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## Using chloroform as a preservative for trivalent foot and mouth disease vaccine in comparison to thiomersal

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### Abstract:

**Background:** Chloroform has a potential value as a substitute for thiomersal as a preservative due to its high antibacterial and antifungal activity.

**Objective:** Comparative analysis of the preservative efficacy of chloroform and thiomersal in ISA206 trivalent foot and mouth disease vaccine concerning the antimicrobial activity and vaccine potency.

**Method:** This study was conducted on 5 prepared ISA206 trivalent foot and mouth disease vaccines, one vaccine prepared with 0.01% v/v thiomersal and four vaccines prepared with different concentrations of chloroform 0.1%, 0.25%, 0.5% and 0.75% v/v. Each vaccine was monthly evaluated by safety and sterility tests for 12 months. Three cattle were vaccinated intramuscularly (I/M) by each vaccine. Serum samples were collected monthly for 12 months. The humeral immune responses were monitored by Serum Neutralization Test (SNT) and Enzyme Linked Immunosorbent Assay (ELISA). The antimicrobial activity of chloroform and thiomersal in the five vaccines were determined 12 months post preparation against nine different gram negative and gram positive bacterial strains and three fungal stains. The bacterial strains were Bacillus

subtilis, Staphylococcus aureus , Micrococcus luteus, Pseudomonas aeruginosa, Escherichia coli, Salmonella typhi, Shigella flexneri, Salmonella para typhi A and Proteus mirabilis and fungal strains were Aspergillus flavus, Aspergillus niger and Aspergillus terreus. Agar well diffusion method was followed in this study. The 12 months comparative analysis of antibacterial activity reflects that among these five vaccines. shows thiomersal as well

**Results:** Our results show that the incorporation of as 0.5% and 0.75% chloroform into ISA206/FMDV vaccine are as effective as thiomersal as a preservative.

**Conclusion:** Finally we recommended using 0.5% chloroform as a substitute for thiomersal as a preservative in foot and mouth disease vaccine.

**Key words:** FMD vaccine, chloroform , thiomersal , preservative.

## **INTRODUCTION:**

Foot and mouth disease (FMD) is an acute contagious viral disease of cloven footed animals (*Orsel et al., 2007*). The causative agent is a single stranded positive- sense RNA virus that belongs to the genus Aphthovirus in the family Picornaviridae. There are seven immunologically distinct serotypes of FMD virus, namely ; O, A, C, , SAT1, SAT2, SAT3 and Asia1 (*Belsham, 1993*).

In Egypt, The history of FMDV goes back to 1950 (*Mousa et al., 1974*), only serotype O was reported in Egypt (*Zahran (1960), and Farag et al., (2005)*), with the exception of 1972 when type A was introduced from Sub-Saharan Africa (*Abdel-Rahman et al.,(2006) ,Knowles et al., (2007) , Ghoneim et al., (2010)* ). Series of outbreaks predominantly caused by serotype O, and with a dramatic upsurge in FMD SAT 2 outbreaks during 2012 were reported (*Ahmed et al., (2012), Kandeil et al.,*

(2013), *Shawky et al.*,( 2013) , *El-Shehawy et al.*, (2014 ). Serotypes O, A and SAT2 have been circulating in the country since 2012, and Serotype O is considered the predominant serotype (*FAO 2012*).

The control of FMD in animals was considered to be important to effectively contain the disease in endemic areas, so that vaccination of animals is effective in limiting the spread of FMD (*Nair and Sen, 1992*). The proper use of good quality vaccines has been a significant factor in the control and / or eradication of FMD (*Allende et al., 2003*).

Preservatives are chemical substances whose role is to protect food products, stimulants, medicinal products and cosmetics against harmful changes caused by microorganisms (*Rybacki and Stozek 1980*). When added in proper concentrations, preservatives inhibit the growth of microorganisms during manufacturing and use of medicinal products (*Martindale 2007*). In concentrations used, they should be soluble and non-toxic as well as physiologically and chemically compatible. Chloroform and Methanol have antibacterial activities and thus have curative properties against pathogens (*Nweze et al., 2004*).

