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Stereotyping and Genotype of Poliovirus Aetiology of Acute Flaccid Paralysis by Using Intratypic Differentiation in Iraq.

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Abstract

The current study aims to determine the causes of Acute Flaccid *Paralysis* AFP cases for ages less than 15 years. Four hundred eighty stool specimens were collected during the period from January to December 2019 and received to National Polio Laboratory (NPL\ IRAQ) that is responsible for the laboratory part of AFP surveillance project specimens collected from all Iraqi provinces.

The results showed that 65 (13.5%) sample from total samples are non –polio viruses that have specific cytopathic effects (CPE) on cell culture in RD cells only, while 11 (2.3 %) samples were positive for polio virus which isolated from AFP cases, all these cases showed CPE on L20B cells only. All these samples were from patient vaccinated with the type of OPV in varying number of orally bivalent oral polio vaccine (bOPV) dose. The results also showed that 404 (84.2%) that were (Negative) no appears CPE on both cells.

All positive Poliovirus cases 11 (16.9%) were serotyping by use rt qPCR method ,the current study confirmed that Sabin like Poliovirus serotype three (SLPV3) 7 cases (63.6%) from all positive Poliovirus cases followed by Sabin like Poliovirus serotype one (SLPV1) 3 cases (27.2%), while this study showed one case 1 (9%) Sabin like Poliovirus serotype one (SLPV1) is negative (discordant) in ITD method. The results also showed no Wild polio virus or Vaccine derived poliovirus (VDPV) isolates.

Keywords: AFP , VDPV, CPE , ITD, SLPV1, poliovirus. SLPV3

1- Introduction

Poliovirus is highly infectious cause Acute flaccid paralysis(AFP) on children below age five years a clinical syndrome' of AFP was described by quick onset of weakness (**Pallansch et al., 2013)** and affecting the muscles then developing to maximum severity within numerous days to weeks. Poliovirus is a category of enterovirus denotation entered by

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concluded the mouth and nasal cavity(oropharynx)**(Khan et al., 2018**) and at that time, the submucosal tissue of the pharynx is replicated then the digestive tract. The virus extents outside through direct connection with an infected human, contact with their infected sputum or mucus from the mouth or nose, or touching infected feces (**Zaffran et al.,2018).**

Poliovirus (genus Enteroviruses, family *Picornaviridae*) are amongst the greatest mutual viruses infect human all over the world (**Scalise, 2010).** Enteroviruses related per various clinical syndrome extending from slight febrile infection to severe. possibly lethal circumstances(e.g., aseptic meningitis. Encephalitis. Paralysis. acute flaccid paralysis. especially in child (**Ooi et al., 2009)**.

Myocarditis and neonatal enterovirus sepsis might be link per the progress of certain chronic disease, For example, diabetes type 1 along with Dilation of the heart muscle (**Khetsuriani and Parashar 2003)**. Severe form of polio symptomatic called paralytic polio (PP) the affect only a slight ratio of those occupied thru the polio virus **(Mueller *et al.,* 2005).**PP occurring in around 1 percent of cases. In this case, the virus come into motor neurons where it replicate then destroy of cells (**Ooi et al., 2009)**.

polioviruses are divided to 3 different classes: polioviruses (types PV1. PV2, and PV3) (**Knowles et al., 2012).** . Via the gastrointestinal (GI) and respiratory tract, the enterovirus reaches the human host. The infection can development to CNS association during the main viremia phase or at a late time (**Schwartz at el., 2014).**

Antibody creation in responses to enteroviral infection occur in the first 7-10 days .Epidemiologic investigations of enteroviruses in healthy subject are prevalent in the stool specimens of healthy children "normal "permanent viral intestinal flora (**Rotbart, 2002)** then transiently inhabit the (GI) and are most frequently isolated before the age of four years (**Mueller et al., 2005)** .

Vaccine associate paralytic poliomyelitis (VAPP) is an adversative result after contact to oral polio vaccine (OPV) (**Pedreira et al., 2017).** Vaccine derived polioviruses (VDPVs) by compare, polioviruses that have different genetic possessions indicative of lengthy duplication or transmission **(CDC.2013; Skinner, 2008).** (VAPP) is periodic and intermittent, happening in several states by using OPV at similarly low rates. (**Pallansch et al., 2013).** Within the U.S. The risk of VAPP was about 1 case per 900 000 first circulated doses (**Zhao et al., 2017).**

The current study aims to determine the causes of poliomyelitis and determine the genotypes and serotypes in Iraq

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2- Materials and Methods

A total of four hundred and eighty (480) stool specimens investigated were collected during the period (January to December 2019) collection of stool samples from children afflicted with acute flaccid paralysis in Iraq.

