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Review article

Full length articles

Short Communications

Review article

Diagnostic and Therapeutic approach to a Chronic Kidney Diseases in Dogs

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INTRODUCTION

Chronic Kidney Disease (CKD) is defined as the presence of structural or functional abnormalities in one or both kidneys that have been present for an extended period usually from three months or more. Nephron damage associated with CKD is usually irreversible and progressive and characterized by a wide spectrum of disease, ranging from a minor structural lesion in a single kidney to extensive loss of nephrons affecting both kidneys (Polzin, 2011).CKD is a major cause of morbidity and mortality, especially in older dogs and cats, which were recorded with an overallincidence of 1- 3 per cent in cats and 0.5 – 1.5 per cent in dogs (Brown, 2007).

Azotemia is defined as an abnormal concentration of urea, creatinine, and other nonprotein nitrogenous substances in blood, plasma, or serum. Azotemia is a laboratory finding with several fundamentally different causes. Since non-protein nitrogenous compounds (including urea and creatinine) are endogenous substances, abnormally elevated concentrations in serum may be caused by an increased rate of production (by the liver for urea; by muscles for creatinine), or by a decreased rate of loss (primarily by the kidneys).

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When the structural and functional integrity of both kidneys has been compromised to such a degree that polysystemic signs of kidney failure are clinically manifested, the relatively predictable symptom complex called uremia appears, regardless of underlying cause. In some instances, uremic crises may suddenly be precipitated by prerenal disorders or, less commonly, postrenal disorders in patients with previously compensated primary kidney failure. Uremia is characterized by multiple physiologic and metabolic alterations that result from impaired kidney function.

Classification and Staging of CKD

There are several classification systems like AKIN, KDIGO etc. have been existed and categorized the renal diseases in human medicine, whereas International Renal Interest Society (IRIS) has created to advance the scientific understanding of kidney disease in small animals particularly to help practitioners better diagnose, understand, and treat canine and feline renal disease. The IRIS has been classified into four stages of renal patients in pet animals primarily based on the serum creatinine levels and sub-staging was done considering the levels of proteinuria and systemic blood pressure (Table 1-3).

The stage of CKD is based on the level of kidney function as measured by the patient has been fasted and is well hydrated. serum creatinine concentrations. Staging should be based on a minimum of two serum creatinine values obtained when the

Table. 1: International Renal Interest Society Staging of Chronic Kidney Disease in Dogs and Cats based on the Serum Creatinine levels.

IRIS Stage of CKD	Serum Creatinine Values (mg/dl)	
	Dogs	Cats
Stage I	<1.4 (<125mmol/L)	<1.6 (<140mmol/L)
Stage II	1.4 – 2.0 (125-179 mmol/L)	1.6 – 2.8 (140-249 mmol/L)
Stage III	2.0 – 5.0 (180-439 mmol/L)	2.9 – 5.0 (250-439 mmol/L)
Stage IV	>5.0 (>440 mmol/L)	>5.0 (>440 mmol/L)

***http://www.iris-kidney.com.**

Table. 2: International Renal Interest Society sub staging of Chronic Kidney Disease in Dogsand Cats based on Proteinuria.

Classification	Urine Protein: Creatinine ratio	
	Dogs	Cats
Nonproteinuria	≤0.2	≤0.2
Borderline proteinuria	0.2-0.5	0.2-0.4
Proteinuria	>0.5	>0.5

***Based on ACVIM consensus statement on proteinuria (Lees, 2005).**

Table. 3: International Renal Interest Society sub staging of Chronic Kidney Disease in Dogsand Cats based on the blood pressure.

Hypertensive stage	Arterial Pressure	
	Systolic BP (mmHg)	Diastolic BP (mmHg)
Risk	<150	<95
Mild	150 - 159	95 - 99
Moderate	160 - 179	100 - 119
Severe	≥180	≥120

***http://www.iris-kidney.com.**

Proteinuria is an important risk factor for the development of azotemia in cats and the progression of azotemia and decreased survival in both dogs and cats. Presence or absence of proteinuria is used to substage CKD (Table 2) in the IRIS staging system. Renal proteinuria can be glomerular and/ or tubular in origin (ie, excessive filtration, decreased tubular reabsorption, or both). Renal proteinuria is persistent—with at least 2 positive tests separated by 10 to 14 days—and associated with inactive urine sediments. Urine protein/creatinine ratios (UPCs) > 2 suggest glomerular-range proteinuria, which is rare in cats compared with dogs.

IRIS blood pressure substaging is based, in part, on risk of target organ—eye, brain, heart, and kidney—damage (Table 3). In the absence of target organ damage, persistence of hypertension should be documented. Systolic blood pressure is typically measured by the Doppler methodology in dogs and cats.

Diagnosis of Chronic Kidney Disease in dogs

As the kidney performs several functions in the body, dysfunction of kidneys exerts abnormalities in multiple systems. Hence, we needs to consider several factors while assessing functional status and stage of dysfunction in renal diseases. It includes physical parameters, renal function tests, serum electrolyte concentrations, acid-base status, urinalysis, renal biomarkers and renal imaging studies.

Physical Assessment

In dogs polyuria (PU) and polydipsia (PD) may be the first indication of CKD. Cats maintain their urine concentrating ability further into the disease process than dogs; therefore, PU/PD is often not recognized in early stages of CKD in cats. As urine concentrating ability is lost later as the disease progresses, cat owners are more likely to recognize PD than PU. In addition, dogs and cats in IRIS stages 3 and 4 often present with nonspecific signs, including poor body condition, weight loss, decreased appetite, lethargy and dehydration. Intermittent vomiting secondary to uremic gastric ulceration may occur.

Physical examination findings in CKD patients will vary depending on the stage of disease. Early in the disease (IRIS stages 1 and 2), physical examination may be within normal limits. Palpable renal abnormalities may be detected especially in cats with polycystic kidney disease. As CKD progresses to IRIS stages 3 and 4, clinical signs will become more apparent and reflect the chronic nature of the disease. General physical examination findings include poor body condition, rough hair coat, dehydration and palpable kidney abnormalities. Oral examination may reveal pale mucous membranes, ulcers and/or uremic breath. Secondary systemic hypertension may

Selected non-invasive markers of kidney disease.

Marker	Information provided[a]	Measured in serum/plasma or urine[b]
Serum creatinine	Estimate of GFR	Serum/plasma
Cystatin C	Estimate of GFR	Serum/plasma
Symmetric dimethylarginine	Estimate of GFR	Serum/plasma
Gel electrophoresis(e.g.SDS-PAGE)	Glomerular and/or tubular damage/dysfunction	Urine
Urine protein:creatinine	Glomerular and/or tubular damage/dysfunction	Urine
Albumin	Glomerular and/or tubular damage/dysfunction	Urine
C-reactive protein	Glomerular damage/dysfunction	Urine
Immunoglobulins A,G and M	Glomerular damage/dysfunction	Urine
N-acetyl-β -D-glucosaminidase	Tubular damage/dysfunction(but also increase with glomerular damage/dysfunction	Urine
Clusterin	Tubular damage/dysfunction	Urine
Cystatin C	Tubular damage/dysfunction	Urine
Gamma glutamyl-transpeptidase	Tubular damage/dysfunction	Urine
Kidney injury molecule-1	Tubular damage/dysfunction	Urine
Neutrophil gelatinase-associated lipocalin	Tubular damage/dysfunction	Urine
Retinol binding protein	Tubular damage/dysfunction	Urine
Tamm-Horsfall protein	Tubular damage/dysfunction	Urine

SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis;GFR,glomerular filteration rate.

[a] For all protein measurements in urine,interpretation assumes absence of post-renal disease(i.e.inactive sediment).

[b] For UPC and all individual urinary biomarkers,urine creatinine concentration must also be measured for normalization purposes.

Hemato-biochemical Parameters in CKD dogs and cats

Common abnormal findings on the CBC and chemistry panel include azotemia (elevation of BUN and creatinine), hypoproteinemia, hypoalbuminemia, nonregenerativeanemia, hyperphosphatemia, hypercalcemia or hypocalcemia, hypokalemia (in cats) and metabolic acidosis.

Role of biomarkers in the early diagnosis of the renal diseases

A diagnosis of CKD is typically straight forward once the disease is in its later stages and there is clinical suspicion based on history and physical examination findings, azotemia evident on biochemical profile and loss of urine concentrating ability (<1.030 in dogs and <1.035 in cats). However, recognition of CKD can be challenging early in the course of disease since clinical signs may be absent, mild or attributed to another concurrent condition. Additionally, azotemia does not typically develop until approximately 75% loss of nephron function, and in cats especially, PU/PD may not be evident or noticed by owner.

Serum creatinine and blood urea nitrogen (BUN) are routinely used biochemical tests to help diagnose kidney disease. BUN can be influenced by several extrarenal factors, including dehydration, protein content of the diet, gastrointestinal bleeding and liver insufficiency. Creatinine is a breakdown product of muscle and is a better indicator of glomerular filtration rate (GFR) than BUN, but it can be influenced by a reduction in muscle mass, which is not uncommon especially in older animals with CKD.

It is generally accepted that creatinine does not increase until 75% of renal function is lost, and measuring GFR is done infrequently in the private practice setting. Clearly, there is a need for a more sensitive test of renal function.

Symmetric dimethylarginine (SDMA) is a relatively newly discovered renal biomarker. SDMA is primarily eliminated by renal excretion. Therefore, it is an endogenous marker of GFR. It is not influenced by muscle mass, which is an advantage in comparison with creatinine. So far, SDMA has been used successfully to diagnose CKD in dogs and cats.

Because SDMA will help clinicians diagnose CKD earlier when dogs and cats are likely to still be in IRIS stage 1 or early IRIS stage 2, early intervention strategies are needed. Early identification of CKD should prompt investigation for an underlying cause, giving the potential for specific treatment. It will allow substaging of the CKD so that proteinuria and hypertension can be detected and managed earlier in the disease process. Early management of CKD may slow progression of the disease. Closer monitoring will help identify progression and when additional therapies should be initiated.

