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Full length articles

Short Communications

Full Length Article

Quality Evaluation of Orange Peel (Flavedo) incorporated Ice Cream

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ABSTRACT

By-products of fruits possess antioxidant polyphenols, anti-mutagenic, cardio preventive and antiviral activities. The consumers are aware of the health benefits of nutraceutical foods and the demand for such foods is increasing in recent years. Orange peels are rich in flavanones, powerful antioxidants that help to reduce oxidative damage and fight free radicals. Hence, an attempt was made to develop a value added ice cream with orange peel. Orange peel powder was incorporated in ice cream at varying levels viz. 1.5, 2.5, 3.5 and 5 per cent. Based on texture, sensory attributes and physio-chemical properties, sensory evaluation of the product revealed that incorporation of orange peel powder at 2.5% level found to be acceptable.

Key Words: Ice cream - Orange peel – Flavour - Sensory attributes.

INTRODUCTION

Ice cream is a delicious, wholesome, nutritious, frozen dairy food consumed around the world and relished by all age groups. A typical composition range for the components used in ice cream mix is milk fat 10-16%, milk solids not fat 9-12%, corn syrup solids 4-6%, stabilizers/emulsifiers 0.5%, total solids 36-45% and water 55-64% (Goff, 1997). Flavour and stabilizer usually used are of synthetic origin and this can be partly replaced by orange peel which is a waste by product in juice industry as orange peel has a higher level of vitamin C than the pulp and also contribute cellulosic material , essential oils, paraffin waxes, steroids and triterpenoids, fatty acids, pectin pigments besides flavour.

Orange constitutes about 60 per cent of the total citrus production (Lucia and Calogera, 2008). Peel represents between 50 and 65 per cent of total weight of the fruits and was the primary by-product (Ashbell and Donahaye, 1984).

According to Soukoulis *et al.* (2009), the quality of ice cream was influenced by several sensory attributes viz. flavour, texture, melting quality, package, and appearance. These attributes also affect the consumer preference for different variants of ice cream.

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Ice cream was a well-known dessert among all ages in many countries. Warke *et al.* (2000) stated that ice cream was one of the major products in the dairy industry and continues to dominate attention of a large segment of the population. Its mixture was made of dairy products (e.g. whole milk, condensed milk, milk powder, cream), sweeteners (sugar, glucose), stabilizers, emulsifiers and colourings. Ice cream could also serve as a topping, add-on or mix-in for other desserts.

Considering the above facts, an attempt has been made to develop an innovative ice cream utilizing the orange peel which contains good flavour and health benefits.

MATERIALS AND METHODS

Preparation of ice cream

The quantity of milk, cream, skim milk powder, sucrose, sodium alginate and GMS required for a batch (i.e. 6 kg of ice cream mix) was calculated by serum point method (Marshall *et al*., 2003). The composition of the experimental mixes were 6.0 % fat, 11.5 % MSNF, 14.0 % sucrose, 0.1 % sodium alginate and 0.2 % GMS. The composition of control was 10% fat, 11% MSNF, 15% sucrose, 0.25% sodium alginate and 0.15% GMS. The calculated quantities of liquid ingredients viz. whole milk and cream for each treatment were weighed, mixed and blended thoroughly in a stainless steel vessel for ice cream preparation.

The prepared ice cream mix was homogenized at 2500psi (I stage) & 500psi (II stage), then pasteurised at 72º C for 30 min and cooled to room temperature and kept for ageing at 4º C. Just before freezing, dried orange peel powder at 2.5% was added to the mix. The frozen ice cream was dispensed to cups and stored at -25º C for further studies.

Orange peel was dried in a fluidized bed dryer with below 60ºC temperature for 50-55 min, powdered in a mixer sieved in 40 size mesh sieve was used for incorporation in ice cream. Organoleptical evaluation of the peels revealed that orange peel dried by the fluidized bed dryer was preferred over hot air oven for incorporation in ice cream.

Physico-chemical and sensory evaluation of ice cream and ice cream mixes

Ice cream samples viz. control, T_1, T_2, T_3 and T_4 were evaluated for physiochemical and sensory characteristics. Protein, total solids, pH and acidity were calculated according to AOAC (1990). Gerber's method (Davide, 1977) was used for fat estimation. The total solids of the ice cream were determined by the standard procedure as described for milk using 2 g of sample (ISI Standards).

The titratable acidity of the ice cream was determined by the standard method suggested in ISI Handbook of Food Analysis (1989). The pH of the ice cream mixes was determined at 25°C using a Systronic digital pH meter, Model 335, Systronic Ltd., Ahmedabad, India. The protein content of the ice cream mixes was determined by Kjeldahl method (Menefee and Overman, 1940).

Ice cream samples were organoleptically evaluated for appearance,

taste, flavour, body & texture, and overall acceptability, following the 9-point hedonic scale (Larmond, 1977).

The results obtained were statistically analysed by applying one way ANOVA in IBM SPSS (US) software (version 20) as per the Snedecor and Cochran (1994).

RESULTS AND DISCUSSION

The sensory analysis revealed that the flavour differ between the ice cream samples (Table 1). The ice cream flavour and aftertaste scores were negatively affected by addition of orange peel powder, which was also evidenced by the low scores of these attributes by Karaca *et al*. 2009. The acceptance mean values of the colour of the orange peel incorporated ice creams were higher when compared to that of the control ice cream. The decrease in taste might be due to the increase in addition of orange peel powder contributed by the higher fibre content in the orange peel as per Gorinstein *et al.* (2001). The contents of total, soluble and insoluble dietary fibre in peels were significantly higher than in peeled fruits ($P< 0.05$ in all cases).

Table – 1. Sensory evaluation of Ice cream using 9-point hedonic scale@

Sensory attributes	Control (C)	T_1	T_2	T_3	T_4	F- value
Colour & Appearance	7.04^{e}± 0.05	7.2^{d}± 0.06	7.35^{c}± 0.16	7.38^{b}± 0.17	7.4^{a}± 0.12	45.213**
Taste	6.5^{e}± 0.04	7.29^{d}± 0.14	7.92^{a}± 0.12	7.54^{b}± 0.05	7.31^{c}± 0.14	68.102**
Flavour	6.8^{e}± 0.07	7.32^{c}± 0.10	8.24^{a}± 0.18	7.45^{b}± 0.14	7.24^{d}± 0.17	54.923**
Body & Texture	7.10± 0.03	7.12± 0.21	7.16± 0.14	7.18± 0.15	7.25± 0.45	62.357^{NS}
Overall acceptability	6.88^{e}± 0.02	7.23^{d}± 0.14	7.66^{a}± 0.19	7.38^{b}± 0.14	7.30^{c}± 0.12	69.314**

@Average of six trials

Mean with different superscripts within a same row differ significantly from each other (P<0.01)

NS – Non significant (P > 0.05)

** Highly significant (P < 0.01)

T_1- Ice cream incorporated with 1.5% orange peel

T_2- Ice cream incorporated with 2.5% orange peel

T_3- Ice cream incorporated with 3.5% orange peel

T_4- Ice cream incorporated with 5% orange peel

Regarding the scores for overall acceptability, (T_2) 2.5% incorporation was found to be best as compared to other treatments because as the percentage of incorporation increase the flavour and taste scores decreased due to the increase in addition of orange peel contributed by the higher fibre content in the orange peel as per Gorinstein *et al.* (2001) and hence, T_2 was considered to be the optimum level of addition of orange peel in preparation of orange ice cream. The flavour, taste and overall acceptability were found to score higher for T_2 among others in consumer panel on evaluation using 9 point hedonic scale.

Table – 2. Physico-chemical properties of ice cream@

Attributes	Control (C)	T_1	T_2	T_3	T_4	F - value
Fat (%)	10.23^{e}± 0.12	11.54^{d}± 0.14	11.63^{c}± 0.23	12.02^{b}± 0.20	12.41^{a}± 0.16	7.123^{NS}
Protein (%)	4.73^{b}± 0.32	4.52^{e}± 0.21	4.61^{d}± 0.20	4.72^{c}± 0.12	4.89^{a}± 0.11	2.872^{NS}
Total solids (%)	38.77^{e}± 0.13	40.21^{d}± 0.20	42.65^{c}± 0.22	43.87^{b}± 0.21	44.36^{a}± 0.30	17.932**
Acidity (% LA)	0.12^{e}± 0.01	0.15^{d}± 0.02	0.18^{c}± 0.03	0.21^{b}± 0.03	0.24^{a}± 0.04	12.865^{NS}
pH	6.2^{a}± 0.13	6.1^{b}± 0.11	6.0^{c}± 0.12	5.9^{d}± 0.14	5.7^{e}± 0.15	32.321^{NS}

@Average of six trials
Mean with different superscripts within a same row differ significantly from each other (P<0.01)
NS – Non significant (P > 0.05)
** Highly significant (P < 0.01)

The average compositional values, acidity and pH of control as well as experimental samples (T_1, T_2, T_3 and T_4) are presented in Table 2. There was an increase in the total solids content of the mix with an increase in level of orange peel addition which is from 40.21 in T_1 to 44.36 in T_4. Citrus peel is rich in nutritional ingredients such as soluble sugars (46.241±0.015 g/100g d.b), proteins (8.120±0.120 g/100g d.b) and minerals (3.170±0.035 g/100g d.b) that tends to increase the total solids (M'hiri *et al*., 2015).

Incorporation of orange flavedo at different levels also tend to increase the fat content. These findings also correlate the findings of Hanan *et al.* (2012). All the experimental samples had no significant difference in protein content compared to the control. Incorporation of orange flavedo at higher levels tend to increase the acidity and decrease pH of mixes significantly (P<0.05). Similar observations were also reported by Desai *et al.* (2010), while studying the effect of malted ragi flour in cake preparation. There was a decrease in pH and increase in titratable acidity as the quantity of orange peel increased due to its ascorbic acid content. Savita *et al.* (2010) revealed that the ascorbic acid content in fresh peel was 82.45mg/ 100 g. Ice cream productions can be made at acidity 0.7% lactic acid with no loss of ice cream quality. Higher levels than 0.7% lactic acid contribute to progressive loss of sensory quality and hence lower stability with increase in acidity as the higher inclusion levels.

The results obtained in this study were similar to the findings of Nasser *et al.* (2008) and Magda *et al.* (2008) revealed that crude fibre and carbohydrate increased as the inclusion level of orange peel increased. Our findings also correlated with the

findings of Hanan *et al.* (2012) who found that incorporation of 10 per cent citrus peel powder in wheat biscuits increased crude protein, crude fat content as well as crude fibre, moisture and calorie value. The total citrus solid extract was characterized by a low fat content (0.48 to 4.00 g/100 g d.b) (Kammoun *et al.,* 2011; Ghanem *et al.*, 2012; Marin *et al.*, 2007).

Table – 3. summarizes the results of textural analysis of ice cream viz. hardness, adhesiveness, springiness, cohesiveness, and gumminess. @

Textural Attributes	Control (C)	T_1	T_2	T_3	T_4	F - value
Hardness (g)	$1528.33^{a}\pm 0.81$	$1625.21^{e}\pm 0.42$	$1726.84^{d}\pm 0.52$	$1842.14^{c}\pm 0.61$	$1934.78^{b}\pm 0.72$	675.986**
Adhesiveness (g)	$-258.96^{e} \pm 0.14$	$-286.58^{d}\pm 0.23$	$-309.66^{c}\pm 0.35$	$-324.12^{b}\pm 0.30$	$-352.54^{a}\pm 0.39$	78.931^{NS}
Springiness (g)	$0.1277^{e}\pm 0.02$	$0.1364^{d}\pm 0.01$	$0.2789^{c}\pm 0.01$	$0.3256^{b}\pm 0.02$	$0.4256^{a}\pm 0.01$	3.452**
Cohesiveness (g)	$0.1078^{e}\pm 0.01$	$0.1123^{d}\pm 0.01$	$0.1168^{c}\pm 0.02$	$0.1134^{b}\pm 0.02$	$0.1756^{a}\pm 0.03$	4.879^{NS}
Gumminess (g)	$563.00^{e}\pm 0.22$	$613.12^{d}\pm 0.36$	$647.84^{c}\pm 0.22$	$715.21^{b}\pm 0.31$	$729.53^{a}\pm 0.42$	58.730**

@Average of six trials
Mean with different superscripts within a same row differ significantly from each other (P<0.01)
NS – Non significant (P > 0.05)
** Highly significant (P < 0.01)

The lowest hardness values were observed in the control ice cream. In the ice cream samples with ($p \leq 1.0$) of fibre significantly differ from ($p \leq 0.05$) the control ice cream. The addition of greater amount of orange peel powder as a fat replacer significantly increased the hardness of ice cream ($p \leq 0.05$). The effect of fat content on hardness of ice cream was evaluated by Roland *et al.* (1999) and Rossa *et al.* (2012), who opined that the hardness was inversely proportional to the fat content.

The adhesiveness values of control ice cream (Table 3) were not significantly different ($p \leq 0.05$) from those of most fat reduction in ice cream with added orange fibre. These results were not in agreement with those findings of Aime *et al.* (2001) and Prindiville *et al.* (1999), who found that low fat ice cream had the lowest adhesiveness.

The ice cream with 5% orange peel powder showed higher springiness values than those of the other samples. However, the ice cream with 1.5 % orange peel powder did not differ significantly from the control. The cohesive values did not differ between different ice creams, indicating that fibre addition did not affect this parameter, which agreed with the results obtained by Hwang *et al.* (2009).

The gumminess of control ice cream were significantly lower ($p \leq 0.05$) than those of the reduced-fat ice creams, but different values were obtained by Prindiville *et al.* (1999).

It was concluded that the ice cream with 2.5 per cent T_2 was found to be the best among all treatments by sensory and textural qualities. By utilizing the orange peel a waste of juice industry could be incorporated in ice cream to enhance the flavour, texture and to enrich the product acceptability by the consumers. T_2 was judged as the best by the overall acceptability scores though there was no significant difference between the scores of body and texture.

The scores diminished gradually which might be due to hard texture of ice cream as the inclusion levels of orange peel increased due to the pectin present in orange peel.