All children were under the age of 15 years, the method approved for AFP investigation and Laboratory analysis of fecal specimens isolation besides typing of isolates. intratypic differentiation procedures (ITD) as defined by WHO Polio Laboratory Guide **(WHO, 2016)** and supplementary Manual in 2006 **(WHO, 2015)** relating the New Algorithm Method (Figure-1)

Per each AFP case two fecal specimens was collected. 24 hours apart, then Fourteen days after the onset of paralysis. All specimens were sent to the Iraqi National Polio / Intratypic Separation Laboratory in the cooler box with frozen packets. Where they were preserved at -20C^0 until tested.

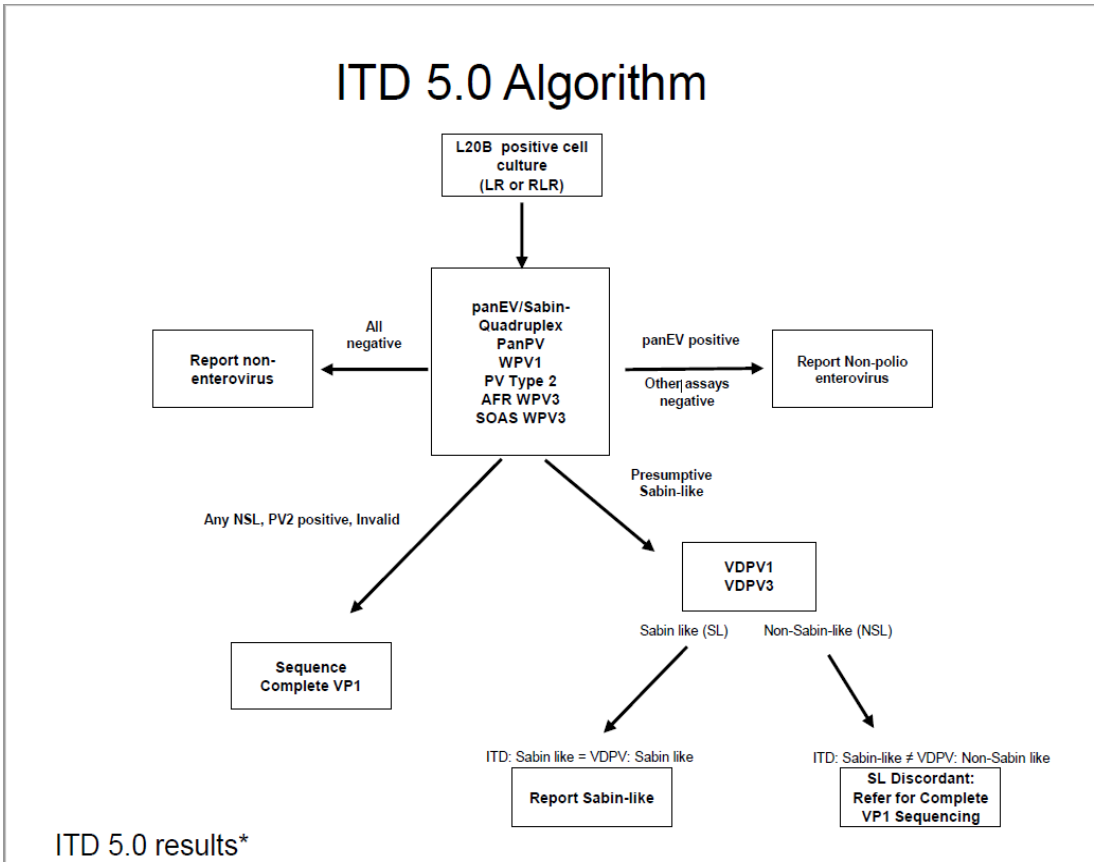

Figure-1 : New Algorithm for Picornavirus Isolation.

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Viruses isolation and Processing of sample

Pre-treated of Fecal samples with chloroform earlier being injected on a well mono-layer of L20B and RD cells lines in maintenances medium (MEM enhanced with 2% fetal bovin serum (FBS) (**Hemeda et al., 2014).**

The cell was cultured 48 hours earlier in a growing medium culture tube (Eagle's MEM increased by 10 percent FCS) (**Teterina et al., 2016**). Each sample of stool was injected into equally tubes culture L20B cell and RD cell. The injected monolayer were perceived daily for the distinguishing enteroviruses cytopathic.

