ORIGINAL ARTICLE

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.

Objectives: 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.

Methods: 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, Pseudo-monas aeruginosa, Escherichia coli, Salmonella typhi, Shig-ella flexneri, Salmonella para typhi A and Proteus mirabilis and fungal strains were Aspergillus flavus, Aspergillus nigar* and *Aspergillus pterus*. Agar well diffusion method was followed in this study. The 12 monthes comparative analysis of antibacterial activity reflects that among these five vaccines shows thiomersal as well as 0.5% chloroform exhibiting maximum anti-bacterial and antifungal activity.

Results: Our results show that the incorporation of chloroform into ISA206/ FMDV vaccine is as effective as thiomersal as a preservative.

Conclusion: Finally we recommended using 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. 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 Asia [1].

In Egypt, The history of FMDV goes back to 1950, when an outbreak caused by serotype SAT2 was reported. Between 1964 and 2005, only serotype O was reported in Egypt, with the exception of 1972 when type A was introduced from Sub-Saharan Africa. Series of outbreaks predominantly caused by serotype O, and with a dramatic upsurge in FMD SAT 2 outbreaks during 2012 were reported. Serotypes O, A and SAT2 have been circulating in the country since 2012, and Serotype O is considered the predominant serotype.

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.

The proper use of good quality vaccines has been a significant factor in the control and/or eradication of FMD.

Preservatives are chemical substances whose role is to protect food products, stimulants, medicinal products and cosmetics against harmful changes caused by microorganisms. When added in proper concentrations, preservatives inhibit the growth of microorganisms during manufacturing and use of medicinal products. In concentrations used, they should be soluble and non-toxic as well as physiologically and chemically compatible.

It is well established [1] that multidose 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 [2] 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 [3]. 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

Vaccines

Five inactivated oil adjuvanated FMD Vaccines were formulated from FMD virus local strains (O/pan Asia2, A/ Iran 05 and SAT2/ Egypt 2012) according to Barnett et al. 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 [4,5].

Animals

18 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) [6].

Quality control of the prepared vaccines

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

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culturing of the tested vaccine on nutrient agar, thioglycolate broth and Sabauraud's dextrose agar [7].

Safety test for the formulated FMD vaccines: The inactivated FMD virus was tested for safety in vitro on BHK-21 cell line and the whole prepared vaccines in vivo in susceptible cattle and baby mice (OIE 2000).

Sterility and safety of the prepared vaccines were done according to (Code of Federal regulation of USA 1986. Henderson 1970 and OIE 2000).

Serum Neutralization Test (SNT)

The end point was calculated and expressed as log10 TCID50 as described by Reed and Muench.

Enzyme Linked Immunosorbant Assay (ELISA)

The test was carried out using the micro technique described by OIE by using flat bottom tissue culture microtitre plate. SPSS18.0 was used for data analysis, DNA sequencing was used as the reference pattern. The kappa coefficient was used to analyze the consistency of these two results. Kappa ≥ 0.75 was considered highly consistent, 0.4 ≤ kappa < 0.75 was moderately consistent, and kappa < 0.4 was poorly consistent. Mc-Nemar test was also performed between these two methods, $P<0.05$ indicating the difference between these two methods was statistically significant [8].

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. antifungal activity of the 5 preservatives was tested against three fungi;

TABLE 1

Safety of trivalent FMD vaccine with different preservatives tested

Months post preparation	Trivalent FMD Vaccine				
	0.01% thiomersal	Chloroform			
		0.10%	0.25%	0.50%	0.75%
1	safe	safe	safe	safe	safe
2	safe	safe	safe	safe	safe
3	safe	safe	safe	safe	safe
4	safe	safe	safe	safe	safe
5	safe	safe	safe	safe	safe
6	safe	safe	safe	safe	safe
7	safe	safe	safe	safe	safe
8	safe	safe	safe	safe	safe
9	safe	safe	safe	safe	safe
10	safe	safe	safe	safe	safe
11	safe	safe	safe	safe	safe
12	safe	safe	safe	safe	safe

TABLE 2

Immune response of trivalent FMD vaccine with 0.01% thiomersal

Months post vaccination	SNT	ELISA
1	2.7	2.7
2	2.85	2.85
3	3	3
4	2.4	2.7
5	2.4	2.25
6	1.8	1.95
7	1.8	1.8
8	1.65	1.65
9	1.5	1.5
10	1.2	1.35
11	1.05	1.05
12	0.9	0.9

TABLE 3

Immune response of trivalent FMD vaccine with 0.1% chloroform

Months post vaccination	SNT	ELISA
1	**2.4**	2.4
2	2.7	2.85
3	3	3
4	3	3.15
5	2.7	3
6	2.4	2.85
7	2.4	2.4
8	2.1	2.1
9	1.8	1.8
10	1.65	1.5
11	1.5	1.05
12	1.05	0.9