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Forecasting Livestock and Poultry Production in India

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ABSTRACT

The challenges faced by India in achieving the food and nutritional security to the fast growing population need a concerted approach for growth of livestock sector. Various forecasting models like combinations of ARIMA models and Exponential Smoothing models like Simple, Holt, Brown and Damped trend were used to identify the growth patterns and to predict the future trends in livestock and poultry production in India. Time series data on milk production from 1950-51 to 2017-18 were used to forecast the milk production in India up to 2050-51. Brown Exponential Smoothing was the best fit model for forecasting of milk production in India. The forecast obtained showed that milk production would increase to 207.57, 311.86, 416.16 and 520.45 MT respectively during 2020-21, 2030-31, 2040-41 and 2050-51 from 121.80 MT during the year 2010-11. ARIMA (0,1,1) was the best fit model for forecasting meat and wool production, the forecasted values of meat production showed that meat production would increase from 1.08 MT in 2000-01 to 5.18, 7.63, 10.07 and 12.52 MT, respectively during 2020-21, 2030-31, 2040-41 and 2050-51. Wool production would increase appreciably over years with a production of 46.26, 52.06, 58.59 and 65.17 million kg, respectively during the years 2020-21, 2030-31, 2040-41 and 2050-51 from 43.00 million kg in 2010-11. The Brown Exponential Smoothing model was the best fit model for forecasting egg production in India. The forecasted values obtained from the best fitted Brown ES was 103341.99, 144374.02, 185406.04 and 222334.86 million numbers during the years 2020-21, 2030-31, 2040-41 and 2050-51, respectively.

Key words: Livestock and Poultry Production, India, Forecasting

INTRODUCTION

India's livestock sector is one of the largest in the world with a holding of 11 per cent of world livestock population. It ranks first in buffalo population, second in cattle and goat, third in sheep, fifth in ducks and chicken and tenth in camel in the world.

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India continues to be the largest producer of milk in the world, by producing 13.1 per cent of the total world milk output, with the production of milk of 17 million tons in 1950-51 rising to 176.35 million tons in 2017-18. As a result, livestock sector has become one of the fastest growing agricultural subsectors in India. Its share of agricultural GDP was 25.6 per cent in 2016-2017. Livestock sector in India has undergone perceptible changes in its size, composition and productivity in the last few decades, transforming itself from a low-profile backyard venture to the status of a promising industry. This phenomenal growth is driven by the rapidly increasing demand for livestock products, owing to the population growth, urbanization and increasing incomes in the country.

The growing demand for livestock products, as they have income elastic demand in the country, underlines the fact that there is a greater opportunity for enhancing livestock production. It is now understood that initiatives in globalization and economic liberalization across the world would continue to exert more pressure on livestock production to be efficient to meet the challenges of global competition. To be able to effectively plan potential strategies for optimizing livestock production to ensure the attainable socio-economic benefits to the rural poor in the country, a more complete exploration of data and further extrapolation for the future using advanced forecasting tools would be highly helpful. Various sophisticated forecasting models developed would be able to provide a more comprehensive outlook into the dynamics of livestock sector, in terms of livestock production in the country. This study attempts to unravel the future state of livestock production by taking into account the past and present trends.

MATERIALS AND METHODS

Data to be used for the study were collected only from secondary sources. Data on livestock production in India (from 1951 to 2012) were collected from various reliable secondary sources like Basic Animal Husbandry Statistics (BAHS), Food and Agriculture Organization (FAO), Agricultural and Processed Food Products Export Development Authority (APEDA) and Agri. Stat. Although provisional figures of livestock population are now available for the year 2017, they were not considered for analysis to avoid the possible ambiguity in the results. Data pertaining to livestock production including production of milk, meat, egg and wool from 1950-51 to 2016-17 and State wise data from 2008-09 to 2015-16 were collected from BAHS and FAO.

Various statistical tools and time series forecasting models were employed to identify the growth patterns and to predict the future trends in livestock production. Different forecasting models were compared so as to identify the best fit model. Among various time series forecasting models, Auto Regressive Integrated Moving Average [ARIMA] - p, d, q and Exponential Smoothing [ES] models were fitted to choose the best fit model for forecasting of livestock production.

Various combinations of ARIMA models like ARIMA (1,1,1), (1,1,0), (0,1,1), (0,1,0), (0,1,2), (1,1,2), (2,1,0), (2,1,1),

(2,1,2), (1,2,1), (0,2,1), (1,2,0), (0,2,0), (0,2,2), (1,2,2), (2,2,0), (2,2,1), (2,2,2) and Exponential Smoothing models like Simple ES, Holt ES, Brown ES and Damped trend ES were used for finding the best fit model. By using the best fit model, two kinds of forecasts were performed: Sample period forecasts and post-sample period forecasts. The former was used to develop confidence in the model and the latter to generate genuine forecasts for use in planning and other purposes. In this study, both ARIMA models and ES models were applied to yield these kinds of forecasts. Forecasting accuracy was identified by using measures of indices like MAE (Mean Absolute Error) and MAPE (Mean Absolute Percentage Error).

ARIMA model

The data used in the study were non-stationary and non-seasonal. ARIMA model is a combination of an Auto Regressive (AR) process and a Moving Average (MA) process applied to a non-stationary data series. A combined model that contains p (AR) term and q (MA) term is called ARMA (p,q). If the object series is differenced 'd' times to achieve stationary, the model is classified as Autoregressive Integrated Moving Average (ARIMA) (p, d, q) model as discussed by Box *et al.* (2007). The basic criteria for choosing the best fit of ARIMA (p, d, q) model are given in Table 1.

Table - 1. Criteria for choosing the appropriate forecasting model

Selection Criterion	Notation
Mean Square Error =	MSE
Bayesian Information Criterion = n log(MSE) +K log n	BIC
Coefficient of Determination $= \frac{1}{n-k}\sum \hat{\varepsilon}_t^2 - \frac{Error\ sum\ of\ square}{Total\ sum\ of\ squares}$	R^2
Root Mean Square Error = $\sqrt{\frac{1}{n-k}\sum \hat{\varepsilon}_t^2}$	RMSE
Mean Absolute Error = $\frac{1}{n}\sum_{t=1}^{n}\left\|\hat{\varepsilon}_t\right\|$	MAE
Mean Absolute Percent Error = $\frac{1}{n}\sum_{t=1}^{n}\left\|\frac{\hat{\varepsilon}_t}{y_t}\right\| \times 100$	MAPE
where, k = Number of parameters in the model; n = Sample size ; y_t = Observed value; and $\hat{\varepsilon}_t$ = Difference between the observed and estimated values.	

The general form of ARIMA model of order (p, d, q) is

$$Y_t = \hat{\varepsilon}_t Y_{t-1} + \emptyset_2 Y_{t-2} + ... + \emptyset_p Y_{t-p} + \mu - \square_1 \square_{t-1} - \square_2 \square_{t-2} - ... - \square_q \square_{t-q} + \square_t$$

Where,

Y_t = Value at t^{th} year;

ε_t's = Error terms which are independently and normally distributed with mean zero and constant variance σ^2 for t =1, 2..., n;

μ = Constant and

ϕ_s and θ_s = Coefficients to be estimated.

Simple Exponential Smoothing

The Simple Exponential Smoothing (SES) model is a time series forecasting technique that can be defined using an additive model used to analyze data which have no trend and seasonal pattern. This is a method of estimation of forecasts of single weight or parameter. Greater weights are assigned to recent observation and smaller weights to distant observation (Sharpe *et al.,* 2010). The model is as given below:

$$F_{t+1} = F_t + \alpha(y_t - F_t)$$

New forecast value at time t+1 = Old forecast at time t + α (Error in the last forecast)

The smoothing constant (α) value is selected based on error minimization approach (Talwar and Goyal, 2019)

Brown's Linear (Double) Exponential Smoothing model

The double exponential smoothing model is used to model time series data which have trend, but not seasonality (Brown, 1963). Here, F' denotes a simple smoothed value and F" denotes a double smoothed value:

$$F_t^{'} = \alpha Y_t + (1-\alpha)F_{t-1}^{'}$$

$$F_t^{"} = \alpha Y_t^{'} + (1-\alpha)F_{t-1}^{"}$$

$$a_t = F_t^{'} + \left(F_{t+}^{'} - F_t^{"}\right) = 2F_t^{"} - F_t^{"}$$

a_t denotes the estimated smoothed level at time t

$$b_t = \frac{\alpha}{1-\alpha}\left(F_t^{'} - F_t^{"}\right)$$

And b_t shows the estimated trends at the end of time period t, for m period ahead forecast

$$F_{t+m} = a_t + mb_t$$

Holt's Linear (Double) Exponential Smoothing model

Holt's method can be implemented for the time series data demonstrating a trend (Hanke and Wichern, 2008). This method is appropriate for non stationary data and to make short term forecast. In this technique, level and trend components are smoothed separately using different parameters α and β. Holt's double exponential smoothing method uses three equations one each for level, trend and forecast.

$$L_t = \alpha Y_t + (1-\alpha)(L_{t-1} + b_{t-1})$$

$$b_t = \beta(L_t - L_{t-1}) + (1-\beta)b_{t-1}$$

$$F_{t+m} = L_t + b_t m$$

where,

L_t = Level of time series at period t

b_t= trend (slope) estimate of time series at time period t

F_{t+m}= forecast at m period ahead of time t

α and β are smoothing constants for level and trend with their values lying between 0 and 1.

Damped Trend Exponential Smoothing method

The forecasts generated by Holt's linear method display a constant trend (increasing or decreasing) indefinitely into the future. Since empirical evidence indicated that this method tended to over-forecast, especially for longer forecast horizons, Gardner and McKenzie (1985) introduced a parameter that dampens the trend to a flat line sometime into the future.

The smoothing equations are,

$$L_t=\alpha Y_{t+} (1-\alpha) (L_{t-1}+\varphi T_{t-1})$$

$$T_t= \gamma (L_t-L_{t-1}) + (1- \gamma) \varphi T_{t-1}$$

The m-step-ahead prediction equation is $\hat{Y}_{t+m} = L_t + \sum_{i=1}^{m} \varphi^i T_i$

This is the forecast y, m-steps ahead by taking the last available estimated level state and multiplying the last available trend (slope) T_i, with φ^i=damping factor.

RESULTS AND DISCUSSION

Time series data on milk production from 1950-51 to 2017-18 were used to forecast the milk production in India up to 2050-51. The time series prediction models like ARIMA and Exponential Smoothing were compared and based on their fitness, forecasting of milk production was done. The model was estimated using SPSS software (ver.26). The criteria adopted for model selection for forecasting of milk production are given in Table 2, like the values of R^2, RMSE, MAPE, MAE and normalized BIC. On perusal of the table, it can be known that Brown Exponential Smoothing was the best fit model for forecasting of milk production in India. The normalised BIC value for the model was 0.885, which was the lowest among the models, with a higher R^2 value of 0.999. For this model, RMSE (1.491), MAPE (2.007) and MAE (1.043) values were also found to be lower than that of other models.

Table - 2. Criteria for model selection for milk production

Model	R^2	RMSE	MAPE	MAE	BIC
ARIMA (1,1,1)	0.999	1.482	1.883	1.002	1.137
ARIMA (1,1,0)	0.999	1.500	1.946	1.014	1.073
ARIMA (0,1,1)	0.999	1.586	1.984	1.080	1.184
ARIMA (0,1,0)	0.999	1.526	1.988	1.061	1.195
ARIMA (0,1,2)	0.999	1.526	1.964	1.045	1.164
ARIMA (1,1,2)	0.999	1.579	1.679	1.089	1.147
ARIMA (2,1,0)	0.999	1.564	1.948	1.265	1.146
ARIMA (2,1,1)	0.999	1.523	1.989	1.145	1.254
ARIMA (2,1,2)	0.999	1.541	2.004	1.254	1.321
ARIMA (0,2,0)	0.999	1.645	2.013	1.002	1.562
ARIMA (1,2,0)	0.999	1.518	1.901	0.997	1.102
ARIMA (0,2,1)	0.999	1.416	1.638	0.924	0.962
ARIMA (1,2,1)	0.999	1.406	1.736	0.920	1.037
ARIMA (1,2,2)	0.999	1.641	1.945	0.978	1.004
ARIMA (2,2,0)	0.999	1.625	1.769	1.046	1.065
ARIMA (2,2,1)	0.999	1.643	1.948	1.087	1.047
ARIMA (2,2,2)	0.999	1.523	1.766	1.065	0.964
ARIMA (0,2,2)	0.999	1.499	1.989	1.098	0.894
Holt ES	0.999	1.491	2.007	1.043	0.885
Simple ES	0.999	1.495	1.812	1.009	0.977
Brown ES	0.999	1.491	2.007	1.043	0.885
Damped trend ES	0.999	1.514	1.807	1.012	1.088

ARIMA model was used by Jaisankar and Prabakaran (2012) for forecasting milk production in Tamil Nadu, based on time the series data from 1978 to 2008. Paul *et al.* (2014) also used milk production data from 1979-2007 and found that ARIMA (1, 1, 0) model could fit in well for forecasting. Deshmukh and Paramasivam (2016) concluded that among ARIMA and Vector Auto Regression (VAR) models, ARIMA (1, 1, 1) was the most suitable method for forecasting milk production in India.

After this model selection, the model parameter of Brown Exponential Smoothing was estimated and the results are presented in Table 3. Based on the best fit Brown Exponential Smoothing model, milk production was predicted for the years 2020-21, 2030-31, 2040-41 and 2050-51 and the forecasted values are given in Table 4. The forecast obtained from the fitted Brown Exponential Smoothing model showed that milk production would increase to 207.57, 311.86, 416.16 and 520.45 MT respectively

during 2020-21, 2030-31, 2040-41 and 2050-51 from 121.80 MT during the year 2010-11. It needs emphasis that these predictions are more likely to come true, as the prediction for the year 2010-11 is almost similar to the actual figure, unlike the prediction by Paul *et al.* (2014) whose prediction is far from the actual figure.

Table - 3. Estimates of the Brown Exponential Smoothing model for milk production

Model	Estimate	SE	T	Sig.
Alpha (Level and Trend)	0.790	0.073	10.760	0.000

Table - 4. Forecasts of milk production (in MT)

Year	Actual	Predicted	LCL (95 %)	UCL (95 %)	Residual
2000-01	80.60	81.32	78.31	84.33	-0.72
2010-11	121.80	120.77	117.76	123.78	1.03
2020-21	-	207.57	198.88	216.26	-
2030-31	-	311.86	254.94	368.78	-
2040-41	-	416.16	288.52	543.79	-
2050-51	-	520.45	305.34	735.56	-

The time series data on meat production from 1998-99 to 2015-16 were used for forecasting of meat production for future years in India. Various statistical models like 18 combinations of ARIMA model and four ES models were selected and among which the best fit model for the forecasting was selected for forecasting of meat production based on the criteria specified in Table 5. From the table, it could be found that ARIMA (0,1,1) was the best fit model for forecasting milk production, since it was having the lowest normalised value of BIC -4.558 and relatively lowest RMSE (0.085), MAPE (6.135) and MAE (0.061) values and higher R^2 value (0.995). Hossain and Hassan (2013) forecasted milk, meat and egg production in Bangladesh using time series data and they in contrary to this study, revealed that the cubic model was the best fit model for forecasting milk and meat production.\

After the model selection, the model parameters were estimated and the results of the estimates are given in Table 6. Based on the best fit ARIMA (0,1,1) model, forecasting of meat production for the years 2020-21, 2030-31, 2040-41 and 2050-51 were done and the results are presented in Table 7. The forecasted values of meat production obtained by using ARIMA (0,1,1) showed that meat production would increase from 1.08 MT in 2000-01 to 5.18, 7.63, 10.07 and 12.52 MT, respectively during 2020-21, 2030-31, 2040-41 and 2050-51.

