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Sensitivity of Different Methods Used In Determination of Formalin Residues in Inactivated Veterinary Vaccines

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Abstract: Formaldehyde concentration is very crucial in the potency of inactivated vaccines and its adverse effects. High level of formaldehyde will affect on potency of the vaccine by masking the B and T cell epitopes. Determination of formaldehyde concentration can be measured by visual method (phenyl hydrazine) and by spectrophotometric methods (phloroglucinol and ferric chloride methods). In the current study, the sensitivity and specificity of these methods were investigated. The visual method is not accurate where it is depended on naked eye in matching of a coloured complex product while the two spectrophotometric methods gave nearly the same values and required inexpensive instrumentation(spectrophotometer) with simple operation. The ferric chloride quantitative colorimetric method depends on complicated principle, easily used in routine analysis and the used chromogenic agent is fairly expensive. In addition, this method is highly sensitive and accurate compared to phloroglucinol method where it could determine the formaldehyde concentration of all inactivated veterinary vaccines either bacterial or viral. Also, it could differ between the oil adjuvanted and the gel adjuvantedinactivated vaccines during the operating process.

Key words: Formaldehyde · Ferric Chloride · Phloroglucinol · Spectrophotometer · Phenyl Hydrazine · Vaccines

INTRODUCTION

Formaldehyde is a colourless gaseous chemical available as 37% aqueous solution, commonly referred to as "formalin" [1]. It has been produced commercially since 1889 by the catalytic oxidation of methanol. Various specific methods of production are widely used currently like the silver catalyst process and the metal oxide catalyst process [2-4].

Formaldehyde is widely present in the environment; it originates from both natural sources, e.g. forest fires and direct human sources, such as automotive combustion and industrial uses. Formaldehyde is a potential mutagen and carcinogen in laboratory animal for its serious toxicological properties [5].

Formaldehyde is highly irritant to mucous membranes. Also, there were toxico- pathological effects of different levels of formalin fed to the broiler chicks [6], the same results in female quils [7].

Formaldehyde is considered as a preservative for human and veterinary drugs and biological materials as viral vaccines which contain 0.05% formalin as an inactivating agent [8].

Formaldehyde should be added during the vaccine production within permissible limits where incomplete inactivation by formaldehyde was the probable cause of the outbreaks of some diseases as formaldehyde inactivating Venezuelan equine encephalomyelitis vaccines in central America in 1969- 1972 [9] and alsovaccines with higher level than the recommended formalin concentration lower hemagglutination inhibition antibody levels (mean depression in immunity) and induce an imbalance in estradiol secretion, resulting in degenerative change in ovarian follicles and uterus. Hence, new H5N1 vaccines with recommended formalin levels are urgently needed where there were rapid drop in egg production and a high culling rate in hens are associated with using four avian influenza (AI) inactivated vaccines [10]. Also, the use of therapeutic

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dose of formalin as a disinfectant is efficient against many fish infections but their use should be done under strict regulation to avoid its pathological side effects [11]. During vaccine production, several quality control tests are performed to ensure that vaccines have been made under optimal circumstances, at the end of vaccine production process many tests must be done on the bulk product and the final vaccine. Such tests include safety, potency, sterility, identity as well as residual chemical constituents particularly formaldehyde which is used as inactivating agent during manufacture. Formaldehyde is considered as toxigenic or carcinogenic material for both animals and human beings. Tests have been formulated for each vaccine according to [12-14]which are generally accepted allover the world.

A lot of methods for the determination of formaldehyde have been reported, which include spectrophotometry [15-18], high-performance liquid chromatography (HPLC) method [19, 22], gas chromatography method [21, 22], fluorimetry [23, 24], polarography [25] andpotable formaldehyde sensors-based spectrometric [26, 27] and electrochemical [28, 29] principles. Various chromogenic agents, such as chromotropic acid, pararosaniline, 4-amino-3-pentene-2-one (Fluoral-P), acetylacetone, 4-amino-5-hydrazine-3-mercapto-1,2,4-triazole (AHMT) and 3-methyl-2-benzothiazolone hydrazone (MBTH) have been used. AHMTmethod [30] and MBTH method [31] are getting popular due to their higher sensitivity compared to other methods.

