Studies on the using of 2-Phenoxyethanol as an alternative to Thiomersal as a preservative in Foot-and-Mouth Disease vaccine

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Abstract

The control of foot-and-mouth disease (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 the disease. The progress in FMD vaccine production directed primarily towards the safety of the vaccine, purity of the antigen and selection of proper additives as adjuvants and preservatives. Thimerosal (Merthiolate) has been used as a preservative since 1930. Nevertheless, Thiomersal itself proved to be very toxic because it contains mercury. Hence, the current article discussed the cause and the prevention measures of the pyrogen-free colored sediment that might appear in the vaccine formula. Where the etiology might appear in the biological product was approached and solved. Besides, 2-phenoxyethanol was examined as an alternative preservative in FMD vaccine. The results of the examined samples (virus, vaccine, and milk), using the later preservative, showed no formation of sediment and no changes in colors. In addition, 2-phenoxyethanol had no cytotoxic effect on BHK. It showed safety and efficacy as substitution preservative.

Keywords: Foot-and-Mouth disease virus, Thiomersal, 2-phenoxyethanol

1. Introduction

Foot-and-Mouth Disease Virus (FMDV) is the etiologic agent of one of the most devastating diseases that affect cloven-hoofed livestock. It is small, non-enveloped single-stranded, positive sense RNA virus belongs to family *Picornaviridae* and has seven serotypes: O, A, C, Asia 1, and Southern African Territories (SAT) 1, 2 and 3, all of which cause highly contagious vesicular disease ***(Alexandersen et al., 2003)***. Within these serotypes, over 60 subtypes have also reported. Because of this diversity, there are no universal vaccines which present challenges during selection of vaccine strains ***(Brown, 2003 and Arzt et al., 2011).*** Infection with FMDV causes acute disease that spreads very rapidly and is characterized by fever, lameness and vesicular lesions on the feet, tongue, snout, and teats, with high morbidity rates but low mortality ***(Grubman and Baxt, 2004).*** Although vaccines have been extensively used to control FMD, there is no antiviral therapy available to treat ongoing infections with FMD V ***(Grubman, 2005).***

Preservatives are added to vaccines should formulation to ensure sterility of vaccine during shelf life. They do not change or alter the nature of antigens present in the vaccine formulation. They should be non-toxic in the concentration used and do not reduce the immunogenicity of the vaccine itself. The commonly used preservatives are phenol, benzethonium chloride, 2-phenoxyethanol and Thiomersal (Merthiolate) ***(Arif Khan 2015).*** Thiomersal is an organic-mercury (Hg)-containing compound (sodium ethyl-

mercury (Hg), $C_9H_9HgNaO_2S$) Thiomersal is the most widely used preservative in multi-dose vaccines due to its low cost and high effectiveness in killing bacteria. However, it is not an ideal preservative. Higher concentrations not recommended because it might reduce vaccine potency or pose a danger to individuals receiving the vaccine. As a result, the investigators suggested that those administering thimerosal containing vaccines should not rely on its effectiveness, but instead should apply particular attention to sterile technique when using multi-dose vials ***(Khandke et a.,l 2011***). In 1999, Food and Drug Administration (FDA) was required by law to assess the amount of mercury in all the products the agency oversees, not just vaccines. The U.S. Public Health Service decided that as much mercury as possible should be removed from vaccines, and thimerosal was the only source of mercury in vaccines. Even though there was no evidence that thimerosal in vaccines was dangerous, the decision to remove it was a made as a precautionary measure to decrease overall exposure to mercury ***(Ball et al., 2001 and Atkins 2001)***.

2-Phenoxyethanol (2-PE) is a broad spectrum preservative, which has an excellent activity against wide range of Gram-negative and Gram-positive bacteria, yeast, and mold ***(EU, 2016)***.

