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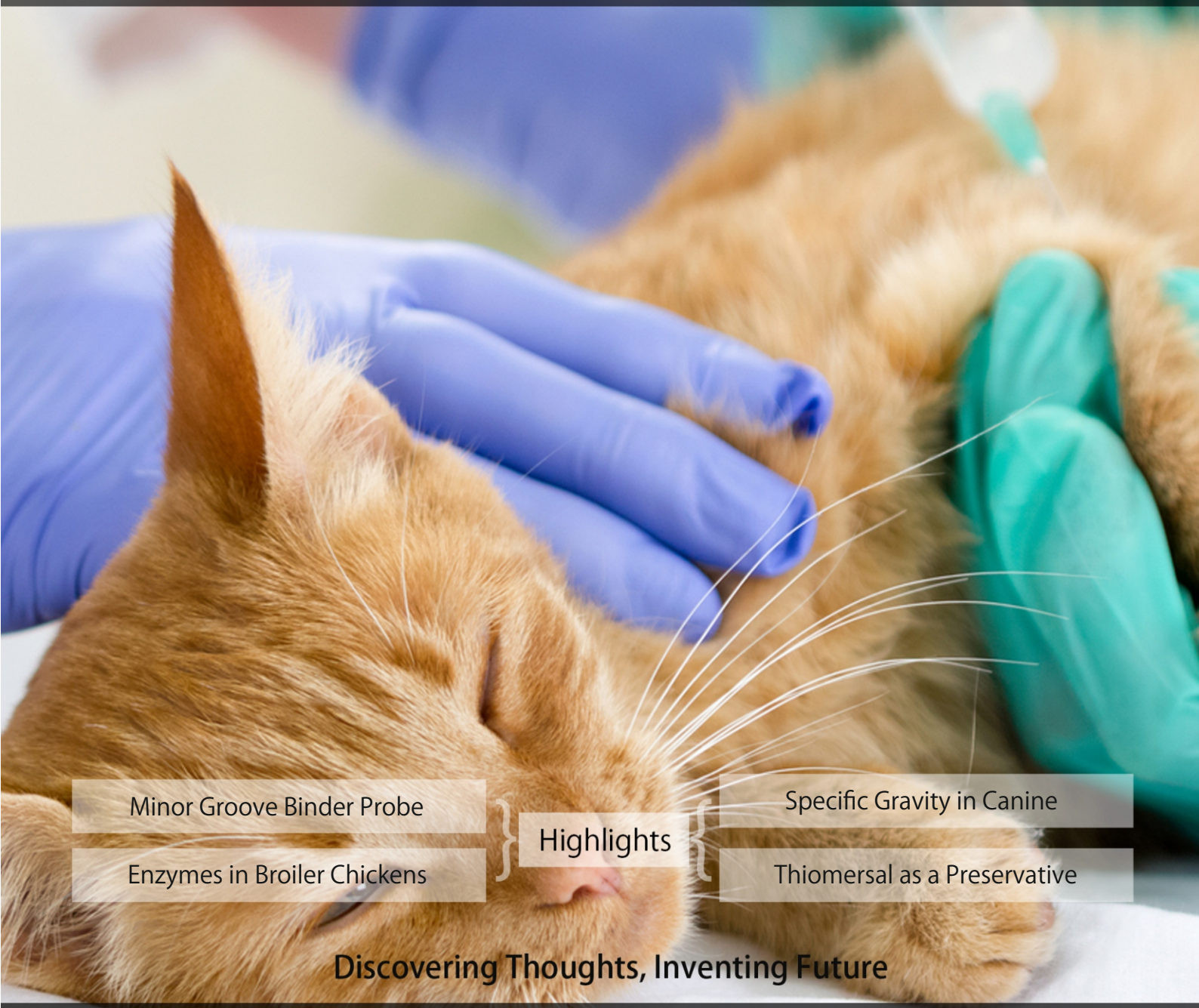

Minor Groove Binder Probe

Enzymes in Broiler Chickens

Highlights

Specific Gravity in Canine

Thiomersal as a Preservative

Discovering Thoughts, Inventing Future

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Studies on the using of 2-Phenoxyethanol as an Alternative to Thiomersal as a Preservative in Foot-and-Mouth Disease Vaccine

By Hany I Abu-Elnaga, Sonia A Rizk, Hind M Daoud, Akram Z Hegazy & Walaa S Shabana

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Keywords: *foot-and-mouth disease virus, thiomersal, 2-phenoxyethanol.*

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Hany I Abu-Elnaga [α], Sonia A Rizk [σ], Hind M Daoud [ρ], Akram Z Hegazy [ω] & Walaa S Shabana [¥]

Abstract- **The progress in foot-and-mouth disease (FMD) vaccine production directed primarily towards the safety of the vaccine, purity of the antigen, selection of proper additives, as adjuvant and preservative. Thimerosal (Merthiolate) has been used as a preservative since 1930. Nevertheless, it is important to note that 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 examined as an alternative preservative in FMD vaccine, where it showed safety and efficacy as substitutional.**

Keywords: foot-and-mouth disease virus, thiomersal, 2-phenoxyethanol.

I. Introduction

Foot-and-Mouth Disease Virus (FMDV) is the etiologic agent of one of the most devastating diseases that can affect cloven-hoofed livestock. It is a small, non-enveloped single-stranded, positive sense RNA virus related 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 a 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 thus presenting challenges in the selection of vaccine strains *(Brown, 2003 and Arzt et al., 2011).* Infection with FMDV causes an 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 but low mortality *(Grubman and Baxt, 2004).* Although vaccines have been extensively being used to control FMD, there was no antiviral therapy available to treat ongoing infections with FMD virus *(Grubman, 2005).*

Preservatives are added to vaccines formulation to ensure sterility of vaccine during its shelf life. They do not change or alter the nature of antigens present in the vaccine formulation. They are non-toxic in the concentration used and do not reduce the immunogenicity of the vaccine itself. Some of 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), $C_9H_9HgNaO_2S$) this is 49.55% Hg by weight. Historically, it was added to many multi-dose vials of vaccine as a preservative till now *(Tan and parkin 2000 and Geier et al., 2017*).

Thiomersal has been the most widely in multi-dose vaccines due to its low cost and high effectiveness in killing bacteria. 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., I 2011*). In 1999, the 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 excellent activity against a wide range of Gram-negative and Gram-positive bacteria, yeast, and mold *(EU, 2016)*. 2-Phenoxyethanol 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 of 2-phenoxyethanol was the least toxic compounds among preservative compounds as it's 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-phenoxyethanol 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

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well as yeast *(Lowe and Southern 1994).* Using of 2-Phenoxyethanol 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 multi dose 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-phenoxyethanol) 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. Hence, the current article discussed the cause and the prevention measures of the pyrogen-free colored sediment that might appear in the vaccine formula. Besides, 2-phenoxyethanol examined as an alternative preservative.

II. Materials and Methods

a) FMDV, Cells and lab animal

FMD virus, O Pan Asia 2, locally isolated strain of cattle origin. The virus was typed at Veterinary Serum and Vaccine Research Institute, Abbasia, Cairo and confirmed by Pirbright, International Reference Laboratories, United Kingdom, with a titer of 10^7 $\log_{10}$ $TCID_{50}$/ml. For detection the cytotoxicity test, Baby Hamster Kidney cell line (BHK21) Clone 13 maintained in FMD Department, Veterinary Serum and Vaccine Research Institute, Abbasia, Cairo according to the technique described by *Macpherson and Stocher (1962)* using Eagle's medium with 8-10% sterile new bovine serum obtained from Sigma, USA was used. Additionally, twenty healthy adult albino Guinea pigs of approximately 400-500 grams body weight used in safety test.

b) 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)*.

c) 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.

d) 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 added solely to samples from FMDV O harvest, its different vaccine formula and full sterile milk (3% fat) involved 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 observed after centrifugation.

e) 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 one was the previous mentioned Traditional prepared Merthiolate solution, whereas, Thiomersal solution aliquot two was prepared and used avoiding heat and light. The two aliquots applied on the samples, in addition to negative controls without Merthiolate were involved.

f) 2-Phenoxyethanol preservative

The former samples (virus, vaccine, milk) 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 cell controls, 100µl of media without 2-phenoxyethanol added. The cell cultures incubated at 37 °C for 24 h. After incubation, cytotoxicity determined by examining cellular morphology depending on microscopic detection of morphological alterations. Also, safety test for 2-Phenoxyethanol in Guinea pigs carried out. It 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 assessed after 24 and 48 hours. Microbial inspection using bacterial and fungal growth media for 2-Phenoxyethanol preservative performed as previously mentioned.

III. Results

Microbial inspection of FMD virus and vaccine showed that the bacterial and fungal growth media included Agar, Broth, Brain-Heart infusion medium, Thioglycollate broth, Thioglycollate broth with Tween 20 and Sabouraud agar did not detect contaminant in the aspirated sediment in both the virus harvest sample and its vaccine formula that had an added traditional prepared Merthiolate solution. Hence, the sediments isolated from the virus harvest sample and various vaccine formula were pyrogen-free. Thiomersal solution aliquot one showed discoloration, where, aliquot two showed non-discoloration (Fig. 1). Samples and negative controls, inspected after centrifugation for an apparent chemical reaction of Merthiolate aliquot one and two, did not reveal color difference, except the one with added Merthiolate aliquot one that 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 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 the virus harvest sample and its vaccine formula that had an added 2-Phenoxyethanol solution.

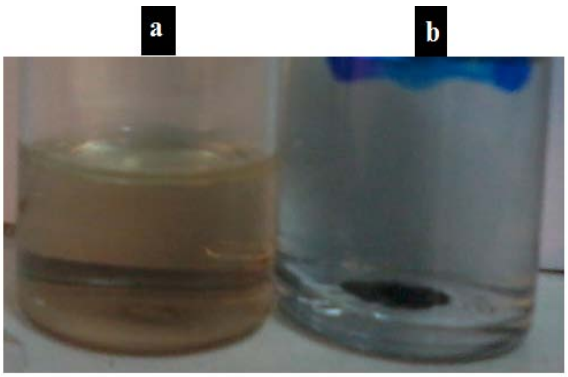

Fig. 1: Thiomersal *exposed* or *avoided* light and heat Thiomersal solution a- aliquot1 (100%) showed discoloration and b- aliquot two (10%) showed non-discoloration.

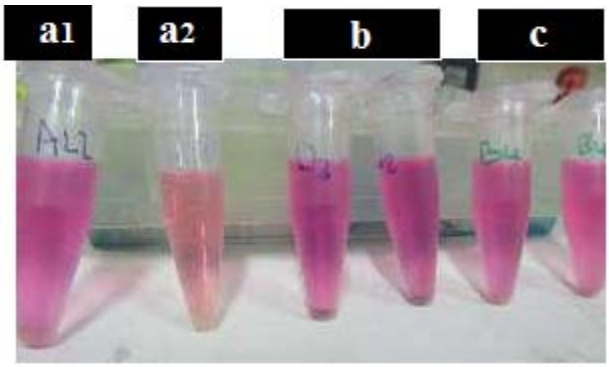

Fig. 2: Thiomersal (0.02%) effect on *virus harvest*. *Sterile virus harvest* showed micro-tubes with a_1- non-discolored sediment and no Thiomersal added, used as negative control containing virus harvest, while a_2 non-discolored sediment and no Thiomersal added, used as negative control containing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

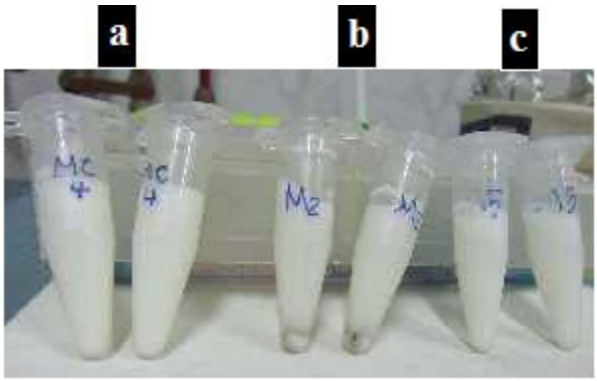

Fig. 3: Thiomersal (0.02%) effect on *milk Sterile milk* showed micro-tubes with a- non-discolored sediment and no Thiomersal added, used as negative control; b-discolored sediment and Thiomersal solution aliquot 1 added; c- non-discolored sediment and Thiomersal solution aliquot two added.

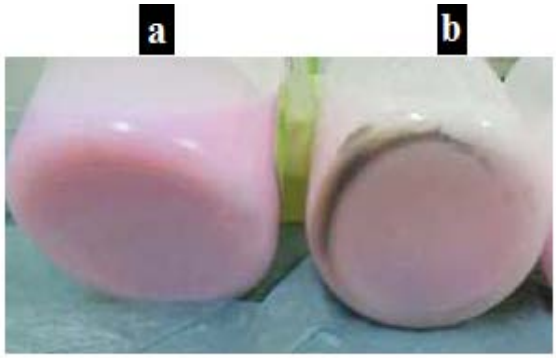

Fig. 4: *Sterile vaccine formula* showed a-non-discolored and b-discolored sediment. The discoloration was due to chemical cause.

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.

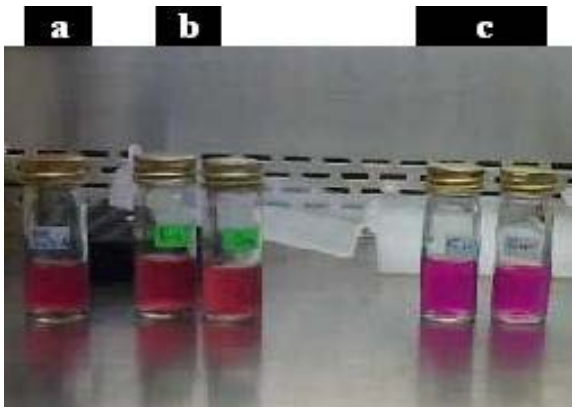

Fig. 6: Thiomersal (0.01%) effect on *pH* exposed to 37°C & 4°C *for 2 days. Sterile virus harvest* with phenol red (pH indicator). The McCartney:

a. After kept at 37°C for one day with no Thiomersal added, followed by 4°C 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 37°C for one day with Thiomersal added (0.01%), followed by 4°C for one day where pH color *changed* from pink to red.
c. With Thiomersal added (0.01%) *before* kept at 37°C for one day, followed by 4°C for one day where pH color *unchanged* from pink (pH value more than 8).

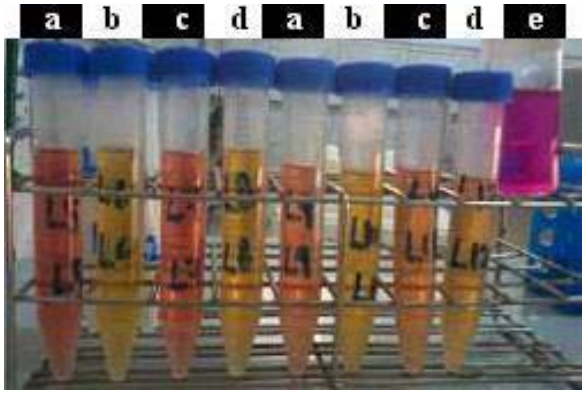

Fig. 7: Thiomersal effect (0.1%) on *pH* exposed to 37°C, room temperature (during the study was 28 °C) and 4°C *for 6 day. Sterile virus harvest* and *aseptic cells suspension* with phenol red (pH indicator) were shown. The tubes (the 1st four tubes contained cells, while the 2nd four tubes contained virus harvest) were:

a. With cells or virus harvest kept at 37°C for one day, followed by 4°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°C for one day, followed by 4°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°C for six days with no Thiomersal added, used as a control, where pH color *unchanged* from pink (pH value more than 8).

IV. 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*). 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, results of 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 *(Khandke et al., 2011.* Where, it is suitable for use as a preservative vaccine (Eiji et al., 2002).

