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Gamma Interferon Assay

Studying the Presence of Adultery

Highlights

Review on Major Factors

Cellular Immune Response in Cattle

Discovering Thoughts, Inventing Future

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Gamma Interferon Assay for Cellular Immune Response in Cattle Vaccinated With FMD Vaccine Adjuvanted with Different Montanide Oils

By Sonia Ahmed Rizk, Wael Mossad Gamal EL-Din, Safy Eldean Mahdy, Ehab EL-Sayed Ibrahim & Hiam Mohamed Fakhry

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Gamma Interferon Assay for Cellular Immune Response in Cattle Vaccinated With FMD Vaccine Adjuvanted with Different Montanide Oils

Sonia Ahmed Rizk [α], Wael Mossad Gamal EL-Din [σ], Safy Eldean Mahdy [ρ], Ehab EL-Sayed Ibrahim [ω] & Hiam Mohamed Fakhry [¥]

Abstract- Cell-mediated immunity is critical for the prevention and control of Foot and Mouth Disease (FMD). Despite significant advancements in modern vaccinology, inactivated whole virus vaccines for FMD remain the mainstay for prophylactic and emergency uses. Emergency vaccination as part of the control strategies against foot-and-mouth disease virus (FMDV) has the potential to limit virus spread and reduce large-scale culling. Many efforts are currently devoted to improve the immune responses and protective efficacy of these vaccines. Adjuvants, which are often used to potentiate immune responses, provide an excellent mean to improve the efficacy of FMD vaccines.

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All of the prepared vaccines were capable of stimulating a systemic gamma interferon response. Montanide ISA-61 adjuvanted vaccine induced early response and produced higher IFN-gamma as compared to the two other adjuvants, while no systemic IFN-gamma was detected in plasma samples from the unvaccinated cattle.

Conclusion: Finally, conclusion from the obtained results through the present study it could be concluded that, all of the prepared vaccines were capable of stimulating a systemic gamma interferon response. Montanide ISA-61 adjuvanted vaccine induced early response, high cellular and humeral immunity and produced higher IFN-gamma as compared to the two other adjuvants, while no systemic IFN-gamma was detected in plasma samples from the unvaccinated cattle.

I. Introduction

Foot-and-mouth disease virus (FMDV) causes foot-and-mouth disease (FMD), a contagious and fatal disease in cloven-hoofed animals, characterized by vesicles in the mouth, tongue, hoofs, and nipples and increase in body temperature and appetite loss Depa et al., (2012). the natural route of infection is via the upper respiratory tract or through ingestion of the virus. Initial virus replication usually occurs in the pharyngeal epithelium resulting in primary vesicles Alexandersen and Mowat (2005). Fever and viraemia can occur within 1–2 days resulting in virus excretion from the respiratory tract, faeces, urine, saliva, milk and semen. Virus entering the blood disseminates to various predilection sites such as the mouth and nose, hooves and also sometimes teats and udder, in which secondary vesicles occur, and from which further virus is released Grubman (2005) and Diaz-San et al., (2009). The progress in FMD vaccine production was primarily directed towards safety of the vaccine, purity of the antigen, selection of proper adjuvant and endurance of immunity Osama (1992). Adjuvants, also can prolong the immune response and stimulate specific components of the immune response either humoral or cell mediated immunity Lombard et al., (2007) and Cao (2014). Currently, the double oil emulsion vaccines are preferred for FMD prevention as they can be used to protect all susceptible species, particularly dur-ing an outbreak situation Cox and Barnett (2009). Also, the oil adjuvant vaccines generate higher and long lasting immune responses, and show less inter-ference from maternal antibodies than the aqueous vaccines Selim et al., (2010). In particular, the Montanide™ ISA series of oil-adjuvants (SEPPIC France) have shown superior efficacy for inactivated FMD vaccines in different susceptible animal species Iyer et al., (2000). Recently, SEPPIC has developed a new adjuvants (Montanide ISA-201 and Montanide ISA-61) and claim that those adjuvants induce better immune responses (particularly CMI responses) Seppic. Montanide ISA 201 VG-ready to use oil adjuvant for veterinary vaccines and Sébastien et al., (2013). The ability to stimulate cell-mediated immunity (CMI) and conse-quent inhibition of sub-clinical infection in ruminants or otherwise induction of sterile immunity is usually insufficient Moonen et al., (2004) Interferons belong to cytokines. They are glycoproteins with multifaceted signal effects on cellular functions among which the antiviral effects belong to the early and non-specific defense mechanisms of organisms against infections Vilcek and Sen (1996).

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Interferons (IFNs) are the first line of the host innate immune defense against important, derives from its immunostimulatory and immunomodulatory effects Samuel (2001) and Delcenserie et al., (2008). The assay system has proven to be a rapid, sensitive and inexpensive method for measuring antigen specific cell-mediated reactivity when compared with the more traditional lymphocyte proliferation assay. The IFN-gamma assay is the first in-vitro cellular assay to be used as a routine diagnostic test in veterinary medicine Rothel et al., (1992). The production of interferon-gamma by stimulated helper T lymphocytes regulates production of immunoglobulin in vaccinated animals Green et al., (2015). IFN-gamma is a modulator of T-cell growth and functional differentiation. It is a growth-promoting factor for T-lymphocytes and potentiates the response of these cells to mitogens or growth factors. The production of IFN-gamma or IL-4 by subsets of helper T lymphocytes reciprocally regulates production of IgG2a and IgG1.The minimum detectable dose of IFN-gamma is typically less than 5 pg/ml Cubillos et al., (2008) and Bucafusco et al., (2015), while the protective level is more than 38% Sample to Positive (SP %) Gurung et al., (2014).

It has been suggested that cell-mediated immunity may be involved in the clearance viral infection so the importance of Interferon-Gamma (IFN-gamma) in the immune system stems in part from its ability to inhibit viral replication directly, but, most of persistent virus Ilott et al., (1997) and Childerstone et al., (1999) and it has been hypothesised that the initiation of FMDV persistence is correlated with the amount of interferon produced in the cells Phillips and Dinter (1963). FMDV strains modified by passage in alternate hosts or repeated passage in cell cultures have reduced virulence in cattle and, in contrast to more virulent wild-virus, will induce the production of interferon Zhang et al., (2014) with a correlation between lack of virulence in cattle and increased IFN production Alexandersen et al.,(2002).

The present work aims to evaluate the FMD virus-specific cell-mediated immunity in cattle vaccinated with FMD vaccine adjuvanted with different Montanide oils using interferon-gamma Assay, in order to determine to any extent FMD trivalent vaccine is able to elicit a sterile immunity.

II. Material and Methods

a) Cell and virus

Baby Hamster Kidney cell line (BHK21) Clone 13 maintained in FMD Department, Veterinary Serum and Vaccine Research Institute, Abbasia, Cairo according to the technique described by Macpherson and Stocher (1962) using Eagl's medium with 8-10% sterile new bovine serum, obtained from Sigma, USA, used for virus propagation and application of serum neutralization test.

b) Virus propagation and concentration

FMD viruses O PanAsia2, A/Iran 05 and SAT2/2012, are locally isolated strains of cattle origin. The viruses were typed at Veterinary Serum and Vaccine Research Institute, Abbasia, Cairo and confirmed by Pirbright, International Reference Laboratories, United Kingdom. FMD viruses then concentrated using polyethylene glycol 600 (PEG-600) according to Killington et al., (1996). The viral suspension was concentrated at 25,000 rpm, for 5 hours at 4℃ in a high-speed centrifuge (Avanti J25, Beckman Coulter, and Fullerton, CA, USA), the virus in the bottom was removed and polled. The virus was further concentrated in ultracentrifuge 35,000 rpm/min, 3 hours at 4℃, the viral pelted polled and aliquots of the concentrated virus preserved at -80℃.

c) FMD viruses inactivation

The concentrated virus stock completely inactivated using Binary Ethyleneimine (BEI) according to Bahnemann (1975), 1%M BEI in 0.2N NaOH was added to the virus suspension to give final concentration of 0.001M of BEI. The virus and BEI mixture were mixed well and the pH adjusted to 8.0 by sodium bicarbonate. The virus was placed in the incubator at 37°C for 24 hours for inactivation to occur. Sodium thiosulphate was added to give a final concentration of 2% to neutralize the BEI action. The killed vaccine kept at -80℃, to use in preparation of vaccine formulation with different Montanide Oil adjuvants (ISA 206,201 and 61) for animal immunization according to FAO. (2012).

d) Montanide ISA 206

This is a mineral oil based adjuvant which has been developed for the manufacture of Water-in-Oil-in-Water (W/O/W) emulsions mixed with antigen 50% w/w. It was obtained from Seppic, Paris, France.

e) Montanide ISA 201

This is a mineral oil based adjuvant that has been developed for the manufacture of Water-in-Oil-in-Water (W/O/W) emulsions mixed with antigen 50% w/w. It was obtained from Seppic, Paris, France.

f) Montanide ISA 61

This is a mineral oil based adjuvant that has been developed for the manufacture of water-in-oil (W/O) emulsions mixed with antigen 60% w/w. It was obtained from Seppic, Paris, France.

g) Trivalent FMD vaccines preparation

i. *FMD oil adjuvanted vaccine formulated with Montanide ISA 206*

Formulation with oil phase carried out according to the method described by Wael et al., (2014), where the oil phase consisted of Montnide ISA

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206 mixed with the inactivated viruses as equal parts of an aqueous and oil phase (50% w/ w) and mixed thoroughly.

ii. *FMD oil adjuvanted vaccine formulated with Montanide ISA 201*

Formulation with oil phase carried out according to the method described by **Dar et al., (2013) and Ehab et al., (2015)** where the oil phase consisted of Montnide ISA 201 mixed with the inactivated viruses as equal parts of an aqueous and oil phase (50% w/ w) and mixed thoroughly.

iii. *FMD oil adjuvanted vaccine formulated with Montanide ISA 61*

Formulation with oil phase carried out according to the method described by **Gurung et al., (2014)** where the oil phase consisted of Montnide ISA 61 mixed with the inactivated viruses as 60% of an aqueous and oil phase (60% w/w) and mixed thoroughly.

h) Animal groups

.Twelve calves (local breed) were clinically healthy and free from antibodies against FMD virus as proved by using SNT and ELISA were used in this study.

Calves used in experimental vaccination were classified into four groups:

Group A: (3 animals) inoculated subcutaneously (S/C) with 3ml of inactivated FMD vaccine djuvanted with Montanide oil ISA 206.

Group B: (3animals) inoculated subcutaneously (S/C) with 3ml of inactivated FMD vaccine adjuvant with Montanide oil ISA 201.

Group C: (3 animals) inoculated subcutaneously (S/C with 3ml of inactivated FMD vaccine adjuvanted with Montanide oil ISA 61.

Group D: (3 animals) non vaccinated and used as control group.

i) Samples collection

Blood samples were collected on 3rd post vaccination every three days for 2 weeks and later every week up to 10 weeks. Serum samples were collected weekly post vaccination for one month then every 2 weeks post-vaccination till the end of experiment. The immune response was evaluated through the detection of INF-gamma and humoral immune level using Bovine IFN-gamma ELISA assays, SNT and ELISA.

i. *Detection of interferon gamma (IFN-gamma) using Bovine IFN-gamma ELISA kits*

It was applied according to **Barnett et al., (2004).** The cytokine IFN-gamma was measured in plasma samples from all cattle groups at various time points before and following vaccination using Bovine IFN-γ ELISA kit **(Mabtech- Sweden – code/3115-1H-20).** High protein binding ELISA plates were coated with mAb bIFN-γ-1 diluted to 2μg/ml in PBS, PH 7.4, by adding 100 μl/well incubated overnight 4-8◦C according to the manual technique. The plates were washed with PBS (200μl/well) before blocking with PBS containing 1% bovine serum albumin for 30 min at room temperature. Blocked plates then were washed five times with PBS containing 0.05% Tween 20(Incubation buffer).

Bovine IFN-gamma was prepared standard by reconstituting content of vial in 1ml PBS to give concentration of 0.5μg/ml and leaved at room temperature for 15 minutes, then vortex the tube and spin down and use immediately. Samples or standards diluted in incubation buffer added as 100μl/well and incubated for 2 hours at room temperature, then washed as before. Then 100μl/well of mAb PAN-biotin at 0.1g/ml in incubation buffer was added, incubated for 1 hour at room temperature and then washed as washing step. Then 100μl/well of Streptavidin-Horse Radish Peroxidase (Streptavidin-HRP) diluted 1:1000 in incubation buffer was added and incubated at room temperature for 1 hour.

Appropriate substrate solution was added as 100μl/well. Finally measured the optical density in an ELISA reader after suitable developing time, absorbance values were read at 492 nm and the results were calculated according to kits typical data. Optical density values were normalized across plates using the following calculation:

Sample-to positive (SP %) = [(Mean sample OD) – (Mean negative control OD) /[(Mean positive control OD) – (Mean negative control OD) × 100 **Gurung et al., (2014)**.

ii. *Serum neutralization test (SNT)*

The test was performed by the microtechnique as described by **Ferreira (1976)** in flat bottom tissue culture microtitre plates.

iii. *Enzyme linked immunosrobent assay (ELISA)*

It was carried out according to the method described by **Voller et al., (1976).**

Serum samples were examined for FMD viral specific IgG antibodies using in-house developed ELISA assay.

