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Review article

GUIDELINES FOR THE DIAGNOSIS OF HUMAN LEPTOSPIROSIS*

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INTRODUCTION

Leptospirosis is under diagnosed and under reported in developing countries due to lack of diagnostic facilities. The diagnosis of leptospirosis is complex, because the gold standard tests are not easily available. Though rapid tests are available for the past two decades, they have to be combined with the existing gold standard tests for confirmation of diagnosis. It should be adequate to diagnose leptospirosis with rapid tests. I intend to review the literature from developing countries in Asia, Latin America and Africa and suggest the need for utilizing rapid tests for diagnosis.

Leptospirosis can be diagnosed only by diagnostic tests and epidemiological data, such as incidence/prevalence data cannot be obtained without these tests. Without these data, funding cannot be obtained for diagnostic, treatment and control programs, as without diagnostic tests, leptospirosis would be considered a rare disease. In this article, the various guidelines for the diagnosis of leptospirosis will be discussed.

*Dr. Srinivasan Memorial endowment lecture delivered on 15.03.2018 at Madras Veterinary College, Chennai - 600 007

DATA FROM DEVELOPING COUNTRIES

It has been estimated that the global incidence of leptospirosis is about one million cases with a mortality of 59,000 cases1. The actual number of diagnosed cases would be about 50,000 cases, with a large number of cases from Asia, followed by Latin America.

The disparities between estimated and ctual cases are lack of awareness about the disease and lack of diagnostic facilities in developing countries. There is recently a greater awareness of this disease and leptospirosis is considered as an important cause of non malarial acute febrile illness in many Asian countries. In addition, rapid tests are frequently used in Sri Lanka and India for the diagnosis of leptospirosis. In Sri Lanka, studies have revealed that Leptocheck is used as a screening test and confirmed by Elisa IgM or MAT.

LEPTOSPIROSIS IN ASIA

The data given below are from the following Asian countries, where Leptospirosis has been reported in large numbers2-9 (Table 1).

Table 1: Data from Asian Countries

Country	No. of cases	Year
Thailand	**14285**	**2000**
Sri Lanka	**7406**	**2008**
Philippines	**5522**	**2012**
Malaysia	**7806**	**2014**
Indonesia	**877**	**2007**
India	**<10000**	**2013**

In Asia, Thailand, India, Sri Lanka, Philippines, Malaysia and Indonesia report large number of cases and cases have also been reported from Nepal, Bangladesh and Cambodia[2-12].

These are probably underestimated data, because of inadequate diagnostic facilities. Leptospirosis contributes to about 15-20% of acute febrile illness and is usually mild and recovers early with empiric therapy.

It is an important cause of non-malarial AFI. Therefore, they may not be investigated due to lack of awareness of the disease or lack of diagnostic facilities by primary care doctors, unless severe form with complications, such as jaundice, acute kidney injury or pulmonary hemorrhage occurs.

The incidence of leptospirosis is estimated to be 10-100/100,000 cases/year in developing countries. By this estimate, India should report 0.1 - 1 million cases per year, but less than 10,000 cases are reported8,9. Only four states (Kerala, Gujarat, Tamil Nadu and Maharashtra) report more than 500 cases per year. Andaman, Andhra Pradesh, Assam, Goa, Delhi, Karnataka, Orissa, Puducherry and Uttar Pradesh also report cases. Kerala has reported leptospirosis cases from all districts and this disease is the leading cause of mortality, among the infectious diseases. Gujarat has reported cases from the southern districts of Surat, Valsad and Navasari. Chennai and Mumbai are large cities from which leptospirosis has been reported. Recently, West Bengal, Punjab, Haryana and Himachal Pradesh have reported cases of leptospirosis. The difference between the estimated and actual incidence suggests lack of awareness of the disease and lack of diagnostic facilities in many states of the country.

LEPTOSPIROSIS IN THE AMERICAS

The annual incidence of leptospirosis in the Americas during the period 1996 - 2006 reported from 24/48 countries is 4713.5 of which only 3 countries reported 3920 cases (83.1%)13.

- Brazil : 3165.5 cases
- Cuba : 558.5 cases
- Costa Rica : 196 cases

- 8 countries reported 380 death, of which 349 (83.1%) were reported from Brazil

Recent data (2014) from Latin America report that 28/48 countries had leptospirosis and the number of cases were 10,702 human infections with Brazil (40.2%), Peru (23.6%), Colombia (8.2%) and Ecuador (7.2%) being the important nations with large number of cases. Leptospirosis also occurs in Bolivia, Nicaragua, Venezuela, Argentina, Uruguay and Caribbean countries[14,15]. Many cases have been observed in the south pacific islands.

LEPTOSPIROSIS IN AFRICA

Leptospirosis is being recognized as an important cause of non malarial Acute Febrile Illness (AFI) in many African countries16-18. The prevalence of human leptospirosis ranged from 2.3% to 19.8% of AFI.

The Incidence rate of leptospirosis in the following countries is:

- Tanzania : 75-102/100,000
- Seychelles : 60-101/100,000
- Reunion : 31-120/100,000

There is scarce epidemiological data from many countries, because of lack of awareness of the disease and lack of diagnostic facilities.

CRITERIA FOR DIAGNOSIS OF HUMAN LEPTOSPIROSIS

The clinical indications for investigations to= diagnose human leptospirosis are:

1. Acute Febrile Illness (AFI) in the tropics with high risk environmental factors/high risk occupations/high risk recreational activities.
2. AFI with Jaundice and Acute Kidney Injury
3. AFI with Pulmonary Hemorrhage/ ARDS
4. AFI with Meningo - encephalitis
5. AFI with Thrombocytopenia

The diagnosis of leptospirosis is made by the following tests:

1. Culture of blood, urine and tissues.
2. PCR
3. MAT (Microscopic Agglutination Test)
4. Rapid tests - Elisa IgM , Macroscopic Slide Agglutination Test (MSAT), ICT Lateral flow assay (Leptocheck IgM), Latex Agglutination test, Lepto-dipstick.

Culture, PCR and MAT are the gold standard tests and are available in specialized labs in tertiary care centers. The rapid tests are simple and can be done at smaller centers. These tests have been found to be more sensitive in the early diagnosis of leptospirosis by many studies and should be suitable in developing countries for diagnosis of leptospirosis. Samples can be sent to higher centers for the gold standard tests.

Laboratory support is needed

1. To confirm the Diagnosis

2. For epidemiological and public health reasons, to determine which serovar caused the infection, the likely source of infection, potential reservoir and its location.

- In Developing countries, to confirm the diagnosis, simple rapid diagnostic tests are adequate. They can be confirmed by Elisa IgM.

- For epidemiological reasons, the following tests are necessary - Culture, PCR and MAT. They need specialized laboratories to do these tests.

GUIDELINES FOR DIAGNOSIS

The following guidelines will be evaluated.

1. CDC 2013 Criteria for Diagnosis of Leptospirosis19

2. WHO LERG criteria for diagnosis of Leptospirosis (2011)20

3. Modified Faines Criteria (2012)8

1. CDC 2013 Criteria for Diagnosis of Leptospirosis

A. Clinical criteria

B. Laboratory criteria

Supportive

1. MAT : 1:200 or more to less than 1:800

2. IgM Antibodies detection

3. Demonstration of leptospira by DFM

Confirmed

1. Culture - Isolation of organism

2. MAT : Seroconversion / Fourfold rise in titre /Single Titre 1:800 or more.

3. PCR : Positive

C. Epidemiological linkage : flooding, adventure sports

Case Classification

1. Probable : Clinical / Epidemiological / laboratory tests without confirmation (supportive criteria)

2. Confirmed : Same as above with confirmed tests

2. WHO LERG criteria for diagnosis of Leptospirosis (2011)

Confirmed

Clinical signs and symptoms consistent with leptospirosis and one of the following:

1. Culture of the organism
2. MAT : Seroconversion or four fold rise / single MAT titre : 1:400 or more
3. PCR positive (for pathogenic DNA)
4. Detection of leptospira by histology, histochemical and Immunostaining
5. Demonstration of Leptospira

Probable

1. MAT : Titre of 1:100 or more
2. Elisa IgM positive
3. IgM or four fold rise by IFA

The CDC 2013 guideline has used a MAT titre of 1:800 or above to confirm the diagnosis of leptospirosis and a titre of 1:200 to <1:800 as supportive evidence.

The LERG criteria 2011 has used a MAT titre of 1:400 or above to confirm the diagnosis in single samples and a titre of 1:100 or above as probable leptospirosis.

There is always confusion, whether single samples high titre can confirm the diagnosis, as this titre can represent a rising titre of a current infection or declining titre of a past infection. These high titres can persist for a number of years, as they can be due to both IgM and IgG antibodies and therefore IgM antibody detection based rapid tests, such as Elisa IgM may be valuable to confirm current infection. Combining Elisa IgM with MAT is ideal to confirm diagnosis after 5 days of fever due to leptospirosis and at this stage, even low titres of MAT (1:100) is acceptable. Titres of 1:400 or above is acceptable after 10 days of AFI.

3. Modified Faines Criteria (2012)

Table 2 : Modified Faines Criteria (2012)

Clinical Data (PART A)		Epidemiological Factors (PART B)		Bacteriological and Lab Findings (PART C)	
Headache	2	Rainfall	5	Isolation of leptospira in culture – Diagnosis certain	25
Fever	2	Contact with contaminated Environment	4	PCR	
Temp >39°C	2			Positive Serology Elisa IgM	15
Conjunctival suffusion	4	Animals Contact	1	Positive* SAT – Positive* Other Rapid Tests**	15
Meningism	4	Total	10	MAT – Single positive High titre*	15
Myalgia	4			MAT – Rising titre / Seroconversion (paired sera)	25
Conjunctival suffusion + Meningism + Myalgia	10				
Jaundice	1			* Any one of the tests only should be scored. **Latex agglutination test/Lepto Tek Dri-Dot /Leptodipstick/Lepto Tek lateral flow/Lepto check test	
Albuminuria/Acute Kidney Injury (AKI)	2				
Hemoptysis / Dyspnea	2				

Presumptive diagnosis of leptospirosis is made of:

- Part A or Part A and Part B score : 26 or more
- Parts A, B, C (Total) : 25 or more
- A score between 20 and 25 suggests leptospirosis as a possible diagnosis.

This criteria is a modification of the Faine's criteria (1982) and modified faines criteria (2004)21,22. This includes clinical, epidemiological and laboratory criteria. In this criteria, rapid diagnostic test have been included for diagnosis of leptospirosis (Table 2).

The Modified Faine's Criteria has 2 objectives:

1. Confirmation of leptospirosis utilising laboratory tests. The clinical features of leptospirosis are non-specific and hence confirmation by diagnostic tests is essential. Simple rapid tests such as Elisa, MSAT and other rapid tests have been included along with MAT and cultures to confirm the diagnosis with appropriate scores (A+B+C = 25 or more)22,23,24. This is the most important aspect of the criteria. It is very important that rapid tests

Table 3 : Role of Diagnostic tests for Leptospirosis25-38

Culture	PCR	MAT	MSAT / Elisa IgM and other rapid screening tests
❖ Isolation of leptospira organism by culture of blood, CSF and Urine are the most definite way of confirming leptospirosis ❖ Culture does not contribute to an early diagnosis as results come late, weeks or even months after inoculation of culture medium.	❖ PCR is the only available diagnostic test available in the first week of leptospirosis ❖ It is a complicated and expensive test ❖ The serovar cannot be identified by this test.	❖ Gold Standard ❖ Complicated, DFM required ❖ Titres peak late (2nd or 3rd week), but persist longer (5 to 10 years) ❖ Valuable in sero-epidemiologic studies ❖ Less sensitive for current diagnosis ❖ Repeat samples required for confirming diagnosis ❖ Requires 24 live serogroup cultures ❖ Cut-off titres controversial ❖ **Interpretation of MAT** ❖ Single Titre ➢ 1:100 – significant criteria ❖ Diagnostic criteria ➢ Endemic area – 1:400 (1:800, 1:1600) ➢ Non- endemic area – 1:100, 1:200 ➢ Serosurvey – 1:50 ❖ Repeat titr ➢ four fold rise / sero conversion	➢ Single positive sample adequate for diagnosis. ➢ Simple, sensitive & specific tests. ➢ Becomes positive earlier than MAT. ➢ Cannot identify the serogroup. ➢ Can be done also in small rural hospitals. ➢ Can be easily done for a large number of patients during an epidemic. **Other Rapid Tests are:** ❖ Latex agglutination test ❖ Lepto dipstick ❖ Lepto Tek lateral flow ❖ Lepto Tek Dri-Dot test ❖ Lepto check

Abbreviations: CSF - Cerebrospinal fluid; PCR - Polymerase chain reaction; MAT - Microscopic agglutination test; MSAT - Macroscopic slide agglutination test

are made easily available in both urban and rural hospitals.

2. Since these tests become positive only after a week, a scoring based on clinical and epidemiological criteria has been used for the first week (A+B = 26 or more).
This scoring system is valuable in diagnosis of severe leptospirosis. But this has less sensitivity than A+B+C as milder cases tend to be missed. Therefore, it is very essential that investigations to diagnose leptospirosis are definitely done. The A+B criteria should be used to start empiric therapy even for possible leptospirosis (A+B = 20 - 25).

It is essential to combine both clinical features and epidemiological risk factors to make a possible diagnosis of leptospirosis. For example, a patient with fever, headache, myalgia and conjunctival suffusion during the monsoon month who gives the history of wading through flood water would have a score of 21 which would be diagnostic of possible leptospirosis.