2-PE used as a preservative in cosmetics, pharmaceuticals and liquid protein concentrate. Investigators described the toxicity levels of commonly used preservatives in vaccines and biologics. The results showed that 2-PE was the least toxic compounds among preservative compounds as its relative toxicity expressed as 4.6 fold while it is 330 fold in case of Thiomersal and 12.2 fold for phenol (***Geier et al., 2010***). The activity of the antimicrobial preservatives, 2-PE and Thiomersal, were compared in diphtheria, tetanus, and pertussis (adsorbed) vaccine. Both chemicals were equally effective in inactivating challenge doses of Gram-negative and Gram-positive microorganisms, as well as yeast ***(Lowe and Southern 1994).*** Using of 2-PE as a preservative at a concentration of 5 mg/dose was stable and met ***European Pharmacopoeia*** (EP) recommended criteria for antimicrobial effectiveness tests when the formulation kept over 30 month. In contrast a dose of Thiomersal, as a comparator, or other preservatives did not meet EP antimicrobial effectiveness acceptance criteria. The results indicate that 2-PE provides superior antimicrobial effectiveness over thimerosal for this vaccine formulation ***(Khandke et al., 2011)***. Also, PCV13 vaccine formulated with 2-phenoxyethanol in multidose vials safe and immunogenic when administered according to the routine schedule **(*Idoko et al., 2017*).** Antibiotics are inadequate for preventing the growth of heavy contamination with bacteria or light contamination with fungi in biological products. The addition of 0.375% of 2-phenoxyethanol as a preservative to the vaccine furnished a stable mixture of preservatives (streptomycin, neomycin, and 2-PE) was inhibitory to both bacteria and fungi. This mixture was completely effective to preserve vaccine ***(Hilliard et al., 1964).***

The traditional preservative Merthiolate was used as in veterinary vaccine in developing countries with its adverse effects in human (**Geier et al., 2015**) and may discolor on exposure to light.

So, this study aimed to discuss the cause and the prevention measures of the pyrogen-free colored sediment that might appear in the vaccine formula. Besides, examination of 2-PE as an alternative preservative in vaccine production.

2. Materials and methods

2.1. FMDV, Cells and lab animal:

FMD virus type, O PanAsia 2, is a local strain of cattle origin. The virus was typed at Veterinary Serum and Vaccine Research Institute (VSVRI), Abbasia, Cairo and was confirmed by Pirbright, International Reference Laboratories, United Kingdom, the titer of the virus strain is 10^7 $\log_{10}$ $TCID_{50}$/ml. For detection of cell cytotoxicity, Baby Hamster Kidney cell line (BHK-21) Clone 13 was maintained in FMD Department, (VSVRI), according to the technique described by ***Macpherson and Stocher (1962)*** using Eagle's medium supplement with 8-10% sterile new bovine serum obtained (Sigma, USA). Additionally, twenty healthy adult albino Guinea pigs of approximately 400-500 grams body weight used in safety test.

2.2. Thiomersal and 2-phenoxyethanol:

Thiomersal ≥97% (HPLC) powder, Sigma Prod. No. T5125. The rate of oxidation of Thimerosal in solution is greatly increased by traces of copper ions. In slightly acidic solution Thimerosal may be precipitated as the corresponding acid which undergoes slow decomposition with the formation of insoluble products. Sodium chloride has been shown to adversely affect its stability. Thimerosal should be stored at room temperature protected from light. It is reportedly stable in air but not in sunlight. While, 2-Phenoxyethanol ≥99% (Phenylglycol), 77699 Sigma-Aldrich of molecular weight 138.16 was a viscous liquid, soluble and clear. Used at a concentration of 0.5% ***(Khandke et al., 2011)***.

2.3. Microbial inspection of FMD virus and vaccine

FMDV O serially inoculated onto BHK cells. Virus harvest exposed to sterilization using a 0.2µm filter. Monovalent oil emulsion FMDV O vaccine formula prepared. Traditional prepared Merthiolate solution prepared in a 1L glass bottle, autoclaved, kept in room temperature. It added to a sample from FMDV O harvest inactivated with Binary BEI and to the vaccine formula. Within time, the FMDV O harvest sample and vaccine formula showed somewhat colored sediment. The sediment aspirated and spread on bacteriological media and agar for pyrogenic agent inspection.