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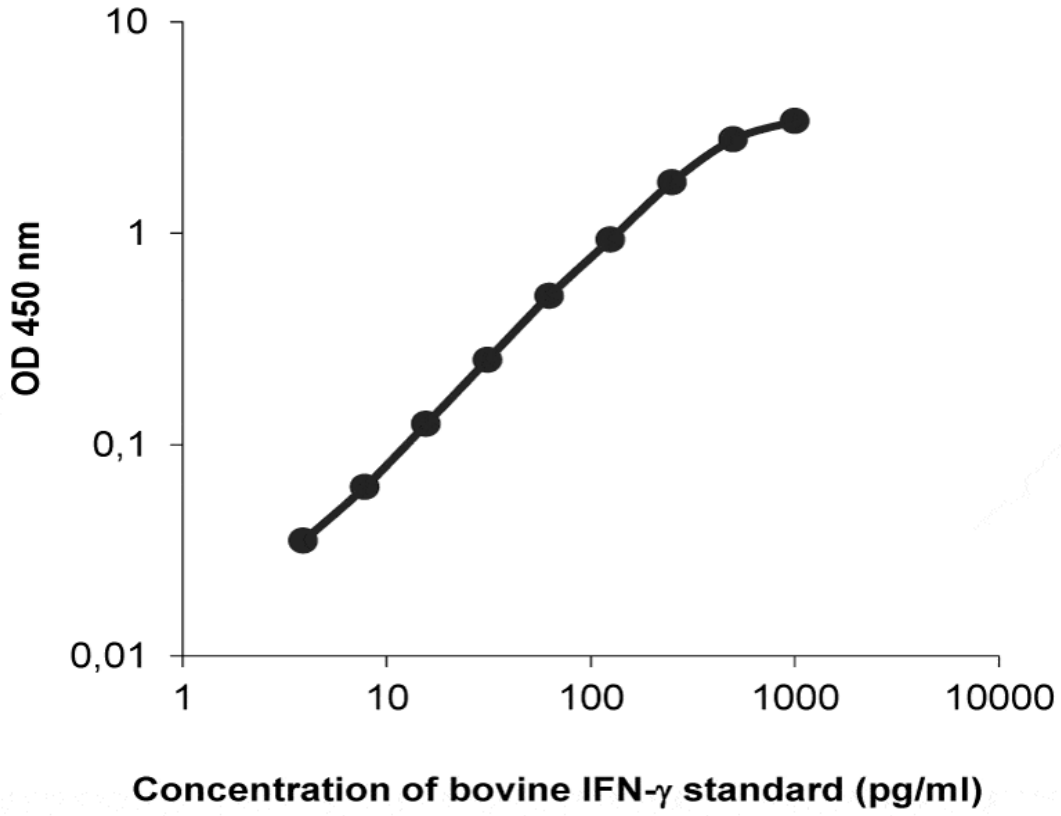
III. Results

Table (1) : Typical data using Bovine IFN-gamma ELISA Kits.

Standard	Bovine IFN-gamma Concentration (pg/ml)	O.D. at 492 nm	Mean O.D. at 492 nm
1	1000.0	1.983 - 1.977	1.980
2	500.0	1.701 - 1.790	1.746
3	250.0	0.881 - 0.876	0.879
4	125.0	0.462 - 0.485	0.479
5	62.5	0.252 - 0.258	0.255
6	31.3	0.144 - 0.149	0.147
7	15.6	0.093 - 0.096	0.095
8	7.8	0.067 - 0.067	0.067
Blank	0	0.031 - 0.028	0.030

Chart (1) : Standard curve for typical data using Bovine IFN-gamma ELISA Kits



a) Interferon gamma (IFN-gamma) ELISA

The cell mediated response to inactivated trivalent FMD vaccine adjuvanted with different Montanide oils was evaluated by measuring of IFN-gamma in plasma of vaccinated and control cattle groups. IFN-gamma for Montanide ISA 206 group (Group A) detected at 7th day following vaccination. Mean IFN-gamma cons. was (200pg/ml), Optical density (O.D.) was 0.763 and SP% was (40%.) The highest level was at 14Th day, with a cons. of (450pg/ml) and O.D. of 1.572 and SP% (84%). It was at protective level (>38%) till 35 days post vaccination. **Tables (2)**

IFN-gamma for Montanide ISA 201 group (Group B) detected at 3rd day following vaccination. Mean IFN-gamma cons. was (200pg/ml), Optical density (O.D.) was 0.765 and SP% was (40%). The highest level was at 14Th day, with a cons. of (500pg/ml) and O.D. of 1.743 and SP% (93%). It was at protective level (>38%) till 42 days post vaccination. **Tables (3)**

IFN-gamma for Montanide ISA 61 group (Group C) detected at 3rd day following vaccination. Mean IFN-gamma cons. was (250pg/ml), Optical density (O.D.) was as 0.881 and SP% was (46%). The highest level was at 14Th day, with a cons. of (800pg/ml) and O.D. of

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1.847 and SP% (97.5%). It was at protective level (>38%) till 56 days post vaccination. **Tables (4)**

IFN-gamma level of was undetectable in plasma of control unvaccinated group (Group D).

Table (2) : Interferon gamma (IFN-gamma) responses in cattle vaccinated with inactivated trivalent FMD vaccine adjuvanted with MontanideISA 206 oil

Days	IFN-gamma	Vaccinated cattle				Mean Control group
		A1	A2	A3	Mean	
0	*cons.	0	0	0	0	0
	O.D	0.031	0.030	0.029	0.030	0.030
	**SP%	0%	0%	0%	0%	0%
3	cons.	125.0	125.0	62.5	100	0
	O.D	0.462	0.462	0.258	0.394	0.030
	SP%	23%	23%	12%	19.5%	0%
7	cons.	200.0	200	200	200	0
	O.D	0.761	0.765	0.763	0.763	0.030
	SP%	40%	40%	40%	40%	0%
10	cons.	250.0	250.0	250.0	250.0	0
	O.D	0.881	0.881	0.881	0.881	0.030
	SP%	46%	46%	46%	46%	0%
14	cons.	450	450	450	450	0
	O.D	1.572	1.572	1.572	1.572	0.030
	SP%	84%	84%	84%	84%	0%
21	cons.	450	450	450	450	0
	O.D	1.572	1.572	1.572	1.572	0.030
	SP%	84%	84%	84%	84%	0%
28	cons.	400	400	400	400	0
	O.D	1.402	1.400	1.401	1.400	0.030
	SP%	74%	74%	74%	74%	0%
35	cons.	250.0	250.0	250.0	250.0	0
	O.D	0.881	0.881	0.881	0.881	0.030
	SP%	46%	46%	46%	46%	0%
42	cons.	125.0	125.0	62.5	100	0
	O.D	0.462	0.462	0.258	0.394	0.030
	SP%	23%	23%	12%	20%	0%
49	cons.	100	100	100	100	0
	O.D	0.396	0.392	0.394	0.394	0.030
	SP%	19.5%	19.5%	19.5%	19.5%	0%
56	cons.	62.5	62.5	62.5	62.5	0
	O.D	0.258	0.254	0.256	0.256	0.030
	SP%	12%	12%	12%	12%	0%

**IFN-gamma cons. (pg/ml)*

O.D. at492 nm

*** Sample-to positive %*

SP% protection cutoff > 38%

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Table (3) : Interferon gamma (IFN-gamma) responses in cattle vaccinated with inactivated trivalent FMD vaccine adjuvanted with MontanideISA 201 oil

Days *	IFN-gamma	Vaccinated cattle				Mean Control group
		A1	A2	A3	Mean	
0	*cons.	0	0	0	0	0
	O.D	0.031	0.030	0.029	0.030	0.030
	**SP%	0%	0%	0%	0%	0%
3	cons.	200.0	200	200	200	0
	O.D	0.764	0.765	0.766	0.765	0.030
	SP%	40%	40%	40%	40%	0%
7	cons.	250.0	250.0	250.0	250.0	0
	O.D	0.881	0.881	0.881	0.881	0.030
	SP%	46%	46%	46%	46%	0%
10	cons.	500.0	500	500	500	0
	O.D	1.745	1.742	1.742	1.743	0.030
	SP%	93%	93%	93%	93%	0%
14	cons.	250.0	250.0	250.0	250.0	0
	O.D	0.881	0.881	0.881	0.881	0.030
	SP%	46%	46%	46%	46%	0%
21	cons.	500.0	450	500.0	650	0
	O.D	1.742	1.572	1.742	1.685	0.030
	SP%	92%	83%	92%	89%	0%
28	cons.	400	400	400	400	0
	O.D	1.402	1.402	1.402	1.402	0.030
	SP%	74%	74%	74%	74%	0%
35	cons.	250.0	250.0	250.0	250.0	0
	O.D	0.881	0.881	0.881	0.881	0.030
	SP%	46%	46%	46%	46%	0%
42	cons.	250.0	250.0	250.0	250.0	0
	O.D	0.881	0.881	0.881	0.881	0.030
	SP%	46%	46%	46%	46%	0%
49	cons.	125.0	125.0	125.0	125.0	0
	O.D	0.460	0.462	0.464	0.462	0.030
	SP%	23%	23%	23%	23%	0%
56	cons.	100	100	100	100	0
	O.D	0.392	0.394	0.396	0.394	0.030
	SP%	19.5%	19.5%	19.5%	19.5%	0%

**IFN-gamma cons. (pg/ml)*

O.D. at492 nm

*** Sample-to positive %*

SP% protection cutoff > 38%

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Table (4) : Interferon gamma (IFN-gamma) responses in cattle vaccinated with inactivated trivalent FMD vaccine adjuvanted with Montanide ISA 61oil.

Days *	IFN-gamma	Vaccinated cattle				Mean Control group
		A1	A2	A3	Mean	
0	*cons.	0	0	0	0	0
	O.D	0.030	0.030	0.030	0.030	0.030
	**SP%	0%	0%	0%	0%	0%
3	cons.	250.0	250.0	250.0	250.0	0
	O.D	0.881	0.881	0.881	0.881	0.030
	SP%	46%	46%	46%	46%	0%
7	cons.	500.0	500	500	500	0
	O.D	1.745	1.745	1.745	1.745	0.030
	SP%	93%	93%	93%	93%	0%
10	cons.	1000	650	800	800	0
	O.D	1.981	1.673	1.887	1.847	0.030
	SP%	105%	88%	100%	97.5%	0%
14	cons.	800	800	500.0	800	0
	O.D	1.887	1.887	1.750	1.841	0.030
	SP%	100%	100%	92%	97.5%	0%
21	cons.	500.0	450	500.0	650	0
	O.D	1.742	1.572	1.742	1.685	0.030
	SP%	92%	83%	92%	89%	0%
28	cons.	500.0	450	500.0	650	0
	O.D	1.742	1.572	1.742	1.685	0.030
	SP%	92%	83%	92%	89%	0%
35	cons.	400	400	400	400	0
	O.D	1.402	1.400	1.401	1.400	0.030
	SP%	74%	74%	74%	74%	0%
42	cons.	400	400	400	400	0
	O.D	1.402	1.400	1.401	1.400	0.030
	SP%	74%	74%	74%	74%	0%
49	cons.	250.0	250.0	250.0	250.0	0
	O.D	0.881	0.881	0.881	0.881	0.030
	SP%	46%	46%	46%	46%	0%
56	cons.	250.0	250.0	250.0	250.0	0
	O.D	0.881	0.881	0.881	0.881	0.030
	SP%	46%	46%	46%	46%	0%

**IFN-gamma cons. (pg/ml)*

O.D. at492 nm

*** Sample-to positive %SP% protection cutoff > 38%*

b) *Evaluation of humeral immune response in calves vaccinated with FMD vaccines using SNT against FMDV serotypes (O,A&SAT2)*

The humeral immune response of calves vaccinated with trivalent FMD vaccines(formulated with Montanide oil ISA 206,201 and 61) using SNT for FMD virus showed that protective neutralizing serum antibody titer for Montanide ISA 206 started at the 2nd week post vaccination with average antibody titer of (1.5-1.6 &1.5 $\log_{10}$) for (O, A & SAT2) respectively. The obtained antibody titer reached to the peak level at 10th week post vaccination with average titers of (2.4 – 2.7 &2.6 $\log_{10}$). The protective neutralizing serum antibody titer for Montanide ISA 201 started at the 1st week post vaccination with average antibody titer of (1.5-1.6&1.5 $\log_{10}$) for (O, A&SAT2) respectively. The obtained antibody titer reached to the peak level at 10th week post vaccination with average titers of (3.05– 3.1 &3.05

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log_{10}).The protective neutralizing serum antibody titer for Montanide ISA 61started at the 1st week post vaccination with average antibody titer of (1.7, 1.8 &1.7 log_{10}) for (O, A&SAT2) respectively. The obtained antibody titer reached to the peak level at 10th week post vaccination with average titers of (3.1-3.4&3.1 log_{10}). **Tables (5)**

Table (5) : Neutralizing antibody titers of calves vaccinated with inactivated trivalent FMD vaccine using SNT against FMDV serotype (O , A & SAT2).

