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Original Article

Evaluation of Adventitious Agents in MMR & Oral Poliomyelitis Vaccines

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Abstract

Background and Aims: Adventitious agents, especially viral agents are among the most important concerns in viral vaccines. Viral contamination of biological products may arise from the original source of the reagents such as, serum, trypsin, animal or human derived media components or cell culture and working seed or cross contamination of vaccine during production. In this study, adventitious agents were evaluated in the MMR and oral polio vaccines.

Methods: For detection of adventitious agents, two techniques were used. The suspensions that were harvested after inoculation on MRC-5 were neutralized with specific antisera and inoculated into Vero, HeLa and MRC-5 cells (in vitro tests). The suspensions that harvested after inoculation of CHEF primary cell culture were neutralized with specific antisera and inoculated in to Vero, MRC-5, CHEF, chorioallantoic membrane and yolk sac of SPF embryonated eggs (in vivo tests).

Results: As indications of viral contamination, CPE in cell culture and the viability of egg embryo were observed and haemadsorption and haemagglutination test were performed. Finally, data were analyzed by exact binomial test (version 2.8.1). There was not any CPE in cell culture, the inoculated embryos were viable and there was not any haemadsorption & haemagglutination in all of the samples.

Conclusion: The major focus of this Study will be to ensure that vaccines are devoid of adventitious agents. There were not any signs of viral agents in the samples and the preparations could be used for vaccine production.

Keywords: Adventitious Agents; Measles; Mumps; Rubella; Poliomyelitis

Introduction

A major concern in production of a biological product specially, live viral vaccine is the risk of viral contaminations that could have serious clinical consequences. Adventitious agents are microorganisms that have been unintentionally introduced into the manufacturing process of a biological product and administration of the contaminated biological product could be detrimental to the patient.

Vaccine contamination can arise through the introduction of adventitious (accidentally introduced) viruses during the manufacturing process. The likely sources of contamination include the use of contaminated cell culture media, a breakdown in GMP allowing operator or other external contamination or the use of contaminated reagents used in the process. These contaminations require the development of suitable analytical techniques to ensure the absence of human and animal adventitious

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viruses. In this study Biological approaches include in vitro and in vivo tests were used for detection of adventitious viral agents in OPV and MMR vicinal bulk. OPV and MMR vaccine are live-attenuated trivalent vaccines. OPV includes type 1, 2 and 3 of polio virus and MMR vaccine includes measles, mumps and rubella virus against these diseases.

Methods

Sampling and neutralization

After titration of vaccinal bulk (WHO TRS, 840, 1994 and 904, 2002), for the bulk viruses that had titer equal or more than 10^4 $CCID_{50}$/ml, the size of sample was selected to contain at least 500 human doses to 50 ml for the bulk viruses that had titer less than 10^4 $CCID_{50}$/ml. The sample size was calculated on the basis of WHO guideline (WHO TRS, 878, 1998). 9 batchs of each virus bulk includes polio virus type 1, 2 & 3, rubella and measles (prepared in MRC5) and 15 batchs of mumps and measles (prepared in CHEF (chicken embryo fibroblast) (totally 75 batches) were sampled.

The polyclonal antiserum was produced in goat. (Keshavarz *et al* 2008). For virus neutralization, the volume of antiserum was calculated (WHO TRS, 878, 1998) and virus and antiserum were mixed and incubated at room temperature for 2 hours.

Detection of adventitious agents in vaccine bulks which prepared in MRC-5 as cell substrate (in vitro tests)

0.5 ml of each neutralized virus was inoculated in to each 75 cm^2 flask of HeLa (ATCC CCL-2) and Vero (ATCC CCL-81) as line cell cultures and MRC-5(ATCC CCL17) as a diploid cell culture (Table 1). 0.5 ml of each virus without antiserum was inoculated similar to test samples as positive control. After two weeks of incubation at 36°C and daily observation, the original cultures of supernatants or lysates from cell banks were sub cultured into fresh cells and were observed for at least an additional two weeks. This subculture into fresh cells might help to distinguish between non-specific CPE and virus-induced CPE, as toxic effects of the initial specimen. Because of the possibility of contamination with human or simian cytomegaloviruses were a consideration, the cell cultures was observed for at least 4 weeks.

At the end of observation period, for detection of non cytopathic viruses, hemadsorption and hemagglutination tests were performed for hemadsorbing and hemagglutinating viruses: 1/3 of the tests and control flasks were inoculated with 0.32% pigeon RBC, 1/3 were inoculated with 0.5% guinea pig RBC and the remaining monolayer cultures were inoculated with 0.5% of monkey RBC (for detection of certain respiratory viruses) and the flasks were incubated at 2-8°C for 30 minutes and 30 minutes at room temperature (22-25°C). They were observed for hemadsorption and hemagglutination respectively (Haemadsorption, vsop45 and FDA, Feb. 2010).