2.4. Chemical inspection of FMD virus and vaccine

The previous observed colored sediment posed to inspect most prominent chemicals used in the preparation steps of the virus harvest and its vaccine. These chemical include the pH adjusting buffer, sodium bicarbonate, Binary Ethyleneimine (BEI, Aziridine) in NaOH solution, Sodium Thiosulhate, Antimirobial agent (Neomycin and Nystatin), in addition to, the vaccine preservative agents, Merthiolate and formalin. All the previous chemical compounds and solutions were added solely to samples from FMDV O harvest, its different vaccine formula and full sterile milk with 3% fat (used as a control). Also, negative control without the previous chemicals. Each sample was adjusted to 1.5 ml and centrifuged at highest speed in a high-speed cooling centrifuge. The physical color appearance of each sample was observed after centrifugation**.**

2.5. Thiomersal preservative

The samples (virus, vaccine, milk) were further exposed to Thiomersal solution stress as following. Two aliquots of Thiomersal solution were used. Thiomersal solution **aliquot 1 (one)** (100% i.e. 100g/100ml) was the Traditional prepared Merthiolate solution, whereas, Thiomersal solution **aliquot 2 (two)** (10% i.e. 10g/100ml) was prepared and used avoiding heat and light. The two aliquots were applied on the samples. Negative controls without Merthiolate were also involved in the assay.

2.6. 2-Phenoxyethanol preservative

The former samples (virus, vaccine, milk) were inspected versus to 2-Phenoxyethanol**.** Cytotoxic assay of 2-phenoxyethanol on BHK-21 cells was performed as follow**.** BHK-21 cells seeded in 96–well micro-titer plates (Greiner-Bio one, Germany), for 24 h at 37°C. The medium removed from each well and replenished with 100 µl two-fold serial dilutions of 2-phenoxyethanol in fresh medium containing 2% fetal calf serum. For the cell control, 100 µl of media without 2-phenoxyethanol were added. The cell cultures incubated at 37 °C for 24 h. After incubation, cytotoxicity was determined by examining cellular morphology depending on microscopic detection of morphological alterations. Also, safety test for 2-Phenoxyethanol in Guinea pigs was carried out. It was performed using intradermal injections of 2-Phenoxyethanol solution (0.5, 1, 2, 4 %) in 0.9% saline in Guinea pigs, five animal for each concentration. Reactions were assessed after 24 and 48 hours. Microbial inspection using bacterial and fungal growth media for 2-Phenoxyethanol preservative was performed as previously mentioned.

3. Results

Microbial inspection of FMD virus and vaccine performed using, bacterial growth medium (Agar, Broth, Brain-Heart infusion medium, Thioglycollate broth and Thioglycollate broth with Tween 20), and fungal growth media (Sabouraud agar).

The results showed that there were no bacterial or fungal contaminants in the aspirated sediment for both the virus harvest sample and its vaccine that formulated with traditional prepared Merthiolate solution. Hence, the sediments isolated from the virus harvest sample and various vaccine formulas were pyrogen-free. Also, the results from (**Fig.** 1) showed clear discoloration of Thiomersal solution aliquot **one**, but there were non-discoloration in case of aliquot **two**. The inspection of tested samples and negative controls, after centrifugation, did not reveal color difference, except the one with added Merthiolate aliquot **one,** which showed discolored sediment in comparison to the aliquot **two** and negative control (**Fig.** 2-5). Furthermore, discolored sediment appeared in contaminated vaccine formula but accompanied by breaking of the emulsion into layers with changed colors (**Fig.** 6). Moreover, the effect of two Thiomersal concentrations (**0.01 and 0.01**%) on the **pH** was observed (**Fig.** 6-7).

For 2-Phenoxyethanol preservative, samples (virus, vaccine, milk) were inspected versus to 2-Phenoxyethanol and showed no apparent discoloration sediment and no alteration in their pH. Inoculation onto BHK cells for toxicity revealed that at concentration of 4%, the cytotoxicity was about 100% and when the concentration was decreased till reaching 0.5% there was no cytotoxicity found in the treated cells. In context of 2-Phenoxyethanol, safety test, necrotic skin lesions were induced by 4%, 2% and 1% solutions of 2-Phenoxyethanol. At the concentration of 0.5% there were no lesions. Microbial inspection for media showed no bacterial and fungal growth media on the virus harvest sample and its vaccine formula that had an added 2-Phenoxyethanol solution.