It is well established (*Kallings et al.,( 1966)*, *Public Health Laboratory Service Working Party (1971)*, *Pharmaceutical Society Working Party (1971)*, *Committee of Official Laboratories and Drug Control Services ( 1980)*) that multi dose vaccines should be effectively preserved against microbial growth. Thiomersal and chloroform, which are widely employed in the form of a 0.01% v/v and 0.25% v/v respectively, have been reported (*Lynch et al., 1977*) to be a reasonably effective bactericide against vegetative organisms provided that its concentration does not fall below 0.01% and 0.20% respectively. The rate of loss of chloroform from mixtures by volatilization is difficult to predict since it depends upon the initial concentration of chloroform, the frequency with which

the container is opened and the conditions of storage. In addition, the safety of thiomersal or chloroform is a subject of controversy and their use is restricted in some countries (*The Pharmaceutical Codex (1979)*).

The objective of this study was to evaluate the use of chloroform as a potential substitute for thiomersal as a preservative in ISA206 trivalent foot and mouth disease vaccine.

## **MATERIALS AND METHODS:**

### **1. Vaccines:**

Five inactivated oil adjuvanted FMD Vaccines were formulated from FMD virus local strains (O /pan Asia2 , A/ Iran 05 and SAT2/ Egypt 2012) according to *Barnett.et.al. (1996)*. Preservatives in the 5 vaccines were thiomersal 0.01% v/v and chloroform 0.1% ,0.25%, 0.5% and 0.75% v/v .The ratio of the aqueous antigen to the oil adjuvant was 50:50 according to *OIE Manual (2000)*.

### **2. Animals:**

23 cattle were clinically healthy and free from antibodies against FMD virus strains as proved by SNT. 15 animals was divided into 5 groups, each group of 3 animals, one group vaccinated intramuscularly (I/M) with trivalent FMD-thiomersal 0.01% v/v vaccine, second group vaccinated intramuscularly (I/M) with trivalent FMD-chloroform 0.1% v/v vaccine, third group vaccinated intramuscularly (I/M) with trivalent FMD-chloroform 0.25% v/v vaccine, fourth group vaccinated intramuscularly (I/M) with trivalent FMD-chloroform 0.5% v/v vaccine, fifth group vaccinated intramuscularly (I/M) with trivalent FMD-chloroform 0.75% v/v vaccine, three cattle were used as negative control (non-vaccinated) and for safety test.

### **3- Unweaned baby mice**

30 Swiss Albino suckling mice (three to five days old were) classified into six groups, supplied by the Lab. animals farm of

Veterinary Serum and Vaccine Research Institute, Abassia, Cairo, Egypt.

#### 4-Quality control of the prepared vaccines:

i. **Sterility test** : It was applied to confirm that vaccine is free from any bacterial or fungal contaminations. Sterility of the examined vaccine was done by culturing of the tested vaccine on nutrient agar, thioglycolate broth and Sabouraud's dextrose agar .

ii. Safety test: **for the five formulated FMD vaccines: one vaccine prepared with 0.01% v/v thiomersal and four vaccines prepared with different concentrations of chloroform 0.1%,0.25%,0.5% and 0.75% v/v. were tested for safety in susceptible cattle and baby mice .**

Sterility and safety of the prepared vaccines were done according to *Code of Federal regulation of USA ( 1986 ) , OIE( 2000)*.