TABLE 4

Immune response of trivalent FMD vaccine with 0.25% chloroform

Months post vaccination	SNT	ELISA
1	3.15	2.55
2	2.85	3
3	3.15	3
4	2.85	2.7
5	2.7	2.7
6	2.4	2.55
7	2.4	2.4
8	2.1	2.4
9	1.8	2.1
10	1.65	1.8
11	1.5	1.65
12	1.05	1.5

TABLE 5

Immune response of trivalent FMD vaccine with 0.5% chloroform

Months post vaccination	SNT	ELISA
1	2.55	2.4
2	3.15	2.85
3	3	2.7
4	3	2.7
5	2.85	2.55
6	2.7	2.4
7	2.55	2.25
8	2.25	2.1
9	2.1	1.95
10	1.8	1.8
11	1.65	1.65
12	1.5	1.5

TABLE 6

Immune response of trivalent FMD vaccine with 0.75% chloroform

Months post vaccination	SNT	ELISA
1	2.55	2.4
2	3.15	3
3	3	2.85
4	2.85	2.85
5	2.55	2.7
6	2.4	2.55
7	2.4	2.4
8	2.25	2.1
9	2.1	2.1
10	1.8	1.95
11	1.65	1.8
12	1.5	1.65

TABLE 7

Sterility testing of trivalent FMD vaccine 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 8

Sterility testing of trivalent FMD vaccine with 0.1% chloroform

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 9

Sterility testing of trivalent FMD vaccine with 0.25% chloroform

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 10

Sterility testing of trivalent FMD vaccine with 0.5% chloroform

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 11

Sterility testing of trivalent FMD vaccine with 0.75% chloroform

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 12

Preservative challenge test of trivalent FMD vaccine with 0.01% thiomersal 12 months post preparation

Bacteria and fungi	Trivalent FMD vaccine with 0.01% thiomersal
Bacillus subtilis	negative
Staph. aureus	negative
Micrococcus luteus	negative
Pseudo-monas aeruginosa	negative
Escherichia coli	negative
Salmonella typhi	negative
Shig-ella flexneri	negative
Salmonella para typhi A	negative
Proteus mirabilis	negative
Aspergillus flavus	negative
Aspergillus nigar	negative
Aspergillus pterus	negative

TABLE 13

Preservative challenge test of trivalent FMD vaccine with 0.1% chloroform 12 months post preparation

Bacteria and fungi	Trivalent FMD vaccine with 0.01% thiomersal
Bacillus subtilis	negative
Staph. aureus	negative
Micrococcus luteus	negative
Pseudo-monas aeruginosa	negative
Escherichia coli	negative
Salmonella typhi	negative
Shig-ella flexneri	negative
Salmonella para typhi A	negative
Proteus mirabilis	negative
Aspergillus flavus	negative
Aspergillus nigar	negative
Aspergillus pterus	negative

TABLE 14

Preservative challenge test of trivalent FMD vaccine with 0.25% chloroform12 months post preparation

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

TABLE 15

Preservative challenge test of trivalent FMD vaccine with 0.5% chloroform12 months post preparation

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

TABLE 16

Preservative challenge test of trivalent FMD vaccine with 0.75% chloroform12 months post preparation

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

Aspergillus flavus, Aspergillus nigar and Aspergillus pterus using poison plate method according to Shastri and Varudkar.

RESULTS AND DISCUSSION

Results in Table 1 showed that the five prepared vaccines were safe for use during the whole experiment time. These results were in agreement with (OIE 2000).

Results in Tables 2-6 showed that the five prepared vaccines were potent during the whole experiment time. These results were in agreement with Wisniewski et al., Hamblin et al., and OIE 2009.

Results in Tables 7-11 showed that the five prepared vaccines were sterile during the whole experiment time. These results were in agreement with (OIE 2000).

Tables 12 showed that the thiomersal 0.01% v/v is active against all the tested microbes 12 months post preparation.

Tables 13 and 14 showed that the chloroform 0.1 and 0.25% v/v respectively, are not active against all the tested microbes 12 months post preparation.

Table 15 and 16 showed that the chloroform 0.5 and 0.75% v/v respectively, are active against all the tested microbes 12 months post preparation.

These results are matching with Lynch et al. [2] who mentioned that chloroform has effective antimicrobial activity against vegetative organisms provided that its concentration does not fall below 0.2% v/v.

CONCLUSION

Chloroform 0.5% v/v could be safely used instead of thiomersal 0.01% v/v as a preservative in FMD vaccine in Egypt.

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