List of Urinary biomarkers

Urinary marker	Causes of urinary marker elevetion
Markers of glomerular impairment	
Albumin	X-linked hereditary nephropathy (before the onset of overt proteinuria) (Lees 2002)
	Glomerular disease in Soft Coated Wheaten Terriers (Vaden et al.2001)
	Chronic kidney disease, CKD with hypertension (Basic et al.2010)
	Hypertension without CKD (Surman et al.2012)
	Systemic disease which may secondarily affect the kidneys (Whittemore et al. 2006)
	Diabetic nephropathy in dogs and cats (Struble et al.1998:Al-Ghazlat et al.2011)
	Severe inflammatory response syndrome (Schaefer et al.2011)
	Lymphoma and osteosarcoma in dogs (Pressler et al.2003)
	Hypercortisolism (Smets et al.2012)
Immunoglobulin G	X-linked hereditary nephropathy (Nabity et al.2012)
	Snake envenomation (Hrovat et al.2013)
	Pyometra (Maddens et al.2011)
	Leishmaniasis (Solano-Gallego et al.2003;Zaragoza et al.2003a)
	Leptospirosis (Zaragoza et al.2003b)
	Hypercortisolism (Smets et al.2012)
C-reactive protein	Pyometra (Maddens et al.2010b)
	Babesiosis (Defauw et al.2012)
	Leishmaniasis (Martinez-Subiela et al.2013)
Markers of tubular impairment	
N-acetyl-β -D-glucos-aminidase	Leishmaniasis (Palacio et al.1997)
	X-linked hereditary nephropathy (Nabity et al.2012)
	Heartworm disease with cardiac impairment (Uechi et al.1994b)
	Pyometra (Maddens et al.2010b)
	Experimental immune complex glomerulonephritis (Bishop et al.1991)
	Acute renal failure experimentally induced in cats (NAG-B isoenzyme) (Sato et al.2002a)
	CKD in cats (Jepson et al.2010a)
	Hypercortisolism (Smets et al.2012)
Gamma-glutamyl transpeptidase	Aminoglycoside-induced nephrotoxicity (Greco et al.1985;Grauer et al.1995;Rivers et al.1996)
	Renal insufficiency associated with pyometra (De Schepper et al.1989)
	Heartworm disease with cardiac insufficiency (Uechi et al.1994b)
	Leishmaniasis (Palacio et al.1997)
	Envenomation by the common European adder (Palviainen et al.2013)
	Experimental immune complex glomerulonephritis in cats (Bishop et al.1991)
Neutrophil gelatinase-associated lipocalin	Acute kidney injury (Hsu et al.2014;Segev et al.2013;Zhou et al.2014)
	Chronic kidney disease (Hsu et al.2014a;Steinbach et al.2014)
Retinol binding protein	Chronic kidney disease (Smets et al.2010b)
	Pyometra related renal impairment (Maddens et al.2010b)
	Babesiosis (Defauw et al.2012)
	Severe inflammatory response syndrome (Schaefer et al.2011)
	Envenomation by cytotoxic or neurotoxic snakes (Hrovat et al.2013)
	X-linked hereditary nephropathy before the onset of azotemia (Nabity et al.2012)
	Chronic renal failure and hyperthyroidism in cats (Van Hoek et al.2008)
	Hypercotisolism (Smets et al.2012)
Beta2-microglobulin	Early stages of X-linked hereditary nephropathy (Nabity et al.2012)
Cystatin C	Severe CKD in dogs with leishmaniasis (Garcia-Martinez et al.2015)
Cauxin	Tubular impairment (Myiazaki 2007)
Clusterin	Drug-induced acute kidney injury (Zhou et al.2014)

IRIS TREATMENT RECOMMENDATIONS

Stage 1 CKD Patients

1. Identify and correct any prerenal or postrenal disorders. Dehydration is the most common prerenal abnormality encountered, especially if urine-concentrating ability is compromised. Any clinical or suspected subclinical dehydration should be corrected with isotonic, polyionic replacement fluid solutions, such as lactated Ringer's solution either IV or SC.
2. Identify and treat any treatable primary disease processes (eg, renal lymphoma and hypercalcemia) or complicating disorders (eg, urinary tract infections and ureteroliths).
3. Pursue additional diagnostics recommended for Stage 1 CKD patients, including: Urine culture and sensitivity.
4. Identify and treat hypertension and renal proteinuria. Dietary sodium and protein reduction (eg, a renal diet) combined with ACE inhibitors, CCAs, and ARBs are used to reduce hypertension and proteinuria.
5. Discontinue all potentially nephrotoxic drugs.
6. Assess CKD stability or progression by monitoring patients at least twice a year. Dogs and cats with Stage 1 CKD are at risk for kidney disease progression; however, not all Stage 1 CKD patients progress to become azotemic. Those with borderline hypertension and proteinuria should be monitored closely.

Stage 2 CKD Patients

Dogs and cats with mid to late Stage 2 CKD often have progressive loss of renal function, although the rate of renal disease progression can be variable.

Reduce phosphorus intake with renal diets and enteric phosphate binders (if needed to meet goals).—This is a major treatment goal for dogs and cats with Stage 2 and beyond CKD.

Consider calcitriol supplementation—a potentially renoprotective treatment in dogs and cats. In dogs and cats receiving calcitriol, avoid use of calcium containing enteric phosphate binders or monitor patients closely for hypercalcemia.

Monitor patients for metabolic acidosis. Stage 2 CKD patients should be monitored for metabolic acidosis by measuring serum bicarbonate or total CO2 concentrations. If necessary, renal dietary therapy may be supplemented with oral sodium bicarbonate or potassium bicarbonate in order to maintain serum bicarbonate concentrations in the 18 to 24 mmol/L range.

Assess CKD stability or progression by monitoring patients for every 3 to 6 months.

Stage 3 CKD Patients

Continue renoprotective treatments (eg, renal diets, antihypertensive and antiproteinuric treatments) as Stage 3 CKD patients have progressive renal disease and it is important—as in State 2 CKD patients—to slow disease progression.

Initiate symptomatic treatment to improve quality of life because many dogs and cats with Stage 3 CKD, especially late Stage 3 CKD, begin showing clinical signs.

Stage 4 CKD Patients

In both dogs and cats, pursue all treatments for Stage 1, 2, and 3 CKD.

Continue renoprotective treatments (eg, renal diets, antihypertensive and antiproteinuric treatments) as these treatments are still important in early Stage 4 CKD patients but invariably the management focus shifts to making the patient as comfortable as possible given its renal failure.

Continue symptomatic treatment to improve quality of life. Owners frequently—and rightfully—equate nausea, decreased appetite, vomiting, and weight loss with poor quality of life.

Stop the catabolic spiral of calorie malnutrition— one of the primary management goals in Stage 4 CKD (Table 4). Appetite stimulants, antiemetics, and gastric acid blocking drugs become important in these patients (Table 7), but correction of metabolic deficits (eg, dehydration) and excesses (eg, hyperphosphatemia) is a higher priority.

Monitoring of serum creatinine and urea levels.Re-evaluation for every 1-2months.

TABLE 4 Diagnostic &Treatment Considerations Linked to IRIS CKD Stages

OPTIMUM IRIS STAGES	DIAGNOSTIC & TREATMENT FOCUS	CONSIDERATIONS	
		POTENTIAL PROBLEMS	DIAGNOSTICS/TREATMENT
Stage 1 Stage 2 Early Stage 3	Assess primary disease and complicating disorders Monitoring at least Q 6 months	Renal infiltrative disease Renal lymphosarcoma	Radiographs ,ultrasound±FNA, chemotherapathy
		Obstructive uropathy Ureteral obstruction	Radiographs, ultrasound± FNA, chemotherapy, SC uretral bypass
		Hypercalcemic nephropathy	Serum Ca and Ica assessment, NaCl fluid therapy, furosemide dieresis
Stage 2 Stage 3 Early Stage 4	Assess CKD stability or progression Monitoring at least Q 3 months	Nephrocalcinosis	Renal diets ,intestinal phosphorus binders
		Hypertension	CCAs, ACE inhibitors, ARBs
		Proteinuria	ACE inhibitors, CCAs, ARBs
Late Stage 3 Stage 4	Assess patient problems Monitoring at least Q 1-2 months	Anorexia,nausea,vomiting	Appetite stimulants,antiemetics, H2 receptor blocker, proton pump blockers
		Metabolic acidosis	Dietary alkalization
		Potassium depletion	Potassium supplementation
		Dehydration	Fluid therapy
		Anemia	Recombinant erythropoletin
		Calorie malnutrition	Appetite stimulants,dietary variety,feeding tube placement
		Uremia	Enteric dialysis

ACE= angiotensin-converting enzyme; ARB=angiotensin receptor blocker; Ca=calcium; CCA=calcium channel antagonist; CKD=chronic kidney disease; FNA=fine-needle aspiration; iCa=ionized calcium; NaCl=sodium chloride

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Full Length Article

Analysis of Single-Stranded Conformational Polymorphism at 3'UTR of *SLC11A1*Gene in Jersey Crossbred Cattle

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ABSTRACT

Intracellular bacterial diseases cause significant economic losses in livestock industries. Constraints in eradicating infectious diseases indicate the need for selection and breeding of animals based on natural disease resistance. Solute Carrier Family 11 Member 1(SLC11A1) gene expressed in macrophages has significant involvement in innate immune mechanism.Investigation of polymorphism of 3'untranslated region (3'UTR) of SLC11A1 gene was conducted and the Polymerase Chain Reaction - Single-Stranded Conformational Polymorphic (PCR-SSCP) pattern at 3'untranslated region (3'UTR) of SLC11A1 gene in Jersey crossbred cattle was analyzed. A fragment of 175bp encoding 3'UTR was amplified by PCR and the genotyping was done by using PCR-SSCP. SSCP pattern of 3'UTR revealed the presence of four genotypes, viz. AB,BB, CC and CB with a frequency of 0.29, 0.40, 0.17 and 0.13, respectively with allelic frequencies of A (0.144), B (0.615) and C (0.24). The result showed that the population was not in Hardy-Weinberg equilibrium for SLC11A1 gene. SLC11A1 gene was found to be highly polymorphic in the Jersey crossbred cattle population studied.