#### 5. Serum Neutralization Test (SNT):

Serum neutralization test was done according to (Ferreira, 1976). Simply, the used FMD strains were O /pan Asia2 , A/ Iran 05 and SAT2/ Egypt 2012, and obtained kindly from FMD department ,veterinary serum and vaccine research institute, it was performed in flat bottomed tissue culture microtitre plate 96 wells. Serum samples were serially diluted (1:2) in modified Eagle Medium (MEM), 50  $\mu$ L from each dilution was distributed into the wells and 50  $\mu$ L of 100 TCID<sub>50</sub> FMDV were added to each well, then the plates put on shaker for 10 minutes. Then it was incubated at 37 °C for one hour, 100  $\mu$ L of BHK21 cell suspension were added to each well, in each plate cell control and virus control, then the plate was sealed with pressure sensitive adhesive cellulose tape. Plates were incubated at 37C for 48hrs and reading by inverted microscope, the serum neutralization titer was expressed as log<sub>10</sub> of the reciprocal dilution which protected 50% of the cells as calculated by *Karber method (1931)*.

#### 6. Enzyme Linked Immunosorbant Assay (ELISA):

The test was carried out using the micro technique described by *OIE (2009)* by using flat bottom tissue culture microtitre plate.

#### **7. Antimicrobial Activity (Preservative challenge test) :**

Antimicrobial activity of the 5 preservatives was determined against nine different gram positive and gram negative bacteria. Agar well diffusion assay was used to evaluate the antibacterial activity according to *Gatsing et al., (2006)* .

antifungal activity of the 5 preservatives was tested against three fungi; *Aspergillus flavus*, *Aspergillus nigar* and *Aspergillus pterus* using poison plate method according to *Shastri and Varudkar (2009)* .

#### **RESULTS:**

The Safety of trivalent FMD vaccine with different preservatives tested elicited in table (1) showed that no viable viral residues in prepared vaccines when tested for safety in cattle and un weaned baby mice , so the vaccines were safe to use. Also, The results of culturing sterility test were shown in tables (2&3) revealed that the vaccines free from any pathogenic or non-pathogenic microorganisms with 0.01% thiomersal and 0.5% and 0.75% of chloroform.

**Table (4)** represent the antibacterial activity of thiomersal 0.01% v/v which is active against all the tested microbes and fungus in FMD vaccine .

While the antibacterial activity of chloroform was observed to be in dose dependent manner chloroform 0.1 v/v respectively, was not active against *Staph.aureus*, *Pseudo-monas aeruginosa* , *Escherichia coli* , *Salmonella typhi* and *Salmonella para typhi A*. Also chloroform 0.25 % v/v was not active against *Staph. aureus* and *Salmonella typhi*. The antifungal studies of chloroform 0.1 and 0.25 % v/v exhibits most efficacious results against *Aspergillus nigar* and *Aspergillus pterus*. Its activity is against *Aspergillus flavus* is quite low.

The excellent results were shown with the chloroform 0.5 and 0.75 % v/v which active against all the tested microbes and fungus 12 months post preparation. as Shown in table (5)

**Table(1): Safety of trivalent FMD vaccine with different preservatives tested.**

Animals	Trivalent FMD Vaccine				
	Thiomersal	Chloroform			
	0.01%	0.1%	0.25%	0.5%	0.75%
Cattle	safe	safe	safe	safe	safe
un weaned baby mice	safe	safe	safe	safe	safe

**Table(2): Sterility testing of trivalent FMD vaccin with 0.01% thiomersal.**

Months post vaccination	Nutrient agar	Thioglycolate broth	Sabauraud's dextrose agar
1	negative	negative	negative
2	negative	negative	negative
3	negative	negative	negative
4	negative	negative	negative
5	negative	negative	negative
6	negative	negative	negative
7	negative	negative	negative
8	negative	negative	negative
9	negative	negative	negative
10	negative	negative	negative
11	negative	negative	negative
12	negative	negative	negative