Detection of adventitious viruses in vaccine bulks which were prepared in CHEF (in vivo tests)

After performing of in vitro tests like as described using Vero as a line cell culture, MRC-5 as a diploid cell and CHEF as a primary cell, the next steps for mumps and measles suspensions was performed by in vivo tests:

Inoculation in chorioallantoic fluid: 0.5 ml of each neutralized virus was inoculated to embryonated SPF eggs 10 to 11 days old. 0.5 ml of each virus without antiserum was inoculated similar to test samples as positive control. Following incubation at 35°C for 72 hours, the allantoic fluids was harvested, pooled, and passaged by the same route into fresh, embryonated eggs, same days old and was incubated at 35°C for 72 hours.

Inoculation in to yolk sac: 0.5 ml of each neutralized virus was inoculated in to embryonated SPF eggs 6 to 7 days old. Following incubation at 35°C for at least 9 days, the yolk sacs were harvested and pooled. A 10% suspension of yolk sacs was passaged by the same route into the yolk sacs of fresh embryonated eggs, 6 to 7 days old, and was incubated at 35°C for at least 9 days.

Table 1. Plane of viral agent detection.

Suspensions type	Cell cultures				Embryonated eggs	
	vero	HeLa	MRC-5	CHEF	CAM	Yolk Sac
Poliomyelitis (type 1, 2, 3) Rubella and Measles (on MRC-5)	✓	✓	✓	-	-	-
Mumps and Measles (on CHEF)	✓	-	✓	✓	✓	✓

Both the initial pool and the passaged harvests were tested for the presence of hemagglutinating agents using 0.5% red blood cells from guinea pigs and chicken.

Statistical calculation

For analysis of the result, the data was analyzed by exact binomial test by R statistical software (version 2.8.1).

Results

Cell culture microscopic observation showed, in all of the test samples there was not any CPE and any cell abnormality in comparison with negative and positive controls (table3 and 4).

After haemadsorption and heamagglutination tests, all of the test samples were negative (without Haemadsorption and heamagglutination) (table 2, 3, 4 and figure 1, 2).

In inoculated eggs with viruses by chorioalantoic and yolk sac inoculation, all of the embryos were normal and there was not any abnormality and death of embryo (table 2).

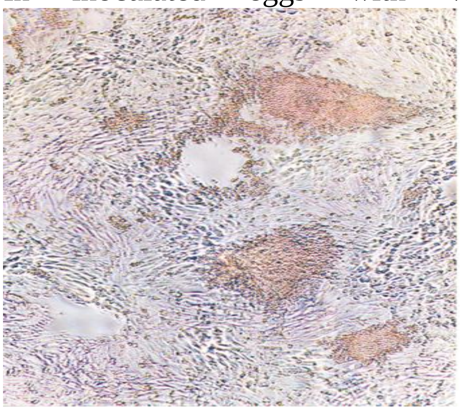

Fig. 1. Haemadsorption in cell culture with Mumps virus (Positive Control).

In all of the positive controls there was specific CPE related to specific inoculated virus**.**

The result of statistical analysis is:

Exact binomial test: Data: 0 and 75, p=0, alt='greater'

Number of successes= 0 Number of trials = 75

p-value = 1

Alternative hypothesis: True probability of success is greater than 0

95 percent confidence interval: 0.1

Sample estimates: probability of success 0.

Discussion

Maximal vaccine benefit is achieved when vaccination rates are sufficiently high to achieve herd immunity. Because vaccines are administered to healthy children, it is especially important that parents, pediatricians

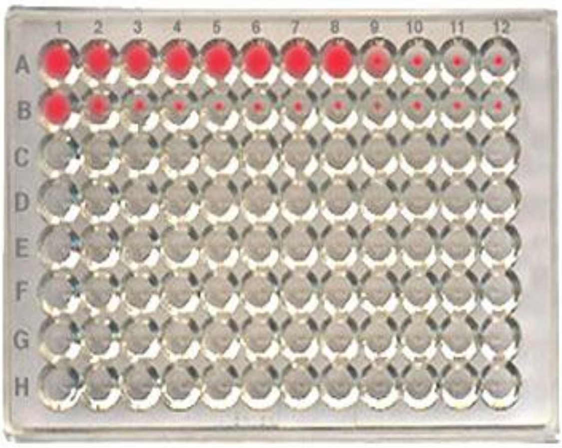

Fig. 2. Haemagglutination with guinea pig RBC by Mumps virus (Positive Control).