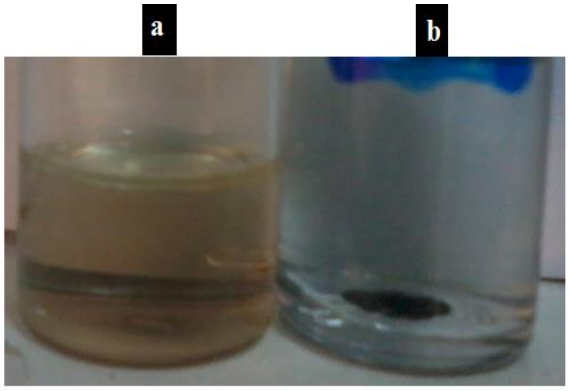

Fig. 1 Thiomersal ***exposed*** or ***avoided*** light and heat.
Thiomersal solution a- **aliquot 1** (100%) showed discoloration and b- **aliquot 2** (10%) showed non-discoloration.

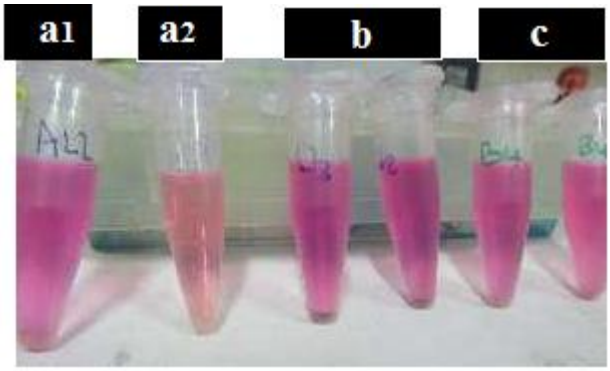

Fig. 2 Thiomersal (0.02%) effect on ***virus harvest***.
Sterile virus harvest showed micro-tubes with a_1- non-discolored sediment and no Thiomersal added, used as negative control containing virus harvest, while a_2 non-discolored sediment and no Thiomersal added, used as negative control containing aseptic cell suspension; b-discolored sediment and Thiomersal solution with **aliquot 1** added; c- non-discolored sediment and Thiomersal solution with **aliquot 2** added.

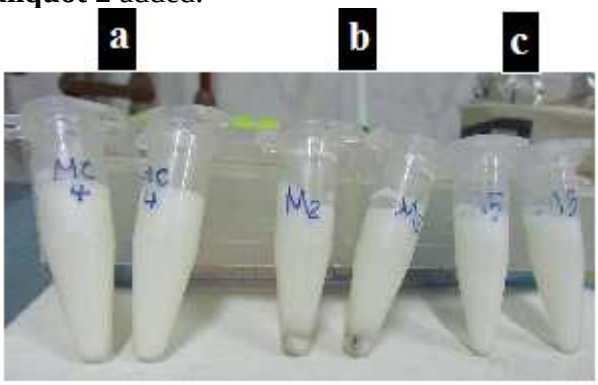

Fig. 3 Thiomersal (0.02%) effect on ***milk.***
Sterile milk showed micro-tubes with a- non-discolored sediment and no Thiomersal added, used as negative control; b-discolored sediment and Thiomersal solution with **aliquot 1** added; c- non-discolored sediment and Thiomersal solution with **aliquot 2** added.

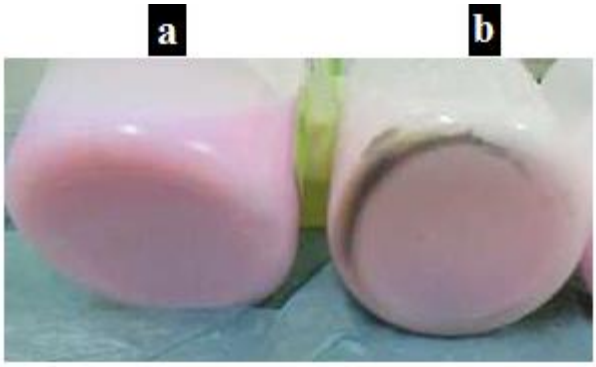

Fig. 4 ***Sterile vaccine formula*** showed a-non-discolored and b-discolored sediment.
The discoloration was due to aseptic chemical cause.