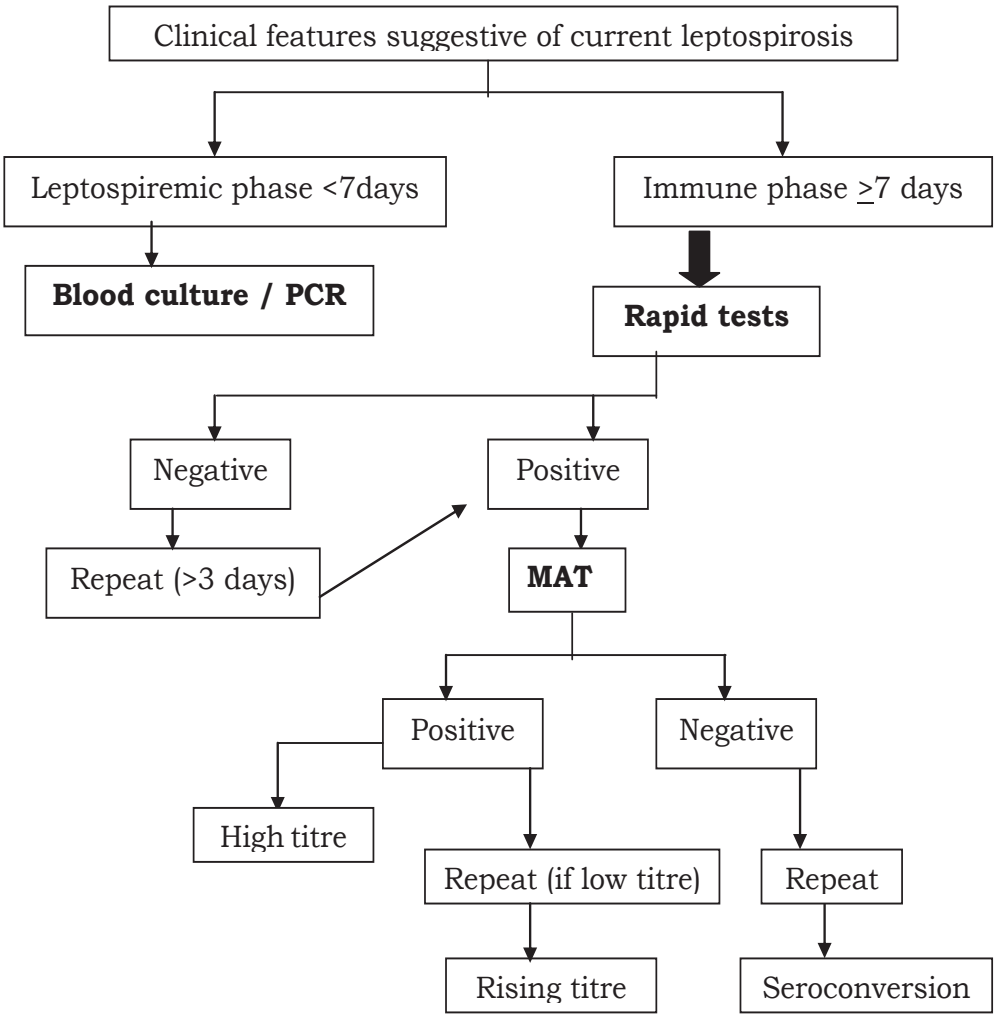
Therefore, in the first week clinical and epidemiological factors (A+B) should be used to diagnose leptospirosis, while in the second week, laboratory tests should be used to confirm the diagnosis. The role of diagnostic tests for leptospirosis and the epidemiological risk factors are shown in table 3 and 4.

Table 4 : Epidemiological Risk Factors

Environmental Risk Factors	Occupational Risk Groups	Recreational activities
◆ Rainfall and flooding ◆ Contaminated environment ❖ Poor Sanitation. ❖ Inefficient solid waste disposal. ❖ Inadequate drainage facilities and open drains. ❖ Presence of rodents, cattle, pigs & dogs. ❖ Walking bare foot. ❖ Wading through contaminated water. ❖ Absence of indoor toilets. ❖ Living in overcrowded residential areas (urban slums). ❖ Outdoor manual work.	❖ Farmers ❖ Rice, Sugarcane, Vegetables, Cattle, Pigs ❖ Sewage workers ❖ Abattoir workers, Butchers ❖ Vetenarians, Lab staff ❖ Miners ❖ Fishermen – Inland ❖ Soldiers	❖ Swimming in fresh water ❖ Sailing ❖ Marathon runners ❖ Gardening ❖ Adventure travel ❖ Water sports ❖ Eco tourism

In urban areas of developing countries, rainfall and flooding39 are the most important risk factors in combination with environmental factors which affect the citizens, mostly from the lower socio-economic groups. In rural areas, occupational risk factors play an important role40. Recreational activities affect travele in developing countries. The approach to diagnosis of leptospirosis is shown in Fig.1

Fig 1 : Approach to Diagnosis of Leptospirosis9



1. In the first 5 days of acute illness, PCR is valuable in the diagnosis of leptospirosis.

2. In the late phase of acute illness (5-10 days), Rapid tests (Elisa IgM) are useful and are more sensitive than MAT. Low titre of MAT (1:100 or 1:200) can be accepted for diagnosis.

3. MAT is done after 10 days in the early convalescent phase, to obtain the best results41,42. High titre of MAT (1:400 or above) in single samples is diagnostic of leptospirosis. It would be preferable to have repeat sample to demonstrate fourfold rise in titre. Combining PCR + Rapid tests or Rapid tests + MAT is a good strategy in acute phase or early convalescent phase. But PCR and MAT can be done in higher centers only.

Rapid tests play an important role in the diagnosis of Leptospirosis in developing countries. Elisa IgM is a popular rapid test for diagnosis of Leptospirosis, with good sensitivity and specificity. This can be done in centers with facilities to do this test. Simple tests such as Lateral flow assay (Leptocheck) and Latex agglutination test (Lepto Dri Dot) can be done at peripheral medical centers as bed side tests & have been highlighted in the references as sensitive tests compared to Elisa IgM. It is very essential that these tests are made easily available in both urban & rural health centers for diagnosis of Leptospirosis. National guidelines should accept that these tests are adequate for diagnosis. The gross under reporting due to under diagnosis of Leptospirosis in developing countries can be solved by utilizing these tests and funding to do these tests should be easily available.

An administrative structure is needed for the control of Leptospirosis. This should consist of clinicians, microbiologists, epidemiologists and public health staff of both medical and veterinary faculty. Their role should be to recommend the development of laboratory services for providing reliable diagnosis, collection of clinical cases in both humans and animals, to investigate the source of infection and mode of transmission, to educate the relevant professionals/public and suggest an effective control program. This should be at the regional and national level. They should publish their data regularly in the relevant journals.

To conclude, the following 3 tier system can be applied.

1. Peripheral hospitals in urban and rural areas:

- Rapid tests are adequate to diagnose leptospirosis in acute febrile illness.

2. Larger hospitals with microbiology department (Medical College Hospitals):

- These institutes can do ELISA IgM & MAT (if available).

- Samples from peripheral hospital should be sent to these institutes for further evaluation and confirm the diagnosis.

3. Regional Reference laboratories:

- These centers can do the cultures, MAT and PCR to identify the serovars and guide the other centers.

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Full Length Research Articles

EFFECT OF DIFFERENT ACIDULANTS ON SHELF LIFE OF CHHANA PODO AT AMBIENT TEMPERATURE

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ABSTRACT

Present study was undertaken to evaluate the shelf life of chhana podo prepared from 1% citric acid and 2% lactic acid and stored at ambient temperature (30± 2 °C). Microbial, physico-chemical and sensory qualities of product were studied during storage. The chhana podo were analyzed Standard Plate Count (SPC), yeast and mould count on alternate days. The SPC, yeast and mould count increased significantly ($P \leq 0.05$) with the progress of storage at ambient temperature in 1% citric acid and 2% lactic acid products. No significant change in SPC, yeast and mould count was observed up to 2nd day storage but later there was remarkable increase in SPC (2.79 & 2.55) as well as yeast and mould count (12.40 & 12.30) at the end of day four. The significant reduction in pH, moisture and fat content in 1% citric acid and 2% lactic acid products during the progress of storage of 8 days, while protein content showed an increasing trend. All the sensory quality attributes of products made by 1% citric acid and 2% lactic acid were comparable up to 2 days of storage from then on declined significantly with progress of storage. Colour, body & texture, flavour and overall acceptability score of channa podo declined significantly up to the 4th days of storage. Thus, the findings of the study indicated that the channa podo could be safely stored upto 4 days at ambient temperature.

Keywords : Ambient temperature, citric acid, lactic acid, microbial quality, physico-chemical quality, sensory quality.

INTRODUCTION

Channa podo is a channa based Indian delicacy much popular in eastern region of India, which is prepared by baking. It is made from chhana, sugar and semolina (suji)/ refined wheat flour (maida). It is often garnished with nuts, cloves and cardamoms. The chhana podo has been served to Lord Jagannath in Puri as offering prasad for hundreds of years. Since the product is presently confined to Orissa, its characteristic taste and appeal may find wider acceptance in other parts of India.

Traditionally, it is made by smouldering chhana sugar mix wrapped in sal leave (Shorea robusta) or any other large leaves on slow fire. The wrapped podo is repacked

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in polyethylene pouches. In urban areas, polyethylene sheets are used as primary packaging material followed by cardboard boxes as secondary packaging material. Polystyrene containers are also used in some places. The traditional method of packaging is exposed to contamination and the product has a short shelf life at ambient temperature. Further, the entire process of chhana podo is manual and production is on small scale. Nature of spoilage is mainly microbial, primarily fungal (mould). Due to the high sugar content of the product, only osmophilic bacteria thrives on it. With better hygienic process, packaging and good storage condition (controlled temperature), it is expected that shelf life of chhana podo could be extended considerably. Therefore, the present study was conducted to assess the shelf life of chhana podo at ambient temperature.

MATERIAL AND METHODS

Milk: Fresh cow milk was procured from the livestock unit of College of Veterinary and Animal Sciences, Parbhani.

Coagulants: Citric acid and lactic acid were used in different concentrations for preparation of chhana.

Chhana making: Chhana was prepared from standardized cow milk content 4 % fat as per the method described by De (1980). Milk was divided into different lots as per the treatment. Each lot of milk was subjected to boiling and subsequently cooled to 80 °C. Different coagulants viz. 1% citric acid and 2% lactic acid was used. Before addition, coagulants were heated at 80°C and slowly added with continuous stirring till complete coagulation occurred and the clear whey was observed. The coagulated mass was kept undisturbed for 2 min. and then transferred to muslin cloth for drainage of whey. After 20 min. the chhana obtained from each lot was collected and weighed.

Preparation of chhana podo: Chhana podo was prepared from chhana made from standardized cow milk.

a. Preparation of dough: Standardization of dairy ingredients to be incorporated in chhana for making chhana podo was done as under. Chhana dough was made by mixing the weighed quantity of chhana with non dairy ingredients viz. 5 % suji using 25 per cent sugar.

b. Baking of dough: The kneaded mixture was spread on a flat, dry and clean pan smeared with ghee to a thickness of about 2cm. The pan was kept in hot air oven maintained at 180 ^{0}C for 60 min to obtain a baked product. At the end, a puffed, brown, spongy textured product was obtained which was subsequently cooled to room temperature.

Standardized milk (4% Fat)
↓
Boiling
↓
Cooling (80 °C)
↓
Addition of coagulant
(1% citric acid &2% lactic acid)
↓
Straining of coagulated mass
↓
Channa
↓
Addition of non-dairy ingredients
(5.0 % suji and 25 % sugar)
↓
Kneading
↓
Blending
↓
Moulding
↓
Baking
(180 ^{0}C for 60 min)
↓
Cooling
↓
Packaging
↓
Storage (30±2^{o}C)

FLOW CHART OF CHANNA PODO PROCESS

Storage of chhana podo: Chhana podo so prepared stored at ambient temperature (30±2^{o}C) in polythene pouches to evaluate quality of product at every second day.

Microbial analysis

Ten gram chhana podo sample was removed aseptically and was cut into small pieces and transferred in the 90 ml sterile normal saline solution then tenfold serial dilutions were made using sterile NSS up to 10^{-3}dilution.

For evaluation of Standard Plate Count (SPC), standard pour plate technique was followed as per the (A.O.A.C. 1995). Yeast and mould count was done according to the procedure recommended by ISI (1969).

Physical examination

Estimation of pH: All the chhana podo samples were analysed for estimation of pH using digital pH meter.

Estimation of Moisture and Fat: Moisture content of chhana podo was determined by standard gravimetric method (IS: SP: 18, 1981) and fat content were determined by Gerbers method (IS: SP: 18, 1981)

Estimation of protein: Total protein content of chhana podo were determined according to methods of AOAC (1995).

Sensory evaluation: Representative samples from the two categories of chhana podo were randomly selected and evaluated for organoleptic qualities by panel of judges. Chhana podo were judged for various sensory attributes (flavour, body and texture, colour, appearance and overall acceptability) using 9 point hedonic scale (Amerine et al., 1965). -

Statistical methods: The data were subjected to analysis of variance using Completely Randomized Design and standard deviation was computed as described Snedecor and Cochran (1989).

RESULT AND DISCUSSION

1. Determination of pH and microbial quality of chhana podo:

The change in pH was inversely proportional to storage period. With the progress of storage, significant decline in pH was observed for both the products. Sharp reduction in pH was seen after 4th day of storage. The reduction in pH may be attributed to sharp increase in microbial count particularly after 4th day of storage making the product unacceptable thus limiting the product shelf life in between 2 to 4 days.

The SPC, yeast and mould count increased significantly with the progress of storage at room temperature (30 $\pm$ 2 ^{o}C). However, the differences in SPC as well as yeast and mould count were considerable with progress of 2nd day of storage without any appreciable change. Further, from 4th day onward there was tremendous increase in SPC as well as yeast and mould count making the product unacceptable which might be due to formation of slime on the surface of the product with formation of green and black mould spots. While on the 6th and 8th days of storage SPC count, yeast and mould count rising massively in product. The present findings were in close observation of Jalamkar (2002) who reported that the presence of sufficient moisture during the storage of the product greatly promotes microbial growth including yeast and mould count. In contrast Bikash et al. (2008) who reported significantly increasing SPC, yeast and mould count in chhana podo as progress of storage.

2. Determination of chemical quality of chhana podo at ambient temperature:

The moisture and fat content recorded in this study decline significantly during storage period of 8 days in both 1% citric acid and 2% lactic acid chhana podo but the protein content showed an rising trend. No appreciable changes were recorded up to 2nd day of storage in moisture, fat and protein and the values did not differ significantly. This indicates that the chemical quality of chhana podo on 2nd day was almost similar to that of day zero for both the product. As the storage time increasing the moisture and fat content declined significantly but no significant changes were observed in protein content from 4th day of storage in both 1% citric acid and 2% lactic acid chhana podo. The observation to Jalamkar (1999) who also observed a declining trend in fat and moisture content and increased protein content during storage of chhana podo.