and the public at large feel confident that the vaccines are safe. So it is the ethical obligation of any manufacturer of a pharmaceutical to provide a drug that is safe as well as effective, after all a drug should cure, not harm the patient (Richard S. Woodward, 1995). It is more important in the case of biological product. Production of viral vaccines generally involves inoculation of a cell substrate with a vaccine seed and purification of bulk product from these cells after a sufficient time for replication of the virus or production of vaccine proteins. Other row materials (e.g. tissue culture reagents, stabilizers) may be added to the product at various stages of production. Thus adventitious agents could theoretically enter a viral vaccine through any of these ingredients (Jens P.G., 2008). Close control of the vaccine manufacturing environment (by producing vaccines in sophisticated modern facilities), appropriate testing of the raw materials, and testing of both the bulks and final products can help ensure that adventitious agents have not entered the vaccine. The adventitious agents formed from human source such as: HIV, hepatitis, cytomegalovirus, epstein barr, herpes, parvovirus B19, reoviruses, polyoma, coronaviruses, papilloma, influenza, enteroviruses, parainfluenza and respiratory syncytial virus and from animal source such as: bovine viral diarrhea virus, adeno and adenoassociated viruses, parvovirus, reovirus, rabies, bluetongue, circovirus, orthopoxviruses, bovine immunodeficiency virus and lymphocytic choriomeningitis virus (Caroline k. y. fong *et al* 1992).

Contamination with adventitious viral agents in tissue culture cells, especially endogenous viruses in primary cells, has been known for many years (Philip R. Krause, 2001). For examples simian viruses in monkey kidney cells (Hsiung, G.D. 1968) (some polio vaccines prepared from 1954 to 1961 was contaminated with infectious SV40 (Cutrone R. *et al* 2005), It has been assumed that all polio vaccines were SV40 free in the united states after 1961 and in other countries after 1962 (Cutrone R. *et al* 2005), measles vaccine can be contaminated with pestivirus (Philip R. Krause 2011), guinea pig herpes virus in guinea pig kidney or embryo cells (Hsiung, G.D and Kaplow, L.S.

Table 2. Summary results of the Viral Agents detection in Measles & Mumps by Chicken Embryo.

Virus	Number	Culture	Specification	Result	
				Haemagglutination	Viability
Measles (on CHEF)	3	Chicken Embryo (Chorio Allantoic)	Viable & No Haemagglutination	No Haemagglutination	Viable
	3	Chicken Embryo (Yolk sac)			
Mumps	3	Chicken Embryo (Chorio Allantoic)	Viable & No Haemagglutination	No Haemagglutination	Viable
	3	Chicken Embryo (Yolk sac)			
Total	12	-	-	-	-

Table 3. Summary results of the Viral Agents detection in Measles & Mumps by cell culture.

Virus	**Cell Culture**	**Number**	**Specification**	**Result**				
				Observation	Haemadsorption (Guinea pig RBC)	Haemadsorption (Monkey RBC)	Haemadsorption (pigeon RBC)	Haemagglutination
Measles (on CHEF)	Vero	3	*No CPE and No Haemadsorption*	No CPE	No Haemadsorption	No Haemadsorption	No Haemadsorption	No Haemagglutination
	MRC5	3						
	CHEF	3						
Mumps	Vero	3	*No CPE and No Haemadsorption*	No CPE	No Haemadsorption	No Haemadsorption	No Haemadsorption	No Haemagglutination
	MRC5	3						
	CHEF	3						
Total	-	18	-	-	-	-	-	-
Virus	**Cell Culture**	**Number**	**Specification**	**Result**				
				Observation	Haemadsorption (Guinea pig RBC)	Haemadsorption (Monkey RBC)	Haemadsorption (pigeon RBC)	Haemagglutination
Measles (on CHEF)	Vero	3	*No CPE and No Haemadsorption*	No CPE	No Haemadsorption	No Haemadsorption	No Haemadsorption	No Haemagglutination
	MRC5	3						
	CHEF	3						
Mumps	Vero	3	*No CPE and No Haemadsorption*	No CPE	No Haemadsorption	No Haemadsorption	No Haemadsorption	No Haemagglutination
	MRC5	3						
	CHEF	3						
Total	-	18	-	-	-	-	-	-

1969), equine herpes virus in horse kidney cells (Hsiung , G.D *et al* 1969), contamination of yellow fever vaccine with hepatitis B virus in the 1940s because a human-derived excipient contained hepatitis B virus (Philip R. Krause 2011), adventitious agents in smallpox vaccine (Frederick, A. 2005) and in a cell culture-derived subunit influenza vaccine (Gregersen JP, 2008 and Novartis behring, Emil von behring, 2008). In addition adventitious viral agents in continuous cell lines (endogenous retroviruses) (Kajima, M. *et al* 1967) and bovine serum, which is essential major component of cell culture medium, have been reported (Boone, C.W. *et al* 1971, Fong, C.K.Y. *et al* 1975, Swack, N.S. *et al* 1975).