Impact of detaching the round, refractive cell from the tube surface (**Dhere et al., 2017**). The tubing and CPE above 75 percent were obtained and allocated at -20 ° C to become a passageway in the other cell line's current monolayer to raise the titer. Whereas such negative ill cells were re-passed on the same cell lines later than 5 days of cultivation (**Zurbriggen et al., 2008).** Those who found negative were avowed negative later on another 5-day cultivation cycle, The positive tube used for poliovirus was freeze-thawed three times with a particular cytopathic effect. Rotated at 4 ° C (according to rpm and times) and aliquoted and reserved freezing of supernatants at -20 ° C as poliovirus isolate 22-24 (**Chen et al., 2013).**

rtq PCR

RNA extraction. The RNA extractions method for feces sample. A mini kit RNeasy (Qiagen, Valencia, CA) was utilized by the producer's convention. For each series of extractions, positive-and negative monitor responses were recalled. extracted RNA arrangements was put away at - 80°C

Reverse transcription. Reverse transcription is carried out to create cDNA after extraction of viral RNA. Five extricated microliters were combined with 1 µl of arbitrary hexamer primer.(3 µg/µl; Invitrogen, Carlsbad, CA), 1 µl 10 mM deoxynucleoside triphosphate (dNTP) blend (10 mM each dNTP), and 5 µl distill water. This mixture of responses was warmed for 5 min to 65 ° C and then snappy chilled on ice afterwards. 4 microliters 5x first-strand buffer (Invitrogen, Carlsbad, CA), 2 µl 0.1 M dithiothreitol, and 1 µl RNase Out recombinant RNase inhibitor (40 units/µl; Invitrogen, Carlsbad, CA) were added, blended in with delicate pipetting, and hatched at 25°C for 2 min. At last, 1 µl (200 units/l) of SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA) was added and blended in with delicate pipetting. The combination was brooded at 25°C for 10 min, 42°C for 50 min, and 70°C for 15 min. Until proceeding with the PCR measure, 45 microliters of

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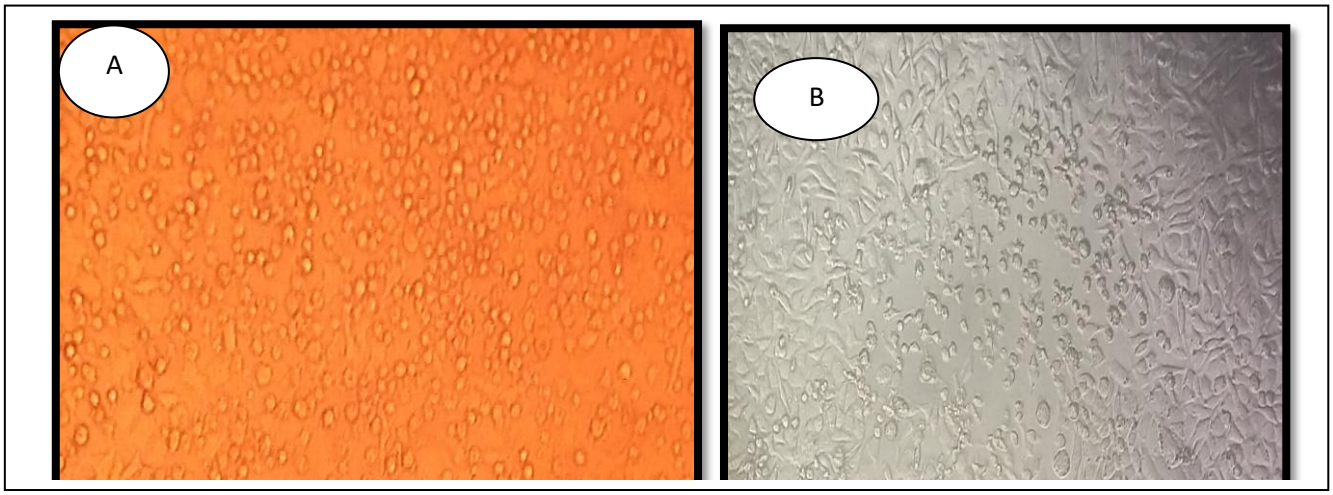
refined water were applied to the response mix. For each set of responses, positive and negative controls were implemented.