Finally, 2-phenoxyethanol could use 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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Carcass Characteristics, Hematology, Serum Chemistry, and Enzymes in Broiler Chickens Fed Maggot Meal as a Protein Substitute for Fishmeal

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Keywords: *maggot meal, hematology, serum chemistry, enzymes, carcass characteristics.*

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Carcass Characteristics, Hematology, Serum Chemistry, and Enzymes in Broiler Chickens Fed Maggot Meal as a Protein Substitute for Fishmeal

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Abstract- Conventional protein sources used in poultry farming are extensively competed for, by livestock and humans leading to high prices and reduced returns. Focus on better utilization of available alternative feed resources with little or no negative impacts on the health of broilers and consumers is useful. The objective of this research was to assess the performance of carcass characteristics, hematology, serum chemistry, and enzymes in broiler chickens fed maggot meal as a protein substitute for fishmeal. 225 Tropical Broc day old chicks brooded for two weeks and fed the control diet, were distributed in a completely randomized block design with five treatments and three replicates each consisting of the starter and finisher phases and the experiment conducted for eight weeks. Diets were compounded with maggot meal (MM) replacing FM at 0%, 25%, 50%, 75% and 100%. On the last day of week 8, 30 birds, 2 from each replicate, were randomly selected and weighed, each bird slaughtered and allowed to bleed for 2 minutes while blood samples collected from one bird per replicate were put into 2 tubes (one with EDTA and the other without) for studies of Hb, WBCS and RBCs, total protein, albumin, and globulin, AST and ALT. Dressed, eviscerated, carcass parts, liver, and gizzard, weights were taken. Then averages from each replicate statistically analyzed for any significant differences. Results showed better carcass characteristics and lower amounts of WBCs in the treatment groups with maggot meal, stable values of RBCs and Hb, no defined trend in variations of total protein, globulin, and enzymes studied between the various treatments. Given that broilers with the best carcass characteristics performance were those with 100% maggot meal inclusion and that physiological parameters were not deviated from normal values in birds fed experimental diets, it can be concluded from this study that maggot meal can completely replace fish meal at 5% in broiler feed for better carcass characteristics, and a stable physiological profile.

Keywords: maggot meal, hematology, serum chemistry, enzymes, carcass characteristics.

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I. Introduction

Broiler production is one of the main areas in animal farming that involves quite a large section of the population either skilled or unskilled. One important advantage in this is the fact that poultry meat is very rich in unsaturated fatty acids as against saturated fatty acids. Both turkey and chicken have about 30% saturated fatty acids, 43% monounsaturated fatty acids, and 22% polyunsaturated fatty acids. The ratio is a clear indication that poultry meat may stand a better position as a more healthful alternative for red meat (Encyclopaedia Britannica, 2014). Notwithstanding there are some challenges in areas of nutrition, health, and management (Awoniyi, 2004). Feeding alone accounts for 60-70% of the total production cost in modern poultry production systems (Smith, 1990; Church, 1991; Wilson and Beyer, 2000). Conventional protein sources used are extensively competed for, by other livestock and humans (Gadzirayi *et al*., 2012), thereby leading to very high prices and reduced returns. Any attempt to improve commercial poultry production and increase its efficiency, therefore needs to focus on better utilization of alternative but available feed resources. Knowledge of nutritional characteristics of these feeds and optimal levels of inclusion in rations and optimum combination of ingredients is useful (Kamalzadeh *et al*., 2008). Competition for conventional protein sources has prompted researchers to embark on research for alternatives like MM that are cheaply available and comparable to FM. The crude protein content of maggots is high (39-63%) (Aniebo and Owen, 2010) and akin to that of fish meal (Veldkamp *et al*., 2012).

Even though MM may reduce competition between man and other livestock, there is very high need to investigate its health implications in broiler and humans as well the effects on performance of broilers (Awoniyi, 2004). A study of the carcass characteristics, blood indices and enzymes should correlate the benefits of MM as a protein source in poultry feed vis-à-vis the physiological status of the birds. The study of these

physiological parameters will serve to bridge the gap as work done in Cameroon on this aspect is scanty.

II. Materials and Methods

a) Study area

The study was carried out at Muyuka Agro-Industrial Farm situated between latitudes $4^0$16″ and $4^0$23″N and longitudes $9^0$21″ and $9^0$28″E in the fourth agro-ecological zone of Cameroon (AEZ IV). Muyuka on the windward side of mount Cameroon, experiences very high temperatures ranging from 25°C during the rainy season (March to September) to about 30°C in the dry season (October to March). The climate is typical of the equatorial type. The monthly rainfall ranges between 9.2mm to 374.1mm, the lowest realized in January while the heaviest is in August.

b) Birds

Two hundred and twenty-five Tropical Broc day-old broilers used for the experiment fed on the control diet (Table 1) during the brooding period of two weeks. Coal pots provided heat supplementation and prophylactic measures employed. Daylight served as the main lighting source during the day and electric current at night; lanterns served as the illuminating source in cases of power outage.

c) Experimental design

The trial used a completely randomized block design (CRBD) in which two weeks old chicks were randomly allocated to 5 treatments, each containing 45 birds; and each treatment had three replicates with 15 birds each. Diets formulated using maggot meal (MM) substituted fishmeal (FM) at graded levels; 0%, 25%, 50%, 75% and 100% at both the starter and finisher phases. Mineral and vitamin premixes customary to poultry production, oyster shell, salt, and bone served as complements (Tables 1 and 2).

Birds lived on deep litter while enjoying natural ventilation, Feed, and water *ad libitum*. Broiler starter, 23% crude protein, sustained for four weeks followed by broiler finisher, 19% crude protein from the end of the 4^{th} week till slaughter (8^{th} week).

d) Ration formulation for experimental diets

The feed ingredients used in this study included: Yellow maize, fishmeal, maggot meal, groundnut cake, kernel cake, soybean cake, wheat bran, and premixes. Proximate analysis gave crude protein and ME values for maggot meal while the nutrient master plan (*livestock feeds*) provided those for other ingredients. Levels of inclusion of protein (maggot meal and fishmeal) and energy sources to meet the protein and energy requirements were manipulated using Pearson's Square method and the various percentages calculated as indicated in Tables 1 and 2 with the help of a nutrient master plan which gave the protein and energy contents of each ingredient.

After all the ingredients were measured and put together at the same spot on the cardboard paper, they were mixed with the hand, making at least three complete turns to ensure proper mixing, and then put into bags with treatment labels.

Table 1: Percentage inclusion levels and chemical composition of experimental diets for broiler starter (weeks 1-4)

Ingredients	Treatment Composition				
	Control (T_0, 0%)	T_1 (25%)	T_2 (50%)	T_3 (75%)	T_4 (100%)
Maize	48.00	48.00	48.00	48.00	48.00
Soya bean cake(SBC)	12.50	12.50	12.50	12.50	12.50
Fishmeal (FM)	5.00	3.75	2.50	1.25	0.00
Maggot meal (MM)	0.00	1.25	2.50	3.75	5.00
Groundnut cake (GNC)	17.00	17.00	17.00	17.00	17.00
Palm kernel cake (PKC)	5.00	5.00	5.00	5.00	5.00
Wheat bran (WB)	10.50	10.50	10.50	10.50	10.50
Premix	0.75	0.75	0.75	0.75	0.75
Bone meal (BM)	0.50	0.50	0.50	0.50	0.50
Salt	0.25	0.25	0.25	0.25	0.25
Oyster shell	0.50	0.50	0.50	0.50	0.50
Total	100.00	100.00	100.00	100.00	100.00
Crude Protein (%CP)	23.20	22.99	22.79	23.58	22.37
Metabolizable energy (ME) Kcal/Kg	2882.72	2888.25	2893.78	2899.31	2904.85

Table 2: Percentage inclusion levels and chemical composition of experimental diets for broiler finisher weeks 5-8

Ingredients	Treatment Composition				
	Control (T_0 0%)	T_1 (25%)	T_2 (50%)	T_3 (75%)	T_4 (100%)
Maize	48.00	48.00	48.00	48.00	48.00
Soya bean cake(SBC)	9.00	9.00	9.00	9.00	9.00
Fishmeal (FM)	5.00	3.75	2.50	1.25	0.00
Maggot meal (MM)	0.00	1.25	2.50	3.75	5.00
Groundnut cake (GNC)	10.00	10.00	10.00	10.00	10.00
Palm kernel cake (PKC)	15.00	15.00	15.00	15.00	15.00
Wheat bran (WB)	10.00	10.00	10.00	10.00	10.00
Premix	0.75	0.75	0.75	0.75	0.75
Bone meal (BM)	1.00	1.00	1.00	1.00	1.00
Salt	0.25	0.25	0.25	0.25	0.25
Oyster shell	1.00	1.00	1.00	1.00	1.00
Total	100.00	100.00	100.00	100.00	100.00
Crude Protein (%CP)	19.53	19.36	19.12	18.91	18.70
Metabolizable energy (ME) Kcal/Kg	2811.57	2817.10	2822.63	2828.16	2833.70

e) Carcass characteristics

Determination of carcass characteristics took place on the last day of the 8th week. After recording live weights of 30 randomly selected birds, two from each replicate, we slaughtered each bird and allowed to bleed for about two minutes before putting in hot water for almost a minute to soften the skin for easy plucking. Dressed weight represented the bulk after removal of the shanks, crop, entrails and other organs. The carcass parts consisted of head, neck, wings, breast, back, thigh and drumstick.

We discarded entrails and weighed eviscerated birds, livers, gizzards, and carcass parts. Then averages from each replicate statistically analyzed for any significant differences between treatments.

f) Studies of hematological parameters, serum chemistry, and enzymes

Studies of hematological parameters (hemoglobin, white and red blood cells), serum chemistry (total protein, albumin, and globulin), and enzymes (aspartate amino-transaminase and alanine amino-transaminase) were carried out at the end of the experiment. Blood was collected at the time of slaughter for carcass analysis into 30 tubes from 15 birds; fifteen tubes had the anticoagulant ethylene diamine tetra-acetate (EDTA) to prevent clotting, and the rest of the cylinders had no anticoagulant. Two hoses (one with EDTA and the other without the anticoagulant) were used to collect blood from one bird per replicate.

g) Method of data processing and analysis

Data were organized in Microsoft Office Excel Version 2010 and analyzed using SPSS 17.0 (SPSS Inc, 2008). Data screened for exploration using Kolmogorov Smirnov and Shapiro Wilk tests revealed that the data departed from the normal distribution. The non-parametric test, notably Kruskal Wallis test, was then used to compare groups for significant differences (Nana, 2012) and the Dunnett T_3 test used for paired comparisons. We took measurements of central tendencies and dispersion, presented the data using statistical tables and charts, and discussed at the 95% CL (Alpha=0.05).

III. Results and Discussion

a) The chemical composition of experimental diets

Table 3 reveals a drop in the CP value of analyzed components for all the treatments, except for the 100% MM. There was a reasonable increase in ME values, especially in 75% and 100% MM.

Table 5 shows a drop in the crude protein content of the control and 25% MM and a slight increase in the rest of the analyzed feed composition compared to the calculated feed composition. There was a considerable increase in ME values of the evaluated components compared to the premeditated constituents across the treatments.

The differences observed in the calculated and the analyzed compositions for both the starter and finisher diets may have been due to variations between the tabulated nutrient content values used in the calculations and the actual nutrient contents of the ingredients used in the experiment. Doing proximate analysis for all components before formulating and compounding the various feeds for the trial keeps this situation in check. Increase in ME with increasing maggot meal in the diets may be explained by the high fats in the maggots which release a lot of energy when oxidized (Adeniji, 2007).

Table 3: Percentage inclusion levels and chemical composition of experimental diets at the starter phase (weeks 1-4)

Ingredients	Control (T_0 0%)	T_1 (25%)	T_2 (50%)	T_3 (75%)	T_4 (100%)
Maize	48.00	48.00	48.00	48.00	48.00
Soya beans cake (SBC)	12.50	12.50	12.50	12.50	12.50
Fishmeal (FM)	5.00	3.75	2.50	1.25	0.00
Maggot meal (MM)	0.00	1.25	2.50	3.75	5.00
Groundnut cake (GNC)	17.00	17.00	17.00	17.00	17.00
Palm kernel cake (PKC)	5.00	5.00	5.00	5.00	5.00
Wheat bran (WB)	10.50	10.50	10.50	10.50	10.50
Premix	0.75	0.75	0.75	0.75	0.75
Bone meal (BM)	0.50	0.50	0.50	0.50	0.50
Salt	0.25	0.25	0.25	0.25	0.25
Oyster shell	0.50	0.50	0.50	0.50	0.50
Total	100.00	100.00	100.00	100.00	100.00
Calculated composition					
Crude Protein (%CP)	23.20	22.99	22.79	23.58	22.37
Metabolizable energy (ME) Kcal/Kg	2882.72	2888.25	2893.78	2899.31	2904.85
Analyzed composition					
Crude Protein (%CP)	20.4	18.9	20.4	20.7	22.4
Metabolizable energy (ME) Kcal/Kg	3114.9	3121.7	3121.5	3359.8	3205.5

Table 4: Percentage inclusion levels and chemical composition of experimental diets at the finisher phase (weeks 5-8)

Ingredients	Control (T_1 0%)	T_2 (25%)	T_3 (50%)	T_4 (75%)	T_5 (100%)
Maize	48.00	48.00	48.00	48.00	48.00
Soya beans cake (SBC)	9.00	9.00	9.00	9.00	9.00
Fishmeal (FM)	5.00	3.75	2.50	1.25	0.00
Maggot meal (MM)	0.00	1.25	2.50	3.75	5.00
Groundnut cake (GNC)	10.00	10.00	10.00	10.00	10.00
Palm kernel cake (PKC)	15.00	15.00	15.00	15.00	15.00
Wheat bran (WB)	10.00	10.00	10.00	10.00	10.00
Premix	0.75	0.75	0.75	0.75	0.75
Bone meal (BM)	0.50	0.50	0.50	0.50	0.50
Salt	0.25	0.25	0.25	0.25	0.25
Oyster shell	0.50	0.50	0.50	0.50	0.50
Total	100.00	100.00	100.00	100.00	100.00
Calculated composition					
Crude Protein (%CP)	19.53	19.36	19.12	18.91	18.70
Metabolizable energy (ME) Kcal/Kg	2811.57	2817.10	2822.63	2828.16	2833.70
Analyzed composition					
Crude Protein (%CP)	17.1	16.0	20.4	19.0	20.0
Metabolizable energy (ME) Kcal/Kg	3253.4	3166.6	3015.7	3418.1	3296.6

b) Carcass analysis

Table 5 indicates the effects of graded level inclusion of MM in broiler diets on carcass characteristics. Live weight, dressed weight, eviscerated weight, carcass characteristics and organs were all significantly different ($P<0.001$) between treatments except for the liver which was not significantly different ($P>0.05$). Generally, the weight of carcass parts increased from T0 to T4. This increase in weight agrees with the findings of Hwangbo *et al*. (2009). The general increase in bulk of the carcass parts with increase inclusion of MM in the diets may have been due to the live weight which also increased with increased levels of maggot inclusion. Agbede and Aletor (2003) found no significant change in all carcass characteristics and organs except for the relative weights of the neck and heart which were significantly higher in diets containing 7.24% of gliricidia leaf protein concentrate in place of FM. Awoniyi *et al.* (2003) instead reported that equal replacement of FM with MM in broiler chicken diet had

no significant effect on the relative length, breadth or weight of muscles of key economic importance in chickens. His report is similar to that of Quinton (2011). Amal *et al*., (2013), Meseret *et al*., (2012) and Bello *et al*., (2012) found no significant ($p>0.05$) differences between all treatments groups in Live weight, eviscerated weight of carcass cuts, dressing percentage, edible inner organs (liver, gizzard, and heart).

The weight of the liver was not significantly different between the control and experimental diets, although higher ($P>0.05$) in treatment groups than in non-experimental diet. Hwangbo *et al*. (2009) and Okah and Onwujiariri (2012) also found no significance ($P>0.05$) in weight of the liver amongst treatments. This indifference in bulk of the liver may have been an indication that there was no infection in the maggot meal that could cause undesirable effects on the nutrition of broilers as indicated by Hwangbo *et al*. (2009) and Okah and Onwujiariri (2012). These results differed from those of Teguia *et al*. (2002) who obtained proportional increases in weights of the liver and gizzard from the control through treatment groups and linked it to toxicity. Live and dressed weights were significantly higher ($P<0.001$) in treatment groups than in control. T_4 was, in turn, higher ($P<0.001$) than the rest of the treatment groups which didn't differ significantly ($P>0.05$) between themselves in live and dressed weights. Eviscerated weight in T_4 was significantly ($P<0.001$) higher than in the rest of the treatments which were, in turn, greater than the control though not significantly ($P>0.05$) different. The above findings agree with those of Gadzirayi *et al*., (2012) using mature *Moringa oleifera* leaf meal as a protein supplement to soybean meal but goes contrary to the outcome of Yisa *et al*., (2013) who stated that complete withdrawal of FM implied poorer development of the meat yielding parts.