Table - 5. Criteria for model selection for meat production

Model	R^2	RMSE	MAPE	MAE	BIC
ARIMA (1,1,1)	0.996	0.084	6.145	0.060	-4.456
ARIMA (1,1,0)	0.995	0.085	6.208	0.062	-4.556
ARIMA (0,1,1)	0.995	0.085	6.135	0.061	-4.558
ARIMA (0,1,0)	0.995	0.092	6.982	0.074	-4.465
ARIMA (0,1,2)	0.995	0.094	6.741	0.072	-4.423
ARIMA (1,1,2)	0.995	0.085	6.654	0.081	-4.426
ARIMA (2,1,0)	0.995	0.086	6.684	0.069	-4.259
ARIMA (2,1,1)	0.995	0.089	6.642	0.076	-4.378
ARIMA (2,1,2)	0.995	0.091	6.959	0.081	-4.356
ARIMA (0,2,0)	0.994	0.100	7.202	0.075	-4.099
ARIMA (1,2,0)	0.993	0.105	8.035	0.086	-4.117
ARIMA (0,2,1)	0.994	0.095	7.116	0.071	-4.332
ARIMA (1,2,1)	0.994	0.086	7.156	0.084	-4.269
ARIMA (1,2,2)	0.994	0.091	7.189	0.087	-4.489
ARIMA (2,2,0)	0.994	0.094	7.325	0.091	-4.256
ARIMA (2,2,1)	0.994	0.093	7.849	0.098	-4.226
ARIMA (2,2,2)	0.994	0.097	7.965	0.123	-4.550
ARIMA (0,2,2)	0.994	0.123	7.465	0.089	-4.213
Simple ES	0.994	0.093	7.307	0.077	-4.510
Brown ES	0.994	0.097	10.056	0.085	-4.539
Holt ES	0.977	0.184	11.537	0.158	-3.266
Damped trend ES	0.994	0.092	11.965	0.245	-4.550

Table - 6. Estimates of ARIMA (0, 1, 1) for meat production

Model	Parameters	Estimate	SE	t	Sig.
ARIMA (0, 1, 1)	Constant	23.330	30.251	8.771	0.000
	MA(1)	0.190	7.413	2.026	0.000
	Difference	1.000			

This increasing trend in meat production implies the fact that the ever rising demand for protein rich meat would continue to be there due to the changing tastes and preferences among medium and high income groups especially in urban and semi urban areas, the creation of awareness among all sections of the society about protein malnutrition and the comparative advantage the country possesses on the export potentials of this products, owing to the presence of appreciable livestock wealth.

Table - 7. Forecasts of meat production (in MT)

Year	Actual	Predicted	LCL (95 %)	UCL (95 %)	Residual
2000-01	1.08	0.89	0.70	1.08	0.19
2010-11	2.65	2.71	2.52	2.90	-0.06
2020-21	-	5.18	4.61	5.75	-
2030-31	-	7.63	5.35	9.91	-
2040-41	-	10.07	5.48	14.67	-
2050-51	-	12.52	5.13	19.92	-

Time series data on egg production from 1950-51 to 2015-16 were used to forecast the egg production in India for the years 2020-21, 2030-31, 2040-41 and 2050-51. Forecasting models such as ARIMA and Exponential Smoothing were used and among which, the best fit model was selected for forecasting. The criteria used for selecting best fit model are given in Table 8. Comparing ARIMA and ES, it was found that Brown ES was the best fit model for forecasting egg production in India, since its BIC value (14.455) was the lowest among all models and it had higher R^2 value (0.997) like others and relatively lower values of RMSE (1316.987), MAPE (7.058) and MAE (14.455). In contrary to this, ARIMA (0, 1, 0) was the best fit model for forecasting egg production in India as reported by Chaudhari and Tingre, (2015). The estimate of the parameter of the selected best fit Brown Exponential Smoothing model is given in Table 9.

By using the best fitted Brown ES model, forecasting of egg production were done for the years 2020-21, 2030-31, 2040-41 and 2050-51 and the results are presented in Table 10. On perusal of the table, it is evident that the egg production would increase in a linear trend in the future years. The forecasted values obtained from the best fitted Brown ES was 103341.99, 144374.02, 185406.04 and 222334.86 million numbers during the years 2020-21, 2030-31, 2040-41 and 2050-51, respectively. Unlike the prediction by Chaudhari and Tingre (2015) who showed it would be 61993 million numbers during 2010-11, prediction in this study of 63228 million numbers is found to be close to the actual figure.

Table - 8. Criteria for model selection for egg production

Model	R^2	RMSE	MAPE	MAE	BIC
ARIMA (1,1,1)	0.997	1255.345	4.977	915.201	14.633
ARIMA (1,1,0)	0.997	1241.058	4.962	913.613	14.519
ARIMA (0,1,1)	0.997	1241.077	4.960	913.507	14.519
ARIMA (0,1,0)	0.997	1298.046	4.996	923.564	14.652
ARIMA (0,1,2)	0.997	1287.265	4.956	921.132	14.745
ARIMA (1,1,2)	0.997	1297.146	4.912	915.263	14.635
ARIMA (2,1,0)	0.997	1246.713	4.994	913.215	14.598
ARIMA (2,1,1)	0.997	1213.146	4.978	913.485	14.689
ARIMA (2,1,2)	0.997	1227.694	4.989	894.651	14.598
ARIMA (1,2,1)	0.997	1202.773	4.892	844.649	14.554
ARIMA (1,2,0)	0.996	1513.693	4.911	987.091	14.921
ARIMA (0,2,1)	0.997	1254.072	4.851	908.767	14.545
ARIMA (0,2,0)	0.997	1270.914	4.927	909.366	14.748
ARIMA (1,2,2)	0.997	1217.208	4.965	861.668	14.670
ARIMA (2,2,0)	0.997	1869.452	5.697	915.621	14.984
ARIMA (2,2,1)	0.997	1795.648	5.689	915.621	14.862
ARIMA (2,2,2)	0.997	1643.989	6.123	925.173	14.834
ARIMA (2,2,2)	0.997	1725.213	6.145	926.156	14.498
Simple ES	0.988	2482.038	8.137	1931.126	15.723
Brown ES	0.997	1316.987	7.058	863.081	14.455
Holt ES	0.997	1280.906	5.622	832.990	14.489
Damped trend ES	0.997	1698.479	8.269	812.659	15.889

Table - 9. Estimate of Brown Exponential Smoothing model for egg production

Model	Parameter	Estimate	SE	t	Sig.
Brown ES	Alpha (Level and Trend)	0.571	0.066	8.596	0.000

Table - 10. Forecasts of egg production (in million numbers)

Year	Actual	Predicted	LCL (95 %)	UCL (95 %)	Residual
2000-01	36632	31378.11	28718.40	34037.81	5253.89
2010-11	63024	63228.03	60568.32	65887.74	-204.03
2020-21	-	103341.99	94058.98	112625.01	-
2030-31	-	144374.02	109263.90	179484.13	-
2040-41	-	185406.04	115125.93	255686.15	-
2050-51	-	222334.86	114128.84	330540.88	-

Time series data on wool production from 1950-51 to 2015-16 were used to forecast wool production for the next three decades, i.e. up to 2050. Time series forecasting models like ARIMA and exponential smoothing were compared and the best fit model was chosen for forecasting wool production. The criteria used for selecting the best fit model are given in Table 11.

Table - 11. Criteria for model selection for wool production

Model	R^2	RMSE	MAPE	MAE	BIC
ARIMA (1,1,1)	0.954	1.375	2.186	0.913	0.999
ARIMA (1,1,0)	0.953	1.364	2.170	0.907	0.892
ARIMA (0,1,1)	0.954	1.357	2.194	0.917	0.882
ARIMA (0,1,0)	0.949	1.402	2.257	0.953	0.857
ARIMA (0,1,2)	0.954	1.124	2.246	0.987	0.889
ARIMA (1,1,2)	0.949	1.235	2.217	0.954	0.915
ARIMA (2,1,0)	0.954	1.257	2.297	0.978	0.921
ARIMA (2,1,1)	0.954	1.264	2.136	0.965	0.992
ARIMA (2,1,2)	0.954	1.285	2.145	0.986	0.999
ARIMA (1,2,1)	0.938	1.506	2.350	0.988	1.188
ARIMA (1,2,0)	0.931	1.571	2.446	1.030	1.180
ARIMA (0,2,1)	0.933	1.544	2.432	1.026	1.145
ARIMA (0,2,0)	0.928	1.582	2.424	1.014	1.102
ARIMA (1,2,2)	0.954	1.564	2.465	1.045	1.102
ARIMA (2,2,0)	0.954	1.516	2.415	1.103	1.232
ARIMA (2,2,1)	0.954	1.521	2.478	1.123	1.215
ARIMA (2,2,2)	0.954	1.549	2.314	1.135	1.232
ARIMA (2,2,2)	0.954	1.521	2.579	1.231	1.153
Simple ES	0.946	1.494	2.805	0.999	0.892
Brown ES	0.946	1.499	2.388	0.996	0.899
Holt ES	0.950	1.461	2.340	0.968	0.936
Damped trend ES	0.954	1.456	2.654	0.998	1.324

From the selection criteria given in the table, it was observed that ARIMA (0, 1, 1) was the best fit model for wool production forecasting since it had the lowest BIC value (0.882), higher R^2 value and lower values of RMSE (1.357), MAPE (2.194) and MAE (0.917) values. After model selection, the model parameters were estimated and the results of the estimates are given in Table 12. Based on the best fit ARIMA (0, 1, 1) model, forecasting of wool production was done for 2020-21, 2030-31, 2040-41 and 2050-51 and the results are given in Table 13. On perusal of the table, it can

be observed that wool production would increase appreciably over years with a production of 46.26, 52.06, 58.59 and 65.17 million kg, respectively during the years 2020-21, 2030-31, 2040-41 and 2050-51 from 43.00 million kg in 2010-11.

Table - 12. Estimates of ARIMA (0, 1, 1) model for wool production

Model	Parameters	Estimate	SE	t	Sig.
ARIMA (0, 1, 1)	Constant	0.116	0.005	2.109	0.041
	MA(1)	0.124	6.459	4.013	0.000
	Difference	1.000			

Table - 13. Forecasts of wool production in India (in million kg)

Year	Actual	Predicted	LCL (95 %)	UCL (95 %)	Residual
2000-01	48.40	48.47	45.21	51.90	0.00
2010-11	43.00	43.61	40.68	46.70	-0.01
2020-21	-	46.26	39.53	53.81	-
2030-31	-	52.06	39.51	67.42	-
2040-41	-	58.59	40.90	81.53	-
2050-51	-	65.17	42.73	95.54	-

Although the predicted milk production presents a rosy picture, unless the required infrastructure in terms of facilities for production of adequate feed and fodder, breeding, disease control, etc. are ensured, it will be highly infeasible to reach the predicted levels of production. The increasing trend in meat production implies the fact that the ever rising demand for protein rich meat would continue to be there due to the changing tastes and preferences among medium and high income groups especially in urban and semi urban areas.

In spite of promising trends in production of livestock and poultry in the country, the export potentials of livestock and poultry products from India to other countries only look gloomy. This points to the fact that the country has to effectively tackle challenges it faces in food safety issues. Considering the results obtained and conclusions drawn from the study, certain policy options are required to address the issues and challenges, so as to ensure higher productivity in the sector and assured food security to the consumers:

1. It needs to be ensured that necessary development programmes are framed and implemented for enhancing livestock population and productivity. These may include placing greater stress on feed and fodder availability and disease control.

2. Region specific programmes need to be formulated and introduced, where certain categories of livestock can be given greater thrust.

3. Best practices models may be drawn and popularised among farmers, so as to effectively exploit the potential benefits of livestock and poultry.

4. With the surplus production of milk and egg is the remarkable feature of Indian livestock and poultry industry, unless food safety issues are strictly considered and addressed, the country will not be able to fully exploit the export potentials in the near future. Also, exploring all possible avenues for further processing and value addition of livestock and poultry products will help to achieve the desired results.

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VP2 Gne based Molecular Characterization of Blue tongue Virus Serotype 16 Isolated in 2017 from Andhra Pradesh, India

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ABSTRACT

Bluetongue virus (BTV) genome consists of 10 segmented double-stranded ribonucleic acid (dsRNA). Of these, genome segment 2 (Seg-2), which encodes outer capsid protein VP2 is the primary determinant of virus serotype. In the present study, partial VP2 gene of KRL2/2017 (BTV-16) isolate collected from Kurnool district of Andhra Pradesh was amplified to know the evolutionary relationship of this isolate with previously reported Indian and global BTV-16 isolates. Seg-2 sequence data of KRL2/2017, showed >98% nucleotide identity with other Indian isolates. Comparison of this isolate with BTV-16 isolates across the world, showed higher sequence homology to isolates of Japan followed by Greece and China than to those of isolates from South Africa, Italy, UK, Australia and Indonesia. Phylogenetic analysis suggest that the isolate of the current study belong to eastern topotype similar to the viruses recovered from Japan, Greece and China reflecting their common eastern origin.

Key Words: Bluetongue, serotype-16, VP2, topotype

INTRODUCTION

Bluetongue (BT) is an economically important, infectious, non-contagious, haemorrhagic disease caused by bluetongue virus (BTV), which is classified as type species of the genus *Orbivirus* in the family *Reoviridae* and subfamily *Sedoreovirinae.* BTV can infect both domestic and wild ruminants as well as some carnivores (MacLachlan, 1994 and Alexander, 1994). Currently, 27 distinct serotypes of BTV have been recognised worldwide and two additional putative novel serotypes were detected in a Capripox vaccine in the Middle East (BTV-28) and in an alpaca in South Africa (BTV-29) (Zientara *et al.,* 2014; Wright, 2014 and Maan *et al.,* 2015). BTV is primarily transmitted among susceptible hosts through the bite of a competent *Culicoides* midge (Du Toit, 1944). However, transplacental transmission is demonstrated in naturally and experimentally infected cattle and in experimentally-infected sheep with BTV-8 (Saegerman *et al.,* 2011).