Key Words: Disease resistance, *SLC11A1* gene, PCR-SSCP, allele frequencies, Jersey crossbred cattle,

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INTRODUCTION

In India, diseases like Johne's disease (JD) is endemic and high prevalencehas been reported in domestic ruminants (Kennedy and Benedictus, 2001). Treatment of intracellular bacterial diseasesare expensive and also not successful (Sweeney*et al*., 2012). In these circumstances, genetic selection for disease resistance can be an effective strategy for the control of incurable and highly pathogenic diseases (Donagh*et al*., 2011).Earlier studies have

shown that considerable genetic variation in *SLC11A1* gene has an association with disease suscepatability. *SLC11A1* gene is also known as *NRAMP1,* primarily expressed in phagosomes. The gene product exhibits pleiotropic effects on iron transport, early innate immune response against intracellular bacterial growth through inducible nitric oxide synthase (iNOS) expression in mice (Alter-Koltunoff *et al*.,2008). Natural Resistance Associated Macrophage Protein 1 (*NRAMP1*/*SLC11A1*) is a candidate gene in regulating resistance and susceptibility towards a number of antigenically different microorganisms including *Mycobateriumbovis*, *Salmonellasp* and *Leishmaniasp* (Juste *et al*., 2005 and Reddacliff *et al*., 2005).Several reports shows that GT repeats in 3'UTR of *SLC11A1* gene are significantly associated with disease resistance and are also considered as disease resistance locus (Zhang *et al*.,2009). Allelic differences localized in the 3'UTR of the gene have been associated with macrophage function and resistance to disease in cattle and buffalo (Capparelli *et al*.,2007, Ganguly *et al*., 2008 and Kumar *et al*.,2011). In the present study, the allele frequency in 3'UTR of *SLC11A1* gene was detected by Polymerase Chain Reaction - Single-Stranded Conformational Polymorphism (PCR-SSCP) analysis in Jersey crossbred population.

MATERIALS AND METHODS

Population under study were 52 (40 from JD suspected Jersey crossbreds and 12 normal crossbreds) in and around Namakkal district. Animals were categorized as being suspected based on symptoms of JD that included diarrhea, weakness, weight loss. Genomic DNA was isolated fromblood samples using standard phenol chloroform extraction method by Montgomery and Sise (1990). Quality of DNA was checked by 1% agarose gel electrophoresis and concentration measured by Nanodrop, Thermoscientific. The ratio of optical density at 260/280nm of DNA for the above genetic group ranged from 1.8 to 1.89 respectively. Samples with DNA concentration above 50 ng/µl were taken for study. Serum samples were processed to identify the presence of MAP (*Mycobacterium avium* subspecies *paratuberculosis*) by employing the direct IS900 PCR as per the procedure described by Pillai and Jayarao (2002) using two primer sequence was 5'-CCGCTAATT GAGAGATGCGATTGG-3' and the 3' primner sequence was 5'-AATCAACTCCA GCAGCGCGGCCTCG-3' for detection of insertion sequence of 900 (IS900) of MAP which amplifies a 229 bp fragment PCR product.

Primers of 3'UTR forward 5'-AAGGCAGCAAGACAGACAGG-3' and 3'UTR reverse 5'-ATGGAACTCA CGTTGGCTG-3' were used for the amplifing the 3'untranslated region (3'-UTR) of *SLC11A1* gene as described by Zhang *et al.* (2009).Polymerase chain reaction (PCR) was conducted in a final volume of 25 µl reaction mixture containing 12.5 µl of amplicon redeye master mix, 10 pM of each forward and reverse primer and 50 ng of genomic template DNA. The Bio-Rad thermal cycling profile for the reaction include initial denaturation at 95°C for 3 min followed by 35 cycles of denaturation at 95° C for 30 sec, annealing at 64° C for 30 sec, extension at 72° C for 1 min and final extension at 72° C for 10min.

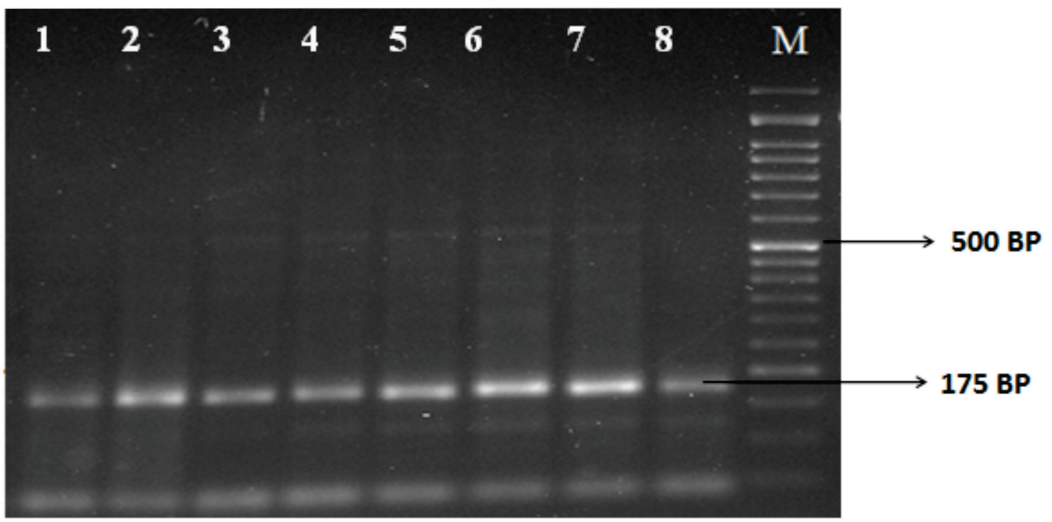
The PCR products were checked by 2 percent agarose gel electrophoresis using 50 bp ladder (Himedia) and visualized in UV transilluminator (Bio Rad, USA) after staining with ethidium bromide. The PCR products were further processed for SSCP by mixing the 5µl of PCR product with 15µl of SSCP dye. The mixture was subjected to denaturation at 95°C for 10 minutes, then immediately transfer to cool down at -20°C for 10 minutes.

To explore genetic polymorphism in 3'UTR of *SLC11A1* gene, after denaturation the PCR productswere subjected to 8% Polyacrylamide gel electrophoresis (29:1 Acrylamide: Bisacrylamide crosslinking). The pre run was given to Polyacrylamide gelat 100V for 30 minutes and then SSCP samples were loaded. Overnight Polyacrylamide gel Electrophoresis was carried out at 200V. After the run was completed, silver staining was carried out according to Bassam *et al*. (1991) with certain modifications to visualize the banding patterns. The allele and genotype frequencies were calculated and Hardy-Weinberg equilibrium was tested by comparing expected and observed genotype frequencies using a Chi-square (χ^2)-test.

RESULT AND DISCUSSION

Johne's disease was confirmed as negative in all the suspected animals based on PCR technique and ELISA method. The PCR amplification yielded products at 175 bp (Figure 1) as expected for 3'UTR of *SLC11A1* gene. PCR amplicons were subjected to SSCP analysis to detect the polymorphic patterns of *SLC11A1*gene in Jersey crossbred cattle. PCR-SSCP analysis of *SLC11A1* gene (Figure 2) revealed AB, BB, CC and CB genotypes with predominance of BB genotype. The genotype frequencies of AB, BB, CC and

Figure-1. Resolution of SLC11A1 (3'UTR region) PCR product on 2 % agarose gel.



{Lane: 1 to 8 samples; Lane M: Marker (50 bp)

Figure-2. SSCP patterns of SLC11A1 gene (3'UTR region) in 8 % non-denaturing gels

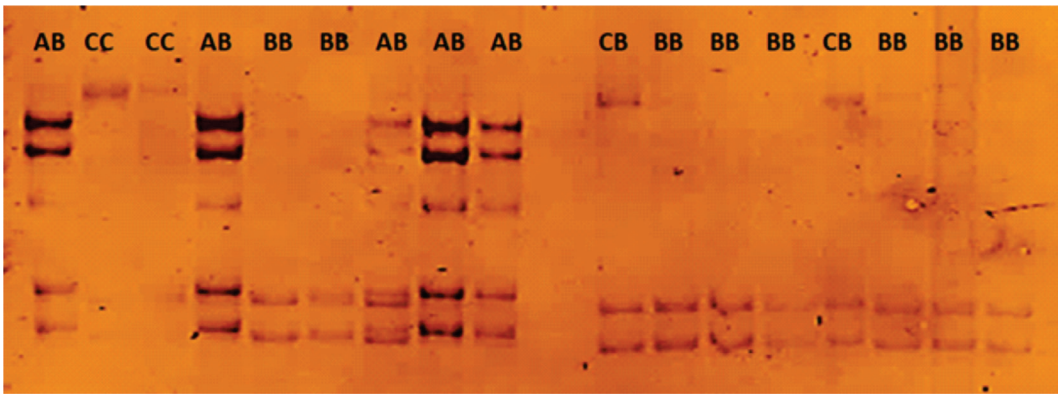


Table-1. Genotype and allele frequencies of SLC11A1 gene in Jersey crossbred cattle

Genotypic	Number of animals N=52	Observed Genotypic frequency	Allele frequency		Expected Genotype frequency	χ2 value (df = 2)
AA	0	0.00	**A**	0.14	0.021	21.32*
AB	15	0.288	**B**	0.615	0.177	
BB	21	0.404	**C**	0.24	0.378	
CC	9	0.173			0.058	
CB	7	0.135			0.295	

χ^2_{tab} value at 5% level and 2 df is 0.103. ($\chi^2_{cal} > \chi^2_{tab}$ value)

*Significant differences between observed and expected number of genotypes

CB were in the order of 0.29, 0.40, 0.17 and 0.14, respectively. The A, B and C allele frequencies were 0.14. 0.62 and 0.24 respectively (Table-1). The genotype AA was absent in the population studied. The present population was notin Hardy-Weinberg equilibrium for *SLC11A1*loci.

The variation of GT repeats in 3'UTR of *SLC11A1* gene have been reported to be associated with the resistance against Brucella and tuberculosis infection in cattle and buffalo (Ganguly *et al*. 2008, Capparelli *et al.,*2007), but natural resistance against mastitis could not be established (Zhang *et al*. 2009). Korou *et al.,* (2010) identified two polymorphic regions in the 3'UTR end of the *SLC11A1* gene and B_7 allele [AAGG$(GT)_7$GCACAC] was significantly associated with being ELISA negative for *Paratuberculosis* infection in goat.

Single-Stranded Conformational Analysis (SSCA) revealed a highly significant association of polymorphic $(GT)_n$ microsatellite identified in the 3'untranslated region (3'UTR) of bovine *NRAMP1* with natural resistance to *Brucellosis*(Adams and Templeton, 1998). The presence of polymorphism in 3'UTR

of this gene in the Jersey crossbred was reportedto be due to GT repeat sequence variation at targeted segment of *SLC11A1* gene(Adams and Templeton, 1998).