Table 5: Comparison of carcass parts between treatments

Carcass parts	Average Weight (Mean ± SE) g					
	T_0 (N=24)	T_1 (N=24)	T_2 (N=24)	T_3 (N=24)	T_4 (N=24)	Kruskal Wallis Test
Live weight (g)	1466.667±13.03	1616.667±13.003^{ab}	1616.667±4.915^{ac}	1666.667±34.403^{bc}	1900.000±22.522	X^2=77.517 P<001
Dressed weight (g)	1366.667±13.03	1483.333±17.720^{ab}	1483.333±4.915^{ac}	1516.667±35..441^{bc}	1800.000±22.522	X^2=69.471 P<001
Eviscerated weight (g)	1166.667±21.43^{abc}	1216.667±13.003^{ade}	1233.333±4.915^{bdf}	1266.667±38.385^{cef}	1416.667±13.003	X^2=48.809 P<001
Head (g)	41.840±0.236^{a}	43.867±0.455^{bc}	43.913±0.210^{bd}	45.257±1.322^{acde}	47.167±0.615^{e}	X^2=34.850 P<001
Neck (g)	74.870±0.845^{a}	79.912±0.805^{bc}	84.153±0.299^{d}	84.000±03.37^{abde}	80.787±0.943^{ce}	X^2=28.617 P<001
Wing (g)	126.077±2.321	136.137±2.022^{a}	146.297±0.232^{b}	142.663±3.145^{ab}	167.890±1.997	X^2=73.667 P<001
Breast (g)	252.380±4.603^{a}	260.150±7.789^{abc}	280.483±3.282^{bd}	286.823±6.730^{cd}	364.863±4.096	X^2=67.717 P<001
Back (g)	218.307±5.862^{abc}	220.352±3.941^{ade}	214.233±1.086^{bdf}	208.947±8.047^{cef}	240.160±2.341	X^2=20.117 P<001
Drumstick (g)	156.213±2.658	175.232±3.426^{ab}	172.470±0.321^{ac}	173.907±4.142^{bc}	213.957±2.982	X^2=65.450 P<001
Thigh (g)	170.117±2.789^{abc}	174.413±3.178^{ade}	173.470±0.908^{bdf}	187.540±7.708^{cefg}	197.750±2.706^{g}	X^2=29.750 P<001
Liver (g)	38.587±1.310^{abcd}	41.147±0.368^{aefg}	41.737±0.296^{behi}	39.483±1.409^{cfhj}	41.643±0.459^{dgij}	X^2=10.483 P=.033
Gizzard (g)	44.847±0.185^{a}	47.523±.556b^{c}	43.090±0.696^{a}	49.005±1.121^{bd}	47.4467±.284^{cd}	X^2=31.167 P<001

$^{a, b, c, d, e, f, g, h, I, j}$ Dunnett T_3: Paired comparison between treatments and within weeks; pairs with the same letter are not significantly different at the 0.05 Level.

c) Hematology, serum chemistry, and enzyme studies

The results presented in Table 6 reveal that there was a significant difference ($P<0.001$) between treatments in the hematological parameters, serum chemistry, and enzymes studied, except for albumin which was the same throughout the control and treatment groups. T_2 recorded the lowest total protein and globulin and T_3 the highest. ALT was significantly lower ($P<0.001$) in control compared to the rest of the treatment groups and topmost in T_2, but not significantly different ($P>0.05$) from the rest of the treatment groups.

Table 6: Comparison of hematology, serum chemistry, and enzyme parameters between treatments

Parameters	Average (Mean ± SE)					
	T0 (N=6)	T1 (N=6)	T2 (N=6)	T3 (N=6)	T4 (N=6)	Kruskal Wallis Test
Total protein (g/dl)	34.667±0.428^{abc}	34.000±1.228^{adef}	30.667±1.322^{bdg}	36.333±1.095^{ce}	32.667±0.354^{fg}	X^2=11.241 P=0.024
Albumin (g/dl)	1.000±0.000	1.000±0.000	1.000±0.000	1.000±0.000	1.000±0.000	X^2=0.000 P=1.000
ALT (U/L)	5.333±0.260	9.000±0.590^{abc}	10.333±0.260^{a}	8.333±0.098^{b}	9.333±0.098^{c}	X^2=67.419 P<001
AST (U/L)	211.333±1.722^{ab}	222.333±2.871^{c}	185.000±4.283	219.333±3.589^{acd}	211.000±0.742^{bd}	X^2=49.885 P<001
Globulin (g/dl)	34.333±0.491^{ab}	33.000±1.228^{acde}	29.667±1.322^{cf}	35.333±1.095^{bd}	31.000±0.450^{ef}	X^2=15.509 P<001
Hb (g/dl)	13.000±0.170	11.667±0.260^{abc}	11.000±0.170^{ade}	11.667±0.260^{bdf}	11.667±0.260^{cef}	X^2=31.657 P<001
RBC/L (×10^6)	2.237±3.490×10^{4abcd}	1.400±4.500×10^{4aefg}	2.000±5.898(×10^{4behi}	1.967±9829.464^{cfhj}	1.767±9829.464^{digj}	X^2=81.987 P<001
WBC/L	1300.000±17.025^{ab}	1200.000±34.050^{acd}	800.000±128.537^{ce}	950.000±118.260^{bde}	1666.667±26.006	X^2=54.345 P<001

$^{a, b, c, d, e, f, g, h, I, j}$ Dunnett T_3: Paired comparison between treatments and within weeks; pairs with the same letter are not significantly different at the 0.05 Level.

ALT= Alanine Amino-Transaminase, AST= Aspartate Amino-Transaminase, RBC= Red Blood Cell, WBC= White Blood Cell, Hb= Hemoglobin.

RBC and WBC were low in T_1 and T_2 and highest in T_0 and T_4 respectively. Generally, the values of WBC presented in this study (0.800×103-1.667×103/L) are very low compared to those (2.35×106-3.39×106/L) reported by Nworgu *et al.* (2007) using fluted pumpkin leaf extract. The low WBC in this report is an indication of the absence of infections in these birds, supporting the fact that MM caused no health hazards. RBCs reported by Nworgu *et al.* (2007) were higher than those in this study, the highest (2.237×106/L) being the control, even though not significantly different from the rest of the treatment groups. The range of RBCs obtained in this study falls within the range for broilers (1.5 to 4.5 x 106 cells/µL) as stated by Martinho (2012). Hemoglobin was higher (P<0.001) in control than in the treatment groups which did not differ (P>0.05) amongst themselves, but generally it was still higher than the value (8.22g/dl) obtained by Orawan and Aengwanich (2007) for broiler chickens. However, these values fell within the normal range of 7-13g/dl (Bello *et al*., 2012). Total protein values in this experiment were high surely as a result of higher levels of globulin. Total protein range (3.10-3.70g/dl) and globulin (1.10-1.40) reported by Nworgu *et al.* (2007) are far lower than those obtained in this study (30.667-34.667g/dl and 29.667-34.333g/dl respectively). AST was lowest in T_2 and highest in T_1. The values of alanine transaminase (ALT) reported in this study (5.333-10.333U/L) were very low compared to 23.00-24.84U/L obtained by Nworgu *et al.* (2007); meanwhile, aspartate transaminase (AST) was exceedingly higher (185.000-222.333U/L) than what Nworgu *et al.* (2007) obtained (17.000-21.110U/L). The exceptionally high AST in this report could be the result of oxidative stress.

IV. Conclusion

Conclusions derived from this study follows thus: Better carcass characteristics in treatments with maggot meal is an indication that maggot meal is not inferior to fishmeal. Lower amounts of white blood cells in the treatment groups with maggot meal than in control indicate the absence of any infection in the system attributable to MM. The stable values of RBCs and Hb were an indication that the birds were not suffering from anemia, thereby indicating that MM did not upset the physiological status of the birds. The values of total protein, globulin, and enzymes studied did not show any defined trend in their variations between the various treatments. Given that broilers with the best carcass characteristics performance were those with 100% maggot meal inclusion and that physiological parameters were not deviated from normal values in birds fed experimental diets, it can be concluded from this study that maggot meal can entirely replace fish meal at 5% in broiler feed for better carcass characteristics, and a stable physiological profile.

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Oedematous Skin Disease (OSD) Transmission among Buffaloes

By Arafa M. I., Hamouda S. M., Rateb H. Z., Abdel-Hafeez M. M. & Aamer A. A.
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Abstract- During buffaloe OSD spread in a village affiliating to Assiut Governorate-Egypt, 44 buffaloe cows hosted and owned sporadically were subjected to the study. From 43 buffaloe cows (had closed lesions either edematous or nodular) and a buffaloe cow (had open ulcerative lesion), Corynebacterium pseudotuberculosis equi (C. ps. equi) as 72% and Corynebacterium pseudotuberculosis ovis (C. ps. ovis) as 28% were isolated and identified. Blood sucking insects hosted on the infected buffaloe cows (no. 22) louse fly(Hippobosca equine) and 20 Haematopinus eurysternus lice were included during the study where both C. ps. equi and C. ps. ovis were isolated from Hippobosca equina (H equina) but failed to isolate any biovar of C. ps. from Haematopinus eurysternus lice (H. eur.). Moreover, C. ps. equi was isolated from two H equina pupae – lab deposited – as well as a H equina second generation fly concluding that there is endosymbiosis nature of C.ps. limited only to H. equina fly which can transmit C.ps. vertically.

Keywords: *buffaloes, oedematous skin disease, corynebacterium pseudotuberculosis, hippobosca equina.*

GJMR-G Classification: *NLMC Code: WA 360*

Strictly as per the compliance and regulations of:

Oedematous Skin Disease (OSD) Transmission among Buffaloes

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I. Introduction

Buffaloe OSD is an endemic disease in Egypt caused by C. ps. (Khalel et al 1995, Ali & Zaitoun 1999,Selim 2001) biovar II named C. ps. equi (Khater et al., 1983; Barakat et al., 1985,Selim 2001, Soares et al 2013, Selim et al 2016, Almeida et al 2017) which is toxogenic producing diphtheria toxin (Selim et al 2016) characterized mainly by reduction nitrates to nitrites (Selim 2001, Abdel-Latif 2011, Selim et al 2016, Oliviera et al 2016, Almeida et al 2017), by the action of nitrate reductase gene (nar G) (Soares et al 2013, Almeida et al 2017). The disease is initiated by intradermal inoculation of C. ps. in buffaloe skin through biting of the blood-sucking insects (Khater et al., 1983; Barakat et al., 1985,Selim 2001, Soares et al 2013) especially H. equina fly (Ghoneim et al., 2001, Selim 2001, Moussa et al 2016, Viana et al 2017).Many studies on open or ruptured lesions suggested that transmission may be mechanically with contaminated environment (soil & water) with C. ps. mainly by Musca domestica (Abou-Zaid and Hammam, 1994; Sayed, 2001; Yeruham et al., 2003 and Spier et al., 2004). The work aimed to study the role of H. equina (adult fly) and its life cycle stages in OSD transmission.

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II. Material & Methods

a) Insects sampling

Through a private clinic in Fayama village (15 Km. east north from Assiut city, Assiut Governorate) the study conducted on 44 buffaloe cows - owned and hosted sporadically - suffering from OSD ectoparasite infested, where 70 adult blood sucking insects (40 H. equina flies & 30 H. eur. lice) were gathered.22 H. equina flies as well as 20 H. eur. lice were used for bacteriological examination and the rest were used in parasitological investigation.

The insects were collected directly using sterile forceps from infected buffalo or donkeys hosted together. They were kept into plastic sacs or wide-naked bottles for laboratory examination where they taxonomically identified (Soulsby, 1982 and Kettle, 1984). 22 H. equina flies as well as 20 H. eurysternus lice were used for bacteriological examination and the rest were used in parasitological investigation.

b) Lab reared H. equina flies

18 flies were still alive for 24 hours where some of them deposited their larvae (Full mature larvae) inside the collected sacs untill larvae pupated. The achieved pupae were incubated at room temperature in plastic sand containers covered with a piece of gauze until giving adult fly (Baraka, 1983). These different stages were photomicrograph.

c) Bacteriological examination

Bacteriological examination was conducted with 22 flies gathered in sterile plastic sacs from infested animals (17 affected buffaloes, 4 cattle and a donkey hosted together). In addition to two pupae (lab deposited) and one laboratory developed fly (second generation) as well as 20 H. eur. lice for bacterial existence as follows:

1. Fly was inoculated as it is into a sterile nutrient broth tube for bacterial isolation from body surface, legs and external mouth parts contamination (EBS).
2. The forceps catched fly was washed several times using sterile distilled water to rinse the rest of external contamination.
3. The washed fly was crushed, destructed and macerated into another nutrient broth tube for isolation of gut bacterial content (IBC).
4. The above mentioned three procedures were performed on lice (20) from only affected buffaloes

5. To obtain their external (EBS) and internal (IBC) bacterial contents.
6. From the laboratory deposited pupae, 2 were burst into nutrient broth to isolate their bacterial contents.

d) Bacteriological examination

The above mentioned test tubes were overnight incubated aerobically at 37°C, and then were streaked onto 10% sheep blood agar (24 - 48 h). Growing colonies were purified and identified morphologically by Gram's stain. Biochemically tested for motility, glucose and maltose fermentation, catalase activity and nitrate reduction were adopted (Quinn et. al., 2011).

III. Results

Parasitologically, all diseased buffaloes were infested with dark leathery flies identified as H. equina. Adult flies were more abundant on stabled diseased animals. They mainly aggregated under the tail, on the udder, around genitalia and inner aspect of thighs. Flies were dark brown in color measuring 9 x 4.5- 10.5 x 5.0 mm. Their abdominal segmentation was indistinct. Wings were longer than body length while wing veins crowded towards the anterior border. Flies had three pair of feet is provided with strong claws (fig: 1). Five diseased buffaloes were infested also with sucking lice identified as H. eur. having a relatively short head and broad thorax and abdomen measuring about 4 x 2 – 5 x 2.5 mm (fig: 2).

Fig. (1)

Fig. (2)

Out of 8 full mature H. equina larvae creamy in color, oval shape measuring 1.5 x 2.5 mm provided with a small spine posteriorly, 5 pupated (6-10) hours (fig: 3). Pupation period was 30 days where the pupa is broadly oval with two round postero-lateral spiracular lobes producing a full mature fly. Pupa was yellowish in color measuring 4.0 x 2.5- 4.5 x 3 mm. It was soft and covered with sticky layer but at 24 hours later, it became dark red to black in color and quit hard (fig: 4).

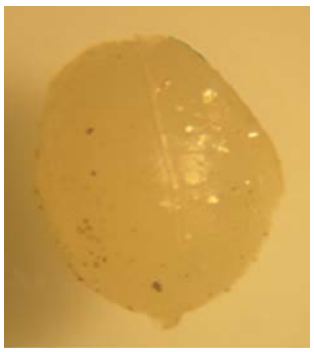

Fig. (3)

Fig. (4)

Bacteriological investigation results are tabulated in tables (1 & 2).

Table (1): Nitrate reduction (NR) test of *C. ps.* isolates.

Source of bacterial isolation	No.	*C. ps. equi*		*C. ps. ovis*	
Skin lesion samples	35	24	68.5%	11	31.5%
H. equina flies	13	10	76.9%	3	23.1%
Pupae	2	2	100%	0	0%
Total	50	36	72%	14	28%

Table (2): Bacterial species isolated from buffaloe OSD and hosted blood sucking insects.

Bacterial isolate species	Lesion exudate Samples	*H. equina* buffaloe hosted		*H. eurysternus* buffaloe hosted		Pupae lab. deposited	*H. equina* Lab. Developed	
+ve bacterial isolation	40	EBS	IBS	EBS	IBS		EBS	IBS
C. ps.	32	2	6	-	-	2	-	-
C. ps. + S. epid.	3	-	-	-	-	-	-	-
C. ps. + Anthr.	-	1	2	-	-	-	1	1
S. epid.	5	-	-	-	-	-	-	-
S. sapr.	-	-	2	-	3	-	-	-
Anthr. spp.	-	19	12	9	7	-	-	-
S.sap. + Anthr.	-	-	-	11	10	-	-	-
-ve bacterial isolation	4	22		20		2	1	
Total	44							

IV. Discussion

OSD appears mainly among buffaloes and occasionally cattle in Egypt confined to Lower Egypt as a result of the suitable climatic conditions (Selim 2001, Mohamad and Reda 2015), especially during late spring and early summer (Yeruham et al 1997, Mohamad and Reda 2015, Sokó and Michalski 2015) correlating with H. equina breeding season (Barakat et al 1985,Selim 2001, Syame et al 2008,Sokó and Michalski 2015) and lesions are associated with its predilection seats of infestation (Selim 2001,) (hairless areas as axilla and groin (Syame et al 2008), inner aspect of limbs and under tail) (Selim 2001,).