Table 4. Summary results of the Viral Agents detection in Polio, Measles & Rubella by cell culture.

Virus	Cell Culture	Number	Specification	Result				
				Observation	Haemadsorption (Guinea pig RBC)	Haemadsorption (Monkey RBC)	Haemadsorption (pigeon RBC)	Haemagglutination
Polio Type 1	MRC5	3	*No CPE and No Haemadsorption*	No CPE	No Haemadsorption	No Haemadsorption	No Haemadsorption	No Haemagglutination
	HeLa	3						
	vero	3						
Polio Type 2	MRC5	3	*No CPE and No Haemadsorption*	No CPE	No Haemadsorption	No Haemadsorption	No Haemadsorption	No Haemagglutination
	HeLa	3						
	Vero	3						
Polio Type 3	MRC5	3	*No CPE and No Haemadsorption*	No CPE	No Haemadsorption	No Haemadsorption	No Haemadsorption	No Haemagglutination
	HeLa	3						
	vero	3						
Measles (on MRC5)	MRC5	3	*No CPE and No Haemadsorption*	No CPE	No Haemadsorption	No Haemadsorption	No Haemadsorption	No Haemagglutination
	HeLa	3						
	vero	3						
Rubella	MRC5	3	*No CPE and No Haemadsorption*	No CPE	No Haemadsorption	No Haemadsorption	No Haemadsorption	No Haemagglutination
	HeLa	3						
	vero	3						
Total	-	45	-	-	-	-	-	-

Knowing that vaccines are free from adventitious agents is a large component of this confidence (Philip R. Krause 2001). The challenge of identifying potential adventitious agents in vaccines closely parallels the challenge of identifying the agents causing particular emerging infectious diseases (Philip R. Krause 2001). For a substance to be considered ‘free’ of an adventitious agent, assays must demonstrate that a defined quantity of a vaccine is negative for that agent at a defined level of sensitivity (Keith Peden 2010).

There is no any study about polio and MMR vaccines adventitious agents. Thus, the major focus of this discussion will be the approaches used in a regulatory setting to ensure that polio and MMR vaccines are devoid of adventitious agents. In in vitro systems the methods were based on the ability of cell cultures to grow a wide array of pathogens; also based on their extensive use in diagnostic laboratories to detect human pathogens. This cell-culture tests can detect a variety of adventitious viruses, including cytopathic viruses, hemadsorbing viruses, and hemagglutinating viruses (adenoviruses, alphaviruses, coronaviruses, entroviruses, flaviviruses, herpesviruses by using primary and diploid cells and arenaviruses, bunyaviruses, filoviruses, entroviruses, flaviviruses (by blind passage), by using cell lines). Selection of the cell line depends upon the potential exposure to agents (species and tissue type of cell substrate; human diploid cells; monkey kidney cells). In in vivo system the tests detect viruses, not readily detected in other systems. For in vivo tests vaccine bulks were inoculated into chorioalantoic membrane for detection of orthomyxoviruses, paramyxoviruses, alphaviruses and vesiculoviruses and yolk sac for assessing of herpes viruses, pox viruses and rhabdoviruses as well as rickettsiae, mycoplasma and bacteria (WHO TRS, 878, 1998 and FDA guidance, 2010).

In all of the tests in this study, there were not any signs of viral agents in the vaccine suspensions. So the ratio of viral agent in the statistical analysis in all of the 75 samples, were zero. These results showed, the vaccine bulks had not any adventitious viral agents and could be used in production of vaccines. It means 100% vaccine bulks had only expected viruses.

The tests is considered satisfactory, because there was no evidence of adventitious agents in virus bulks and is valid because, at least 80% of the cultures and embryos (both the initial test and the passage) were available for observation at the end of the observation period (FDA, 2010).

The limitation of these tests is, unknown sensitivity of in vivo tests for detection of wild-type strains, because this method was usually established with laboratory-adapted strains and in vitro tests only could detect agents that can infect and propagate in indicator cells and many pathogenic viruses for humans do not infect or replicate readily in eggs or cell cultures (e.g., HPV, HCV). So, we should use others techniques such as transmission electron microscopy, reverse transcriptase (RT), assays for retroviruses and molecular methods such as PCR and RT PCR. Comparison between PCR and biological assay showed that the biological assays are more sensitive for large samples and greater potential to detect the unknown agents. So, adventitious agent detection relies on the use of multiple overlapping strategies and detection methods for adventitious agents continue to evolve and represent improvements in technology, including sensitivity and throughput.

Finally, in production of vaccines, using of suitable sources of material, valid equipment and facility and selection of valid tests for evaluation of adventitious agent are very important together.

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