The PCR-SSCP analysisin Jersey crossbred cattle study revealed that *SLC11A1* gene was highly polymorphic and four PCR-SSCP patterns were observed. The highest *SLC11A1* genotypic frequency was observed for BB genotype (0.4). The allele frequency of B was 0.62. The detected polymorphic pattern can be used for identification of SNPs by sequencing the target region and association analysis with disease incidence caused by intracellular pathogens in larger population of Jersey crossbred cattle.

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Studies on preparation of smart curd

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In India, curd forms one of the essential item and efficient source of probiotics supplement. Probiotics are a group of beneficial lactic acid producing bacteria which constitute the major portion of the gut micro floral population providing invaluable B-complex vitamins. However, the beneficial effects of these probiotics depend on their nutrient availability, especially prebiotics. Prebiotics are non-digestible food ingredients that target selected groups and selectively enhances the growth of probiotics (DiRienzo, 2000). In the present study, the prebiotic potential of commercially available Fructo-oligosaccharide (FOS) targeting, *Bifidobacterium spp.* and *Lactobacillus spp.* was utilized to prepare smart curd and the same was examined for chemical, microbiological, sensory evaluation and compared with control curd.

The curd was prepared by addition of different levels of FOS as a prebiotic and sensory evaluation was carried out. The physical and chemical properties were also estimated. Fresh cow milk was procured and pasteurized at the temperature of 95°C for 15 min, then cooled to 40°C. FOS was incorporated at various levels such as 0.5%, 1%, 1.5% and the mesophilic culture inoculated at 2% level. The mix was transferred to the cups and incubated at 37°C for 4 hours, then stored at refrigerated temperature (4°C). The sensory qualities like pH, acidity, fat, proteins and total solids were estimated at different period of time interval. Microbiological properties like total count, coliform count, yeast and mould count were also assessed. The sensory evaluation was also carried out with different parameters like appearance, sourness, flavour and overall acceptability. The chemical, microbiological and sensory evaluation of smart curd was carried out and the same was compared with control curd (without addition of FOS).

The chemical composition of the smart curd and control curd is presented in table 1. The pH and titrable acidity was found to increase with the increased addition of FOS to the curd. From the present study, it was observed that FOS did not affect the fat percentage of the product. The present findings were in agreement with findings of Guven *et a1.* (2005) who found that the use of inulin as a fat replacer did not significantly affect the pH values. Similarly, Barrantes *et al.* (1994) reported that fat replacers did not negatively change the activity of yogurt starter bacteria.

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Table 1. Chemical composition of the smart curd

Variables	Control curd	Smart curd	F values
pH	4.20	4.30	8.00*
Acidity	1.32	1.17	9.65*
Fat	0.326 ± 0.003	0.315 ± 0.006	2.22^{NS}
Protein	3.673^{a} ± 0.017	3.615^{b} ± 0.067	5.51*
Lactose	4.511^{b} ± 0.018	4.648^{a} ± 0.011	36.42**
Total solids	12.570^{b} ± 0.073	13.827^{a} ± 0.086	96.80**

It was observed that the lactose percentage was significantly ($p<0.01$) higher in smart curd group when compared to control curd. The protein and total solids content of the different product showed significant difference between the different products. It was observed that protein content decreased with increase in inclusion of FOS. Tamime and Robinson (1999) also reported that the variation of total solids could be due to increase in the total viable count of these bacteria.

Microbial qualities of the smart curd at different storage period are presented in table 2.

Table 2. Microbial qualities of the smart curd during different storage periods

Microbial qualities (log_{10} cfu/g)	Smart curd			
	0 day	3rd day	7th day	F values
Total count	8.15 ± 0.37	8.43 ± 0.40	8.55 ± 0.30	0.66^{NS}
Coliform count	NIL	NIL	NIL	-
Yeast and mould count	NIL	NIL	1.65 ± 0.27	0.22^{NS}

It was observed that there was no significant difference between treatment groups with respect to total count, coliform count and yeast and mould count during the different storage periods. The increased total count may be due to the enhanced availability of simple sugars mainly glucose, fructose and minerals which act as growth promoters (Analie Lourens Hattingh and Viljoen, 2001). Coliforms were found to be absent in smart curd which indicates that the product was prepared in hygienic condition and the present results were in agreement with the findings of Abou-Donia *et al.* (1991) and Vijayalakshmi *et al.* (2005).

Sensory characteristics of different curds are furnished in Table 3. It was observed that, there were no significant differences between the different types of curd with respect to appearance and flavour scores while significant difference was noticed with respect to sourness and overall acceptability. It was also noted that FOS was not contributing any flavour in general, so there was no significant difference in the product. The sourness of the product was decreased with increase in inclusion level of FOS to the curd, whereas, the overall acceptability increased with increase in inclusion levels of FOS.

Table 3. Sensory evaluation of different treatment of curd (Mean ± SE)

Treatment curd	Appearance	Flavour	Sourness	Overall acceptability
CC	8.16 ± 0.31	8.33 ± 0.21	8.50^{a} ± 0.22	6.83^{b} ± 0.40
SC3	7.33 ± 0.49	7.50 ± 0.43	6.83^{b} ± 0.40	8.83^{a} ± 0.17
F values	0.65^{NS}	0.82^{NS}	3.42I*	6.16**

CC : Control Curd SC3 : Smart Curd3
NS : Non significant * : Significant
** : Highly significant

The cost of production of CC and SC3 were 0.9 and 2.64 rupees respectively. The cost of production of smart curd was higher than the control curd because of the cost of FOS, but this can be offset when compared to the benefits. Hence, from the present study it may be concluded that addition of FOS improves the sensory parameters and did not affect the microbiological parameters and can be deemed safe for consumption.

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Isolation and Identification of Bacteria of Normal Flora in Handfed Exotic Psittacine Birds at Tamil Nadu, India

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ABSTRACT

Bacterial normal flora were identified in handfed psittacine birds (lory, amazons, lorikeet, cockatoo, conure, grey parrot, macaw, eclectus and African love birds) at Cavin estate exotic bird farm, Chennai, Tamil Nadu. A total of 54 cloacal swabs were collected from different species of psittasine birds for bacteriological screening to identify the microorganisms by morphological and biochemical characteristics. In the present study, four Gram positive organisms such as *Staphylococcus, Enterococcus, Bacillus* and *Corynebacterium* and ten Gram negative bacteria such as *Escherichia coli, Enterobacter* sp.*, Klebsiella* sp.*, Yersinia* sp.*, Providencia* sp.*, Erwinia* sp.*, Citrobacter* sp.*, Serratia* sp.*, Acinitobacter* sp. and *Buttiauxella* sp.*,* were identified as the normal flora in apparently healthy handfed psittacine birds. The frequency of occurrence of *Staphylococcus* sp.*,* and *E.coli* was higher when compared to others.

Key Words: Psittacines, Normal flora, Cloacal swab, hand feeding

INTRODUCTION

Parrots are found all over the world and popular as pets due to their sociable and lovable nature, intelligence, beauty and ability to imitate human voices.

Economically also avian pets are very much beneficial to communities as a source of income through pet trade. Mostly parrots are kept in a cage or aviary. Species of parrot vary in their temperament, noise level, cuddliness with people and talking ability (Akhter et al., 2010).

Cavin estates exotic birds farm, Chennai is located on the south–eastern coast of India in the north–eastern part of Tamil Nadu on a flat coastal plain known as the Eastern Coastal Plains. Here, so many exotic bird varieties like speaking amazons, beautiful macaws are available. Other than this, lories, lorikeets, cockatoos, conures, monk, gray parrot, eclectus are also available.

Maintaining a healthy environment for birds is very important to prevent

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spreading of infectious diseases which are the major threat for avian species. Routine screening of birds for bacterial infection is also necessary for proper treatment and to understand the epidemiology of the disease (Machado et al., 2018).

Isolation, identification and characterisation of microorganisms at the time of disease outbreaks are the key issues in determining whether the organism is pathogenic or non-pathogenic. Based on that, therapeutic and preventive measures are to be standardised to get the highest success rate in establishing a healthy farm. Keeping all these in view, microbiological investigation was carried out to know the normal flora of bacteria in exotic psittacine birds at Cavin estates exotic bird farm, Chennai, Tami Nadu.

MATERIALS AND METHODS

Sample collection:

A total of 54 cloacal swab samples were collected from apparently healthy handfed psittacine birds of lorys (green napped lory, red lory, yellow bipped lory, violet necked lory, chattering lory and olive swainson lory), amazons (red lord amazons, Cuban amazon, vinaceous breasted amazon and blue fronted amazon), cockatoos (major mitchell cockatoo, galah cockatoo and umberlla cockatoo), conures (jandey conure), parrots (gray parrot, African gray parrot, rupella parrot, timney grey parrot, caique), macaws (hyacinth macaw, blue gold macaw and green wing macaw), eclectus and African love birds at Cavin estate exotic bird farm, Chennai, Tamil Nadu. This exotic bird farm consists of more than 400 psittacine birds and is reared in highly sophisticated cages with maximum possible flight space than the recommended standards, sprinklers to create suitable microenvironment, natural perches and sufficient numbers of breeding nest boxes of different size and shape to mimic the natural nest. The birds are provided with a highly nutritious and balanced feed consisted of seeds, grains, nuts, fresh fruits, vegetables, greens and fresh fruit juices as per the standard recommended requirements for each species of birds. The birds are regularly and periodically dewormed with intalben suspension (albendazole 2.5% w/v) or alzonic (albendazole 3% w/v + niclosamide 10% w/v) at the dose of 5ml per litre in drinking water to whole night water deprived exotic pet birds.

Isolation of bacteria and morphological characterization:

Collected cloacal swab samples were inoculated in nutrient broth and incubated at 37°C overnight. The bacterial culture obtained was plated onto nutrient agar plate and incubated at 37°C for 24 hrs. Based on the colony morphology, Individual colonies were subcultured and stained by Gram's method to study the morphology and staining characters (Gram, 1884).

Biochemical characteristics:

Oxidase and catalase test:

Oxidase test was carried out by Himedia oxidase disc and catalase test was performed using three per cent hydrogen peroxide.