Basing on nitrate reduction test, the identification of bacterial isolates all over the study revealed that both C. ps. biovars were recovered as C. ps. Equi and C. ps. Ovis represented 72 & 28 %, while from buffaloe lesions resembled 68.5 & 31.5% respectively (Table 1). Some studies stated that OSD is associated only with C. ps. Equi (Selim 2001, Selim et al 2016, Viana et al 2017), while others detected both C. ps. biovars as causative agents in cattle ulcerative lymphangitis (Yeruham et.al.,1997 Yeruham et.al., 2003, Yeruham et.al., 2004).C. ps. transmission among buffaloe is a conflicting issue since some studies concluded that it is only mechanical by insects (Khalel et al., 1995; Zaghawa and El-Gharib, 1996; Ali and Zaitoun, 1999, Doherr et al., 1999) mainly blood sucking insects - as mentioned above - (Khater et al., Selim 2001,; Barakat et al. 1985 Soares et al 2013) as having a protrusion that can pierce the thick buffalo skin. These conditions coordinates with H. equina features which remain for long periods on their hosts and are not easily disturbed in addition it has very long mouthparts are adapted for piercing thick skin (Selim 2001). In the present study, out of 44 affected buffaloe cows, 43 showed closed (edematous or nodular) lesions avoiding suggestion of mechanical transmission unless through piercing the whole skin thickness to contract the pathogens from the infected subcutaneous tissues, the condition presented only related to H. equine (Selim 2001) when be with contaminated piercing mouth parts. Even, from the blood sucking H. eur. lice with piercing mouth part just a vessel feeder could not reach to the infected subcutaneous tissue(Roberts and Janovy 1996), the present study failed to isolate any C. ps. strain from H. eurysternus lice infesting the infected buffaloes in different locations (Table 1) suggesting that it cannot act as a mechanical or biological vector for C. ps. These finding concluded that not any blood sucking insect has role in transmission, but among blood sucking insects, it is associated with H. equine (Selim 2001, Ghoneim et al 2001). Musca domestica (house flies) with mouth part adapted only for a liquid diet not to pierce host skin(Kettle 1990) have been confirmed as potential vectors for C. ps. equi among horse (Yeruham et al., 2003 and Spier et al., 2004) or cattle(Abou-Zaid and Hammam, 1994; Sayed, 2001)with ulcerative lesions mechanically. C. ps. equi survival inside the fly's gut experimentally - on feeding house flies on C. ps. equi broth - revealed that the pathogen presented in fly droppings for only up to 4 h and in saliva up to 3 h post infection (Yeruham et al 1996). Some studies investigated the existence of the C. ps.equi inside Musca domestica by PCR detection of its phospholipase D (PLD) exotoxin gene (Spier et al 2004, Barba 2015) with great disadvantage that detection of PLD did not inform about the viability of pathogens. In the present study bacterial isolation of C. ps. biovar equi from all H. equine life stages(adult flies, their pupae either gathered or lab deposited as well as the second generation flies) viable up to 30 days post collection ascertained that there is endosymbiosis nature of C.ps. limited only to H. equina fly which can transmit C.ps. vertically.

The study concluded that both C. ps. biovars (equi & ovis) could be isolated from buffaloe OSD lesions. Its transmission is associated only to H. equina fly, the mechanical and biological vector for buffaloe OSD, since it is proved that there is endosymbiosis nature of C.ps. limited only to H. equina fly which can transmit C.ps. vertically.

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Minor Groove Binder Probe Real-Time RT-PCR for Detection of Foot-and-Mouth Disease Virus in Egypt

By Hany I Abu-Elnag

Abstract- Shorter, more specific minor groove binders (MGBs) probes are dsDNA-binding agents attached to the 3' end of Taq Man probes that could be designed strictly to the invariant region. Application and assessing of a new trend for viral detection in Egypt depending on MGB probe real-time RT-PCR (rRT-PCR) applied on local FMDV serotypes O, A, and SAT2. Moreover, FMDV O was detected using two serotype specific primer sets by SYBR Green real-time RT-PCR assaying rapid formats. The limit of detection of diluted RNAs using MGB probe rRT-PCR assay reached to≤ 6 FG/ul. Besides, the high specificity of it was clear. In contrary, the employing of FMDV O specific primer pairs in SYBR Green real-time RT-PCR showed less sensitivity and specificity, particularly one of them displayed poor performance illustrating important cause of the false negative results in the conventional PCR. Lastly, the local financial cost of MGB probe is considered the obvious hinder in my country.

Keywords: *foot-and-mouth disease virus; rRT-PCR; MGB probe.*

GJMR-G Classification: *NLMC Code:QW 70*

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I. Introduction

Foot-and-mouth disease (FMD) is the most economically significant animal viral disease worldwide affecting cloven-hoofed animals and caused by Foot-and-mouth disease virus (FMDV). The virus is a picornavirus (genus *Aphthovirus*, family *Picornaviridae*). The virus has a linear single-stranded RNA genome [1]. Seven antigenically distinct forms of the virus are known, called serotypes, but serotype C has not been detected anywhere for many years and may now be extinct. The serotypes have been further divided into topotypes (except for serotype Asia-1 viruses, which comprise a single topotype), genotypes, lineages, and sublineages, which are usually restricted to specific geographical regions [2].

The rapid and precise detection of FMD virus is a prerequisite. Conventional reverse transcriptase polymerase chain reaction (RT-PCR) [3-6] and real-time RT-PCR assays [7-10] have been developed to complement primary diagnostic techniques for the detection of FMDV. Real-time RT-PCR recommended by the World Organization for Animal Health (Office International des Epizooties, OIE) for detection of FMDV incorporate universal primers and fluorescent-labeled probes that recognize conserved regions within the 5-UTR & 3D polymerase [11].

The usage of a panel of rRT-PCR assays is imperative, as RNA, viruses are prone to mutation. If one assay is rendered ineffective due to a catastrophic mutation in the primer or probe binding regions or as a result of the event of contamination problems, results from a other assay will still be valid especially when using assays targeting different areas of the genome [12]. The MGB molecule involved in the detector probe design increase the Tm of the probe. This shortens the probe sequence and enables it to be designed strictly to in the variant region. Moreover, the dynamic range of MGB-NFQ probes is larger because of its increased fluorophore quenching, efficiency and resulting low fluorescent background compared to FAM-TAMRA probes [9, 13].

FMDV serotype O is the most ancient well-identified worldwide type [14]. Also, in Egypt it the classical enzootic and the most prevalent serotype pose many outbreaks [15, 16]. Therefore, application and assessing of a new trend for viral detection in Egypt depending on minor groove binder probe (MGB) real-time RT-PCR (rRT-PCR) was applied on local FMDV serotype O, A, and SAT2 with special handling of FMDV O serotype specific primer sets in SYBR Green real-time RT-PCR for the rapid and precise detection of the virus

II. Materials and Methods

a) Viruses

Strains designated O/EGY/2009 iso1 (cell culture grew virus), A/EGY/2009 iso-Cai (clinical isolate) and SAT2/EGY/H1Ghb/2012 (bovine tongue epithelium suspension from Gharbia, Egypt), local strains of serotypes O, A and SAT2, respectively [17-19] used. They were initially employed in the validity of Minor groove binder probe real-time RT-PCR to detect different Egyptian FMDV serotypes. Moreover, Other FMD viruses representing FMDV serotypes found in Egypt, previously type discriminated by RT-PCR assay and nucleotide sequence used as unknown samples in the performance of the probe rRT-PCR assays. These FMD viruses were clinical and culture viruses, O (n=10), A (n=4) and SAT2 (n=2). The context of that, a related vesicular viral disease that cause mucosal lesion with excessive salivation accompanied by lameness in

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chronic infection also incorporated in the assays. The virus belongs to the family Flaviviridae, causative agent is bovine viral diarrhea virus (BVDV). The genome consists of a positive-sense, single-stranded RNA molecule of approximately 12.3 kb; two BVDV genotypes are recognized, its strains divided into distinct biotypes (cytopathic or non-cytopathic) according to their effects on tissue cell culture.

b) RNA purification and analytic sensitivity

QIAamp® Viral RNA kit (Qiagen, Germany) for RNA extraction used according to the manufacturer's instruction. Extracted RNAs from three FMDV O were quantitated by ultraviolet (UV) spectrophotometry and used as; in-house Standard (a candidate culture propagated virus in 2017 with titer ~7.3 $TCID_{50}$/ml on BHK), Positive control 1 (semi-purified concentrated culture grown virus in 2012) and Positive control 2 (cell adapted FMDV isolated in 2009). The standard was imperative to obtain the standard curve. Bulks of extracted RNAs from the standard and positive control two were divided in two aliquots for each. From one aliquot of the standard RNA, seven times, serial 10-fold dilutions in RNAse-free water were performed to obtain the Standard RNA Dilutions (SRD) to have values that were used to construct a standard curve to calculate unknown sample concentrations.

An archived Stock virus (SV) RNA [20], kept for a complete 6-years in an ordinary kitchen fridge and previously assessed by SYBR Green rRT-PCR, was examined in the current article for assessment rRT-PCR assays on RNAs that suffered storage for a long duration. Briefly, 10-fold serial dilutions of stock virus (SV) in minimum essential medium (MEM) with Hank's salts in the range of 10^{-1}-10^{-8} performed. Each dilution was exposed to RNA isolation procedures to prepare SV RNAs. The previous different RNAs preparation formats viz. SRD and SV RNA was used in analytic sensitivity. Negative controls included: no template control, NTC, which was RNAse free water; Negative control one that was RNA from healthy BHK cells; and Negative control 2 that was RNA from non-infected BHK cells showed contamination. For quantification the mass concentration of RNA, two spectrophotometer instruments utilized, one was the conventional spectrophotometer (Milton Roy 601 Spectronic 335104, USA) and other was the modern spectrophotometer (Nano Drop 2000c Spectrophotometer, Thermo Fisher Scientific, USA).

Repeatability assay of MGB probe rRT-PCR sensitivity was performed after one-month interval on FMDV O RNA standard, its 1st dilution (10^{-1}) and positive control two, which considered as an old exhausted aliquot RNA after using for one-month. Where the standard and control two had been exposed to repetitive freezing and thawing for ten and six times, respectively.

Using samples and depending on the current primers flanked the MGB probe, each MGB and SYBR green rRT-PCR were extra re-assessed with the previous old aliquot RNA, but also, new aliquots of the standard, positive control two and primers/probe set were additionally involved in the assay.

c) Real-time RT-PCR (rRT-PCR)

All the extracted RNAs tested on the real-time PCR system Rotor-Gene Q 2 (Corbett Life Science, a QIAGEN Company, Germany) using either QuantiTect Probe RT-PCR Kit or QuantiTect SYBR Green RT-PCR Kit (Qiagen, Germany). Primer set: PF-5-GTT TTG TTC TTG GTC ACT CCA T-3'; PR-5'-ACG GAG ATC AAC TTC TCC TGT A-3' and a labeled FAM, 5' conjugated minor groove binder (MGB) probe CTC TCC TTT GCA CGC C, 5'- FAM _ 3'-NFQ/MGB, were employed in MGB rRT-PCR investigations with approximately 163 bp target genome fragment amplification [12]. The primers were purchased from either Metabion, Germany or Bioneer, Korea; while, the Probe designed by Applied Biosystems, Life Technologies, Thermo Fisher Scientific, USA. The serotype O specific primer pair (our lab termed PH1/PH2), designed from the 1D and 2AB regions of the viral genome as described previously [4] to give 402 bp expected band sizes used in SYBR Green rRT-PCR assay for further MGB comparative assessment. Also, for auxiliary performance evaluation, the previously [21] documented oligos O-1C124 (ARS4)/NK61 of an expected amplicon of approximately 1126 bp were implemented by SYBR rRT-PCR method. For TaqMan MGB rRT-PCR, cycling conditions were: 1 cycle at 45°C for 30min, 1 cycle at 95°C for 15min and 45 cycles at 95°C for 30s, 51°C for 30s, and 72°C for 30s. For the SYBR Green rRT-PCR methods, the optimized reaction contained 5% RNA template. The cycling parameters were as described previously [4, 21] and for 45 cycles. At least one of the positive controls and negative controls was involved in every assay.

III. Results

MGB rRT-PCR assay were valid to detect the specific target genome fragment of different Egyptian FMDV serotypes strains. Besides, various negative controls and the tested BVDV produced neither threshold cycle (C_T) values nor the expected amplification sizes in agar gel electrophoresis. Using MGB amplification, RNA sensitive detection of the standard and all its dilution extended to the theoretical mass quantification of six hundred attograms (ag) RNA per microliter (μl).Also, sensitivity were attained in examining the archived stock virus (SV) RNA, MGB fluorogenic signals were attained in dilutions from 10^{-1} to 10^{-4} and 10^{-6}. On the other hand, MGB probe and primers failed to hybridize to the respective templates in dilutions 10^{-5}, 10^{-7} and 10^{-8} (Fig. 1 and 2).

SYBR green rRT-PCR method using primer pair for FMDV serotype O (Fig. 3), showed specific sensitivity reached to sixty femtogram RNA per microliter utilizing PH1/PH2 primer set. Furthermore, quantification of six

hundred picograms (might barely reach 60 pg in repeatability) of RNA per microliter were obtained when employing alternative oligos for FMDV O, 1C124 (ARS4)/NK61 (Fig. 4).

Likewise, Repeatability of MGB rRT-PCR assay revealed variability in the sensitivity between aliquots and replicates with a highlighted effect of RNA degradation and an approximately negligible impact of the primers /probe hybridization regression. C_T values variability using MGB probe between the replicates for each sample in comparison with the mean was ±3.5 with values difference reached 6.5, 5.7, and 2.9 for Positive Control 2, Standard and Standard 10^{-1}, respectively (Fig. 5 and 6) and Table 1. Accordingly, it was suggested to be the cut off ≤ 45 cycles for MGB probe due to its high sensitive and specific detection.

Using the primer set that flanked the MGB probe in either Probe or SYBR rRT-PCR methods, successively at the same day, clearly showed the performance of the standard and its dilution were promising in Probe in comparison to SYBR assays, where the signals were linear in the exponential phase. The standard curve efficiency for the MGB probe was 0.89, while for the SYBR PH1/PH2 primers was 0.79. Using Taqman MGB Probe, the standard virus and its serially 10-fold dilutions showed a 2.8-4.4 increment increasing of C_T values between undiluted virus until its 10^{-6} dilution (undiluted & 6 dilution series). Whereas, there were no prominent changes in C_T value at the 10^{-7} dilution. Furthermore, using SYBR Green PH1/PH2 oligos protocol, the standard virus and its serially 10-fold dilutions gave C_T values of 3.3-4.6 differences between undiluted virus until its 10^{-6} dilution (undiluted & 6 dilution series). Melt curve peak (*T*m) showed that specific amplification giving the expected peak, affirmed by yielding the anticipated fragment size on agarose based electrophoresis. The negative samples controls either did not exhibit the anticipated specific former *T*m of the primers or showed the expected peak, but they all had higher C_T values above the last positive standard dilation.

IV. Discussion

Shorter, more specific minor groove binders (MGBs) probes are dsDNA-binding agents attached to the 3' end of TaqMan probes to increase the Tm value (by stabilization of hybridization) and to design shorter probes. Shorter probes make it easier to use short conserved or unique sequences for hybridization. MGBs also reduce background fluorescence and increase dynamic range due to increased efficiency of reporter quenching due to shorter distances between the reporter and quencher and the use of non-fluorescent (dark) quenchers (NFQ) at the 3' end instead of fluorescence dyes like TAMRA. MGB probes have more sequence specificity for better mismatch recognition.

The format of standard and its dilutions (SRD) in the current article was selected according to what comprehend from the thermal cycler manufacture's recommendation, which mentioned that the DNA used in the standard curve should be derived from similar DNA in the samples being measured. It was recommended that the concentration of at least one DNA sample be determined using ultraviolet spectrophotometry and that this sample be used as the standard. The minimum number of standards used should be three (with replicates). Importantly, DNA standards used in fluorescence detection are only linear within the range of 100 nano-grams per micro-litre to 1 nano-gram per micro-litre. That is, within this range, if the concentration of DNA is halved, so is the fluorescent reading. The confidence intervals for any concentration outside this range are very broad due to non-linearity in the chemistry. In addition, differences have been observed in the measurement of various forms of DNA. For example, genomic DNA compared with plasmid DNA. Therefore, it is recommended that only alike DNA are measured together, and the use of plasmid DNA as a standard be avoided when measuring genomic DNA.

The oligonucleotide probe with a 5' conjugated minor groove binder (MGB) ligand as a reporter in real-time PCR. The hybridization of the probe triggered fluorescent. MGB probe rRT-PCR was the best specificity and sensitivity than the other two primer pairs used in SYBR Green RT-PCR protocols. Relevant amplification of the homologous templates were implemented, whereas, the heterologous templates were mismatched. Consequently, the specificity of this fluorogenic probe was very satisfactory for FMDV investigation and quantification. The fluorogenic MGB probes were more specific for single base mismatches and fluorescence quenching was more efficient, giving increased sensitivity [13]. Result revealed lower C_T values, in addition to, higher detection specificity and sensitivity when using FMDV O specific primers that produce smaller amplification size, in comparison to, O specific oligos amplifying larger fragment. In real-time PCR with TaqMan probes, the amplicon size directly influenced detection: the larger the amplicons, the later the detection. Earlier detection and a higher fluorescence level (plateau phase) were generally observed for shorter amplicons [22]. On the other side in SYBR Green methods, melting curve analysis was considered as a tool to verify the specificity of the amplified product, although it is a common indicator used in fluorescence rather than flurorophore-based RT-PCR assays. Besides, agar gel electrophoresis support the amplicon specificity. In contrary to MGB probe, the serotype specific oligos, 1C124 (ARS4)/ NK61, trial in SYBR rRT-PCR format gave the poorest analytical specificity and sensitivity.