Time post vaccination	SNT titers of vaccinated animal groups									Control group
	Group A (ISA 206)			Group B (ISA 201)			Group C (ISA 61)			
	O	A	SAT2	O	A	SAT2	O	A	SAT2	
0	0.15*	0.12	0.12	0.3	0.3	0.3	0.3	0.3	0.3	0.3
1 week	0.9	1.2	0.9	1.5	1.6	1.5	1.7	1.8	1.7	0.3
2 week	1.5	1.6	1.5	1.7	1.8	1.7	1.8	2.1	1.8	0.3
3 week	1.8	1.8	1.8	2.1	2.4	2.1	2.4	2.4	2.4	0.3
4 week	2.1	2.1	2.1	2.4	2.4	2.4	2.7	2.7	2.7	0.6
6 week	2.1	2.4	2.1	2.4	2.7	2.4	2.7	2.9	2.7	0.9
8 week	2.4	2.4	2.4	2.7	2.9	2.7	3.05	3.1	3.05	0.9
10 week	2.4	2.7	2.6	3.05	3.1	3.05	3.1	3.4	3.1	0.9
12 week	2.4	2.4	2.4	2.7	2.9	2.7	3.05	3.1	3.05	0.6
14 week	2.1	2.1	2.1	2.7	2.7	2.7	2.8	2.8	2.8	0.6
16 week	2.1	2.1	2.1	2.4	2.7	2.4	2.7	2.8	2.7	0.6
18 week	2.1	2.1	2.1	2.4	2.4	2.4	2.6	2.7	2.6	0.6
20 week	1.8	1.8	1.8	2.1	2.4	2.1	2.4	2.7	2.4	0.6
22 week	1.8	1.8	1.8	2.1	2.1	2.1	2.4	2.4	2.4	0.3
24 week	1.8	1.8	1.8	1.8	2.1	1.8	2.1	2.4	2.1	0.3

** = Antibody titers expressed as log_{10} serum neutralizing antibody titer.*

Protective level (1.5)

c) *Evaluation of humeral immune response in calves vaccinated with FMD vaccines using ELISA against FMDV. serotypes (O,A&SAT2)*

The protective antibody titer for FMD vaccine formulated with Montanide ISA 206 started at the 2st week post vaccination with average antibody titer of (1.40 -1.50& 1.50 log_{10}) for O,A & SAT2 respectively . The obtained antibody titer reached to the peak level at 10th week post vaccination with average titers of (2.90 – 2.92 & 2.92log_{10}) for (O, A&SAT2) respectively.

The protective antibody titer for Montanide ISA 201 started at the 1st week post vaccination with average antibody titer of (1.93 -1.95 &1.93 log_{10}). The obtained antibody titer reached to the peak level at 10th week post vaccination with average titers of (3.12- 3.15 &3.13 log10). The protective neutralizing serum antibody titer for Montanide ISA 61 started at the 2nd week post vaccination with average antibody titer of (1.97- 1.99 & 1.96 log_{10}). The obtained antibody titer reached to the peak level at 10th week post vaccination with average titers of (3.32 -3.34 &3.33 log10).**Table (6).**

Table (6) : Antibody titers of calves vaccinated with inactivated trivalent FMD vaccine using ELISA against FMDV serotype (O, A and SAT2).

Time post vaccination	ELISA titers of vaccinated animal groups									Control group
	Group A (ISA 206)			Group B (ISA 201)			Group C (ISA 61)			
	O	A	SAT2	O	A	SAT2	O	A	SAT2	
0	0.24*	0.27	0.27	0.18*	0.21	0.21	0.11	0.21	0.21	0.3
1 week	1.40	1.50	1.50	1.93	1.95	1.93	1.70	1.70	1.69	0.0
2 week	1.90	1.92	1.90	2.12	2.12	2.11	1.97	1.99	1.96	0.0
3 week	2.19	2.19	2.16	2.42	2.42	2.41	2.61	2.62	2.61	0.3
4 week	2.43	2.43	2.43	2.47	2.47	2.46	2.43	2.49	2.48	0.6
6 week	2.44	2.44	2.44	2.73	2.73	2.73	2.73	2.79	2.79	0.7
8 week	2.80	2.80	2.78	2.92	2.92	2.92	2.92	2.95	2.95	0.6
10 week	2.90	2.92	2.92	3.12	3.15	3.13	3.32	3.34	3.33	0.6

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12 week	3.10	3.10	3.10	3.15	3.15	3.15	3.15	3.19	3.19	0.6
14 week	2.49	2.49	2.49	2.85	2.85	2.85	2.97	2.99	2.99	0.0
16 week	2.52	2.52	2.52	2.67	2.67	2.67	2.75	2.78	2.76	0.6
18 week	2.43	2.43	2.43	2.66	2.66	2.65	2.69	2.71	2.71	0.0
20 week	2.19	2.19	2.19	2.34	2.34	2.34	2.60	2.62	2.62	0.6
22 week	2.10	2.11	2.11	2.31	2.32	2.32	2.44	2.46	2.46	0.7
24 week	2.09	2.10	2.10	2.34	2.34	2.34	2.43	2.46	2.46	0.3

** = Antibody titers expressed as log_{10} ELISA antibody titer*

Protective level (1.9)

IV. Discussion

The first use of an oil adjuvant inactivated FMD vaccine was stated by **Cunliffe and Graves (1963)**. Such vaccine was found to induce higher immune levels and protection in vaccinated cattle than that induced by the conventional aluminum hydroxide vaccines. So it could be considered an important tool in the control programs of FMD **Bahnemann and Mesquita (1987) and Iyer et al., (2000)**. An adjuvant may act in one or more of five ways, based on current knowledge; namely, immune-modulation, presentation, induction of CD8+cytotoxic T-lymphocyte (CTL) responses, targeting, and depot generation. Addition to that adjuvant plays an important role in production of different lympho-kines such as various interleukins and INF-gamma according to **Barnett et al., (2004) and Ebeid et al., (2011)**. The innate immune response induced by a viral infection in the upper respiratory tract, the macrophages present in the respiratory tract produce interferons (IFNs) upon stimulation of pattern recognizing surface receptors, causing alterations in local vascular walls, and providing recruitment and activating stimuli to antigen presenting cells and phagocytes **Wilkins and Gale (2010)**. IFNs are also known as viral IFNs and secreted by virus infected cells with the function of blocking spread of virus to uninfected cells and have an important role in the host response to FMDV **Summerfield et al., (2009)** and that the ability of the virus to induce an IFN response may be related to the pathogenicity of different isolates of FMDV **Santos et al., (2006) and Stenfeldt et al., (2011)**. To better characterize the immune response to FMD vaccines and to search for early markers predictive of induction of immune memory; must analyze the kinetics and magnitude of the antibody and cell-mediated immune responses to FMD vaccinesand further characterization of the antigen-specific $CD4^+$ T-cell response better to be attempt by measuring IFN-gamma production **Carr et al., (2013)**.

So, this study was performed for evaluation of FMD virus-specific cell-mediated immunity in cattle vaccinated with FMD vaccine adjuvanted with different Montanide oils using interferon-gamma Assay, in order to determine to any extent FMD trivalent vaccine is able to elicit a sterile immunity.

Table (1) and Chart (1) show the typical data using Bovine IFN-gamma ELISA Kits for 8 slandered solutions beside the blank one. Bovine IFN- gamma Concentration (pg/ml) with the respectively O.D. at 492 nm.

From **Tables (2, 3 and 4)** no systemic IFN-gamma was detected in plasma samples from the unvaccinated cattle. IFN-gamma for Montanise ISA 206 group (Group A) detected at 7th day following vaccination, that results agreed with **Stenfeldt et al., (2011)** who observed that within seven days of vaccination with FMD oil vaccine, IFN-gamma production was observed and supported with **Cavalcanti et al., (2012) and Bucafusco et al., (2015)** they found that on day 7 both $CD4^+$ and $CD8^+$ T cell populations produced IFN-gamma .The obtained results also in agreement in some points with **Habjanec et al., (2008)** who stated that ISA206 formulations were less effective in inducing INF-gamma. IFN-gamma for Montanise ISA 201 group (Group B) detected at 3rd day following vaccination and that results agreed with **Dar et al., (2013)** who observed that Montanide ISA-201 adjuvanted vaccine induced earlier and higher immune response in vaccinated animals, and supported with **Gurung et al., (2014)** who reported that vaccine formulation with the antigen and Montanide™ ISA 201 adjuvant produced strong specific IFN-gamma responses in a high proportion of the vaccinated animals. IFN-gamma for Montanide ISA 201 group (Group B) detected at 3rd day following vaccination and that results agreed with **Dar et al., (2013)** who observed that Montanide ISA-201 adjuvanted vaccine induced earlier and higher immune response in vaccinated animals, and supported with **Gurung et al., (2014)** who reported that vaccine formulation with the antigen and Montanide™ ISA 201VG adjuvant produced strong specific IFN-gamma responses in a high proportion of the vaccinated animals. The results also come parallel and in agreement with what obtained by **Dong et al., (2013)** who reported that the efficacy of the FMD vaccine emulsified with ISA 201 was better than which with ISA 206. IFN-gamma for Montanide ISA 61 group (Group C) detected at 3rd day following vaccination and that results agreed with **Gurung et al., (2014)** who observed that vaccine formulated with ISA 61 showed the highest specific IFN-gamma responses among the

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different ISA oil formulations, which can be observed at 9 weeks post vaccination. The results also showed that great variation was observed between the vaccinated animal groups in INF-gamma production level depending on the adjuvant. From previous results, the quantity of IFN-gamma produced was significantly the highest in group (C) compared to the other groups from day 3 till day 63 post-vaccination .Also, the quantity of IFN-γ produced in the plasma samples from vaccinated animals was significantly higher than the quantity produced in the samples from the unvaccinated control animals. From tables **(5and 6)** the results revealed that SNT and ELISA titers for different oil FMD vaccines agreed with **Dar et al., (2013)** who showed that Montanide ISA-201 adjuvanted vaccine induced earlier and higher neutralizing antibody responses as compared to the two other oil adjuvants, also were supported by **Parida et al., (2006)** who recorded that IFN- production assay could be used to support the established serological assays to confirm infection in a previously vaccinated herd. Our results also go in hand with the results obtained were consistent with the statement of **Hamblin et al., (1986)** who explained that the SNT measures those antibodies which neutralize the infectivity of FMD virion, while ELISA probably measure all classes of antibodies even those produced against incomplete and non-infectious virus.

The obtained results were in agreement with **Parida et al., (2006) and Barnett et al., (2004) w**ho showed that in a vaccine IFN-gamma response could be a useful indicator of the ability of a FMD vaccine to elicit a so-called sterile immunity in which subclinical infection is prevented. This early IFN-gamma production probably comes from NK cells activated by macrophage derived cytokines as part of the innate immune response.

Our results also were supported by **Wu et al., (2003) and Diaz-San et al., (2010)** who suggested that there is a complex interplay between IFN-induced immunomodulatory in protection of animals against FMDV.

Finally, conclusion from the obtained results through the present study it could be concluded that, all of the prepared vaccines were capable of stimulating a systemic gamma interferon response. Montanide ISA-61 adjuvanted vaccine induced early response, high cellular and humeral immunity and produced higher IFN-gamma as compared to the two other adjuvants, while no systemic IFN-gamma was detected in plasma samples from the unvaccinated cattle.

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Studying the Presence of Adultery, Fraudulent Imitation and Food Pathogens within Processed Meat Products (Such as Salami, Sausage, Braised Meat) using DNA Typing and PCR Procedures*

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Abstract- Adulteration of meat with cheaper ambiguous meats of different origin during preparation of meat products is a common practice in many countries. Because meat adulteration and mislabeling are illegal and raise many health, religious, cultural and economic issues. In this study, 500 ready to eat raw meat samples (minced meat, lahmacun ingredients, kebap, stew and meatball samples / 100 samples for each type) were collected from different types of plants that were located in Istanbul. The samples were explored if they had different animal originated DNA residues (pork, chicken, cattle, sheep, horse, donkey, cat, dog, mouse, cockroach and house fly) by PCR procedures. According to the results, total of 52 samples were determined as adulterated and different originated animal DNA samples were found (chicken, horse and sheep DNA residues). It was concluded that to apply total quality management and food security systems are very important to decrease the risk factors for both products and the public health.

