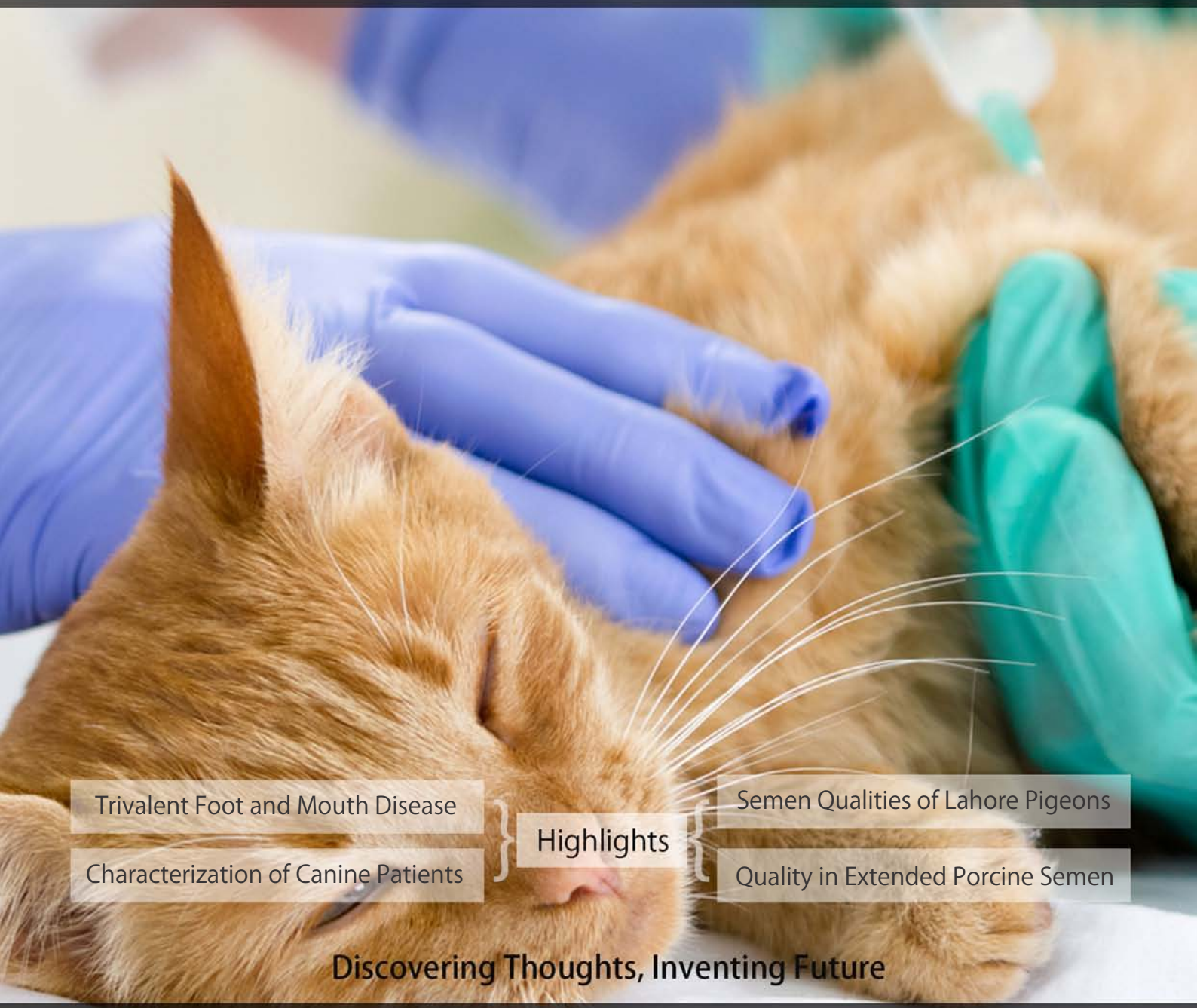
Online ISSN : 2249-4618
Print ISSN : 0975-5888
DOI : 10.17406/GJMRA

GLOBAL JOURNAL

OF MEDICAL RESEARCH: G

Veterinary Science & Veterinary Medicine



Trivalent Foot and Mouth Disease

Characterization of Canine Patients

Highlights

Semen Qualities of Lahore Pigeons

Quality in Extended Porcine Semen

Discovering Thoughts, Inventing Future

VOLUME 18 ISSUE 3 VERSION 1.0

Global Journal of Medical Research: G
Veterinary Science and Veterinary Medicine

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Veterinary Science and Veterinary Medicine
Volume 18 Issue 3 (Ver. 1.0)

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Contents of the Issue

Global Journal of Medical Research: G
Veterinary Science and Veterinary Medicine
Volume 18 Issue 3 Version 1.0 Year 2018
Type: Double Blind Peer Reviewed International Research Journal
Publisher: Global Journals
Online ISSN: 2249-4618 & Print ISSN: 0975-5888

Using Emulsigen®-D as Recent Adjuvant in Trivalent Foot and Mouth Disease Vaccine

By Walaa Shabana, Ismail A., Fathy A., Hind Mohamed & Mossad W.

Abstract- The immunity and protective capability produced by vaccines can vary remarkably according to the kinds of adjuvant being used. Through this work three formulae of the inactivated trivalent FMD vaccine (O pan Asia, A Iran O5 , and SAT2 / EGY/2012) were prepared using different adjuvants including Emulsigen®-D; Montanid ISA 206 and Emulsigen®-D (ED) with aluminum hydroxide gel (ALOH). All of these vaccine formulae were found to be free from foreign contaminants and safe. Also, each vaccine formula was injected in a separate sheep group and serum samples were collected along 38-week post-vaccination for tracing of antibodies against FMDV serotypes by serum neutralization test (SNT) and enzyme-linked immune sorbent assay (ELISA).

Keywords: FMD, SNT, ELISA, emulsigen®-D; montanid ISA 206.

GJMR-G Classification: NLMC Code: QW 70

Strictly as per the compliance and regulations of:

Using Emulsigen®-D as Recent Adjuvant in Trivalent Foot and Mouth Disease Vaccine

Walaa Shabana [α], Ismail A. [σ], Fathy A. [ρ], Hind Mohamed [Ω] & Mossad W. [¥]

Abstract- The immunity and protective capability produced by vaccines can vary remarkably according to the kinds of adjuvant being used. Through this work three formulae of the inactivated trivalent FMD vaccine (O pan Asia, A Iran O5 , and SAT2 / EGY/2012) were prepared using different adjuvants including Emulsigen®-D; Montanid ISA 206 and Emulsigen®-D (ED) with aluminum hydroxide gel (ALOH). All of these vaccine formulae were found to be free from foreign contaminants and safe. Also, each vaccine formula was injected in a separate sheep group and serum samples were collected along 38-week post-vaccination for tracing of antibodies against FMDV serotypes by serum neutralization test (SNT) and enzyme-linked immune sorbent assay (ELISA). Results of SNT and ELISA revealed that the onset of protective antibody titer was achieved early in the Emulsigen® and Emulsigen® with ALOH gel vaccinated groups as it starts at 2nd -week post-vaccination while the onset of protective antibody titer in Montanide ISA 206 vaccinated group started at 3rd-week post-vaccination. Concerning the highest peak antibody titer values were induced by Emulsigen®-D with aluminum hydroxide gel on 8th-week post- vaccination followed by Emulsigen®-D on 10th-week post-vaccination and lastly for Montanid ISA 206 on 12th-week post-vaccination. Concerning the duration of protective immunity against the three serotypes of FMDV included in the vaccine, the results revealed that the longest duration was achieved by the Emulsigen® D alone and with the ALOH adjuvanted vaccines as it lasts for 36-week post vaccination as recorded by the SNT values. The Montanide ISA 206 adjuvanted vaccine group protective SNT antibody titer against the three serotypes lasts for 32- weeks post vaccination. Depending on these findings, it could be concluded that Emulsigen®-D and with aluminum hydroxide gel induce the superior immune response of sheep to the trivalent FMD vaccine over the Montanide ISA 206 adjuvanted trivalent FMD vaccine.

Keywords: FMD, SNT, ELISA, emulsigen®-D; montanid ISA 206.

I. Introduction

Foot-and-mouth disease (FMD) is a viral infectious disease that forms vesicles in the mouth and hooves of artiodactyls, such as pigs, cattle, sheep, and goats resulting in weight loss, reduced milk production and growth delays. The disease can be spread rapidly not only by the excrement of infected animals but also by contaminated feed, vehicles, and humans. Efforts directed to the eradication and prevention of FMD centering on stamping-out policies are controversial and the prevention, and control of the disease using vaccines have become areas of extreme interest Min-Eun et al 2016. Thus, the economic damage is substantial once an outbreak occurs. Therefore, FMD is subject to international regulations for the global trade of both livestock and their products Kitching 1999, Meyer and Knudsen 2001. The administration of vaccines is a highly effective method for preventing FMD.

The causative agent is the FMD virus which has seven serological types identified as O, A, C, SAT1, SAT2, SAT3, and Asia1 Doel and Baccarini 1981, Barnett and Carabin 2002. FMD is characterized by fever, lameness and vesicular lesions on the feet, tongue, snout, and teats with high morbidity and low mortality Satya 2009. The disease is enzootic in Egypt, with many outbreaks having been reported since 1950. The present serotypes of FMD virus in Egypt now are SAT2, A and O. Serotype O was lastly reported Aidaros 2002. Serotype A was firstly recorded in Egypt in 2006 through importation of live animals and resulted in sever clinical signs in cattle and buffaloes Abd El- Rahman et al 2006. The recent FMDV serotype introduction is the serotype SAT2 in 2012, also from the importation of live animals. All these serotypes were isolated and typed by Veterinary Serum and Vaccine Research Institute (VSVRI) and confirmed by World Reference Laboratory (WRL) for FMD, Pirbright Institute, United Kingdom Abd El-Aty et al 2013. Vaccination is the corner stone and effective method for preventing FMD. The selection of an appropriate adjuvant is the most important factor in determining the efficacy of potent vaccines to ensure a protective immunity enables susceptible animals to withstand the disease outbreaks Min-Eun et al 2016.

Emulsigen®-D is an oil-in-water emulsion contains uniformly dispersed micron-size oil droplets, which ensure maximum emulsion stability and decreased viscosity. Micron-size oil droplets also increase the surface area available to antigens, reducing the quantity of oil required in the final produced vaccine. Emulsigen®-D reduces the undesirable side effects associated with other oil-in-water or water-in-oil adjuvants while eliciting a rapid and strong immune response Technologies M. Emulsigen®-D Technical Bulletin 2012. Emulsigen®-D as an adjuvant produces increased immunogenicity because it incorporates dimethyl-dioctadecyl ammonium bromide (DDA), which is a T-cell immune stimulator in Emulsigen®. Its efficacy as an adjuvant was proved in Toxoplasma gondii and

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rabies Hiszczynska-Sawicka et al 2010 and Kaur et al 2010. According to Kaur et al 2010 the DDA contained in Emulsigen®-D induces enhancement of immune responses by increasing the surface area of antigens in oil-in-water emulsions so that antigen spread slowly. Therefore, protection against Aujeszky's disease virus is increased when infected animals have been vaccinated with Emulsigen® plus DDA. Also, aluminum compounds have been known to be the most frequently used adjuvant in veterinary vaccines Gupta 1998. These compounds have been found to induce memory cell responses and long-lasting protection when animals have been inoculated with vaccines, thereby enhancing immune reactions Rimaniol et al 2004. Among them, aluminum phosphate and aluminum hydroxide are the only adjuvants approved for routine use in humans because of their relatively low toxicity Li and Nookala 2007.

In this study, we evaluate comparatively the efficacy of experimental batches of FMD trivalent vaccine (including O pan Asia, A Iran O5 and SAT2 / EGY/2012) using various adjuvants as Emulsigen®-D alone, and with Aluminium hydroxide gel and Montanide ISA 206 aiming to determine the best vaccine formula is having the optimum antigenicity and immunogenicity. The efficacy of prepared vaccine formulae will be tested in dairy sheep as one of the susceptible animal species for FMD.

II. Material and Methods

a) Ethical Approval

The experiment was carried out according to the protocol of the Institutional Animal Ethics Committee, and the authors had permission of the animal owners at the private farms.

b) FMD Virus Strains

Local Foot and Mouth disease virus serotypes O pan Asia, A Iran O5 and SAT2 / EGY/2012 propagated in Baby Hamster Kidney (BHK_{21}) cell line monolayer which was supplied by the Department of Foot and Mouth Diseases Research, Veterinary Serum and Vaccine Research Institute. The titer of the three serotypes was expressed as log $_{10}TCID_{50}$/ml as described by Reed and Muench 1938 and the complement fixation test was carried out according to Health Protection Agency 2009 These viruses were used for the preparation of trivalent inactivated vaccine as well as in serological tests.

c) Animals

i. *Sheep*

Twenty native breed sheep in a private farm free from FMD antibodies as screened by serum neutralization test were divided into four groups (5 animals/group).Each of 3 experimental FMD trivalent vaccines adjuvanted with Emulsigen®-D, Emulsigen®-D with ALOH, Montanide ISA 206, was inoculated as each in a sheep group keeping one group without vaccination as a negative control. The vaccine dose was 1.5 ml/animal inoculated subcutaneously where each dose contains 109 TCID50 of each type of Foot and mouth disease virus serotype.

ii. *Suckling Baby Mice*

Suckling Swiss baby mice, two to four days old, (Charles River Strain, USA) were used for testing the safety of the inactivated viruses according to OIE 2017.

d) Serum Samples

Serum samples were obtained from all sheep groups at the time of vaccination (zero time) then every week till four weeks, every two weeks for 16 weeks, every four week till 32 weeks post vaccination and lastly every two weeks till the end of the experiment (38 - weeks post vaccination). These samples were subjected for estimation of FMD antibodies in vaccinated animals using SNT and indirect ELISA.

e) Cell Culture

Baby Hamster kidney cell line (BHK21) was supplied by Veterinary Serum and Vaccine Research Institute, Abbasia, Cairo using Eagle's medium supplemented with 8-10% bovine serum as described by Xuan et al 2011 and used for application of serum neutralization test, virus titration ,and vaccine preparation.

f) Virus Clarification and Inactivation

Each FMD virus serotype (O, A and SAT2) at the 7th passage on BHK monolayer was treated with chloroform at a concentration of 1.5% (Volume / Volume) as a clarification method before inactivation. Inactivation was occurred using combination 1mM of BEI and 0.04% FA (BEI-FA) according to the method described by Barteling and Cassim 2004 and Ismail et al 2013. Sodium thiosulphate 20% in final concentration of 2% and sodium bisulphite 20% in final concentration of 2% were added after the inactivation process to neutralize the excess of BEI and formalin residues.

g) Formulation of the Prepared Experimental Vaccine Batches

The antigens were added to each of the following adjuvants:

1. Emulsigen®-D (Emulsigen®-D; MVP Technologies, NE, USA),
2. ISA 206 (Montanidetmisa 206 VG; SEPPIC, France)
3. Emulsigen®-D with aluminum hydroxide gel (Rehydragel®HPA; General Chemical, NJ, USA).

The ratio of adjuvant to total volume was 20:80 for Emulsigen®-D as recommended by Technologies M. Emulsigen®-D Technical Bulletin 2012 and Min-Eun et al 2016 and was 50:50 for ISA 206(volume (v/v) as mentioned by El-Sayed et al.2015. For the oil/gel

adjuvant mixture, we added 10% aluminum hydroxide gel. The mixture was stirred at 300 rpm for10 min at 30◦C in a water incubator to form a water-in-oil-in-water blend.

h) Evaluation of the Prepared FMD Trivalent Vaccine

i. *Sterility and Safety Testing*

The prepared vaccine batches were tested for their freedom of aerobic and anaerobic bacteria; fungal and mycoplasma contaminants where vaccines samples were cultured on thioglycolate broth, Sabouraud's, Nutrient agar; phenol dextrose media and mycoplasma medium. The safety of the prepared vaccines was done in baby mice according to OIE 2017.

i) The Potency of the Prepared Vaccines

i. *Evaluation of the Humeral Immune Response*

Serum samples collected from the vaccinated sheep were tested for monitoring of the exhibited FMD antibody titers against the three serotypes by serum neutralization test (SNT) using the technique described by Ferreira 1976 and indirect enzyme-linked immune sorbent assay (ELISA) according to Voller et al 1976.

III. Results and Discussion

The control of FMD is dependent on the vaccination of susceptible animal species with inactivated whole virus vaccines Rodriguez and Grubman 2009. Vaccination with good quality FMD vaccines helps in the prevention of livestock production losses and reduces the overall incidence of the disease Hunter 1998. The selection of adjuvant in FMD vaccine formulation is important for both early and long-lasting immunity and protection. Hence, efforts are focused on developing adjuvant that can promote protective immunity through induction of enhanced and more durable antibody responses Dar et al 2013.

Attention is often directed to improve the potency of FMD vaccine aiming to provide the highest immune level in vaccinated animals to be able to withstand virus infection and accordingly avoid the suggested dramatic economic losses.

Emulsigen®-D is a unique oil-in-water emulsion and contains uniformly dispersed micron-size oil droplets. These Micron-size oil droplets increase the surface area available to antigens, reducing the quantity of oil required in the final vaccine. Emulsigen®-D incorporates dimethyl-dioctadecyl ammonium bromide (DDA) which is a T-cell immune stimulator. According to Kaur et al. 2010, the DDA contained in Emulsigen®-D induces the enhancement of immune responses by increasing the surface area of antigens in oil-in-water emulsions so that antigens spread slowly. The use of ALOH gel in combination with oil is attributed as it is the most commonly used adjuvant in commercial vaccines Rimaniol et al 2004 and a previous report showed that AL induces Th2-type responses in animal models, facilitating the dissemination of antibodies from the injected region Gupta et al 1995 and Brewer et al 1996. Also, the gel was shown to play an important role in memory responses by inducing the differentiation of macrophages Min-Eun et al 2016. The combined components of oil and AL have been used to protect against rabies in bovines Reddy, and Srinivasan 1997. So in this study, we apply the use of Emulsigen®-D and the use of ALOH gel in combination with oil as an adjuvant in foot and mouth disease vaccine and tracing the humeral immune response of sheep upon using these adjuvants.