3. Result on sensory quality of chhana podo stored at ambient temperature

Sensory scores of chhana podo declined significantly for all the quality attributes during storage of 8 days. The extent of decline was not significant up to 2 days of storage but later scores declined tremendously making the product unacceptable. Even the product stored on 4th day was disliked by the judges which might be due to increased browning, brittleness and formation of slime with initiation of rancid flavour. Chhana podo prepared from

1% citric acid and 2% lactic acid exhibits significant changes in sensory quality of products at all the sensory attributes. Further, non significant deterioration in sensory quality with regards to colour, body & texture, flavour and overall acceptability were observed in chhana podo stored for 2 days at ambient temperature but the deterioration in quality was much faster making the product unacceptable on 4th day of storage. The significant reduction in scores with progress of storage might be due to more browning, much brittle texture and surface spoilage by yeast and mould. Surender Kumar et al. (2002) reported shelf life of chhana podo at 30°C was 3 days maximum. Ghosh et al. (2002) reported the shelf life of podo, which varied from 3 to 6 days depending upon the type of podo.

CONCLUSION

It can be concluded that the chhana podo prepared from cow milk chhana coagulated by 1% citric acid and 2% lactic acid with incorporation of 2.5 % suji + 2.5 % maida along with 25 % sugar and baked at 180 ºC for 60 min and stored at ambient temperature (30 ± 2 °C) is microbiologically safe and remain acceptable upto the 4th days.

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Table. 1 Changes in pH and microbial count during storage of chhana podo from 1% citric acid

Storage period (days)	pH	Standard plate Count (CFU x 10^3/gm)	Yeast & Mould (CFU x 10^3/gm)
0	6.45[a]	0.63[a]	0.70[a]
2	6.38[b]	1.72[ab]	3.50[b]
4	6.12[c]	2.79[b]	12.40[c]
6	6.07[c]	8.53[c]	15.60[d]
8	5.96[d]	19.40[d]	18.50[c]

Common superscripts indicate treatments are not significant at 5 per cent and 1 per cent level of probability.

Table 2: Changes in pH and microbial count during storage of chhana podo from 2% lactic acid

Storage period (days)	pH	Standard plate Count (CFU x 10^3/gm)	Yeast & Mould (CFU x 10^3/gm)
0	6.25[a]	0.24[b]	0.60[b]
2	6.18[a]	1.36[b]	2.40[b]
4	6.07[b]	2.55[a]	12.30[a]
6	5.99[b]	8.31[a]	15.30[a]
8	5.86[b]	17.35[a]	18.30[a]

Common superscripts indicate treatments are not significant at 5 per cent and 1 per cent level of probability.

Table 3: Storage related changes in chemical quality of chhana podo made from 1% citric acid

Storage period (days)	Moisture	Fat	Protein
	Percentage		
0	26.23[a]	22.70[a]	18.57[a]
2	26.15[ab]	22.68[a]	18.57[a]
4	26.01[b]	22.64[ab]	18.96[b]
6	25.92[bc]	22.57[b]	18.96[b]
8	25.81[b]	22.38[c]	19.14[b]

Common superscripts indicate treatments are not significant at 5 per cent and 1 per cent level of probability.

Table 4: Storage related changes in chemical quality of chhana podomade from 2% lactic acid

Storage period (days)	Moisture	Fat	Protein
	Percentage		
0	25.56[a]	22.81[a]	18.68[a]
2	25.53[ab]	22.81[a]	18.75[a]
4	25.38[b]	22.76[b]	19.01[b]
6	25.16[c]	22.70[c]	19.03[b]
8	25.02[c]	22.65[d]	19.08[b]

Common superscripts indicate treatments are not significant at 5 per cent and 1 per cent level of probability.

Table 5: Storage related changes in sensory quality of chhana podo made from 1% citric acid chhana

Period of storage (days)	Colour	Body and texture	Flavour	Overall acceptability
0	8.32[a]	8.36[a]	8.32[a]	8.52[a]
2	8.25[a]	8.30[a]	8.12[a]	8.32[a]
4	6.12[b]	5.96[b]	5.20[b]	5.36[b]
6	5.80[c]	5.00[c]	4.12[c]	4.12[c]
8	4.36[d]	4.24[d]	2.44[d]	3.40[d]

Table 6: Storage related changes in sensory quality of chhana podomade from 2% lactic acid

Period of storage (days)	Colour	Body and texture	Flavour	Overall acceptability
0	8.48[a]	8.56[a]	8.48[a]	8.64[a]
2	8.28[a]	8.50[a]	8.30[a]	8.44[a]
4	6.28[b]	6.12[b]	5.36[b]	5.48[b]
6	5.74[c]	5.12[c]	4.28[c]	4.20[c]
8	4.56[d]	4.56[d]	2.64[d]	3.76[d]

Effect of Different Acidulants on Physico-Chemical, Microbial and Sensory Quality of Chhana Podo

Treatments	Storage period (days)				
	0	2	4	6	8
	pH				
T1	6.45^{a}	6.38^{b}	6.12^{c}	6.07^{c}	5.96^{d}
T2	6.25^{a}	6.18^{a}	6.07^{b}	5.99^{b}	5.86^{b}
	Standard plate count (CFU x 10^3/gm)				
T1	0.63^{a}	1.72^{ab}	2.79^{b}	8.53^{c}	19.40^{d}
T2	0.24^{b}	1.36^{b}	2.55^{a}	8.31^{a}	17.35^{a}
	Yeast & mould (CFU x 10^3/gm)				
T1	0.70^{a}	3.50^{b}	12.40^{c}	15.60^{d}	18.50^{c}
T2	0.60^{b}	2.40^{b}	12.30^{a}	15.30^{a}	18.30^{a}
	Proximate composition Moisture				
T1	26.23^{a}	26.15^{ab}	26.01^{b}	25.92^{bc}	25.81^{b}
T2	25.56^{a}	25.53^{ab}	25.38^{b}	25.16^{c}	25.02^{c}
	Fat				
T1	22.70^{a}	22.68^{a}	22.64^{ab}	22.57^{b}	22.38^{c}
T2	22.81^{a}	22.81^{a}	22.76^{b}	22.70^{c}	22.65^{d}
	Protein				
T1	18.57^{a}	18.57^{a}	18.96^{b}	18.96^{b}	19.14^{b}
T2	18.68^{a}	18.75^{a}	19.01^{b}	19.03^{b}	19.08^{b}
	Sensory attributes Colour				
T1	8.32^{a}	8.25^{a}	6.12^{b}	5.80^{c}	4.36^{d}
T2	8.48^{a}	8.28^{a}	6.28^{b}	5.74^{c}	4.56^{d}
	Body and texture				
T1	8.36^{a}	8.30^{a}	5.96^{b}	5.00^{c}	4.24^{d}
T2	8.56^{a}	8.50^{a}	6.12^{b}	5.12^{c}	4.56^{d}
	Flavour				
T1	8.32^{a}	8.12^{a}	5.20^{b}	4.12^{c}	2.44^{d}
T2	8.48^{a}	8.30^{a}	5.36^{b}	4.28^{c}	2.64^{d}
	Overall acceptability				
T1	8.52^{a}	8.32^{a}	5.36^{b}	4.12^{c}	3.40^{d}
T2	8.64^{a}	8.44^{a}	5.48^{b}	4.20^{c}	3.76^{d}

T1: 1% citric acid; T2: 2% lactic acid, Common superscripts indicate treatments are not significant at 5 per cent and 1 per cent level of probability.

EFFECT OF SEX AND BODY REGION ON THE FOLLICULAR PROFILE OF CORRIEDALE SHEEP

Running Title: Follicular Profile of Corriedale Sheep

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ABSTRACT

The quality and quantity of wool production is a function of number and type of wool follicles. Hence a study was conducted to evaluate the follicular characteristics of corriedale sheep, a dual purpose breed established well in the temperate climatic conditions of Kashmir valley. Skin samples of corriedale sheep were collected ethically from different body regions viz; neck, shoulder, thorax, flank and back of both sexes and preserved in 10% neutral buffered formalin. The samples were processed for histomorphlogy. Primary and secondary follicle density and their diameter was recorded. Secondary to primary follicle ratio was also calculated. The study revealed the difference in the follicular density and S/P follicle ratio between the sexes as well as between the body regions. In general, the follicle density and S/P ratio was found higher in the flank and thorax region respectively. Evaluation of the follicular profile at flank and thorax during early period will serve as a reliable predictor for the wool yield and quality.

Key words: Corriedale, Follicles and S/P Ratio.

INTRODUCTION

The market demand of the fibre and its products is determined by its quality in terms of its physical characteristics (Hatcher *et al.,* 2010 & Swan, 2010), processing performance, durability (Swan *et al.,* 2008) and textile attributes (Warn *et al.,* 2006). Wool is a natural animal fiber that grows from the wool follicles of sheep's skin. The unique characteristics and virtues of wool have, through the years, enabled it to hold its position of prominence in competition with products of plant origin and the invasion of innumerable synthetic materials. However, its physical characteristics vary depending upon the sheep genetics, environment and management strategies (Poppi and McLennan, 2010) and classified as apparel, carpet and coarse wools.

The fibers grow from the follicles present in the skin which is an epidermal appendage, penetrating deep into the underlying dermis (Dellman, 1998). Histologically, wool follicles are of primary

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and secondary types. The quality and yield of wool is a function of the type and number of the follicles. Hence, study of these follicles has been focus of research. Marked differences have been noted in the wool quality and yield between different sheep breeds, individuals and different body regions (Tabbaa *et al.,* 1998). In general, fine fibres grow from secondary follicles while coarse fibres grow from primary follicles. Secondary to primary (S/P) follicles ratio is the major determinant of fibre quality and varies between body regions, individuals and breeds (Kazmi, 2014). Since follicular characteristics are genetically determined, it may provide an early indication of wool quality and yield, and serve as a marker for selection.

Corriedale, a dual purpose sheep breed, had its origin in New Zealand and Australia. It is known for outstanding efficiency to produce more amount of meat and wool per kg of body weight as compared to other range breeds. It produces average fine wool with an average diameter of 26.30 µ, staple length of 4.75 cm and greasy fleece yield of 1.02 kg (Singh *et al.*, 2008). The breed has established well in the temperate climatic conditions of Kashmir valley in relation to both mutton and wool productivity (Singh *et al*., 2004; Singh *et al.,* 2005; Afzal *et al.,* 2009). The study of the follicular characteristics and their quality in the temperate region of the state of J&K shall be helpful in framing future wool production strategies. Hence, the current study was carried out with an objective to study the effect of sex and body regions on the follicular profile of Corriedale sheep.

MATERIAL AND METHODS

A total of twelve corriedale sheep, six each from both sexes were taken for the study. Skin samples were obtained ethically from five body regions of each animal viz; back, neck, shoulder, flank and thorax and preserved in separate vials containing 10% neutral buffered formalin till processed. The samples were processed by paraffin embedding technique for histo-morphology (Luna, 1968). Sections of 5-6µm thickness were cut and 12 sections/samples were stained with Harris haematoxylin and eosin method (Luna, 1968). The sections were observed under the microscope equipped with the Prog Res Capture Pro Camera (Version 3.8.0 Win; Make: Jenoptik). The density of primary and secondary follicle/ mm^2 of skin was calculated as per Clark (1960). The follicle was identified by their size (Oznurlu *et al.,* 2009 & 2011) and associated structures (Ansari Renani *et al.,* 2011a). S/P follicle ratio was calculated by dividing number of secondary follicles by primary follicles. Diameter of the primary and secondary follicle was calculated using image analyzing software (Video Test.-5). Data obtained from the experiments was grouped as per sex and body regions and analyzed statistically by one way ANOVA and T test following the method of Snedecor and Cochran (1994).

RESULTS AND DISCUSSION

The follicles in the Corriedale sheep were observed in the form of groups, consisting of primary follicles and associated secondary follicles. The follicle groups, called trioses included both complete (3 primary and associated

secondaries) and incomplete (1 or 2 primary and associated secondaries) ones. The basic criteria for distinguishing primary follicle from secondary was the presence of sweat glands (Ansari *et al.,* 2011a) and their larger size (Oznurlu *et al.,* 2009 & 2011).

The results pertaining to the follicular profile in terms of primary follicle density (PFD), secondary follicle density (SFD), total follicle density (TFD) per mm^2 of skin and secondary to primary follicle density (S/P ratio), primary follicle diameter (PFDia) and secondary follicle diameter (SFDia) of Corriedale breeds of both sexes from different body regions are presented in Table 1. In Corriedale males and females, the average PFD was found as 1.18±1.11 & 1.85±0.21; 1.21±0.19 & 1.08±0.15; 1.61±0.12 & 1.28±0.15; 2.15±0.27 & 3.43±0.71; and 1.34±0.11 & 2.86±0.21 from neck, shoulder, thorax, flank and back regions with an overall mean of 1.52±0.42, 1.14±0.12, 1.45±0.11, 2.79±0.41 and 2.10±0.26, respectively. In males, flank region showed significantly ($P<0.05$) higher PFD than neck, shoulder, thorax and back, whereas in females, flank showed significantly ($P<0.05$) higher PFD than neck, shoulder and thorax and non-significant difference ($P>0.05$) with back. Females showed significantly ($P<0.05$) higher PFD only at neck and back region than males. The observations in current study were in agreement with Mobini (2013) wherein he also observed higher PFD in flank region than other regions of Iranian Baktiari lambs. The variation in the primary follicle density between different body regions was similar to the observations in Lori sheep (Abbasi *et al.,* 2008), Bakhtiari sheep (Mobini, 2012); Tuj breed (Kocamus and Aslam, 2004); German Black Head, Hampshire Down, Lincolon long wool, White Karman, Awassi and Konya Merino (Kurtdele and Asti, 1999). The difference in the follicle density between the male and female within the breed was similar to the observations in Merino (Andrew *et al*., 1998); Lori (Abbasi *et al.,* 2008) and Madras Red sheep (Mir *et al.,* 2011). The gender related changes of skin are attributed to endogenous androgen stimulation at puberty (Yeruham *et al.,* 1997).