The BTV genome is composed of ten linear segments of double stranded ribonucleic acid (dsRNA) and enclosed

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within a three layered icosahedral protein capsid, consisting a total of seven structural proteins (VP1-VP7) and four non-structural proteins (NS1, NS2, NS3/NS3a and NS4) (Verwoerd *et al.,* 1970; Mertens *et al.,* 1984 and Ratinier *et al.,* 2011). BTV outer capsid proteins VP2 and VP5are encoded by genome Seg-2 and -6, respectively. The VP2 gene particularly contains epitopes that bind to neutralizing antibodies generated during infection of the mammalian host and is main determinant of virus serotype (Mertens *et al.,* 2004). Topotypes were proposed for segments of BTV based on the high degree of nucleotide similarity among BTV isolates from the same geographical region (Gould and Pritchard, 1990). Sequence analysis of all segments of BTV revealed two broad topotypes. The BTV isolates from Africa, the Mediterranean and America were considered as 'western' types, and those from Australasia, the Middle East and the Mediterranean were considered as 'eastern' types (Maan *et al.,* 2010). Virus isolation and serum or virus neutralization tests (SNT or VNT) are slow, taking weeks whereas molecular methods like RT-PCR, sequence analysis, phylogenetic comparison etc. are time saving, in the detection of virus (Maan *et al.,* 2012a). Occurrence of multiple serotypes and presence of antigenic diversity within serotypes of BTV is a major constraint for developing an effective vaccine. Hence, there is a felt need to identify and characterize currently circulating BTV serotypes. In this study, we describe VP2 gene based molecular characterization of BTV-16 isolated from the samples collected during 2017 BT outbreak from Andhra Pradesh state. We report the evolutionary relationship of BTV-16 isolate from the current study with global BTV-16 isolates.

MATERIALS AND METHODS

Virus isolate

Blood sample was collected aseptically in Ethylene diamine tetraacetic acid (EDTA) vial from suspected field outbreak of BT disease in sheep during 2017 that occurred in Kurnool district of Andhra Pradesh and designated as KRL2/2017. Blood was collected from sheep exhibiting BT symptoms like pyrexia, oral lesions, swollen face, nasal discharges, frothy salivation and torticollis.

Virus dsRNA extraction and cDNA synthesis

Virus from blood sample was isolated by initially inoculating the washed erythrocyte lysate on to *Culicoides sonorensis* (KC) cell line and after 10 days, the KC cell culture adapted sample was passaged three times in BHK-21 cell line (Clavijo *et al.,* 2000). Viral dsRNA was isolated from infected BHK-21 cell line showing cytopathic effect (CPE) using TRIZOL method and then subjected to 1% agarose gel electrophoresis, for testing *Orbivirus* like pattern. cDNA was

synthesized using PrimeScript™ 1st strand cDNA Synthesis kit (TaKaRa) using random hexamers. The resulting cDNA was used as template in PCR.

Polymerase chain reaction

The PCR was carried out by using EmeraldAmp® GT PCR Master Mix (TaKaRa) with group specific primer pair; Seg10F-TTGGAYAAA GCRATGTCAAA;Seg10R-ACRTCATC ACGAAACGCTTC targeting Seg-10, and serotype-specific primer pairstargeting Seg-2 as described previously (OIE, 2012 and Reddy *et al.,* 2016). PCR was carried out with initial denaturation at 94^0C for 3 min followed by 35 cycles of 30 sec denaturation at 94^0C, 30 sec of primers annealing at 55^0C for all sets of group specific and serotype specific primers except for Seg-2 of BTV-16 where 50^0C was used, followed by extension at 72^0C for 1 min and then final extension at 72^0C for 10 min. PCR products were visualized by agarosegel electrophoresis. Amplified PCR product with VP2 gene primers of BTV-16(1196 bp) was purified using XcelGen® PCR Purification MiniKit as per the manufacturer's instructions.

Sequencing and Phylogenetic analysis

Sequencing was done from Xcelris Labs Ltd, Gujarat using VP2 gene specific primers as mentioned above for purified PCR products. The partial sequences of VP2 gene of KRL2/2017 isolate obtained after sequencing was compared with other available sequences in GenBank using NCBI BLAST (www.ncbi.nlm.nih/gov/blast). Phylogenetic trees were generated using the Neighbor-Joining (NJ) method with the Tamura 3-parameter model and bootstrapped on the set of 1,000 replicates with MEGA version 7.0 software to study the evolutionary relationship of isolate from the current study with other BTV-16 isolates from various geographical regions of the world.

RESULTS AND DISCUSSION

Virus BTV-16 (KRL2/2017) was isolated by initially inoculating the blood sample collected from sheep suspected with BT on to KC cells for 10 days followed by culturing in BHK-21 cell line. CPE was noticed after three passages in BHK-21 cell line. BTV nucleic acid was extracted from the infected cell culture fluid by TRIZOL method when the cells were showing above 75% CPE (Fig. 1A&B). The extracted RNA was subjected to 1% agarose gel electrophoresis and 10 segmented pattern of BTV RNA was observed (Fig. 1C). The isolate was confirmed as BTV by Seg-10 based RT-PCR (Fig. 1D). When subjected to Seg-2 based serotype specific RT-PCR, the isolate was confirmed as BTV-16. The size of the amplified product was1196 bp(Fig. 1E); it was purified and sequenced. No amplification of similar size was observed in negative control indicating that the amplified product was specific for VP2 gene ofBTV-16.

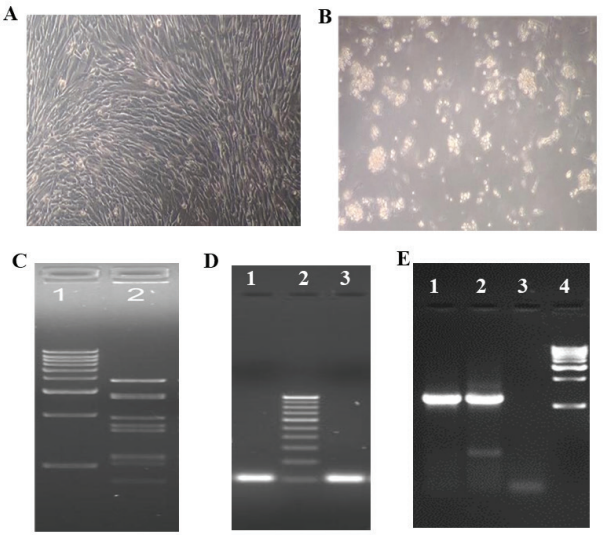

Fig 1:Molecular characterization of BTV-16 (KRL2/2017).*A-Uninfected BHK-21 cell line (10X); B-Infected BHK-21 cell line exhibiting CPE (10X) like cell rounding, clumping and detachment of cell bunches; C-Segmented pattern of BTV dsRNA, Lane 1- 1Kb Ladder, Lane 2- Segmented dsRNA of BTV-16 isolate (KRL2/2017); D-NS3group-specific RT-PCR (98 bp), confirming BTV, Lane 1- positive control, Lane 2-100 bp DNA ladder and Lane 3- BTV-16 isolate (KRL2/2017); E-VP2 gene specific RT-PCR (1196 bp) confirming BTV serotype-16E, Lane 1- BTV-16 isolate (KRL2/2017), Lane 2- Positive control, Lane 3- Negative cotrol and Lane 4-1 Kb ladder.*

PCR product of BTV-16 was sequenced and analysed. The BTV-16 Seg-2 sequence data (1196 bp; nucleotide421-1616) for KRL2/2017showed 98.5% nucleotide identity with Indian isolates (GeneBank Acc. No. KX302636; KC751423; KC751424 & KC751425), 98% nucleotide identity with isolates (GenBank Acc. No. JQ924821; JX007924; MG710531; KC751419; MG710529; JN572917; KF664134; KY934051; KF664104; KY934050 & MG7105530). Comparison of this isolate with previously reported isolates across the world showed 97.3%, 96.9%, 96.5%, 96.4%, and 96% nucleotide identity with isolates of Japan (GenBank Acc. No. AB686220; AB686226 & AB686225), Greece (Gene bank Acc. No.KP820989; KP820990 & AM773709), China (Gene bank Acc. No.KP195134 & KP195134), Israel(GenBank Acc. No.KP820992), UK (GenBank Acc. No.KP820985; KP820986 & KP820987) and Turkey (GenBank Acc. No.AJ585146; AJ585147 &AJ585148), respectively.BTV-16 isolates of UK (GenBank Acc. No. KP820988), Italy (GenBank Acc. No. KF387522; MH990434; MH990424 & MK014493) and South Africa reference strain (Gene bank Acc. No. AJ585137) showed 95.29% nucleotide identity with isolate from the current study whereas isolates of

Australia (GenBank Acc. No. MF384481 & MF384480) and Indonesia (GenBank Acc. No. AJ585151) showed 91.58% nucleotide identity. Maximum nucleotide identity of 96.5-97.3% was showed by Indian BTV-16 isolates with isolates of Japan followed by Greece and China than to those of isolates from South Africa, Italy, UK, Australia and Indonesia having 91.58-95.29% nucleotide similarity. Sequence homology and phylogenetic analysis of Seg-2 indicated that the BTV-16 isolate of the current study belonged to eastern topotype (Fig. 2).

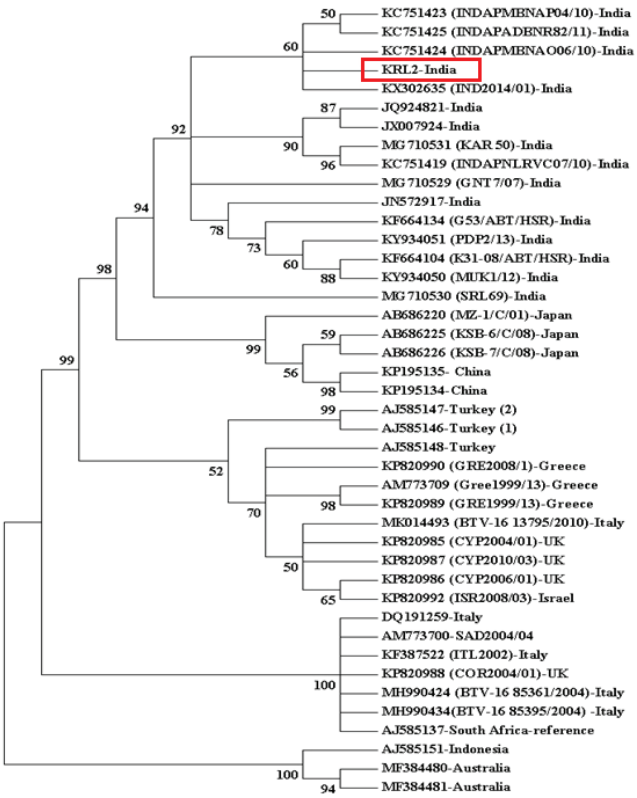

Fig 2: Phylogenetic analysis of BTV-16 (KRL2/2017) isolate against reference BTV-16 isolates isolated worldwide. Neighbor joining (NJ) tree showing relationship between KRL2/2017 (BTV-16) isolate sequenced in the current study and other 41 global isolates of BTV-16 based on nucleotide sequences of VP2 region of BTV using MEGA 7.0. The optimal tree with sum of branch length = 0.20209871 and the bootstrap test (1000 replicates) is shown. Based on the analysis the current isolate can be categorised as eastern topotype of BTV.

Sequence analysis of BTV segments have been extensively used for genetic characterization of isolates from various geographic regions of the world (Shirafuji *et al.,* 2012; Maan *et al.,* 2007 and Maan *et al.,* 2015). VP2 encoding genomic segments of *Orbivirus* are the most variable between serogroups as well as between serotypes of a serogroup (Gould and Pritchard, 1990). The sequence information on genome Seg-2 encoding outer capsid protein VP2 (the primary determinant of virus serotype), have been useful for understanding molecular epidemiology, phylogenetic relationship of BTV and are also capable of determining the geographic origin of a virustopotype (Gould and Hyatt, 1994, Maan *et al.,* 2015).

Most of the BT serotypes were isolated from Africa whereas BTV-16 was first isolated from the Indian subcontinent (Hazara, West Pakistan) in 1960 (Howell, 1970**)**. The sequence analysis of BTV-16 (KRL2/2017) isolate from Andhra Pradesh state, presented here showed very high sequence homology of >98% nucleotide identity with previous BTV-16 isolates circulating in India and when compared with BTV-16 isolates from different geographical regions of the world showed an overall nucleotide identity of 91.58-97.3%.This similar observation was reported by Saxena *et al.* (2018) based on genetic and phylogenetic analysis of the outer capsid protein genes (VP2 and VP5) of Indian isolates ofBTV-16. Phylogenetic analysis based on VP2 gene segregated Indian BTV-16 isolates at proximity to the viruses recovered from Japan, Greece and China than to those of isolates from South Africa, Italy, UK, Australia and Indonesia. Supporting our data, Shafiq et al. (2013) previously reported the Indian isolates are at close proximity to Japan and Greece isolates based on phylogenetic analysis of Seg-2 of BTV-16. In this study a higher percentage of nucleotide similarity of VP2 gene of most of the globally isolated BTV-16 indicate that the eastern segment might have evolved from a common ancestor. Sequencing analysis and phylogenetic studies based on VP2 gene showed that isolate characterized from the current study belonged to eastern topotype. This observation was in accordance with Maan *et al.* (2012b) and Kumar *et al.* (2016), who reported the complete genome sequence analysis of BTV-16 isolates of India and identified as eastern topotype.

It may be concluded that BTV typing by molecular methods is considerably faster and cheaper than serological techniques. The VP2 gene based sequence analysis and phylogenetic approach will be useful for understanding molecular epidemiology, predicting BTV origin, patterns of distribution, variations within the serotypes, genotyping, and phylogenetic relationships. BTV-16 isolate from this study, could be grouped under eastern topotype. It will be possible to determine the origin of virus and genome segments with high degree of confidence only with complete genome sequence of BTV.

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Improvement of Farmers Economy through Frontline Demonstrations conducted at VUTRC, Trichy

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ABSTRACT

Proven scientific technologies related to the Animal Husbandry practices are generally demonstrated in the field for the benefit of the farmers. Veterinary University Training and Research Centre, Tiruchirappalli has conducted three frontline demonstration programmes (FLD) during the year 2015 - 2018. By these programmes, it is observed that farmers get benefitted economically. Twenty farmers were benefitted in the programme conducted in 2015-16 on the "Impact of TANUVAS mineral mixture on the milk yield of dairy cows". Mineral mixture supplementation improved the milk quality and milk quantity. Hence each farmer obtained additional improved returns of Rs.150/month/cow. In the year 2016-17 a programme on "Scientific intervention in to Improve Production Performance of Backyard native chicken" was conducted and four farmers were benefitted. They reported that the scientific technologies like TANUVAS desi chicken feed, deworming and vaccination demonstrated in this programme increased body weight gain, reduced mortality, increased egg production and improved hatchability. Each farmer profited Rs.500/- for 5 birds additionally. Six farmers participated and benefitted in the programmes conducted in 2017-18 on "Azolla pinnata as cattle feed supplement". Supplementation of azolla reduces the cost of production of milk and improves the milk quality. In this study it is observed that there was a reduction in cost of production of about Rs.2.50 / litre of milk. Also there was an increase in returns of milk since improved quality of milk.