Keywords: *PCR, species identification, ready to eat meat products.*

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Özet- Et ve ürünlerinde taklit ve tağşiş uygulamaları gerek kar amacını yükseltmek amacı ile illegal bir biçimde yapılmakta, gerekse birden fazla et ürünü işleyen işletmelerde kaza / yetersiz hijyen ve san istasyon uygulamaları sonucu meydana gelebilmektedir. Et ve ürünlerinde taklit ve tağşişler ekonomik, dini inançlar, sağlık, kültürel, tüketiciyi aldatma yönünden önemli sorunlara yol açabilmektedir. Bu çalışmada 500 adet tüketime hazır halde satışa sunulmuş olan çiğ et örneği (kıyma, lahmacun iç malzemesi, kebap, köfte ve sulu yemeklerde kullanılmak üzere hazırlanmış etler olmak üzere) İstanbul'da bulunan farklı satış noktalarından toplanılmış ve söz konusu örneklerde 9 adet farklı hayvana ait (domuz, tavuk, sığır, koyun, at, eşek, kedi, köpek, fare, hamamböceği ve ev sineği olmak üzere) DNA örnekleri PCR prosedürleri kullanılarak araştırılmıştır. Elde edilen sonuçlara göre 52 adet örnekte farklı hayvan türlerine ait (tavuk ,at ve koyun olmak üzere) DNA kalıntıları saptanmıştır. Sonuç olarak özellikle et ve ürünlerini üreten işletmelerde toplam kalite yönetimi ve optimal hijyen uygulamalarının kontrollü bir biçimde uygulanmasının taklit ve tağşiş uygulamalarının minimize edilebileceği sonucuna varılmıştır.

Anahtar sözcükler: PCR, tür tayini, tüketime hazır et ürünleri.

I. Introduction

The composition of food is a major concern of consumers today. In the case of adulterated meat product consumption, several factors including economic, food safety (allergy) and moral reasons (religious belief), trigger such apprehensions. Among these concerns, consumers are most sensitive because of religious factors and do not tolerate even trace amounts of adulteration of meat products with forbidden meats like pork. Hygiene and right labeling notified on the label of any food stuff are very important criteria especially for public health.

Although food safety practices is one of the top priority policies of European Union, the information on the labels of meat and meat products does not provide food safety guarantee for the period "from the stable to table" (1,2).

According to the latest "Meat and Meat Products Manifest announced in our country in February 2013 (3), production of meat products containing meat from different animal species has been banned.

Meat and meat products are species-wise safe if they are acquired from healthy animals and processed under hygienic conditions. However, in the frauds and adulterations which are used in order to cut down the costs and increase the profits, meat from inappropriate animals (horse, donkey, and hog) might be mixed in the aforementioned meat and meat products. Besides, in facilities which process several animal products (like facilities processing both cattle and poultry), foreign animal meat might be indeliberately adulterated in the meat products. Besides, due to poor hygienic standards, there may be a possibility of meat and meat products to be adulterated by the wastes and/or tissues of mice and / or insects.

Before the introduction of DNA (Deoxyribonucleic Acid) typing method, methods such as Ouchterlony method, SDS-PAGE, ELISA, isoelectric

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focusing (IEF), immune diffusion tests, chromatography, mass spectrophotometry analysis, HPLC analysis based on fractioning hemoglobin or fatty acids have been used to specify the animal type in meat and meat products. Some of these are based on protein analysis and immunological tests (4,5). However, in case of cooked and processed meat products, heat and continuity of temperature causes the denaturation of type-specific proteins and this decreases the reliability of these methods. PCR (Polymerize Chain Reaction) procedures based on DNA isolation are relatively more stable and are considered to be the most reliable method to specify the animal species of meat and meat products, especially for the short primary strands consisting of specific locus in heat treated products (6).

This study aimed to examine various meat and meat products (kebaps, lahmacun ingredients, minced meat, stews, various meat balls etc.) which are presented in various sales points (restaurants, butcher shops, groceries etc.) in Istanbul region, to determine their ingredients through DNA typing method and to specify the different animal tissues / residuals in these products.

II. Materials and Methods

a) Specimen Handling

Random sampling method has been used in this study. From 500 different sales points in the Istanbul region (250 sales points from Asian side and 250 sales points from European side), 500 meat and meat product samples have been collected. As required by the asepsis and antisepsis norms, samples have been placed in sterile containers and transferred to the laboratory in these containers which have +4°C internal heat.

Table 1 : Detailed information about specimen handling

Tablo 1 : Örnek toplama programına ait detay bilgiler

Region	Sample name	Sales point	Total number of samples
İstanbul Europe	Lahmacun ingredients	Kebap shop/restaurant	50
İstanbul Europe	Minced Meat	Butcher shop	50
Istanbul Europe	Kebap	Kebap shop/pedlar wrap point	50
İstanbul Europe	Meat balls	Restaurant	50
İstanbul Europe	Stews	Restaurant	50
Istanbul Asia	Lahmacun ingredients	Kebap shop/restaurant	50
İstanbul Asia	Minced meat	Butcher shop	50
Istanbul Asia	Kebap	Kebap shop/pedlar wrap point	50
İstanbul Asia	Meat ball	Restaurant	50
İstanbul Asia	Stew	Restaurant	50
TOTAL			500

b) DNA Extraction

DNA of all the isolates are extracted using commercial DNA extraction kits and in accordance with kit protocol. Extracts have been kept at –20°C, to be used as target DNA in PCR process.

c) PCR

50-100 mg tissue from the meat samples have been put into a microcentrifuge tube as small pieces. 400 µL solutions SH has been added and blended with vortex. 8 µL Proteinase K and 40 µL solution SLS have been added to the mixture. After blending properly, the mixture has been kept waiting for two hours at 60°C, in order for the cells to stretch. After the incubation at 60°C, 300 µL Solution SP has been added and blended with vortex for 30 seconds. The mixture has been centrifuged at 12.000 rpm for 30 minutes. The supernatant has been transferred to a clean tube and 500 µL isopropanol has been added.

Table 2 : Type-specific primer sets used in PCR procedure (15,16,17,18,19).

Tablo 2 : PCR prosedüründe kullanılan türe spesifik primer setleri (15,16,17,18,19)

Type	Primer Direction	Sequence
Pork	Forward / Reverse	5'-CTTGCAAATCCTAACAGGCCTG-3'/5'-CGTTTGCATGTAGATAGCGAATAAC-3'
Chicken	Forward / Reverse	5'-TCTGGGCTTAACTCTCATACTCACC-3'/5'-GGTTACTAGTGGGTTTGCTGGG-3'
Cattle	Forward / Reverse	5'-CCCGATTCTTCGCTTTCCAT-3'/5'-CTACGTCTGAGGAAATTCCTGTTG-3'
Sheep	Forward / Reverse	5'-CCTTATTACACCATTAAAGACATCCTAAGGT-3'/5'–GGGTCTCCAGTAAGTCAGGC-3'
Horse	Forward / Reverse	5'-CAGCCAATGCGTATTCGTACTCT-3'/5'-GTGTTCCACTGGCTGTCCG-3'

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Donkey	Forward / Reverse	5'-CATCCTACTAACTATAGCCGTGCTA-3'/5'-CAGTGTTGGGTTGTACACTAAGATG-3'
Cat	Forward / Reverse	5'-CATGCCTATCGAAACCTAACATAA-3'/5'-AAGAAGCTGCAGGAGAGTGAGT-3'
Dog	Forward / Reverse	5'-GATGTGATCCGAGAAGGCACA-3'/5'-TTGTAATGAATAAGGCTTGAAG-3'
Mice	Forward / Reverse	5'-CCAAGTCGACATGCACRTGTATACATAGTAAC–3'/5'TTATGTAAAACGACGGCCAGT-3'
Cocroach	Forward / Reverse	5'-GTGGAACTGGCTGGACTT-3'/5'-GAGACATGTGTAATCAGG-3'
Fly	Forward / Reverse	5'-CACAAGGATCGCTTCAAG-3'/5'-TGTTGGTATCATTGTCGG-3'

After blending with vortex, the mixture has been incubated for an hour at -20 ℃. Then, it has been centrifuged at 12.000 rpm for 20 minutes. Supernatant has been removed. The remaining pellet has been gently vortexed by 1 ml 70% ethanol and has been distributed, then centrifuged at 13.000 rpm for 5 minutes. Ethanol has been removed and the subsided DNA has been left to dry. After ethanol completely vaporized, 150 µL Solution SE has been added to the pellet and kept waiting for one night at room temperature, in order for the DNA to dissolve. The dissolved DNA has been measured with UV Spectrometers and diluted to the point of 50 ng/µL concentration. After that, heat treatment protocol has been applied for 10 seconds at 95°C and 15 seconds at 60°C. The second and third steps are repeated for 5 times as 3 cycles (7,8,9,10,11).

III. Results

18 (3.6%) of the samples showed chicken DNA, 33 (6.6%) of them showed sheep DNA and 1 (0.2%) of them showed horse DNA. None of them showed pork, donkey, cat, dog, mice, cockroach and fly DNA. The detailed refraction of the results can be seen in Table 3. The positive results have been determined through Real-time PCR procedures.

Table 3 : Extraneous DNAs (other than cattle DNA) determined ın the samples

Tablo 3 : Toplanılan örneklerde tespit edilen yabancı türlere ait (sığır DNA'sı dışında olmak üzere) DNA kalıntıları

Region	Sample (RAW)	Sales point	Extraneous DNA	DNA positive samples
Istanbul Europe - İstanbul Asia	Lahmacun ingredients	Kebap shop	Chicken	11
İstanbul Europe - İstanbul Asia	Minced meat	Butcher shop	Chicken	5
İstanbul Europe	Kebap	Kebap shop	Chicken	2
İstanbul Europe - İstanbul Asia	Kebap	Kebap shop	Sheep	30
İstanbul Europe	Minced meat	Butcher shop	Sheep	3
Istanbul Asia	Minced meat	Butcher shop	Horse	1
TOTAL				52

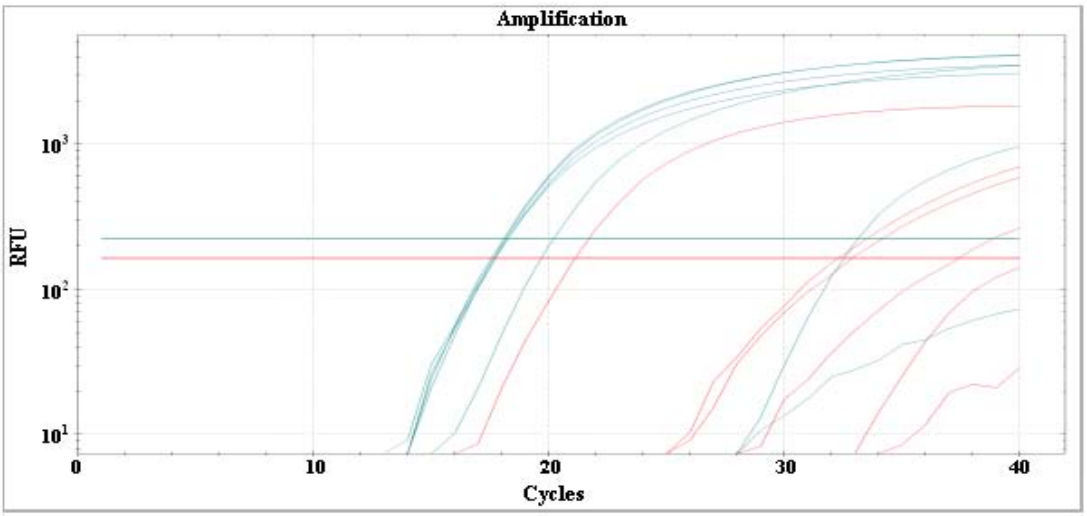

Figure 1 : Real time PCR Horse DNA amplification samples

Şekil 1 : Real time PCR at DNA'sına ait ampflikasyon örneği

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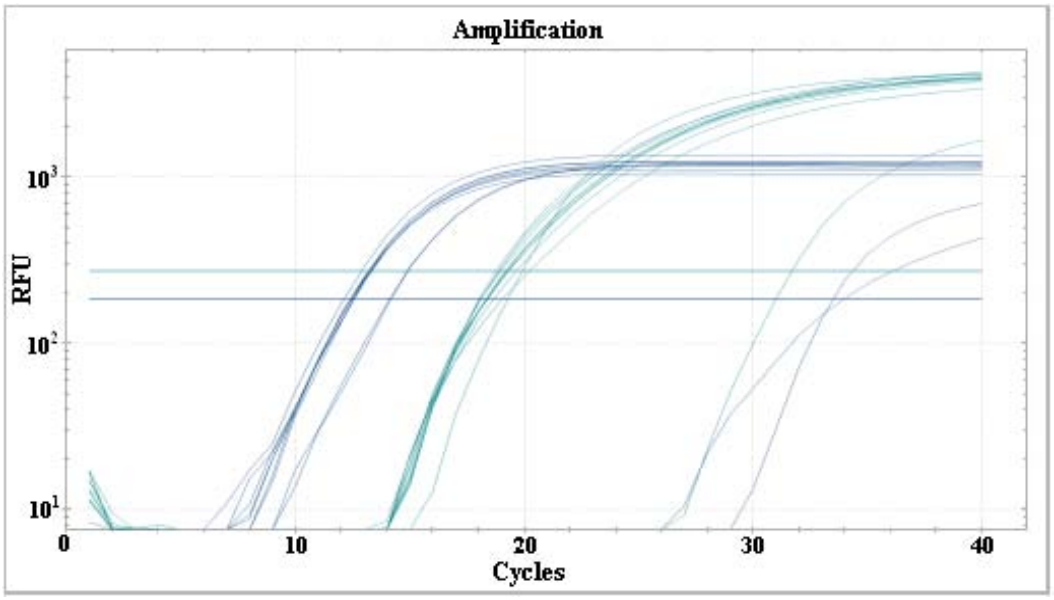