Analytical sensitivity was a trial to detect variable genome of FMDV of different serotype to overcome the possibility of false negative result due to serotype unspecificity. In the absence of a target molecule, the MGB probe does not fluoresce, as there is

sufficient interaction between the reporter fluorophore and the quencher to prevent a fluorescent signal. Hybridisation to a complementary target molecule triggers an increase in fluorescence due to the separation of the fluorophore and quencher [12]. Probe with minor grove binder (MGB) form stable, higher melting temperature interactions with their target sequences. The positive result of rRT-PCR in this article are mainly C_T values. Where there is general correlation between them and quantity of input nucleic acid. The more target template is present in the reaction, the fewer cycles it requires to reach logarithmic growth and end point of RT-PCR (i.e. lower C_T values) [9].

After one month of 1st assessment of the performance and the analytic sensitivity of MGB rRT-PCR, an anticipated detection limit decreased by 10-fold, with 2.6-5.5 increment difference in C_T values between undiluted virus until its 10^{-6} dilution, where higher increment values in lower dilutions and lower increment values in higher dilution. The operator for faulty cost saving did not carry out re-assessment of dilution 10^{-7}. However, result was satisfactory by ending the re-assessment by dilution 10^{-6} and not exceed to dilution 10^{-7}, because the signal curve of dilution 10^{-6} was at the border of the threshold ($C_T = 43$) that predict if dilution 10^{-7} was done, the C_T value would be weak positive or negative.

Six-years before the current assay, SYBR Green rRT-PCR had detection limit for the stored Stock virus (SV) RNA that was extended to 10^{-7} dilutions using 0.2 RNA template/rxn volume (Azab et al., 2012). In context, the current MGB probe was still cable to detect RNA in the archived RNA across approximately 6-log range of input template with 1 $\log_{10}$ regression, taking in consideration that the RNA template input in this paper was 4x lesser. Also, the detection signals produced as a result of SYBR Green DNA incorporation were generally more earlier as revealed in repeatability assay when MGB probe complementary primers used in SYBR Green investigation. This result was satisfactory when investigation a long period storage of RNA template.

The nucleic acid amplification detection on the real-time PCR platform was verified by agarose gel electrophoresis that revealed the expected positive band. Serial dilution of the virus RNA control could be used as one of the viable reference for relative FMDV quantification. The virus RNA control sample was of cultured derived virus, not a wild virus to minimize the possibility of contamination by non-specific fragment. Furthermore, in future, we hope to use in-vitro synthetic FMD RNA fragment of the primer/probe target sequence as a positive amplification control.

The drawback of the current MGB probe (FAM dye–labelled, with NFQ), in comparison to the non-MGB assay (FAM dye–labeled, with BHQ) in the poor developing country is the relative comparative higher cost of MGB probe. That might be reached to 1.7x the price of the non-MGB probe that will be translated to thousands of EGP (or hundred of USD), in consequence of that, rising the finance of the quantity detection assay of the unknown samples.

Finally, MGB RT-PCR assay provided a rapid, sensitive, specific and less labor for detection of FMDV with subsequent early planning for a control strategy in case of an outbreak with liberating FMDV free animals from quarantine measure.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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Table 1: C_T values of three samples exposed to different conditions

Conditions 1 Time; Probe or SYBR	1st day probe same run		2nd day probe same run				2nd day SYBR same run				
Conditions 2 rxn mixture content	old primers /probe aliquot		old primers /probe aliquot		new primers /probe aliquot		old primers aliquot		new primers aliquot		
Conditions 3 rxn mixture content	old RNA aliquot	new RNA aliquot	old RNA aliquot	new RNA aliquot	old RNA aliquot	new RNA aliquot	old RNA aliquot	new RNA aliquot	old RNA aliquot	new RNA aliquot	
Replicate no	1	2	3	4	5	6	7	8	9	10	Mean using Probe
Positive Control 2	29.6	27.7	27	23.1	27	23.3	20.4	18.8	19.6	17.3	26.2
Standard	25	25.2	25.9	20.2	22.1	23.4	16.2	13.5	15.3	13.8	23.6
Standard, 10^{-1}	28.5	ND	26.5	ND	25.6	ND	20	ND	19.7	ND	26.8
NTC	-	-	-	-	-	-	42.4	42.4	42.4	42.4	-

.ND=not done, Reading was CT values recorded in various conditions

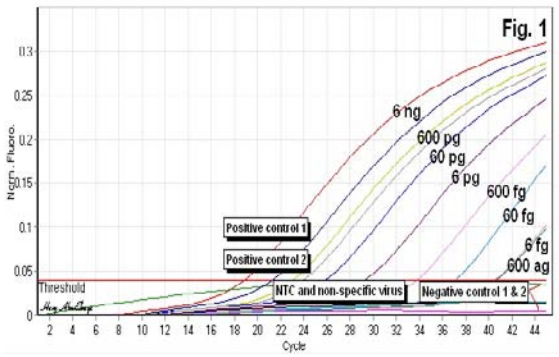

Fig. 1A

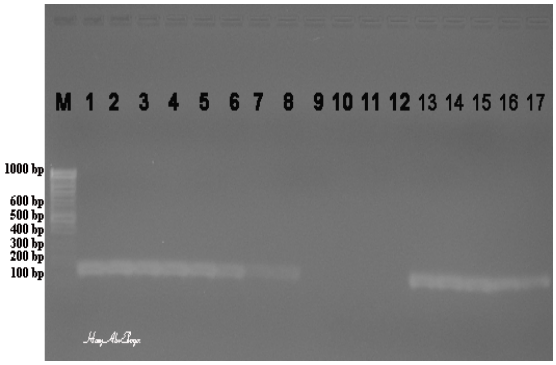

Fig. 1B

Fig. 1: Amplification sensitivity of MGB probe rRT-PCR for FMDV O RNA pre-quantified, 6 ng/µl, and serially 10-fold diluted until theoretically reach 6 ag/µl. The former virus was used as standard. Nomenclature on the real-time figure is representing curves either by intersecting the 1st digit (i.e. 6) in the quantity number of the RNA masses or by having an annotation with a black shadow pointed to it. The threshold is the horizontal red line intersecting the curves. (A) Quantitation data of the standard and its dilutions. Two positive controls, two negative controls, no template control (NTC) and non-specific virus (BVD) were involved in the assay run. Positive C_T values were curves peaks above the threshold, while negative values were peaks at the borders or below the threshold (B) Agar-based electrophoresis of the amplified products by the MGB probe. M: 100 bp ladder. Expected fragment size were approximately 163 bp of FMDV 3D gene. Lanes 1-8: The standard dilutions from 6 ng/µl to 6 ag/µl, Lanes 9-10: negative control 1 & 2, Lanes 11: BVD, Lane 12: NTC, Lanes 13-17: Unknown samples, that were skipped in Quantitation data figure to not hide the illustrated standard curves. Positive controls were not shown.

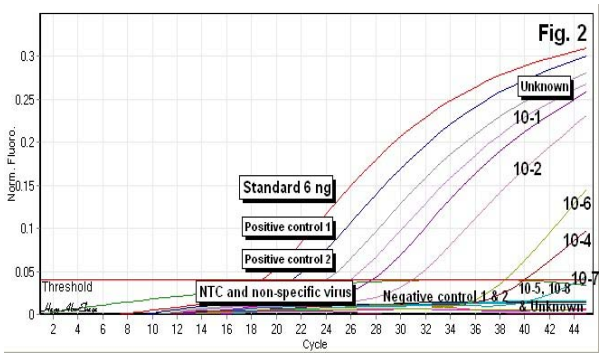

Fig. 2A

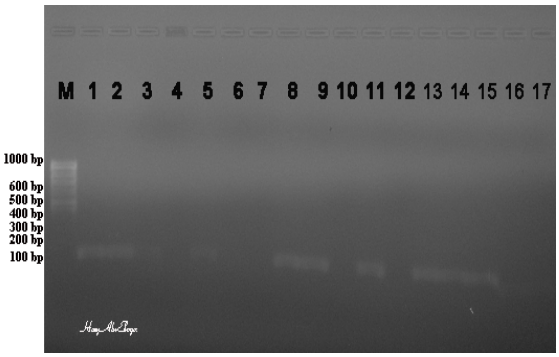

Fig. 2B

Fig. 2: (A) Detection of an archived FMDV stock virus (SV) RNA by MGB probe rRT-PCR. SV dilutions 10^{-1}-10^{-8}, except 10^{-3}, showed various C_T values with amplification cycles. Two positive and negative controls, NTC, non-specific virus (BVD) and unknown samples were involved in the test. (B) Detection of stock virus (SV) RNA with anticipated 163 bp. M: 100 bp ladder. Lanes: 1-7 SV dilutions 10^{-1}-10^{-8}, except the not done dilution 10^{-3}. Lanes 8 & 13 were positive control 1& 2. Other lanes were unknown samples, Lanes 16-17: showed a very faint bands of an archived unknown samples, which were amplified by another TaqMan probe of an expected size of 107 bp and were used as a control of electrophoresis.

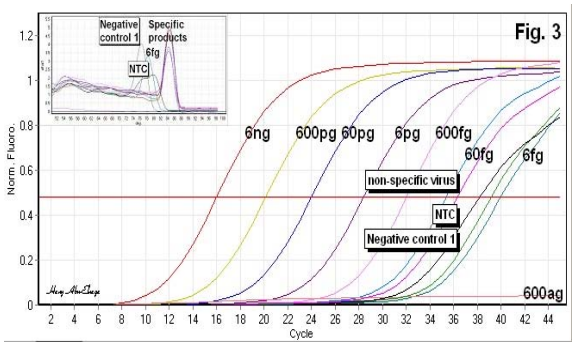

Fig. 3A

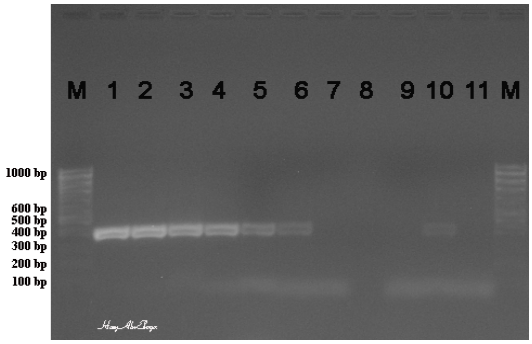

Fig. 3B

Fig. 3: Implementation of SYBR Green rRT-PCR using oligos targeting FMDV O 1D gene of an expected 402 bp for minimum detecting of FMDV O RNA pre-quantified as 6 ng/μl, serially 10-fold diluted and theoretically extended to 6 ag/μl. The former virus was used as standard. (A) Graphical representation of real time one-step RT-PCR data of the standard and its dilutions. Negative control, NTC and non-specific virus, BVD, were involved in the assay run. Positive C_T values were curves peaks above the threshold, while negative values were peaks at the borders or below the threshold (B) Electrophoresis on agarose gel of the rRT-PCR assay samples using SYBR Green. M: 100 bp ladder. Positive bands were approximately 402 bp of FMDV O 1D gene. Lanes 1-8: The standard dilutions from 6 ng/μl to 6 ag/μl, Lane 9: negative control 2, Lane 10: BVD, Lane 11: NTC, Insets show melting curve analysis.

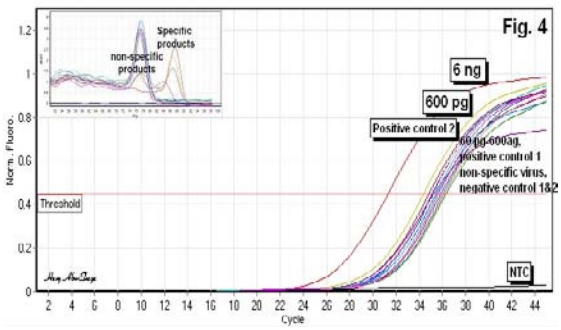

Fig. 4A

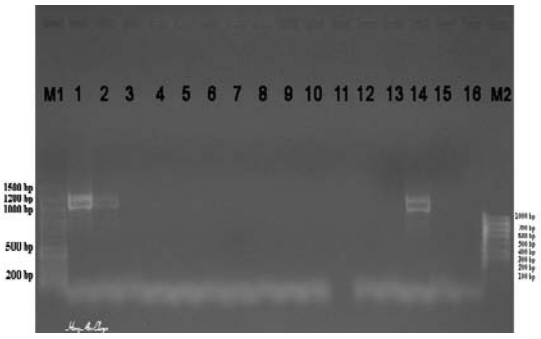

Fig. 4B

Fig. 4: SYBR Green rRT-PCR amplification of an expected 1301 bp embarrassing FMDV O 1D gene of for assesment the detection limit of FMDV O RNA pre-quantified as 6 ng/μl, serially 10-fold diluted and theoretically extended to 6 ag/μl. The former virus was used as standard. (A) Graphical representation of real time one-step RT-PCR data of the standard and its dilutions. Two positive controls, two negative controls, no template control (NTC) and non-specific virus (BVD) were involved in the assay run.Positive C_T values were curves peaks above the threshold, while negative values were peaks at the borders or below the threshold (B) Electrophoresis on agarose gel of the rRT-PCR assay samples using SYBR Green. M: 100 bp ladder. Positive bands were approximately 1301 bp of FMDV O 1D gene. Lanes 1-8: The standard dilutions from 6 ng/μl to 6 ag/μl, Lane 9-10: negative control 1 &2, Lane 11: NTC, Lane 12: BVD, Lanes 13-16: unknown samples, Melting curve analysis (insets) revealed amplification specificity.

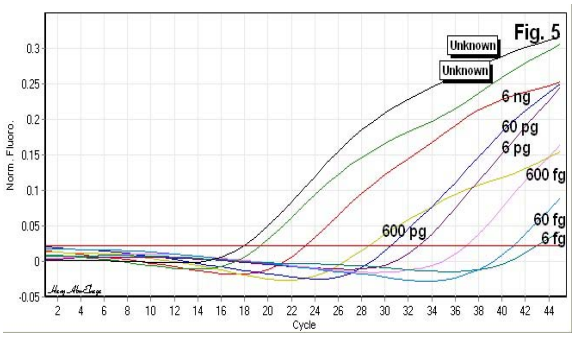

Fig. 5A

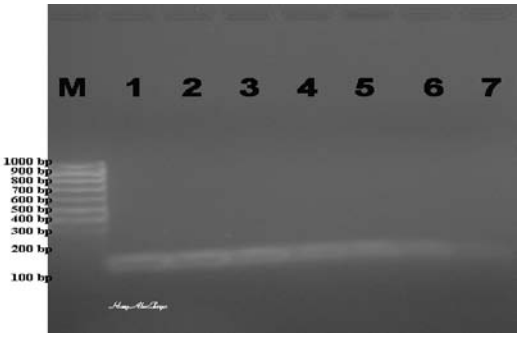

Fig. 5B

Fig. 5: Re-assessment (1-month later from previous evaluation) of the sensitivity of MGB probe rRT-PCR for FMDV O RNA standard (6 ng/μl, and serially 10-fold diluted). (A) Quantitation data of the standard and its dilutions. Unknown samples were involved in the assay run. (B) Agar-based electrophoresis of the amplified products by the MGB probe. M: 100 bp ladder. Expected fragment size were approximately 163 bp of FMDV 3D gene. Lanes 1-7: The standard dilutions from 6 ng/μl to 6 fg/μl. Unknown samples were not shown.

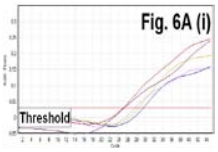

Fig. 6A (i)

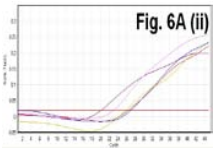

Fig. 6A (ii)

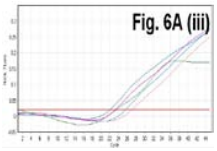

Fig. 6A (iii)

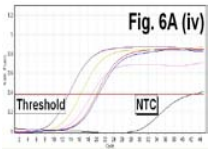

Fig. 6A (iv)

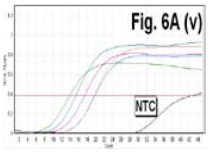

Fig. 6A (v)

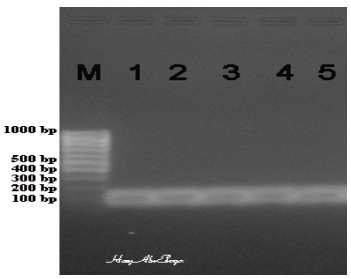

Fig. 6B (i)

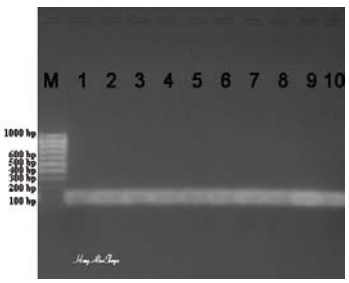

Fig. 6B (ii)

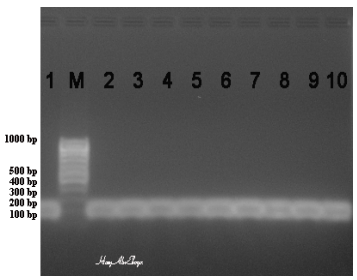

Fig. 6B (iii)

Fig. 6: Re-testing, 1-month later from previous current article evaluation, of a standard & its 1st RNA dilution for investigate the efficiency of a MGB probe rRT-PCR under variable factors. Positive control 2 using the primers either with MGB probe rRT-PCR Fig 6A (i)-(iii), Fig 6B (i) or with SYBR Green rRT-PCR Fig 6A (iv)-(v), Fig 6B (iii) depending on an old & new RNA aliquot of the standard and positive control 2, also an old & new aliquots of primers & probe. (A) Real-time detected signals curve (B) Agar gel based electrophoresis.