Fermentation test for bacterial identification

Pure bacterial colonies were plated on Mannitol salt agar (MSA), MacConkey agar (MA) and Eosin methylene blue agar (EMB) to identify the fermentation of glucose, sucrose, lactose, dextrose and mannitol (Abbas et al., 2016).

Hydrolysis test:

Hydrolysis test was carried out for further identification of bacteria. Pure colonies were plated over bile esculin and urease media to identify the hydrolysis of bile escullin and urea.

IMVic

Biochemical tests such as indole, methyl red, Voges-Proskauer and citrate were carried out as a secondary bacterial identification method and interpretation of the results was carried out (Bergy's manual of bacteriology and ABIS online tool, George et al., 2004).

RESULTS AND DISCUSSION

In this present study, 14 bacteria were isolated which included Gram positive (four) and Gram negative (10) micro organisms. Four Gram positive organisms such as *Staphylococcus* sp., *Enterococcus* sp., *Bacillus* sp., and *Corynebacterium* sp., and ten Gram negative microorganisms such as *Escherichia coli*, *Enterobacter* sp., *Klebsiella* sp., *Yersinia* sp., *Providencia* sp., *Erwinia* sp., *Citrobacter* sp., *Serratia* sp., *Acinitobacter* and *Buttiauxella* sp. were identified as the normal flora in apparently healthy psittacine birds (Table 1). Birds are appeared healthy, there was no clinical symptoms observed in the birds.

Table 1 Organisms isolated from handfed birds

Sl. No.	Species of birde	No.of Birds	Organism Isolated
1.	**Amazon**		
	Red lord amazon	4	*Enterobacter aerogen, Klebsiella, Enterococcus, Staphylococcus aureus* and *Serratia*
	Cuban amazon	4	*Staphylococcus, E.coli, Serratia, Buttiauxella* and *Acinetobacter*
	Vinaceous breasted amazon	2	*Bacillus*
	Double yellow headed amazon	1	*Staphylococcus*
	Blue fronted amazons	1	*E.coli*
2.	**Macaw**		
	Hyacinth macaw	5	*Staphylococcus* and *E.coli*
	Blue gold macaw	2	*Corynebacterium, Citrobacter* and *Klebsiella*
	Green wing macaw	1	*Staphylococcus* and *E.coli*

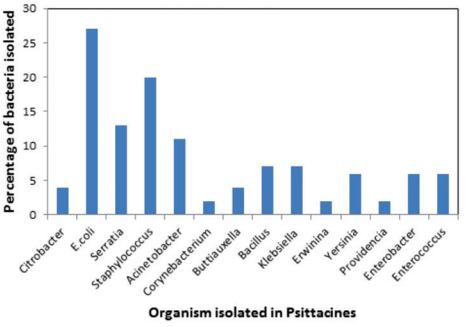
3.	**Lories and Lorikeets**		
	Green napped lory	1	*Klebsiella* and *Buttiauxella*
	Red lory	1	*Serratia, Staphylococcus* and , *Enterococcus*
	Yellow bippedlory	2	*Serratia* and *E.coli*
	Violet necked lory	1	*E.coli*
	Chattering lory	2	*E.coli*
	Olive Swainsonlory	2	*Erwinia, Yersinia*, and *Staphylococcus*
4.	**Cockatoo**		
	Medium sulphur cockatoo	1	*E.coli*
	Galah cockatoo	3	*E.coli, Serratia* and *Providencia*
	Triton cockatoo	1	*Citrobacter*
	Major Mitchell cockatoo	2	*Acinetobacter* and *Serratia*
	Umberlla cockatoo	1	*E.coli* and *Bacillus*
5.	**Eclectus**	8	*Staphylococcus, Yersinia, Proteus, Enterobacter, Enterococcus, E.coli* and *Serratia.*
6	**Parrots(Timney grey pattor)**	9	*Staphylococcus, Yersinia, Enterobacter, Enterococcus, Acinetobacter* and *Klebsiella*

Most of the bacteria can be identified by primary screening like growth on selective and differential media. Full MSA is a selective medium for S*taphylococcus aureus*. It produced yellow colony surrounded by an yellow zone. *Escherichia coli*, *Klebsiella* and *Enterobacter* were identified using a EMB agar. *Escherichia coli* showed metallic sheen, *Enterobacter* sp. revealed pink colour colonies without sheen and *Klebsiella* sp., presented pink mucoid colonies on eosin methylene blue agar.

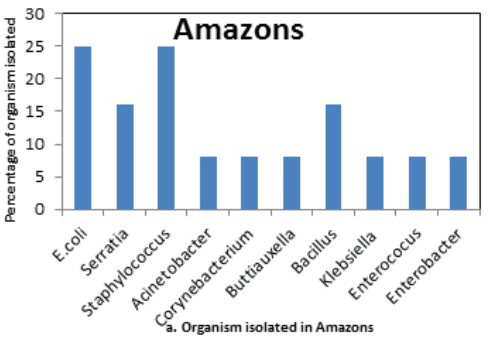
Citrobacter was identified by acid butt and acid slant with gas and H_2S production on TSI slant. Swarming growth in nutrient agar was observed for *Proteus* sp. Other bacteria such as *Serraia* sp., *Yersinia* sp., *Acinetobacter* sp., *Buttiauxella* sp., *Erwini* sp., *Enterococcus* sp., and Co*rynebacterium* sp. were identified by their biochemical characteristics.

Out of 14 bacteria isolated from psittacine birds, *E. coli* (27%) was found to be more followed by *Staphylococcus* sp. (20%), *Acinetobacter* sp. (11%), *Buttiauxella* sp. (7%), *Bacillus* sp. (7%), *Klebsiella* sp. (7%), *Serratia* sp. (7%), *Enterobacter* sp. (6%), *Enterococcus* sp. (6%), *Yersinia* sp. (6%), *Citrobacter* sp. (4%), *Providencia* sp. (2%), *Erwinia* sp. (2%), and *Corynebacterium* sp. (2%) were

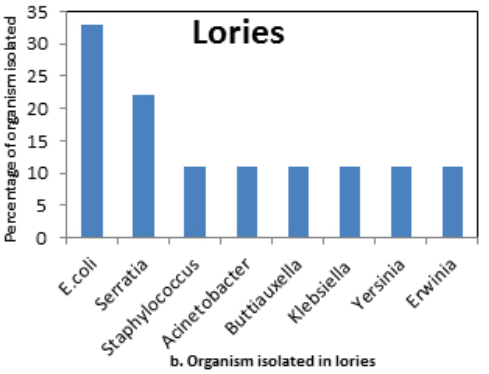
isolated (Table. 2). Among these organisms, *E. coli* and *Staphylococcus* sp. was isolated at highest percentage from all the species of psittacine birds. *Corynebacterium* sp. was isolated only from Macaw, *Providentia* sp. from cockatoo and *Erwinia* sp. from lory (Table.1 and Fig.1).



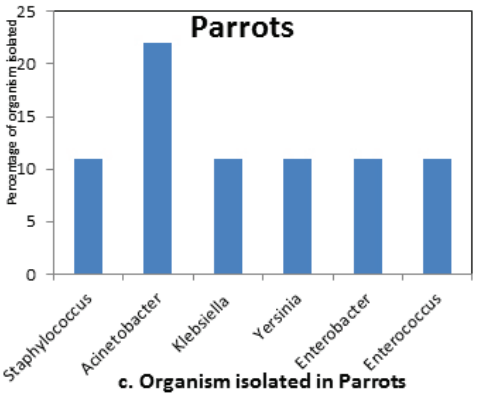
Out of the 54 psittacine birds, 12 amazon species were screened for bacterial isolation. It includes red lord amazon, Cuban amazon, vinaceous breasted amazon and double yellow headed amazon. Among all bacteria, *E.coli* and *Staphylococcus* sp. were isolated at a higher percentage (25%) followed by *Serratia* sp. and *Bacillus* sp. (16%) and other organisms were isolated at a lower percentage (8%) (Fig. 2a).



Out of 54 psittacine birds, nine lory species were analysed in this study which includes, green napped lored lory, yellow bipped lory, violet necked lory, chattering lory, olive swainson lory. *Escherichia coli* was found to be more (33%) followed by *Serratia* sp. *(*22%) and other organisms (11%). In comparison with other species, *Erwinia* sp. was isolated from lorry only (Fig. 2b).

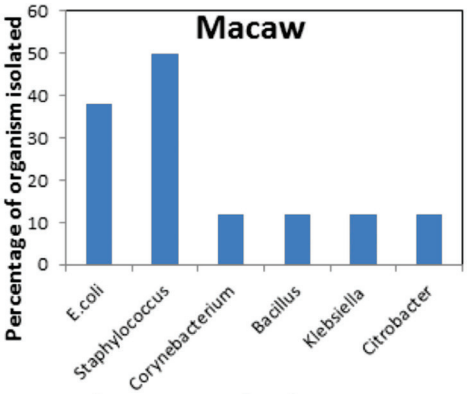


In parrots (9/54), *Acinetobacter* sp. was isolated at a higher percentage (22 %) followed by *Staphylococcus* sp., *Klebsiella* sp., *Yersinia* sp., *Enterobacter* sp. and *Entercoccus* sp. (11%) (Fig. 2c).



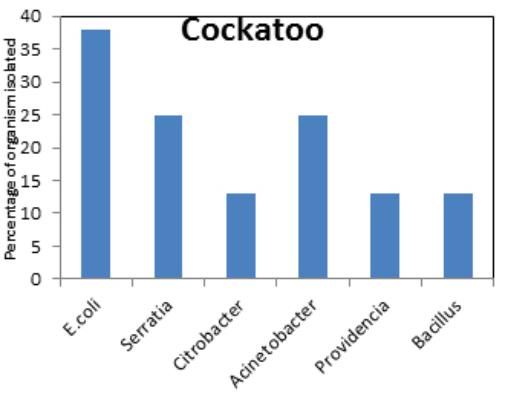
Among Macaws (8/54), hyacinth macaws, blue gold macaws and green wing macaws were included in this study. *Staphylococcus* sp. (50%) and *E. coli* (38%) were isolated at a higher percentage followed by *Corynebacterium* sp., *Bacillus*

sp., *Klebsiella* sp. and *Citrobacter* sp. (12%). In comparison with other species, *Corynebacterium* was isolated only from macaw (Fig. 2d).