In males, flank (21.45±2.91) showed significantly ($P<0.05$) higher SFD than neck (12.55±0.72), shoulder (8.80±0.54) and thorax (15.38±1.35) and non-significant difference with back (18.01±0.88). In females, flank (20.11±2.29) showed significantly ($P<0.05$) higher SFD than shoulder (11.45±0.89) and non-significant difference with neck (18.48±1.67), thorax (15.29±1.56) and back (17.42±1.35). Higher secondary follicle density found in the flank region in both the male and females of Corriedale sheep than other regions was in disagreement with the reports of Mobini (2013) where he showed higher density in hip followed by neck. Between sexes, females showed significantly ($P<0.05$) higher SFD than males at neck and shoulder region only. In general, females showed higher density than male which is not in agreement with the observations of Mobini (2012 and 2013) in Bhaktiari sheep wherein he reported higher density in males. This could be because of the difference in the genetic makeup and agro climatic conditions. The gender related changes of skin are attributed to endogenous androgen stimulation at puberty (Yeruham *et al.,* 1997).

In Corriedale males, flank region (23.59±3.09) showed significantly (P<0.05) higher TFD than neck (13.73±0.82), shoulder (10.00±0.72), thorax (16.99±1.39) and back (17.69± 1.53). Within females, non-significant results were found between shoulder (12.53±1.04) and thorax (16.58±1.67); thorax and back (20.30±1.50); whereas neck (29.69±1.57) showed significantly higher TFD than all other regions. Between the sexes, females showed significantly (P<0.05) higher TFD (29.69±1.57) than males (P<0.05) from neck region only. The results obtained in Corriedale were more or less in agreement with that obtained in Suffolk, Romney Marsh and Lincolon (Singh, 1997), while in disagreement in fine Merino and Polworth (Singh, 1997). TFD of flank region reported in Corriedale was in agreement with the findings of Mobini (2013) who reported TFD in flank region of Bakhtiari sheep as 21.67.

The S/P follicle ratio in male and female Corriedale sheep from neck, shoulder, thorax, flank and back region were 10.89±0.69 & 10.57±1.55; 7.85±0.75 & 11.11±0.72; 9.68±0.83 & 12.33±1.12; 10.33±1.16 & 7.14±1.56; and 12.53±1.44 & 6.09±0.36, with an overall ratio of 10.72±0.80, 9.47±0.69, 11.00±0.77, 8.73±1.04 and 9.31±1.20, respectively. S/P follicle ratio in males from back region showed significantly higher ratio than shoulder and non-significant difference with neck, thorax and flank. In females, flank and back showed non-significant difference (P<0.05) among each other and had significantly lower S/P ratio than neck, shoulder and thorax region. Between sexes, males had significantly (P<0.05) lower S/P ratio than females at shoulder region whereas females showed significantly (P<0.05) lower ratio at back region than males. The results obtained were in agreement with those reported by Singh (1997) in Corriedale while in disagreement with the observations of Britt *et al., (*1985) in Merino sheep and Crook and Purvis (1995) in pippin merino sheep. The variation in the S/P follicle ratio is influenced mainly by hereditary factors but also influenced by diet considerably (Gifford *et al.,* 1995).

The average PFDia (µ) of male and female Corriedale sheep were 190.79±8.03 & 126.59±6.42; 155.56±15.02 & 172.73±19.66; 174.56±5.21 & 161.80±9.78; 159.46±6.26 & 120.82±16.31; and 161.93±14.18 & 136.24 ± 8.70 from neck, shoulder, thorax, flank and back, respectively. In males, only neck differed significantly (P<0.05) from shoulder region. In females shoulder region showed significantly higher values than neck and flank. Comparison between sexes revealed significantly (P<0.05) higher values (190.79±8.03) in males than females (126.59±6.42) at neck region only. The results were more or less in agreement with the findings of Raichev and Khristova, (1990) in Kotel, Stranja, Sarkar and in disagreement with the findings of Genkovski and Gerchev (2007) in Bakhtiari lambs and Tsigai ewes.

The average values of SFDia in male and female Corriedale sheep from neck, shoulder, thorax, flank and back regions were 63.22±3.45 & 63.24±1.27; 58.93±3.21 & 75.98±1.74; 63.02± 1.13 & 61.57±2.87; 54.74±2.33 & 56.56±2.65; and 53.86±2.72 & 55.04± 2.14, , respectively. In males neck and thorax region differed significantly

($P<0.05$) from back region. In females shoulder region showed significantly higher values than all other regions whereas neck differed significantly ($P<0.05$) from shoulder and back only. Between the sexes only shoulder region showed significantly ($P<0.05$) higher SFDia in females than males. The results were more or less in agreement with Genkovski and Gerchev (2007) in Bakhtiari lambs and in disagreement with the findings of Raichev and Khristova (1990) in Duben, Kotel, Stranja, Sarkar and Central Rodopi ewes

The study revealed that the follicular profile of corriedale sheep in terms of density and S/P ratio varies between sexes, individuals as well as between body regions. Evaluation of the follicular profile at flank and thorax during early period will serve as a reliable predictor for the wool yield and quality.

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Table 1: Effect of sex and body regions on the follicular Profile of Corriedale sheep

Body regions	Primary Follicle Density			Secondary Follicle Density			Total Follicle Density			S/P Follicle ratio			Primary Follicle Diameter			Secondary Follicle Diameter		
Sex	Male	Female	Overall	Male	Female	Overall	Male	Female	Overall	Male	Female	Overall	Male	Female	Overall	Male	Female	Overall
Neck	1.18± 1.11^{aA}	1.85± 0.21^{abB}	1.52± 0.42	12.55± 0.72^{abA}	18.48± 1.67^{bB}	15.51± 1.25	13.73± 0.82^{abA}	29.69± 1.57^{dB}	21.70± 2.54	10.89± 0.69^{ab}	10.57± 1.55^{b}	10.72± 0.80	190.79± 8.03^{bA}	126.59± 6.42^{aB}	158.68± 10.84	63.22± 3.45^{b}	63.24± 1.27^{b}	63.22± 1.75
Shoulder	1.21± 0.19^{a}	1.08± 0.15^{a}	1.14± 0.12	8.80± 0.54^{aA}	11.45± 0.89^{aB}	10.12± 0.64	10.00± 0.72^{a}	12.53± 1.04^{a}	11.26± 0.71	7.85± 0.75^{aA}	11.11± 0.72^{bB}	9.47± 0.69	155.56± 15.02^{a}	172.73± 19.66^{b}	164.14± 12.07	58.93± 3.21^{abA}	75.98± 1.74^{cB}	67.45± 3.10
Thorax	1.61± 0.12^{a}	1.28± 0.15^{a}	1.45± 0.11	15.38± 1.35^{bc}	15.29± 1.56^{ab}	15.34± 0.98	16.99± 1.39^{b}	16.58± 1.67^{ab}	16.78± 1.03	9.68± 0.83^{ab}	12.33± 1.12^{b}	11.00± 0.77	174.56± 5.21^{ab}	161.80± 9.78^{ab}	168.17± 5.62	63.02± 1.13^{b}	61.57± 2.87^{ab}	62.29± 1.48
Flank	2.15± 0.27^{b}	3.43± 0.71^{c}	2.79± 0.41	21.45 2.91^{d}	20.11± 2.29^{b}	20.78± 1.78	23.59± 3.09^{c}	23.53± 2.64^{c}	23.55± 1.93	10.33± 1.16^{ab}	7.14± 1.56^{a}	8.73± 1.04	159.46± 6.26^{ab}	120.82± 16.31^{a}	140.14± 10.16	54.74± 2.33^{ab}	56.56± 2.65^{ab}	55.64± 1.70
Back	1.34± 0.11^{aA}	2.86± 0.21^{bcB}	2.10± 0.26	18.01± 0.88^{cd}	17.42± 1.35^{b}	17.71± 0.77	17.69± 1.53^{b}	20.30± 1.50^{bc}	18.99± 1.09	12.53± 1.44^{bA}	6.09± 0.36^{aB}	9.31± 1.20	161.93± 14.18^{ab}	136.24± 8.70^{ab}	149.08± 8.82	53.86± 2.72^{a}	55.04± 2.14^{a}	54.45± 1.65
Total	**1.50± 0.98**	**2.10± 0.22**	**1.80± 0.13**	**15.24± 1.03**	**16.55± 0.87**	**15.89± 0.68**	**16.40± 1.10**	**20.52± 1.31**	**18.46± 0.89**	**10.26± 0.51**	**9.45± 0.66**	**9.85± 0.41**	**168.46± 4.99**	**143.63± 6.63**	**156.04± 4.41**	**58.75± 1.34**	**62.48± 1.65**	**60.617± 1.082**

Means across the rows in a same column with different lower case superscript differ significantly.
Means between sexes with different upper case superscript differ significantly.
P level 0.05;
No. of observations (n=6 each)

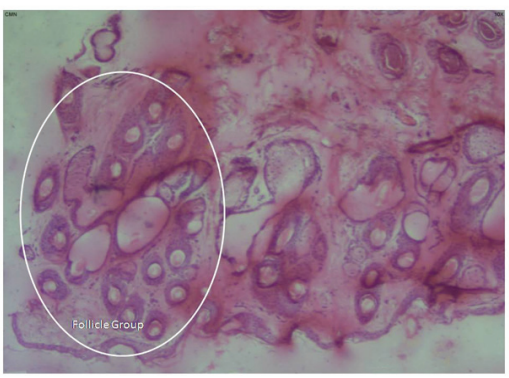

Fig 1: Follicle Group of Corriedale sheep comprising of Primary and associated secondary follicles

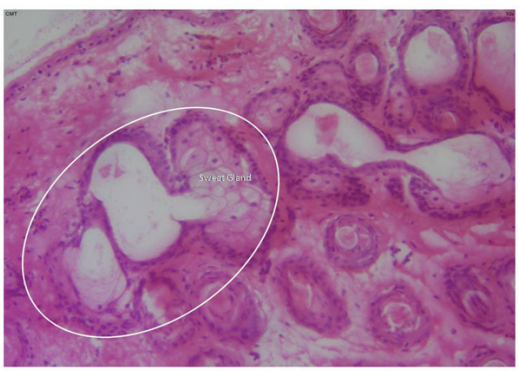

Fig 2: Primary Follicles of Corriedale sheep along with associated sweat gland.

IN VITRO ANTI-BACTERIAL AND BIOLOGICAL PROPERTIES OF MAGNETRON SPUTTERED SILVER NANOPARTICLES CONTAINING TITANIUM IMPLANTS

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ABSTRACT

Bacterial infection followed by implant fixation is a common complication after surgery. In order to reduce the incidence of implant-associated infections, several biomaterial surface treatments have been proposed. In this study, the effect of *in vitro* antibacterial activity of magnetron sputtered silver nanoparticles was studied. Sputtering was done using radiofrequency magnetron sputtering technology. Film applicator coating assay was used to assess the antibacterial effect of the coated titanium implants. SEM examination revealed successful deposition of silver nanoparticles on the titanium surface. The average diameter of the nanoparticles was 40-60 nm. SEM examination before incubation and after incubation of bacteria was done. The bactericidal ratio between the uncoated and coated implant was determined.

Key words: Infection, silver nanoparticles, antibacterial effect

Introduction

Infections associated with orthopaedic implants are a challenge to the long-term "survival" rate of implants and may cause the treatment to fail.[1,2] The titanium surfaces of implants are appropriate for microbial colonization and biofilm formation.[3]Moreover, there are boundaries to the use of aseptic surgical techniques and prophylactic antibiotics. Therefore, strategies involving the delivery or incorporation of antibiotics such as gentamicin and vancomycin on some titanium implants have been tried to reduce infections.[4, 5]Non-conventional antibiotics have been developed in an attempt to handle these problems since there is increasing concerns on antibiotic-resistant pathogens in orthopaedic field. Silver is effective against a broad spectrum of bacterial and fungal species, including strains that are resistant to antibiotics.[6] Silver nanoparticles are considered to be even more active due to theirlarge surface area to volume ratio.[7] Previous work has indicated that silver nanoparticles embedded in titanium may be highly effective in inhibitingboth *Staphylococcus aureus* and *Escherichia coli*.[8]

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In veterinary practice, a common complication following open reduction and internal fixation using plates and screws to treat compound fracture is osteomyelitis. Compound fractures are very difficult to treat since they are already contaminated and infected with microorganisms. The common organism found in compound fracture is *Staphylococcus aureus* organism. This study is conducted in an attempt to eradicate infection in compound fracture and as a prophylactic tool.

MATERIALS AND METHODS

Preparation of titanium plate

Commercially available pure titanium implants were ultrasonically cleaned with acetone and distilled water. In this study, 2.0 mm titanium reconstruction implants were used to test the antibacterial effect of silver nanoparticles.

Deposition of silver nanoparticles on the titanium surface

Silver nanoparticles were deposited on the titanium surface using radiofrequency magnetron sputtering technology. Sputter coating machine delivers uniform amount of silver nanoparticles on the titanium surface.

Operating Characteristics of a sputtering machine

Parameters used to coat a titanium implant in a radiofrequency magnetron sputter coating machine are shown in the below table.

Table 1: Parameters required to surface coat an implant (Moseke et al. 2011)

Parameter	Details
Target	Silver
Power	40 W
Deposition time	8 min
Inert gas	Argon
Size of the nanoparticle	40-60 nm
Base pressure	10^{-5}
Working pressure	2×10^{-3}
Characterisation	SEM

Characterization of the silver nanoparticles

The morphology of the silver nanoparticles coated surface of the titanium implants was characterized using SEM (HITACHI S-4800, Tokyo, Japan).

Antibacterial tests

The antibacterial activity of the silver nanoparticles coated titanium surface was tested against the Gram-positive *Staphylococcusaureus* (MTCC).

Zones of inhibition (ZoI) tests were carried out to decide the degree of silver ion discharge from the coated titanium surface. In the ZoI test, the concentration of *Staphylococcus aureus*was adjusted to 1x 10^6 colony-forming units (CFUs)/mL and spread evenly on Luria-Bertani medium agar plates. Titanium coated with silver nanoparticles implant and the non-coated control titanium implant were placed on the above prepared agar plates separately. The plates were incubated at 37°C in an aerobic

petri dish for 24 hours and photographed to record the results.