This work deals with three prepared formulae of inactivated trivalent FMD vaccine (O pan Asia, A Iran O5 and SAT2 / EGY/2012) were prepared using three different adjuvants including Emulsigen®-D; Montanid ISA 206 and Emulsigen®-D with aluminum hydroxide gel The present obtained results revealed that all the prepared FMD trivalent vaccine formulae are free from foreign contaminants and safe inducing no abnormal post vaccination signs in vaccinated sheep in agreement with what recommended for such vaccine OIE 2017.

The antibody titer against the three serotypes (O, A and SAT2) were monitored in the serum samples using the serum neutralization and ELISA tests. Before vaccinating the different sheep groups, we ensure that all sheep involved in the experiment are free from antibody titer against the foot and mouth disease virus.

The results as tabulated in tables no. (1 & 2) and demonstrated by the figures (1-6) revealed that the onset of protective antibody titer was achieved early in the Emulsigen® and Emulsigen® with ALOH gel vaccinated groups as it starts at 2nd week post vaccination while the onset of protective antibody titer in Montanide ISA 206 vaccinated group started at 3rd week post- vaccination. Concerning the highest peak antibody titer values were induced by Emulsigen®-D with aluminum hydroxide gel on 8th-week post-vaccination (3.1, 3.2 & 3.21 log 10 for serotypes O, A & SAT-2 respectively); followed by Emulsigen®-D on 10th-week post-vaccination (2.9, 3.05 & 2.95 log 10 for type O, A & SAT-2 respectively) and then for Montanid ISA 206 on 12th week post-vaccination (2.8; 2.9 and 2.6 log10 for type O, A & SAT-2 respectively) as evaluated by SNT. Concerning the duration of protective immunity against the three serotypes of FMDV included in the vaccine, the results revealed that the longest duration was achieved through the Emulsigen®-D alone and with the ALOH adjuvanted vaccine as it lasts for 36 weeks post-vaccination as recorded by the SNT values. The Montanide ISA 206 adjuvanted vaccine group protective SNT antibody titer against the three serotypes lasts for 32 weeks post-vaccination. So from these results there is a two weeks protection duration difference between the different vaccinated groups.

ELISA results as a confirmatory test came in a parallel manner with those results obtained by SNT. From these results, it is clear that the use of Emulsigen®-D adjuvant and, the addition of ALOH gel have a positive impact on the onset, peak and duration of protective immunity.

The previous results come in parallel with that obtained by Min-Eun et al 2014 as he mentioned that a high level of neutralizing antibodies in the ED + AL or ISA 201 groups exhibited a statistically significant difference from that in the ISA206 group. Regarding cell-mediated immune responses, the ED and ED + AL vaccination groups exhibited statistically significant increases after antigen stimulation in both Th1 and Th2 cytokines, although they exhibited a low level of cytokines.Th1 reactivity was stronger in the ED + AL vaccination group than the ED-only vaccination group. Also, he found that a high level of neutralizing antibodies developed in a short period in the group of dairy goats inoculated with combined ED + AL, proving that Emulsigen®-D in combination with aluminum hydroxide enhances the immune response in both pigs and dairy goats against foot and mouth disease virus.

In conclusion for the present work we found that the use of Emulsigen®-D in sheep has an improvement immunogenicity effect over the use of the Montanide ISA 206 and also the use ALOH in combination potentiate the effects of ED adjuvants in the trivalent FMD vaccine.

Table 1: Mean FMD Serum Neutralizing Antibody Titers (log10 /ml) in Sheep Vaccinated with Trivalent FMD Vaccine using different Adjuvants.

WPV*	Mean FMD Serum Neutralizing Antibody Titers (Log10/Ml) in Sheep Group Vaccinated with Trivalent FMD Vaccine Adjuvanated with								
	Montanide ISA 206			Emulsigen®-D			Emulsigen®-D with ALOH Gel		
	O	A	SAT2	O	A	SAT2	O	A	SAT2
0	0.12	0.1	0.2	0.2	0.15	0.16	0.1	0.13	0.12
1	0.46	0.65	0.49	1.4	1.5	1.15	1.48	1.42	1.52
2	1.23	1.29	0.86	1.62	1.62	1.58	1.71	1.69	1.62
3	1.55	1.56	1.5	1.74	1.81	1.71	1.8	1.82	1.9
4	1.8	1.91	1.76	1.9	1.96	1.95	2.1	2.21	2.3
6	1.95	2.16	1.91	2.32	2.39	2.21	2.76	2.69	2.71
8	2.16	2.43	2.25	2.56	2.79	2.49	3.1	3.2	3.21
10	2.45	2.72	2.49	2.9	3.05	2.95	2.94	3	3.07
12	2.8	2.9	2.6	2.91	2.8	2.9	2.76	2.7	2.9
14	2.74	2.92	2.58	2.64	2.66	2.72	2.61	2.54	2.72
16	2.34	2.68	2.43	2.44	2.43	2.61	2.55	2.4	2.5
20	2.1	2.34	2.21	2.3	2.12	2.44	2.4	2.21	2.31
24	1.95	2.05	2.05	2.05	1.96	2.31	2.25	2.05	2.08
28	1.83	1.8	1.94	1.93	1.8	2.24	2.1	1.86	1.88
30	1.62	1.59	1.6	1.78	1.69	2.05	1.89	1.8	1.8
32	1.5	1.53	1.49	1.66	1.6	1.82	1.72	1.69	1.74
34	1.35	1.46	1.32	1.62	1.55	1.7	1.6	1.6	1.62
36	1.21	1.3	1.05	1.57	1.53	1.6	1.51	1.51	1.55
38	1.05	0.95	0.86	1.51	1.34	1.48	1.35	1.2	1.09

**WPV= week-post-vaccination*

Table 2: Mean FMD ELISA Antibody Titers in Sheep Vaccinated with Trivalent FMD Vaccine using different Adjuvants

WPV*	Mean FMD ELISA Antibody Titers in Sheep Group Vaccinated with Trivalent FMD Vaccine Adjuvanated with								
	Montanide ISA 206			Emulsigen® D			Emulsigen® D with ALOH Gel		
	O	A	SAT2	O	A	SAT2	O	A	SAT2
0	0.4	0.36	0.51	0.51	0.46	0.44	0.32	0.4	0.45
1	0.71	0.92	0.76	1.67	1.81	1.43	1.74	1.7	1.8
2	1.51	1.54	1.13	1.9	1.93	1.86	2	2.07	1.92
3	1.81	1.81	1.76	2.05	2.1	2	2.13	2.12	2.19
4	2.05	2.15	2.02	2.21	2.24	2.22	2.39	2.49	2.71
6	2.23	2.43	2.15	2.6	2.66	2.5	3.04	3	3.02
8	2.42	2.71	2.5	2.81	3.04	2.76	3.41	3.51	3.5
10	2.72	3.05	2.75	3.28	3.32	3.27	3.2	3.19	3.33
12	3.05	3.15	2.86	3.2	3.15	3.14	3.03	3.05	3.21
14	3	3.2	2.85	2.9	2.92	3	2.9	2.81	3.04
16	2.62	2.86	2.71	2.71	2.7	2.92	2.81	2.66	2.77
20	2.38	2.64	2.5	2.62	2.4	2.7	2.71	2.5	2.6
24	2.23	2.29	2.3	2.31	2.24	2.61	2.52	2.3	2.34
28	2.1	2.13	2.21	2.2	2.13	2.53	2.41	2.14	2.15
30	1.9	1.82	1.87	2.02	1.92	2.3	2.15	2.05	2.06
32	1.77	1.79	1.76	1.94	1.86	2.13	2.03	2	2.02
34	1.64	1.74	1.6	1.9	1.8	1.96	1.91	1.89	1.91
36	1.5	1.58	1.31	1.82	1.8	1.91	1.83	1.8	1.81
38	1.29	1.19	1.13	1.79	1.62	1.72	1.61	1.52	1.37

**WPV= week-post-vaccination*

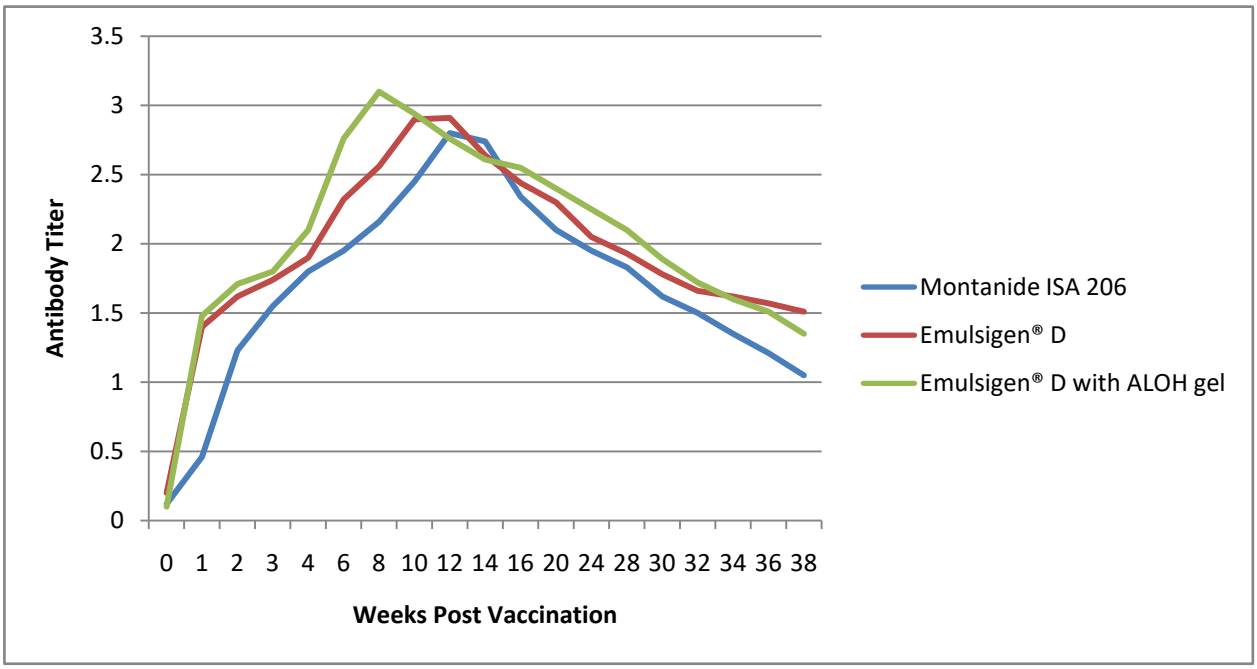

Fig. 1: Mean FMD Serum Neutralizing Antibody Titers (Log /Ml) against Serotype (O) in Sheep Group Vaccinated with Trivalent FMD Vaccine using different Adjuvants

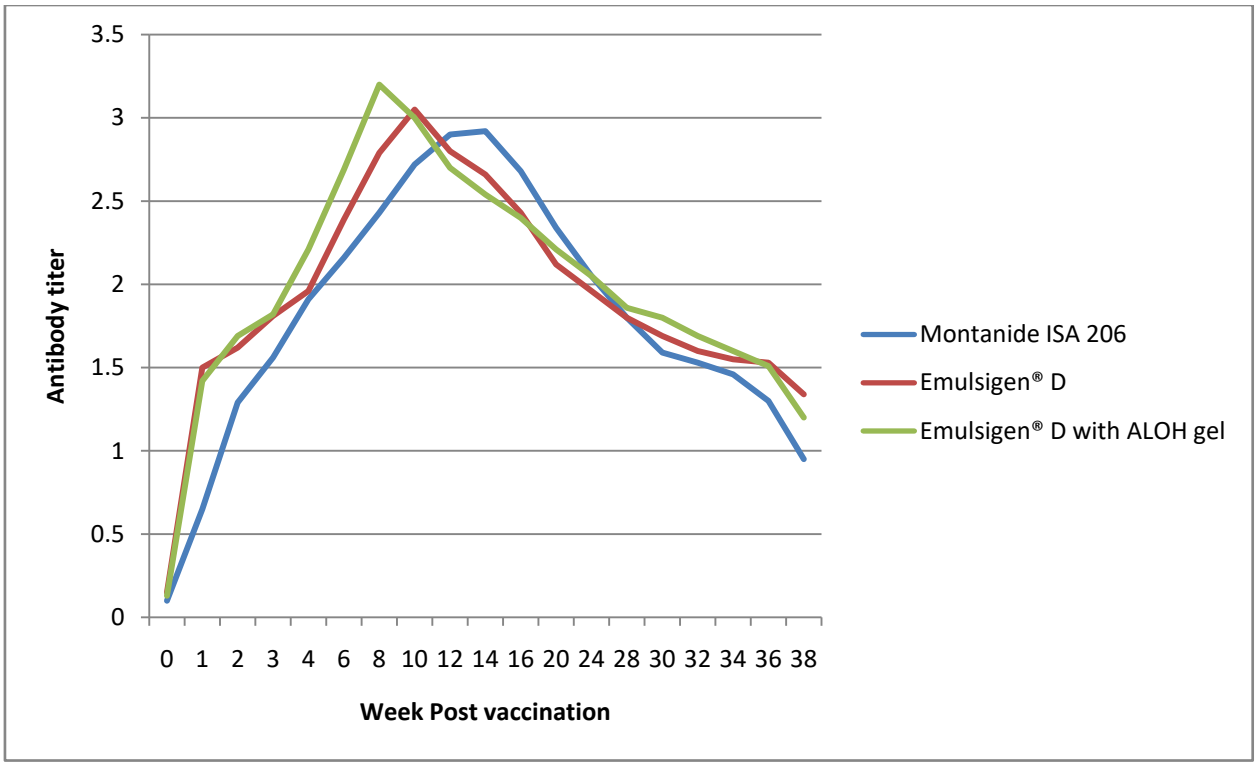

Fig. 2: Mean FMD Serum Neutralizing Antibody Titers (Log /Ml) against Serotype (A) in Sheep Group Vaccinated with Trivalent FMD Vaccine using different Adjuvants

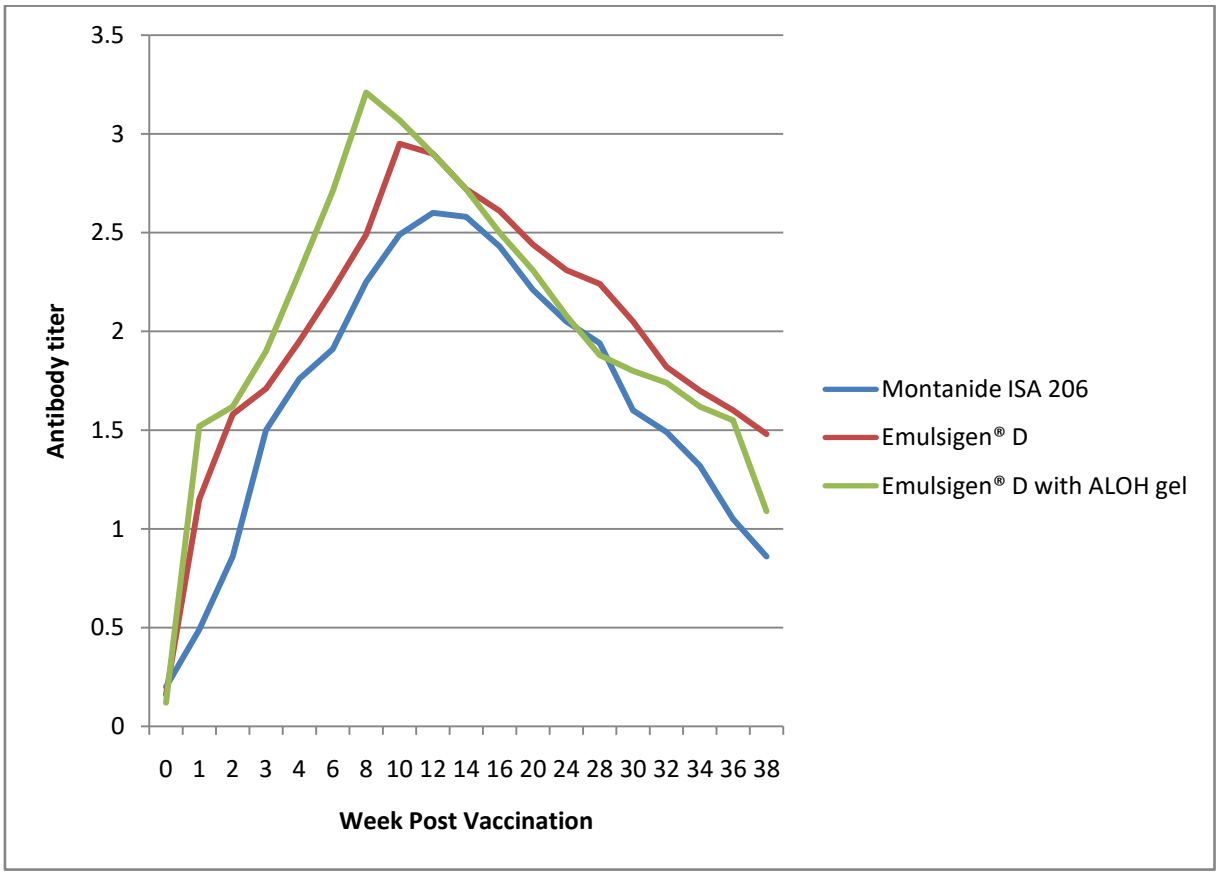

Fig. 3: Mean FMD Serum Neutralizing Antibody Titers (Log /Ml) against Serotype (SAT2) in Sheep Group Vaccinated with Trivalent FMD Vaccine using different Adjuvants