Rt qPCR technique. To each reverse transcription, creation before go through real-time PCR examination. 5 microliters of the reverse transcription response combination was mixed with 10 µl TaqMan general PCR master mix lacking uracil-N-glycosylase (Applied Biosystems, Foster City, CA), 0.05 µl TaqMan probe (Applied Biosystems, Foster City, CA), 0.9 µl each of forward in addition to reverse primers. and 3.15 µl distill water, Pedaling circumstances were as defined previously. Samples which returned normal cycle threshold (CT) standards of >35 from copy measures were viewed as negative, because of an expanded number of bogus positive outcomes over this CT.

3- Results and discussion

Cell lines RD was recommended to use and to isolate all types of Entero Virus from patients suffering from AFP. Figure 2 - (A) .All positive cases on RD cell line were cultured on L20B cell line. Poliovirus could grow on L20B. Figure 2 - (A,B) . While, other enteroviruses could not grow on this cell line (**Adeniji et al., 2017)**.



Figure(2). A-RD cell lysis by Non polio ENterovirus . (B) L20B Cells lysis by polio virus after 24 hours of incubation (400 x).

The results showed that 65 (13.5%) sample from total samples are non –polio viruses that have specific cytopathic effects (CPE) on cell culture in RD cells only, while 11 (2.3 %) samples were positive for polio virus which isolated from AFP cases, all these cases showed CPE on L20B cells only. All these samples were from patient vaccinated with the type of

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OPV in varying number of orally bivalent oral polio vaccine (bOPV) dose. The results also showed that 404 (84.2%) that were (Negative) no appears CPE on both cells. such as table(1)

Table (1) result of cultures AFP sample on tissue cultures.

types	**number**	**%**
non –polio viruses cultured in RD cells	65	13.5
polio virus which cultured on L20B cells	11	2.3
(Negative) no appears CPE on both cells	404	84.2
total	480	100

RT PCR to differentiated Sabin in addition non-Sabin like Poliovirus isolate.

All PolioViruses isolate in L20B cell cultures are recognized through reverse-transcription quantitative, polymerase chain reaction (RT-qPCR) analyses that usage enterovirus -specific and poliovirus group –specific, serotype-specific, and Sabin strain–specific primer arrays (**Abbasian et al., 2011).**

Figure 2 :- Diagnostic RT- qPCR for Intratypic Differentiation of Poliovirus isolate .

Lab Sample No.	**Primers (ITD 5.0)**									**ITD Results**
	PanEV	**PanPV**	**Sabins**			**WPV1**	**WPV3-AFR**	**WPV3-SOAS**	**PV Type 2**	
			S1	**S2**	**S3**					
NTC	neg.	neg.	neg.	neg.	neg.	neg.	neg.	neg.	neg.	
PC	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	
Sample 1	pos.	pos.	neg.	neg.	pos.	neg.	neg.	neg.	neg.	SL3
Sample 2	pos.	pos.	neg.	neg.	pos.	neg.	neg.	neg.	neg.	SL3
Sample 3	pos.	pos.	pos.	neg.	neg.	neg.	neg.	neg.	neg.	SL1
Sample 4	pos.	pos.	neg.	neg.	pos.	neg.	neg.	neg.	neg.	SL3
Sample 5	pos.	pos.	pos.	neg.	neg.	neg.	neg.	neg.	neg.	SL1
Sample 6	pos.	pos.	neg.	neg.	pos.	neg.	neg.	neg.	neg.	SL3
Sample 7	pos.	pos.	pos.	neg.	neg.	neg.	neg.	neg.	neg.	SL1
Sample 8	pos.	pos.	neg.	neg.	pos.	neg.	neg.	neg.	neg.	SL3
Sample 9	pos.	pos.	neg.	neg.	pos.	neg.	neg.	neg.	neg.	SL3
Sample 10	pos.	pos.	pos.	neg.	neg.	neg.	neg.	neg.	neg.	SL1
Sample 11	pos.	pos.	neg.	neg.	pos.	neg.	neg.	neg.	neg.	SL3

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Vaccine-related poliovirus isolate are additional mark off for VDPVs by via a RT-qPCR analyze directing sequence that characteristically return during replication of OPV in the human intestines (**Adu et al., 2007)**. current study finding and confirm that Sabin like Poliovirus serotype three (SLPV3) 7 cases (63.6%) was the highest number of poliovirus followed by Sabin like Poliovirus serotype one (SLPV1) 3 cases (27.2%) Table 2 ,