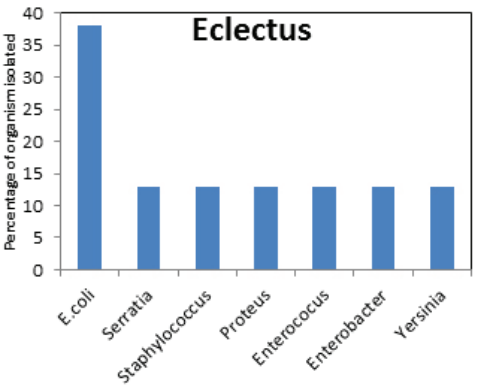
d. Organism isolated in Macaw

In cockatoos (8/54), medium sulphur cockatoo, galah cockatoo, Triton cockatoo, major mitchell cockatoo, umberlla cockatoo used for this study. Organism isolated form cockatoos were *E. coli, Serratia* sp., *Citrobacter* sp., *Acinetobacter* sp., *Providencia* sp. and *Bacillus* sp. *Escherichia coli* was isolated at a highest percentage (38) followed by, *Serratia* sp. and *Acinetobacter* sp. (25 each) and other organisms (13%) (Fig. 2e).



e. Organism isolated in cockatoo

From eclectus, *E.coli* was isolated at a highest percentage of 38 and other organisms were isolated as shown in fig. 2f. It was reported that the presence of Gram-negative bacteria, including those belonging to the Enterobacteriaceae in their intestinal microbiota has been considered as an indication of potential diseases (Bangert et al., 1988; Mattes et al., 2005). Microbiological studies in psittacine have increasingly reported Enterobacteria in healthy birds (Serafini et al., 2015; Lopes et al., 2015; Machado et al., 2016). In the present study, we have isolated more numbers of negative microorganisms in healthy birds. Birds look apparently healthy and there were no clinical symptoms of illness.



f. Organism isolated in Eclectus

The isolation of microorganisms from the birds in this study could be explained by the fact that young birds were colonised by microbes immediately after hatch, and acquired a microbial biomass by contact with environmental microorganisms. This could be the main sources of microbial transmission to growing birds (Mills et al., 1999). Moreover, the young birds might have low immunity and acquired this harmful microbial load by direct contact

with other animals that might have visited the artificial nests, such as mice, bats, and other avian species, or by indirect contact with their secretions (Allgayer et al., 2009; Serafini et al., 2015).

The absence of *Salmonella* sp. was also observed in apparently healthy handfed birds maintained in incubator at 37°C. Mostly psittacines are very sensitive to avian salmonellosis, particularly at young ages (Marietto-Gonçalves et al., 2010). The absence of Salmonella sp. in the present study suggests that the birds were not exposed to this pathogen (Godoy et al., 2007). This study gives the general knowledge on the normal microbial flora of apparently healthy handfed exotic pet birds and it is very essential to differentiate them from the pathogenic microorganisms isolated at the time of disease outbreaks.

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Isolation of Bacteria From Dead - In - Shell Chick Embryos of Psittacines

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ABSTRACT

The objective of the present study is to explore the bacteria associated with the dead-in-shell eggs of psittacines. A total of 132 dead-in-shell eggs of different psittacine breeds (Macaws, Lories, Conures, Amazons, Eclectus, Cockatoos, Grey parrots and Jardine parrots) were obtained from the incubation unit of Cavin estate exotic bird farm, Chennai, Tamil Nadu and subjected to microbological analysis. The overall prevalence of bacteria isolated from the dead-in-shell was 80.30% (106/132). Among these, Gram-negative bacteria were predominant (49.24%, 65/132) was observed followed by Gram-positive organisms (28.78%, 38/132) and Gram-positive filamentous organisms (2.27%, 3/132) were also isolated from dead in shell embryos. Among the Gram-positive bacteria, *Staphylococcus sp.* (21.12%) was found to be more followed by *Bacillus* sp. (3.78%), *Corynebacterium* sp. (2.2%) and *Enterococcus* sp. (1.51%). Similarly, among the Gram-negative bacteria, *Escherichia* sp. (25%) was identified more followed by *Pseudomonas* sp. (6.60%), *Serratia* sp. (6.60%), *Acinetobacter* sp. (4.5%), *Enterobacter* sp. (3.78%), *Yersinia* sp. (3.77%), *Citrobacter* sp. (2.27%), *Buttiauxella* sp. (1.52%), *Klebsiella* sp. (1.51%), *Cedeacae* sp. (1.51%) and *Erwinia* sp. (0.75%). Among the psittacine species, more numbers of the dead in shell cases were found to be in conures (37%) and lories (14.5%). Commonly found bacterial isolates were subjected to antibiotic sensitivity test. All the isolates were found to be sensitive to enrofloxacin and cefotaxime whereas, most of the bacteria showed resistance to trimethoprim + sulphamethoxazole and kanamycin. Resistance to other antibiotics were variable. Multiple resistance was not observed for any of the isolate.

Key words: Dead in shell, Embryos, Psittacine, Lories, bacterial isolates

INTRODUCTION

Psittacines or parrots and parakeets, which belong to the order Psittaciformes are popular display animals and pets due to their colourful behaviours and widely recognised intelligence. Psittacine birds are characterised by having a

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strong, curved bill, an upright stance, strong legs, and clawed zygodactyl feet (Reavill and Dorrestein, 2018). They are distributed mostly in the tropical and subtropical continents and regions including Australia, south east Asia, central and South America, Africa, Caribbean and Pacific islands. Many parrots are brightly coloured, and some are multi-coloured. Despite these unique and attractive characters, psittacines lack in parenting which may affect the production of live young birds. Further, natural incubation of captive psittacines depends on many factors including parent's breeding behaviour, health, diet, species, origin (wild caught or hand raised), experience, environment, egg integrity, and nest box design (Martin and Romagnano, 2006).

Artificial incubation is now being practiced which has tremendous insight in the field of psittacine breeding. This is more beneficial when compared to the natural incubation as it prevents the cross contamination of disease from the parents to off-springs and therefore the probability of developing healthy chicks increased to a higher extent.

A dead-in-shell embryo is of the major emerging problem with the artificial incubation. Bacteria represent primary or secondary agent of dead-in-shell embryos. A number of general bacteria especially in the family *Enterobacteriaceae* can be isolated from dead embryos, dead-in-shell, weak chicks or infected yolk sacs of young birds (Barrow, 1994). Trans-shell transmission, translocation from the digestive tract and vertical transmission are the ways in which bacteria gain access to the developing embryo (Bruce and Drysdale, 1994). Therefore, microbiological examination is required to study the reason behind the failure in hatching. Literature available on the etiological agent responsible for dead-in-shell embryos in artificial incubation of psittacines is limited. The objective of the study is to identify and explore the plethora of bacterial pathogens that are associated with the dead in shell mortalities.

MATERIALS AND METHODS

Sample collection

Cavin Estates Exotic birds farm, Chennai is located on the south–eastern coast of India in the north–eastern part of Tamil Nadu on a flat coastal plain known as the Eastern Coastal Plains. A wide variety of exotic birds *viz.* Amazons, Macaws, Lories, Lorikeets, Cockatoos, Conures and Eclectus are available at Cavin estates. The birds are maintained in aviaries with nest box facilities to enhance breeding. A separate incubation unit is functioning in cavin estates for artificial incubation of eggs collected from those captive psittacines. The incubators are maintained at a temperature of 37.3ºC and humidity of 40 to 60%. The eggs are subjected to 10 -12 rotations per day

A total of one hundred and thirty two dead-in-shell eggs were collected from the incubation unit of cavin estates farm from June to December 2018. Eggs collected were preserved in refrigerator. Each egg was carefully opened under sterile conditions and swabs from the unabsorbed yolk, egg white and visceral organs of the embryos were aseptically collected and stored for bacteriological examination.

Bacteriological screening

The yolk swabs were inoculated in nutrient broth and incubated at 37ºC for 18 hrs. The broth cultures were then streaked on nutrient agar and incubated at 37ºC for 24 hrs. After incubation, the agar plates were examined for cultural characteristics by determining the size, shape, elevation, edges, surface, and colour of the colonies. Well isolated colonies were subcultured on to selective media to obtain pure colonies. The pure colonies were subjected to Gram's staining and examined under the oil immersion (100x) to classify them as Gram positive or Gram negative (Merchant and Packer, 1967 and Cheesbrough, 1991)

Gram positive bacteria were identified by their morphology (rods, cocci, spore forming), mannitol fermentation, haemolysis on blood agar, catalase test and bile aesculin hydrolysis. Gram negative bacterial colonies were inoculated on MacConkey agar to differentiate lactose fermenters from non lactose fermenters and then subjected to a set of standard biochemical tests *viz.* indole, methyl red, Voges-Proskauer, citrate utilisation, triple sugar iron agar test, lysine decarboxylase, bile aesculin hydroloysis, urease test and oxidase test. *Salmonella* sp. was confirmed by production of black centered colonies on XLD agar and *E. coli* was confirmed by growth of colonies with green metallic sheen on EMB agar. The results were analysed as per the guidelines of Bergey's manual of systematic bacteriology (Bergey et al*.,* 1994) and ABIS online analytical tool in the following website address.

(http://www.tgw1916.net/bacteria_abis.html).

All media used in this study were prepared following the manufacturer's (Himedia) recommendations. Antibiotic sensitivity test for the bacterial isolates was carried out using the standard disk diffusion method (Bauer et al., 1966) using Mueller Hinton agar plates. A total of six antibiotic discs were placed at equal distance and plates were incubated at 37 °C for 18 to 24 hrs. The sensitivity and resistant patterns of the isolates were derived by measuring the diameter of inhibition zone around the antibiotic discs following the manufacturer's (Himedia) instructions. Antimicrobial agents used in the study were cefataxaime 30 mcg (CTX), enrofloxacin 5 mcg (ENR), tetracycline 30 mcg (T30), kanamycin 30 mcg (K), gentamicin 10mcg (GEN) and trimethoprime + sulphamethoxazole 1.25/23.75 mcg (COT).

RESULTS AND DISCUSSION

The overall prevalence of bacateria isolated from the dead-in-shell chick embryos of psittacines was 80.30% (106/132). Among these, high level of Gram-negative organisms (49.24%, 65/132) was observed followed by Gram-positive organisms (28.78%, 38/132) and Gram-positive filamentous bacteria (2.27%, 3/132) were also isolated from the dead in shell chick embryos (Table 1; Figure 1). Among the Gram-positive bacteria, *Staphylococcus* sp. (21.12%) was found to be more followed by *Bacillus* sp. (3.78%), *Corynebacterium* sp. (2.2%), and *Enterococcus* sp. (1.51%). Similarly, among the Gram-negative bacteria *Escherichia* sp. (25%) was identified more followed by *Pseudomonas* sp. (6.60%), *Serratia* sp. (6.60%), *Acinetobacter* sp.