The present work aimed to identify the most sensitive and accurate method among the three currently methods which are visual method, phloroglucinol method and ferric chloride method used for determination of the free formaldehyde concentration in inactivatedveterinary vaccines.

Table 1: Relationship between formaldehyde concentrations (%) and their absorbance (O.D.) by using phloroglucinol method

Formaldehyde Concentration (%)	Absorbance (O.D.)
0.05	0.1031
0.10	0.2059
0.15	0.3178
0.20	0.4418
0.25	0.6147

MATERIALS AND METHODS

Tested Vaccines: A total number of 72 batches of inactivated vaccines were used in this study. These batches represent locally produced and imported vaccines; 48 viralpoultry vaccines, 8 bacterial poultry vaccines, 8 viral large animal vaccines and 8 bacterial large animal vaccines.

Determintion of the Formaldehyde Concentation

Phenyl Hydrazine Method (Visual): It was carried out according to Quality control of vaccines[13] and Shrivastaw and Singh [32].

Phloroglucinol Method: It was performed according to the method described by Gayathriand Balasubramanian [33]:

Preparation of Standard Curve: In five test tubes containing 1 ml of 1% phloroglucinal, 0.50, 1.0, 1.50, 2.0 and 2.5 ml of 1 ppm standard formaldehyde solution was added separately. Four ml concentrated sulphuric acid was added carefully to each tube using a long stem funnel. The solution was allowed to stand for 20 min., to attain room temperature. The solution was then transferred into 10 ml standard flask, washed with 1ml of 9 M sulphuric acid and diluted to the mark with the same acid. Absorbance was measured at 435 nm against reagent blank prepared according to the same procedure (Table 1, Fig. 1).

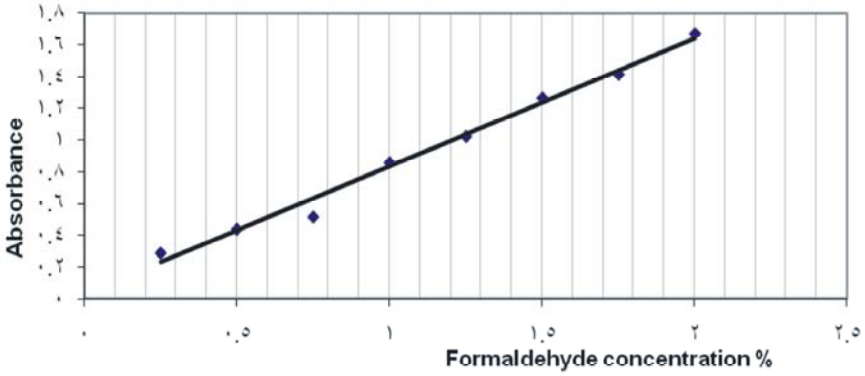

Fig. 1: Standard curve of different formaldehyde concentration (%) and their absorbance by using phloroglucinol method

Determination of Formaldehyde in Inactivated Vaccines: one ml of the inactivated vaccine was diluted to 100ml with distilled water. The diluted solution was filtered and used for analysis. One ml of the sample solution was analyzed for formaldehyde content following the procedure described before.

Calculation: Formaldehyde concentration (%) was obtained from the standard curve based on the following equation:

O.D. of sample/ O.D. of standard □Concentration of standard

Residual Formaldehyde- Ferric Chloride Method: It was described in [14, 34-38]. This test is based on the reaction of formaldehyde with methylbenzothiazolonehydrazone hydrochloride (MBTH) which combined with formaldehyde to give one product. The oxidation of excess MBTH to give another product and these two products combined to give blue chromophore.

Sample and Standards Preparation:

- Formaldehyde standards of 0.25, 0.50, 0.75, 1.00, 1.25, 1.50, 1.75 and 2.00 g/L were prepared by diluting formaldehyde solution with water in suitable volumetric flasks.
- If vaccine to be examined is an oil emulsion, the emulsion should be broken by a suitable separation method in which 1.00 ml of vaccine was added, to 1.0 ml of isopropyl myristate and mix. To the mixture, 1.3 ml of 1 M hydrochloride acid was added, 2.0 ml of chloroform and 2.7 ml of sodium chloride (9 g/L aqueous solution) mixed thoroughly and centrifuged at 15000 xg for 60 min. The aqueous phase was transferred to a 10 ml volumetric flask and diluted to volume with water.