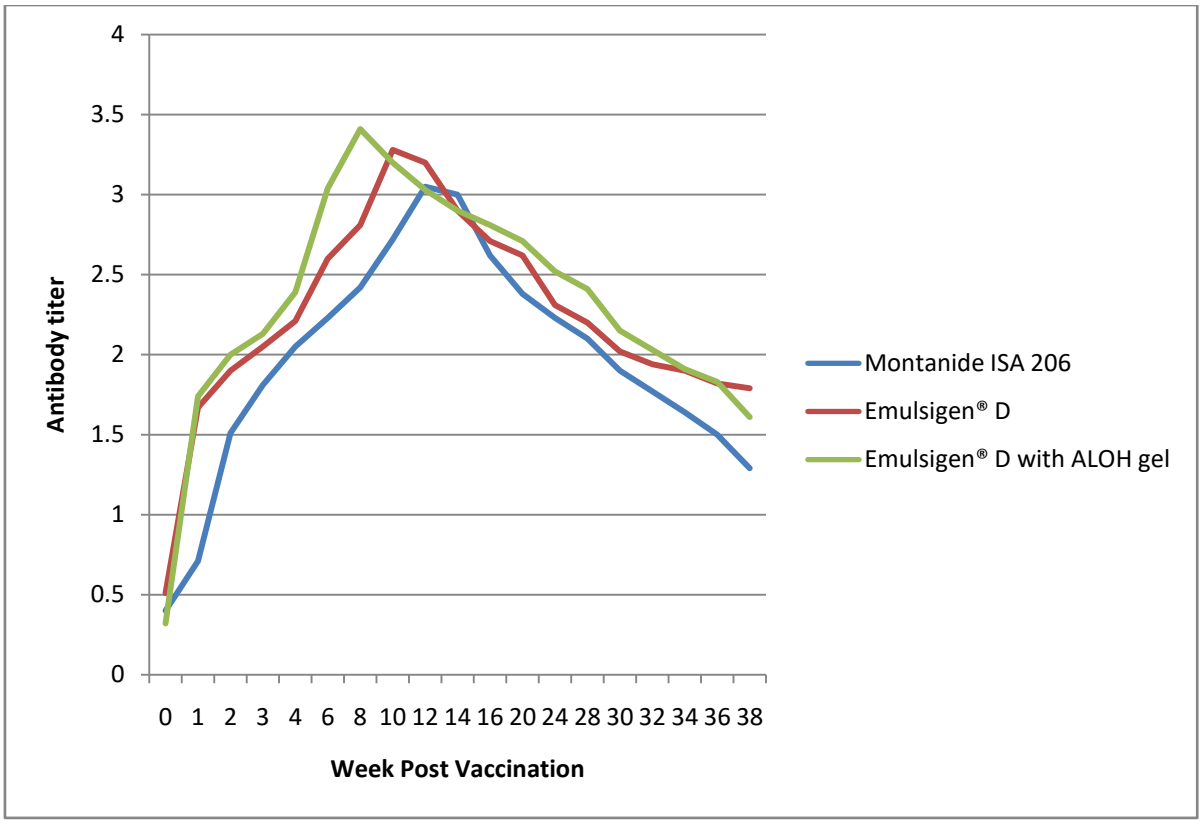

Fig. 4: Mean FMD ELISA Antibody Titers against Serotype (O) in Sheep Group Vaccinated with Trivalent FMD Vaccine with different Adjuvants

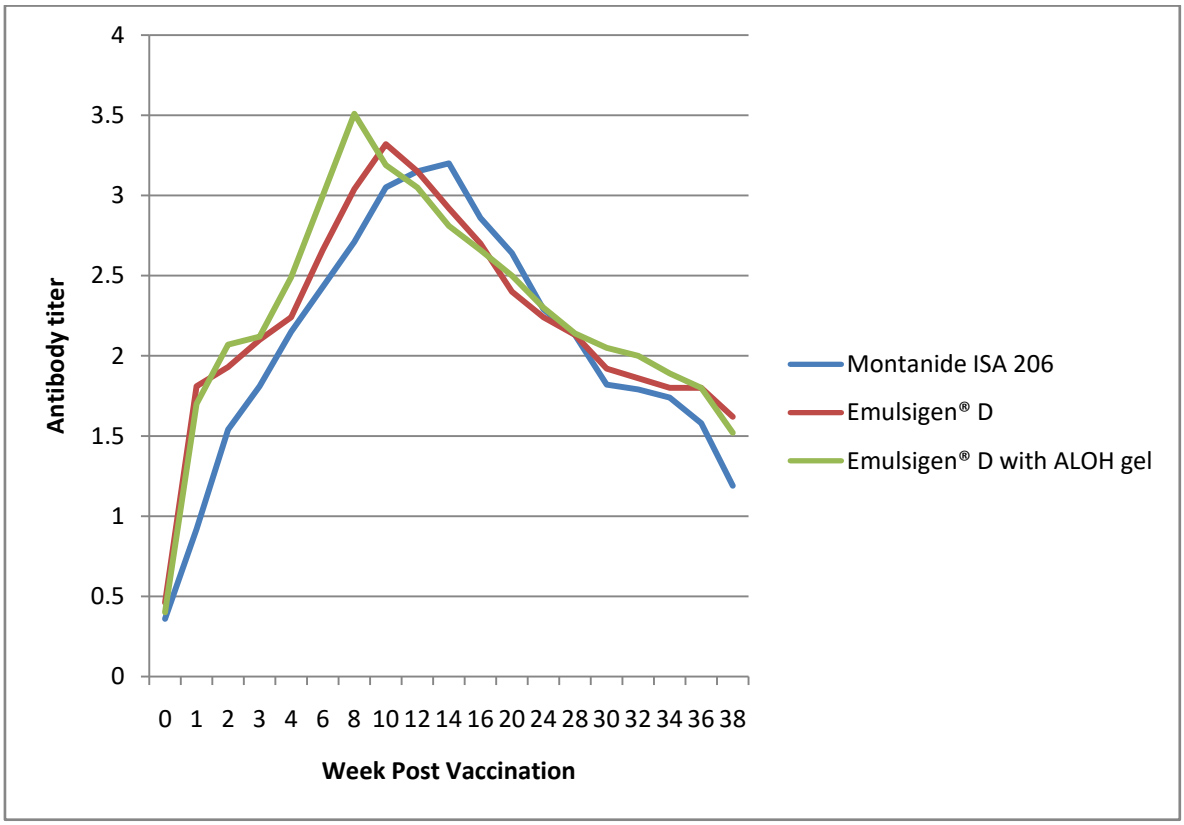

Fig. 5: Mean FMD ELISA Antibody Titers against Seritype (A) in Sheep Group Vaccinated with Trivalent FMD Vaccine with different Adjuvants

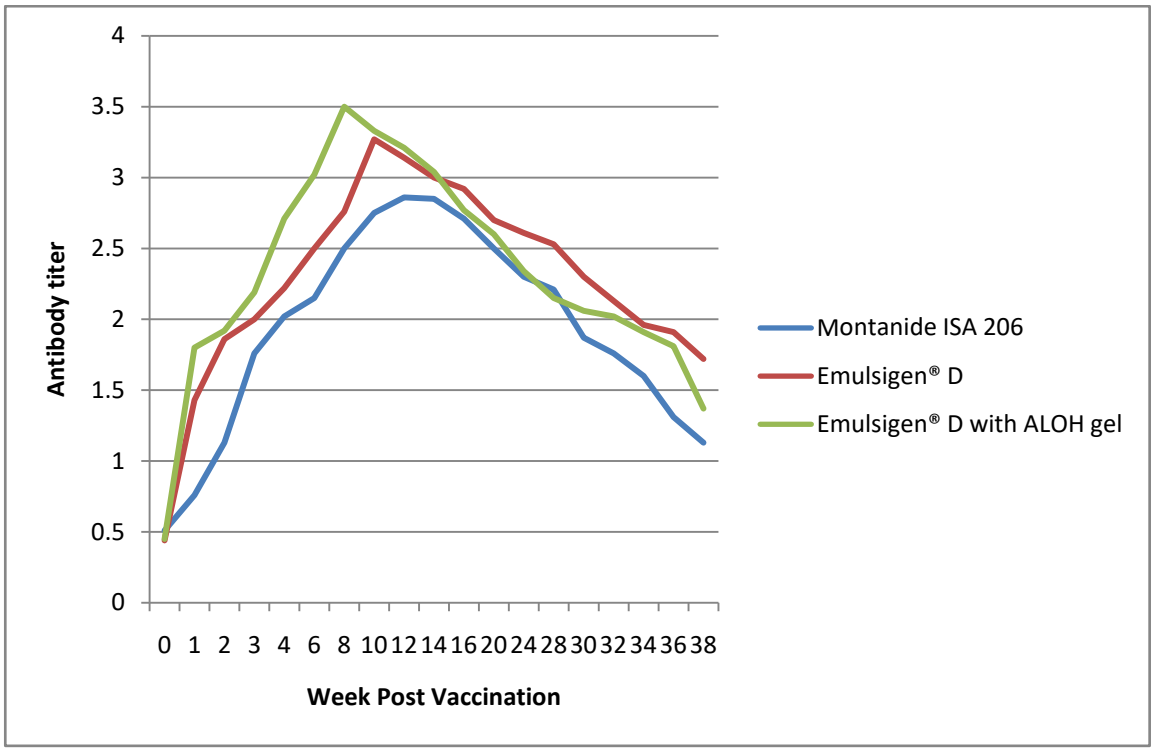

Fig. 6: Mean FMD ELISA Antibody Titers against Serotype (SAT2) in Sheep Group Vaccinated with Trivalent FMD Vaccine with different Adjuvants

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Global Journal of Medical Research: G
Veterinary Science and Veterinary Medicine
Volume 18 Issue 3 Version 1.0 Year 2018
Type: Double Blind Peer Reviewed International Research Journal
Publisher: Global Journals
Online ISSN: 2249-4618 & Print ISSN: 0975-5888

Studies of Semen Qualities of Lahore Pigeons (Columba Livia)

By K. Athis Kumar

Abstract- Ejaculation capabilities and semen characteristics depending on seasonal variations are presented in this paper. The mean temperature range in the study area was 25.6°C (February) -30.5°C (May) and the mean humidity range was correspondingly between 62.5–80.4% (February) and 77.6–83.3% (May). The photoperiod was minimum in February (13h/day) and maximum in May (15.6h/day). Out of 927 collection attempts, 509 attempts ended with successful ejaculation and hence the semen collection rate was 54.9±8.8%. The highest ejaculation rate was 65% in March followed by 63.7% in November, whereas it was low (44.7%) in September. Average volume of semen ejaculated from a bird in one collection was 9.3±1.5μl and the range was 6.1 ± 0.2 μl (September) - 10.8 ± 0.7 μl (November)/ collection ($p>0.05$). The average semen concentration was $4.01\pm0.5x10^9$/ml.

Keywords: *lahore pigeon, semen quality, semen ejaculation and season.*

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

Strictly as per the compliance and regulations of:

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Keywords: lahore pigeon, semen quality, semen ejaculation and season.

I. Introduction

Reproductive performance of male pigeons is directly related to the semen quality as has been recognized in the poultry science that for selecting breeding males or for routinely monitoring their reproductive performance, evaluation of semen is of the intrinsic worth in poultry breeding. The analysis of pigeon's semen characteristic has hardly been possible due to the lack of adequate information about the qualities of the semen. The semen has been collected from birds such as drakes (Setioko and Hetzel, 1984), cocks (Saeid and Al-Soudi, 1975, Lake and Stewart, 1978), ganders (Pawluczuk and Grunder, 1989), turkeys (Burrows and Quinn, 1937; Noirault and Brillard, 1999), pheasants (Mantovaniet al., 1993) and racing pigeons (Cheng et al., 2002) and its qualities have been evaluated in the breeding industry (Sexton, 1977; Setioko and Hetzel,1984). Manual massage is the most frequently used method of semen collection from fowls and turkey (Burrows and Quinn, 1937) and pigeons (Cheng et al., 2002), but use of artificial vagina in ganders (Pawluczuk and Grunder, 1989) and electro ejaculation in drakes (Setioko and Hetzel, 1984) were also described systematically. The first paper regarding the yield of pigeon semen by manual massage was documented as early as 1941 (Owen); this method is safe, undisruptive, and not stressful to the donor. Cheng et al (2002) have evaluated the annual change in the semen qualities of racing pigeons but there is no report regarding the semen characteristic of Lahore pigeons so far. Since racing pigeons take more amount of feed than the Lahore pigeons, the semen qualities of the latter would possibly vary from the former.

The semen of birds has a significant effect on their fertility and reproductive potential (Sexton, 1983), which is affected by environmental conditions (McDaniel et al., 1995, 1996) and nutrition provided to the birds (Athis Kumar and Anatha Rajan, 2016). Similar studies conducted in domestic fowls reveal that semen quality is influenced by seasons (Saeid and Al-Soudi, 1975). Likewise, ambient temperature greater than 31°C notably affects the rooster sperm motility, viability, and fertilizing potential of domestic fowls (McDaniel et al., 1996). Further, change in atmospheric temperature at ejaculation is an important exogenous physiological factor that affects the sperm motility (Ashizawa and Sano, 1990; Wishart and Wilson, 1999). There was a negligible effect of a high ambient air temperature (50-60°C) on the breeding potential of rock pigeons (Arieli et al., 1988) in the nature, but deviation in the ejaculation performance of males in response to ambient temperature has not been made clear. The increase in fertility percentage of eggs is related to higher sexual efficiency and better semen quality of males (Mariety, 2005). The quality and quantity of semen produced by birds are the deciding factors for the fertility of eggs (Sexton, 1983), which usually varies depending on environmental conditions (McDaniel et al., 1995). According to Owen (1941), the semen quantity of domestic pigeons is not at all related to the size of the

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bird but it appears to be related to the seasons of year. The semen is of superior quality during the spring and summer, so that the fertility of eggs is relatively high during these two seasons (Cheng et al., 2002; Athis Kumar, 2017).

Evaluation of semen qualities of Lahore pigeons has hardly been known so far. The semen quality evaluation in Lahore pigeons (Columba livia) and ejaculation performance in terms of submission of male birds and the colour of their cloacal mucosal secretion during collection were investigated and presented in this paper.

II. Materials and Methods

a) Experimental Design

Two year old male Lahore pigeons (Columba liviadomestica; family: Columbidae; order: Columbiformes) weighing about 350g were chosen as the experimental birds for this study and grown in the Animal House at the Zoology Department of Sivanthi Anthithanar College situated at 77°13'E and 8°29'N in Tamilnadu. 20 pigeons were housed in separate lofts of 40 x 30 x 50cm size. The lofts were constructed with wooden frame, steel plated roof and wire mesh floor and lateral sides. These lofts were kept at a height of 2.5′ from the ground level for reducing dampness facilitating the rapid spreading of pathogenic germs. All the lofts were equipped with a wind insulation to the north under native environmental conditions (24°07′ N, 120°40′E), where birds received a natural photoperiod of 12L: 12D to 15L: 9D throughout the experiment. Feed mixture (in Table 1) was given at the rate of 45 grams / bird / day and drinking water was provided at the rate of 60 ml/ bird/day. Vitamins required for the birds were provided along with the drinking water at the rate of 5ml of Vimeral® (vitamin mix)/ 1 liter water. This feed composition was maintained throughout the study period for feed uniformity in the experimental pigeons groups.

Table 1: Composition of Basal Feed

Ingredients	Percentage
Wheat Grains	35 %
Finger Millet	15 %
Pearl Millet	15 %
Green Pea	30 %
Grid*	4.97 %
Vimeral ® **	0.5 ml / Pair

** Grid: I kg contains 100 g charcoal, 100 g egg shell, 75 g limestone, 150 g table salt and 575 g brick powder; ** Vimeral ®: 1ml contains vitamin A - 12,000 IU; Vitamin B_{12} - 20 mcg; vitamin D_2 - 6,000 IU; and vitamin E - 40 mg.*

Pigeons were maintained in the lofts for 2 weeks period to enable them to adjust the experimental conditions and to be friendly enough to the operator. Semen collection was performed for over a 2-weeks period and those pigeons which did not produce semen by the end of the training period were excluded from the experiment. Finally, 10 pigeons with optimal ejaculation performance were used throughout the study. Semen was collected from each male pigeon twice in a week (Monday and Thursday) between 8.00 AM and 10.00 AM.

b) Semen Collection

Semen collection was performed from each and every bird all the times only by the investigator to avoid variations in handling performance of pigeons. The technique described by Owen (1941) was employed while collecting the semen. The pigeon was caught and held with its chest positioned against the collector's belly. Tail feathers around the cloaca were clipped before semen collection to prevent contamination of the ejaculate and to have clear view of the cloacal opening. Its wings were stretched and then closed to relax the birds while fixing the main feathers in order. Afterward, massage was exerted by applying pressure with thumbs and index fingers, up and down the exterior of the pygostyle. Thumbs were rhythmically pressed on the rump region, while the index fingers massaged the opposite abdominal region in the vicinity of cloaca for a few rounds. Concurrently, the middle finger of the right hand pressed the os pubis, just below the vent. If the positions of the fingers were correct, the cloaca opened wide showing two fleshy folds. Then, the little finger on the left hand was hooked under the right wing while the other fingers were extended along the donor's back. The donor was then turned onto its back and lay on the left palm. The tail of the pigeon was free to move and spread. Thereafter, the thumb and index finger of the left hand were rhythmically pressed against the opposite sides of the caudal bone. The milking action of thumb and index finger could exert on the base of the cloacal projection while the middle finger was pressed deeply between the pubis bones. After 2 seconds of massage, a small drop of white semen flew from the opening of the cloaca. Massage was continued for about 30 seconds and semen was aspirated into a micropipette equipped with a fine tip. The quantity of semen was directly measured with the micropipette. If there was no ejaculate within 30 seconds, the donor was returned to his loft, and a second attempt was made after 15 min.

c) Submission Test

Responses of the pigeons while collecting the semen were noted and classified into submissive, tolerant, and resistant types. In the submissive response, pigeon was calm and waving the tail to show the cloacal opening; in the tolerant response, it was slightly struggling but allowed semen collection; and in the resistance, pigeon struggled by flapping and attempted to escape.

d) Testing the Cloacal Mucosa Colour

The colour of the cloacal mucosa was tested at the time of massaging for semen collection. The colour of the everted membrane folds of cloacal opening was recorded, which was either red or pink or pale depending on the redness of the membrane.

e) Semen Quality Evaluation

Volume of semen was measured directly when it was aspirated from the cloacal vent using a micropipette. The semen was examined under a compound light microscope for contamination with blood, feces, and uric acid. To estimate the sperm motility, semen samples were diluted 20-fold using Beltsville poultry semen extender (Sexton, 1977) and 10μl of the diluted semen was taken and placed over 10 slides which were then covered by a cover glass (18 x 18 mm) and viewed under the microscope. Motility was expressed as the percentage of motile spermatozoa with moderate to rapid progressive movement. To estimate the sperm concentration, semen was diluted to 1:200 with a weak eosin solution (Brillard and McDaniel, 1985) and sperm cells were counted using a hemocytometer. Sperm morphology was examined microscopically (500x) in smears stained with nigrosin and eosin (Blom, 1950). Viable cells which were impermeable to eosin and dead cells which were permeable to eosin were assessed from the total count of 100 spermatozoa/ slide.

f) Statistical Analysis

Data obtained from this experiment was subjected to one-way ANOVA, using SPSS (1997) computer software. The significant differences among the means of different dietary treatments were analyzed with the Duncan multiple range test (Duncan, 1955).