"Seeing is believing" concept is the major outline of the Front line demonstration programmes. Hence farmers of the villages where the programmes were conducted also were benefitted apart from the beneficiaries.

Key Words: Front line demonstration, Mineral mixture, Azolla, Scientific practices

INTRODUCTION

Front line demonstrations are very effective in teaching the concept to the farmer by "seeing is believing" and this will be motivating the farmers to adopt the technology/scientific practice. Tamilnadu Veterinary and Animal Sciences University is practicing front line demonstrations of proven technologies/scientific innovations through its outreach centres for the benefit of farming community. Veterinary University Training and Research Centre,

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Tiruchirappalli is one of the outreach centre in TANUVAS situated in Trichy town whereby farmers can be reached easily for their technical advices. Apart from the regular on campus and off campus training programme, on farm trial and front line demonstrations are very easier to reach the farm personnels. So far three programmes were conducted at VUTRC Trichy from 2015 and the improvement in farmers economy was recorded.

MATERIALS AND METHODS

Impact of TANUVAS mineral mixture on the milk yield of dairy cows

Twenty beneficiaries were involved in this project who are having milch animals. Two groups of having 20 numbers of lactating cows which are in second order of lactation were selected for this trial: one group for control and another one for treatment group. All the animals were recently calved (within one month). All the beneficiries were gathered and instructions about supplementation of mineral mixture was given by the programme coordinator and co-coordinator. TANUVAS mineral mixture was supplemented @ 50g/day/cow with feed for a period of six months. All the other parameters like feeding, grazing, milking etc were remaining same since the beneficiary group belonged to the same village and community. Milk production and milk quality were assessed in every two months for a period of six months. For recording the milk quality (fat, protein, lactose and SNF) we used the milk o scanner available in nearby society. The data has been tabulated and analysed.

Scientific Intervention to Improve Production Performance of Backyard Native Chicken

Four beneficiaries were involved in this project who are having desi chicken not less than 10 in numbers. Five birds of each farmer totally 20 birds were undertaken for scientific intervention in this study. Other birds of the beneficiaries were treated as control. All the beneficiries were taught about scientific intervention in backyard native chicken rearing. Each beneficiary was given with 87.5 kg of TANUVAS desi chicken feed. All the 20 birds were dewormed and vaccinated against Ranikhet disease with Oral Pellet Vaccine. Method demonstration of dipping was done for ectoparasitic control. Beneficiaries feed their chicken (20 Nos) with TANUVAS desi chicken feed @ 100g/day/bird with regular grazing for a period of six months. The mortality rate and production performance (body weight, egg weight and no. of eggs produced & hatched) of the native chicken were assessed at monthly intervals for the period of 6 months. The data has been tabulated and analysed.

Azolla pinnata as cattle feed supplement

Six numbers of Azolla units were established at farmer's field. A total number of six demonstrations were done to cultivate the *Azolla pinnata* at the farmer's field with a gathering of farmers. Awareness campaign was conducted to create the awareness about the azolla as cattle feed supplement. Milk production details and quality of milk were assessed through farmer's record. Cost of production per litre of milk before and after intervention of azolla was worked out.

RESULTS AND DISCUSSION

Mineral mixture supplementation improves the milk production significantly (P>0.05) in the field milch cows (Table 1). Quantity of milk production has been significantly increased in the TANUVAS mineral mixture supplemented group 6.70±0.11 over the control group (6.55±0.12) at 4th month of lactation. Milk production has been reduced in the 6th month of lactation in both control (6.15±0.25) and supplemented group (6.25±0.11). This is mainly due to stage of lactation of milch cows. All the nutrient values like fat, SNF, Protein and lactose values were reported significantly (P>0.05) higher when compared to control group. Economically each farmer is getting Rs.150/Cow/month additionally due to mineral mixture supplementation. These findings are similar to those of Shivdeep Singh (*et al.* 2016) Hackbart *et al.* (2010) who reported an increase in milk production in dairy cattle. In Indian conditions, Tiwari *et al.* (2013) has reported an increase in milk production as well as increased in total lactation length in cattle post area specific mineral mixture supplementation. Nocek *et al.* (2006) observed an increase in milk production during second lactation as compared to first lactation post mineral supplementation. Hence it can be concluded that mineral mixture supplementation increases the milk production. In contrast with the findings of the present study, Wu *et al.* (2000), Sharma *et al.* (2002), Rabiee *et al.* (2010) and Begum *et al.* (2010) reported no significant changes between supplemented and non-supplemented groups in milk components such as milk lactose, milk protein, milk fat and milk SNF.

Table – 1. Impact of mineral mixture on the milk yield

Parameters		Milk production (L/day)	Fat (%)	SNF (%)	Protein (%)	Lactose (%)
2nd month	Control	6.52±0.25	3.01±0.15	8.12±0.05	3.13±0.01	4.03±0.27
	Treatment	6.62 ±0.31	3.23±0.11	8.36±0.25	3.35±0.21	4.26±0.24
4th month	Control	6.55 ±0.12	3.11±0.05	8.17±0.25	3.11±0.12	4.05±0.14
	Treatment	6.70 ±0.11	3.48±0.05	8.56±0.17	3.41±0.05	4.28±0.20
6th month	Control	6.15 ±0.25	3.26±0.32	8.12±0.14	3.15±0.11	4.21±0.12
	Treatment	6.25 ±0.11	3.71±0.05	8.60±0.02	3.55±0.16	4.58±0.20

In this study, it is observed that average body weight of scientifically grown desi chicken had improved over the control birds (Table 2). The body weight at first lay and 40th week were 1.25±0.14 and 1.75±10.12 respectively. This in accordance with the findings of Nath and Pathak (2013) and Faruque *et al.* (2013).

The egg production at 24th, 32nd and 40th week egg production were 11.84±1.06, 13.84±1.16 and 14.36±1.06 respectively.

Mortality was lower in scientifically reared desi chicken compared to control desi chicken. Mortality of control desi birds upto 40th week was recorded as 6.61 ±1.32 and for scientifically reared desi chicken was 2.02 ±0.12.4. This is in contradicting with the following finding of Tanveer Akhtar, 2015 who reported that survivability of desi bird was 61.40% under village conditions. This contradiction in this study may be due to awareness of good management practices by this FLD programme in this Punganur village, Ramji Nagar, Trichy. Average egg weight of desi chicken at 32nd and 40th week of control and 24th, 32nd and 40th week of treatment were 29.45±2.31, 32.95±1.98 and 37.1±1.41, 40.80±1.12 and 42.00±1.18 respectively. These results are in concordance with the findings of Mahapatra and Pandey (1989) and Sharma (1995). Hatchability in scientifically reared chicken was noted as 78.27±2.48 (28th week)and 80.26±2.16 (36th week). Overall performance of scientifically reared desi chicken improved the farmer's economy which was recorded as Rs.500/5 birds additionally

Table -2. Effect of scientific management in desi chicken in production parameters

Parameters	Control						Treatment					
Weeks	20	24	28	32	36	40	20	24	28	32	36	40
Mortality (%)	6.61 ±1.32						2.02 ±0.12					
Body Weight (Kg)	0.85± 0.03	0.98± 0.12	1.12± 0.32	1.26± 0.13	1.28± 0.14	1.28± 10.04	0.85± 0.02	1.25± 0.14	1.52± 0.03	1.61± 0.01	1.78± 0.12	1.75± 10.12
Egg production	-	-	-	10.52 ± 1.30	-	9.4± 1.64	-	11.84± 1.06	-	13.84± 1.16	-	14.36± 1.06
Egg weight	-	-	-	29.45± 2.31	-	32.95± 1.98	-	37.1± 1.41	-	40.80± 1.12	-	42.00± 1.18
Hatchability (%)	-	-	-		62.24± 2.48		-		78.27± 2.48		80.26± 2.16	

Farmers advised to feed the azolla as cattle feed supplement. Before starting the azolla supplementation, milk production details were collected and taken as control. This study was conducted for six weeks duration (Table 3). All the animals were having second order of calf and between 3rd to 4th month of lactation. It is observed that there was an increase in milk production and quality. There was a significant change in fat and SNF after one week of supplementation of azolla. After 3rd week, no significant changes observed in milk production, fat and SNF. Hence the farmers were advised to continue the supplementation to all their cows for increased milk production and quality.

Table -3. Impact of azolla feeding on milk production and quality

Duration (after azolla supplementation)	Milk production (litre/day)	Fat	SNF
Control	10.52±0.51	3.81±0.11	8.31±0.05
Ist week	10.71±0.25	3.82±0.15	8.52±0.02
2nd week	11.12±0.32	3.65±0.25	8.65±0.11
3rd week	11.52±0.25	3.72±0.21	8.75±0.21
4th week	11.56±0.26	3.80±0.15	8.75±0.25
5th week	11.45±0.18	3.78±0.20	8.75±0.23
6th week	11.52±0.13	3.75±0.25	8.78±0.15

Table 4. Cost effectiveness in milk production

Cost of feed/day	Control	Treatment (with Azolla supplement)
Green fodder 15 Kg	60.00	60.00
Paddy straw 5 kg	10.00	10.00
Concentrates 6 kg	120.00	90 (4.5 kg)
Azolla 750 g		1.00
	190.00	161.00

Cost of production of 12 litres of milk in control animals was Rs.190/- whereas azolla supplemented animal it is around Rs.161/-. Hence there was a reduction in cost of production in azolla supplemented animal which is Rs.2.5 /litre of milk (Table 4).

In each front line demonstration, the technology/ scientific practices reached the farmers. Since the participated farmer's economy improved, other farmers were also getting motivated by seeing the demonstrations. Hence it is concluded that front line demonstrations provided the knowledge as well as increased income to the farmers.

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Superovulation and Embryo Yield after GNRH Pretreatment in Crossbred Cows

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ABSTRACT

The effect of GnRH pretreatment on superovulatory response (SOR) and embryo yield in crossbred cattle was studied. Six crossbred cows were subjected to three superovulatory treatments: i.Control: Four day FSH schedule was initiated on Day 10 of the cycle ii.Gn-D8: GnRH was administered on Day 6 and FSH schedule was initiated on Day 8 and iii. Gn-D10: GnRH was administered on Day 6 and FSH schedule was initiated on Day 10. In control group, the mean SOR was 13.7 $\pm$ 5.8 CL. In Gn-D8 group, the SOR is inconsistent (5.0 $\pm$ 1.7) with recovery of poor quality embryos, which was attributed to significantly higher progesterone concentration on the day of superovulatory oestrus. In Gn-D10 group, SOR (11.0 $\pm$ 1.6) and embryo yield were comparable to the control group. Transferable quality embryos were significantly higher in Gn-D10 group which could be attributed to the follicular maturation under favourable endocrine environment. Thus, GnRH pretreatment in superovulation protocol ensured consistent SOR and increased yield of transferable quality embryos in crossbred cattle.

Key Words: GnRH pretreatment, superovulatory response, embryo yield, crossbred cows

INTRODUCTION

Gonadotropin induced superovulation is the basic and efficient method of obtaining multiple embryos from the genetically valuable females. However, the superovulatory response (SOR) is highly unpredictable and variable between treatments thus affecting the efficiency of the technology and limiting its practical application (Adams *et al.,* 1993). Variability in ovarian response has been attributed to various exogenous factors such as donor parity and production status, season, hormone preparations and their dose (Lee *et al.,* 2012; Vieira *et al.,* 2015; Abdelatty *et al.,* 2018). Above all, success of ovarian response is dependent on the nature of dominant follicle (DF) and the availability of gonadotropin sensitive follicles at the time of initiation of treatment (Driancourt, 2001). Nasser *et al.* (1993) opined that SOR was found to be higher when gonadotropin treatment was initiated at the time of follicular wave emergence (FWE). However, difficulty in predicting the day of FWE during an oestrous cycle poses a major problem. To obviate this problem, an alternative approach is to control the FWE

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and initiate the superstimulation treatment at the most favourable time that is optimal for recruited follicles to respond for exogenous gonadotropins.

The technique of synchronizing FWE in crossbred cattle was standardized by administering GnRH on Day 6 of the cycle (Satheshkumar *et al.,* 2008 and Satheshkumar *et al.,* 2012). Based on these previous findings, the present research was designed with the objective to study the effect of GnRH pretreatment on SOR and embryo yield in crossbred cattle.

MATERIALS AND METHODS

Experimental animals

Six healthy, pluriparous, non-lactating and regularly cycling crossbred cows aged between 5-6 yrs maintained at the Centralized Embryo Biotechnology Unit, Department of Animal Biotechnology, Madras Veterinary College, Chennai were utilized for the study. The cows were housed together and maintained under same conditions throughout the study. All the experimental cows were monitored regularly for their cyclicity and oestrus (Day 0) was confirmed by gynaeco-clinical and ultrasound examination.

Superovulation treatment

All the six animals are subjected for three different superovulatory treatments as mentioned below, with an interval of two months between each treatment.

Treatment 1- Control: Superstimulatory treatment was initiated on Day 10 of the cycle. The schedule included total dose of 400 mg NIH-FSH-P1 (Folltropin-V; Bioniche, USA) administered in equally divided doses (50 mg each; i.m.) twice daily at 12 h interval over a period of four days. Superovulatory oestrus (SO) was induced with two injections of prostaglandin (PG) -Dinoprost tromethamine (Inj. Lutalyse; 25 mg each, i.m.; Pfizer, Belgium) given at 48 and 60 h after first FSH injection. Animals were inseminated during SO thrice at 12 h interval, from 48h post PG administration. To control the potentially confounding effect of handling stress on SOR, cows received an intramuscular injection of saline (2.5 ml) on Day 6, simulating the GnRH administration in other treatment groups.

Treatment II - Gn-D8: The animals were injected with GnRH analogue- Buserelin acetate (Inj. Receptal; 10 µg i.m.; Intervet International, GmbH, Germany) on Day 6 of the cycle and FSH schedule for superovulation (as mentioned in control group) was initiated 48 h after GnRH i.e., on Day 8, the day of synchronized FWE (Satheshkumar *et al.,* 2012).

Treatment III - Gn-D10: GnRH analogue was administered on Day 6 of the cycle and superovulation schedule was initiated 96 h after GnRH i.e., on Day 10, two days after the synchronized FWE and before the deviation of DF (Satheshkumar *et al.,* 2012).

Assessment of follicular characteristics during superovulation treatment

Number of follicles of various size categories (Class I - $\leq$ 5 mm, Class II - > 5 -

< 9 mm and Class III - ≥ 9 mm) on the day of initiation of FSH treatment and on the day of SO were determined by ultrasound scanning using a real time B-mode ultrasound scanner (Sonovet 600, Universal Medical Systems) equipped with a 7.5 MHz rectal probe.