Film applicator coating **(**FAC) was used to test the antimicrobial effect by directly incubating microbial cells on Ti-nAg surfaces. In the FAC assay the concentration of each bacterial strain was adjusted to $1x10^4$ cells/mL in phosphate buffer solution and Luria Bertani Medium. The titanium implants were then placed on these culture plates and kept in wet boxes. Then 10 µL bacterial suspensions were applied to the Ti-nAg and the titanium control implants, respectively. Following incubation at 37°C in an aerobic petri dish for 24 hours, the bacterial suspensions on the coupons were then transferred separately into tubes containing 10 mL of sterilized PBS, followed by vigorous vortex mixing for 5 minutes. Following this 10 µl of bacterial solutions from the mixtures were then spread on Luria Bertani medium broth-agar plates. The plates were incubated aerobically for 24 hours. The viable cells on each of the plates were counted by quantifying the CFUs.

The antibacterial effect in each group was determined as bactericidal ratio and was calculated as follows:

Bactericidal ratio (%) = [(CFU of CG - CFU of EG)/CFU of CG] × 100%

CFU represents colony-forming unit; CG - titanium implants (control group) and EG - silver nanoparticles coated titanium implants (experimental group).

RESULTS AND DISCUSSION

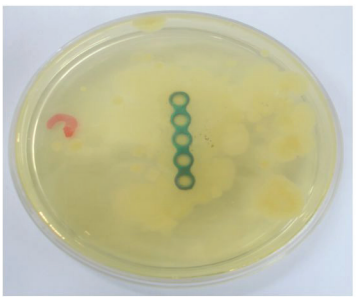
Zones of inhibition (ZoI)

There was no zone of inhibition noticed around either the silver nanoparticles coated titanium plate or the non-coated plate.

The Zones of Inhibition (ZoI) test was inconclusive in both control and silver nanoparticles coated titanium implants. This result may be due the Zone of Inhibition was based on the escape of silver ions from the surface the inhibition of the bacterial growth depends on a sufficient concentration of silver ions in the surrounding aqueous environment and the elemental silver has a very low rate of dissolution in an aqueous environment, it is possible that the silver dissociated from the titanium surface did not reach a concentration sufficient to inhibit bacterial growth.[9]

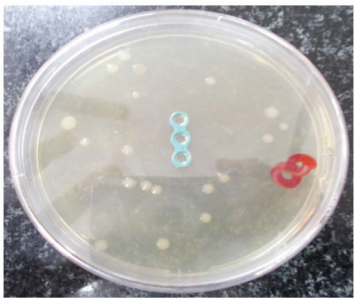
dissociated from the titanium surface did not reach a concentration sufficient to inhibit bacterial growth.[9]

Figure 1



CONTROL

Figure 2



COATED

Fig 1 and 2: Zone of inhibition tests showing no discernible line of inhibition in both control and silver nanoparticles coated titanium implants

Film applicator coating (FAC) assay

Film applicator coating (FAC) assay was done to determine the bactericidal ratio of silver nanoparticles coated titanium surface. The coated titanium implant showed remarkable antibacterial effect against *Staphylococcus aureus* organism. The bactericidal ratio was found to be 95 per cent.

Bactericidal ratio (%) = [(CFU of CG - CFU of EG)/CFU of CG] × 100%

The mean ± SD values obtained from the study was depicted in the bar diagram below.

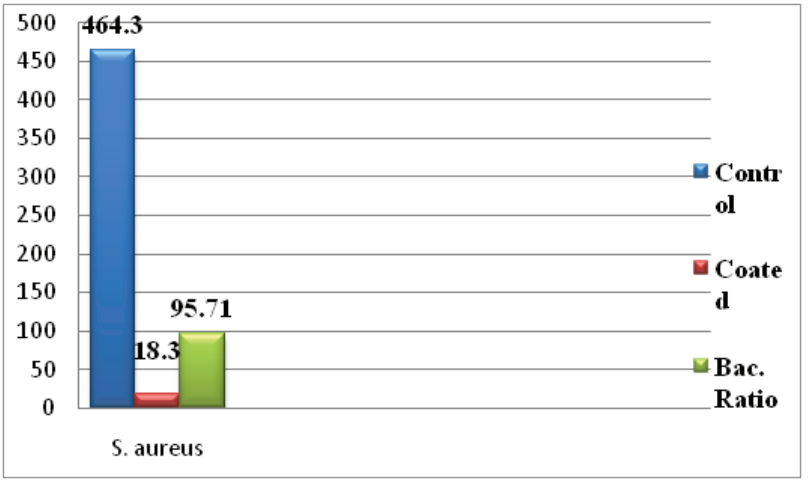


Figure 3: Counts of CFU (mean ± SD) and bactericidal ratio obtained from FAC

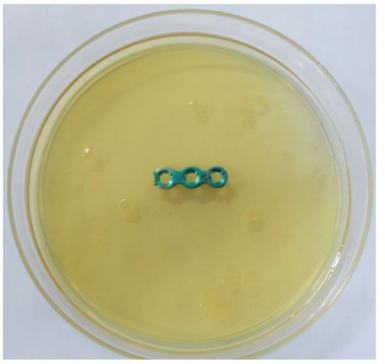
In contrast to the Zone of Inhibition test, the silver nanoparticles coated titanium plate surface unveiled a robust antibacterial property. Following 24-hour incubation of bacteria on Silver nanoparticles coated titanium plate surface (Ti-nAg surface) resulted in a depletion of bacteria which is more than 95 per cent. Ti-nAg specimens significantly inhibited the growth of both Staphylococcus aureus than non-coated Titanium specimen. The incongruity between ZoI and FAC tests may be due to the difference of the antibacterial mechanism. The former relied on leaching of the silver ions from the surface, whereas the latter mediated an inhibitory effect by

direct contact. The SEM examination of anti-adhesive test showed that there was much less bacteria adherent to Ti-nAg surface than to the non-coated control titanium surface. This was due to the direct contact inhibition and the anti-adhesive properties of the Ti-nAg surface. Therefore it was suggested that the silver nanoparticles coated titanium plate (Ti-nAg) surface reduces the risk of bacterial colonization. This was in accordance to the study done by Chen et al. (2006) where he observed that deposition of silver-hydroxyapatite (Ag-HA) coatings on titanium implant surfaces by magnetron sputtering exhibited high antibacterial activity against S. aureus. Juan et al. (2010) found similar observation where he proposed that the silver nanoparticles modified titanium surface provided effective contact with microorganism and exhibited strong antibacterial properties against S. aureus organism. Silver nanoparticles coated titanium surface showed strong antibacterial properties against infection causing organisms which was in accordance with Zhao et al. (2011), Goodman et al. (2013) and Jematet al. (2015).

Figure 3

CONTROL

Figure 4



COATED

Fig 4 and 5: Film applicator coating assay tests showing bacterial growth in control and silver nanoparticles coated titanium implants

Characterization of the silver nanoparticles on titanium surface

The Scanning Electron Microscopy (SEM) micrographic pictures showed silver nanoparticles uniformly deposited on the titanium surface, with a diameter of approximately 40 - 50 nm at 500 x magnificationsand at 1000 x magnifications. Some of the silver nanoparticles formed aggregates/agglomerates together.

In the control titanium surface after the incubation there was numerous amount of *Staphylococcus aureus* organism present at 500 x magnification. In the silver nanoparticles coated titanium implant there was absence of *Staphylococcus aureus* organism at 500 x magnifications.

A noticeable change after the coating of titanium implants with silver nanoparticles

was appreciable amount of silver macroscopically deposited uniformly over the implant. The SEM micrograph showed silver nanoparticles sparingly deposited on the titanium surface, with a diameter of 40-60 nm. This finding was similar to the study done by Lkhagvajav et al. (2011) where he demonstrated colloidal silver nanoparticles with a size range of 25 to 45 nanometer exhibiting antibacterial activity against *S. aureus* and *E. coli* organisms. This was contrary to the study done by Moroneset *al.* (2005) where he opined that the bactericidal properties of the silver nanoparticles were size dependent and direct interaction with the bacteria occurred preferentially at a diameter of 1 to 10 nanometer size.

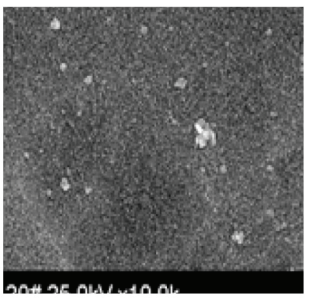


Figure 6: SEM micrograph of silver nanoparticles coated titanium

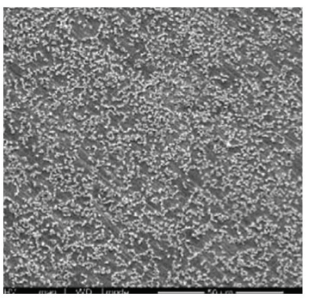


Figure 7: SEM micrograph of control titanium showing numerous bacterial growth

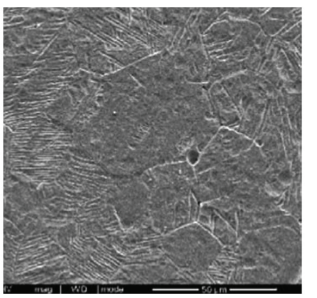


Figure 8: SEM micrograph of silver nan oparticles coated titanium showing no or absence of bacterial growth

CONCLUSION

In the present study, silver nanoparticles coated titanium implants were prepared using radiofrequency magnetron sputtering technology. Deposition was successful which was confirmed by Scanning Electron Microscope. SEM images showed uniform deposition of silver nanoparticles on the titanium surface. The average diameter was 40-60 nanometers. Film applicator coating assay and Zone of inhibition tests were performed and the anti-bactericidal effect of the nanoparticles was studied. The bactericidal ratio of the silver nanoparticles coated titanium implants was 95.41 percent. Nanoparticles showed remarkable antibacterial properties against *Staphylococcus aureus* organism. These data suggests that silver nanoparticles coated titanium implants will be a promising tool in preventing implant associated infections in near future.

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ASSESSMENT OF MICROBIOLOGICAL QUALITY OF KHOA BASED SWEETS MARKETED IN THRISSUR CITY

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ABSTRACT

Bacteriological quality of khoa based sweets marketed through different retail outlets of Thrissur city was evaluated based on total viable count, coliform count, yeast & mould count and staphylococcal count. Altogether 72 samples of khoa based sweets such as peda, burfi and gulabjamun were evaluated for their bacteriological quality. Out of the total peda samples tested, coliforms were prevalent in 37.5%, yeast and mould in 91.7% and staphylococcal organisms in 41.7% of the samples. In burfi, coliforms were prevalent in 66.6%, yeast and mold in 95.8% and staphylococcal organisms in 83.3 % of the samples tested. In gulabjamun, coliforms, yeast and mould and staphylococcal organisms were present in 45.8%, 91.6% and 70.8% respectively of the samples tested. The highest rate of contamination was recorded in burfi while the lowest was recorded in peda. Strict hygienic measures during preparation and storage are recommended to ensure the quality of the products.

Key words: Khoa based sweets, bacteriological quality, Peda, Burfi, Gulabjamun.

INTRODUCTION

Traditional milk sweets have been developed to preserve the nutritional goodness of milk and to extend its shelf life under high ambient temperature. The market for Indian milk based sweets is expanding overseas also. The total Indian sweet market is around Rs. 520 billion. Khoa is a major intermediate base material used for the preparation of a variety of milk sweets such as peda, gulabjamun, burfi, kalakand and milk cake. In India, these milk sweets are an indispensable part of the socio-cultural life. They also have high commercial significance because of their popularity throughout the country. Khoa based milk sweets have high nutritive value. Due to the complex biochemical composition and high water content, these products also serve as an excellent medium for the growth and multiplication of different microorganisms particularly when stored at ambient temperature. The manufacture of these products is mostly done on a small-scale in unorganized sector based on traditional method without much regard to the quality of raw material or hygienic packaging and storage. Under such conditions, many microorganisms including some pathogens find access to the sweets and pose great threat to the health

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of the consumer. In view of public health significance, the study was designed to examine the bacteriological quality of khoa based sweets marketed in Thrissur city.

MATERIALS AND METHODS

Collection of samples

Altogether 72 samples (Peda, Burfi and Gulabjamun-24 each) were collected from different retail outlets in and around Thrissur city within eight Kilometer radius during monsoon season (June to October). The samples were brought to the laboratory in sterile containers and processed for microbiological analysis. All the samples were evaluated for their microbiological quality based on total viable count, coliform count, yeast and mould count and *staphylococcal* count.

Total viable count and coliform count of each sample was estimated by pour plate technique, as described by Wehr and Frank (2004) by using Standard Plate Count agar and Violet Red Bile Agar (VRBA) respectively. Yeast and mould count of khoa based sweet samples were determined according to the procedure described by the Bureau of Indian Standards (1980) by using Potato dextrose agar (PDA). *Staphylococcal* count was done by using Baird Parker agar as per the procedure described by Wehr and Frank (2004).

Data obtained in the study were subjected to statistical analysis as per the method suggested by Snedecor and Cochran (1994). One sample t-test was used to check whether the mean values of different parameters meet the standards specified by BIS.

RESULTS AND DISCUSSION

Microbiological quality of Peda

The total viable count of peda samples ranged from 5.05-5.30 log cfu/g with a mean value of 5.17 ± 3.63 log cfu/g. The coliform count ranged between 0 and 3.78 log cfu/g with a mean value of 3.06 ± 2.56 log cfu/g. Yeast and mould count varied from 0 to 4.62 log cfu/g with a mean value of 4.04± 3.33 log cfu/g and *Staphylococcal* count ranged between 0 and 3.43 log cfu/g with a mean value of 2.73 ±2.20 log cfu/g (Table-1).

Garg and Usha (1984) reported that the total viable count of khoa based milk sweets collected from local vendors were high (1.1×10^3 to 5.6×10^5cfu/ gm) as compared to the private manufacturers ($1.9x10^3$ to $2.3x10^5$) and organized dairies ($2x10^3$ to $1.1x10^3$). According to Grewal and Tiwari (1990), *Staphylococcus* sp. was the most frequently occurring organism in sweet based milk products such as khoa, rabri and gulabjamun. Riadh and Tahiri (2005) have reported higher coliform counts in milk sweets.