Figure 2 : Real time PCR Sheep DNA amplification samples

Şekil 2 : Real time PCR koyun DNA'sına ait ampflikasyon örneği

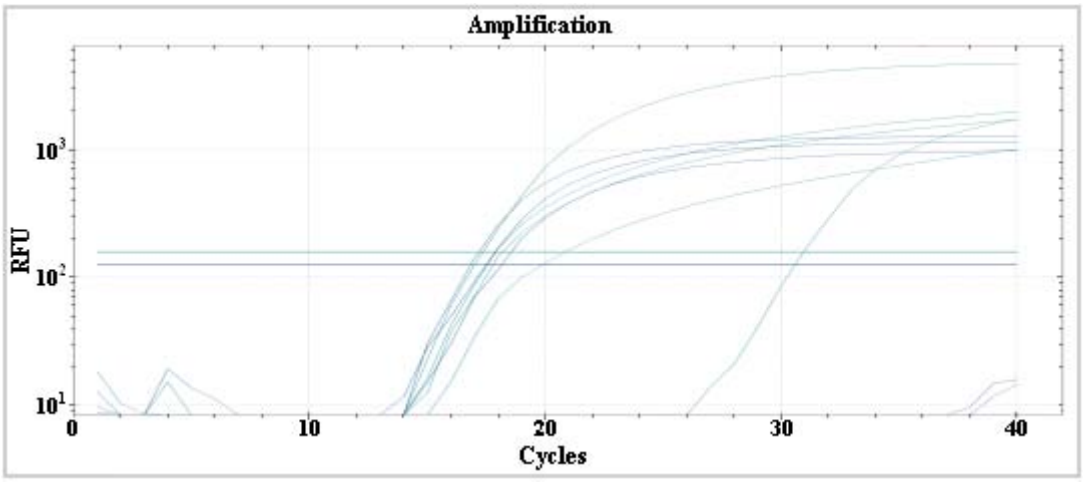

Figure 3 : Real time PCR Chicken DNA amplification samples

Şekil 3 : Real time PCR tavuk DNA'sına ait ampflikasyon örneği

IV. Discussion

The nutritious choices are determined by life styles, religious beliefs, cultures, diets and health conditions. Pursuant to community health, customs, traditions and beliefs, to determine the source of animals of the consumed meat and meat products has been one of the main research subjects for food scientists (12). In many countries, food fraud and adulteration in food products, especially in meat and meat products are done either deliberately in order to increase the profit margin or involuntarily as a result of not following the food safety standards, especially in facilities which process more than one animal species.

A study conducted in USA (United States of America) has analyzed raw minced meat and determined 15.9% of the samples to be containing extraneous animal DNAs . Hsieh et al. (13) has conducted another study in USA in 1996 and reported that 90% of the minced meat samples has been adulterated with poultry, either deliberately or unintentionally. Turkyılmaz et al. (14), studied 121 meat and meat product samples using the AGID method and determined horse meat in 3 (2.5%) of them and pork meat in 2 (1.7%). Turk et al. (15), studied 223 samples and determined pork meat in 16 (7.1%), horse meat in 12 (5.3%) and mixture of pork and horse meat in 6 (2.6%). The results of our study in general examination are lower than the results of Hsieh et al. (16), similar to those of Turkyılmaz et al. (14) and Turk et al. (15) The different results which have been reported in world and Turkey literature may originate from many reasons, such

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as the physical conditions of the sales points, whether the food safety products have been applied or not, the differences in the supervision processes, the deficiencies of the facilities which process more than one animal species and/or usage of the same equipment, the deliberateness of adulterations and the staff's lack of information about the procedures.

In this study, the highest extraneous DNA in the bovine meat samples was sheep DNA (6.2%). 96% (30 of the 31 mutton positive samples) of these positive samples have been collected from kebap shops. Since mutton meat is used commonly in kebap shops, mixture of bovine and mutton meat can be a microbiological threat to consumers.

Out of the 500 samples collected, 68 (13.6%) were determined to be risky for human consumption according to the plate count parameter. 39 (57.4%) of these "risky" samples contain meat from different animal species. On the other hand, 29 (42.6%) of these samples contained only one type of meat. Plate count is an indicator of not only food hygiene but also of the tools used in production, food contact surfaces and hands of the staff who contact food. If the plate count is high, it may mean that food, contact surfaces, tools and hands may be carrying potential pathogens and saprophytes.

In a study conducted to determine the food intolerance reactions, 22% of the subjects showed food intolerance and if the foods causing the intolerance are consumed again, the reactions repeated themselves in 15% of the subjects (17,18). Food intolerance may cause chronic inflammatory diseases such as chronic headache, abnormal weight gain, abnormal weight loss, dermatological problems, autoimmune diseases, fibromyalgia, migraine, stomach diseases, bowel diseases such as inflammatory bowel disease (IBD), malabsorptions, rheumatic diseases, shortness of breath, asthma, depression, anxiety, Type 2 diabetes, hypertension, metabolic syndrome, hypothyroidism, chronic rhinitis, eczema, acne, edematous eyelids, urinary diseases, Crohn's disease, cardiovascular diseases (19,20). Literature shows intolerance against food of animal origin. The intolerance, which is determined to be more common in males can cause the abovementioned clinical symptoms and some of them can be life threatening. According to WHO (World Health Organization), half of the world population has food intolerance and 1 billion people have been diagnosed with it. WHO predicts that by the year 2015, the count would reach 2.5 billion (21).

Whether done deliberately in order to increase the profit margin or accidentally by the facilities which process meat from more than one animal species, adulteration is an illegal practice which deceives the consumer in the sense of health, religion, culture and economy. Another point to be kept in mind is that adulterated meat and meat products pose a greater microbiological risk for consumer health as well. DNA typing also used in our study is a very efficient way of detecting foreign meat species in meat and meat products.

Whatever the reason of the adulteration maybe, it results in deficient hygiene conditions and this is a serious threat for the facility, staff and product and consumer health. Besides, microorganisms which reproduce in meat and meat products because of hygiene deficiency can quickly develop single or multi resistance to antibiotics through complex genetic interactions. Our study shows that adulterated products pose a statistically meaningful higher risk for consumer health than unadulterated products. Total quality management systems and food safety practices should be applied together with the official inspection of the state authorities; programs to raise consumer awareness and continuous training programs for the staff responsible for food production should also be carried into effect. All these would be beneficial to reduce the incidence of the adulteration practices.

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Review on Major Factors Affecting the Successful Conception Rates on Biotechnological Application (AI) in Cattle

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Key Words: *heat detection, artificial insemination, inseminator, conception rate.*

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Review on Major Factors Affecting the Successful Conception Rates on Biotechnological Application (AI) in Cattle

Hamid Jemal [α] & Alemayehu Lemma [σ]

Summary- Cows become fail to conceive with various factors including management failures, nutritional status, postpartum reproductive health, semen quality and other miscellaneous factor and hence reduce efficiency of AI service. Mainly heat detection skill by farmers and timing of insemination are the major factors that determine the success and failure of AI programme. Am/Pm rule is the way which helps to determine relative insemination times achieved in practice, since maximum fertility to artificial insemination occurs when cows are bred near the end of "standing heat". Ovulation occurs about 12 hours after the end of standing heat. Management limitations also synergize other factors like delivery problems which prone the AI service to have inefficient and poor result. Conservative stocking rate, a sensible year round feeding and herd health plan and adequate AI service are important to improve reproductive efficiency, and hence, economically benefit from the crossbreeding activities. Skill of inseminator is an important element in the success of the artificial insemination program and regular practice at inseminating time is required to maintain high conception rates. Besides to that site of semen deposition has an important role in achieving conception of AI in cattle. So that the deposition of semen in the uterine body resulting in higher non-return rate than cervical deposition. Cows are inseminated just into the short uterine body.

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I. Introduction

The role of livestock in general and cattle in particular in the national economy is more significant than what the official production figures would suggest when their contributions for farm traction, farm fertilization and fuel (through manure) are considered (Hassen *et al.*, 2007). Ethiopian cattle population is ranked first in Africa as cited by Hassen *et al.* (2007) and estimated about 43.12 million (Demeke, 2010) of which 55.41% are females. Out of the total female cattle population, only 151,344 (0.35%) and 19,263 (0.04%) heads are hybrid and exotic breeds, respectively (Demeke, 2010). Cattle production is an integral part of almost all farming systems in the highlands, and the major occupation in the lowlands. The arid, semi-arid and sub-humid zones are homes for 14% of the cattle population each while 6% and 52% of the cattle population inhabit the humid zones and the highlands of the country, respectively. The majority of the cattle population is found in the highlands of Ethiopia where 43.6% of the human agricultural population is residing which indicates that cattle have a very important role in the Ethiopian economy (Belihu, 2002).

Artificial insemination (AI) is the manual placement of semen in the reproductive tract of the female by a method other than natural mating and it is one of a group of technologies commonly known as "assisted reproduction technologies" (ART), whereby offspring are generated by facilitating the meeting of gametes (spermatozoa and oocytes). ART may also involve the transfer of the products of conception to a female, for instance if fertilization has taken place *in vitro* or in another female. AI has been used in the majority of domestic species, including bees, and it revolutionizing the animal breeding industry during the 20th century (Milad, 2011).

As reported by Katherine and Maxwell (2006), the first successful attempt at artificial insemination (AI) was conducted in 1776 by Italian physiologist Spallanzani who inseminated a bitch producing three pupies. The first lamb born in Australia from AI was in 1936. Both the puppies and lamb were born from AI with fresh semen; since these early attempts there has been considerable effort in the development of semen preservation and AI technology. At present, fresh, chilled and frozen-thawed semen is used extensively for AI in animal breeding and production throughout the world. It was not until around 1900 that serious attempts were made to develop the technique in farm animals. The work was carried out by Ivanov and colleagues in Russia, and by 1930 they had achieved success with cattle and sheep. Within the next ten years AI was in commercial use for cattle in the USA and the UK (Ball and Peters, 2004).

As livestock genetic improvement, AI has become one of the most important and successful reproductive biotechnology ever devised for the genetic improvement of farm animals which enable to use bulls of high genetic merit available to all (Mukassa-Mugerwa, 1989; Webb, 1992). As mentioned by Webb (1992)

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artificial insemination (AI) plays an important role in the development of the dairy industry; however, follow-up of cows that have been inseminated and assessing success of AI by regular pregnancy diagnosis is a problem. The usual method is by rectal palpation, which can be performed only 2–3 months following AI and requires an experienced inseminator or veterinarian (FAO/IAEA, 2007). These limitations result in long waiting periods before non-pregnant cows are detected, leading to long calving intervals and economic losses to the farmers. Therefore, a technique for early detection of non-pregnancy is required to shorten the interval between an unsuccessful insemination and the subsequent breeding (NAIC, 1995; FAO/IAEA, 2007). Selective breeding is a highly effective and sustainable approach for increasing animal productivity in the long-term. Reproductive technologies such as artificial insemination (AI) allow single animals to have multiple progeny, reducing the number of parent animals required and allowing for significant increases in the intensity of selection, and proportional increases in genetic improvement of production (FAO/IAEA, 2007).

According to NAIC (1995), AI has been introduced to Ethiopia in the early 1930's, however, it was interrupted because of World war II and in 1981 National Artificial Insemination Center (NAIC) has been established to coordinate the overall AI activities throughout the country, even though a cross breeding program has been introduced to Ethiopia at a wider scope in late 1960's (Brannang *et al*, 1980).

An achievement in an increasing milk and meat production by improving the genetic merit of indigenous cattle has been one of the primary livestock development objectives of Ethiopia (Heinonen, 1989). Improvement in livestock resources have been achieved through the implementation of an efficient and reliable AI service, in parallel with proper feeding, health care and management of livestock (Meles and Henonen,1991). Hence to cope up with effective AI service trained man power, facility, follow up and linkage with those involved animal management and breeding while a lack in one of these have been resulted in a failure of the service or in its effect (Shiferaw *et al*., 2002). In Ethiopia even though AI program has been started and continued for several decades, the genetic improvement achieved throughout the country is still unsatisfactory due to several factors. Very few studies in limited part of the country have been conducted to evaluate the success rate of AI.

Therefore, the objectives of this literature review are to provide some insights on: types of major factors that hinder the achievement of pregnancy post insemination and effects of these limiting factors on efficiency of AI.