Superovulatory response and embryo yield

On the day of embryo collection (Day 7 post SO), the SOR was assessed by estimating the number of corpus luteum (CL) and anovulatory follicles (AF) by rectal palpation and confirmed by ultrasound scanning. Based on which animals were categorized as either responders (animals having > 2 CL) or non-responders (animals having ≤ 2 CL) (Vieira et al., 2015). Embryos / ova were recovered non-surgically by flushing the uterine horns using two-way Foley's catheter as described by Kathiresan *et al.* (1997). The embryos were morphologically scored for quality, colour and developmental stage (Lindner and Wright, 1983). The grade 1 (Excellent) and 2 (Good) embryos were considered as transferable, while grade 3 (Fair) and 4 (Poor) were non-transferable quality embryos. Apart from the unfertilized oocytes (UFO), embryos in earlier developmental stages than morulae were categorized as 'arrested or degenerated'.

Plasma progesterone (P_4) concentration

Blood samples were collected on the day of initiation of FSH treatment, day of PG, day of SO and day of embryo collection in all the experimental groups. Plasma was separated by centrifuging the blood sample and stored in duplicate vials at -20^{O}C until assayed. The P_4 concentration was measured with solid-phase radio immuno assay kit (Coat – A – Count, Immunotech SAS, France) and the radioactivity was counted in I^{125} gamma counter (STRATEC, Germany). The sensitivity of the P_4 assay was 0.05 ng / ml and intra-assay co-efficient variation was 6.5. Hormone assay was carried out at Department of Veterinary Physiology, Veterinary College and Research Institute, Namakkal, Tamilnadu.

Statistical analysis

Data on follicular characteristics, SOR, embryo yield and plasma P_4 concentrations in superovulatory cycles were analyzed by Student's *t*-test and by Analysis of Variance (ANOVA) with completely randomized design. SPSS.10.0® software was used for analysis of data. Analysis of data was carried out as per Snedecor and Cochran (1994).

RESULTS AND DISCUSSION

Various categories of follicles during the course of superovulatory protocol in control and GnRH treated groups are presented in Table 1. Perusal of the data revealed that GnRH pretreatment favoured the availability of more number of FSH-responsive follicles at onset of protocol than the control group in concurrence with the earlier findings of Satheshkumar *et al.* (2008) and Satheshkumar *et al.* 2012).

Table – 1. Mean ± SE of follicular numbers during and recruitment and development in superovulatory cycles of crossbred cows

Treatment Groups	FOLLICULAR POPULATION (Range within parenthesis)								
	Day 6			Day of I FSH			Day of SO		
	Class I	Class II	Class III	Class I	Class II	Class III	Class I	Class II	Class III
Control (n = 6)	0.5 ± 0.2 (0 – 2)	0.8 ± 0.1 (0 – 2)	1.0 ± 0.0 (1)	6.5 ± 0.9 [a] (3 – 9)	0.7 ± 0.3 [a] (0 – 2)	0.5 ± 0.2 (0 – 1)	0.0 (0)	5.1 ± 0.8 [a] (2 – 8)	9.8 ± 1.2 [a,b] (7 – 15)
Gn-D8 (n = 6)	0.4 ± 0.3 (0 -2)	0.7 ± 0.2 (0 – 1)	1.0 ± 0.0 (1)	11.5 ± 0.6 [b] (8 – 14)	0.8 ± 0.3 [a] (0 – 2)	0.0 (0)	0.0 (0)	9.2 ± 1.3 [b] (0 - 18)	8.3 ± 2.8 [a] (5 – 13)
Gn-D10 (n = 6)	0.5 ± 0.2 (0 – 2)	0.8 ± 0.3 (0 – 2)	1.0 ± 0.0 (1)	5.2 ± 0.5 [a] (4 – 8)	7.5 ± 0.6 [c] (5 – 8)	0.0 (0)	0.0 (0)	3.7 ± 0.4 [a] (2 – 5)	11.0 ± 0.9 [b] (9 – 15)
Significance	#	#	#	**	**	#	#	*	*

Class I - ≤ 5 mm; Class II - > 5 - < 9 mm; Class III - ≥ 9 mm

Values within the column with different superscripts differ significantly ** ($P < 0.01$) * ($P < 0.05$)

 # - Statistically not comparable

The plasma P_4 concentrations during various stages of the superovulation programme are presented in Table 2. On the day of initiation of FSH treatment, the mean plasma P_4 concentration in Gn-D10 group was significantly ($P < 0.01$) higher than the control group and non-significantly higher than Gn-D8 group. The increased P_4 concentrations in Gn-D10 group could be attributed to the presence of developing GnRH induced accessory corpus luteum (ACL). Mapletoft *et al.* (2009) indicated the importance of a functional CL with sufficient P_4 concentrations at the time of initiation of gonadotropin treatment in achieving a better SOR.

Table – 2. Mean ± SE of Plasma progesterone concentrations in superovulated crossbred cows

Treatment groups	PROGESTERONE CONCENTRATION (ng / ml)			
	Day of I FSH	Day of PG	Day of SO	Day of collection
Control	5.3 ± 0.9 [a]	8.5 ± 0.3 [a]	0.4 ± 0.0 [a]	18.7 ± 1.2 [b]
Gn-D8	6.7 ± 0.5 [a,b]	9.4 ± 1.1 [a]	4.3 ± 0.9 [b]	6.2 ± 1.4 [a]
Gn-D10	8.8 ± 0.8 [b]	10.0 ± 0.9 [a]	0.6 ± 0.1 [a]	17.0 ± 1.0 [b]
Significance	**	N.S	**	**

Values within the column with different superscripts differ significantly ** ($P < 0.01$) N.S – Not significant ($P > 0.05$)

In Gn-D8 group, the P_4 concentration was significantly ($P < 0.01$) higher on the day of SO than the other groups. Interestingly, a prominent luteal tissue of ACL could be detected on the day of SO in four animals (66.7%) of this group which was also supported by suprabasal levels of P_4 (4.3 ng / ml). It was reported that the immature CL (< 5 days old) was not consistently responsive to $PGF_2\alpha$ due to

luteal insensitivity or insufficient numbers of PG receptors (Duchens *et al.,* 1994). In the present study, the ACL was immature (4 days old) at the time of PG administration and hence would not have responded resulting in incomplete luteolysis. On the contrary in Gn-D10 group, the ACL was mature enough (6 days old) to respond to PG and hence complete lysis could be accomplished as indicated by low P_4 concentration (0.6 ng / ml) on the day of SO.

The SOR as indicated by number of CL and AF in various experimental groups are presented in Table 3. All the animals (100%) subjected for superovulatory treatment in control group and Gn-D10 groups responded with more than two ovulations, but in Gn-D8 group, only four (66.7%) animals responded to the superovulation treatment. Among these four responders only two animals had more than 10 ovulations and the remaining two animals had three ovulations each. Eventhough more numbers of FSH responsive follicles were present initially in Gn-D8 group than the control group, majority of them failed to ovulate reducing the overall SOR. Similarly, Rajamahendran and Calder (1993) and Farin *et al.* (2008) also failed to show improvement in the SOR when the cows were superovulated two days after hCG or GnRH treatment. Significantly more numbers of Class II follicles were recorded than Class III follicles in this group on the day of SO, which might be an indication that follicles have not matured sufficiently to complete the ovulation process, as suggested by D'Occhio *et al.* (1997). Supra-basal levels of P_4 on the day of SO would have inhibited the LH surge and prevented the ovulation (Duchens *et al.,* 1994). Thus the highly inconsistent SOR in Gn-D8 group could be attributed to the aberrant endocrine milieu during the course of superovulatory cycle.

Table – 3. Mean ± SE of superovulatory response and embryo yield in superovulated crossbred cows

Treatment groups	**No. of animals responded** (% in parenthesis)	**Superovulatory response** (Range within parenthesis)		**No. of animals yielded embryos / oocytes** (% in parenthesis)	**No. of embryos / oocytes recovered** (Mean % - in parenthesis)
		CL	**AF**		
Control (n = 6)	6 (100.0)	13.7 ± 5.8 [b] (9 – 21)	2.0 ± 0.4 [a] (1 – 3)	6 (100.0)	8.7 ± 2.0 (63.4 ± 9.7) [a]
Gn-D8 (n = 6)	4 (66.7)	5.0 ± 1.7 [a] (3 – 11)	12.8 ± 4.6 [b] (2 – 23)	3 (50.0)	3.0 ± 1.6 (36.6 ± 16.4) [b]
Gn-D10 (n = 6)	6 (100.0)	11.0 ± 1.6 [b] (10 – 14)	3.5 ± 0.2 [a] (3 – 4)	6 (100.0)	6.8 ± 0.87 (61.8 ± 4.2) [a]
Significance		**	**		**

Values within the columns with different superscripts differ significantly ** ($P < 0.01$)

The SOR in Gn-D10 group was comparable to control group, but had significantly higher number of ovulations than Gn-D8 group. Fortune *et al.* (2001) stated that till the point of deviation, the future DF and subordinate follicles were similar in FSH receptors on their granulosa cells. Ginther *et al.* (2003) also concluded that all follicles in the common growth phase have the potential for future dominance and administration of FSH early in wave prevented deviation phenomenon and induced several follicles to become dominant in cattle. Contrary to the Gn-D8 group, decreased P_4 levels on the day of SO would have favoured the follicular maturation (as indicated by increased numbers of Class III follicles) and ovulation in Gn-D10 group. Thus it could be determined that majority of the recruited follicles of synchronized wave could be rescued from atresia and incorporated into the cohort that attains ovulatory capability when treatment was initiated before the deviation process.

The data on embryo recovery in control and GnRH treated groups are presented in Table 3. Embryos could be recovered from all the animals (100%) in control and Gn-D10 groups, while only three (50%) animals yielded embryos in Gn-D8 group. There was no significant difference in the embryo recovery rate between the control and Gn-D10 groups but the recovery rate was drastically reduced (36.6%) in the Gn-D8 group, which was in corroboration with the previous findings of Kohram *et al.* (1998) and Deyo *et al.* (2001). The Gn-D8 group had a significantly increased number of AFs on the day of SO when compared with other groups. As suggested by Sato *et al.* (2005), abnormally high levels of oestrodiol secreted by the persistent AFs might have hindered the ova transport in the oviducts adversely affecting their recovery in Gn-D8 group.

Various grades of embryos / ova recovered from superovulated animals are presented in Table 4. The percentage of transferable embryos in Gn-D10 group was significantly ($P < 0.01$) higher than Gn-D8 group and non-significantly ($P > 0.05$) higher than control group. The increased concentrations of P_4 at the onset of treatment would have enhanced the quality of follicular inventories and embryo quality thereon. On the other hand, non-transferrable embryos and UFO constituted the major proportion of ova recovered in Gn-D8 group. High levels of E_2 secreted by AFs might have affected the fertilization and subsequent embryo development in this group (Sato *et al.,* 2005).

Table - 4. Mean ± SE of quality of embryos recovered from superovulated crossbred cows

Treatment groups (Number of ova/embryos in parenthesis)	QUALITY OF EMBRYOS (%) (Number of ova / embryos in parenthesis)							
	Grade 1	Grade 2	Transferable (Gr 1+ Gr 2)	Grade 3	Grade 4	Non-transferable (Gr 3 + Gr 4)	Arrested / Degenerated	UFO
Control (52)	49.7 ± 4.5 [a] (26)	29.7 ± 4.8 [a] (15)	79.4 ± 4.7 [b] (41)	2.1 ± 2.1 [a] (1)	6.4 ± 3.0 [a] (4)	8.4 ± 2.6 [a] (5)	6.7 ± 3.9 [b] (4)	4.4 ± 2.3 [a] (2)
Gn-D8 (18)	4.2 ± 2.2 [#] (1)	4.2 ± 2.2 [#] (1)	8.3 ± 2.2 [a] (2)	8.3 ± 3.3 [#] (2)	20.8 ± 6.3 [#] (3)	29.1 ± 4.8 [b] (5)	16.6 ± 12.3 [c] (3)	45.8 ± 22.7 [b] (8)
Gn-D10 (41)	56.8 ± 5.4 [a] (23)	30.4 ± 7.4 [a] (11)	87.2 ± 6.2 [b] (34)	5.2 ± 3.4 [b] (2)	5.2 ± 3.3 [a] (2)	10.3 ± 3.3 [a] (4)	3.3 ± 1.3 [a] (2)	1.8 ± 0.8 [#] (1)
Significance	*	N.S	**	*	N.S	**	*	**

Values within the columns with different superscripts differ significantly ** ($P < 0.01$) * ($P < 0.05$)

N.S – Not significant ($P > 0.05$); # - Statistically not comparable

Based on the developmental stages, Gn-D10 group recorded increased percentage of early blastocysts and blastocysts than the control group (Table 5). Thus homogenous recruitment and development of healthy follicles under an appropriate endocrine milieu would have contributed for the better developmental quality of embryos in Gn-D10 group as suggested by Yadav *et al.* (1986).

Table – 5. Percentage of different developmental stage embryos recovered from superovulated crossbred cows

Treatment groups (Number of embryos in parenthesis)	DEVELOPMENTAL STAGES OF EMBRYOS (%) (Number of embryos in parenthesis)			
	Morula	Compact morula	Early Blastocyst	Blastocyst
Control (46)	34.8% (16)	36.9% (17)	23.9% (11)	4.3% (2)
Gn-D8 (7)	14.3% (1)	42.9% (3)	28.6% (2)	14.3% (1)
Gn-D10 (38)	15.8% (6)	36.8% (14)	42.1% (16)	5.3% (2)

The study proved the hypothesis that GnRH pretreatment ensured increased availability of gonadotropin responsive follicles on the day of initiation of superstimulation treatment. However, initiating the gonadotropin treatment two days post synchronized FWE (before the deviation) had the advantage of proper follicular maturation and favourable endocrine environment in achieving satisfactory SOR and embryo yield, rather than initiating the treatment on the day of synchronized FWE . The problems recorded in Gn-D8 group during the study might be the reasons for limited success by the previous researchers (Kohram et al.,1998 and Deyo *et al.* 2001) when GnRH pretreatment was included in superovulation schedule.

From This study, it was concluded that administration of GnRH on Day 6 of the cycle and initiation of FSH treatment on Day 10 resulted in homogenous recruitment of healthy follicular inventories. Under a favourable endocrine environment satisfactory SOR and embryo yield could be achieved. With the recovery of increased percentage of transferable quality and better developed embryos than the conventional method, it is suggested that GnRH pretreatment could be successfully incorporated in the superstimulation protocol of cattle.