Karthikeyan and Dhanalakshmi (2010) had isolated *Aspergillus* sp. more frequently (70.90 per cent) in khoa based milk sweets than the *Penicillium* sp. (15.11 per cent). Thaker *et al.* (2013) have isolated 10 strains of (6.25%) of *S. aureus* from peda.

The total viable count serves as an important criterion for evaluating the microbial quality of various foods and also the degree of freshness of food. The findings in the present study indicate that all samples of peda collected from different parts of the

city were highly contaminated with bacteria and fungi. The unhygienic conditions of preparation of these khoa based milk sweets, poor microbial quality of raw materials and poor quality water used for washing the utensils might have enhanced the bacterial load of these products. A high incidence of *Staphylococcus* in peda samples indicate lack of hygienic practices during manufacture and handling.

Percentage of prevalence of different microbial groups in Peda

The percentage of prevalence of different microbial groups in peda is presented in Table 2. Out of the total samples tested, coliforms were prevalent in 37.5% of peda samples. Yeast and mould were present in 91.7% of the samples and *staphylococcal* organisms were present in 41.7% of the total samples tested.

Higher prevalence of yeast and mould in milk based sweets were also reported in earlier studies. Jatkar *et al.* (1982) studied the microbiological quality of market milk sweets in twin cities of Hydrabad and Secunderabad. They have observed that 90 per cent of peda, 75 per cent of kalakand and 100 per cent of rasogollas were contaminated with yeast and mould.

Microbiological quality of Burfi

The total viable count in burfi samples varied between 5.08-5.48 log cfu/g with a mean value of 5.22 ± 3.92 log cfu/g. Coliform count ranged from 0 to 3.52 log cfu/g with a mean value of 3.11 ± 2.40 log cfu/g. Yeast and mould count varied from 0 to 4.97 log cfu/g with a mean value of 4.51 ±2.85 log cfu/g. *Staphylococcal* count ranged between 0 and 3.36 log cfu/g, with a mean value of 3.11 ±2.19 log cfu/g. (Table 3).

Kumar and Sinha (1989) recorded higher coliform count in khoa based sweets obtained from local vendors when compared with organized sector. The coliform count of khoa based milk sweet samples of local vendors was in the range of $4x10^2$ - $3.1x10^3$ cfu/g with a mean value of $1.9x10^3$±3.85 respectively.

Ghodeker *et al.* (1980) had reported that the yeast and mould count of burfi varied between 20 and 3,700 cfu/g. Ranganathan (1984) and Mandokhot and Garg (1985) reported high number of pathogens in burfi.

The total viable count of khoa based milk sweets obtained from local vendors, private manufacturers and organized dairies ranged from $1.2x10^5$ to $8x10^5$, $1.9x10^3$ to $2.3x10^5$ and $8x10^2$ to $3.1x10^4$ respectively. Similarly the coliform count ranged from $4x10^2$ to $3.1x10^3$, $2x10^2$ to $1.1x10^3$ and 2x10 to $1x10^2$ respectively (Karthikeyan and Pandiyan, 2013).

The higher microbial counts obtained for burfi in the present study when compared to the earlier reports can be attributed to the unclean hands of workers, inferior quality of raw materials, unhygienic conditions of manufacturing units and improper storage conditions.

Percentage of prevalence of different microbial groups in Burfi

The percentage prevalence of different microbial groups in burfi is presented in Table 4. In burfi, coliforms were prevalent

in 66.6% of total samples tested. Yeast and mould and *staphylococcal* organisms were prevalent in 95.8% and 83.3% respectively of the total samples tested.

Kumar and Prasad (2010) isolated *Staphylococcus* and *E.coli* from milk products such as gulabjamun, burfi and khoa. The results showed that, out of 135 samples tested, 25 samples were found to be contaminated with *Staphylococcus* and *E.coli*. The highest rate of contamination was recorded in burfi. Similar reports were made by Bajaj *et al.* (2013).

The presence of coliform organisms in khoa based sweets is an clear indication of post production contamination which might have occurred due to widespread unhygienic conditions in manufacturing units coupled with faulty storage measures adopted by the retailers.

Microbiological quality of Gulabjamun

The total viable count of gulabjamun ranged between 5.02-5.37 log cfu/g with a mean value of 5.21 ±3.75 log cfu/g. The coliform count of gulabjamun varied from 0 to 3.52 log cfu/g with a mean value of 3.07 ±2.44 log cfu/g. Yeast and mould count ranged between 0 and 4.93 log cfu/g with a mean value of 4.37 ± 3.83 log cfu/g. The *staphylococcal* count ranged from 0 to 3.85 log cfu/g with a mean value of 3.37 ± 2.69 log cfu/g (Table 5).

The study conducted by Grewal and Tiwari, (1990) indicated that *Staphylococcus* species was the most frequently occurring organism in milk based sweet product gulabjamun. Similar results were reported by Hamama and Tatini (1991). Soomro (2003) reported that indigenous milk based sweets like Khoa, gulabjamun and rasogolla are highly susceptible to contamination by a variety of microorganisms because of high nutritive value and complex chemical composition.

The total viable count of Khoa based sweets obtained from from local vendors, private manufacturers and organized dairies ranged from $1.2x10^5$- 8 x 10^5 cfu/ g^{-1},$1.9x10^3$-$2.3x10^5$ cfu/g^{-1} and $8x10^2$ - $3.1x10^4$ cfu/g^{-1} respectively(Karthikeyan and Dhanalakshmi 2010).

The higher microbial counts of gulabjamun in the present study may be attributed to the unhygienic practices followed during preparation, inferior quality of raw materials, faulty storage conditions and inadequate knowledge regarding food safety among the workers.

Percentage of prevalence of different microbial groups in Gulabjamun

The percentage of prevalence of different microbial groups in gulabjamun is presented in Table 6. In gulabjamun, coliforms were present in 45.8% of the total samples tested. Yeast & mould and staphylococcal organisms were prevalent in 91.6% and 70.8% respectively of the samples tested (Table 6).

According to Yadav *et al.* (1993), higher yeast and mould count in khoa based sweets might be due to the usage of khoa stored for long period for making sweets.Kumar and Prasad (2010) isolated *Staphylococcus* and *E.coli* from milk

products such as gulabjamun, burfi and khoa. The results revealed that out of 135 samples, 25 samples were found to be contaminated with *Staphylococcus* and *E.coli*.

The higher microbial load observed in the present study may be attributed to the contamination during post-preparation handling, transportation and storage of the finished product.

CONCLUSION

From the results of the study, it can be concluded that the bacteriological quality of khoa based sweets (peda, burfi and gulabjamun) marketed in Thrissur city is generally poor. Out of the 72 samples tested, 50 percent of samples were found to be heavily contaminated with coliforms, yeast and moulds and *Staphylococcus* which indicates public health risk. The highest rate of contamination was recorded in burfi while the lowest was recorded in peda. The study reveals the need for good hygienic practices during preparation; handling and storage are recommended to ensure the quality and safety of milk sweets.

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Table 1. Microbiological quality of Peda (Log cfu/g)

Microbiological parameter	Minimum	Maximum	Mean ± Standard Error
Total viable count	5.05	5.30	5.17±3.63
Coliform count	0	3.78	3.06 ± 2.56
Yeast and mould count	0	4.62	4.04± 3.33
Staphylococcal count	0	3.43	2.73 ±2.20

Table 2. Percentage of prevalence of different microbial groups in Peda

Bacterial count	No. of samples	Positive	Negative	Percentage of prevalence (%)
Coliform count	24	9	15	37.5
Yeast and mould count	24	22	2	91.7
Staphylococcal count	24	10	14	41.7

Table 3. Microbiological quality of Burfi (Log cfu/g)

Microbiological quality	Minimum	Maximum	Mean ± Standard Error	BIS Standards for burfi
Total viable count	5.087	5.48	5.22 ± 3.92**	30,000/g
Coliform count	0	3.52	3.11 ± 2.40	
Yeast and mould count	0	4.97	4.51 ±2.85**	10/g
Staphylococcal count	0	3.36	3.11 ±2.19	

**Significantly greater than standard value at 1 per cent level

Table 4. Percentage of prevalence of different microbial groups in Burfi

Bacterial count	No. of samples	Positive	Negative	Percentage of prevalence (%)
Coliform count	24	16	8	66.6
Yeast and mould count	24	23	1	95.8
Staphylococcal count	24	20	4	83.3

Table 5. Microbiological quality of Gulabjamun (Log cfu/g)

Microbiological quality	Minimum	Maximum	Mean ± Standard Error
Total viable count	5.02	5.37	5.21 ±3.75
Coliform count	0	3.52	3.07 ±2.44
Yeast and mould count	0	4.93	4.37 ± 3.83
Staphylococcal count	0	3.85	3.37 ± 2.69

Table 6. Percentage of prevalence of different microbial groups in Gulabjamun

Bacterial count	No. of samples	Positive	Negative	Percentage of prevalence (%)
Coliform count	24	11	13	45.8
Yeast and mould count	24	22	2	91.6
Staphylococcal count	24	17	7	70.8

Short Communications

EFFECT OF DELTAMETHRIN ON REPRODUCTIVE PERFORMANCE IN MALE ZEBRAFISH

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Pesticides and herbicides are commonly use dinagriculture to control and eradicate pests and weeds. They are used to control mosquitoes, flies, and termites which are responsible for disease spread. The indiscriminate, heavy use of chemical pesticides results in ecological degradation, deleterious effects on water taste and odour, lethal effects on non-target organisms in agro ecosystems, and direct toxicitytousers (AnsariandKumar1988, Kalavathy*et al*., 2001). Deltamethrin is highly toxic to aquatic life, fish and aquatic invertebrates. Deltamethrin was found in aquatic sediments in California (Johnson *et al.,*2010).and it was detected in sediments in 2 ponds treated withdeltamethrin at Department of Fisheries and Oceans, Freshwater Institute, Winnipeg, Canada. Higher concentrations were found (3.5-5 ng/g) at 306 days post treatment to deltamethrin.(Muir *et al*.,1985)

Deltamethrin is suspected endocrine disruptor (European commission, 2000) and more study and investigation is needed to check the potential of deltamethrin as endocrine disruptor compound.The European Commission has prepared a strategy for EDC. One of the initiatives of the strategy is to compile a list of potential EDC. It includes three categories in which category 1 is for the substances for which endocrine activity have been documented in at least one study of a living organism. Deltamethrin is included in this category. These substances are given the highest priority for further studies.Hence present study was conducted.

The zebrafish were maintained at zebrafish facility, Department of Pharmacology and Toxicology, Bombay veterinary college.A pilot study was conducted using minimum of 10 adult male zebrafish including one control group without exposure and four different concentrations of deltamethrin which were 0.01 µg/L, 0.1 µg /L, 1 µg /L and 2 µg /L. During the period of 21 days, the zebrafish were observed for mortality and nervous signs and also the spawn count were recorded for control as well as exposure groups. After the analysis of results of pilot study,two concentrations were selected viz0.5 micrograms/ liter and 1 micrograms/ litre of deltamethrin and zebrafish were exposed to these concentrations Total 48 male zebrafish and 96 female zebrafish were used for the study. They were divided into two groups. Each group was divided into three subgroups/replicates. Each replicate/ subgroup contained 8 males and 16 females of breeding age (4-12 months).Fish were

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fed thrice with pellets, live brine shrimp and flakes feed.Room temperature was maintained between25-28^{0}C. Tanks were cleaned regularly in order to remove debris and maintain fish in hygienic conditions. Fish had to be netted from their tanks and transferred temporarily to a separate tank during the cleaning. Dirty water from fish tanks had to be drained off. The circadian cycle of 14 hours of light and 10 hours of darkness was strictly maintained. Technical grade deltamethrin (98.5%) was used during study. Deltamethrin was stored in a dry, amber coloured bottle, away from sunlight. Each timeit had to be introduced into the test groups, solution was freshly prepared by weighing and then dissolving in DMSO & ethanol (1:1) and diluting to selected concentrations.

Males and females were randomly selected from breeding stock prevailing in the zebrafish facility. They were maintained in separate tanks for three days and on the fourth day mating was allowed early in the morning for three hours in three different breeding tanks each containing eight males and 16 females. Once the spawning of the fish was confirmed by the presence of eggs at the bottom of the breeding tank, the males and females kept for breeding were separated and maintained in the separation tanks. The male zebrafish separated in the separation tanks were exposed continuously to deltamethrinfor 21 days except their weekly mating period of three hours.

For assessing the possible effect of deltamethrin on the endocrine system and reproductive performance, the assessment of reproduction was done by observing the spawn count, livabilityof embryos up to 96 hours post fertilization. The per cent reduction in spawn count, per cent fecundityand per cent embryo mortality were calculated for both Group I and Group II and plotted on graph for comparison.

1. Per cent reduction in spawn count :

   $$\frac{\text{Reduction in spawn count}}{\text{Spawn count (day 01)}} \times 100$$

2. Fecundity percent :

   $$\frac{\text{Live count (Respective week)}}{\text{Spawn count (Respective week)}} \times 100$$

3. Mortality percent :

   $$\frac{\text{Total mortality (0-96 hpf)}}{\text{Spawn count (Respective week)}} \times 100$$

Completely Randomized Design was applied for comparison of pre-exposure and post-exposure values at weekly intervals within each group and Students T test was used to compare between groups.

Results show significant reduction in total count of embryos over 21 days.Mean (±SE) of per cent reduction in spawn count in Group I was 39.27± 1.10, 69.97±1.00 and 85.39±0.08 at 7, 14 and 21 days respectively post exposure of male zebrafish to deltamethrin. Similarly, in Group II it was 36.49 ± 0.54, 72.74±0.09 and 86.37±0.06 at 7, 14 and 21 days respectively post-exposure of male zebrafish to deltamethrin. Comparison of spawn count within both the groups to their respective pre-exposure spawn count values indicated significant ($p \leq 0.05$ and 0.01) reduction at all three recording periodsThere was no significant difference between the groups at day 8 and day 15 . There was significant difference

(p≤0.05 and 0.01) between per cent spawning count reduction at day 22.