III. Results

Seasonal variations in the ejaculations and semen collection rates are shown in the table-1. The mean temperature range in the study area was 25.6°C (February) -30.5°C (May) and the mean humidity range was correspondingly between 62.5–80.4% (February) and 77.6–83.3% (May). The photoperiod was minimum in February (13h/day) and maximum in May (15.6h/day). Out of 927 collection attempts, 509 attempts ended with successful ejaculation and hence the semen collection rate was 54.9±8.8%.The highest collection rate was 65% (52/80) in March followed by 63.7% (51/80) in November, whereas significantly lowest collection rate was 44.7% (34/76) in September.

Table 1: Annual Variation in the Semen Collection and Volume of Semen in 2017

Month	Average Temperature (°C)	Relative Humidity (%)	Average Photo Period (Hours/Day)	Collection Attempts	Ejaculations (N)	Collection Rate (%)
January	27.0 (24.2–30.2)	68.4–75.0	13.5	80	38	47.6
February	25.6 (21.7–29.2)	62.5–80.4	13.0	70	40	57.1
March	27.5 (25.6–33.1)	63.8–72.4	13.7	80	52	65
April	27.7 (24.7–32.5)	60.5–70.9	14.3	74	46	62.2
May	30.5 (27.2–32.3)	77.6–83.3	15.6	80	45	56.3
June	29.4 (24.4–29.1)	77.1–82.7	15.5	70	41	58.6
July	28.6 (24.6–29.1)	75.1–82.8	15.4	79	46	58.2
August	29.2 (24.5–29.3)	63.5–79.4	14.7	80	37	46.3
September	29.4 (25.1–31.4)	74.0–81.8	14.0	76	34	44.7
October	29.5 (24.5–30.4)	71.1–86.2	13.2	80	36	45.0
November	29.1 (23.7–30.8)	67.9–71.1	12.3	80	51	63.7
December	28.6 (23.4–30.2)	67.1–72.2	12.4	78	43	55.2
Total/Mean ± SEM				927	509	54.9±8.8

[a] denotes the significance (P < 0.05) and [b] denotes the significance (P > 0.05).

Table-2 clearly depicts the seasonal changes in the semen characteristics of Lahore pigeons. Average volume of semen ejaculated from a bird in one massage collection was 9.3±1.5μl and the range was 6.1 ± 0.2 μl (September) - 10.8 ± 0.7 μl (November)/ collection ($p>0.05$). The average semen concentration was $4.01\pm0.5x10^9$/ml. The highest semen concentration ($4.5\pm0.3x10^9$/ml) was observed in March and November and the least semen concentration was observed in July ($3.4\pm0.2x10^9$/ml). The statistical significance was $p<0.05$. The mean value of sperm motility was 76.3±8% per ejaculate, the highest motility (83±3%) was noted in March and November ($p<0.05$) while the lowest motility (70±2%) was in July and October ($P>0.05$). The average sperm viability was 71.8±10.2% and the viability range was 61±3% (September) - 78±5% (March and November). The statistical significance was $p<0.05$. Sperm viability and sperm motility were positively correlated ($r=0.94$; $P<0.05$). The overall ejaculation performance was best during March and November compared to other months of a year but very poor in September. There were 5 -16 granular crystals of uric acid per ml of semen, which were in large proportions during March ($p>0.05$) and least proportion

during September ($p<0.05$). The average number of uric acid crystals was 8.4±5.6/ml. The mean blood cells count, which included Red blood cells, pus cells, epithelial cells and testicular cells, in the semen was 25.2±3x10^3/ml and it was the highest (29.1±3x10^3/ml) during August while lowest (22.0±3x10^3/ml) during November. The average number of faecal droplets in the semen was 14.6±2.1/ml; the highest level of faecal contamination was 17±3/mlin August ($p<0.05$) and lowest level of contamination was 12±2/ml in November ($p>0.05$). Uric acid crystals, faecal droplets and blood cells were contaminants found in almost all the ejaculates but they were in low level. Faecal droplets were relatively in higher proportions in the ejaculates of tolerant and resistant types. Abnormal spermatozoa with coiled, hooked, or ruptured heads and coiled or tailless spermatozoa were found in almost all ejaculates but they accounted only 10% of total cell counts in the samples.

In the total of 509 ejaculates, 57% of donors (290) were found to be submissive to the massage method of semen collection, 28% of donors (142) were tolerant and the remaining 15% of donors (77) were resistant during semen collection (Fig. 1). Pigeons became more and more submissive to the semen collector as they gained experience of handling in course of time.

While examining the colour of cloacal membrane during semen collection, 39% of donors (198) showed reddish cloacal membrane, 43% of donors (219) had pink coloured cloacal membrane and 18% of donors (92) had pale coloured cloacal membrane (Fig. 2).

Table 2: Seasonal Changes in the Semen Characteristics of Lahore Pigeon

Month	Semen Volume (µl/D/Bird)	Concentration of Sperms (10^9/Ml)	Sperm Motility (%)	Sperm Viability (%)	Uric Acid Crystals (No./Ml)	Blood Cells (10^3/Ml)	Faecal Droplets (No./Ml)
January	9.2±0.3^a	4.0±0.2^a	79±3^a	78±6^a	10±2^a	26.4±3^a	15±3^a
February	9.2±0.2^b	4.1±0.2^b	79±2^b	76±5^a	11±2^a	25.1±6^a	16±3^b
March	10.7±0.6^b	4.5±0.3^a	83±3^a	78±5^a	14±2^b	23.1±2^b	13±2^a
April	10.4±0.3^b	4.3±0.2^b	82±2^a	76±6^b	12±3^b	24.3±3^b	13±3^b
May	8.4±0.3^a	4.1±0.2^b	79±2^b	75±5^b	11±2^a	26.7±5^b	14±2^b
June	10.3±0.6^a	3.6±0.3^a	73±2^b	70±3^b	8±2^b	27.4±6^a	15±3^a
July	10.8±0.6^a	3.4±0.2^a	70±2^a	68±5^b	7±3^a	28.2±4^b	16±2^a
August	6.4±0.3^b	4.1±0.2^a	68±3^a	62±4^a	6±2^b	29.1±3^a	17±3^a
September	6.1±0.2^b	4.2±0.1^b	69±4^b	61±3^a	5±2^a	24.3±4^b	17±1^b
October	10.2±0.4^b	3.5±0.2^a	70±2^b	63±3^a	6±1^b	22.1±2^a	13±2^a
November	10.8±0.7^b	4.5±0.3^a	83±3^a	78±5^b	13±1^a	22.0±3^b	12±2^b
December	9.4±0.3^a	4.1±0.2^b	79±3^b	73±6^b	10±1^a	24.2±5^b	14±1^b
Total/Mean ± SEM	9.3±1.5	4.01±0.5	76.3±8	71.8±10.2	8.4±5.6	25.2±3	14.6±2.1

^{a} denotes the significance ($P < 0.05$) and ^{b} denotes the significance ($P > 0.05$).

IV. Discussion

As in racing pigeons, in the Lahore pigeons also, there is a heavy fluctuation in the semen quality and quantity. Ejaculation performance of domestic pigeons was considerably affected by season, even though pigeons (Columba livia) are in fact not typically seasonal breeders (Arieli et al., 1988; Johnston, 1998). Breeding activity of pigeons is high during the spring and summer while low in autumn and winter and hence many birds laid single eggs during the autumn and winter (Riddle, 1971; Janiga, 1985; Janiga and Kocian, 1985), which is mainly due unfavourable weather conditions and shortening of photoperiod that inhibits the gonads development in pigeons (Lofts et al. 1966, Murtonet al. 1973). Similar effect of photoperiod was also demonstrated in Mallards in which increasing photoperiod in the spring and summer has promoted the gonadal growth and increasing plasma testosterone levels (Hasse, 1983). Likewise, most domestic chickens produce high volume of semen during the spring (Saeid and Al-Soudi, 1975). The present study reveals that the ejaculation performance of Lahore pigeons is high during the spring and summer seasons which have characteristic longer photoperiods and low in the autumn and winter seasons which have almost neutral photoperiods under South Indian conditions. The results of present study therefore coincide with the findings of Lofts et al. (1966), Riddle (1971), Murtonet al. (1973), Saeid and Al-Soudi (1975), Hasse (1983), Janiga, (1985), Janiga and Kocian (1985), Johnston (1998) and Cheng et al (2002). For that reason, the major reproductive periods are during these two seasons, even though pigeons will breed throughout the year. After building nests, pigeons perform copulation several times daily, but semen can be obtained hours after first successful ejaculation on the same day. In some cases, pigeons failed to ejaculate and often became aspermic

after first success. However, sperm count is restored after at least 2 days of rest from sex. Therefore, daily semen collection would definitely lead to inferior quality semen from pigeons.

According to Owen (1941), the normal semen quantity of household pigeons is 10 - 20 μL per ejaculate, containing roughly 5-6 million spermatozoa. In racing pigeons the volume of ejaculate is 8.5 -13.5μL (Cheng et al., 2002). In the meantime, in Lahore pigeons the volume of semen per ejaculate is 6.1 -10.8μL, which is comparatively less than the ejaculate of racing pigeons. In general, racing pigeons feed more amounts of food grains than the Lahore pigeons and remain vigorous in the flight activities, so that the semen volume might be higher in comparison to Lahore pigeons. A similar view was also reported by Owen (1941) and Cheng et al (2002). Owen (1941) further concluded that in domestic pigeons, the volume of semen per ejaculate does not appear to be related to the size of birds, which also agrees with the results of present study. But, in chicken and turkey, the volume of semen per ejaculate varies greatly depending on the bird's size and nutrition provided to them because of their large sized internal and external reproductive organs (Lake and Stewart 1978; de Reviers and Williams, 1984), which have variable number of Sertoli cells in testis, that determine the rate of semen production (de Reviers and Williams, 1984).

It is generally accepted that high concentration of spermatozoa is slightly directly proportional to the volume of semen per ejaculate. High volume of semen in the ejaculate contains more amount of seminal plasma while low volume of semen in the ejaculate contains relatively low amount of seminal plasma which is the deciding factor of sperm concentration. All the experimental animals are young adults, semen volume and sperm concentration are always positively correlated ($r = +0.32$; $p<0.005$). Since the reproductive activity is high during the spring and summer, the sperm concentration is relatively high in these seasons compared to that during the winter and autumn. In this context, the present study is in confirmation with the findings of Murtonet al. (1973), Janiga, (1985), Janiga and Kocian (1985), Johnston (1998) and Cheng et al (2002).

This study makes out a clear point that physiological activities of birds also affect the semen volume per ejaculate and sperm concentration therein. Semen volume and sperm concentration were comparatively low during February, August and September, in which the natural molt occurred in pigeons. At the time of molting, major portion of metabolic energy is diverted to the re-growth of remiges (Decuypere and Verheyen, 1986; Brake, 1993) and decline in the testosterone level in the blood (Zeman et al., 1990), so that there was a marked decrease in semen volume and sperm concentration (Zeman et al., 1990). This decline in testosterone adversely affects the semen quality and reproductive activities (Zeman et al., 1990; Stunden et al., 1998). Similarly, in the male ring dove, semen volume and sperm concentration have been decreasing during the molting period because of reduced level of androgen (Cheng, 1979). Molting is influenced by slightly long photoperiod, health of the bird, decreased plasma thyroxine and increased triiodothyronine (Decuypere and Verheyen, 1986; Brake, 1993). These altered physiological conditions may disturb the reproductive processes, resulting in marked reduction in semen volume and sperm concentration. This study confirms that 3/10 of the male Lahore pigeons experienced a reduced semen volume and sperm count during molting.

Cheng et al (202) demonstrated that ambient temperature in which pigeons are living affects the ejaculation, semen volume and sperm concentration. Low ambient temperature (<15 °C) creates cold stress that forces the bird to reserve energy for maintenance of body temperature, resulting in a lack of sex drive. Similarly, heat stress (>32°C) creates heat induced sub-fertility, promoting non-viability of sperms (McDaniel et al., 1996), but this condition is reversed when the birds are exposed to suitable temperature range. This rapid response of the domestic fowl to elevated ambient temperatures is not related to impaired sperm formation (McDaniel et al., 1996) because spermatogenesis takes 10 to 12 days followed by 1 to 5 days for sperm to pass through the excurrent ducts (Lake, 1984). However, the ambient temperature has only a negligible effect on the pigeon sperm concentration as recorded by Cheng et al (2002). Thus, thermal stress disturbs semen output rather than production. According to Cheng et al (2002), temperatures between 19 and 24°C are critical for optimal ejaculation performance. Ejaculated spermatozoa of many bird species showed decreased in vitro motility at 40°C, but motility was restored at 30°C (Ashizawa and Sano, 1990; Wishart and Wilson, 1999). Pigeon spermatozoa diluted with Beltsville poultry semen extender exhibited vigorous motility at 30°C, as in other species. The ambient temperature range under Tamilnadu condition is 21.7 -32.5°C and hence there would be no possibility for inhibition either by heat stress or by cold stress. The ejaculation performance, semen volume and semen count are mainly due to the combined effects of temperature condition and photoperiod.

For breeding purposes, the challenge is to maximize the production of viable or functional spermatozoa per ejaculate (Sexton, 1983). Sexton (1983) and Cheng et al (2002) reported that, for effective fertilization and egg production, the semen should contain 50-80% of viable motile sperms. In the present study, the percentages of motile sperms are within the range of 70±2 - 83±3% and those of viable sperms are within the range of 61 ±3 - 78.5±3%, which are in the ideal range for maximum reproductive performance of Lahore pigeons.

In addition to sperms, there were uric acid crystals, blood cells, and faecal droplets in the semen. About 6-14 urate crystals were observed per ml of semen, which indicated that uric acid was released during the collection of semen or partly due to retention of a few drops of uric acid in the cloacal pouch before collection. Likewise, 12-16 fecal droplets were noted in every one ml of semen, which denotes that fecal matter comes to the semen either by their release during the collection of semen or by retention of such fecal granules in the cloacal pouch before collection. Ciliates that are the products of rete testis (de Reviers and Williams, 1984) are also found in the ejaculates, but these ciliates do not seem to affect pigeon fertility (Owen, 1941). Blood cells in the semen are probably the result of rupture of capillaries in the mucosal wall of the cloaca, which are visualized in the semen when it is examined under a microscope. Red blood cells, epithelial cells, testicular cells and pus cells were found in the semen, but Owen (1941) and Chang et al (2002) strongly believe that these cells do not affect the fertility of pigeons. From the present observation, it is clear that such contamination of semen with uric acid, blood cells and fecal droplets is generally low in submissive pigeons, and that semen of tolerant and resistant pigeons is more contaminated with these things. Since, the feces, uric acid and semen are simultaneously discharged through cloacal pouch; such contamination cannot be avoided in birds (Tan, 1980). Semen contaminated with fecal droplets and uric acid is generally not good for artificial insemination because contaminated semen can hamper the fertility in ducks (Tan, 1980). Therefore, it is advised that to avoid contamination with uric acid or feces, the collection frequency should be at least twice weekly and submissive birds should be selected for semen collection (Sexton, 1983; Noirault and Brillard, 1999).

In avian species, ejaculation is initiated by sexual arousal, starting with the ejection of semen from the muscular portion of vas deferens into the urodeum; the erection of fleshy cloacal folds is the sign of a sexually aroused male. Having reached the urodeum, semen gets transported into proctodeum where the edematous phallic folds are visible and mucosal folds are engorged with lymphatic fluid (Lake, 1981; Knight et al., 1984). The filling of the sinus network under the phallic folds resulted in varying degrees of redness of the cloacal mucosa surface, indicating the level of sexual arousal. Sexual arousal is the maximum when the cloacal membrane is red in colour because of passage of more blood to sinus networks in the phallic folds. Sexual arousal is enough for semen collection when the cloacal membrane is pink coloured. A pale membrane indicated that sexual arousal was not sufficiently initiated in these males. Ejaculation rarely occurred when the mucosal membranes were pale, and less semen was ejaculated in an attempt.

The submissive pigeons allow the easy semen collection using manual massage and the tolerant ones let the collectors to collect samples but with little difficulty. On the other hand, the resistant ones get nervousness during semen collection. This phenomenon was more noticeable during the molt stages. Some resistant pigeons became submissive during the successive attempts due to gradual decrease in nervous temperament; this nervous temperament of certain pigeons impaired the sexual arousal, and no ejaculate could be obtained (Lee et al., 1999). These pigeons normally exhibited signs of fear (Craig et al., 1983; Shabalina, 1984; Lee et al., 1999), which is a stress factor associated with poor semen production in roosters pigeons (Lee et al., 1999). In ducks, this stress also inhibited the reflex response to massage (Tan, 1980).