II. Major Factors Affecting Success of AI Delivery

The successful outcome of artificial insemination (AI) in cattle depends on a number of intrinsic and extrinsic factors which have deleterious effect. An understanding of the impact of such factors on the probability of success when performing AI is of basic importance to established correction measures (Haugan *et al*., 2005). Reproductive efficiency is poor in most cattle production systems, mainly cows fail to become pregnant with various factors including management failures, nutritional status, postpartum reproductive health, semen quality and other miscellaneous factors. So that the extension service must ensure that farmers get adequate information on the input required to benefit from crossbreed dairy cows and from those of higher genetic merit (Mekonene, 2010).

a) Effect of AI Delivery System

Absence of appropriate collaboration and communication between the NAIC, regional agriculture bureaus and other stakeholders, absence of recording system, lack of clearly defined share of responsibilities among stakeholders, poor integration of AI service with livestock health and feed packages, poor motivations and skills of inseminators due to of lack of on job training, lack of support and readily available inputs such as liquid nitrogen. All these factors make the delivery service poor and some of the farmers move their cows for long distance in search of AI service. This is happening in many areas and the reason is AI technicians are unable to get transport facilities like motor bicycles and fuel.but insemination is time dependent job, in which during this long journey/waiting time, heat period is passed away before the service have been given (Lemma, 2010).

b) Factors related to heat detection

Compared with other factors accuracy of heat (estrus detection) is one of the major factor that determine AI program. Heat detection in cows carried out by experienced herd persons/inseminator who can able to identify those animals which would be in heat stand while being mounted/ridden by other female cows or vasectomized bulls, since the period is the shortest period between two successive oestrus cycles (Iftikhar *et al.*, 2009: Arthur,2001). As shown in the table (1) below, those animals manifest behavioral symptoms like frequent urination, bellowing raised tail, restlessness and licking of external genitalia besides to different visible external changes like vulvular edema and absence of wrinkles on vulvular lips, vaginal hyperemia, wetness and mucus discharge also observed. Roelofs *et al*. (2006) mentioned that expression of estrus can be influenced by many factors such as heritability, number of days postpartum, lactation number, milk production,

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and health are known to influence estrus expression. Environmental factors like nutrition, season, housing, herd size, etc. also play a role in estrus expression.

Estrus in cattle is commonly referred to as heat which occurs every 18 to 24 days in sexually mature, open (non pregnant) female cattle when they are receptive to mounting activity by bulls or other cows or heifers. According to Jane *et al.* (2009) standing heat can occur any time in a 24-hour period (table 1). However, the most likely time for a cow or heifer to show heat signs is at night but the season of the year can influence this, with more cows showing heat at night in hot weather and more showing heat during the day in cold weather. Hot weather, high production, crowded conditions, and high stress environments may reduce mounting activity. Observers must distinguish among cattle coming in to heat, in standing heat, and going out of heat. Females that are in standing heat, were in standing heat yesterday, or will be in standing heat tomorrow are the most likely herd mates to mount other cows or heifers in heat. Observe cows away from the feed bunk so feeding behavior does not interfere with heat detection. Cattle need nonslip footing and ample room to interact freely. Dirt footing increases mounting and standing activity (Jane *et al., 2009).*

Table (1) Timing of Estrus Detection in Cattle

Timeline for Heat Signs in Cattle			
	Coming into Heat	*Standing Heat*	*Going out of Heat*
	(8 hours)	*(18 hours)*	*(14+ hours)*
Heat Signs	Stands and bellows Smells other cows Headbutts other cows Attempts to ride other cows but will not stand to be mounted Red, moist, slightly swollen vulva Clear mucous discharge from vulva	Stands to be mounted Rides other cows Bellows frequently Nervous and excitable	Attempts to ride other cows but will not stand to be mounted Smells other cows Clear mucous discharge from vulva

*Source: Jane et al. (*2009)

The optimal time at which insemination should take place relative to ovulation (insemination–ovulation interval = IOI) depends mainly on the fertile lifespan of spermatozoa and on the viable lifespan of the oocyte in the female genital tract (Roelofs *et al*., 2006). For conception to occur, insemination must take place at the correct stage of the cow's estrus cycle since ova remains viable for about 12-18 hrs after ovulation (Bekana, 1991; Rodriguez-Martinez, 2000).

Successful fertilization highly depends on the time interval from insemination to ovulation meaning that insemination takes place too early, the sperm is aged and by the time ovulation occurs it cannot fertilize the ovum and if insemination takes place too late, the egg is aged and fertilization and formation of a viable embryo is not likely. Indications exist that, in practice, an enormous variability exists in the timing of insemination relative to ovulation (Roelofs *et al.*, 2005).

Oestrus is short in the cow, with ovulation occurring 10–12 hours after the end of oestrus (Arthur, 2001). During the next 6 hours the oocyte travels about a third of the way down the uterine tube, during which time fertilisation occurs, about 30 hours after the onset of oestrus. The best conception rates occur if insemination is carried out in the middle to the end of standing oestrus, i.e. 13–18 hours before ovulation. Cows may conceive if they are inseminated at the beginning of oestrus or even 36 hours after the end of oestrus but conception rates are reduced (Arthur, 2001).

There are various reports that indicate low rates of service in artificially inseminated cattle, mainly due to problems in the detection of estrus. While few cows are detected in heat losses occur in significant herd reproductive efficiency. This is higher in *Bos indicus* cattle (Galina, 1996) since have special breeding features - heat of short duration with a high percentage of expression during the night and also depend on social cues. The secretion of estrogen, a manifestation of oestrus LH surge and ovulation are closely related and well known. With follicular growth, the amount of estrogen secreted increases to a peak serum concentration, triggering a preovulatory LH surge, follicular maturation and ovulation, lasting 27 hours. The goal of increased concentrations of estrogen is triggering hormonal cascade of events that includes the LH surge and a series of changes that promote follicular ovulation, and sexual behaviors associated with acceptance of mounts. The main characteristic of estrus is the posture of immobility assumed by acceptance of the cows and ride. High milk producing cows manifest estrus of shorter duration than cows with lower production. Females of childbearing age are pregnant or in the luteal phase of the cycle (under the domain of progesterone) are less likely to mount other females in estrus. Almost 86% of females who ride other females are in estrus and proestrous (under the domain of the estrogen) (Milad, 2011; Arthur, 2001).

When natural service is used there are no problems, since a cow will only stand for the bull when she is in oestrus, and under free-range conditions a cow may be served several times at each oestrus. Several literatures review (O'Connor, 1993; Milad, 2011; Arthur, 2001; Jane *et al 2009*; Hafez,1993*)* on the correct timing of artificial insemination which is a dependent upon true, accurate and early identification of oestrus, the accurate identification of the individual animal and informing the

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inseminator at the correct time. A cow that is first seen in oestrus in the morning is usually inseminated in the afternoon of the same day, whilst a cow that is first seen in oestrus in the afternoon is inseminated early the next day (Arthur, 2001). Frequently, where large numbers of cows are inseminated at the incorrect time, the oestrus detection rate is poor, thus generally reflecting a poor standard of herd management. In such circumstances, some of the methods described above should be used to improve the oestrus detection rate in the herd (Arthur, 2001; Hafez, 1993).

Among the management problems, poor heat detection skill by farmers and timing of insemination are the major factors that determine the success and failure of AI programme (Mukasa-Mugerewa, 1989). Reproductive efficiency is thus poor in most cattle production systems, mainly because cows either fail to become pregnant and require high number of services per conception. Among the varous problems, poor heat detection skill by farmers and timing of insemination are the major factors that determine the success and failure of AI programme (Mukasa-Mugerewa, 1989; Mekonen *et al.,* 2010).

As Richard J. (1998) stated that when AI was being developed and validated, there were several studies that were designed to determine the optimal time of AI in relation to estrus. The data suggested that optimal pregnancy rate per AI (PR/AI) would be achieved from midestrus until a few hours after the end of estrus. Since then, the recommended practice has been AI 12 h after the first observed estrus (a.m.-p.m. breeding) (Arthur, 2001, Galina, 1996). However, because of the variability of interval between the onset and the observation of estrus, it is difficult to define the ideal time of AI in relation to ovulation. A protocol has been developed using GnRH and PGF2a that synchronizes the time of ovulation within an 8-h period (24 to 32 h after the second injection of GnRH) with PR/AI similar to a.m.-p.m. breeding. This precise synchrony of ovulation allows for an effective test to determine the optimal time of AI in relation to ovulation (Richard, 1998).

NAAB (2011) reported that embryonic quality and accessory sperm numbers can be effected by time of insemination. Using varying quality and quantities of semen, the number of accessory sperm was highest when insemination occurred 24 hours after onset of estrus. The quality was the best when insemination occurred at heat onset, but fertilization rates are lower at this time. The optimum insemination time to maximize pregnancy rates is approximately 12 hours after onset of heat. Therefore, AI timing should be performed at 12 hours after estrus detection for maximized pregnancy rates. Loss of pregnancy rate to early inseminations is due to fertilization failure (but embryo quality is high). Whereas, loss to late insemination is due to embryonic failure (but fertilization rate is high). Thus optimum insemination time appears to be a compromise (NAAB, 2011).

c) Intrinsic Factors related to the cow

i. *Reproductive health*

Cows with uterine infection in the early postpartum period generally have lower conception rates at subsequent breeding. Studies confirm that even mild uterine infections adversely affect conception rates (O'Connor, 1993). As Smith (1982) on his finding in the following table described that health and reproductive disorders to post-calving seriously affect conception rate. Calving and post-calving reproductive disorders seriously affect conception rates as illustrated by the following table (Table 1). Thus, the key to maximizing conception rates must lie in the *prevention* of disorders, not treatment after they have occurred. Some evidences indicate cows suffering from metabolic disorders, like milk fever, may have a higher incidence of reproductive disorders and lower conception rates. Smith (1982) reported that first service conception rate was lower (38%) in cows treated for milk fever than in cows not suffering from this disorder (47%). Besides to that a higher incidence of cystic ovaries (20%) in cows treated for milk fever than in cows that were not (4%).

Table (2) Effect of calving and post calving disorders on conception rate

Disorder	Incidence (%)	1st SCR*(%)
None	77	49
Difficult Calving	1	43
Retained Placenta	4	42
Uterine Infection	14	36
Cystic Ovaries	4	35

*SCR-Service per Conception Rate

Source: Smith (1982).

High reproductive efficiency is dependent on obtaining normal uterine involution, early resumption of ovulation, high efficiency of oestrous detection and high conception rates per service as shown in the following table(3) (James, 2006).

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Table (3) The postpartum (pp) reproductive targets to be met to obtain high reproductive efficiency and the associated key risk factors affecting these targets

Reproductive process	Target to be achieved	Risk factors affecting targets
Normal uterine involution	Day 50pp	Dystocia, RFM , Uterine infection
Resumption of ovulation	90% by day 42	Loss of > 0.5 BSC unit, Low feed intake, Uterine health
High oestrous detection	85% per cycle	Infrequent checks, Sub-oestrus, High yield
High conception rate to AI	50% per breeding	Excess BCS loss, Prior uterine problems Low P4 days 4–7 of pregnancy

Source James (2006)

Early Embryonic Loses

Reduced conception rates could due to early embryonic mortality which contributes to reproductive inefficiency in lactating dairy cows because fertility assessed at any point during pregnancy is a function of both conception rate and pregnancy loss. Conception rates at 28 to 32 days post-AI in lactating dairy cows according to James (2006) ranged from 40 to 47%, whereas conception rates in dairy heifers nearly 75% where as 50-55% around day 42(Jonathan, 2009). The fertilization rate after AI in beef cows is 90%, whereas embryonic survival rate is 93% by Day 8 and only 56% by Day 12 post AI. In dairy cattle, only 48% of embryos were classified as normal on Day 7 after AI. Thus, substantial pregnancy loss probably occurs within two weeks post AI (Grimard *et al.,* 2006).

On the other hand conception rate to first service is the combined consequence of fertilization, early embryonic, late embryonic and foetal development, and each of these steps in establishing pregnancy may be affected. Grimard *et al.* (2006) cited that fertilization failure and early embryonic loss, late embryonic/foetal loss and late abortion represent 20–45%, 8–17.5% and 1–4% of pregnancy failure, respectively. Jonathan (2009) at the following table shows the condition of pregnancy rate after fertilization.

Table (4) : Frequency of embryonic mortality at different stages of pregnancy

Day 0	Fertilization rate in cattle----90%	
Day 10-13	Pregnancy Rate ----80% Failure in embryonic development	-Poor oocyte quality
		-Delayed ovulation
		-In adequate pattern of P_4 rise
Day-19	Pregnancy rate-----60-65% Failure of embryo to prevent lueolysis	-Poor embryo quality and developmental potential
		-"lack of synchronization "between mother and the embryo
Until day 42	Pregnancy rate ----50-55% Late embryonic loss	-Infectious factors, directly affecting the embryo or placental function

Source: Jonathan (2009).

d) Factors related to management

i. *Nutrition*

Fluctuation in season which have effect on availability of feed, high environmental temperature and other environmental factors (Haugan *et al*., 2005) cause stress and the challenge of high disease risk in cross breed cows contribute for high number of services per conception, late age at first calving and first service, and longer calving interval which are all the major areas of reproductive loss in cattle (Mukasa-Mugerewa, 1989).

Most investigations conducted on effect of nutrition at the later part of gestation (Smith, 1982) because of the limited nutrient requirements of the fetus because of the limited nutrient requirements of the fetus for growth and development during the first one-half of gestation. But according to Smith (1982) and Anzar (2003) energy status is generally considered to be major nutritional factor that influence reproductive performance. Also total dietary intake can affect fertility, both at the level of the oocyte and embryo, which means it is important to differentiate between optimum conditions for follicle growth (both in terms of number and follicles and paracrine environment) and optimum conditions for embryo survival. So that nutrition has effect on quality of follicle, oocyte and embryo (Maurice, 2003). Maurice (2003) briefly described the blastocyst

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formation is as a key developmental process in the growth of an embryo and the blastocoels cavity forms as a consequence of fluid transport across the trophectoderm. This process is partially facilitated by Na/K-ATPase. Messenger RNA for this enzyme has been identified in day-7 bovine embryos. Dietary intake and diet type can alter the expression of transcripts of genes involved in early embryo development, such as Na/K-ATpase. and CU/Zn SOD. A decreased in invitro secretion of interferon-tau in day-15 embryo from undernourished ewes and an increase in the invitro secretion by endometrial tissue of $PGF_{2\alpha}$ was evident in the same animals. Thus, nutrient requirements for optimum follicle growth and embryo development may be quite different. Based on this, he indicates that the importance of diet around the time of mating and in particular the significance of extreme underfeeding post mating in regulating pregnancy rate.