(4.5%), *Enterobacter* sp. (3.78%), *Yersinia* sp. (3.77%), *Citrobacter* sp. (2.27%), *Buttiauxella* sp. (1.52%), *Klebsiella* sp. (1.51%), *Cedeacae* sp. (1.51%) and *Erwinia* sp. (0.75%) (Table 2). Among the psittacine species, more numbers of the dead in shell cases were found in conures (37%) and lories (14.5%) depicted in figure. 2. Commonly found bacterial isolates were subjected to antibiotic sensitivity test. All the isolates were found to be sensitive to enrofloxacin and cefotaxime whereas, most of the bacteria showed resistance to trimethoprim + sulphamethoxazole and kanamicin. Resistance to other antibiotics were variable. Multiple resistance was not seen among the isolates.

Table 1 Bacterial isolates obtained from shell chick embryos of psittacines

Bacterial isolates obtained	Number of sample positive
Gram negative bacteria	65
Gram positive bacteria	38
Gram positive with filamentous type	3
Total	**106**

Fig. 1 Bacterial isolates obtained from shell chick embryos of psittacines

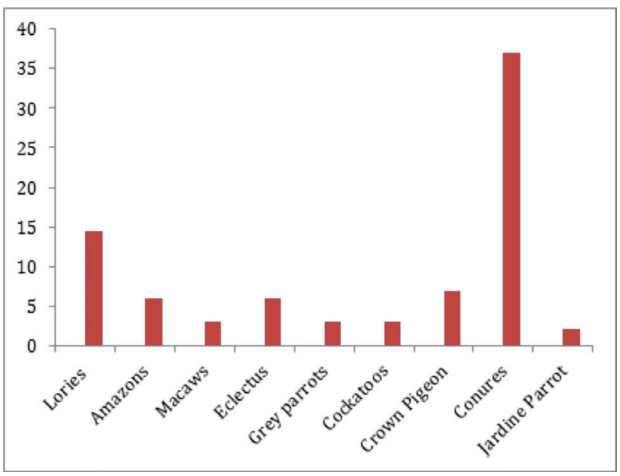


Table 2 List of Gram positive and gram negative bacteria isolated from different Psittacine species

Gram negative bacteria	Lories	Gray parrot	Amazon	Conures	Cockatoos	eclectus	Crown pigeon	Macaws	Total
Acinetobacter sp.		2		3		1			6
Butiauxella sp.			1						1
Citrobacter sp.	2		1						3
Escherichia coli	4	2		26	1				33
Yersinia sp.	1	1	1	2			1		6
Enterobacter sp.	3							1	4
Pseudomonas sp.						1			1
Serratia sp.	2							3	5
Erwinia sp.							1		1
Cedeace sp.							2		2
Klebsiella sp.		1		1					2
Gram positive bacteria									
Bacillus sp.		1	1	1		2			5
Staphylococcus sp.	6	1		14	1		6		28
Enterococci sp.				2					2
Corynebacterium sp.	1	2							3
Gram positive with filamentous type			1	2					3

Dead-in-shell in artificial incubation due to bacterial contamination is a critical issue and should be considered important as the exposure of eggs to environment is minimal when compared to that of natural incubation. The possible reasons for dead-in-shell in artificial incubation are faecal contamination of the egg shell, design and ventilation of hatchery, high stocking density, poor sanitation of hatchery room and their equipment resulting in poor hatchability. Out of the 132 samples screened, 106 samples showed bacterial contamination due to both Gram positive and Gram negative bacteria. A total of 41 Gram positive bacteria and 65 Gram negative bacteria were isolated. The percentage of Gram negative bacteria isolated was higher than that of the Gram positive genera. This is correlated well with the results of Osman et al. (2013). *Escherichia* sp. was isolated most commonly among the Gram negative (25%) organism which is in accordance with the findings of Cortes *et al.* (2004) and Amer et al. (2017). The higher rate of *Escherichia coli* isolation in the dead-i-in shell may be due to the presence of *E. coli* in faecal contaminated water which may

contaminate the egg shells (Cortes et al., 2004).

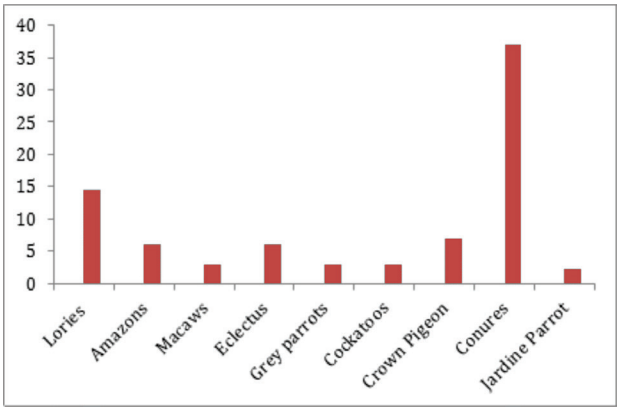
The other Gram negative bacteria isolated in higher percentage were *Acinetobacter* sp. (4.5%) and *Yersinia* sp. (3.75%). This result goes in parallel with the work of Osman et al. (2013) and Casadio et al. (2014). *Pseudomonas* sp., reported as a potential pathogen causing embroyonic moratlities (Walker et al., 2002) was isolated in a higher percentage (6.60%) when compared to the other Gram negative genera next to *Escherichia* sp. In this study, the incidence of *Salmonella* sp. is zero and this was in agreement with the results of Jahantigh. (2010). However Amer et al. (2017) and Osman et al. (2013) reported low percentage of S*almonella* sp. in their studies. The other Gram negative bacteria isolated were *Enterobacter* sp. (3.78%), *Citrobacter* sp. (2.27%), *Buttiauxella* sp. (1.52%), *Klebsiella* sp. (1.51%), *Cedeacae* sp. (1.51%) and *Erwinia* sp. (0.75%). The number of Gram positive and negative bacteria isolated and their percentage are listed in table 2.

Table 3 Dead in shell mortality due to bacterial growth

Species	Number of samples examined	Number of showed bacterial growth	Positive percentage
Lories	24	19	79
Amazons	10	5	50
Macaws	09	4	44
Eclectus	12	4	33
Grey parrots	10	10	100
Cockatoos	04	2	50
Crown Pigeon	11	11	100
Conures	52	51	98
Total	**132**	**106**	

Among the Gram positive genera, *Staphylococcus* sp. was found to be in higher percentage (21.12%). This is similar to the results of Cortes et al. (2004). The other Gram positive bacteria isolated were *Bacillus* sp., *Enterococcus* sp. and *Corynebcaterium* sp. as reported by many authors (Osman *et al*., 2013, Razmyar and Zamani, 2016 and Amer et al., 2017). Most of the dead in shell cases were reported in Conures, followed by lories (Fig .2). No bacterial contamination was detected in 26 dead-in-shell cases. This may be due to malposition of the egg and irregular humidity levels in the incubation room as reported by other authors (Sanctuary et al., 1925) or fluctuating humidity levels (Jordan, 2001).

Fig 2 Overall dead in shell mortality percentage in different species of psittacine



In this study, it was observed that the frequency of isolation of pathogenic bacteria *viz. Pseudomonas* sp. and *Salmonella* sp. were lower or nil when compared to the *E. coli* and *Staphylococcus* sp. The results suggest that the egg contamination in artificial incubation may be due to improper handling of egg without sanitisation during shifting of eggs from the cage to incubator and moving eggs with in the incubator. The surface contaminants may penetrate the egg shell and enter the yolk sac which may probably results in contamination. The ability of bacteria *viz*. *Acinetobacter* sp., *Staphylococcus* sp. and *Serrratia* sp. to penetrate ostrich egg shell has been studied (Knobl et al., 2012).

The antibiotic sensitivity profile of the most frequently isolated bacteria and bacteria of pathogenic importance was studied. Variable sensitivity/ resistant patterns were observed among the isolates. Except *Staphylococcus* sp. and *Acinetobacter* sp., which showed sensitivity to all the antibiotics tested, the others showed resistance to two or more antibiotics. The highest rate of resistance was against kanamicin and trimethoprim + sulphamethoxazole followed by gentamicin and tetracycline. All the isolates were found to be sensitive for cefotaxime and enrofloxacin. Most of the isolates showed resistance to kanamicin and trimethoprim + sulphamethoxazole. None of the isolates showed multidrug resistance. However, 100% resistance to three antibiotics were observed in *Klebsiella* sp. (gentamicin, kanamicin, trimethoprim + sulphamethoxazole) and *E.coli* (kanamycin, tetracycline and trimethoprim + sulphamethoxazole.).

It can be concluded that bacterial contamination was found to be the major challenge in artificial incubation in psittacine species. Thus, the study recommends the use of clean room facilities for artificial incubation and establishment of hygienic practices to avoid bacterial contamination thereby enhancing hatchability of eggs.

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Comparison of Effect of Nanocoating Against Biofilm Forming Bacteria on Mild Steel

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ABSTRACT

Copper has been known to possess antimicrobial properties since as far back as the Phoenician era where ship hulls were copper sheathed to prevent the inevitable effects of biofouling. As a consequence of evolving scientific research and development, the realization of novel materials and agents has enabled new scientific branches, such as nanotechnology. In this paper, we investigate the performance of different forms of copper coating for application as antifouling materials. Samples were deployed in Tuticorin-New harbour for four weeks and analyzed for evidence of biofouling. It was found that copper in its nanoform, produced the greatest antifouling effectiveness in mild steel compared to other forms ofantifouling coating.