V. Conclusion

The present study concludes that, though copulation is going on several times every day, manual semen collection can be done twice in a week in pigeons for the purpose of artificial insemination, and that in Lahore pigeons, as in the racing pigeons, ejaculation of semen is high during the spring and summer because of ambient temperature and long photoperiods. Ejaculation performance is high in March and November and low in September. The volume of ejaculated semen, sperm concentration, motile sperms and viable sperms are high during March and November but least in September. Semen qualities such as percentage of motile sperms and viable sperms are between 70 and 86% in the Lahore pigeons, so that semen collected in all the months are ideal for artificial insemination after dilution with Beltsville poultry semen extender. This paper recommends that for breeding purpose semen should be collected from submissive donors which are providing a large volume of semen containing a large proportion of motile and viable sperms.

Acknowledgements

The author is thankful to the Principal - Sivanthi Athithanar College, Pillaiyarpuram, Kanyakumari District, Tamilnadu - for providing necessary facilities and to Dr. R. Anantha Rajan - Pioneer Kumaraswamy College, Nagercoil, Kanyakumaridistrict, Tamilnadu - for valuable technical instructions.

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Global Journal of Medical Research: G
Veterinary Science and Veterinary Medicine
Volume 18 Issue 3 Version 1.0 Year 2018
Type: Double Blind Peer Reviewed International Research Journal
Publisher: Global Journals
Online ISSN: 2249-4618 & Print ISSN: 0975-5888

Characterization of Canine Patients with Transmissible Veneral Tumor in Veterinary Hospital of San Carlos of Guatemala University of the Year 2016

By Rivera-Guirola, Eduardo-Andrés, Villatoro-Chacón, Daniela Mariel
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Abstract- Transmissible venereal tumor (TVT) is a sexually transmitted neoplasm that affects the external genitalia mainly although extragenital presentation has been reported. Its prevalence is variable, with developing countries presenting the highest rates due to large populations of stray dogs and poor breeding control. In the present study the prevalence of TVT was determined in the population that attends the Veterinary Hospital of the San Carlos of Guatemala University, being this the only university in the country that provides veterinary service to the public. We analyzed 1,125 medical records of patients who attended an outpatient clinic in 2016. The prevalence of TVT was 1.8%.

Keywords: transmissible venereal tumor, tvt, dogs, Guatemala.

GJMR-G Classification: NLMC Code: WA 360

Strictly as per the compliance and regulations of:

Characterization of Canine Patients with Transmissible Veneral Tumor in Veterinary Hospital of San Carlos of Guatemala University of the Year 2016

Caracterización De Pacientes Caninos Diagnósticados Con Tumor Venéreo Transmisible En El Hospital Veterinario De La Universidad De San Carlos De Guatemala En El Año 2016

Rivera-Guirola, Eduardo-Andrés [α], Villatoro-Chacón, Daniela Mariel [σ] & Arizandieta-Altán, Carmen Grizelda [ρ]

Resumen- El tumor venéreo trasmisible (TVT) es una neoplasia de transmisión sexual que afecta principalmente los genitales externos, aunque se ha reportado la presentación extragenital. Su prevalencia es variable, siendo los países en desarrollo los que presentan mayores índices, debido a grandes poblaciones de perros vagabundos y pobre control de crianza. En el presente estudio se determino la prevalencia de TVT en la población que asiste al Hospital Veterinario de la Universidad de San Carlos de Guatemala, siendo esta la única universidad en el país que presta servicio veterinario al público. Se analizaron 1,125 registros médicos de pacientes que asistieron a consulta externa en el año 2016. La prevalencia de TVT fue de 1.8%. Se observaron más casos de hembras respecto a los machos en un rango etario entre 2-7 años. Los pacientes sin raza definida presentaron con mayor frecuencia la enfermedad al igual que la presentación genital. El promedio de terapias efectivas utilizando el quimioterapéutico vincristina fue de 7.6 ± 0.812.

Palabras Clave: tumor venéreo transmisible, TVT, perros, Guatemala.

Abstract- Transmissible venereal tumor (TVT) is a sexually transmitted neoplasm that affects the external genitalia mainly although extragenital presentation has been reported. Its prevalence is variable, with developing countries presenting the highest rates due to large populations of stray dogs and poor breeding control. In the present study the prevalence of TVT was determined in the population that attends the Veterinary Hospital of the San Carlos of Guatemala University, being this the only university in the country that provides veterinary service to the public. We analyzed 1,125 medical records of patients who attended an outpatient clinic in 2016. The prevalence of TVT was 1.8%. More cases of females were observed compared to males and the age 2-7 years was the most affected. Patients without a defined race presented with greater frequency of the disease as well as genital presentation. The average number of effective therapies using the chemotherapeutic agent vincristine was 7.6 ± 0.812.

Keywords: transmissible venereal tumor, TVT, dogs, Guatemala.

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I. Introducción

El tumor venéreo transmisible (TVT) es una neoplasia que afecta a perros, siendo los callejeros o ambulatorios sexualmente activos los que reportan mayores prevalencias (Benavides, Murcia, Quevedo, Suaza, 2017; Alvarado y Sánchéz, 2013). El tumor se clasifica dentro de los tumores de células redondas, siendo éstas células de origen reticuloendotelial e histiocítico (Ortega-Pacheco, Acevedo-Arcique, Sauri-Arceco, Bolio-González y Gutiérrez-Blanco, 2003). Algunos investigadores han considerado la probabilidad de que la enfermedad tenga un componente viral ya que han observado partículas similares a los virus, pero aún no han podido transmitirlo a filtrados libres de células (Salamanca, Santander, Triana,, Rondon, 2008). La principal vía de presentación es la genital, aunque se ha reportado de forma extra genital. Su transmisión es principalmente a través del coito seguido de mordeduras o lameduras de las masas o lesiones (Alvarado y Sánchez, 2013). Para el diagnóstico es importante la historia clínica, visualización macroscópica de la masa, presencia de secreciones y localización anatómica de la masa. Sin embargo, el diagnóstico definitivo debe realizarse con biopsia o citología (Boscos, Tontis, Samartzi, 1999).

El TVT es de distribución mundial. Se han reportado prevalencias en zonas urbanas tropicales y subtropicales de diversos países del norte, centro y sur América, así como en el continente Europeo (Nielsen u Kennedy, 1990 y Richardson, 1981). Por otra parte, los perros callejeros o ambulantes tienen un papel importante para la diseminación y perpetuidad de la enfermedad (Pineda, Romero, Mendoza, García, Plata,

Martínez y Ramírez, 2010). Por esta razón, se hace necesario conocer la casuística de la enfermedad en cada región para la adopción de medidas de control y mitigación. El objetivo del presente estudio fue conocer la prevalencia y caracterizar la población de pacientes positivos a TVT que se presentaron en el Hospital de Veterinaria de la Universidad de San Carlos de Guatemala en el año 2016; considerando que éste es un centro de referencia a nivel nacional, siendo la única Universidad del país que brinda este servicio al público.

II. Métodos

El estudio se realizó en el Departamento de Ayudas Diagnósticas, Hospital Veterinario de la Universidad de San Carlos de Guatemala, ubicado en la zona 12 capitalina cuyas coordenadas son latitud 14.626187 y longitud -90.499797.

Se realizó un estudio retrospectivo evaluando 1,125 registros médicos de los pacientes que asistieron a consulta médica en el año 2016.

Se clasificaron los datos categorizando los pacientes como positivos a TVT y negativos en base a la concordancia del diagnóstico médico y citológico de los pacientes evaluados.

Finalmente, se obtuvo la prevalencia de casos positivos de TVT, y se clasificaron los datos en hojas de registro según las siguientes características: Sexo, edad, raza, procedencia, presentación de la neoplasia y número de dosis de tratamiento aplicadas.

Para el análisis de los datos se utilizó estadística descriptiva realizando distribuciones de frecuencias para las variables (Blair y Taylor, 2008). El software estadístico utilizado fue Past ®.

III. Resultados y Discusión

Se evaluaron 1,125 registros de pacientes atendidos en el año 2016, en los cuales se determinó la prevalencia de TVT siendo esta de 1.8%.

En el cuadro 1 se describe la distribución en cuanto a sexo de los pacientes positivos a TVT.

Cuadro 1: Pacientes positivos a TVT según sexo

Sexo	No.	%
Hembra	13	65
Macho	7	35

Se categorizaron las edades de los pacientes positivos a TVT siendo el grupo de 2-7 años el que presentó mayor frecuencia de la enfermedad (cuadro 2).

Cuadro 2: Pacientes positivos a TVT según edad.

Edad (años)	No.	%
0-1	0	0
2-7	14	70
8-14	4	30

En el cuadro 3 se presenta la distribución de la procedencia de los pacientes positivos a TVT, siendo la zona 12 capitalina en la que se observó mayor frecuencia.

Cuadro 3: Pacientes positivos a TVT según su procedencia

Procedencia	No.	%
Zona 3	3	15
Zona 6	1	5
Zona 7	1	5
Zona 12	6	30
Zona 18	1	5
Zona 21	2	10
Villa nueva	1	5
Mixco	2	10
Santa Catarina Pínula	2	10
Boca del Monte	1	5

De los pacientes positivos a TVT, se observaron pacientes positivos de siete razas. Sin embargo, los pacientes sin raza definida (SRD) fueron los que obtuvieron una mayor frecuencia de la enfermedad (cuadro 4).

Cuadro 4: Pacientes positivos a TVT según raza

Raza	No.	%
Sin raza definida	8	40
Husky Siberiano	4	20
Coker Spaniel	2	10
Akita	1	5
French Poodle	1	5
Golden Retriver	1	5
Schnauzer	2	10
Chow chow	1	5

En cuanto a la presentación anatómica de la enfermedad, el 85% fue de origen genital siendo de menor frecuencia la presentación extragenital (cuadro 5).

Cuadro 5: Pacientes positivos a TVT según presentación anatómica de la masa

Lugar de Presentación	No.	%
Genital	17	85
Extragenital	3	15

En la presentación extragenital, se observó un caso en miembro pélvico izquierdo, otro en cavidad nasal y oral y el último en piel.

De los pacientes positivos a la enfermedad, sólo el 33.3% acepto y siguió con tratamiento. En promedio el número de quimioterapias efectivas para recesión del tumor fue de 7.6 ± 0.812.

IV. Discusión

La prevalencia de tumor venéreo transmisible observada en el estudio fue similar a la reportada por Ortega-Pacheco, et al. (2003). Sin embargo, Bravo,

Cruz-Casallas y Ochoa (2010) y Vivero, Chavera, Perales y Fernández (2013); reportan prevalencias que oscilan entre 6.5% a 18.5% respectivamente. Por otra parte, Ortíz (2005) ha reportado prevalencias del 12% en años previos en la misma institución. Estas variaciones tan amplias pueden deberse a varios factores como la disponibilidad diagnóstica, crianza de los perros en vías públicas sin control de sus dueños, falta de controles reproductivos y abandono de las mascotas en la vía pública (Mendoza, Chavera, Falcón y Perales; 2010). Así mismo, es importante considerar el impacto de la dinámica poblacional sobre los resultados del presente estudio.

En cuanto al sexo, la presentación en hembras fue mayor respecto a los machos. Estos datos son similares a los reportado por Gurel, Kuscu, Gulanber y Arun (2002), Ortega, Acebedo, Sauri, Bolio y Gutierrez (2003), Clavo (1995) y Sousa, Saito, Nardi, Rodaski, Guérios y Bacila (2000). Sin embargo, dichos autores señalan que esta diferencia no parece ser significativa y que el sexo no predispone a la aparición de la enfermedad. Por otra parte, esta tendencia podría deberse a que se reporta mayor número de perras abandonadas versus machos (Sousa, Saito, Nardi, Rodaski, Guérios y Bacila; 2000).

En cuanto a la edad, la mayor frecuencia observada fue en perros de 2 a 7 años. Los hallazgos encontrados son similares a los reportados por otros autores como Mendoza, et al. (2010), Vivero et al, (2013) y Pineda, Romero, Mendoza, García, Plata, Martínez y Ramirez (2010). Esto puede deberse a el inicio de la maduración y actividad sexual. Por tal razón, cualquier perro en pubertad que tiene contacto sexual es propenso al contagio de la enfermedad (Pineda, et al, 2010).

La procedencia de mayor frecuencia fue la zona 12 capitalina, que coincide con la ubicación del Hospital Veterinario. Estos resultados pudieron estar influenciados por la cercanía de los usuarios al Hospital Veterinario.

Los perros sin raza definida fueron en los que se observó mayor frecuencia de la enfermedad. Esto puedo deberse a que comúnmente éstos se encuentran deambulando por la calle o que son adoptados por grupos rescatistas (Mendoza, et al; 2010). Por otra parte en las historias clínicas de perros con raza definida se encontró que estos tenían historial que habían escapado de casa. Estos datos fortalecen la teoría de otros autores en la que se considera a los perros callejeros como parte fundamental de la diseminación y perpetuación de la enfermedad (Pineda, et al., 2010).

La presentación genital de la enfermedad fue la que se encontró con mayor frecuencia en el estudio. Estos datos concuerdan con la forma de transmisión de la enfermedad a través del apareamiento, coincidiendo con reportes de otros autores (Mendoza, et al., 2010; Ndiritu, Mbogwa y Sayer, 1997 y Gurel, Kuscu, Gulanber y Arun, 2002).

El promedio de terapias efectivas utilizando el quimioterapéutico Vincristina fue de 7.6 ± 0.812. Estos datos son similares a lo reportado por otros autores, en los cuales se ha observado hasta un 90% de remisión del tumor a dosis de 0.023 a 0.26 mg/kg (Tomas, Suraniti, Meschiatti y Soberano; 1999).

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Global Journal of Medical Research: G
Veterinary Science and Veterinary Medicine
Volume 18 Issue 3 Version 1.0 Year 2018
Type: Double Blind Peer Reviewed International Research Journal
Publisher: Global Journals
Online ISSN: 2249-4618 & Print ISSN: 0975-5888

Antioxidative Potential of Aqueous Neem Bark Extract (Azadirachtaindica A. Juss) on Spermatozoa Quality in Extended Porcine Semen

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Abstract- The antioxidative potential of Neem bark in relation to spermatozoa quality is not fully understood. Thus, this present study was conducted to investigate the antioxidative potential of aqueous neem bark extract (ANBE) on spermatozoa quality in extended Porcine Semen.

Fresh semen was collected from a mature and intact boar (age, breed, body condition score, health status) using the glove-hand technique. The collected semen samples were diluted and allotted to five treatments with three replicates per treatment in a completely randomized design and evaluated at 0, 24 and 48 h of refrigeration at 17°C. Semen quality parameters such as progressive motility (%), liveability (%), morphology (%), acrosome integrity (%), pH, and lipid per oxidation were evaluated.

GJMR-G Classification: NLMC Code: WJ 834

Strictly as per the compliance and regulations of:

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O. D. Ilori [α], O. A. Shokunbi [σ], F. Alaba [ρ], S. Ajani [ω] & D. Omobayo [¥]

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Fresh semen was collected from a mature and intact boar (age, breed, body condition score, health status) using the glove-hand technique. The collected semen samples were diluted and allotted to five treatments with three replicates per treatment in a completely randomized design and evaluated at 0, 24 and 48 h of refrigeration at 17°C. Semen quality parameters such as progressive motility (%), liveability (%), morphology (%), acrosome integrity (%), pH, and lipid per oxidation were evaluated.

The results of effect of ANBE on spermatozoa quality in extended porcine semen indicate that progressive motility, liveability, morphology, were lowest ($p<0.05$) in the treatment groups than the control group throughout the period of preservation. At 48 hours, significant difference ($P<0.05$) in mean values were observed in spermatozoa progressive motility and liveability across the treatments with T5 given the lowest mean values in progressive motility (48.00±2.00) and liveability (43.33±2.89) respectively. There was no significant difference ($P>0.05$) in morphology across the treatments. However, all the treatments gave mean values within the acceptable normal range.

The results of effect of ANBE on spermatozoa fertilizing potential of extended porcine semen reveal that acrosome integrity and lipid peroxidation were lowest ($p<0.05$) in the treatment groups than the control group throughout the period of preservation. At 48 hours, there was no significant difference ($P>0.05$) in pH across the treatments. Significant difference ($P>0.05$) was observed in acrosome integrity and lipid peroxidation across the treatments. The lower level of lipid peroxidation recorded in this study for all the treatments throughout the period of preservation is an indication of antioxidative potential of ANBE on spermatozoa quality.

The results of this study suggest that 0.75mL of ANBE can be used in boar semen extension up to 48 h as indicated by observed mean values of all parameters, which fall within the acceptable range of normal values indicative of good semen quality.

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I. Introduction

Oxidative stress (OS) due to imbalance between oxidants and antioxidants in the semen can results to sperm damage, impairs the structure and function of spermatozoa and eventually male infertility (Agarwal et al., 2009). Oxidative stress is an important factor which influences fertility potential of spermatozoa by lipid peroxidation which may result in sperm dysfunction (Abasalt, et al., 2013).

The supplementation of a cryopreservation extender with antioxidant has been shown to provide a cryo protective effect on mammalian sperm quality (Amrit et al., 2011).

However, high cost of synthetic antioxidants necessitate a search for novel and more sustainable natural antioxidants to maintain a balance between the reactive oxygen species (ROS) and antioxidants in the body so as to prevent to sperm damage, deformity, and male infertility.

There are abundance of literature on antioxidative potential of Neem bark. Neem bark plays the role of free radical scavenger due to rich source of antioxidants. Hassain et al. (2013) reported that Azadirachtin and nimbolide which are constituents of neem exhibited concentration-dependent antiradical scavenging activity and reductive potential in the following order: nimbolide>azadirachtin>ascorbate. Ghimeray et al. (2009) also justified the antioxidant activity of Leaf and bark extracts of Azadirachtaindica with the results of a study which clearly indicated that all the tested leaf and bark extracts/fractions of neem grown in foothills possess significant antioxidant properties. Cal et al. (2004) reported that phenols are responsible for the variation in the antioxidant activity of Neem bark. Pitchaon et al., (2007) and Pokorney et al., (2001) asserted that neem plant exhibit antioxidant activity by inactivating lipid free radicals or preventing decomposition of hydroperoxides into free radicals.