As Xu *et al*. (2010) reported that the peak of embryo death occurs during the first month of pregnancy, and controlled feed intake is important to reduce mortality of embryos. He has demonstrated that a diet consumed before mating has a major impact on embryo survival and litter uniformity in blastocyst size in pigs. Manipulation of feed intake before mating may affect embryo survival through changes in follicular development by altering oocyte quality (Smith, 1982) and also there are increasing evidences that the feed intake after mating plays a major role on embryo survival. As a result of steroid dependent changes in the uterine environment, embryo survival is affected by different feed intake levels after mating (Maurice, 2003). As mentioned by Robinson *et al.* (2006) the impact of nutrition on embryo survival in ruminants extends beyond the supply of essential nutrients and the modification of the hormones and growth factors that influence embryo development. Numerous micronutrients are involved in embryo development and survival. Those for which deficiencies, and in the case of Vitamin A excesses, are linked to impaired embryo development and poor embryo survival in practical farming systems are shown in Fig. 1

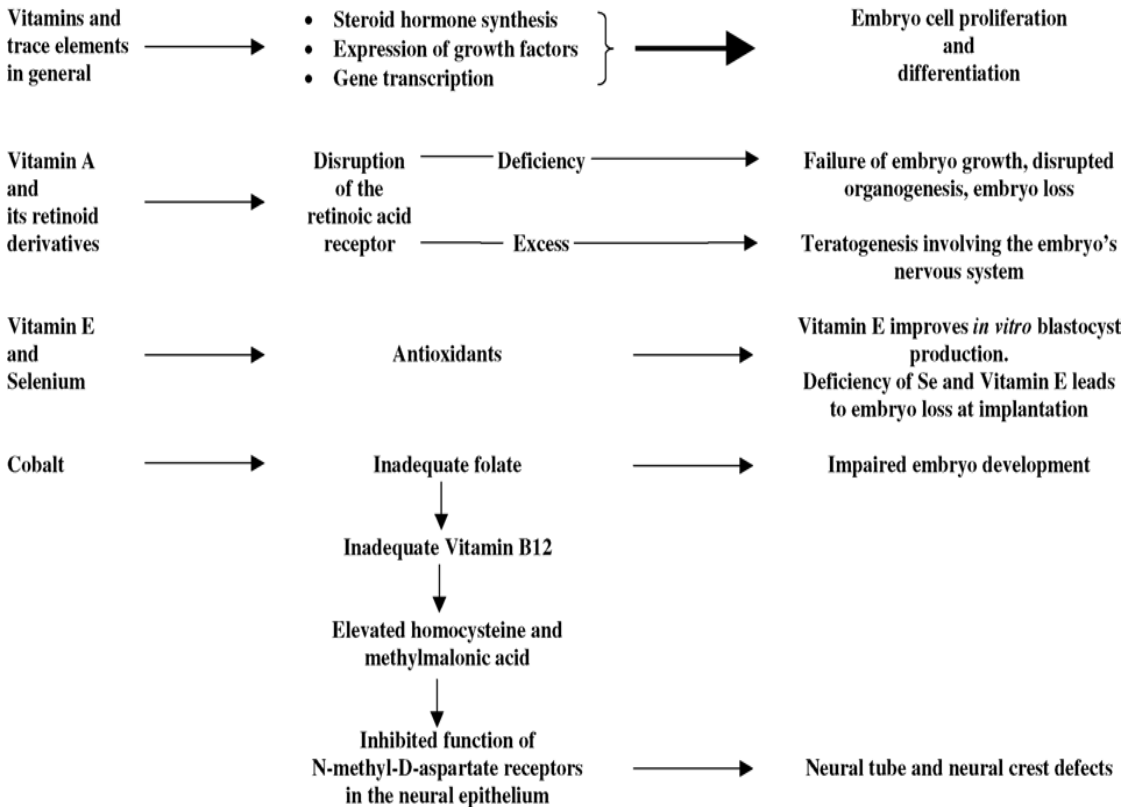

Figure 1: Micronutrient involvement in embryo development and survival

As cited by Dhaliwal (1996), negative energy balance causing sub fertility during the 6-l0-week postpartum period i.e. the period of recovery of energy balance particularly during the first 2-5 oestrous cycles postpartum, cows with low body condition score had lower concentrations of progesterone than those with high body condition score; low progesterone during the cycle preceding insemination results in low pregnancy rates since progesterone hormone is required to maintain pregnancy at all stages.

ii. *Housing condition*

Housing conditions can have an effect on the distribution of heat during a 24-hour period. Estrous behavior expression at any housing arrangement that allows cattle to interact throughout the day provides more opportunity for mounting and standing behavior to

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be expressed which enable to identify estrous cow easly. Especially on cattle housed in tie-stall or stanchion barns must be turned out in order for this behavior to be expressed. L'Oconor (1993) reported under research conducted on high-producing Holstein cows to what extent slippery footing surface could have been affect expression of estrus. The result showed that mounting activity occurs more frequently when cows are on soil rather than concrete. When five estrous cows were individually presented with an opportunity to spend time on soil or on concrete in the presence of a tied cow which was either an estrous cow or a cow not in heat, the test cow spent an average 70 % of the time on ground reported by L'Oconor (1993) and 73% by Milad (2011). The test estrous cow mounted more frequently when a tied estrous cow was on soil rather than on concrete.

e) Factors related to semen quality

As Saacke (2008) considering the economic investment in semen and other inputs, success must be judged on the basis of pregnancy rate to the first AI. Also, a good first service pregnancy rate response usually signifies good conditions for second service. Additional key factors to be considered as impacting pregnancy rate to first service are semen quality (primarily dependent on choice of bulls). In most breeding strategies, whether estrous synchronization is employed or not, semen quality is one of a critical point to a successful pregnancy. The nature of sub fertility due to the male is proving as complex as that due to the female.

Saacke (2008) emphasized that the importance of semen handling and placement to achieve threshold or above threshold number of sperms to the ovum (i.e., approach 10 sperms/ovum) necessary to maximize both fertilization rate and embryo quality for a general population of bulls. Based upon the median number of 2.4 accessory sperms per ovum/ embryo and the threshold requirement of nearly 10 sperms per ovum/embryo to optimize embryo quality, efforts to raise accessory sperm number have been undertaken. While morphological defects are minimum since this will checked before processing.

f) Factors Related to Insemination Techniques

One of the most significant contributions to the successful application of AI in cattle breeding has been made by the highly trained inseminator (Arthur, 2001).The efficiency of cow insemination depends, among other factors, on the ability of the inseminator to deliver the semen to the appropriate site in the reproductive tract at the appropriate stage of estrus. However, there has been a tendency to adopt routine insemination techniques and to ignore inseminator-related factors that can dramatically affect fertility. Although professional inseminators palpate the reproductive tract of numerous cows every day, most are not trained to examine the uterus and ovaries. This poses a serious practical limitation to the success of AI (López-Gatius F., 2011). Animals showing signs of true heat should inseminate using frozen semen thawed at 37 ^{0}C for 30 seconds (Iftikhar *et al.*, 2009; Jane *et al*., *2009*).

Professional technicians are more successful at insemination than inexperienced ones, indicating that selection of a qualified inseminator is an important element in the success of the artificial insemination program and regular practice at inseminating is required to maintain high conception rates. Citation evidence by Gebremedhin (2008) shows that the site of semen deposition has been an important factor in the success of AI in cattle, i.e the deposition of semen in the uterine body resulted in a 10% higher non-return rate than did cervical deposition and An increase in the conception rate has been reported when semen was deposited in the uterine horns rather than the uterine body. cows are inseminated just into the short uterine body(O'connor, 2003). Insemination into the cervix produces a lower fertilization rate, while insemination deeper into the uterus runs the risks of either inseminating into the uterine horn contra lateral to the ovulation site, or scoring the endometrium with the tip of the insemination catheter. Reduced fertility is the consequence of both of the latter two errors (O'connor, 2003; Arthur, 2001). In the early days of AI there was controversy among researchers about the optimum site for semen deposition. A study conducted in Canada cited by O'connor (2003), provided evidence that fertility was highest when semen was deposited in the uterine body. Researchers currently are reexamining insemination technique to determine the proper site of semen deposition. Failure to understand the anatomical and functional relationships among the various tissues and organs of the reproductive system may lead to consistent insemination errors (O'connor, 2003).

The ability to perform an intrauterine insemination in cattle means that a relatively low dose of sperm is required to achieve acceptable pregnancy rates. Typically, of the 20–30 million sperm that are required in each insemination dose, 6–7 million survive freezing, that is generally regarded as the minimum dose compatible with acceptable fertility (Arthur, 2001). Regarding to depth and time of insemination NAAB (2011) recommends that very deep insemination can enhance sperm delivery. However, site of insemination was found to make only small increases in sperm per egg. Deep insemination be used only when the sperm dose is below threshold, or if sexed semen is being used. Also hygien, tawing methods, temperature maintenance between thawing to insemination have also play a factor in achieving pregnancy (Milad, 2011). Rectal palpation and ultrasound examinations should be considered safe procedures when performed correctly,

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and recent evidence gives no indication that ultrasound examination is detrimental to the embryo.

III. Conclusions and Recommendations

Artificial insemination is still now serve as a main tool for dissemination of outstanding germplasm, control of venereal diseases and cost-effective dairy farming approach and through this method it is possible to improve conception rate and reduce number of service per conception by applying proper AI program implementation. The main problems identified are: delayed insemination; low conception rates; which leads to repeat breeding and long calving intervals and resulting overall low AI output. The major reasons for the above problems incriminated cases are: untrained AI technicians, poor heat detection, poor quality of semen, malnutrition, improper AI timing, endometrial problems and other related factors. It is possible to increase CR and decreasing embryo mortality by optimizing the insemination–ovulation interval with a high probability of fertilization. However, the insemination–ovulation interval in which this fertilized oocyte has a high probability of developing into a good embryo is shorter (24–12 h before ovulation).

Based up on the above conclusions, the following recommendations are forwarded:

- ✓ A Successful heat detection methods and subsequent proper timing of insemination should be required in increasing reproductive efficiency. Am-Pm principle should be followed as a rule of thumb approach during insemination service.
- ✓ Technique and site semen deposition is the sole of factor to achieve pregnancy, which inseminators have to given an attention,
- ✓ Through periodical trainings and workshops developing the skill of inseminators is required.
- ✓ Strengthening the extention system and awareness creation on the owners on identification and time of presentation of cattle showing oestrus sign.
- ✓ Strong veterinary intervention is required to maximize conception rates which lie in the prevention of post-calving disorders than treatment trails after they have occurred.
- ✓ To maximize the effectiveness of insemination, post breeding heat detection (detection or return heats) must be high.
- ✓ Extension service must ensure that farmers get adequate information on the input required to benefit from crossbreed dairy cows and from those of higher genetic merit.

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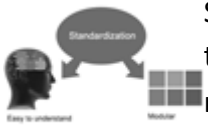

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All manuscripts submitted to Global Journals Inc. (US), ought to include:

Title: The title page must carry an instructive 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) wherever the work was carried out. The full postal address in addition with the e-mail address of related author must be given. Up to eleven keywords or very brief phrases have to be given to help data retrieval, mining and indexing.

Abstract, used in Original Papers and Reviews:

Optimizing Abstract for Search Engines

Many researchers searching for information online will use search engines such as Google, Yahoo or similar. By optimizing your paper for search engines, you will amplify the chance of someone finding it. This in turn will make it more likely to be viewed and/or cited in a further work. Global Journals Inc. (US) have compiled these guidelines to facilitate you to maximize the web-friendliness of the most public part of your paper.

Key Words

A major linchpin in research work for the writing research paper is the keyword search, which one will employ to find both library and Internet resources.

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

Search engines for most searches, use Boolean searching, which is somewhat different from Internet searches. The Boolean search uses "operators," words (and, or, not, and near) that enable you to expand or narrow your affords. Tips for research paper while preparing research paper are very helpful guideline of research paper.

Choice of key words is first tool of tips to write research paper. Research paper writing is an art.A few tips for deciding as strategically as possible about keyword search:

- One should start brainstorming lists of possible keywords before even begin 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 research paper?" Then consider synonyms for the important words.
- It may take the discovery of only one relevant paper to let steer in the right keyword direction because in most databases, the keywords under which a research paper is abstracted are listed with the paper.
- One should avoid outdated words.

Keywords are the key that opens a door to research work sources. Keyword searching is an art in which researcher's skills are bound to improve with experience and time.

Numerical Methods: Numerical methods used should be clear and, where appropriate, supported by references.

