs) are a genus of the Picornaviridae family and responsible for a number of neurological pathologies including acute flaccid paralysis (AFP), encephalitis, and meningitis [4].

Adenoviruses are non-enveloped icosahedral dsDNA viruses. The genus MastAdenovirus comprises mammalian Adenoviruses including 85 genotypes of Human Adenoviruses (HAdV), which are classified into seven species, HAdV-A to HAdV-G. *Adenovirus* infection in immunocompetent subjects is usually asymptomatic or manifests as a mild respiratory syndrome [5]. Adenoviruses have also been shown to infect

the Central Nervous System (CNS) and have been isolated from patients with acute myelopathies [6] and AFP [7].

The method used to isolate poliovirus and non-polio Enterovirus (NPEV) from fecal samples of cases of acute flaccid paralysis is based on tissue culture of viruses on at least two different cell lines (Rd and L20B). Unlikely, failure to isolate virus in cell lines may underestimate the rate of positive specimens for Enterovirus isolation.

Hence, we aimed to detect Adenoviruses and Enteroviruses in stool specimens of AFP cases by RT-PCR sent to the laboratory for investigation of poliomyelitis as part of the global eradication of polioprogram and to compare the performance of molecular results with those of culture.

Methods

Specimens

In this retrospective study, we analyzed 225 patients with clinical features of AFP referred to the National Polio Laboratory of Morocco (NPLM) in 2018-2019, all subjects were under 15 years old. Stool specimens were subjected to chloroform pre-treatment. Briefly, about 2 gr of each sample was mixed with 5 ml of Phosphate Buffer Saline (PBS) and 0.5 ml of chloroform. The solution was shaken vigorously for 20 minutes and centrifuged (20 min, 2100 ×g, 4°C) [8]. The

supernatant or stool suspension was used for cell culture [1].

The analyzed specimens were negative for poliovirus and non-polio Enterovirus detection by cell culture, their stool suspensions were used for nucleic acid suspension.

RNA and DNA extraction

Automated extraction of the viral DNA and RNA has been processed by using the Qiagen Qiacube System with the AllPrep DNA/RNA Mini Handbook kit (Qiagen).

The Real-Time PCR and Retrotranscriptase Real Time PCR were performed using the Enterovirus PCR kits - O-DiaENT™ and Diagenode Adenovirus (R-DiaADV) for Enterovirus and Adenovirus detection in stool specimens respectively; according to the manufactures instructions.

RESULTS

Among the stool samples from AFP cases sent to the national polio reference laboratory during the period 2018-2019, 225 negative cell culture samples were randomly selected for the detection of Enteroviruses and Adenoviruses.

All patients were less than 14 years old.

The Enteroviruses were present in 30/225 samples of AFP patients (13%), whereas Adenovirus were detected in 18/225 samples (8%).

Of note, 5/225 samples (2.2%) showed coinfection with both Enteroviruses and Adenoviruses.

DISCUSSION

The aim of AFP surveillance and laboratory diagnosis is to detect PV and remedy its reimportation into polio-free areas. Virological surveillance is based on detection of poliomyelitis and non-poliomyelitis enteroviruses by cell culture in WHO program certified laboratories [9].

Among the performance indicators of virological surveillance, the annual percentage of non-polio Enteroviruses isolated by cell culture, which must be greater than 10%. However, in our laboratory level, this percentage remains low (2% in 2018-2019). Indeed, cell culture for the detection of Enteroviruses has several limitations: 1- low sensitivity of only 65 to 75%; 2- long turnaround time of 3 to 10 days; 3- high degree of technical expertise required [8]. Also, the viability of viruses may be affected by inappropriate transport or storage of samples [10]. Thus, it is crucial to use a less laborious more rapid and sensitive technique such as RT-PCR for rapid and efficient diagnosis of Enteroviruses.

In the present study, we firstly performed the rRT-PCR for Enteroviruses in the stool of patients with acute flaccid paralysis

diagnosed negative in cell culture for the detection of polio and non-polio Enteroviruses. Enteroviruses were detected in 13% (30/225) of these cases. These results are similar to other studies carried out confirming that RT-PCR is more sensitive than cell culture for Enterovirus detection [10, 11].

Although detection of Adenoviruses is a common practice in the stools of patients with diarrhea, testing for Adenoviruses in AFP, to determine its probable involvement in the pathogenesis, has not been reported previously. Our results showed that Adenovirus were present in 8% of the cases studied. The presence of Adenoviruses cannot be considered as etiological factor in the occurrence of paralysis. Likewise, its presence in healthy subjects does not exclude its ability to cause disease. Therefore, their presence in AFP patients remains inconclusive [3; 5].

Coinfection with both Enteroviruses and Adenoviruses was detected in 2.2% of the samples (5/225). This finding deserves more investigations on a larger number of samples. Based on these results, we suggest to prospectively detect Adenoviruses both in patients' stools and in cerebrospinal fluid to confirm or infirm their implication in the occurrence of AFP [12].

CONCLUSION

As part of the global polio eradication program, virological surveillance of AFP cases is now carried out worldwide.

Surveillance is based on the detection of cases associated with the polio virus or non-polio enteroviruses. However, in a large number of cases, and apart from the non-infectious etiology, the viral agent responsible for the paralysis remains undetermined.

For rapid and efficient diagnosis, RT-PCR must replace cell culture for detection of Enteroviruses. Also it is noted in this study, it is necessary to widen the range of the neurotropic viruses sought in the case of AFP in order to detect the possible causal agents of the disease.

What is already known on this topic:

- Despite the elimination of poliovirus in all five regions of the world, acute flaccid paralysis continues to present a major public health problem
- In one hand, some enterovirus serotypes with the potential to cause neurological disorders are not easily isolated in cell culture systems used for the diagnosis of AFP. In the other hand, long transit time and failure to follow the cold chain can lead to false negative cell culture results.

What this study adds

- We tested for the presence of enterovirus genomes in fecal suspensions previously considered negative by cell culture procedures, using Real Time PCR.
- Several studies have reported the presence of Adenovirus in the stools of AFP patients without a causal link to the disease having been proven.

Competing interests

The authors declare no competing interest.

Authors' contributions

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