Test Performance: To 0.5 ml of a 1:200 dilution of the vaccine to be examined (if emulsion, use 0.50 ml of a 1:20 dilution of the diluted aqueous phase) and to 0.50 ml of 1:200 dilution of each of the formaldehyde standards, 5 ml of MBTH was added. The tubes were shaked and allowed to stand for 60 min. Then 1 ml of ferric chloride- sulphamic acid reagent was added and allowed to stand for 15 min. Absorbance of vaccines was measured and standards on a spectrophotometer at 628 nm in a 1-cm cell, using the reagent blank as compensation liquid.

Calculation and Test Validation: Total formaldehyde concentration (g/L) was calculated from the standard curve using linear regression (acceptable correlation coefficient: r)

RESULTS AND DISCUSSION

The widespread use of formaldehyde and the reports on adverse effects have created the need for specific, sensitive and simple method for its determination.

In this study, three methods were used; the principle of the visual method is based on the reaction of formaldehyde with phenyl hydrazine solution (1%), potassium ferricyanide (5%) in acid solution (HCL)forming a red to faint pink coloured compound, the intensity of which can be matched visually with colours of 1, 0.50, 0.10, 0.05 and 0.01% of formaldehyde standard solutions. The spectrophotometric phloroglucinol method for the determination of formaldehyde in inactivated biological vaccines is based on the reaction of formaldehyde with phloroglucinol as chromogenic agent in acidic solution, this reaction occur rapidly about 20 minutes at room temperature giving unstable intermediate orange product [33] and this result agree with Ziwie *et al*.[39].

The obtained results were illustrated in Table (1) which showed a relationship between formaldehyde concentrations (%) against their absorbance (O.D. optical density) by using spectrophotometric phloroglucinol method.

The ferric chloride method is based on the reaction of formaldehyde with Methylbenzothiazolonehydrazone hydrochloride (MBTH) which combined with formaldehyde to give one product. The oxidation of excess MBTH to give another product and these two products combined to give blue chromophore which is measured at 628 nm against known formaldehyde standard concentration solutions of (0.25, 0.50, 0.75, 1.00, 1.25, 1.50 and 2.00 g/L) and their absorbance ranged between 0.2903 and 1.6698 from the standard curve using linear regression with correlation coefficient (r) = 0.92 as shown in Table (2), Figure (2) and this results agree with results of [14, 34-38] which make a standard formaldehyde concentration curve of correlation coefficient (r) = 0.97. Also, the results of formaldehyde concentration were converted from g/L to percentage (%) depending on 40% formaldehyde solution to easily make comparison with the others methods from the formaldehyde level conversion table as table (3), according toKnight and Tennant [40], Chandler *et al.* [41].

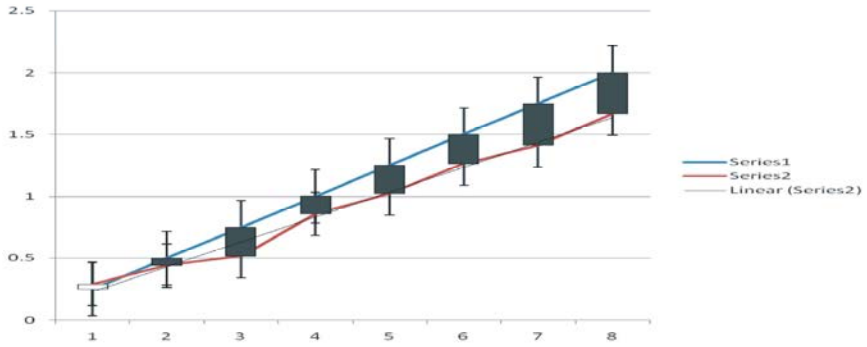

Fig. 2: Standard curve of different formaldehyde concentration (g/L) and their absorbance by using ferric chloride method

Table 2: Relationship between formaldehyde concentrations (g/L) and their absorbance (O.D.) by using ferric chloride method:

Formaldehyde concentration (g/L)	Absorbance (O.D.)
0.25	0.2903
0.50	0.4413
0.75	0.5168
1.00	0.8586
1.25	1.0239
1.50	1.2650
1.75	1.4138
2.00	1.6698

Table 3: Formaldehyde level Conversion table

Formaldehyde concentration (g/L)	Formaldehyde solution concentration%
0.25	0.063
0.50	0.125
0.75	0.187
1.00	0.25
1.25	0.313
1.50	0.375
1.75	0.438
2.00	0.500

The obtained results were illustrated in tables (4,5 and 6) that show determination of residual formaldehyde in 72 randomly selected batches of inactivated vaccines used in this study. These batches represent local and imported vaccines including 48 viral poultry inactivated vaccines, 8 bacterial poultry inactivated vaccines, 8 viral large animal inactivated vaccines and 8 bacterial large animal inactivated vaccines.