Acknowledgements: Please make these as concise as possible.

References

References follow the Harvard scheme of referencing. References in the text should cite the authors' names followed by the time of their publication, unless there are three or more authors when simply the first author's name is quoted followed by et al. unpublished work has to only be cited where necessary, and only in the text. Copies of references in press in other journals have to be supplied with submitted typescripts. It is necessary that all citations and references be carefully checked before submission, as mistakes or omissions will cause delays.

References to information on the World Wide Web can be given, but only if the information is available without charge to readers on an official site. Wikipedia and Similar websites are not allowed where anyone can change the information. Authors will be asked to make available electronic copies of the cited information for inclusion on the Global Journals Inc. (US) homepage at the judgment of the Editorial Board.

The Editorial Board and Global Journals Inc. (US) recommend that, citation of online-published papers and other material should be done via a DOI (digital object identifier). If an author cites anything, which does not have a DOI, they run the risk of the cited material not being noticeable.

The Editorial Board and Global Journals Inc. (US) recommend the use of a tool such as Reference Manager for reference management and formatting.

Tables, Figures and Figure Legends

Tables: Tables should be few in number, 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. Vertical lines should not be used.

Figures: Figures are supposed to be submitted as separate files. Always take in a citation in the text for each figure using Arabic numbers, e.g. Fig. 4. Artwork must be submitted online in electronic form by e-mailing them.

Preparation of Electronic Figures for Publication

Even though low quality images are sufficient for review purposes, print publication requires high quality images to prevent the final product being blurred or fuzzy. Submit (or e-mail) EPS (line art) or TIFF (halftone/photographs) files only. MS PowerPoint and Word Graphics are unsuitable for printed pictures. Do not use pixel-oriented software. Scans (TIFF only) should have a resolution of at least 350 dpi (halftone) or 700 to 1100 dpi (line drawings) in relation to the imitation size. 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.

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6. AFTER ACCEPTANCE

Upon approval of a paper for publication, the manuscript will be forwarded to the dean, who is responsible for the publication of the Global Journals Inc. (US).

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TECHNIQUES FOR WRITING A GOOD QUALITY RESEARCH PAPER:

1. Choosing the topic: In most cases, the topic is searched by the interest of author but it can be also suggested by the guides. You can have several topics and then you can judge that in which topic or subject you are finding yourself most comfortable. This can be done by asking several questions to yourself, like Will I be able to carry our search in this area? Will I find all necessary recourses to accomplish the search? Will I be able to find all information in this field area? If the answer of these types of questions will be "Yes" then you can choose that topic. In most of the cases, you may have to conduct the surveys and have to visit several places because this field is related to Computer Science and Information Technology. Also, you may have to do a lot of work to find all rise and falls regarding the various data of that subject. Sometimes, detailed information plays a vital role, instead of short information.

2. Evaluators are human: First thing to remember that evaluators are also human being. They are not only meant for rejecting a paper. They are here to evaluate your paper. So, present your Best.

3. Think Like Evaluators: If you are in a confusion or getting demotivated that your paper will be accepted by evaluators or not, then think and try to evaluate your paper like an Evaluator. Try to understand that what an evaluator wants in your research paper and automatically you will have your answer.

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

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7. Use right software: Always use good quality software packages. If you are not capable to judge good software then you can lose quality of your paper unknowingly. There are various software programs available to help you, which you can get through Internet.

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9. Use and get big pictures: Always use encyclopedias, Wikipedia to get pictures so that you can go into the depth.

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

12. Make all efforts: Make all efforts to mention what you are going to write in your paper. That means always have a good start. Try to mention everything in introduction, that what is the need of a particular research paper. Polish your work by good skill of writing and always give an evaluator, what he wants.

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17. Never use online paper: If you are getting any paper on Internet, then never use it as your research paper because it might be possible that evaluator has already seen it or maybe it is outdated version.

18. Pick a good study spot: To do your research studies always try to pick a spot, which is quiet. Every spot is not for studies. Spot that suits you choose it and proceed further.

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20. Use good quality grammar: Always use a good quality grammar and use words that will throw positive impact on evaluator. Use of good quality grammar does not mean to use tough words, that for each word the evaluator has to go through dictionary. Do not start sentence with a conjunction. Do not fragment sentences. Eliminate one-word sentences. Ignore passive voice. Do not ever use a big word when a diminutive one would suffice. Verbs have to be in agreement with their subjects. Prepositions are not expressions to finish sentences with. It is incorrect to ever divide an infinitive. Avoid clichés like the disease. Also, always shun irritating alliteration. Use language that is simple and straight forward. put together a neat summary.

21. 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 to your topic. You may also maintain your arguments with records.

22. Never start in last minute: Always start at right time and give enough time to research work. Leaving everything to the last minute will degrade your paper and spoil your work.

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

24. Never copy others' work: Never copy others' work and give it your name because if evaluator has seen it anywhere you will be in trouble.

25. Take proper rest and food: No matter how many hours you spend for your research activity, if you are not taking care of your health then all your efforts will be in vain. For a quality research, study is must, and this can be done by taking proper rest and food.

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

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27. Refresh your mind after intervals: Try to give rest to your mind by listening to soft music or by sleeping in intervals. This will also improve your memory.

28. Make colleagues: Always try to make colleagues. No matter how sharper or intelligent you are, if you make colleagues you can have several ideas, which will be helpful for your research.

29. Think technically: Always think technically. If anything happens, then search its reasons, its benefits, and demerits.

30. Think and then print: When you will go to print your paper, notice that tables are not be split, headings are not detached from their descriptions, and page sequence is maintained.

31. Adding unnecessary information: Do not add unnecessary information, like, I have used MS Excel to draw graph. Do not add irrelevant and inappropriate material. These all will create superfluous. Foreign terminology and phrases are not apropos. One should NEVER take a broad view. Analogy in script is like feathers on a snake. Not at all use a large word when a very small one would be sufficient. Use words properly, regardless of how others use them. Remove quotations. Puns are for kids, not grunt readers. Amplification is a billion times of inferior quality than sarcasm.

32. Never oversimplify everything: To add material in your research paper, never go for oversimplification. This will definitely irritate the evaluator. Be more or less specific. Also too, by no means, ever use rhythmic redundancies. Contractions aren't essential and shouldn't be there used. Comparisons are as terrible as clichés. Give up ampersands and abbreviations, and so on. Remove commas, that are, not necessary. Parenthetical words however should be together with this in commas. Understatement is all the time the complete best way to put onward earth-shaking thoughts. Give a detailed literary review.

33. Report concluded results: Use concluded results. From raw data, filter the results and then conclude your studies based on measurements and observations taken. Significant figures and appropriate number of decimal places should be used. Parenthetical remarks are prohibitive. Proofread carefully at final stage. In the end give outline to your arguments. Spot out perspectives of further study of this subject. Justify your conclusion by at the bottom of them with sufficient justifications and examples.

34. After 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 to 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 in 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 criterion for grading the final paper by peer-reviewers.

Final Points:

A purpose of organizing a research paper is to let people to interpret your effort selectively. The journal requires the following sections, submitted in the order listed, each section to start on a new page.

The introduction will be compiled from reference matter and will reflect the design processes or outline of basis that direct you to make study. As you will carry out the process of study, the method and process section will be constructed as like that. The result segment will show related statistics in nearly sequential order and will direct the reviewers next to the similar intellectual paths throughout the data that you took to carry out your study. The discussion section will provide understanding of the data and projections as to the implication of the results. The use of good quality references all through the paper will give the effort trustworthiness by representing an alertness of 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 the 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 evade

- Insertion a title at the foot of a page with the subsequent text on the next page
- Separating a table/chart or figure - impound each figure/table to a single page
- Submitting a manuscript with pages out of sequence

In every sections of your document

· Use standard writing style including articles ("a", "the," etc.)

· Keep on paying attention on the research topic of the paper

· Use paragraphs to split each significant point (excluding for the abstract)

· Align the primary line of each section

· Present your points in sound order

· Use present tense to report well accepted

· Use past tense to describe specific results

· Shun familiar wording, don't address the reviewer directly, and don't use slang, slang language, or superlatives

· Shun use of extra pictures - include only those figures essential to presenting results

Title Page:

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

Abstract:

The summary should be two hundred words or less. It should briefly and clearly explain the key findings reported in the manuscript--must have precise statistics. It should not have abnormal acronyms or abbreviations. It should be logical in itself. Shun citing 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 approach 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. Yet, use comprehensive sentences and do not let go readability for briefness. You can maintain it succinct by phrasing sentences so that they provide more than 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 maintain the initial two items to no more than one ruling each.

- Reason of the study - 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 quantitative data; results of any numerical analysis should be reported
- Significant conclusions or questions that track from the research(es)

Approach:

- Single section, and succinct
- As a outline of job done, it is always written in past tense
- A conceptual should situate on its own, and not submit to any other part of the paper such as a form or table
- Center on shortening results - bound background information to a verdict or two, if completely necessary
- What you account in an conceptual must be regular with what you reported in the manuscript
- Exact spelling, clearness 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 to comprehend and calculate the purpose of your study without having to submit to other works. The basis for the study should be offered. Give most important references but shun difficult to make a comprehensive appraisal of the topic. In the introduction, describe the problem visibly. If the problem is not acknowledged in a logical, reasonable way, the reviewer will have no attention in your result. Speak in common terms about techniques used to explain the problem, if needed, but do not present any particulars about the protocols here. Following approach can create a valuable beginning:

- Explain the value (significance) of the study
- Shield the model - why did you employ this particular system or method? What is its compensation? You strength remark on its appropriateness from a abstract point of vision as well as point out sensible reasons for using it.
- Present a justification. Status your particular theory (es) or aim(s), and describe the logic that led you to choose them.
- Very for a short time explain the tentative propose and how it skilled 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 with every section. If you make the four points listed above, you will need a least of four paragraphs.

- Present surroundings information only as desirable in order hold up a situation. The reviewer does not desire to read the whole thing you know about a topic.
- Shape the theory/purpose specifically - do not take a broad view.
- As always, give awareness to spelling, simplicity and correctness of sentences and phrases.

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This part is supposed to be the easiest to carve if you have good skills. A sound written Procedures segment allows a capable scientist to replacement 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 for the least amount of information that would permit another capable scientist to spare 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 object, mention the specific item describing a way but draw the basic principle while stating the situation. The purpose is to text 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:

- Explain materials individually only if the study is so complex that it saves liberty this way.
- Embrace particular materials, and any tools or provisions that are not frequently found in laboratories.
- Do not take in frequently found.
- If use of a definite type of tools.
- Materials may be reported in a part section or else they may be recognized along with your measures.

Methods:

- Report the method (not 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 - details how procedures were completed not how they were exclusively performed on a particular day.
- If well known procedures were used, account the procedure by name, possibly with reference, and that's all.

Approach:

- It is embarrassed or not possible to use vigorous voice when documenting methods with no using first person, which would focus the reviewer's interest on the researcher rather than the job. As a result when script up the methods most authors use third person passive voice.
- Use standard style in this and in 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.

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The principle of a results segment is to present and demonstrate your conclusion. Create this part a 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. Carry on to be to the point, by means of statistics and tables, if suitable, to present consequences most efficiently.You must obviously differentiate material that would usually be incorporated in a study editorial from any unprocessed data or additional appendix matter that would not be available. In fact, such matter should not be submitted at all except requested by the instructor.

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Content

- Sum up your conclusion in text and demonstrate them, if suitable, with figures and tables.
- In manuscript, explain each of your consequences, point the reader to remarks that are most appropriate.
- Present a background, such as by describing the question that was addressed by creation an exacting study.
- Explain results of control experiments and comprise 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 in manuscript form.

What to stay away from

- Do not discuss or infer your outcome, report surroundings information, or try to explain anything.
- Not at all, take in raw data or intermediate calculations in a research manuscript.
- Do not present the similar data more than once.
- Manuscript should complement any figures or tables, not duplicate the identical information.
- Never confuse figures with tables - there is a difference.

Approach

- As forever, use past tense when you submit to 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 part.

Figures and tables

- If you put figures and tables at the end of the details, make certain that they are visibly distinguished from any attach appendix materials, such as raw facts
- Despite of position, each figure must be numbered one after the other and complete with subtitle
- In spite of position, each table must be titled, numbered one after the other and complete with heading
- All figure and table must be adequately complete that it could situate on its own, divide from text

Discussion:

The Discussion is expected the trickiest segment to write and describe. A lot of papers submitted for journal are discarded based on problems with the Discussion. There is no head of state for how long a 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 implication of the study. The purpose here is to offer an understanding of your results and hold up for all of your conclusions, using facts from your research and generally accepted information, if suitable. The implication of result should be visibly 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 with prospect, and let it drop at that.

- Make a decision if each premise is supported, 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 the experiment might be personalized to accomplish a new idea.
- Give details all of your remarks as much as possible, focus on mechanisms.
- Make a decision if the tentative design sufficiently addressed the theory, and whether or not it was correctly restricted.
- Try to present substitute explanations if sensible alternatives be present.
- One 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 available information
- Submit to work done by specific persons (including you) in past tense.
- Submit to generally acknowledged facts and main beliefs in present tense.

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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

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