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Protein Profile of Granulosa Cells in Cyclic and Acyclic Buffaloes

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ABSTRACT

The objective of the study is to analyze the alterations in the protein profile of follicular cells in normal cyclic and acyclic anoestrus water buffaloes. Ovaries from sexually mature buffaloes were collected from abattoir and categorized into two groups viz., i. Cyclic and ii. Acyclic. In both the categories of ovaries, the follicular fluid (FF) was aspirated from all large follicles (≥ 9mm diameter) and small follicles (< 9 mm diameter) separately. FF was centrifuged and four categories of pelleted granulosa cells (CSG and ASG: Granulosa cells of small follicles in cyclic and acyclic groups respectively; CLG and ALG: Granulosa cells of large follicles in cyclic and acyclic groups respectively) were subjected for SDS-PAGE analysis. A total of 30 and 18 bands (from 7.1 to 209.0 kDa) were observed in CSG and CLG categories, while 16 bands in each of the ASG and ALG were recorded. It was observed that 40 per cent of bands present in the CSG group were not observed in CLG group. On the contrary, almost all the proteins found in ASG group were retained in the ALG group. It can be inferred that a group of small molecular weight granulosa cell proteins, correlating to IGF / IGFBP system, play a key role in providing a favorable proteo-genomic environment in the early stages of follicular development. In acyclic animals, retaining of such proteins in the large follicles indicated that the non-availability of metabolic factors needed for final maturation of follicles.

Key words: Buffaloes, Anoestrus, Follicular cells, Protein profile analysis

INTRODUCTION

Water buffaloes (*Bubalus bubalis*) are the major contributors of the dairy sector in the south Asian countries. However the milk production parameters of buffaloes are dependent on their reproductive efficiency. Acyclicity due to anovulatory anoestrus is the major disturbance reported to be affecting the reproductive efficiency of buffaloes (Abraham, 2017). Inspite of being acyclic, the anoestrus cattle are found to exhibit usual ovarian follicular wave activity, but the follicles that are able to attain ovulatory size failed to induce oestrus signs and to ovulate (Ghuman *et al.,* 2010; Satheshkumar *et al.,*2012). Khan *et al.* (2013) reported a lower oestradiol: Progesterone ratio in the follicular fluid (FF)

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of acyclic buffaloes. Further anovulatory anoestrus is possibly associated with insufficient production of steroid hormones by the growing preovulatory follicle, which is normally characterized by a high steroidogenic synthesis by the cytochrome P450scc and P450arom enzymes (Diaz *et al.,* 2012). These findings were indicative of failure in follicular maturation and defective steroidogenic activity in the follicular micro-environment which affected the oestrus expression and ovulation thereon.

The functional status of follicular cells is reflected in biochemical and protein profile of FF in buffaloes (Joy *et al.,* 2015). Changes in mRNA expression of various genomic factors (gonadotropin receptors, growth factors and their binding proteins etc.,) associated with different stages of follicular growth and atresia has been appreciated in follicular cells (Mishra *et al.,* 2015). Apart from the changes in mRNA expression pattern, it was hypothesized that the proteomic factors of follicular cells will alter in relation to physiological status of follicular development. Hence the present research was conducted to study the protein profile of granulosa cells in cyclic and acyclic buffaloes by Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and to analyse the electrophoretic patterns between the two groups of animals.

MATERIALS AND METHODS

Source and preparation of ovaries

Ovaries were collected individually from sexually mature buffaloes (*Bubalus bubalis*) from Chennai Corporation abattoir and utilized for the study. The individual pair of ovaries were collected from each animal immediately after slaughter, washed in phosphate buffered saline (PBS) and transported at 37ºC in PBS to the laboratory within 30 minutes after collection.

The pair of ovaries was categorized into two groups viz., i. Cyclic: If prominent corpus luteum (CL) tissue is present in any one of the ovaries and ii. Acyclic: If no CL is present in both the ovaries.

In both the categories of ovaries, the FF was aspirated from all large follicles ($\geq$ 9mm diameter) and small follicles ($<$ 9 mm diameter) separately using a sterile hypodermic insulin syringe and dispensed in separate petri dishes. The FF from one to two large follicles and four to five small follicles were considered as a single aliquot respectively. The aspirated FF was screened and oocytes were recovered. After the oocyte recovery, the FF was transferred to 1.5ml micro-centrifuge tube and centrifuged at 10,000 g for 10-15 minutes at 4ºC. The supernatant FF was stored in separate micro-centrifuge tubes and four categories of pelleted granulosa cells (CSG and ASG: Granulosa cells of small follicles in cyclic and acyclic groups respectively; CLG and ALG: Granulosa cells of large follicles in cyclic and acyclic groups respectively) were stored at -20ºC for protein analysis. A total of 10 aliquots were studied in each category.

Total protein was extracted from granulosa cell pellets of all the four categories as described by Gerard *et al.* (1998). The total soluble protein (TP) was estimated by Bradford protein assay. Based on the TP concentration the extracted samples were evenly corrected to a concentration of 6mg/

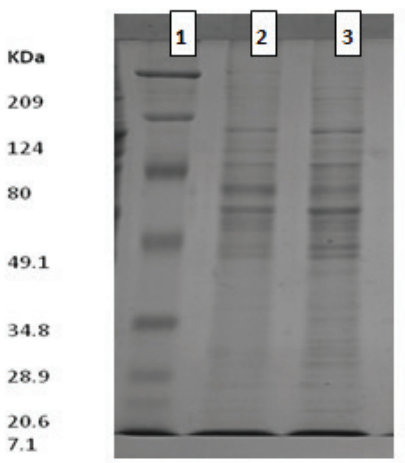
dl with 1X PBS and further diluted to 1:10 ratio with 1X PBS and the protein profile were studied by standard SDS-PAGE method with a 12% separating gel and a 5% stacking gel. Broad range molecular-weight (MW) standards (Bio-Rad) were also routinely loaded. Electrophoresis was performed at a constant intensity of 50 mA / gel. At the end of migration, gels were stained with Coomassie blue stain overnight at room temperature and destained by repeated rinsing in gel de-staining solution. The enhanced chemiluminescence detection system (Amersham Life Science, Buckinghamshire, UK) was used to detect polypeptides.

RESULTS AND DISCUSSION

Comparative SDS-PAGE patterns of granulosa cells of small and large follicles in cyclic and acyclic buffaloes are presented in Table 1, Fig. 1 and Fig.2 respectively.

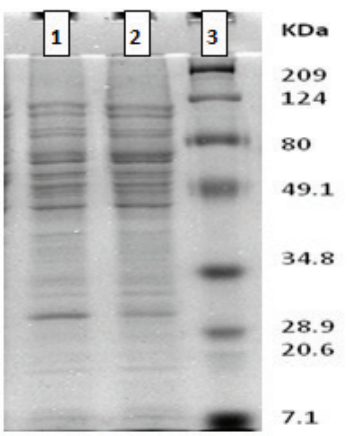
Table – 1. SDS-PAGE patterns of granulosa cells in cyclic and acyclic buffaloes

Molecular weight (kDA)	No. of detectable bands			
	CSG	CLG	ASG	ALG
>124 – 209	4	3	--	--
>80 – 124	4	3	4	4
>49.1 – 80	6	6	4	4
>34.8 – 49.1	6	4	4	5
>28.9 – 34.8	7	1	3	3
>20.6 – 28.9	2	--	--	--
7.1 – 20.6	1	1	1	--
Total no. of bands	**30**	**18**	**16**	**16**



Lane 1 : Protein marker (Broad range)
Lane 2 : Large follicular cells
Lane 3 : Small follicular cells

Fig.1: SDS-PAGE patterns of granulosa cells in cyclic buffaloes



Lane 1 : Large follicular cells
Lane 2 : Small follicular cells
Lane 3 : Protein marker (Broad range)

Fig.2: SDS-PAGE patterns of granulosa cells in acyclic buffaloes

SDS-PAGE analysis of follicular cell proteins revealed differences in the overall electrophoretic pattern between acyclic and cyclic buffaloes. A total of 30 and 18 bands of MW ranging from 7.1 to 209.0 kDa were observed in CSG and CLG categories, while 16 bands in each of the ASG and ALG were recorded. However, Khan *et al.* (2013) reported that there were no qualitative alterations in the protein component of the FF during acyclicity in buffaloes. A maximum of 30 protein bands were detected in the granulosa cell lystaes of cyclic buffaloes in the present study which is in accordance with the findings of Kulkarni (1990) who recorded 26 protein bands in bubaline FF.

In cyclic animals, 40 per cent of protein bands (especially in the range of 20.6 – 34.8 kDa) present in the small follicles obviously disappeared in the large follicles. On the contrary, in acyclic group, all the proteins present in the small follicles were found retained in the large follicles. It can be inferred that a group of small MW granulosa cell proteins play a key role in building up the essential nutrients, hormones and growth factors thus providing a favorable proteo-genomic environment in the early stages of follicular development. Under the gonadotrophin stimulus, these small MW proteins might decrease in concentration and release the sequestered metabolic factors during final stages of follicular development enabling the attainment of dominance. In acyclic animals, retaining of such proteins in the large follicles indicated that the bioavailability of metabolic factors needed for final maturation of follicles was prevented.

A prominent couplet of bands is observed just below the 49.1 kDa protein range in CSG group, which is much faintly expressed in granulosa cells of CLG group. This couplet of bands observed can be correlated to 44–42 kDa native IGFBP-3 as reported by Mazerbourg and Monget (2018). In cyclic animals, the disappearance of IGFBP-3 in the larger follicles signifies the bioavailability of free IGF which ensures follicular maturation. Likewise, an intense band of protein with a MW of about 30 kDa was present in small follicular cells of both the cyclic and acyclic group, which was found to be retained in large follicles of acyclic animals but disappeared in cyclic animals. Thus it could be speculated that the disappearance of that protein might have a major role in terminal follicular development. Similarly, Nandi *et al.* (2006) demonstrated a 30.1 kDa ovine intrafollicular factor that was inhibitory for both cumulus and granulosa cell proliferation *in vitro*. Bridges *et al.* (2002) and Mazerbourg and Monget (2018) stated that the apparent MW of IGFBP-5 ranged between 29 and 30 kDa. The findings confirmed the fact that the concentrations of IGFBPs decrease during final stages of follicular growth, leading to an increase in IGF bioavailability for maturation, oestradiol production and ovulation in normally cycling animals (Fortune *et al.,* 2004). On the other hand, IGFBPs persist in the follicular cells thus sequestering the IGF and preventing the progress in follicular maturation (Braw-Tal *et al.,* 2009). Thus the failure in function of the IGF system in granulosa cells might contribute for the deficient follicular metabolism and steroidogenesis leading to anovulatory anoestrus condition.

No high MW proteins in the range between 124 to 209 kDa were detected in both the follicular categories of acyclic group. On the contrary, 3-4 bands were observed in this range in cyclic animals. In accordance with our findings, Gerard *et al.* (1998) demonstrated presence of a 200-kDa protein in granulosa cells lysates recovered from equine preovulatory follicles. They attributed that this stage specific expression of high MW protein might be involved in the differentiation and maturation mechanisms occurring in the follicle during the preovulatory period. Rivera and Fortune (2003) described Pregnancy-associated plasma protein A (PAPP-A), which is composed of two 200-kDa disulfide-bonded subunits, is the major protease that lyses the IGFBP-4/-5 and has been detected in the FF of bovine preovulatory follicles (Mazerbourg *et al.,* 2001). In light of the present results, it is tempting to speculate that such high MW proteolytic system is active in the cyclic animals, but not in the acyclic animals.

Eventhough we have not conducted specific protein characterization studies, based on the comparisons with previous literatures it could be concluded that low MW proteins in the granulosa cells, correlating to IGF / IGFBP system, play a key role in the follicular maturation. Factors affecting the final stages of development of dominant follicle are closely related to the metabolic status of animals. Reduced plasma concentrations of metabolic hormones (insulin) and related growth factors (IGF) were reported in nutrient-restricted anoestrus cows and these aberrations are thought to have direct effects on follicular dysfunction (Spicer and Echternkamp, 1995). Thus the study confirmed the hypothesis that the proteomic factors of follicular cells were altered in relation to the physiological status of follicular development.

It could be concluded that failure of the intra-follicular IGF / IGFBP system, probably due to deficient nutritional status, lead to ovulatory disturbances and follicular dysfunction in acyclic buffaloes.

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Short Communication

Surgical Removal of Shaving Blade from Stomach of Dog

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ABSTRACT

A 2 years old Labrador dog brought with history of ingestion of a newly paper covered packet shaving blade immediately falling from owner's hand during his shaving one day after the incident. There were no clinical signs observed and the dog was normal. Lateral radiography taken showed radio-opaque shaving blade in the stomach area. Solid food was not given after ingestion of shaving blade till surgical intervention and under general anaesthesia shaving blade was removed surgically. The stomach was sutured with vicryl no. 1/0 in continuous lumbert and cushing pattern in double layer. Post-operatively administration of antibiotics, analgesics and regular dressing of wound was done and dog recovered uneventfully.

Key Words: Dog, History, Radiograph and Anaesthesia.

Dogs are very active and playful and sometime take non-food objects leading to gastric foreign body syndrome (Tripathi *et al.,* 2010).Gastro-intestinal foreign bodies are challenging and difficult to manage and their treatment depends on its location and degree of obstruction. Foreign body fixed with tissue and sharp foreign bodies are not recommended for endoscopic examination. Sharp foreign body might lead to perforation of the organ and anchor in pylorus may require surgical extraction (Tobias, 2006) Stones, bones, coins, balls, clothes, rags, metallic spoons, rubber nipples and magnets are some of the indiscriminately fed objects reported in dogs (Mohindroo *et al.,* 2006; Hayes, 2009). Playing habits and more activity of young dog are responsible for ingestion and subsequent lodgement of foreign body in GIT (Koike *et al.,* 1981; Applewhite *et al.,* 2002; Han *et al.,* 2008).

A 2 years old male Labrador dog weighing 35 kg presented in the clinics with history of ingestion of shaving blade one day before and animal was kept without foods and water. The activity of dog was normal. The temperature, heart rate and respiration rate were not changed. Lateral radiograph showed picture of shaving blade

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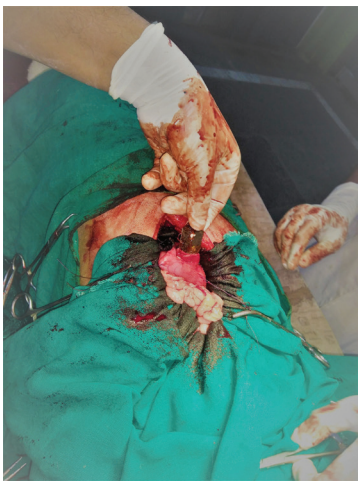
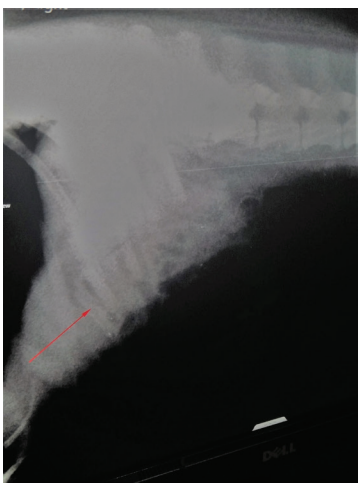
with radio-opaque density in stomach area (Fig. 1). Animal was stabilized with IV DNS-5%. Next day surgical removal was planned.