Table (4) showed the mean of formaldehyde percentages of poultry viral and bacterial vaccines (0.039% and 0.17%)respectively and the mean of formaldehyde percentages of large animal viral and bacterial inactivated vaccines (0.055% and 0.31%) respectively.

Table (5 and 6) showed the mean of formaldehyde percentages of poultry viral and bacterial inactivated vaccines by using phloroglucinol method and the mean of formaldehyde percentages of poultry viral and bacterial inactivated vaccines by using ferric chloride method (0.01%, 0.071%, 0.0089% and 0.01%) and (0.0067%, 0.019%, 0.0064% and 0.012%) respectively.

These results showed the wide range between the formaldehyde concentration percentages where the permissible limit of this visual method is ≤ 1, so it is from the most disadvantage of this method. The visual method by using phenyl hydrazine is simple but it has primitive

Table 4: Determination of formaldehyde concentration (%) in random batches of inactivated vaccines by using phenyl hydrazine(visual method)

Poultry inactivated vaccines						Large animal inactivated vaccines					
Viral vaccines			Bacterial vaccines			Viral vaccines			Bacterial vaccines		
Type	No. of batch	Mean ± SD	Type	No. of batch	Mean±SD	Type	No. of batch	Mean ±SD	Type	No. of batch	Mean±SD
A.I.	17	0.124±0.215	Avian Cholera	2	0.275±0.32	Entero-3	4	0.005±0.00	Clostridia	5	0.113±0.22
N.D.	7	0.005±0.00	M.G.	2	0.05±0.00	BVD	2	0.00±0.00	Clostridia+Mannhei	3	0.502±0.49
A.I.+N.D.	6	0.05±0.00	Avian Coryza	2	0.05±0.00	IBR	2	0.05±0.00			
ND+IB+IBD	5	0.03±0.027	F.P.R.P.	2	1±0.00						
ND+IB+IBD+Reo	4	0.0275±0.026									
ND+IB+IBD+TRT	2	0.005±0.00									
ND+IB+EDS	3	0.05±0.00									
ND+IB	2	0.0275±0.032									
Reo	2	0.0275±0.032									
Total	48	0.039±0.035		8	0.17±0.318	8		0.055±0.00		8	0.31±0.27

Mean±SD (Standard Deviation)F.P.R.P. formalinized poly rabbit pasteurellosis

M.G. *Mycoplasma gallisepticum*

Table 5: Determination of formaldehyde concentration (%) in random batches of inactivated vaccines by using phloroglucinol method

Poultry inactivated vaccines						Large animal inactivated vaccines					
Viral vaccines			Bacterial vaccines			Viral vaccines			Bacterial vaccines		
Type	No. of batch	Mean ± SD	Type	No. of batch	Mean±SD	Type	No. of batch	Mean ±SD	Type	No. of batch	Mean±SD
A.I.	17	0.047±0.001	A. Cholera	2	0.052±0.001	Entero-3	4	0.017±0.002	Clostridia	5	0.031±0.003
N.D.	7	0.032±0.001	M.G.	2	0.016±0.0002	BVD	2	0.052±0.003	Clostridia+Mannhy	3	0.051±0.001
A.I.+N.D.	6	0.106±0.001	A. Coryza	2	0.035±0.001	IBR	2	0.002±0.0003			
ND+IB+IBD	5	0.052±0.001	F.P.R.P.	2	1.021±0.02						
ND+IB+IBD+Reo	4	0.045±0.001									
ND+IB+IBD+TRT	2	0.053±0.003									
ND+IB+EDS	3	0.051±0.001									
ND+IB	2	0.056±0.002									
Reo	2	0.030±0.0002									
Total	48	0.01±0.011		8	0.071±0.022		8	0.0089±0.01		8	0.01±0.01