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Urine Specific Gravity in Canine: Whole or Supernatant Sample?

By Laynez-Herrera, Olinda Maricruz , Villatoro-Chacón, Daniela Mariel , Arizandieta-Altán, Carmen Grizelda , Chávez-López, Juan José & Lepe-López, Manuel Antonio

San Carlos of Guatemala University

Abstract- The ability to concentrate and dilute urine is one of the first functions that are lost as a result of tubular damage. Specific gravity by refractometry is a method used to evaluate this function. Some authors mention that the reactive sediment, as well as the presence of large amounts of high molecular weight substances can give an overestimation of the value of the specific gravity and recommends centrifuging the sample to obtain a reliable value. In the study, 123 urine samples and their specific gravity were evaluated before and after centrifugation. This in order to evaluate if the presence of reactive sediment, glucose and protein affected the value of the density and to obtain a reliable estimate in clinical practice. No significant difference was found between pre and post-centrifugation specific gravity measurement with portable refractometer (W = 8058.5, p-value = 0.3759).

Keywords: specific gravity, refractometer, dogs.

GJMR-G Classification: NLMC Code: WA 360

URINESPECIFICGRAVITYINCANINEWHOLEORSUPERNATANTSAMPLE

Strictly as per the compliance and regulations of:

Urine Specific Gravity in Canine: Whole or Supernatant Sample?

Densidad Urinaria En Caninos: ¿Muestra Entera O Sobrenadante?

Laynez-Herrera, Olinda Maricruz [α], Villatoro-Chacón, Daniela Mariel [σ], Arizandieta-Altán, Carmen Grizelda [ρ], Chávez-López, Juan José [ω] & Lepe-López, Manuel Antonio [¥]

Abstract- The ability to concentrate and dilute urine is one of the first functions that are lost as a result of tubular damage. Specific gravity by refractometry is a method used to evaluate this function. Some authors mention that the reactive sediment, as well as the presence of large amounts of high molecular weight substances can give an overestimation of the value of the specific gravity and recommends centrifuging the sample to obtain a reliable value. In the study, 123 urine samples and their specific gravity were evaluated before and after centrifugation. This in order to evaluate if the presence of reactive sediment, glucose and protein affected the value of the density and to obtain a reliable estimate in clinical practice. No significant difference was found between pre and post-centrifugation specific gravity measurement with portable refractometer (W = 8058.5, p-value = 0.3759). However, since the interpretation of specific gravity is a categorical variable, 8 of 123 samples evaluated showed changes but only one change in their categorization. The findings found are important for the clinical assessment of outpatients or when a small amount of sample is available, allowing refractometry to be a reliable method without the need to centrifuge the urine to obtain the parameter.

Keywords: specific gravity, refractometer, dogs.

Resumen- La capacidad de concentrar y diluir la orina es una de las primeras funciones que se pierden como consecuencia del daño tubular. La densidad urinaria por refractometría es un método que se utiliza para evaluar esta función. Algunos autores mencionan que el sedimento reactivo, así como la presencia de grandes cantidades de sustancias de alto peso molecular pueden dar una sobreestimación del valor de la densidad y recomienda centrifugar la muestra para obtener un valor confiable. En el estudio, se evaluaron 123 muestras de orina y su densidad antes y después de centrifugar. Esto con el fin de evaluar si la presencia de sedimento reactivo, glucosa y proteína afectaba el valor de la densidad y poder obtener una estimación confiable en práctica clínica. No se encontró diferencia significativa entre la medición de la densidad pre y post-centrifugación con refractómetro portátil (W = 8058.5, p-valué = 0.3759). Sin embargo, como la interpretación de la densidad urinaria es una variable categórica 8 de 123 muestras evaluadas mostraron cambios de densidad, pero solo una cambio su categorización. Los hallazgos encontrados, son importantes para la valoración clínica de pacientes ambulatorios o cuando se dispone de poca cantidad de muestra, permitiendo que la refractometría sea un método confiable sin necesidad de centrifugar la orina para la obtención del parámetro.

Palabras clave: densidad urinaria, refractómetro, perros.

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I. Introducción

La capacidad de los riñones para reabsorber en forma selectiva las sustancias químicas y agua a través del filtrado glomerular es una de las funciones más importantes del organismo al igual que la secreción de metabolitos y desechos (Strasinger y Di Lorenzo, 2010; Costa et al, 2010 y Bouda et al, 2007). La capacidad de concentrar y diluir la orina es una de las primeras funciones que se pierden como consecuencia del daño tubular, por consiguiente, la evaluación de esta capacidad es necesaria para el análisis de orina habitual. Esta evaluación se puede realizar mediante la determinación de la densidad urinaria, la cual también brindara información importante sobre el estado de hidratación del paciente (Strasinger y Di Lorenzo, 2010; Costa et al, 2010, Terres y González, 1992; Archer, 2012 y Campuzano y Arbeláez, 2007).

La densidad urinaria (o peso específico) es la relación entre el peso de un volumen dado de orina y el peso del mismo volumen de agua destilada (Costa et al, 2010 y Cortadellas y Fernández, 2012). Depende no solo del número de partículas que hay en solución, sino también de su peso molecular y de la temperatura (Calabia y Arias, 2008).

Se reportan tres métodos para su medición: refractómetro, urodensímetro y tiras reactivas, siendo este último no confiable a diferencia del refractómetro y el urodensímetro (Costa et al, 2010; Archer, 2012 y Cortadellas y Fernández, 2012).

El refractómetro portátil determina la densidad de manera accesible, rápida, fácil y con un volumen de muestra pequeña (Strasinger y Di Lorenzo, 2010). Este instrumento mide el grado de refracción de la luz por parte de los componentes sólidos cuando esta pasa a través de la orina (Bouda et al, 2007 y Archer 2012). El

grado en que se doble el haz de luz es directamente proporcional al número de partículas disueltas en la solución (Wamsley y Alleman, 2007 y Archer, 2012). Sustancias que contribuyen al peso específico de la orina normal son: la urea (20%), el cloruro de sodio (25%), los sulfatos y los fosfatos (Fuller et al, 2005 y Riley y McPherson, 2017). Sin embargo, Wamsley y Alleman (2007), Terres y González (1992) e Idrovo (2018) mencionan que el sedimento reactivo (cristales, células, moco y bacterias) puede dar una sobreestimación de la densidad urinaria debido a que estos materiales también pueden desdoblar el haz de luz incrementando el valor de la densidad. Por esta razón, se recomienda hacer una evaluación de la densidad urinaria utilizando el sobrenadante de la muestra post centrifugación para obtener una estimación real de la capacidad tubular renal. Por otra parte, Calabia y Arias (2008) y Archer (2012) mencionan que se puede dar una sobreestimación de la densidad urinaria cuando la muestra contiene grandes cantidades de glucosa, proteínas, lípidos y material de contraste.

En el presente estudio, se evaluaron 123 muestras de orina y su densidad antes y después de centrifugar. Esto con el fin de determinar si existe diferencia significativa en cuanto al valor de densidad y si la presencia de otros solutos afecta a este parámetro.

II. Materiales y Métodos

Se realizó el estudio en el Laboratorio Clínico del Hospital Veterinario de la Universidad de San Carlos de Guatemala, cuyas coordenadas son 14° 34´58.44´N y 90° 33´10.44 W.

Se colectaron muestras de orina de 123 pacientes caninos que acudieron al Hospital Veterinario durante los meses de abril a julio de 2017; siendo estos de distintas razas, edades, sexo y tipo de recolección de muestra (chorro medio, sondaje, cistocentesis o del ambiente).

Se realizó examen completo a todas las muestras de orina colectadas, el cual consta de: Examen físico (color, olor, aspecto y densidad); examen químico (pH, proteínas, glucosa, cetonas, glucosa, bilirrubina, cetonas, urobilinógeno, sangre y leucocitos); y examen de sedimento (leucocitos, eritrocitos, células epiteliales, cilindros, cristales, bacterias, levaduras y parásitos). Sin embargo, las variables a considerar en el estudio fueron: Densidad, glucosuria, proteinuria con tira reactiva y prueba de Heller y presencia de sedimento reactivo (más de 6 elementos orgánicos o inorgánicos por campo: leucocitos, eritrocitos, células epiteliales, cilindros, cristales, bacterias y levaduras).

Para evaluar la densidad urinaria de la muestra completa y del sobrenadante se utilizó un refractómetro portátil marca Eurolab® previamente calibrado según la técnica descrita por Strasinger y Di Lorenzo (2010).

Para evaluar glucosuria se utilizaron tiras reactivas comerciales (Roche®). La glucosuria se midió a través de una escala de variación de color. Las tiras se colocaron en la orina homogenizada y sin centrifugar a temperatura ambiente y su lectura se realizó 45 a 60 segundos después de sumergidas comparando el cambio de color con la escala cromática provista por el fabricante. La lectura fue realizada visualmente.

Para la evaluación inicial de proteinuria se utilizaron tiras reactivas comerciales (Roche®) debido a que representan el método más usado en medicina veterinaria por su valor económico y facilidad de uso (Beristain et al, 2010). Las tiras son sensibles particularmente a albumina (Cortadellas y Fernández, 2012). Por esta razón se utilizó la prueba de Heller (ácido nítrico al 65%) para confirmar proteinuria (Hutter, 2010).

La evaluación de sedimento reactivo se realizó a través de la observación directa al microscopio binocular, se preparó la muestra como lo describen Diaz (1997) y Bouda, et al (2007).

Se utilizó el programa estadístico de libre acceso R versión 3.3.3 para el análisis de datos. El Test de Shapiro-Wilk expuso que los datos de densidad carecían de normalidad por lo que se utilizó la prueba no paramétrica de Wilcoxon.

III. Resultados

Las 123 muestras evaluadas fueron recolectadas con distintos métodos. En el cuadro 1 se describen el número de muestras según el método de recolección.

Cuadro 1: Método de recolección de muestra.

Método de recolección de muestra	No. De muestras	%
Chorro medio	61	49.59%
Sonda uretral	37	30.08%
Cistocentesis	13	10.57%
Del Ambiente	12	9.76%

En cuanto al sexo, más de la mitad de las muestras fueron de pacientes machos. En el cuadro 2 se presentan la distribución de las muestras según el sexo.

Cuadro 2: Distribución de la población según sexo.

Sexo	No. De muestras	%
Machos	64	52.03%
Hembras	59	47.97 %

El rango de edad de los pacientes fue de 0.5 a 19 años, con un promedio de 7.24 ± 0.38. Según la variable "edad" el primer cuartil agrupó a los individuos menores de 1 año y el tercer cuartil reunió ejemplares mayores de 16 años.

Respecto a la raza, los pacientes sin raza definida (SRD) fueron los pacientes con mayor frecuencia, seguido de los pacientes con raza definida como Poodle, Schnauzer y Shih tzu que obtuvieron mayores frecuencias (cuadro 3). Otras razas que se evaluaron representan el 46.34% siendo estas: Bóxer, Golden Retriever, Labrador Retriever, Beagle, Chow-Chow, Cocker, Husky Siberiano, Chihuahua, Rottweiler, Akita, Alaskan Malamute, Dachshund, Pitbull, American Eskimo, Boston Terrier, Bulldog Inglés, Dálmata, Doberman, Pinscher, Dogo, Lhasa Apso, Mastín Inglés, Viejo Pastor Inglés y West Highland White Terrier.

Cuadro 3: Distribución de la población según raza.

Raza	No. de muestras	%
SRD	24	19.51 %
Poodle	23	18.70 %
Schnauzer	12	9.76 %
Shih tzu	7	5.69 %
Otras razas	57	46.34 %

Respecto a la glucosuria sólo se observó en una pequeña proporción de la población (cuadro 4).

Cuadro 4: Distribución de pacientes con glucosuria

Glucosuria	No, de muestras	%
Sin glucosuria	116	94.31 %
Con glucosuria	7	5.69 %

La proteinuria fue evaluada con tira reactiva y prueba de Heller. En el cuadro 5 se presentan las proporciones según técnica utilizada.

Cuadro 5: Evaluación de proteinuria por técnica

	Técnica			
	Tira reactiva		Reacción de Héller	
Proteinuria	No. de muestra	%	No. de muestra	%
Sin proteinuria	49	39.84 %	58	47.15 %
+ (30mg/dl)	66	53.66 %	65	52.85 %
++ (100mg/dl)	8	6.50 %	0	0 %
+++ (1000mg/dl)	0	0 %	0	0 %
++++ (>1000mg/dl)	0	0 %	0	0 %

En cuanto a la evaluación de sedimento reactivo, más de la mitad de la población fue positivo al mismo (cuadro 6).

Cuadro 6: Presencia de sedimento

Sedimento	No. de muestra	%
Con sedimento	83	67.48 %
Sin sedimento	40	32.52 %

Se realizó la categorización clínica de la densidad urinaria de las muestras evaluadas según los criterios de Wamsley y Alleman (2007). La mayor proporción de los pacientes tanto pre y post centrifugación presento orina bien concentrada (cuadro 7, figura 1).

Cuadro 7: Valorización y categorización clínica de la densidad urinaria pre y post centrifugación del total de la población.

	Pre – centrifugación		Post – centrifugación	
Densidad	No. de muestras	%	No. de muestras	%
Hipostenuria (<1.008)	12	9.76 %	12	9.76 %
Isostenuria (1.008-1.012)	9	7.32 %	9	7.32 %
Concentración Mínima (1.013 -1.029)	33	26.83 %	34	27.64 %
Bien concentrada (> 1.030)	69	56.09 %	68	55,28 %

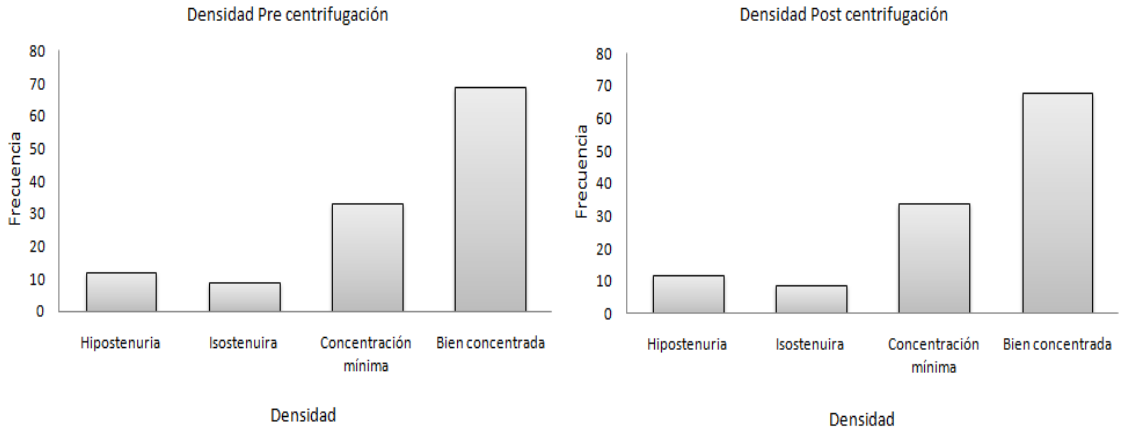

Figura 1: Valoración y categorización de la densidad pre y post centrifugación de la población

Se constató que no existe diferencia estadísticamente significativa entre la medición pre y post centrifugación de densidad urinaria (W = 8058.5, p-valué = 0.3759). Debido a que no se encontró diferencia significativa en la densidad urinaria pre y post centrifugación, no fue posible evaluar el efecto del análisis químico y de sedimento sobre la densidad urinaria.

Sin embargo, ya que el significado de la densidad es categórico, se agruparon los resultados del análisis pre y post centrifugación en una tabla tipo pivote, verificando que únicamente en un individuo se alteró su estado de bien concentrada a concentración mínima después de la centrifugación

IV. Discusión

Del total de la población el 52.03% fueron machos, siendo similar a lo reportado por Alvarado et al (2017). Esto sugiere que tanto machos como hembras son adquiridos de igual manera y la tenencia de hembras no es un problema al disponer de métodos que permiten el control de la fecundidad, como lo son las esterilizaciones quirúrgicas y tratamientos farmacológicos (Ibarra et al., 2003). Así mismo, los caninos de edad adulta fueron quienes presentaron mayor asistencia a consulta veterinaria.