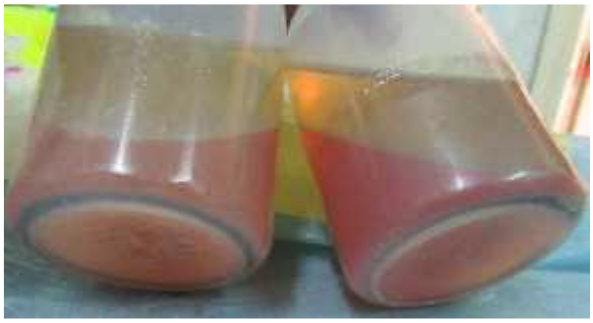

Fig. 5 ***Contaminated vaccine formula*** showed discolored sediment.
The discoloration was due to microbial cause accompanied by breaking of the emulsion into layers with changed colors

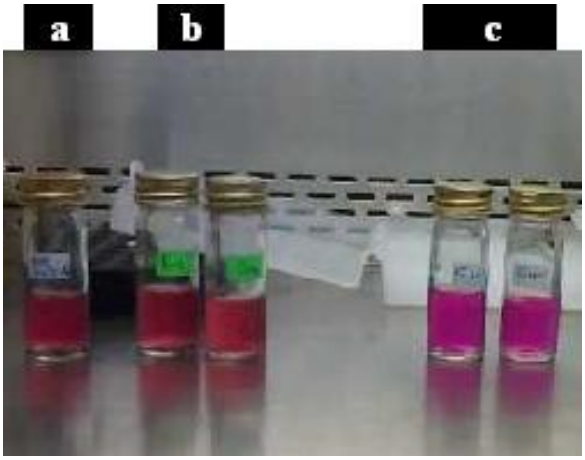

Fig. 6 Thiomersal (**0.01**%) effect on ***pH*** exposed to 37oC & 4oC ***for 2 days.***
Sterile virus harvest with phenol red (pH indicator), where the McCartney:
a- after kept at 37oC for one day **with no Thiomersal added**, followed by 4oC for one day, used as a control, where pH color ***changed*** from pink to red (pH value ~ 7.4-7.6)
b- after kept at 37oC for one day **with Thiomersal added (0.01%)**, followed by 4oC for one day where pH color ***changed*** from pink to red
c- **with Thiomersal added (0.01%)** ***before*** kept at 37oC for one day, followed by 4oC for one day where pH color ***unchanged*** from pink (pH value more than 8)

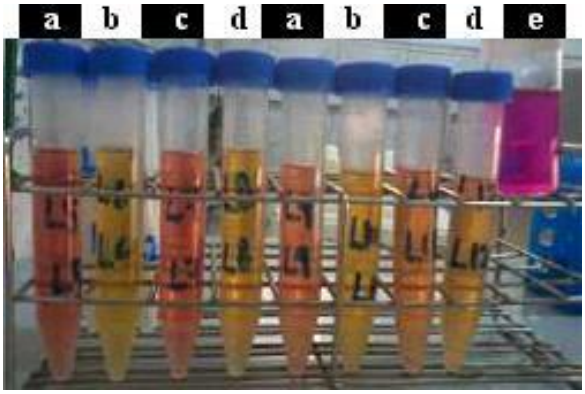

Fig. 7 Thiomersal effect (**0.1**%) on ***pH*** exposed to 37^{o}C, room temperature (during the study was 28 ^{o}C) and 4^{o}C ***for 6 day.***