Hurk*et al.* (1987) demonstrated that steroid glucuronideshave ovulation inducing action synthesized by testes in male zebrafish.The reduction in spawn count in 21 days exposure of deltamethrin in males is indicative of reduced steroid synthesis in males and disruption of endocrine chemicals. In laboratory rat its observed that subcutaneous deltamethrin treatment in rats produces an arrest of spermatogenesis, asignificant disharmony in sex hormones and MDA level rats which is related to dose, length of treatment and to the lipid peroxidation.(Issam*et al.*,2009). Deltamethrin caused a significant reduction in the reproductive organs weights which might be due to the decrease in serum testosterone levels(Oda& El-Maddawy, 2012). In the same way, it has been found that deltamethrin induces a significant decrease in testosterone after 60 days of treatment (Charguie*t al.,* 2009).The reason behind weekly mating lies in reproductive physiology of males which includes 6 days sperm cycle and zebrafish breed normally 5-7 days after previous breeding.

Mean (±SE) per cent fecundity in Group I pre-exposure period was 98.80±0.15 and 95.62±0.20, 86.54±0.24 and 81.56±0.94 at 7, 14 and 21 days respectively post-exposure of male zebrafish to deltamethrin. Similarly, in Group II, for pre-exposure it was 98.62±0.20 and 94.74±0.60, 82.09±0.89, 75.22±0.47 at 7, 14 and 21 days post exposure to deltamethrin. Comparisons of fecundity within group indicated significant reduction (p≤0.05 and 0.01) from pre-exposure values at all three recording periods. There was significant difference (p≤0.05 and 0.01) between the groups at second and third week of observations.The reduction in fecundity indicates that the percent of fertilization had reduced with increased period of exposure. the reduction in per cent fecundity is indicative of the reduced quality of sperm which indirectly implies that the production of sperm is affected possibly as an outcome of hormonal disruption.Male and femalezebrafish, *Daniorerio*(Hamilton), were exposed to 96-h LC5values of Deltamethrin (0.016 µg dm-3) and Achook (0.025 µgdm-3) for three monthsthere was significant reductions in fecundity and hatchability in comparison to the control group. Fecundity was reduced by 54.12% in the fish exposed todeltamethrin.The number of unhatched/ dead eggs increased significantly ($P < 0.05$ for each case),(Sharma and Ansari, 2010)

Mean (±SE) per cent mortality in Group I pre-exposure time was 1.18±0.15 and 4.36±0.20, 13.44±0.24, 18.42±0.94 at 7, 14 and 21 days respectively post exposure of male zebrafish to deltamethrin. Similarly, in Group II, for pre-exposure time was 1.37±0.20 and 5.67±0.67, 17.89±0.89, 24.76±0.47 at 7, 14 and 21 days respectively post exposure of male zebrafish to deltamethrin. Comparison of mortality within both the groups to their respective pre-exposure mortality values indicated significant (p≤0.05 and 0.01) increase at all three recording periods. There was significant difference (p≤0.05 and 0.01) between the groups at second and third week of observation

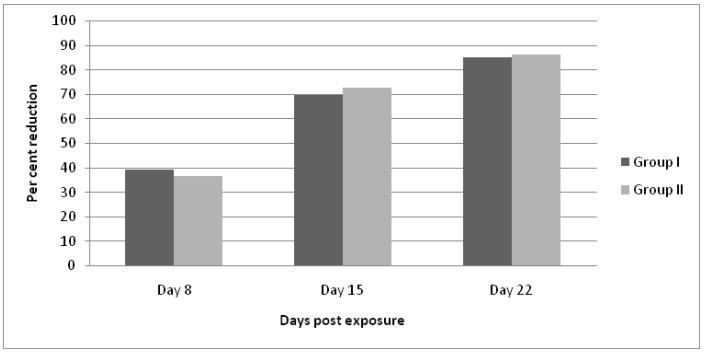

Figure 01: **Per cent reduction in spawn count of zebrafish over 21 days of deltamethrinexposure compared to pre-exposure values**

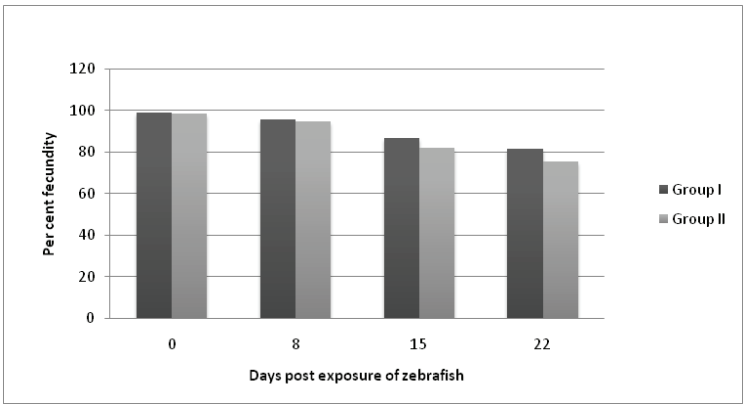

Figure 02: **Fecundity percent in zebrafish based on embryos collected weekly over the period of 21 days of exposure of male zebrafish to deltamethrin.**

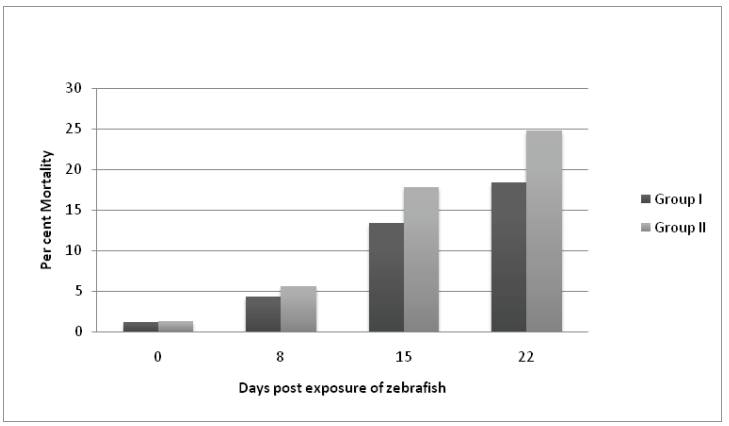

Figure 04: Percent Mortality in embryos recorded during post-spawning observation period of 0-72 hpf of embryos collected weekly

Assessment of male reproductive status considering embryo count, percent embryonic mortality and per centfecundity is an indirect method of assessment of reproductive performance. The direct approach could be semen collection from deltamethrinexposed zebrafish and sperm counts, morphology, motility, live and dead count of sperms and concentration of semen.In zebrafish however, fertilization is *in vitro*. Deposited sperms cannot be collected as they get activated in contact with water and are inactivated within a minute following activation. Thus, random collection of sperms in less than a minute will not give the exact count and may result in errors. Cloacal washings are also difficult to practice as cloacal orifice is too small and washings are contaminated with faecal matter. This indirect approach has also been used previously by Sharma and Ansari, 2010 with lower concentrations and for longer period but present study was carried out with higher concentration along with observations on developmental effects on embryos and it was found practicable and convenient.

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COMPARATIVE OVARIAN BIOMETRY AND OOCYTE RETRIEVAL METHODS IN PIG

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Reproductive success is central to livestock production. Advancement in porcine reproduction has become more important for developing novel biotechnology, as well as for genetic improvement of livestock (Choi *et al.*, 2008). *In vitro* reproductive techniques are powerful tools for studying physiology of maturation, fertilization, development of pre-implantation embryos and increasing production as it gives access to micromanipulation of embryos (Ramsingh *et al*., 2013). For successful *in vitro* production (IVP) of embryos, evaluation of ovaries and efficient collection and grading of oocytes is very important (Islam *et al*., 2007). Large number of oocyte is a prerequisite factor for such studies. However, there has been a limitation to retrieve sufficient number of the oocytes. The total number of oocytes obtained per ovary varies with different collection methods. Therefore, for efficient IVP of embryo from slaughterhouse-sourced ovaries it is necessary to develop a suitable technique that can enhance the oocyte recovery rate. The present study was undertaken for evaluation of slaughterhouse-sourced porcine ovaries for IVP of embryos and relative efficiency of oocyte retrieval methods.

Ovaries of adult crossbred sows, used for the present study, were sourced from organized slaughterhouse (R&D Pork Processing Plant, ICAR-NRC on Pig, Rani, Guwahati) and unorganized slaughterhouses located at Rani, Guwahati. The reported experiment was approved by Institutional Animal Ethics Committee. A total of 178 numbers of ovaries were collected immediately after slaughter and transported to the laboratory within 2 hr at room temperature in normal saline solution (NSS) containing penicillin (100 IU/ml) and streptomycin (100 µg/ml). The ovaries were washed three times with clean water and finally washed in NSS containing penicillin and streptomycin.

Morphometric measurements, e.g. length and width of the ovaries, were measured with a Vernier caliper. The measure from the anterior to posterior end of the ovary was considered as the length, and medial to lateral border of the ovary was considered as width. Numbers of visible surface follicle, corpus luteum and abnormalities (if any) were recorded.

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The oocytes were collected aseptically from the ovaries by following two methods (Fig. 1):

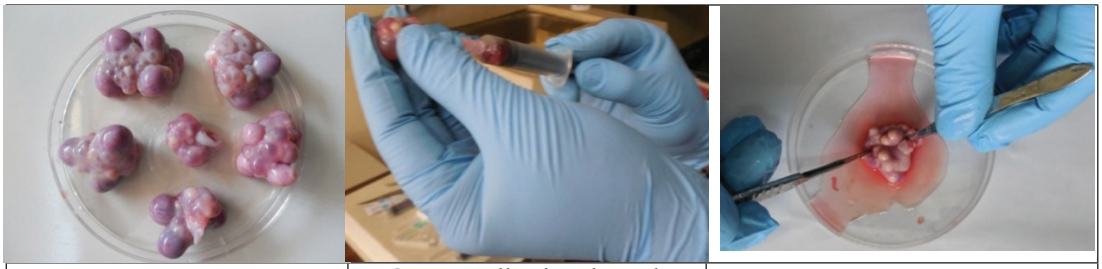

Fig. 1: Pig ovary	Oocyte collection through aspiration	Oocyte collection through slicing

1. Aspiration: The visible follicles (2-8 mm in diameter) present on the surface of the ovary were aspirated with 18G needle fixed to a disposable syringe containing 1 ml of tissue culture medium (TCM) 199 (Sigma). The collected pig oocytes were placed in a 60 mm petridish (Nunc) containing TCM 199 and searched under steriozoom microscope for grading.

2. Slicing: The ovaries were held firmly with the help of a forceps in a sterile glass petridish containing 5 ml of warm (37 ˚C) TCM 199. The ovaries were sliced into possible small sections with a BP blade fixed to a handle. The oocytes containing TCM 199 media were transferred to a searching dish and observed under steriozoom microscope for grading of the oocytes.

Grading of oocytes:

Oocytes were classified on the basis of cumulus layers as follows:

Grade A: Compact, multi-layered (more than three) cumulus with a homogenous cytoplasm

Grade B: Compact cumulus consisting of two to three layers of cells, with a homogenous ooplasm

Grade C: Less compact cumulus, with an irregular ooplasm, containing dark clusters in ooplasm

Grade D: Depleted of complex cells or an expanding cumulus, irregular ooplasm and jelly-like matrix.

Statistical analysis:

Data were analysed using SPSS (ver. 16.0). Morphometric measurements are presented in Mean±SE. Harvest of graded oocytes is presented in percentage. The percentage values were converted using arcsine transformation before two-sample T-test/ANOVA**.**

Biometry and follicular parameters:

The biometry and follicular parameters of ovaries collected from organized and unorganized-slaughterhouses are presented in Table 1. Average length and width of the ovaries sourced from organized slaughterhouse were higher than the ovaries sourced from unorganized slaughterhouses.

Similarly, average numbers of surface and cystic follicles were higher for organized slaughterhouse-sourced ovaries. Number of corpus luteum (CL) was higher for unorganized slaughterhouse-sourced ovaries. However, these differences were not significant. Significant differences ($P<0.05$) in morphometric traits of the porcine ovaries was observed by Naskar *et al.,* (2015) based on source. Number of surface follicles was reported to be significantly higher in unorganized slaughterhouse-sourced ovaries ($P<0.05$) (Naskar *et al.,* 2015). Further, number of CL was higher for porcine ovaries collected from organized slaughterhouses (Naskar *et al.,* 2015). The variation observed in the present study compared to other reports may be due to the fact that number of surface follicles, cystic follicles and CL present in an ovary is influenced by many factors, like breed, stage of reproductive life and reproductive health. Biometry of ovaries and follicular parameters further indicates that female pigs slaughtered in organized slaughterhouses are likely beyond third or fourth farrowing where reproductive worth has already been realized that is common for organized production system. On the other hand, female pigs slaughtered in unorganized slaughterhouses are likely to be slaughtered before third or fourth farrowing, even at prepubertal stage (Dyck and Swierstra, 1983; Bartol *et al.,* 1993; Naskar *et al.,* 2015).

Oocyte recovery and grading:

Average number of oocytes recovered per ovary by slicing (12.93±1.49) was significantly higher ($P<0.01$) than aspiration (6.36±1.02). Overall percentage of different grade of oocytes, namely 'A', 'B', 'C' and 'D', recovered from 178 numbers of ovaries used in our experiment are presented in Table 2. It is important to note that 'A' grade oocytes are the best source material for applications of assisted reproductive technologies (ART). Our study indicates that aspiration method yields more 'A' grade oocytes than slicing ($P<0.01$). On the other hand, slicing method yields more 'B', 'C' ($P<0.01$) and 'D' ($P<0.05$) grade oocytes. These might be due to the fact that oocytes are recovered from mature surface follicle only in aspiration as against predominant retrieval of oocytes from core of cortex of the ovary in slicing which may not have developed enough and thus unfit for ART. Similar results were obtained by Wani *et al*. (2000), Wang *et al.* (2007) and Zeinoaldini *et al.* (2013).