En cuanto a raza, los caninos SRD fueron quienes obtuvieron mayor frecuencia en el estudio. Dentro de las causas puede considerarse el incremento descontrolado de la población canina, el crecimiento de población humana y el aumento de la tasa de migración de las personas del campo a la zona urbana que conlleva un traslado de animales domésticos (Lucio et al., 2017). Del mismo modo, el incremento en la concientización sobre la adopción de perros sin hogar (en su mayoría SRD) por parte de entidades públicas, empresas privadas y asociaciones no gubernamentales (Pacay, 2015). Se estima que en el mundo existen alrededor de 500,000,000 de perros, de los cuales el 75% son no poseen pedigree o no son cruces de dos razas conocidas (Cadena y Lenin, 2013). Por otra parte, la tenencia de razas como Poodle, Schnauzer y Shih tzu puede atribuirse al espacio físico, cuidado y alimentación que estas razas requieren por ser de talla pequeña-mediana (Alvarado et al., 2017).

Respecto a los métodos de recolección de muestra, el método más utilizado fue el chorro medio. Esto pudo deberse a que las muestras colectadas por chorro medio son las más habituales para obtener muestras de tamiz debido a la facilidad de recolección, comodidad para el paciente, ser un método seguro y menos traumático (Strasinger y Di Lorenzo, 2010 y Chew y DiBartola, 1998). Se recomienda que la muestra sea obtenida de la primera orina de la mañana ya que es concentrada y refleja mejor el estado general del paciente, siendo la parte media más representativa ya que la primera fracción puede arrastrar componente de la uretra o del tracto genital (Bouda et al., 2007; Diaz et al., 1997 y Chew y DiBartola, 1998). Por otra parte, las muestras obtenidas por sondaje o cistocentesis en la

mayoría de las situaciones son solicitadas con fines específicos (cultivo bacteriano o citología) conllevan mayor pericia y mayores riesgos (Strasinger y Di Lorenzo, 2010). Las muestras tomadas del ambiente son menos apropiadas y por lo tanto fueron las menos frecuentes. Por ello, se debe recurrir a ellas cuando son la única opción para obtener la muestra considerando la contaminación de las mismas al momento de la interpretación de los resultados (Chew y DiBartola, 1998).

Se observó glucosuria en el 5.69% de las muestras procesadas. Esta prevalencia se asemeja a lo reportado por Mesa y Castillo (2014) y Zamora y Osorio (Zamora et al., 2015). Estos datos sugieren lo poco frecuente de este hallazgo. Sin embargo, ya que la glucosuria está asociada a varias patologías y su origen puede ser renal o extra-renal, se recomienda realizar mediciones seriadas simultaneas de glicemia y glucosuria. Esto permitirá identificar el origen y por consiguiente obtener un diagnostico e implementar un tratamiento adecuado (Campuzano y Arbeláez, 2007 y Zamora et al., 2015).

El 60.16% de los pacientes presentaron proteinuria con el método de tira reactiva, mientras que sólo el 52.85% fue reactivo con la prueba de Heller. Estos datos son similares a otros estudios sugiriendo que la proteinuria es un hallazgo frecuente en perros durante la consulta médica rutinaria (Beristain et al., Gallo y Silva, 2014 y Barros y Carpio, 2017). Sin embargo, la presencia de proteínas en orina no constituye una prueba de nefropatía ni su ausencia la excluye[6]. Por esta razón se debe determinar su origen ya que este suele ser variado y no siempre patológico (Campuzano y Arbeláez, 2007; Cortadellas y Fernández, 2012; Beristain et al., 2010; Zamora et al., 2015 y Barros y Carpio, 2017). Cabe mencionar que en condiciones normales en los perros existe una ligera proteinuria que oscila entre 0.1 a 0.3g/l (Barros y Carpio, 2017). Además, la proteinuria en trazas o 1+ en orina muy concentrada a menudo se atribuye a la concentración de la orina y no a una proteinuria anormal (Grauer, 2016). La eliminación urinaria de una cantidad excesiva de proteínas de forma persistente es un marcador de daño renal (Calabia y Arias, 2008).

El 67.48% de las muestras fueron positivas a sedimento reactivo. Este parámetro pudo estar relacionado al método de toma de muestra (Chew y DiBartola, 1998). Se debe considerar que el sedimento urinario de un individuo sano no está libre de elementos formes, pero contiene un número limitado de estos. Por esta razón, si se encuentran en muy pequeña proporción por campo no se consideran necesariamente anormales (Campuzano y Arbeláez, 2007; Calabia y Arias, 2008 y Díaz et al., 1997). Además, la primera orina de la mañana ofrece un mayor número de elementos celulares y bacterias, que generalmente provienen de uretra, vagina y prepucio. Por este motivo, es de suma importancia una amplia experiencia para su análisis y una adecuada interpretación tomando en cuenta la condición clínica de cada paciente (Zamora et al., 2015).

La densidad que presentó mayor frecuencia fue >1.030. Esto nos sugiere que la mayoría de los caninos evaluados presento correcta capacidad de concentrar la orina. Se encontró que el 26.83% presento concentración mínima mostrando una alteración significativa en la capacidad de concentración renal. Sólo el 7.32% presentó isostenuria lo que indica que el riñón no concentra ni diluye adecuadamente la orina. Sin embargo, la evaluación de la densidad urinaria debe realizarse de forma discreta, ya que un perro sano puede diluir o concentrar la orina entre 1.001 a 1.075 (Watson et al., 2015). Por esta razón, el análisis de la densidad debe evaluarse según las circunstancias clínicas de cada paciente y colectar las muestras por la mañana antes que el animal haya ingerido agua o comida ya que estas muestras son más útiles en la evaluación de la concentración de la orina. De esta manera, se podrá obtener una correlación clínica y patológica de la capacidad de concentración renal (Chew y DiBartola, 1998). Además, debe considerarse que la densidad varía en el curso del día y que una sola lectura no da la información suficiente, por ello se recomienda realizar varias mediciones (Graff, 2007).

Por otra parte, no se encontró diferencia significativa entre la medición de la densidad pre y post-centrifugación con refractómetro portátil. El valor diagnóstico de este hallazgo nos permite realizar una estimación adecuada y confiable de la densidad en muestras sin centrifugar de pacientes ambulatorios con orinas normales o cuando se disponga de poca cantidad de orina (Terres y González, 1992 y Díaz et al., 1997). Dado que en el estudio no se observó diferencia significativa de la densidad urinaria pre y post-centrifugación, las variables proteinuria, glucosuria y sedimento no fueron evaluadas. Sin embargo, autores como Terres y González (1992), Strasinger y Di Lorenzo (2010) y Archer (2012) reportan que la densidad urinaria depende de la naturaleza y del número solutos, por lo que grandes cantidades de sustancias de alto peso molecular (albumina o glucosa) incrementan la densidad urinaria. Este factor da la falsa impresión de que el riñón es capaz de concentrar la orina. Por lo tanto, en estos casos se recomienda realizar una corrección numérica al resultado de la medición o medir directamente la osmolaridad para establecer la verdadera capacidad renal de concentrar y diluir la orina. Sin embargo, a pesar de que la osmolaridad es la medida de elección para evaluar la capacidad de concentración renal, en la práctica clínica, es un método poco accesible a diferencia de la densidad urinaria (Costa et al., 2010; Terres y González, 1992; Calabia y Arias, 2008 y Graff 2007).

V. Conclusion

No se encontró diferencia significativa entre la medición de la densidad pre y post-centrifugación con refractómetro portátil. Por tal razón medir la densidad urinaria en una muestra de orina del sobrenadante o sin centrifugar no afecta el valor diagnóstico en los pacientes. Esto permite que este método brinde datos confiables para la valoración de los mismos en la práctica clínica.

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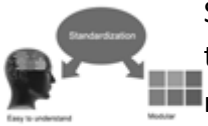

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- The Fellow can organize symposium/seminar/conference on behalf of Global Journals Incorporation (USA) and he/she can also attend the same organized by other institutes on behalf of Global Journals.
- The Fellow can become member of Editorial Board Member after completing 3yrs.
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- • This individual has learned the basic methods of applying those concepts and techniques to common challenging situations. This individual has further demonstrated an in–depth understanding of the application of suitable techniques to a particular area of research practice.

Note :

"

- In future, if the board feels the necessity to change any board member, the same can be done with the consent of the chairperson along with anyone board member without our approval.
- In case, the chairperson needs to be replaced then consent of 2/3rd board members are required and they are also required to jointly pass the resolution copy of which should be sent to us. In such case, it will be compulsory to obtain our approval before replacement.
- In case of "Difference of Opinion [if any]" among the Board members, our decision will be final and binding to everyone.

"

Preferred Author Guidelines

We accept the manuscript submissions in any standard (generic) format.

We typeset manuscripts using advanced typesetting tools like Adobe In Design, CorelDraw, TeXnicCenter, and TeXStudio. We usually recommend authors submit their research using any standard format they are comfortable with, and let Global Journals do the rest.

Alternatively, you can download our basic template from https://globaljournals.org/Template

Authors should submit their complete paper/article, including text illustrations, graphics, conclusions, artwork, and tables. Authors who are not able to submit manuscript using the form above can email the manuscript department at submit@globaljournals.org or get in touch with chiefeditor@globaljournals.org if they wish to send the abstract before submission.

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Authors must ensure the information provided during the submission of a paper is authentic. Please go through the following checklist before submitting:

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2. Authors must accept the privacy policy, terms, and conditions of Global Journals.
3. Ensure corresponding author's email address and postal address are accurate and reachable.
4. Manuscript to be submitted must include keywords, an abstract, a paper title, co-author(s') names and details (email address, name, phone number, and institution), figures and illustrations in vector format including appropriate captions, tables, including titles and footnotes, a conclusion, results, acknowledgments and references.
5. Authors should submit paper in a ZIP archive if any supplementary files are required along with the paper.
6. Proper permissions must be acquired for the use of any copyrighted material.
7. Manuscript submitted *must not have been submitted or published elsewhere* and all authors must be aware of the submission.

Declaration of Conflicts of Interest

It is required for authors to declare all financial, institutional, and personal relationships with other individuals and organizations that could influence (bias) their research.

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- Findings
- Writings
- Diagrams
- Graphs
- Illustrations
- Lectures

- Printed material
- Graphic representations
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- Electronic material
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3. Final approval of the version of the paper to be published.

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The corresponding author should mention the name and complete details of all co-authors during submission and in manuscript. We support addition, rearrangement, manipulation, and deletions in authors list till the early view publication of the journal. We expect that corresponding author will notify all co-authors of submission. We follow COPE guidelines for changes in authorship.

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Unless specified in the notification, the Editorial Board's decision on publication of the paper is final and cannot be appealed before making the major change in the manuscript.

Acknowledgments

Contributors to the research other than authors credited should be mentioned in Acknowledgments. The source of funding for the research can be included. Suppliers of resources may be mentioned along with their addresses.

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Preparing your Manuscript

Authors can submit papers and articles in an acceptable file format: MS Word (doc, docx), LaTeX (.tex, .zip or .rar including all of your files), Adobe PDF (.pdf), rich text format (.rtf), simple text document (.txt), Open Document Text (.odt), and Apple Pages (.pages). Our professional layout editors will format the entire paper according to our official guidelines. This is one of the highlights of publishing with Global Journals—authors should not be concerned about the formatting of their paper. Global Journals accepts articles and manuscripts in every major language, be it Spanish, Chinese, Japanese, Portuguese, Russian, French, German, Dutch, Italian, Greek, or any other national language, but the title, subtitle, and abstract should be in English. This will facilitate indexing and the pre-peer review process.

The following is the official style and template developed for publication of a research paper. Authors are not required to follow this style during the submission of the paper. It is just for reference purposes.

Manuscript Style Instruction (Optional)

- Microsoft Word Document Setting Instructions.
- Font type of all text should be Swis721 Lt BT.
- Page size: 8.27" x 11'", left margin: 0.65, right margin: 0.65, bottom margin: 0.75.
- Paper title should be in one column of font size 24.
- Author name in font size of 11 in one column.
- Abstract: font size 9 with the word "Abstract" in bold italics.
- Main text: font size 10 with two justified columns.
- Two columns with equal column width of 3.38 and spacing of 0.2.
- First character must be three lines drop-capped.
- The paragraph before spacing of 1 pt and after of 0 pt.
- Line spacing of 1 pt.
- Large images must be in one column.
- The names of first main headings (Heading 1) must be in Roman font, capital letters, and font size of 10.
- The names of second main headings (Heading 2) must not include numbers and must be in italics with a font size of 10.

Structure and Format of Manuscript

The recommended size of an original research paper is under 15,000 words and review papers under 7,000 words. Research articles should be less than 10,000 words. Research papers are usually longer than review papers. Review papers are reports of significant research (typically less than 7,000 words, including tables, figures, and references)

A research paper must include:

a) A title which should be relevant to the theme of the paper.
b) A summary, known as an abstract (less than 150 words), containing the major results and conclusions.
c) Up to 10 keywords that precisely identify the paper's subject, purpose, and focus.
d) An introduction, giving fundamental background objectives.
e) Resources and techniques with sufficient complete experimental details (wherever possible by reference) to permit repetition, sources of information must be given, and numerical methods must be specified by reference.
f) Results which should be presented concisely by well-designed tables and figures.
g) Suitable statistical data should also be given.
h) All data must have been gathered with attention to numerical detail in the planning stage.

Design has been recognized to be essential to experiments for a considerable time, and the editor has decided that any paper that appears not to have adequate numerical treatments of the data will be returned unrefereed.

i) Discussion should cover implications and consequences and not just recapitulate the results; conclusions should also be summarized.
j) There should be brief acknowledgments.
k) There ought to be references in the conventional format. Global Journals recommends APA format.

Authors should carefully consider the preparation of papers to ensure that they communicate effectively. Papers are much more likely to be accepted if they are carefully designed and laid out, contain few or no errors, are summarizing, and follow instructions. They will also be published with much fewer delays than those that require much technical and editorial correction.

The Editorial Board reserves the right to make literary corrections and suggestions to improve brevity.

Format Structure

It is necessary that authors take care in submitting a manuscript that is written in simple language and adheres to published guidelines.

All manuscripts submitted to Global Journals should include:

Title

The title page must carry an informative title that reflects the content, a running title (less than 45 characters together with spaces), names of the authors and co-authors, and the place(s) where the work was carried out.

Author details

The full postal address of any related author(s) must be specified.

Abstract

The abstract is the foundation of the research paper. It should be clear and concise and must contain the objective of the paper and inferences drawn. It is advised to not include big mathematical equations or complicated jargon.

Many researchers searching for information online will use search engines such as Google, Yahoo or others. By optimizing your paper for search engines, you will amplify the chance of someone finding it. In turn, this will make it more likely to be viewed and cited in further works. Global Journals has compiled these guidelines to facilitate you to maximize the web-friendliness of the most public part of your paper.

Keywords

A major lynchpin of research work for the writing of research papers is the keyword search, which one will employ to find both library and internet resources. Up to eleven keywords or very brief phrases have to be given to help data retrieval, mining, and indexing.

One must be persistent and creative in using keywords. An effective keyword search requires a strategy: planning of a list of possible keywords and phrases to try.

Choice of the main keywords is the first tool of writing a research paper. Research paper writing is an art. Keyword search should be as strategic as possible.

One should start brainstorming lists of potential keywords before even beginning searching. Think about the most important concepts related to research work. Ask, “What words would a source have to include to be truly valuable in a research paper?” Then consider synonyms for the important words.

It may take the discovery of only one important paper to steer in the right keyword direction because, in most databases, the keywords under which a research paper is abstracted are listed with the paper.

Numerical Methods

Numerical methods used should be transparent and, where appropriate, supported by references.

Abbreviations

Authors must list all the abbreviations used in the paper at the end of the paper or in a separate table before using them.

Formulas and equations

Authors are advised to submit any mathematical equation using either MathJax, KaTeX, or LaTeX, or in a very high-quality image.

Tables, Figures, and Figure Legends

Tables: Tables should be cautiously designed, uncrowned, and include only essential data. Each must have an Arabic number, e.g., Table 4, a self-explanatory caption, and be on a separate sheet. Authors must submit tables in an editable format and not as images. References to these tables (if any) must be mentioned accurately.

Figures

Figures are supposed to be submitted as separate files. Always include a citation in the text for each figure using Arabic numbers, e.g., Fig. 4. Artwork must be submitted online in vector electronic form or by emailing it.

Preparation of Eletronic Figures for Publication

Although low-quality images are sufficient for review purposes, print publication requires high-quality images to prevent the final product being blurred or fuzzy. Submit (possibly by e-mail) EPS (line art) or TIFF (halftone/ photographs) files only. MS PowerPoint and Word Graphics are unsuitable for printed pictures. Avoid using pixel-oriented software. Scans (TIFF only) should have a resolution of at least 350 dpi (halftone) or 700 to 1100 dpi (line drawings). Please give the data for figures in black and white or submit a Color Work Agreement form. EPS files must be saved with fonts embedded (and with a TIFF preview, if possible).