Table 6: Determination of formaldehyde concentration (%) in random batches of inactivated vaccines by using Ferric Chloride method

Poultry inactivated vaccines						Large animal inactivated vaccines					
Viral vaccines			Bacterial vaccines			Viral vaccines			Bacterial vaccines		
Type	No. of batch	Mean ± SD	Type	No. of batch	Mean±SD	Type	No. of batch	Mean ±SD	Type	No. of batch	Mean±SD
A.I.	17	0.039±0.18	A.Cholera	2	0.056±0.072	Entero-3	4	0.0015±0.0	Clostridia	5	0.05±0.20
N.D.	7	0.064±0.20	M.G.	2	0.057±0.007	BVD	2	0.01±0.0	Clostridia+Mannhy	3	0.04±0.14
A.I.+N.D.	6	0.087±0.29	A.Coryza	2	0.018±0.001	IBR	2	0.0069±0.0			
ND+IB+IBD	5	0.021±0.07	F.P.R.P.	2	0.028±0.013						
ND+IB+IBD+Reo	4	0.025±0.12									
ND+IB+IBD+TRT	2	0.03±0.016									
ND+IB+EDS	3	0.012±0.056									
ND+IB	2	0.017±0.106									
Reo	2	0.025±0.01									
Total	48	0.0067±0.020		8	0.019±0.012		8	0.0064±0.0			0.012±0.043

one step reaction and chemicals, also it was done in very short time (the colour was appeared within 5 minutes) and it is visual method so it is probably make a false results due to it depends on naked eye in matching of a coloured complex product formed with formaldehyde by the action of phenyl hydrazine, potassium ferricyanide and chloroform in presence of methanol. So, there is a risk in detection of results depending on coloured reagent. Also the results of formaldehyde concentration percentage were within permissible limit where this method has wide range of permissible limit which is ≤ 1, so, it is not accurate method when compared with two other spectrophotometrical methods.

The two spectrophotometric methods gave nearly the same values where these results are within narrow percentage range which they are the most widely used due to its inexpensive instrumentation, simple operation and fairly good sensitivity one of them using phloroglucinol and the other using ferric chloride. So, it is very clear that the two spectrophotometrical methods are more sensitive than the visual one, where they gave very accurate percentage of formaldehyde while later gave approximate values. The obtained results are in agreement with those obtained by [33] who said that the determination of formaldehyde spectrophotometrically using phloroglucinol is a simple and sensitive method, also ferric chloride method is simple and this was in agreement with results of Ross *et al*. [16] who conducted an international collaborative study of quantitative colourimetric method for determination of formaldehyde in veterinary vaccines products by 15 laboratories in North America, Europe and Japan.

The phloroglucinol method is a simple and sensitive method, formaldehyde reacts with phloroglucinol (1%) as a chromogenic agent in acidic medium producing a yellow dye with λ_{max} at 435nm, the colour development reaction could be conducted easily at room temperature and it needs simple chemicals and short time (it needs about 20 minutes). But the ferric chloride method is quantitative colorimetric method and it depends on complicated principle, easly used in routine analysis applications in the laboratory. It was getting popular due to its higher sensitivity compared to phloroglucinol method this agreement with Pereira and Dasgupta [31], it used MBTH chromogenic agent (0.05%) so it is very low in cost in comparison to using of phloroglucinol chromogenic agent as (1%) and it needs 2 step reactions each one produces a certain compound and require a relative long reaction time, about 1 hour for the first step and 15 minutes for the second step reaction for the final colour development. In

addition, the chromogenic agents are fairly expensive and used in low concentration (0.05%) in ferric chloride method but used in phloroglucinol in high concentration as (1%).

In conclusion, the ferric chloride quantitative colourimetric method is the best method where it is simple operation, used inexpensive instrumentation (spectrophotometer), also the used chromogenic agents are fairly expensive and it is getting popular due to its higher sensitivity compared to other methods due to it needs two-step reactions and requires a relative long reaction time for the final color development, it depends on complicated principle, easly used in routine analysis applications in the laboratory. In addition, it is fairly of good sensitivity where it used to determine the formaldehyde concentration of all inactivated veterinary vaccines either bacterial or viral, also it could differ between the oil adjuvant and the gel inactivated vaccines during the operating method.

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