However, the antioxidative potential of neem bark in relation to spermatozoa quality is not fully understood. Thus, this present study was conducted to investigate the antioxidative potential of aqueous neem

bark extract (Azadirachtaindica A. Juss) on spermatozoa quality in extended Porcine Semen

II. Materials and Methods

a) Location of Study

Collection of semen was carried out at the Piggery Unit of the Teaching and Research Farm, University of Ibadan, Ibadan, South Western part of Nigeria (7°20´N, 3°50´E; 200 m above mean sea level). Preparation of neem extracts and semen analysis were carried out at the Animal Physiology Laboratories of the same institution and the experiment last for 12 weeks.

b) Preparation of Aqueous Extracts from Fresh Neem Leaves

The extracts from fresh neem leaves were prepared immediately after sample collection with the following procedure; 1 kg of fresh leaves was collected, washed with distilled water and then chopped into small pieces. These were soaked into 1000 mL of distilled water in overnight and were then filtered with a cheese cloth. The filtrate was then centrifuged to remove remaining fibre in the extract, thus enhancing the visibility of spermatozoa during the microscopic evaluation and then stored at 5°C (Ilori et al., 2018).

c) Preparation of the Boar, Semen Collection and Extension

Prior to collection of semen, the boar was thoroughly washed and the preputial pouch was cleaned with water by a milking action, to remove urine and other materials that could contaminate semen during collection. Semen was collected using the gloved hand method into a US bag inserted in a collection cup such that the pre and post sperm fractions were separated from the sperm-rich fraction. Semen and extender was mixed in a ratio 1:4, 1:0.25, 1:0.75, 1:0.5, 1:1 as described by (Althouse, 2008). The mixture was refrigerated at 17°C. (Althouse et al., 2000, Althouse, 2008).

d) Semen Evaluation

Semen evaluation was carried out using the following parameters; pH, progressive motility, liveability, morphology, acrosome integrity and lipid peroxidation at 0, 24 and 48 h of preservation (17°C).

e) Progressive Motility

This was assessed by putting a drop of semen on a clean glass slide, covered with a cover slip and examined with a microscope under at 400X (B100,AmScope, USA), The progressive motility of the spermatozoa was subjectively estimated and rated between 0 and 100 (Yi et al., 2008). 0 means low percentage of motile spermatozoa and 100 means a high percentage of motile spermatozoa which indicate that the spermatozoa have not been damaged by the process of dilution and storage (Althouse, 2008).

f) Liveability

This was determined by mixing a drop of semen with a drop of a staining solution (eosin-nigrosin) on a clean glass slide gently and a smear developed using the edge of another clean slide, air-dried and examined with a microscope at 400X (Althouse, 2008).

g) Morphology

This was determined following the same method for liveability. Spermatozoa with coiled or double tail, damaged mid-piece and damaged head were considered abnormal (Levis, 2000).

h) Acrosome Integrity

Sperm was fixed with 1% glutaraldehyde in Beltsville thawing solution (BTS; 3.71 g glucose, 0.60 g trisodium citrate, 1.25 g ethylenediaminetetraacetic acid, 1.25 g sodium bicarbonate, 0.75 g potassium chloride and 100.0 ml distilled water) so as to examine acrosome integrity according to (Yi et al., 2008).

i) pH

A pH meter (Mettler Toledo Switzerland) was used to measure the hydrogen ion concentrations produced by spermatozoa metabolic activities during the storage period.

j) Lipid Peroxidation

Lipid peroxidation was determined by estimating the malondialdehyde (MDA) levels as described by Hunter et al. (1963) modified by Gutteridge and Wilkins (1980).

k) Experimental Treatments and Design

A completely randomized design was utilized for the study, such that diluted semen was allotted to six treatments with three replicates per treatment and evaluated at 0, 24 and 48 h:

Treatment 1 (Control): Semen + Beltsville Thawing Solution (BTS) Extender.

Treatment 2: Semen + BTS + 0.25 mL ANBE.

Treatment 3: Semen + BTS + 0.50 mL ANBE.

Treatment 4: Semen + BTS+ 0.75 mL ANBE.

Treatment 5: Semen + BTS + 1.00 mL ANBE.

III. Results

a) Effect of ANBE on Spermatozoa Quality in Extended Porcine Semen at 0, 24 and 48 Hours

The data on effect of ANBE on spermatozoa characteristics of extended porcine semen at 0, 24 and 48 hours of refrigeration at 17°C is as shown in Tables 1, 2 and 3 respectively.

At 0 hour, there was no significant difference ($P<0.05$) inspermatozoa progressive motility, morphology and liveability across the treatments with the exception of T5 with slight reduction in mean values. Mean vales of T5 were found to be (61.67±2.87), (93.67±3.21) and (90.00±0.00) for spermatozoa

progressive motility, morphology and liveability respectively.

At 24 hours, significant difference (P<0.05) was observed in progressive motility across the treatments with T5 (76.33±5.00) being significantly lower than other treatments. There was no significant difference (P>0.05) in morphology across the treatments with the exception of T5 (83.00±2.00) with slight reduction in mean value. There was no significant difference (P>0.05) in liveability across the treatments with the exception of T4 (84.33±1.15) and T5 (80.33±0.58) with slight reduction in mean values.

At 48 hours, significant difference (P<0.05) in mean values were observed in spermatozoa progressive motility and liveability across the treatments with T5 given the lowest mean values in progressive motility (48.00±2.00) and liveability (43.33±2.89) respectively. There was no significant difference (P>0.05) in morphology across the treatments. However, all the treatments gave mean values within the acceptable normal range.

Table 1: Effect of ANBE on Spermatozoa Quality in Extended Porcine Semen at 0 Hour (Mean ± SD)

	Inclusion Level of ANBE				
Parameters (%)	0 mL	0.25 mL	0.50 mL	0.75 mL	1.00 mL
Progressive Motility	98.00±0.00^{a}	97.00±0.00^{a}	85.33±0.58^{a}	85.00±0.00^{a}	61.67±2.87^{b}
Liveability	98.00±0.00^{a}	97.67±0.58^{a}	97.34±0.24^{a}	95.33±0.58^{a}	90.00±0.00^{b}
Morphology	98.00±0.00^{a}	98.00±0.00^{a}	97.67±0.58^{a}	96.33±0.58^{a}	93.67±3.21^{b}

Mean values on the same row with different superscript (a, and b) are significantly different (p<0.05), SD = Standard Deviation

Table 2: Effect of ANBE on Spermatozoa Quality in Extended Porcine Semen at 24 Hours (Mean ± SD)

	Inclusion Level of ANBE				
Parameters (%)	0 mL	0.25 mL	0.50 mL	0.75 mL	1.00 mL
Progressive Motility	97.67±0.58^{a}	89.67±0.58^{a}	84.33±2.87^{b}	83.33±2.87^{b}	76.33±5.00^{c}
Liveability	89.33±0.58^{a}	89.67±0.58^{a}	88.67±0.58^{a}	84.33±1.15^{b}	80.33±0.58^{b}
Morphology	90.00±0.00^{a}	90.67±0.58^{a}	91.00±0.00^{a}	89.00±1.00^{a}	83.00±2.00^{b}

Mean values on the same row with different superscript (a, b, and c) are significantly different (p<0.05), SD = Standard Deviation

Table 3: Effect of ANBE on Spermatozoa Quality in Extended Porcine Semen at 48 Hours

	Inclusion Level of ANBE				
Parameters (%)	0 mL	0.25 mL	0.50 mL	0.75 mL	1.00 mL
Progressive Motility	75.00±4.04^{a}	64.67±3.51^{a}	59.00±1.73^{b}	52.67±3.06^{b}	48.00±2.00^{c}
Liveability	79.67±0.58^{a}	60.00±0.00^{b}	58.00±0.00^{b}	51.33±5.77^{c}	43.33±2.89^{d}
Morphology	82.00±1.72	80.33±0.58	80.00±0.00	78.67±0.58	76.33±1.53

Mean values on the same row with different superscript (a, b, c and d) are significantly different (p<0.05), SD = Standard Deviation

b) Effect of ANBE on Spermatozoa Fertilizing Potential of Extended Porcine Semen at 0, 24 and 48 Hours

Tables 4, 5 and 6 present the data on effect of ANBE on spermatozoa fertilizing potential of extended boar semen at 0, 24 and 48 Hours of refrigeration at 17°C respectively.

At 0 h, there was no significant difference (P>0.05) in the pH across the treatments. A significant difference (P<0.05) was observed in acrosome integrity and lipid peroxidation across the treatments. However, mean vales of T5 were found to be lower (93.67±3.21) and (0.55±0.18) in acrosome integrity and lipid peroxidation respectively than in the other treatments.

At 24 hours, there was no significant difference (P>0.05) in pH and lipid peroxidation across the treatments. Significant difference (P<0.05) was observed in acrosome integrity with T5 (88.33±2.08) and T4 (91.00±1.00) being significantly lower than the other treatments.

At 48 hours, there was no significant difference (P>0.05) in pH across the treatments. Significant difference (P>0.05) was observed in acrosome integrity and lipid peroxidation across the treatments.

Table 4: Effect of ANBE on Spermatozoa Fertilizing Potential of Extended Porcine Semen at 0 Hour (Mean ± SD)

	Inclusion Level of ANBE				
Parameters	0 mL	0.25 mL	0.50 mL	0.75 mL	1.00 mL
pH	6.96±0.58	6.93±0.58	7.00±0.00	6.96±0.58	6.93±0.58
AI	98.00±0.00[a]	98.33±0.58[a]	97.67±0.58[a]	96.33±0.58[a]	93.67±3.21[b]
LP	0.84±0.24[a]	0.66±0.23[b]	0.63±0.27[b]	0.62±0.27[b]	0.55±0.18[c]

Mean values on the same row with different superscript (a, b, and c) are significantly different (p<0.05), SD = Standard Deviation, AI = Acrosome Integrity, LP = Lipid Peroxidation

Table 5: Effect of ANBE on Spermatozoa Fertilizing Potential of Extended Porcine Semen at 24 Hours (Mean ± SD)

Inclusion Level of ANBE					
Parameters	0 mL	0.25 mL	0.50 mL	0.75 mL	1.00 mL
pH	7.00±0.00	7.03±0.58	7.00±0.10	7.00±0.10	7.03±0.06
AI	96.67±2.31[a]	92.67±0.58[b]	92.00±0.00[b]	91.00±1.00[b]	88.33±2.08[c]
LP	0.79±0.30	0.77±0.20	0.75±0.30	0.71±0.25	0.70±0.09

Mean values on the same row with different superscript (a, b, and c) are significantly different (p<0.05), SD = Standard Deviation, AI = Acrosome Integrity, LP = Lipid Peroxidation

Table 6: Effect of ANBE on Spermatozoa Fertilizing Potential of Extended Porcine Semen at 48 Hours (Mean ± SD)

Inclusion Level of ANBE					
Parameters	0 mL	0.25 mL	0.50 mL	0.75 mL	1.00 mL
pH	7.03±0.06	7.03±0.06	7.06±0.06	7.06±0.06	7.03±0.06
AI	89.33±1.15[a]	82.33±0.58[b]	81.67±1.15[b]	80.00±0.00[b]	79.33±0.58[b]
LP	0.57±0.03[a]	0.47±0.05[b]	0.43±0.05[b]	0.43±0.08[b]	0.21±0.03[c]

Mean values on the same row with different superscript (a, b, and c) are significantly different (p<0.05), SD = Standard Deviation, AI = Acrosome Integrity, LP = Lipid Peroxidation

IV. Discussion

a) *Effect of ANBE on Spermatozoa Quality in Extended Porcine Semen at 0, 24 and 48 Hours*

Spermatozoa progressive motility is one of the major determinants of fertility of male animals such as boar (Haugan et al., 2004). Progressive motility of spermatozoa has always been considered a primary requirement for egg fertilization. It is known to be an important characteristic in predicting the fertilizing potential of an ejaculate (Gadea, 2005). The results of this study showed that ANBE has potential to maintain spermatozoa progressive motility throughout the periods of preservation and this may probably be due to antioxidants activities of neem bark (Gayatri et al., 2010).

A. indica has been reported to contain polyphenolic compounds which possess remarkable antioxidant activities (Siddiqui, et al., 1992; Sultana, et al., 2007; Gayatri et al., 2010). All the treatments gave mean values within acceptable normal range throughout the period of preservation with the exception of 1.00 m Linclusion level of ANBE which gave the mean values below acceptable normal range at 48 hours of storage. The high percentages of motile spermatozoa recorded with the inclusion of ANBE in boar semen is in accordance with findings of Levis, (2000), Roca et, al, (2006); Vytet, al, (2008) who reported that motility above 60% is enough for fertilization to take place provided that all other semen parameters are good.

The antioxidants activities of ANBE was found to enhanced spermatozoa morphology throughout the periods of preservation. Morphological abnormalities of spermatozoa that can severely influenced fertilization and embryonic development was found to be corrected due to presence of polyphenolic compounds in neem barks which possess significant antioxidant activities (Siddiqui, et al., 1992; Sultana, et al., 2007; Gayatri et al., 2010).All the treatments gave mean values within acceptable normal range throughout the period of preservation and this support the findings of Maes et al., (2010) who reported that ejaculates should have greater than 70% normal sperm with no more than 20% sperm with primary abnormalities. This findings agrees with Cerolini et al. (2000) who reported that inclusion of antioxidant into storage diluents could prevent deterioration of boar spermatozoa quality and provided protection to the cells up to 5 days of storage through its prevention of oxidative reduction in the levels of major polyunsaturated fatty acid.

The percent live spermatozoa was found to be enhanced by the antioxidants activities of ANBEthroughout the periods of preservation. All the inclusion level of ANBE gave mean values which fall within the acceptable range of normal values indicative

of good semen quality throughout the period of preservation with the exception of 1.00 Ml inclusion level which gave the mean values below acceptable normal range at 48 hours of storage. This decline in percent spermatozoa liveability at this hour of preservation may indicate a gradual reduction of antioxidant activities of neem barks.However, the high percent live spermatozoa recorded with the inclusion of ANBE in boar semen corroborates the findings of Maes et al., (2010) who reported that semen samples should have more than 70% viable sperm by a vital stain assay prior to processing. This is in line with findings of Cerollini et al., (2000) reported that the inclusion of an antioxidant into the diluent could prevent the significance reduction in viability of cells, and this could lead to high percent live spermatozoa recorded for this study. This indicates that ANBE can be used as exogenous antioxidant in extender to inhibit lipid peroxidation.

b) *Effect of ANBE on Spermatozoa Fertilizing Potential ofExtended Porcine Semen at 0, 24 and 48 Hours*

The antioxidants activities of ANBE was found to enhanced acrosome integrity throughout the period of preservation as indicated by high percentages recorded for acrosome integrity which falls within acceptable range of normal values indicative of good semen quality and this is in line with the findings of Maes et al. (2010) who reported that semen samples with less than 70% sperm with intact acrosomes should be discarded before processing. This finding is agreement with the findings of Ilori et al., (2018) who reported that neem has the potential of maintaining acrosome integrity of boar semen by protecting acrosome from undergoing capacitation during preservation.The antioxidants activities of ANBE was found to maintain the pH throughout the period of preservation. It is important for pH to be maintained because when the pH of the semen is declined; the internal pH of the spermatozoa is also reduced leading to a decrease in sperm metabolism and mobility (Gadea, 2005). This result is in compliance with of Frunza et al., (2008) who recorded a higher proportion of live normal sperm in a neutral and alkaline pHlevel (7.0 and 8.2).

Lipid peroxidation was determined by estimating the malondialdehyde (MDA) levels as described by Hunter et al. (1963) modified by Gutteridge and Wilkins (1980).Malondialdehyde (MDA) is one of the reactive and mutagenic aldehyde products of lipid peroxidation in seminal plasma (Shang et al., 2004). Toxic lipid peroxides are known to cause different impairments of sperm cells and may play a main role in the etiology of male infertility (Abasalt et al., 2013). Malondialdehyde (MDA) is an indicator of lipid peroxidation which may be a diagnostic tool for the analysis of infertility (Tavilani et al., 2008)

High lipid peroxidation levels have been reported to reduced sperm functionality such as motility, acrosomal reaction, fertilization, membrane degradation and sperm oocyte fusion (Abasalt et al., 2013; Goolsbyet al., 2014). However, in this study, the MDA level recorded as an indicator of lipid peroxidation for all the treatments were found to be lower throughout the period of preservation. This could be attributed to antioxidative properties of ANBE such as presence of phenols and other inhibiting compounds which help to inhibit lipid peroxidation throughout the period of preservation. This assertion is in compliance with Cal et al., (2004) who reported that phenols are responsible for the variation in the antioxidant activity of Neem bark.

This is also justified by Pitchaon et al., (2007) and Pokorney et al., (2001) who opined that neem plant exhibit antioxidant activity by inactivating lipid free radicals or preventing decomposition of hydroperoxides into free radicals. Phenolic compounds are considered to be the most important antioxidant components of herbs and other plant materials and a good correlation between the concentration of plant phenolic and total antioxidant capacities has been reported (Madsen et al., 1996;Pellegrini et al., 2000).

The results of this study suggest that 0.75mL of ANBE can be used in boar semen extension up to 48 h as indicated by observed mean values of all parameters, which fall within the acceptable range of normal values indicative of good semen quality.

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Global Journal of Medical Research: G
Veterinary Science and Veterinary Medicine
Volume 18 Issue 3 Version 1.0 Year 2018
Type: Double Blind Peer Reviewed International Research Journal
Publisher: Global Journals
Online ISSN: 2249-4618 & Print ISSN: 0975-5888

Analysis of Various Physiological Values of Growing Miniature Pigs in Isolator

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Abstract- The objective of this study was to provide physiological values on the growing miniature pigs in isolator. Measurements were taken of each miniature pigs' body weight, temperature, hematological and serum biochemistries, and heart rate. The miniature pigs were divided into 2 groups. The piglet group (n = 8), consisting of animals approximately 4 weeks old and weighing 5 to 7 kg, were used to obtain normal physiological values at 6, 10, 14, 18, and 22 weeks of age. The adult group (n = 8), which consisted of animals approximately 40 weeks old weighing 19 to 21.3 kg, was used to obtain normal physiological values for miniature pigs at 43, 47, 51, 55, and 59 weeks of age. None of the animals displayed abnormal behavior or symptoms while they were bred in the isolators. The weight of every animal that was bred in an isolator gradually increased.