Table 3 :- Differentiation Sabin and Non-Sabin Poliovirus Isolate

Poliovirus serotyping	Patient group NO Positive .65	
	Number positive case	Percentage%
PV1	3	27.2
PV3	7	63.6
WILD	0	0
VDPV	0	0
Discordant PV1	1	9
Total	11	100

On the basis of our results in this study isolated of variety of polioviruses from clinical specimens, this is agree with the study in India by **Schmidt et al., (2017).** and Keiser, *et al* **(2016).** that isolated variety of polioviruses Type Poliovirus serotype three (SLPV3) 60/200 and Poliovirus serotype one (SLPV1) (30%) cases of poliovirus .

Our study results disagree with Deborah,*et,al* **(2017**) that found increased shedding of virus serotype SLPV1 component in the OPV dose, which is greater than type SLPV3 components .

Diagnostic RT- qPCR for VDPV screening of Sabin-like Poliovirus

The RT-qPCR VDPV analyze is intended to exploit sensitivity for VDPV recognition, So it's roughly like OPV isolates are identified as candidate VDPVs (**Abbasian et al., 2011).** The molecular assesses have mostly exchanged a selection method that was established on the reflection that VDPVs are characteristically more antigenically different than isolates that are OPV-like (**Adu et al., 2007)**. The finding of one serotype 1 strain in our sample occurs as a discordant outcome in the ITD (Table 3) .

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In our study no Wild polio virus isolate disagree with study in Pakistan by Kalkowska & Thompson (2020), that isolate 4 cases wild type polio virus 1 WPV1 . RT- qPCR for VDPV method show that were all Poliovirus isolates appear positive result serotype three (SLPV3) 7 cases (63.6%) our study results agree with Study by Teterina in Nigeria that also found increase isolated of serotype three (SLPV3) 17 SLPV3 (49%) (**Teterina et al.,2016**).

Current study show 3 cases isolate (27.2%) Sabin like Poliovirus serotype one (SLPV1) .while one case 1 (9%) Sabin like Poliovirus serotype one (SLPV1) are negative (discordant) .This result agree with study by Sebastian *et al.,* **(2008**) in Switzerland that isolated one (SLPV1) negative (discordant) in rRT- PCR for VDPV screening method for Vaccine-related isolates that were found non–vaccine-like antigenic possessions were designated as soft discordant effects Table 4.

Table 4:- Diagnostic Poliovirus VDPV (discordan **)**

	Primers (VDPV 5.0)		**VDPV Result**	**Final Result**
	VP1			
	PV1	**PV3**		
NTC	neg.	neg.		Valid
PC	pos.	pos.		Valid
Sample 1	Neg .	Neg	SL1	**discordan**
Sample 2	pos.	Neg	SL1	Sabin Like
Sample 3	Neg	pos .	SL3	Sabin Like
Sample 4	Neg	Pos	SL3	Sabin Like
Sample 5	Neg	Pos	SL3	Sabin Like
Sample 6	Neg	Pos	SL3	Sabin Like
Sample 7	Pos	Neg	SL1	Sabin Like
Sample 8	Neg	Pos	SL3	Sabin Like
Sample 9	Neg	Pos	SL3	Sabin Like
Sample 10	Neg	Pos	SL3	Sabin Like

The global polio eradication initiative (GPEI) is motionless stressed to eliminate polio in 3 countries where it remnants endemic: Afghanistan, Pakistan and Nigeria. Any of the major problems for realizing this Purpose include powerful battles inside and near places which poliovirus is socially active.

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The oral polio vaccine which is motionless recycled in numerous third-world countries, is prepared from a live polio virus, which transports a danger of causing polio (**Plotkin, 2014).** In addition, the virus throughout the vaccine could mutate into a lethal form, initiating new eruptions. The US started expending an inactivated polio vaccine (IPV) in 1999, after parentages of vaccine-damaged children were fruitful in lobbing for a change in strategy.

While The live oral polio vaccine is often given to children right on the streets in poor countries, without even checking whether or not they have already received it **(Platt et al., 2014).** The withdrawal of OPVs in Iraq began with the transition from trivalent OPV (tOPV) to bivalent OPV (bOPV) in April 2016, with the elimination of category 2 components (OPV2) from immunization systems (W.H.O, (2020).

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