**Table (3): Sterility testing of trivalent FMD vaccine with (0.5&0.75) % chloroform**

Months post vaccination	Nutrient agar	Thioglycolate broth	Sabouraud's dextrose agar
1	negative	negative	negative
2	negative	negative	negative
3	negative	negative	negative
4	negative	negative	negative
5	negative	negative	negative
6	negative	negative	negative
7	negative	negative	negative
8	negative	negative	negative
9	negative	negative	negative
10	negative	negative	negative
11	negative	negative	negative
12	negative	negative	negative

**Table(4): Preservative challenge test of trivalent FMD vaccine with 0.01% thiomersal months post preparation.**

Bacteria and fungi	Trivalent FMD vaccine with 0.01% thiomersal preservative
Bacillus subtilis	negative
Staph. aureus	negative
Micrococcus luteus	negative
Pseudo-monas aeruginosa	negative
Escherichia coli	negative
Salmonella typhi	negative
Shigella flexneri	negative
Salmonella para typhi A	negative
Proteus mirabilis	negative



Aspergillus flavus	negative
Aspergillus nigar	negative
Aspergillus pterus	negative

**Table(5): Preservative challenge test of trivalent FMD vaccine with ( 0.1- 0.25-0.5&0.75) % chloroform 12 months post preparation.**

Bacteria and fungi	Trivalent FMD vaccine with			
	0. 1% chloroform	0.25% chloroform	0.5% chloroform	0.75% chloroform
Bacillus subtilis	negative	negative	negative	negative
Staph. aureus	<b>positive</b>	<b>positive</b>	negative	negative
Micrococcus luteus	negative	negative	negative	negative
Pseudo-monas aeruginosa	<b>positive</b>	negative	negative	negative
Escherichia coli	<b>positive</b>	negative	negative	negative
Salmonella typhi	<b>positive</b>	<b>positive</b>	negative	negative
Shig-ella flexneri	negative	negative	negative	negative
Salmonella para typhi A	<b>positive</b>	negative	negative	negative
Proteus mirabilis	negative	negative	negative	negative
Aspergillus flavus	<b>positive</b>	<b>positive</b>	negative	negative
Aspergillus nigar	negative	negative	negative	negative
Aspergillus pterus	negative	negative	negative	negative

The potency of five FMD vaccines were tested in values using serum neutralization test and ELISA , All calves elicited antibody response which measured at 28<sup>th</sup> dpv till every month until 12<sup>th</sup> month post vaccination. All five prepared vaccines were potent from the first month post vaccination till the 10<sup>th</sup> month post vaccination then declined as shown in tables (6&7)

**Table(6) : Serum neutralizing antibody titre of trivalent FMD vaccine with 0.01% thiomersal and (0.1 - 0.25- 0.5&0.75) % chloroform**

Months post vaccination	Thiomersal	Different Concentration of chloroform in FMD vaccines			
	0.01%	0.1 %	0.25 %	0.5 %	0.75%
Serum neutralizing antibody titre					
1	1.8	1.8	1.8	1.95	1.8
2	2.65	2.7	2.7	2.85	2.85
3	3.0	3.0	3.0	3.0	3.0
4	3.0	3.0	3.0	3.0	3.15
5	2.7	2.55	2.7	2.85	3.0
6	1.8	1.95	2.25	2.7	2.85
7	1.8	1.8	2.1	2.55	2.4
8	1.65	1.8	1.8	2.25	2.25
9	1.5	1.65	1.65	2.1	2.1
10	1.5	1.5	1.5	1.65	1.8
11	1.05	1.35	1.35	1.5	1.5
12	0.9	0.9	1.05	1.35	1.35