Keywords: *isolator, miniature pigs, physiological values.*

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

Strictly as per the compliance and regulations of:

Analysis of Various Physiological Values of Growing Miniature Pigs in Isolator

Young Ah Lee [α] & Jin Soo Han [σ]

Abstract- The objective of this study was to provide physiological values on the growing miniature pigs in isolator. Measurements were taken of each miniature pigs' body weight, temperature, hematological and serum biochemistries, and heart rate. The miniature pigs were divided into 2 groups. The piglet group (n = 8), consisting of animals approximately 4 weeks old and weighing 5 to 7 kg, were used to obtain normal physiological values at 6, 10, 14, 18, and 22 weeks of age. The adult group (n = 8), which consisted of animals approximately 40 weeks old weighing 19 to 21.3 kg, was used to obtain normal physiological values for miniature pigs at 43, 47, 51, 55, and 59 weeks of age. None of the animals displayed abnormal behavior or symptoms while they were bred in the isolators. The weight of every animal that was bred in an isolator gradually increased. Among the center of the animals' foreheads, necks, lateral abdominal areas, and hips, the lateral abdominal areas are showed the highest temperature variation in all tests as determined using an infrared thermometer. The author confirmed that all of the results from the complete blood cell counts (CBCs), serum biochemical tests, and heart rates were within the normal range for miniature pigs.

This study provides a baseline for interpreting physiological data for miniature pigs growing within isolator systems. We expect our study to help other researchers studying miniature pigs and to make a significant contribution in the field of bio-organ transplantation.

Keywords: isolator, miniature pigs, physiological values.

I. Introduction

Pigs have become widely used for biomedical research in recent years since there are many similarities in metabolic and cardiovascular function between swine and humans (Swindle *et al.*, 1988). Miniature pigs specifically make good research animals, since they are easy to deliver, maintain, and utilize in isolators (Mandel and Travnicek, 1987). Their small size relative to that of other large animals facilitates housing and handling, while their ample blood volume allows for more frequent serial blood collections than are possible with rodents. Furthermore, miniature pigs have received attention as potential donors in xenotransplantation because of their ability to be bred in an aseptic environment, their high fertility, and low costs (Park *et al.*, 2006). Isolator systems are used to maintain laboratory and farm animals in a sterile environment to prevent contact-transmitted and airborne infections (Trexler, 1973). While isolators have been largely used in general laboratory animal research, most of the literature contains information pertaining to rodents, and there is little available information regarding optimal isolator design and conditions for miniature pigs.

The establishment of standard basic data is important in the biosciences, because it can reduce and refine the use of laboratory animals (Michael *et al.*, 2006). Although there have been studies to acquire basic data regarding the use of miniature pigs, no data is available regarding their use within an isolator system. Therefore, the aim of this study, in addition to identifying baseline physiological values for miniature pigs growing within an isolator, was to identify optimal isolator designs and conditions for miniature pigs.

II. Materials and Methods

a) Advanced isolator

The miniature pigs were housed in groups of 2 in isolators (1200W × 900D × 950H m/m, SK-ISO-1700HBP600, Three-shine INC, Daejeon, Korea, Fig. 1). The internal temperatures of the isolators were set to 23 ± 2°C, and their relative humidity was kept at 50 ± 10%. The ventilation frequency was 50 times per h, illuminance was 100–150 Lux, and noise levels were kept below 58 phon, when possible. Feeding units and water nozzles were installed in the isolators, which consisted of space for breeding and movable room-dividing sub-frames set on wheels. The sub-frames were equipped with a window for easy animal observation, and gloves for gnotobiotic handling of the animals. Gloves were protected from damage within the isolator by drawing them back into the glove housing after use, and a double-capped transport chamber was used to sterilize the goods coming in and out of the isolator. In short, the inner part of the cap was airtight, so that the outer cap could be removed to either add goods for sterilization and insertion, or extraction of sterilized waste.

The feed unit was securely fixed within the cage, and could be easily washed through a hole in its bottom. Automated watering valves were used to allow *ad libitum* consumption, and a cleaning box containing a water gun was attached for easy cleaning of the isolator's interiors. An uninterruptible power supply was installed to retain operation of the exhaust port in case

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of a blackout, ventilating the isolator via a safety filter to protect against the culling of miniature pigs mid-experiment. The drain under the isolator was installed to make the excrement of the pigs fall downward, and the sub-drain switch valve can be used to remove the body wastes selectively.

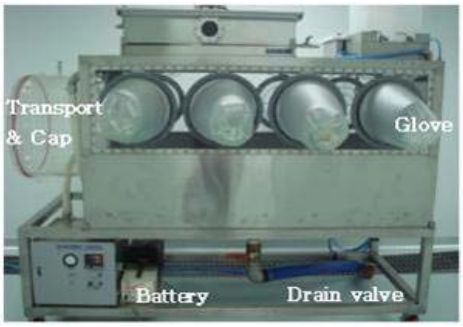

Figure 1: Isolator system for miniature pigs

This system is composed of a stainless steel body containing an entry port, gloves, control box, battery, drain valve, filter boxes, and other variable equipment.

b) Animals

Specific pathogen-free miniature pigs (PWG Micropig, mixed with Yucatan, native pigs, pygmy pigs, and miniature pot-bellied pigs) were obtained from Medi Kinetics Co. (Pyeongtaek, Korea). All of the pigs were quarantined for 4 weeks before use in experiments, and were clinically healthy prior to the study. Miniature pigs were divided into 2 groups. The piglet group (n = 8), consisting of animals approximately 4 weeks old and weighing 5 to 7 kg, were used to obtain normal physiological values at 6, 10, 14, 18, and 22 weeks of age. The adult group (n = 8), which consisted of animals approximately 40 weeks old which weighed 19 to 21.3 kg, was used to obtain normal physiological values for miniature pigs at 43, 47, 51, 55, and 59 weeks of age.

c) Clinical symptoms

The health status of each miniature pig was monitored daily throughout the duration of the study. In case any abnormality was detected, a description of the severity and frequency of the symptoms was recorded by date.

d) Measurements of body weight and body temperature

The miniature pigs were weighed using a balance (HBS-510L; CAS, Korea). We measured the body surface temperature of the miniature pigs using a non-contact infrared thermometer, which allowed for measurement of their body temperatures without causing them any undue stress. Temperatures were measured on the center of the forehead, neck, lateral abdominal area and hip (eye exposure to the beam was avoided).

e) Hematological and serum biochemistry analysis

All pigs were anesthetized prior to blood collection using medetomidine 0.2 mg/kg (Domitor®; Pfizer Korea, Seoul, Korea) intramuscularly and tiletamine–zolazepam 4.4 mg/kg (Zoletil®; Virbac, Carros, France) intramuscularly. All miniature pigs were bled (5 mL) through their jugular veins. All blood samples were analyzed for evaluated using an automated hematology analyzer (FORCYTE; Oxford Science, USA) for CBC and differential blood cell counts. In addition to the red blood cell (RBC), hemoglobin concentration (Hb), and platelet (PLT) counts we did, we also quantified the content of several different cell populations within the white blood cell count (WBC), including: neutrophils (NEU), lymphocytes (LYM), monocytes (MONO), eosinophils (EOS) and basophils (BASO).

All serum samples were analyzed for complete total protein (TP), glucose (GLU), uric acid (URIC), calcium (CA), cholesterol (CHOL), total bilirubin (TBIL), creatine (CREA), alanine aminotransferase (ALT), albumin (ALB), aspatate aminotransferase (AST), and cortisol levels. All serum analysis was measured using a radioimmunoassay (RIA; PerkinElme, Finland) in the clinical laboratory of the Neodin Medical Institute (Seoul, Korea). The mean and SE were calculated for each of the measured parameters. Reference values for the hematology of pigs are documented well in previous literature.

f) Heart rate measurements

Heart rates of the 6- and 43-week-old animals were monitored and recorded. All pigs were anesthetized using medetomidine 0.2 mg/kg (Domitor®; Pfizer Korea, Seoul, Korea) intramuscularly and tiletamine–zolazepam 4.4 mg/kg (Zoletil®; Virbac, Carros, France) intramuscularly prior to collection of their heart rate data. During the 24 h of monitoring, we cleaned the isolators and fed the pigs while they were attached to the holter monitor for examination of their heart rates during their typical daily activities within the isolators. After 24 h, we anesthetized the animals once again, removed the monitors and then compiled the data.

g) Statistical analysis

All statistical analyses were performed using Graph Pad Prism version 4.0 for Windows (Graph Pad Software, San Diego, CA, USA). Data were recorded as mean ± standard error of the mean (SEM).

Comparisons of the two different groups were made by Student's unpaired *t*-tests and P values <0.05 were considered significant.

III. Results

a) Clinical symptoms

All animals showed no clinical signs of disease and appeared healthy throughout the study.

b) Measurements of body weight and body temperature

The recorded body weight measurements are shown in Table 1. The weight of every animal that was bred in an isolator gradually increased. The results of the body temperature measurements are shown in Figures 2 and 3. The center of the forehead, neck, lateral abdominal area, and hip showed the highest temperature variations in all tests. The lowest temperature values occurred in the forehead region for all miniature pigs, and were lower in the 55-week-old miniature pigs (33.0°C) compared to other adult groups. The temperature of the abdominal region was higher in the 6-week-old miniature pigs (35.93°C) than in the other groups, and abdominal skin temperature values were considered to be very to the rectal temperatures obtained of miniature pigs by using an infrared thermometer.

c) Hematological and serum biochemistry analysis

Hematological analysis results are shown in Tables 2 and 3. Total WBCs, neutrophil, and lymphocyte counts of the piglet group tended to decrease with age ($P < 0.05$). The total WBCs and neutrophil counts of the adult group tended to decrease with age, although data was not statistically significant between the age groups. The results of the serum biochemistry analysis are shown in Tables 4 and 5. Cortisol levels of the piglet group decreased with age. The cortisol values for the 6–week-old miniature pigs (18.04 ± 3.18 ug/dL) were higher than those for all other age groups. Although cortisol values of the piglet group tended to be greater than those of the adult group, the differences were not statistically significant. Serum biochemistry values did not differ among the age groups and were not statistically significant between the age groups. All hematological and serum biochemistry values were within the normal reference range.

d) Heart rate measurements

The results of heart rate measurements are shown in Table 6 and Fig. 4. The heart rates of minimum, maximum, and average in 6-week-old animals were 84.17 ± 10.39, 186.55 ± 23.96, and 121.89 ± 16.77, respectively. The minimum, maximum, and average heart rates of 43-week-old animals were 68.57 ± 9.92, 190.96 ± 10.73, and 111.69 ± 12.93, respectively. It should be noted that the isolator pigs' average heart rates increased above a normal heart reference rate (135) during cleaning and feeding periods (data not shown).

Table 1: Body weight changes of miniature pigs within the isolator systems

Group	Age (weeks)	Body weight (kg)
Piglet	6	6.06 ± 0.67
	10	7.94 ± 0.57
	14	9.35 ± 1.31
	18	10.89 ± 2.48
	22	12.24 ± 3.18
Adult	43	20.24 ± 0.67
	47	21.68 ± 1.70
	51	23.56 ± 1.95
	55	25.78 ± 2.69
	59	27.40 ± 1.68

Values are mean ± SE (n = 8).

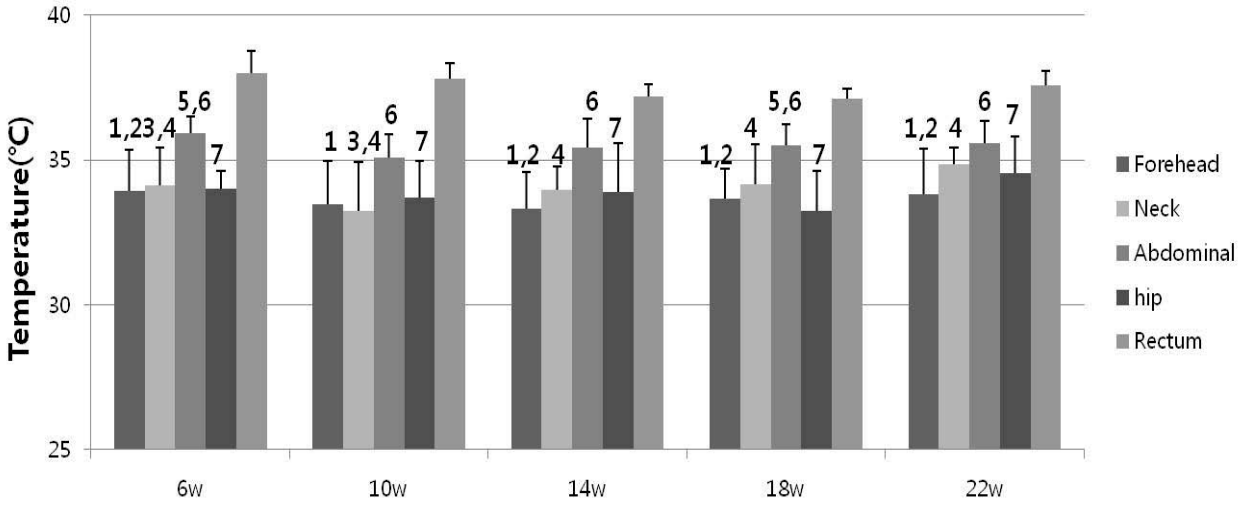

Figure 2: Comparison of body temperatures (℃) from the piglet group within the isolator systems

Bars represent the mean values and standard errors of body temperature (n = 8). One-way analysis of variance (ANOVA) tests revealed significant differences in 5 regions ($P < 0.05$); [1]Significant difference between the forehead and abdomen; [2]Significant difference between the forehead and rectum; [3]Significant difference between the neck and abdomen; [4]Significant difference between the neck and rectum; [5]Significant difference between the abdomen and hip; [6]Significant difference between the abdomen and rectum; [7]Significant difference between the hip and rectum.

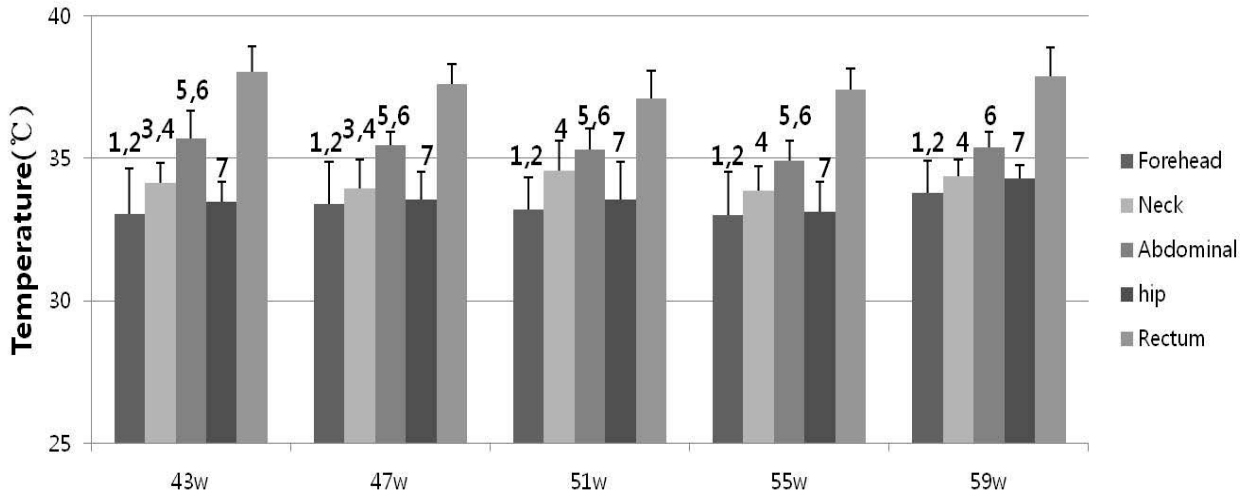

Figure 3: Comparison of body temperatures (℃) from the adult group within the isolator systems

Bars represent the mean values and standard errors of body temperature (n = 8). One-way analysis of variance (ANOVA) tests revealed significant differences in 5 regions ($P < 0.05$); [1]Significant difference between the forehead and abdomen; [2]Significant difference between the forehead and rectum; [3]Significant difference between the neck and abdomen; [4]Significant difference between the neck and rectum; [5]Significant difference between the abdomen and hip; [6]Significant difference between the abdomen and rectum; [7]Significant difference between the hip and rectum.

Table 2: Hematological values from the piglet group within the isolator systems

	Normal Range	6 weeks	10 weeks	14 weeks	18 weeks	22 weeks
White blood cells (K/uL)[1]	4.4-26.4	20.3 ± 6.55[1]	14.28 ± 14.28	12.48 ± 4.50	10.94 ± 2.94	10.18 ± 3.54
Neutrophils (K/uL) [1]	3.1-11.2	9.87 ± 5.95	5.07 ± 5.07	4.96 ± 1.70	4.98 ± 1.56	4.27 ± 1.66
Lymphocytes (K/uL) [1]	4.3-13.6	8.85 ± 2.57	7.52 ± 7.52	6.31 ± 2.99	5.22 ± 1.39	4.84 ± 1.39
Monocytes (K/uL)	0.2-2.2	0.69 ± 0.36	0.69 ± 0.69	0.52 ± 0.15	0.36 ± 0.21	0.52 ± 0.37
Eosinophils (K/uL)	0.1-2.4	0.83 ± 0.74	0.98 ± 0.98	0.69 ± 0.82	0.35 ± 0.19	0.51 ± 0.76
Basophils (K/uL)	0.0-0.4	0.06 ± 0.03	0.04 ± 0.04	0.02 ± 0.01	0.02 ± 0.01	0.03 ± 0.03
Red blood cells (M/uL)	5.3-9.25	6.24 ± 1.51	6.69 ± 6.69	7.21 ± 1.00	5.73 ± 0.66	5.65 ± 1.38
Hemoglobin (g/dL)	9.0-15.8	9.51 ± 2.18	10.25 ± 10.25	10.53 ± 1.42	8.87 ± 1.00	8.73 ± 2.28
Platelets (K/uL)	148-898	374.06 ± 203.9	343.75 ± 343.8	370.13 ± 151.7	336.13 ± 90.0	289.00 ± 148.6

Values are mean ± SE (n = 8). [1] Indicates that the parameter decreased significantly with increasing age ($P < 0.05$). Normal range (Bollen *et al*., 2000; Swindle *et al*., 2007).