The general anaesthesia used in dog was combination of atropine 0.04 mg/kg body weight, xylazine 1mg/kg body weight and ketamine 5mg/kg body weight intramuscularly. Dog was maintained anaesthetic condition by repeated dose of ketamine intermittently with intravenous route. Animal was restrained in dorsal recumbency and behind the post-xiphoid area of abdomen was shaved and prepared aseptically for surgery. Abdominal cavity approached by incision just behind post-xiphoid in direction along linea alba. Stomach was exteriorized. Least vascular area of stomach was incised and stomach kept in outside by stay suture. Shaving blade was explored and removed outside by using alies forceps (Fig. 1). The stomach was sutured by lambert suture followed by cushing to prevent leakage with vicryl 1/0. Muscle and peritoneum were sutured in simple interrupted pattern with vicryl 1/0. Silk was used to suture the skin with horizontal pattern. Dog was treated post-operatively with ceftriazone & sulbactum inj. @ 10 mg/kg body weight I.M. once daily for 8 days and inj. Meloxicam @ 0.2 mg/kg body weight i.m. once daily for three days.Food and water were not given to animal for 4 days. In this period animal was given RL 250 ml and DNS 5% 250 ml intravenously twice daily. Multivitamin inj. 2ml (CB12) was mixed in the fluid for three days. Glucose mixed with water was given from 4th day. Milk was allowed from 7th day onward. Suture removed on 12th postoperative day. Animal recovered uneventfully.



Feeding habit of the dog might be leading to foreign body syndrome at any age of the dog with gastric obstruction but more commonly found in younger dogs. There are many reasons for intestinal obstruction in dog e.g. foreign body, neoplasia and peritonitis. In this case, a newly paper covered packet of shaving blade ingested was surgically removed since the case was recent. All physical parameter was normal and the dog was not showing any symptoms. Foreign body

found in fundus of stomach do not show any symptom and if they are located in pyloric area of stomach, gastric emptying may be impaired (Uma Rani *et al.,* 2010). Foreign bodies cause gastric outflow obstruction, gastric perforation or systemic illness on breakdown and absorption of foreign body (Patil *et al.,* 2010). Gastrotomy is performed for removal of foreign bodies and stomach tumours in treatment of stomach problems (Haragopal and Suresh Kumar 1996.) and early diagnosis and surgery of the organ are important as the chance of perforation and peritonitis increase with delay in surgery.

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Multiple Infections in Pacific White Shrimp (*Penaeus vannamei*) with Black Gill Disease

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ABSTRACT

Penaeus vannamei (Pacific white shrimp) is the most extensively farmed among the crustaceans globally. Disease is the major impeding factor in the commercial culture of *P. vannamei* undertaken in freshwater, brackish and marine aquaculture systems. In this study, *P.vannamei* samples were collected from a commercial shrimp farm at Tiruvallur district, Tamilnadu that has reported black gill disease. The clinical symptoms observed in the shrimp were melanized black gills, reduced feeding, surfacing and mortality in the early morning. Samples of gills were collected aspectically and diagnosed for bacterial Acute Hepatopancreatic Necrosis Disease (AHPND); viral diseases *viz.,* Gill Associated Virus (GAV), White Spot Syndrome Virus (WSSV), Yellow Head Virus (YHV), Infectious Hypodermal and Hematopoietic Necrosis Virus (IHHNV) and fungal infections by conventional diagnostic methods and PCR following confirmation by nucleotide sequencing. The results of the study showed that *P.vannamei* infected with black gill disease had multiple infections with fungus, *Aspergillus niger* and virus WSSV.

Key words: *Penaeus vannamei,* Black gill diseases, WSSV, *Aspergillus* sp.

INTRODUCTION

Sea foods are important and cheap source of protein. As shrimp is considered a delicacy among the consumers in many countries, there is a growing demand for shrimp in the world market (Belton and Thilsted, 2014). India is the one of the leading producer of cultured *P.vannamei* (MPEDA, 2017). Although shrimp farming has grown rapidly, its development and expansion is seriously affected due to various diseases caused by diverse pathogens (Lightner *et al.,* 1997; Durand *et al.,* 2003). Among viral pathogens infecting shrimp, WSSV is considered a highly virulent pathogen causing mortality in shrimp within 3 to 7 days of infection resulting in severe economic losses to the farmers (Zhan *et al.,* 1998; Corbel *et al.,* 2001; Lightner, 2011). Other viral

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pathogens *viz*., Gill-associated virus (GAV), a single-stranded RNA virus and Yellow head virus (YHV) have been reported to cause mass mortalities in penaeid shrimps cultured in Australia and Asia (Spann *et al.,* 1997; Cowley *et al.,* 2000). Diseases caused by *Vibrio* sp. are the most common among the bacterial diseases infecting shrimp (Chatterjee and Halder, 2012). Fungal pathogens have often been reported to be associated with the discoloration and diseases of gills (Rhoobunjongde *et al.,* 1991). Black gill associated with *Fusarium* sp. in Japanese kuruma prawn, *P. japonicus* (Ishikawa *et al.,*1968) and *Aspergillus flavus* has been reported in *P.vannamei* (Dewangan *et al.,* 2015).The objective of this work is to identify the causative of black gill in *P.vannamei* sample from a commercial shrimp farm.

MATERIALS AND METHODS

Sample collection

P. vannamei, Pacific white shrimp (20±2g) exhibiting symptoms of black gill condition were collected from a commercial shrimp farm that had reported a disease outbreak in Tiruvallur district, Tamilnadu (13°19'25.2"N 80°13'44.7"E), India during November 2017. The details *viz.,* stocking density, days of culture (DOC), physico-chemical parameters of water (pH, salinity, DO, alkalinity, hardness, ammonia and H_2S) were recorded at site. Samples of gills from live *P.vannamei* samples (n=10) were collected aseptically for microbiology or fixed in Davidson's fixative for histopathology and 70% ethyl alcohol for PCR.

Isolation and identification of pathogens

Aliquots of the fresh gill tissue were observed under the microscope as wet mounts (40x magnification). For isolation of fungi, the gill tissue were cut into small pieces, rinsed several times in sterile saline (0.85% NaCl) and inoculated in potato dextrose agar (PDA) supplemented with ampicillin and streptomycin and incubated at 25ºC for one to three days. An agar block with the fungal mycelia was placed on PDA (with ampicillin and streptomycin) and incubated at 25ºC in dark (Khoa *et al.,* 2004). The observations such as colour and colony characteristics were recorded. The fungal isolates grown on the media were stained with lactophenol cotton blue and the morphology was observed under the microscope (40x and 100x) and identified (Barnett and Hunter, 1999). Inocula from the gills with black discolouration were also plated on to TCBS agar and incubated for 24 h at 37^0C to facilitate the growth of *Vibrio* spp., the most common bacterial pathogen infecting shrimp.

Molecular identification of viral and fungal pathogens

Total genomic DNA was extracted from the gill samples and fungal isolates using QIAamp genomic DNA kits (Qiagen, Germany) following manufacturer's protocol for the identification of pathogens by PCR amplification and nucleotide sequencing. Total RNA was extracted (Trizol reagent, Sigma) and reverse transcribed to cDNA (High capacity cDNA synthesis kit, Applied Bio systems Inc., USA) following the manufacturer's instructions. PCR amplification was carried out in a thermal

cycler (Biorad T100 Thermal cycler, USA) in a total volume of 25µl reaction mixture containing 2X PCR mastermix (Ampliqon, Denmark), 1 µl (10 pmol) of forward and reverse primer each and 1µl of sample DNA. Published protocols were followed for the PCR diagnosis of WSSV (Takahashi *et al.,* 1996), GAV (Cowley *et al.,* 2000), IHHNV (Tang *et al.,* 2007), and YHV (Cowley *et al.,* 2004). For fungal identification, ITS1 and ITS4 fungal rRNA gene-specific primers were used for PCR amplification (White *et al.,* 1990; and Gardes and Bruns, 1993). Nested PCR reactions were carried out similar to the first step PCR except for the nested primers and 1 µl of the first step product as the template. An aliquot of PCR product was separated on a 1.5 % agarose gel and visualised under UV illumination using a gel documentation system (Biorad, USA).The PCR amplified products of fungal ITS was purified and sequenced using both forward and reverse primers (Eurofins, Bangalore, India). The sequence was compared with the available sequences in the GenBank using BLASTn (Basic Local Alignment Search Tool) and the sequence information was submitted to the GenBank (www.ncbi.nlm.nih.gov).

RESULTS AND DISCUSSION

The observed values of various physico-chemical parameters of the water samples collected from the farm is presented in Table 1.The clinical signs observed in the gills of *P. vannamei* shrimp samples include, black discoloration (Fig. 1), reduced feed consumption and mortality in the early morning. More than 50% of the shrimp population was observed to have black gills condition. Various factors have been reported to be associated with blackening and diseases of gill in shrimp. Presence of various parameters above optimum levels *viz.,* total ammonia (<1.0ppm), H_2S (<0.02ppm), nitrite concentration (2-3 ppm) combined with high organic matter and deposition of debris (Lavilla-Pitogo *et al.,* 2000), nitrate, acids, crude oils, potassium premanganate, cadmium, copper and ozone results in discoloration of gills (Lightner, 1996). High ammonia and H_2S levels in ponds are usually associated with poor pond bottom and favor the growth of fungi and other pathogenic microbes (Soegianto *et al.,* 1999).

Table – 1. Physico-chemical parameters in the water sample from *P.vannamei* pond.

Parameter	Value
Temperature(°C)	30°C
Dissolved oxygen (ppm)	3.5
Salinity (ppt)	30
pH	8.1
Alkalinity (ppm)	150
Total Hardness (ppm)	4500
Ammonia (ppm)	1.2
H_2S (ppm)	0.05

Microscopic observation of wet mounts of affected gills showed dark discolouration with numerous fungal hyphae attached to the gills. Fungal growth was observed in the PDA plates after 3 days. Microscopic observation of the lactophenol blue stained fungal colonies that were yellowish or yellowish green in colour on plates revealed hyaline, septate hyphae, with long conidiophores with globose tip and Phialides around the vesicle which are characteristic of *Aspergillus* sp. (Fig. 2). In this study, we have isolated *A.niger* from *P.vannamei* with black gills from ponds with higher ammonia (1.2ppm) and H_2S (0.05ppm) levels. Infection with fungus *viz., Fusarium* sp., (Hatai and Egusa, 1978; Colorni *et al.,* 1989). *A.niger* (Fernand *et al.,* 2017) and *A. flavus* (Dewangan *et al.,* 2015) have been reported to cause melanized gill filaments resulting in black gill condition or disease. Gills are crucial for respiration, excretion, acid–base balance, and osmotic and ionic regulation (Ramaiah, 2006). As gills are immediately exposed to the external environment and are the first organs to be exposed to pollutants, gill diseases in shrimp may result in their death due to destruction of gills or suffocation caused by mechanical blockage of gas exchange across the surface of the gill lamellae (Lightner, 1996).

Fig.1 *P. vannamei* with black gill condition.

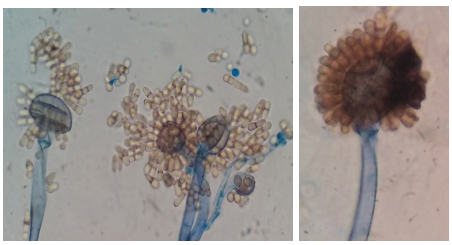

Fig.2 Microscopic observation of *Aspergillus* showing fungal hyphae and conidia (100×).

Histopathological sections of gills showed congestion, fusion of gill lamellae and multifocal mild degeneration and necrosis of secondary gill epithelium with multifocal mild mononuclear cell infiltration in the gill rackers (Fig. 3). Sequence comparison of the fungal isolate from black gill confirmed it as *Aspergillus niger* (Genbank Accession No. MH521172) as it showed higher similarity with other *A.niger* sequences in the BLAST analysis. PCR diagnosis for viral diseases resulted in the amplification of WSSV of expected product sizes (bp) (Fig. 4). No bacterial growth was observed in TCBS thus ruling out the role of *Vibrio* sp. infection. Hence, *P.vannamei* with black gill condition has been confirmed to have multiple infections with the fungus, *Aspergillus niger* and WSSV. Black gill disease in *P.vannamei* caused by *A.flavus* has been reported from India (Dewangan *et al.,* 2015). In addition, shrimps with black gills, when harvested, are not preferred for head-on packing and hence fetch very less market price. Treatment in shrimp with black gills may be treated effectively by bath treatment with 2 to 3 ppm concentration of furazolidone for 2 to 4 days (Shigueno, 1975). Fungal infections pose a significant threat to shrimp culture as

infection in gills would affect the respiration and subsequently their health status, making them susceptible to other diseases. Multiple infections in penaeid shrimp with bacteria and fungi (Kusumaningrum and Zainuri, 2015) and infection due to multiple virus have been reported earlier (Manivannan *et al.,* 2002; Anshary and Baxa, 2017). In our study, we observed that *P.vannamei* shrimp with black gill disease had multiple infections with *A.niger,* and virus WSSV. Reducing the organic load following good management practices (GMPs) will provide a pollution-free healthy environment to ensure the health status in the culture of *P.vannamei* and improves its sustainability in farming practices.

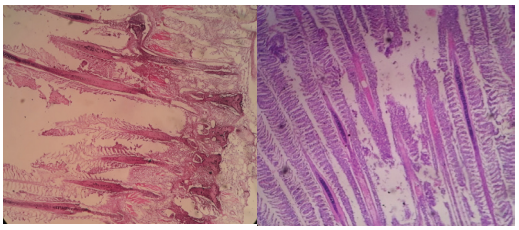

Fig. 3 Tissue sections of gill lamella from black gills showing changes.

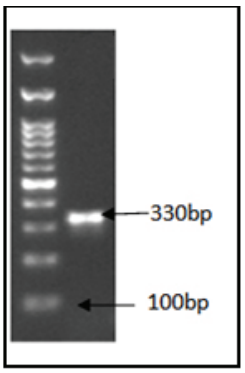

Fig. 4 PCR amplified product WSSV (330bp) in *P.vannamei* with black gill condition.

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All the contributing authors are requested to bestow their personal attention while submitting the revised manuscripts for spelling mistakes and correctness of language.

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