**Table(7): Immune response of trivalent FMD vaccine with 0.01% thiomersal and (0.1 - 0.25-0.5&0.75) % chloroform**

Months post vaccination	Thiomersal	Different Concentration of chloroform in FMD vaccines			
	0.01%	0.1 %	0.25 %	0.5 %	0.75 %
	<b>ELISA antibody titre</b>				
1	2.23	2.29	2.25	2.39	2.28
2	2.85	3.01	3.0	3.22	3.16
3	3.21	3.3	3.28	3.39	3.28
4	3.11	3.39	3.42	3.27	3.41
5	2.86	2.94	3.02	3.14	3.24
6	2.26	2.43	2.53	2.98	3.05
7	1.98	2.22	2.41	2.72	2.76
8	1.83	2.13	2.23	2.42	2.51
9	1.64	2.04	2.0	2.35	2.32
10	1.65	1.88	1.9	2.0	1.97
11	1.05	1.68	1.62	1.85	1.81
12	0.9	1.08	1.10	1.52	1.49

### Discussion :

Foot and Mouth disease (FMD) is an acute, highly contagious viral disease. Routine vaccinations in enzoonotic (non-FMD-free) regions can effective in limiting the spread of FMD .Mostly available FMD vaccines are inactivated whole-virus preparations which contain oil emulsions as an adjuvant to improve their efficacy. The proper use of good quality vaccines has been a significant factor in the control and / or eradication of FMD.

In this work we studied comparative analysis of the preservative efficacy of chloroform and thiomersal in ISA206 trivalent foot and mouth disease vaccine concerning the antimicrobial activity and vaccine potency. From the above

results showed that the five prepared vaccines were safe . during the whole experiment time. sterility test revealed that the vaccines with 0.01% thiomersal or 0.5% or 0.75% of chloroform were free from any pathogenic or non-pathogenic microorganisms , These results were in agreement with **OIE (2000)** FMD vaccine must be free from any living virus.

Also antibacterial activity of thiomersal 0.01% v/v, which were active against all the tested microbes and fungus in FMD vaccine .

The antibacterial activity of chloroform was observed to be in dose dependent manner; chloroform 0.1 v/v respectively, was not active against Staph.aureus,Pseudo-monas aeruginosa , Escherichia coli , Salmonella typhi and Salmonella para typhi A. Also chloroform 0.25 % v/v was not active against Staph. aureus and Salmonella typhi. The antifungal studies of chloroform 0.1 and 0.25 % v/v exhibits most efficacious results against Aspergillus nigar and Aspergillus pterus. Its activity is against Aspergillus flavus is quite low. These results are matching with **Lynch et al., 1977** and **Reddy et al ., 2001** who mentioned that chloroform has effective antimicrobial activity against vegetative organisms provided that its concentration does not fall below 0.3 % v/v.

The excellent results were shown with the chloroform 0.5 and 0.75 % v/v , were active against all the tested microbes and fungus 12 months post preparation. These results are matching with (**Ayyappan et al., (2010)** ; **Parekh et al., (2005)**; and **Rajakaruna et al., (2002)** and **Vedhanarayanan et al., (2015)** who mentioned that The chloroform extracted from plants were most active against gram positive bacteria than the gram negative bacteria

The potency of five FMD vaccine were tested in values using serum neutralization test and ELISA. The protective level were 1.5 log<sub>10</sub> by means of SNT Test and 1.9 by ELISA (**Hamblin et al., 1986**) and (**OIE 2009**) . All

five prepared vaccines were potent from the first month post vaccination till the 10<sup>th</sup> month post vaccination then declined. The constituents of 0.5 and 0.75 % v/v Chloroform in FMD vaccines exhibit sub-stantial activity against Bacillus subtilis ,Staph.aureus, Micrococcus luteus, Pseudo-monas aeruginosa, Escherichia coli ,Salmonella typhi ,Shig-ella flexneri ,Salmonella para typhi A, Proteus observed in previous studies (*Josephin Sheeba and Selva Mohan, 2012; Hema et al., 2013; Vinoth and Manivasagaperumal, 2015*).

It can be concluded that chloroform in conc 0.5 and 0.75 % v/v in FMD vaccine had a potential antimicrobial activity against all the microorganisms tested.

Finally, chloroform 0.5% v/v could be safely used instead of thiomersal 0.01% v/v as a preservative in FMD vaccine ..

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