Biometry and follicular parameters of porcine ovaries collected from organized and unorganized-slaughterhouses are reported in the present study. Relative comparison of oocyte retrieval methods reveals that aspiration yields higher percentage of superior grade oocytes (A) suitable for applications of ART. Further, our study reveals that ovaries sourced from unorganized slaughterhouses can also be used for ART, and use of aspiration method may yield better quality oocytes.

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Table 1: Biometry and follicular parameters of ovaries collected from organized and unorganized-slaughterhouses

Source (n = no.)	Length (mm)	Width (mm)	Follicle (no.)	CL (no.)	Cystic follicle (no.)
Unorg. Slaughterhouse (n=103)	26.74 ± 0.79	18.84±0.55	14.26±1.17	8.43±0.74	0.35±0.19
Org. Slaughterhouse (n=75)	30.50±1.50	21.70±1.27	19.70±4.25	5.70±1.76	1.40±0.98

Table 2: Recovery rate of different grade of oocytes by different methods of oocyte collection

Oocyte collection method	Grade of oocyte (in percentage)			
	A	B	C	D
Aspiration	65.21±5.71[a]	21.32±4.99[a]	3.03±1.63[a]	10.44±2.27[a]
Slicing	34.43±4.08[b]	23.37±2.51[a]	15.16±2.01[b]	27.04±4.65[b]

Means with different superscript within a column differ significantly ($P<0.01$ for A and C grade; $P<0.05$ for D grade)

PERFORMANCE OF LARGE WHITE YORKSHIRE CROSSBRED PREGNANT GILTS FED FOOD WASTES WITH OR WITHOUT SUPPLEMENTATION*

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Tamil Nadu Veterinary and Animal Sciences University

Food wastes of vegetable origin from the restaurants, hotels, and students' mess are generally rich in energy and low in protein and minerals. Hence when food wastes fed to pigs, due to imbalanced nutrient supply during pregnancy and after farrowing, the litter size, birth weight of piglets and survivability of piglets were reduced (Phengsavanh et al., 2010). The food wastes with supplementation of deficit nutrients during the critical period are likely to improve the reproductive performance of gilts / sow and reduce the occurrence of poor birth weight of piglets after farrowing. Supplementation of food wastes with 50% concentrate feed gave better performance than 100% concentrate feed group (Ramesh et al., 2012). Hence this study was proposed to evolve nutrient supplement for food wastes fed to bred gilts to get optimum performance.

Food wastes collected from nearby student mess and restaurants were sampled and estimated for their proximate principles, phosphorus and salt (AOAC, 2005). The pig feed prepared as per NRC (1998) for pregnant gilts (ground maize grain-65 %, wheat bran-16%, de-oiled rice bran-12%, dry fish-4.5%, mineral mixture-2% and salt-0.5%) was also sampled for chemical analysis. The calcium, copper, iron and zinc were determined using Atomic Absorption Spectrophotometer (AAS) Perkin Elmer, Model 3110). The metabolizable energy (ME) was calculated indirectly as per Wiseman (1987).

For this trial, 18 large white Yorkshire crossbred bred gilts with an average body weight of 87.61 ± 2.09 kg were randomly allotted to three treatment groups. The treatment groups were T_1 - Pregnant gilt feed, T_2 - Food wastes as a sole feed and T_3 - Food wastes + evolved supplement. Each treatment group was fed with their respective experimental feeds up to 110th day of gestation. Clean water in *ad-libitum* quantity was supplied to all animals throughout the experiment. Nutrient composition of food wastes used for the study was compared with that of the pregnant gilt feed prepared as per NRC (1998). Suitable nutrient supplement was prepared and included with the food wastes to optimize its nutrient composition (T_3).

* Part of M.V.Sc. Thesis of the first author.

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The parameters like body weights at start of the experiment and 110th day of pregnancy, gestation length, litter performance, feed dry matter intake and economics were measured. Back fat thickness was measured as per the procedure in the reference manual (Renco sonograder 4.2) at the P2 position (above the last rib at approximately 6.5 cm of the midline) using an A- mode ultrasound (Renco sonograder 4.2, Renco Corporation, Minneapolis, MN), at the time of breeding and at 110th day of pregnancy.

The chemical composition of food wastes (Table 1) used in this study was found to have significantly (P<0.05) higher ether extract, metabolizable energy and salt content and lower dry matter, crude protein, crude fibre and total ash content. These findings were comparable with the reports of Kayastha et al. (2013). However, higher crude protein level in food wastes was reported by Kumar et al. (2009) and Saikia and Bhar (2010) might be due to variation in the ingredients used for preparation of food wastes. The formulated pregnant gilt feed (T_1) contained comparable minimum crude protein level recommended by NRC (1998). However, this level was lower than Kumar et al. (2009) and ICAR (2013) recommendations. The calcium content of food wastes observed in this study (0.46%) was comparable with the report of Westerndorf et al. (1998) and significantly (P<0.05) lower than the recommendation. However, higher calcium and phosphorous levels were reported by various workers (Westerndorf and Myer, 2004; Kumar et al., 2009; Kayasstha et al., 2013) in food wastes. The copper and iron content of food wastes observed in this study was lower and zinc level was higher compared to Westerndorf and Myer (2004) and pregnant gilt feed.

The metabolisable energy content of food wastes (3967 Kcal/kg DM) estimated Wiseman (1987) was significantly (P>0.05) higher when compared to formulated pregnant gilt feed (3151Kcal/kg DM), BIS (1986) and NRC (1998). Therefore, the food wastes fed to experimental group had to be optimized by addition of nutrients like crude fibre and minerals to reduce its energy content and increase the minerals through strategic supplementation with feed ingredients to meet the required levels of nutrients as comparable to the pregnant gilt feed (T_1). Hence de-oiled rice bran (33%) to dilute the energy concentration as a fibre source and mineral mixture (2%) as source of minerals (Mineral mixture contained Calcium 23%, phosphorous 12%, Magnesium 6.5%, Iron 0.5%, Iodine 0.026%, Copper 0.077%, Manganese 0.12%, Cobalt 0.012%, Zinc 0.38%, Sulphur 0.5%, Fluorine 0.07%, Selenium 0.3 ppm) were used as supplement (35%) for food wastes (65%) based diets on DM basis.

Data on the performance of pregnant gilts maintained on three treatment diets (T_1, T_2 and T_3) are presented in Table 2. The gestation body weight gain did not differ (P<0.05) significantly among the treatment groups in this study. However, a non-significant higher body weight gain was observed in T_2 correlated to the findings of Long et al. (2010) that higher body weight gain when high energy diets was fed to pregnant gilts. The dietary influence on gestation length was not evidenced in this study. The feed intake measured in terms of

feed dry matter intake per day per animal in this study was higher in T_1 compared to T_2 and T_3. However, the dry matter intake through food wastes in T_2 group was lower than T_3 group. This finding disagreed with Srinivas and Sagar (1991) that food wastes fed group consumed more feed compared to growing pig feed fed groups. The cost of feed per day per animal was reduced in T_2 than the T_3 and T_1. When compared to control (T_1), the cost per day per animal was reduced by 70.51% and 55.11% in T_2 and T_3, respectively. Similar findings were made by Anil et al. (2007) and Kumar et al. (2009).

Data on litter performance of the experimental animals are predicted in Table 3. There was no significant difference in the litter size among the treatment groups. However, numerical increase in litter size (0.84 / litter) in T_2 correlated with the observation of Long *et al*. (2010) fed with high energy diet. But the piglet born alive was also similar in all the treatment groups. The higher stillborns in T_2 compared to T_1 and T_3 did not correlated with the observations of Ramesh *et al.* (2012) and Long *et al*. (2010). There was no significant difference in the litter weight among the treatment groups. However, 9.42% reduction was observed in T_2 compared to T_3. In contrast, higher litter weight in low energy feed fed group was observed by Veum *et al*. (2009) and Long *et al*. (2010).

The average birth weight of piglets was significantly ($P<0.05$) lower in T_2 compared to T_1. Contradictory to this finding, Morales *et al.* (2009) observed heavier piglets at birth with high energy diet during gestation period. Supplementation of food wastes had positive effect on average litter birth weight which was comparable to T_1 and T_2 correlated the observations made by Ramesh *et al.* (2012).

There was no significant difference in the litter size at weaning among the treatment groups. However, the numerical reduction was observed in T_2 might be due to higher per cent mortality (10.86) compared to T_1 (4.54) and T_3 (6.38). The weaning weight of piglets was reduced in T_2 compared to T_1 and T_3. This finding was in agreement with the results of Renaudeau *et al*. (2003) and Smits *et al*. (2008) Supplementation of food wastes increased the litter performance comparable to pig feed fed group.

Sow's body weight after farrowing and at the time of weaning did not differ significantly ($P<0.05$) between the treatment groups (Table 3). The lactation body weight loss observed in this study was also comparable between the treatment groups. However, Coffey *et al.* (1994) observed higher body weight loss during lactation, when fed high energy feed during gestation.

Food wastes may be fed with supplementation of deficit nutrients to pregnant gilts after conducting long term feeding trial and assessing litter performance and subsequent reproductive performance for economic reason.

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Table- 1: Chemical composition (DM basis) of experimental diets fed to bred gilts

Parameters	**Pregnant gilt feed (T_1)**	**Food wastes (T_2)**	**Food wastes + Evolved supplement (T_3)**
Crude protein (%)*	15.50[a]	12.63[b]	15.15[a]
Ether extract (%)*	2.35[c]	8.50[a]	5.83[b]
Crude fibre (%)*	7.13[a]	2.89[b]	8.11[a]
Nitrogen free extract (%)*	67.79[b]	71.33[a]	61.61[c]
Total ash (%)*	7.23[a]	4.65[b]	9.30
Lysine (%)**	0.71[a]	0.46[b]	0.63[a]
Methionine (%)**	0.43[a]	0.34[b]	0.42[a]
ME (Kcal/kg)**	3280[b]	3970[a]	3265[b]
Calcium (%)*	0.76[a]	0.40[b]	0.83[a]
Phosphorus (%)*	0.46[b]	0.18[c]	0.65[a]
Copper (ppm)*	35.54[a]	2.62[b]	28.34[a]
Iron (ppm)*	175.5[a]	61.6[b]	160.2[a]
Zinc (ppm)*	54.91	70.62	68.45

*Mean of 7 observations

**Calculated values

[a,b,c] Values with different superscripts in a row differ significantly ($P<0.05$)

Table- 2 : Performance of pregnant gilts fed experimental diets

Parameters	Pregnant gilt feed (T_1)	Food wastes (T_2)	Food wastes + evolved supplement (T_3)
Body weight at breeding (kg)	90.33 ± 2.56	83.50 ± 2.01	89.00 ± 3.01
Body weight at 110th day of gestation (kg)	142.00 ± 1.57	139.00 ± 1.03	141.83 ± 1.05
110 days gestation weight gain (kg)	51.66 ± 1.42	55.50 ± 2.05	52.50 ± 2.24
Average daily gain (g)	469.69 ± 12.94	504.54 ± 18.66	477.27 ± 20.39
Total DMI / gilt (kg)	280.00	228.64	240.00
DMI / 100 kg body weight (kg)	2.18	1.86	1.89
DMI / day / gilt (kg)	2.54	2.08	2.18
Gestation length (days)	113.16 ± 0.30	112.55 ± 0.42	112.66 ± 0.49
Back fat thickness of pre-bred gilts at breeding	11.83 ± 0.47	13.00 ± 0.36	12.50 ± 0.22
Back fat thickness of gilts at 110th day of pregnancy	12.50 ± 0.42^{b}	14.83 ± 0.30^{a}	14.00 ± 0.36^{a}
Feed cost / kg DM (Rs.)	17.36	6.25	9.08
Feed cost / gilt / day (Rs.)	44.09	13.00	19.79

^{a,b,c} Means with different superscripts in a row differ significantly (P≤0.05)

Table 3 : Effect of experimental diets on the litter performance of primiparous sow (Mean* ± SE)

Parameter	Pregnant gilt feed (T_1)	Food wastes (T_2)	Food wastes + evolved supplement (T_3)
Litter size at birth	7.66 ± 0.33	8.50 ± 0.76	8.33 ± 0.33
Litter weight at birth (kg)	10.97 ± 0.53	9.52 ± 1.28	10.51 ± 0.60
Piglet weight at birth (kg)	1.43 ± 0.07[a]	1.10± 0.07[b]	1.26 ± 0.04[ab]
Piglet born alive	7.33 ± 0.33	7.67 ± 0.61	7.83 ± 0.31
Stillborn (%)	4.34	9.80	6.00
Litter size at weaning	7.00 ± 0.45	6.83 ± 1.01	7.00 ± 0.26
Piglet mortality (%)	4.54	10.86	6.38
Weaning weight of piglet (kg)	8.71 ± 0.28[a]	5.93 ± 0.48[b]	7.25 ± 0.86[ab]
Sows body weight after farrowing (kg)	132.00 ± 2.20	127.33 ± 1.14	130.66 ± 1.78
Sow body weight at weaning (kg)	122.66 ± 2.37	118.0 ± 1.12	120.33 ± 1.74
Lactation body weight loss (kg)	9.33 ± 0.84	9.33 ± 0.86	10.33 ± 0.88
Back fat thickness of sows at weaning (mm)	11.66 ± 0.33	12.50 ± 0.34	12.50 ± 0.42

[a,b,c] Means with different superscripts in a row differ significantly (P≤0.05)

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Journal articles and abstracts

Bardbury, J.M., Mc Carthy, J.D and Metwali, A.Z. (1990). Micro immunofluorescence for the serological diagnosis of avian Mycoplasma infection. *Avian Pathology*, **19**:213-222.

Raja, S., Rani, A., Ravi, M and Kumar. K. (2007). Histopathology of CPV infection. Page no. 120-122….Venue…Date…Place…

Books and articles within edited books

Rundall, C.J. (1991). A colour Atlas of Diseases of the Domestic Fowl and Turkey. 2nd ed. London. Wolf Publishing Ltd. 175 p.

Handbooks, Technical bulletins, Thesis and Dissertations

Callow, L.L and Dalgliesh, R.J. (1982). Immunity and Immunopathology in Babesiosis. In: S. Choen and K.S. Warren (Ed) Immunology of Parasitic Infections. Blackwell, Oxford. pp 475-526.

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