Sterile virus harvest and ***aseptic cells suspension*** with phenol red (pH indicator) were shown. The tubes (the 1st four tubes contained cells, while the 2nd four tubes contained virus harvest) were:

a- with cells or virus harvest kept at 37 ^{o}C for one day, followed by 4 ^{o}C for **five** days **with Thiomersal added**, where pH color ***changed*** from pink to red (pH value ~ 7.4-7.6 for cells or ~ 7-7.2 for virus harvest)

b- with cells or virus harvest kept at 37 ^{o}C for one day, followed by 4 ^{o}C for five days **with no Thiomersal added**, where pH color ***changed*** from pink to yellow (pH value ~ 6.4-6.6)

c- with cells or virus harvest kept at room temperature for **six** days **with Thiomersal added**, where pH color ***changed*** from pink to red

d- with cells or virus harvest kept at room temperature for **six** days **with no Thiomersal**, where pH color ***unchanged*** from pink to yellow

e- virus kept at 4^{o}C for **six** days **with no Thiomersal added**, used as a control, where pH color ***unchanged*** from pink (pH value more than 8)

4. Discussion

The control of FMD relies on stamping out of the infected animals or vaccination with chemically inactivated FMD vaccines. Vaccination has greatly reduced the burden of infectious diseases, and recently the vaccine safety gets more public attention than vaccination effectiveness. In this study, we discussed the cause and the prevention measures of the pyrogen-free colored sediment that might appear in the vaccine formula and tried to improve the adverse influence of Thiomersal. There were neither bacterial nor fungal growth media in virus harvest nor its vaccine formula. However, there was discolored sediment in comparison to the negative control. The discolored sediments were in sterile virus harvest, milk and vaccine formula. The discoloration was due to chemical cause due to the presence of Thiomersal. Previously, Thiomersal leads to the formation of sediment **(*Ludwig et al., 2004*)**. Also, ***Dorea 2017,*** mentioned that Thiomersal had adverse effects as an preservative during vaccine formulation. Furthermore, it was recorded that adding of Thiomersal to FMDV as a preservative leads to dissociation of intact (146S) foot-and-mouth disease virions into 12S particles as assessed by novel ELISAs specific for either 146S or 12S particles **(*Harmsen et al., 2011)*.**

The results of the examination of 2-Phenoxyethanol as a preservative by inspection of samples (virus, vaccine, milk) showed no formation of sediment, and no changes in colors. Also, in case of inoculation of 2-Phenoxyethanol onto BHK cells for toxicity examination, it was clear that at the concentration of 0.5% there was no cytotoxicity in the treated cells. Also, the results of the safety test showed no necrotic skin lesions at the concentration of

0.5%. 2-Phenoxyethanol provides superior antimicrobial effectiveness over Thimerosal for vaccine formulation. Results supported by ***(Khandke et al., 2011)*** who concluded that 2-Phenoxyethanol is the most safe preservative in vaccine formulation**.** Where, it is suitable for use as a preservative vaccine **(*Eiji* et al., 2002)**.

Finally, we can conclude that: 2-phenoxyethanol could be used as an alternative to Thiomersal for safe and effective preservation of FMD vaccine.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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الملخص العربي

دراسات على استخدام 2-فينوكسي إيثانول كبديل للثيومرسال كمادة حافظة في لقاح مرض الحمى القلاعية ثلاثي العترة

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قسم بحوث الحمى القلاعية

ب:131.معهد بحوث الأمصال واللقاحات البيطرية- العباسية-القاهرة-ص

حديثا تم توجيه التقدم في إنتاج لقاح مرض الحمى القلاعية في المقام الأول نحو سلامة اللقاح، ونقاوة المستضد، واختيار مواد مضافة مناسبة مثل الإضافات المساعدة والمواد الحافظة. تم استخدام ثيومرسال كمادة حافظة منذ عام 1930. ومع ذلك، فمن المهم ملاحظة أن الثيومرسال نفسه ثبت أنه سام للغاية لأنه يحتوي على الزئبق. ومن ثم، ناقشت المقالة الحالية السبب والتدابير الوقاية للرواسب الملونة العقيمة التي قد تظهر في صيغة اللقاح. حيث تم التوصل إلى مسببات الرواسب التي قد تظهر في المنتج البيولوجي وحلها. بالإضافة إلى ذلك، تم فحص 2-فينوكسي إيثانول كماده حافظة بديلة في لقاح الحمى القلاعية، حيث أظهرت سلامة وفعالية كبديل.