Table 3: Hematological values from the adult group within isolator systems

	Normal Range	43 weeks	47 weeks	51 weeks	55 weeks	59 weeks
White blood cells (K/uL) [1]	4.4-26.4	11.78 ± 3.95	11.23 ± 3.57	10.21 ± 1.39	10.11 ± 2.74	9.51 ± 3.17
Neutrophils (K/uL) [1]	3.1-11.2	5.22 ± 2.01	4.82 ± 1.67	4.27 ± 1.12	4.03 ± 1.46	3.69 ± 1.53
Lymphocytes (K/uL)	4.3-13.6	5.46 ± 2.18	5.03 ± 2.45	5.21 ± 1.41	5.45 ± 1.17	4.97 ± 1.59
Monocytes (K/uL)	0.2-2.2	0.43 ± 0.23	0.56 ± 0.2	0.41 ± 0.20	0.38 ± 0.18	0.45 ± 0.21
Eosinophils (K/uL)	0.1-2.4	0.62 ± 0.52	0.79 ± 1.19	0.29 ± 0.28	0.24 ± 0.22	0.36 ± 0.45
Basophils (K/uL)	0.0-0.4	0.04 ± 0.03	0.04 ± 0.03	0.03 ± 0.04	0.02 ± 0.02	0.04 ± 0.02
Red blood cells (M/uL)	5.3-9.25	6.43 ± 0.57	6.80 ± 0.83	6.70 ± 1.61	6.29 ± 0.82	6.77 ± 1.86
Hemoglobin (g/dL)	9.0-15.8	9.91 ± 1.87	11.16 ± 1.88	10.42 ± 2.88	10.28 ± 1.72	10.46 ± 2.64
Platelets (K/uL)	148-898	391.9 ± 99.5	311.9 ± 90.7	284.2 ± 110.2	339.6 ± 94.2	323.8 ± 92.3

Values are mean ± SE (n = 8). [1] Indicates that the parameter decreased with increasing age. Normal range (Bollen *et al*., 2000; Swindle *et al*., 2007).

Table 4: Serum biochemistry values from the piglet group within the isolator systems

	Normal Range	6 weeks	10 weeks	14 weeks	18 weeks	22 weeks
Total Protein(g/dL)	2.25 - 8.0	5.08 ± 1.75	4.93 ± 1.97	4.88 ± 1.37	4.74 ± 1.48	5.73 ± 1.25
ALB (g/dL)	1.8 - 3.3	3.23 ± 0.83	3.06 ± 0.77	3.00 ± 0.37	2.89 ± 0.73	3.19 ± 0.52
CREA (mg/dL)	0.5 - 2.1	0.59 ± 0.26	0.63 ± 0.3	0.71 ± 0.27	0.71 ± 0.35	0.85 ± 0.22
URIC (mg/dL)		0.47 ± 0.34	0.38 ± 0.38	0.45 ± 0.32	0.38 ± 0.38	0.37 ± 0.4
Glucose (mg/dL)	43 - 133	112.13 ± 36.05	91.13 ± 37.4	82.38 ± 19.1	72.13 ± 21.24	74.75 ± 20.19
TBIL (mg/dL)	0.0 - 0.3	0.08 ± 0.05	0.08 ± 0.05	0.08 ± 0.05	0.08 ± 0.05	0.08 ± 0.04
AST (IU/L)	16 - 65	55.88 ± 22.42	46.50 ± 17.77	48.63 ± 27.37	41.00 ± 19.15	43.13 ± 17.82
ALT (IU/L)	9 - 43	37.88 ± 11.53	35.00 ± 10.2	38.50 ± 17.57	32.13 ± 16.69	40.00 ± 16.24
CHOL (mg/dL)	18 - 79	47.25 ± 14.82	44.25 ± 21.61	48.50 ± 18.59	41.25 ± 18.82	54.13 ± 15.49
Calcium (mg/dL)	6.5 - 11.4	9.98 ± 1.92	8.70 ± 2.48	8.55 ± 1.41	7.98 ± 2.01	8.95 ± 1.54
Cortisol (ug/dL)		18.04 ± 3.18	4.32 ± 3.81	12.01 ± 5.13	10.43 ± 5.03	9.85 ± 5.73

Values are mean ± SE (n = 8). ALB (albumin), CREA (creatine), URIC (uric acid), TBIL (total bilirubin), AST (aspatate aminotransferase), ALT (alanine aminotransferase), CHOL (cholesterol). Normal range (Bollen *et al*., 2000; Swindle, 2007).

Table 5: Serum biochemistry values from the adult group within the isolator systems

	Normal Range	43 weeks	47 weeks	51 weeks	55 weeks	59 weeks
Total Protein (g/dL)	2.25 - 8.0	6.16 ± 2.23	5.73 ± 2.21	6.45 ± 1.62	6.19 ± 2.04	6.75 ± 1.75
ALB (g/dL)	1.8 - 3.3	2.93 ± 1.02	2.81 ± 1.00	3.31 ± 0.62	3.14 ± 0.8	3.18 ± 0.47
CREA (mg/dL)	0.5 - 2.1	0.69 ± 0.37	0.74 ± 0.32	0.9 ± 0.25	0.97 ± 0.28	0.95 ± 0.1
URIC (mg/dL)		0.38 ± 0.38	0.47 ± 0.36	0.33 ± 0.38	0.35 ± 0.37	0.3 ± 0.37
Glucose (mg/dL)	43 - 133	91.25 ± 55.74	69.38 ± 42.39	90.88 ± 39.44	79.38 ± 45.06	89.13 ± 46.56
TBIL (mg/dL)	0.0 - 0.3	0.09 ± 0.06	0.18 ± 0.3	0.08 ± 0.05	0.1 ± 0.05	0.13 ± 0.05
AST (IU/L)	16 - 65	60.5 ± 28.25	48.88 ± 19.05	39.38 ± 12.98	34.75 ± 14.95	48.38 ± 25.65
ALT (IU/L)	9 - 43	27.38 ± 7.93	30.50 ± 13.85	28.63 ± 6.46	26.63 ± 10.51	31.25 ± 14.06
CHOL (mg/dL)	18 - 79	43.63 ± 21.79	46.25 ± 20.42	47.75 ± 23.56	48.25 ± 20.73	54.25 ± 24.21
Calcium (mg/dL)	6.5 - 11.4	8.98 ± 2.34	8.16 ± 2.37	9.08 ± 1.6	8.86 ± 2.31	9.41 ± 1.8
Cortisol (ug/dL)		9.99 ± 2.33	9.79 ± 3.93	8.2 ± 3.03	9.85 ± 2.81	9.74 ± 6.41

Values are mean ± SE (n = 8). ALB (albumin), CREA (creatine), URIC (uric acid), TBIL (total bilirubin), AST (aspatate aminotransferase), ALT (alanine aminotransferase), CHOL (cholesterol). Normal range (Bollen *et al*., 2000; Swindle, 2007).

Table 6: Heart rate values of miniature pigs within the isolator systems

Age	6 weeks	43 weeks
Minimum	84.2 ± 10.4	68.6 ± 9.9
Average	121.9 ± 16.8*	111.7 ± 12.9
Maximum	186.6 ± 24.0	191.0 ± 10.7

Values are mean ± SE (n = 8). *Significant (P < 0.05) differences between values for 6 and 43 weeks. (Student's unpaired *t*-test, two-tailed)

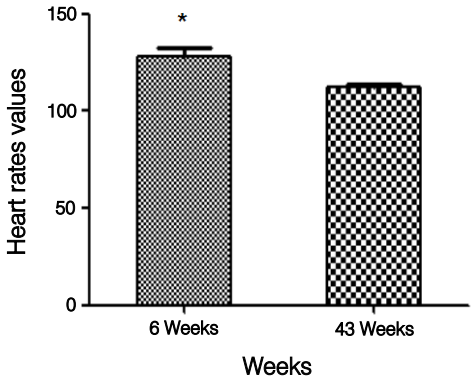

Figure 4: Heart rates of miniature pigs within the isolator systems

Bars represent the mean values and standard errors of the pigs' heart rate values. *Significant (P < 0.05) difference between values at 6 and 43 weeks. (Student's unpaired *t* –test, two-tailed).

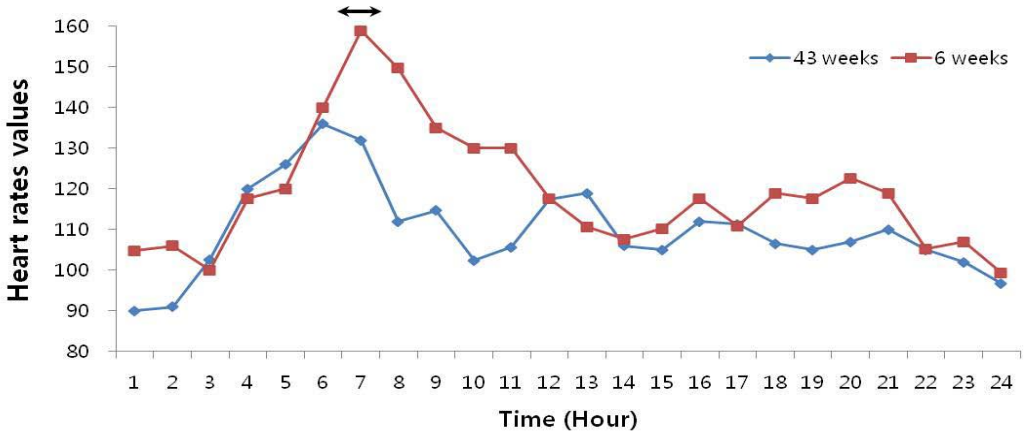

Figure 5: Heart rate trends of miniature pigs within the isolator systems

Hourly heart rates over a 24-h period. The arrow indicates the period during cleaning of the isolator, at which time the heart rate increased immediately.

IV. Discussion

Pigs are exposed to many stress factors that may affect their health and welfare, including housing and management (Ruis *et al*., 1997). Typical signs of stress in pigs include depressed activity levels and decreased feed intake, which typically result in weight loss (Seymour *et al*., 1964; McGlone *et al*., 1987; Christon, 1988). None of the animals used in this study displayed abnormal behaviors or symptoms while they were bred in the isolators, and body weight measurements of all of the animals showed gradual body weight gains.

Temperature measurement is basic for medical evaluation, and is a tool that is frequently used in both clinical and research settings (Quimby *et al*., 2009). Body temperature is most commonly collected rectally in clinical settings, because it is an accurate reflection of core body temperature (Greer *et al*., 2007). This method is very difficult to utilize for animals housed within isolator environments, however, therefore we used a non-contact infrared thermometer. Since non-contact infrared thermometers typically require 2–4°C of calibration, the results should be considered when selecting a particular temperature region for experimental use with a non-contact infrared thermometer in miniature pigs. It is important to note that noninvasive temperature-measurement techniques that are rapidly accomplished could contribute to laboratory animal stress reduction and improved welfare (Chen *et al*., 2006). Results indicated that the areas exhibiting the highest temperatures in all tests were lateral abdominal area.

In order to measure the stress of breeding within an isolator, we monitored the cortisol levels in the animals' blood serum as well as the variation in their heart rates via a holter monitor. There was no significant difference between the average heart rates of 6- and 43-week-old animals. Although heart rates abruptly increased when there were workers outside of the isolators to clean or provide feed, this is likely due in response to cleaning noises and expectations of being fed. Additionally, while gradual increases and decreases in heart rates were observed during the animals' dark-cycle sleeping hours, they were not regarded as abnormal symptoms of stress because reports indicate that heart rate also varies in humans during sleep (Bonnet and Arand, 1997).

Increased cortisol level is an important indicator of stress (Carroll *et al*., 2006), and serum concentrations have been widely used to assess the effects of different stressors on immune function (Bilandzić *et al*., 2006). Cortisol is known to be the primary glucocorticoid released during times of stress in pigs (Kojima *et al*., 2007), and administration of morphine and fentanyl during surgical procedures have been shown to decrease postoperative cortisol concentrations relative to controls in swine weighing 20 kg (Malavasi *et al*., 2006). The results indicate that cortisol levels were elevated in both the piglet and adult groups. Since transportation, weaning, and maternal separation have been shown to increase cortisol concentrations in pigs (Cooper *et al*., 2009; Kojima *et al*., 2008; Nyberg *et al*., 1988; Parrot and Mission., 1989), the author attribute transportation stress to the increased cortisol levels in the 6- and 43-week old animals. Since cortisol levels were the highest in the 6-week-old animals, we assume that their young age caused them to be more susceptible to stress. The data agrees with previous studies, which indicated that increased stress resulted in elevation of WBC numbers and cortisol concentration (Morrow-Tesch *et al*., 1994; Kojima *et al*., 2009). These elevated WBC and cortisol concentrations decreased with increasing age in all of this study's miniature pigs. The author confirmed that all of the results from the CBC and serum biochemical tests were within the normal range. The data presented in this study provide a baseline for interpreting physiologic results of data gathered during the growth of miniature pigs within isolator systems.

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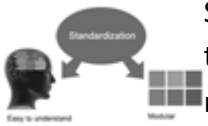

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3. Ensure corresponding author's email address and postal address are accurate and reachable.
4. Manuscript to be submitted must include keywords, an abstract, a paper title, co-author(s') names and details (email address, name, phone number, and institution), 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.

Policy on Plagiarism

Plagiarism is not acceptable in Global Journals submissions at all.

Plagiarized content will not be considered for publication. We reserve the right to inform authors' institutions about plagiarism detected either before or after publication. If plagiarism is identified, we will follow COPE guidelines:

Authors are solely responsible for all the plagiarism that is found. The author must not fabricate, falsify or plagiarize existing research data. The following, if copied, will be considered plagiarism:

- Words (language)
- Ideas
- Findings
- Writings
- Diagrams
- Graphs
- Illustrations
- Lectures

- Printed material
- Graphic representations
- Computer programs
- Electronic material
- Any other original work

Authorship Policies

Global Journals follows the definition of authorship set up by the Open Association of Research Society, USA. According to its guidelines, authorship criteria must be based on:

1. Substantial contributions to the conception and acquisition of data, analysis, and interpretation of findings.
2. Drafting the paper and revising it critically regarding important academic content.
3. Final approval of the version of the paper to be published.

Changes in Authorship

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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Appealing Decisions

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.

Declaration of funding sources

Global Journals is in partnership with various universities, laboratories, and other institutions worldwide in the research domain. Authors are requested to disclose their source of funding during every stage of their research, such as making analysis, performing laboratory operations, computing data, and using institutional resources, from writing an article to its submission. This will also help authors to get reimbursements by requesting an open access publication letter from Global Journals and submitting to the respective funding source.

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:

When you refer to information, differentiate data generated by your own studies from other available information. Present work done by specific persons (including you) in past tense.

Describe generally acknowledged facts and main beliefs in present tense.

The Administration Rules

Administration Rules to Be Strictly Followed before Submitting Your Research Paper to Global Journals Inc.

Please read the following rules and regulations carefully before submitting your research paper to Global Journals Inc. to avoid rejection.

Segment draft and final research paper: You have to strictly follow the template of a research paper, failing which your paper may get rejected. You are expected to write each part of the paper wholly on your own. The peer reviewers need to identify your own perspective of the concepts in your own terms. Please do not extract straight from any other source, and do not rephrase someone else's analysis. Do not allow anyone else to proofread your manuscript.

Written material: You may discuss this with your guides and key sources. Do not copy anyone else's paper, even if this is only imitation, otherwise it will be rejected on the grounds of plagiarism, which is illegal. Various methods to avoid plagiarism are strictly applied by us to every paper, and, if found guilty, you may be blacklisted, which could affect your career adversely. To guard yourself and others from possible illegal use, please do not permit anyone to use or even read your paper and file.

Criterion for Grading a Research Paper (Compilation) by Global Journals

Please note that following table is only a Grading of "Paper Compilation" and not on "Performed/Stated Research" whose grading solely depends on Individual Assigned Peer Reviewer and Editorial Board Member. These can be available only on request and after decision of Paper. This report will be the property of Global Journals.

Topics	Grades		
	A-B	**C-D**	**E-F**
Abstract	Clear and concise with appropriate content, Correct format. 200 words or below	Unclear summary and no specific data, Incorrect form Above 200 words	No specific data with ambiguous information Above 250 words
Introduction	Containing all background details with clear goal and appropriate details, flow specification, no grammar and spelling mistake, well organized sentence and paragraph, reference cited	Unclear and confusing data, appropriate format, grammar and spelling errors with unorganized matter	Out of place depth and content, hazy format
Methods and Procedures	Clear and to the point with well arranged paragraph, precision and accuracy of facts and figures, well organized subheads	Difficult to comprehend with embarrassed text, too much explanation but completed	Incorrect and unorganized structure with hazy meaning
Result	Well organized, Clear and specific, Correct units with precision, correct data, well structuring of paragraph, no grammar and spelling mistake	Complete and embarrassed text, difficult to comprehend	Irregular format with wrong facts and figures
Discussion	Well organized, meaningful specification, sound conclusion, logical and concise explanation, highly structured paragraph reference cited	Wordy, unclear conclusion, spurious	Conclusion is not cited, unorganized, difficult to comprehend
References	Complete and correct format, well organized	Beside the point, Incomplete	Wrong format and structuring

INDEX

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ISSN 9755896