For scanned images, the scanning resolution at final image size ought to be as follows to ensure good reproduction: line art: >650 dpi; halftones (including gel photographs): >350 dpi; figures containing both halftone and line images: >650 dpi.

Color charges: Authors are advised to pay the full cost for the reproduction of their color artwork. Hence, please note that if there is color artwork in your manuscript when it is accepted for publication, we would require you to complete and return a Color Work Agreement form before your paper can be published. Also, you can email your editor to remove the color fee after acceptance of the paper.

Tips for writing a good quality Medical Research Paper

1. Choosing the topic: In most cases, the topic is selected by the interests of the author, but it can also be suggested by the guides. You can have several topics, and then judge which you are most comfortable with. This may be done by asking several questions of yourself, like "Will I be able to carry out a search in this area? Will I find all necessary resources to accomplish the search? Will I be able to find all information in this field area?" If the answer to this type of question is "yes," then you ought to choose that topic. In most cases, you may have to conduct surveys and visit several places. Also, you might have to do a lot of work to find all the rises and falls of the various data on that subject. Sometimes, detailed information plays a vital role, instead of short information. Evaluators are human: The first thing to remember is that evaluators are also human beings. They are not only meant for rejecting a paper. They are here to evaluate your paper. So present your best aspect.

2. Think like evaluators: If you are in confusion or getting demotivated because your paper may not be accepted by the evaluators, then think, and try to evaluate your paper like an evaluator. Try to understand what an evaluator wants in your research paper, and you will automatically have your answer. Make blueprints of paper: The outline is the plan or framework that will help you to arrange your thoughts. It will make your paper logical. But remember that all points of your outline must be related to the topic you have chosen.

3. Ask your guides: If you are having any difficulty with your research, then do not hesitate to share your difficulty with your guide (if you have one). They will surely help you out and resolve your doubts. If you can't clarify what exactly you require for your work, then ask your supervisor to help you with an alternative. He or she might also provide you with a list of essential readings.

4. Use of computer is recommended: As you are doing research in the field of medical research then this point is quite obvious. Use right software: Always use good quality software packages. If you are not capable of judging good software, then you can lose the quality of your paper unknowingly. There are various programs available to help you which you can get through the internet.

5. Use the internet for help: An excellent start for your paper is using Google. It is a wondrous search engine, where you can have your doubts resolved. You may also read some answers for the frequent question of how to write your research paper or find a model research paper. You can download books from the internet. If you have all the required books, place importance on reading, selecting, and analyzing the specified information. Then sketch out your research paper. Use big pictures: You may use encyclopedias like Wikipedia to get pictures with the best resolution. At Global Journals, you should strictly follow here.

6. Bookmarks are useful: When you read any book or magazine, you generally use bookmarks, right? It is a good habit which helps to not lose your continuity. You should always use bookmarks while searching on the internet also, which will make your search easier.

7. Revise what you wrote: When you write anything, always read it, summarize it, and then finalize it.

8. Make every effort: Make every effort to mention what you are going to write in your paper. That means always have a good start. Try to mention everything in the introduction—what is the need for a particular research paper. Polish your work with good writing skills and always give an evaluator what he wants. Make backups: When you are going to do any important thing like making a research paper, you should always have backup copies of it either on your computer or on paper. This protects you from losing any portion of your important data.

9. Produce good diagrams of your own: Always try to include good charts or diagrams in your paper to improve quality. Using several unnecessary diagrams will degrade the quality of your paper by creating a hodgepodge. So always try to include diagrams which were made by you to improve the readability of your paper. Use of direct quotes: When you do research relevant to literature, history, or current affairs, then use of quotes becomes essential, but if the study is relevant to science, use of quotes is not preferable.

10. Use proper verb tense: Use proper verb tenses in your paper. Use past tense to present those events that have happened. Use present tense to indicate events that are going on. Use future tense to indicate events that will happen in the future. Use of wrong tenses will confuse the evaluator. Avoid sentences that are incomplete.

11. Pick a good study spot: Always try to pick a spot for your research which is quiet. Not every spot is good for studying.

12. Know what you know: Always try to know what you know by making objectives, otherwise you will be confused and unable to achieve your target.

13. Use good grammar: Always use good grammar and words that will have a positive impact on the evaluator; use of good vocabulary does not mean using tough words which the evaluator has to find in a dictionary. Do not fragment sentences. Eliminate one-word sentences. Do not ever use a big word when a smaller one would suffice.

Verbs have to be in agreement with their subjects. In a research paper, do not start sentences with conjunctions or finish them with prepositions. When writing formally, it is advisable to never split an infinitive because someone will (wrongly) complain. Avoid clichés like a disease. Always shun irritating alliteration. Use language which is simple and straightforward. Put together a neat summary.

14. Arrangement of information: Each section of the main body should start with an opening sentence, and there should be a changeover at the end of the section. Give only valid and powerful arguments for your topic. You may also maintain your arguments with records.

15. Never start at the last minute: Always allow enough time for research work. Leaving everything to the last minute will degrade your paper and spoil your work.

16. Multitasking in research is not good: Doing several things at the same time is a bad habit in the case of research activity. Research is an area where everything has a particular time slot. Divide your research work into parts, and do a particular part in a particular time slot.

17. Never copy others' work: Never copy others' work and give it your name because if the evaluator has seen it anywhere, you will be in trouble. Take proper rest and food: No matter how many hours you spend on your research activity, if you are not taking care of your health, then all your efforts will have been in vain. For quality research, take proper rest and food.

18. Go to seminars: Attend seminars if the topic is relevant to your research area. Utilize all your resources.

19. Refresh your mind after intervals: Try to give your mind a rest by listening to soft music or sleeping in intervals. This will also improve your memory. Acquire colleagues: Always try to acquire colleagues. No matter how sharp you are, if you acquire colleagues, they can give you ideas which will be helpful to your research.

20. Think technically: Always think technically. If anything happens, search for its reasons, benefits, and demerits. Think and then print: When you go to print your paper, check that tables are not split, headings are not detached from their descriptions, and page sequence is maintained.

21. Adding unnecessary information: Do not add unnecessary information like "I have used MS Excel to draw graphs." Irrelevant and inappropriate material is superfluous. Foreign terminology and phrases are not apropos. One should never take a broad view. Analogy is like feathers on a snake. Use words properly, regardless of how others use them. Remove quotations. Puns are for kids, not grunt readers. Never oversimplify: When adding material to your research paper, never go for oversimplification; this will definitely irritate the evaluator. Be specific. Never use rhythmic redundancies. Contractions shouldn't be used in a research paper. Comparisons are as terrible as clichés. Give up ampersands, abbreviations, and so on. Remove commas that are not necessary. Parenthetical words should be between brackets or commas. Understatement is always the best way to put forward earth-shaking thoughts. Give a detailed literary review.

22. Report concluded results: Use concluded results. From raw data, filter the results, and then conclude your studies based on measurements and observations taken. An appropriate number of decimal places should be used. Parenthetical remarks are prohibited here. Proofread carefully at the final stage. At the end, give an outline to your arguments. Spot perspectives of further study of the subject. Justify your conclusion at the bottom sufficiently, which will probably include examples.

23. Upon conclusion: Once you have concluded your research, the next most important step is to present your findings. Presentation is extremely important as it is the definite medium though which your research is going to be in print for the rest of the crowd. Care should be taken to categorize your thoughts well and present them in a logical and neat manner. A good quality research paper format is essential because it serves to highlight your research paper and bring to light all necessary aspects of your research.

Informal Guidelines of Research Paper Writing

Key points to remember:

- Submit all work in its final form.
- Write your paper in the form which is presented in the guidelines using the template.
- Please note the criteria peer reviewers will use for grading the final paper.

Final points:

One purpose of organizing a research paper is to let people interpret your efforts selectively. The journal requires the following sections, submitted in the order listed, with each section starting on a new page:

The introduction: This will be compiled from reference matter and reflect the design processes or outline of basis that directed you to make a study. As you carry out the process of study, the method and process section will be constructed like that. The results segment will show related statistics in nearly sequential order and direct reviewers to similar intellectual paths throughout the data that you gathered to carry out your study.

The discussion section:

This will provide understanding of the data and projections as to the implications of the results. The use of good quality references throughout the paper will give the effort trustworthiness by representing an alertness to prior workings.

Writing a research paper is not an easy job, no matter how trouble-free the actual research or concept. Practice, excellent preparation, and controlled record-keeping are the only means to make straightforward progression.

General style:

Specific editorial column necessities for compliance of a manuscript will always take over from directions in these general guidelines.

To make a paper clear: Adhere to recommended page limits.

Mistakes to avoid:

- Insertion of a title at the foot of a page with subsequent text on the next page.
- Separating a table, chart, or figure—confine each to a single page.
- Submitting a manuscript with pages out of sequence.
- In every section of your document, use standard writing style, including articles ("a" and "the").
- Keep paying attention to the topic of the paper.
- Use paragraphs to split each significant point (excluding the abstract).
- Align the primary line of each section.
- Present your points in sound order.
- Use present tense to report well-accepted matters.
- Use past tense to describe specific results.
- Do not use familiar wording; don't address the reviewer directly. Don't use slang or superlatives.
- Avoid use of extra pictures—include only those figures essential to presenting results.

Title page:

Choose a revealing title. It should be short and include the name(s) and address(es) of all authors. It should not have acronyms or abbreviations or exceed two printed lines.

Abstract: This summary should be two hundred words or less. It should clearly and briefly explain the key findings reported in the manuscript and must have precise statistics. It should not have acronyms or abbreviations. It should be logical in itself. Do not cite references at this point.

An abstract is a brief, distinct paragraph summary of finished work or work in development. In a minute or less, a reviewer can be taught the foundation behind the study, common approaches to the problem, relevant results, and significant conclusions or new questions.

Write your summary when your paper is completed because how can you write the summary of anything which is not yet written? Wealth of terminology is very essential in abstract. Use comprehensive sentences, and do not sacrifice readability for brevity; you can maintain it succinctly by phrasing sentences so that they provide more than a lone rationale. The author can at this moment go straight to shortening the outcome. Sum up the study with the subsequent elements in any summary. Try to limit the initial two items to no more than one line each.

Reason for writing the article—theory, overall issue, purpose.

- Fundamental goal.
- To-the-point depiction of the research.
- Consequences, including definite statistics—if the consequences are quantitative in nature, account for this; results of any numerical analysis should be reported. Significant conclusions or questions that emerge from the research.

Approach:

- Single section and succinct.
- An outline of the job done is always written in past tense.
- Concentrate on shortening results—limit background information to a verdict or two.
- Exact spelling, clarity of sentences and phrases, and appropriate reporting of quantities (proper units, important statistics) are just as significant in an abstract as they are anywhere else.

Introduction:

The introduction should "introduce" the manuscript. The reviewer should be presented with sufficient background information to be capable of comprehending and calculating the purpose of your study without having to refer to other works. The basis for the study should be offered. Give the most important references, but avoid making a comprehensive appraisal of the topic. Describe the problem visibly. If the problem is not acknowledged in a logical, reasonable way, the reviewer will give no attention to your results. Speak in common terms about techniques used to explain the problem, if needed, but do not present any particulars about the protocols here.

The following approach can create a valuable beginning:

- Explain the value (significance) of the study.
- Defend the model—why did you employ this particular system or method? What is its compensation? Remark upon its appropriateness from an abstract point of view as well as pointing out sensible reasons for using it.
- Present a justification. State your particular theory(-ies) or aim(s), and describe the logic that led you to choose them.
- Briefly explain the study's tentative purpose and how it meets the declared objectives.

Approach:

Use past tense except for when referring to recognized facts. After all, the manuscript will be submitted after the entire job is done. Sort out your thoughts; manufacture one key point for every section. If you make the four points listed above, you will need at least four paragraphs. Present surrounding information only when it is necessary to support a situation. The reviewer does not desire to read everything you know about a topic. Shape the theory specifically—do not take a broad view.

As always, give awareness to spelling, simplicity, and correctness of sentences and phrases.

Procedures (methods and materials):

This part is supposed to be the easiest to carve if you have good skills. A soundly written procedures segment allows a capable scientist to replicate your results. Present precise information about your supplies. The suppliers and clarity of reagents can be helpful bits of information. Present methods in sequential order, but linked methodologies can be grouped as a segment. Be concise when relating the protocols. Attempt to give the least amount of information that would permit another capable scientist to replicate your outcome, but be cautious that vital information is integrated. The use of subheadings is suggested and ought to be synchronized with the results section.

When a technique is used that has been well-described in another section, mention the specific item describing the way, but draw the basic principle while stating the situation. The purpose is to show all particular resources and broad procedures so that another person may use some or all of the methods in one more study or referee the scientific value of your work. It is not to be a step-by-step report of the whole thing you did, nor is a methods section a set of orders.

Materials:

Materials may be reported in part of a section or else they may be recognized along with your measures.

Methods:

- Report the method and not the particulars of each process that engaged the same methodology.
- Describe the method entirely.
- To be succinct, present methods under headings dedicated to specific dealings or groups of measures.
- Simplify—detail how procedures were completed, not how they were performed on a particular day.
- If well-known procedures were used, account for the procedure by name, possibly with a reference, and that's all.

Approach:

It is embarrassing to use vigorous voice when documenting methods without using first person, which would focus the reviewer's interest on the researcher rather than the job. As a result, when writing up the methods, most authors use third person passive voice.

Use standard style in this and every other part of the paper—avoid familiar lists, and use full sentences.

What to keep away from:

- Resources and methods are not a set of information.
- Skip all descriptive information and surroundings—save it for the argument.
- Leave out information that is immaterial to a third party.

Results:

The principle of a results segment is to present and demonstrate your conclusion. Create this part as entirely objective details of the outcome, and save all understanding for the discussion.

The page length of this segment is set by the sum and types of data to be reported. Use statistics and tables, if suitable, to present consequences most efficiently.

You must clearly differentiate material which would usually be incorporated in a study editorial from any unprocessed data or additional appendix matter that would not be available. In fact, such matters should not be submitted at all except if requested by the instructor.

Content:

- Sum up your conclusions in text and demonstrate them, if suitable, with figures and tables.
- In the manuscript, explain each of your consequences, and point the reader to remarks that are most appropriate.
- Present a background, such as by describing the question that was addressed by creation of an exacting study.
- Explain results of control experiments and give remarks that are not accessible in a prescribed figure or table, if appropriate.
- Examine your data, then prepare the analyzed (transformed) data in the form of a figure (graph), table, or manuscript.

What to stay away from:

- Do not discuss or infer your outcome, report surrounding information, or try to explain anything.
- Do not include raw data or intermediate calculations in a research manuscript.
- Do not present similar data more than once.
- A manuscript should complement any figures or tables, not duplicate information.
- Never confuse figures with tables—there is a difference.

Approach:

As always, use past tense when you submit your results, and put the whole thing in a reasonable order.

Put figures and tables, appropriately numbered, in order at the end of the report.

If you desire, you may place your figures and tables properly within the text of your results section.

Figures and tables:

If you put figures and tables at the end of some details, make certain that they are visibly distinguished from any attached appendix materials, such as raw facts. Whatever the position, each table must be titled, numbered one after the other, and include a heading. All figures and tables must be divided from the text.

Discussion:

The discussion is expected to be the trickiest segment to write. A lot of papers submitted to the journal are discarded based on problems with the discussion. There is no rule for how long an argument should be.

Position your understanding of the outcome visibly to lead the reviewer through your conclusions, and then finish the paper with a summing up of the implications of the study. The purpose here is to offer an understanding of your results and support all of your conclusions, using facts from your research and generally accepted information, if suitable. The implication of results should be fully described.

Infer your data in the conversation in suitable depth. This means that when you clarify an observable fact, you must explain mechanisms that may account for the observation. If your results vary from your prospect, make clear why that may have happened. If your results agree, then explain the theory that the proof supported. It is never suitable to just state that the data approved the prospect, and let it drop at that. Make a decision as to whether each premise is supported or discarded or if you cannot make a conclusion with assurance. Do not just dismiss a study or part of a study as "uncertain."

Research papers are not acknowledged if the work is imperfect. Draw what conclusions you can based upon the results that you have, and take care of the study as a finished work.

- You may propose future guidelines, such as how an experiment might be personalized to accomplish a new idea.
- Give details of all of your remarks as much as possible, focusing on mechanisms.
- Make a decision as to whether the tentative design sufficiently addressed the theory and whether or not it was correctly restricted. Try to present substitute explanations if they are sensible alternatives.
- One piece of research will not counter an overall question, so maintain the large picture in mind. Where do you go next? The best studies unlock new avenues of study. What questions remain?
- Recommendations for detailed papers will offer supplementary suggestions.

Approach:

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References	Complete and correct format, well organized	Beside the point, Incomplete	Wrong format and structuring

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