Keywords: Copper, Antifouling coatings, Biofilm, Mild steel

INTRODUCTION

Marine biofouling is one of the chief unanswered troubles at present affecting the shipping industries and industrial equipments. The term 'biofouling' refers to the undesired accumulation of microorganisms, plants and animals on any artificial structures, which are exposed to aquatic environments. The establishment of fouling communities on a wide variety of substrata has been investigated thoroughly and the resulting literatures are vast. Theestablishment of the fouling community has been characterized interms of several stages and some of these stages can overlap oroccur in parallel. In the development of biofouling, any submergedsurface rapidly becomes coated by a conditioning film comprising of organic and inorganic molecules which may act as sourceof nutrients for microorganisms. Formation of this film is immediately followed by the accumulation of microorganisms (bacteria, diatoms), which secrete extracellular polymeric substances (EPS) during attachment, colonization, population growth and the resulting layer is termed as the biofilm (microfilm). The biofilm maypave for the settlement of larvae of higher organisms such as barnacles, mussels and tubeworms which constitute macrofouling.These organisms cause serious technical problems by settling onship hulls, power plant cooling systems, aquaculture systems, fishing nets, pipelines, submerged structures and also oceanographic research instrumentation thereby leading to huge economic losses. In ships, the friction between the hull and water increases, which indirectly increase in fuel consumption (up to 40-50% with

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low-density biofouling) and decrease the speed and manoeuvrability(International Maritime Organization, 1999).

In order to avoid economic losses, associated with accelerated deterioration of the artificial structures in contact with seawater, different types of protections have been used overtime. Among them, it is necessary to specify the copper coatings that began to be used by the Phoenicians, continued to be successful and used on wood ships until the 18th century. When iron ships were first built, paints widely known as "patents" was used, in which the copper sulphate acted as a biocide. The copper based mixture works well for short term and serves as an ideal antifouling agent at least for three years after application (Clare, 1995). When copper is used in nano level, the impacts on the environment is much lesser. The results of scientific studies have revealed that the nanocoatingsprevent biofilm formation, bacterial adhesion besides the attachment of macro foulers. (Szewczyzk, 2010).The present investigation is based on a nanocoating method byincreasing the surface smoothness, which prevents the settlement of bacterial species which are considered to be effective fouling species and responsible for primary film formation

MATERIALS AND METHODS

Test panels

Mild steel of size 15 cm height, 8 cm breadth, and 12mm thickness and weight of 1kg, respectively were prepared and mounted with the help of 4mm polypropylene rope in an iron frame having a dimension of 106.5cm length and 106.5cm width. With the help of a loop provided on the top, the frame was tied with a 12mm polypropylene rope and suspended in the CECRI jetty, inside the Tuticorinnew harbour area at a depth of 1.5m.

Sampling schedule

After immersion in seawater, the study panels were sampled periodically for microbial studies. For microbial analysis, the samples were drawn after 24h of immersion and subsequently after the1st week, 2nd week, 3rd week and the 4th week.

Antifouling coatings

Steel was encasedby adopting Physical Vapor Deposition (PVD) and Spray methods. For comparative studies,the selected boat-building materials were painted with commercially available antifouling paint (NOAH marine paints, Cochin).Various nanocoating methods exposedfor the study are described below.

Physical Vapor Deposition (PVD)

Copper powder was taken in a conicalcontainerwith a flat bottom having a size of 29mm (dia) x 13mm (h)with a capacity of 7CC. The pellet die assembly was then compressed under a pressure of 15 tonto form a pellet within five minutes duration.After pelletformulation, it was placed over the substrate heater i.e. electron gun. The copper pellet targets the panels once thevacuum havecreated inside the chamber arc gas will released. The copper powder is heated above 1789° Celsius, until it gets vapourized. The vaporized atoms get deposits to the panels. The mild steel substrate to be used was cut into proper dimension (75mm x 25mm), so as to

properly fit it into the substrate holder of the sputtering machine. The surface of the mild steel was polished and smoothened with emery sheets of various fineness followed by cleaning with acetone. The respective prepared composite pellets were then placed in the sputtering machine (Hind Hivac, Bengaluru) as targets and the mild steel substrates were coated using RF sputtering in argon atmosphere.

Spray coating

The nanocoating was accomplished for steel by spray method. The synthesized copper nano powder was mixed with enamel paint with amagnetic stirrer at 60^0c for 6 hrs. Subsequently the paint mixed with nano powder was coated with 2-4 bar / 30 psi pressure and dried for 24 hrsat room temperature.

Isolation and Enumeration of biofilm forming bacteria from the test Panels

For the isolation of biofilm forming bacteria, a template of size 5 x 3 cm^2 was placed on each test panel and the bacteria were scraped using ascalpel and placed in a test tube containing 10 ml of sterile saline. Serial dilutions were done with the same diluent. Appropriate dilutions were then inoculated in specific media (Zobell Marine Agar) for the enumeration of biofilm forming bacteria. About 0.1ml of the appropriate dilution was inoculated on the sterile medium and spread plated uniformly with sterile glass spreaders. The plates were left at the room temperature for about 30 min till the sample is completely absorbed by the medium. The petri plates were then inverted, stacked in lots and incubated at a temperature of 37˚C for 24 h. The colonies were counted as biofilm forming bacteria and expressed as CFU/cm^2.For purification of the selected colonies, the predominant colonies were streaked on Zobell agar medium, and the single isolated colonies were picked up and maintained in Zobell agar slants and stored in a refrigerator.

DNA Extraction

From the purified colonies, genomic DNA was extracted (HiMedia DNA extraction Kit). Approximately 1.5ml of overnight growth broth culture was taken in a 2 ml microfuge tube,180 µl lysis bufferand 20 µl of proteinase K were added. After homogenization, the tubes were incubated at 55°C for 30 min in a water bath. Then, an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added. The contents were mixed gently and centrifuged at 9200 rpm for 10 min. The top aqueous layer was then transferred to a new 1.5 ml microfuge tube. The DNA was precipitated by the addition of equal volume of isopropanol and 0.2ml volume of 10M ammonium acetate and by inverting the tube several times. The tube was centrifuged at 13,200 rpm for 10 min. The supernatant was removed and the pellet was washed in 500 µl of chilled 70% ethanol, air-dried and resuspendedin 100 µl sterile water.

Polymerase Chain Reaction

The 16S rDNA region was amplified by PCR from the isolated genomic DNA using the universal primers. Primers used for PCR analysis were, Forward: 5'-AGAGTTTGATCMTGG-3'and Reverse: 5'-ACCTTGTTACGACTT-3'

The amplification was carried out in 25 µl of reaction mixture containing 2.5µl buffer 0.25µl dNTP, 19 µl of molecular grade water, 0.25µl of Taq DNA polymerase, 1µl of each forward and reverse primers and 1µl of template DNA. The PCR protocol comprised of initial denaturation at 95°C for 5 min, denaturation at 94°C for 30 sec, annealing at 52°C for .30 sec, extension at 72°C for 45 sec and afinal extension at 72°C for 10 min. The number of cycles was 35.

Agarose gel electrophoresis

The PCR amplifiedproduct (4 µl) was mixed with 1 µl of 6X loading buffer and subjected to electrophoresis on a 2% agarose gel containing ethidium bromide at a concentration of 0.5 µg/ml in TAE (1X) buffer. The gel was observed under UV transilluminator and photographed in a documentation system.

Purification and sequencing of DNA Samples

Amplified PCR product was purified using column purification kit as per manufacturer's guidelines and used forsequencing. (Unibiosys Lab, Cochin, India) Phylogenetic analysis was done using theMEGA software.

RESULTS AND DISCUSSION

Effect of antifouling coating on Microbes- Steel

In steel panels, initially the biofilm forming bacteria in control, antifouling painted nanocoated and spray coated were 4.3,4.64, 0 and 4.22, logcfu/cm^2, respectively. The counts increased with the duration and reached 4.66, 5.7, 4.65 and 4.98 logcfu/cm^2, respectively after the 4th week.Overall analysis on antifouling, showed that nanocoatedvapor deposition worked on first three weeks and it showed arapid decrease in its antifouling activities due to changes in surface topography. Next to vapor deposition, spray coating shows reduction in colonies formation compared to antifouling painted (Table1).

TABLE: 1 Effect of antifouling coating on Microbes- Steel

Panel type	In weeks (log CFU/cm^2)			
	1st	2nd	3rd	4th
Steel Control	4.37	4.66	4.44	4.3
Vapor deposition	0	0	0	4.65
Antifouling painted	4.64	5.07	4.69	5.7
Spray coated	4.54	4.22	4.66	4.98

Isolation of biofilm forming bacteria

A total of three isolates were selected from the test panels (H_1, A_2, and A3). Two of the isolates (H_1, A_3) were present in all the test panels. Secondisolate (A_2) was isolated particularly from the nanocoated panel (Table 1).

Table-2: Details of BLAST analysis, percentage of similarity and NCBI accession numbers of marine biofilm forming bacteria isolated from different kinds of coated panels.

S.NO	Assigned Code	Sequence length (bp)	Similarity (%)	BLAST results	NCBI's accession
1.	*H1*	977	99	*Pseudomonas aeruginosa*	JQ659528
2.	*A2*	989	99	*Ferrimonasfuttsuensis*	JQ799090
3.	*A3*	1007	99	*Vibrio alginolyticus*	KJ872832

Amplification of 16s rDNA from biofilm forming bacteria

16S rDNA gene amplification of the isolates H, A_2, and A_3 amplified the gene in all the bacterial species resulting in a 1200 bp product, which was clearly visualized in the agarose gel electrophoresis. The first isolate and the third isolate was identified as *Pseudomonas aeruginosa* (H_1) and *Vibrio alginolyticus* (A_3) based on 99% similarity with the available sequence in the NCBI data base. These two species were present in all type of antifoulingcoated panels. The second isolate was identified as *Ferrimonasfuttsuensis* (A_2), which was present in steel control and nanocoated panels

Phylogenetic analysis methods

The overall phylogenetic analysis, of the selected sixteen biofouling bacterial sequences showed two major divergences found among the species in Minimum Evolution method (Fig3). Inner level divergences were observed in all the bacterial species. Bacterial species, such as *Pseudomonas aeruginosa, Ferrimonas futtsuensis, Vibrioalginolyticus* are significantly diverged from other twelve species. These three diverged species have closeness measured as less 0.1 in divergence scale and *Vibrio alginolyticus*were found in same branch whereas *Pseudomonas aeruginosa, Ferrimonas futtsuensis*are diverged in two distinct inner nodes. Apart from the subjected sequences, *Proteus mirabilis, Halomonasaquamarina, Halteleaalkalilenta*are diverged from the first node and further diverged internally. The other distinct divergence observed was *Arthrobactersp, Micrococcus sp, Micrococcus luteus. Exiguo bacteriumaestuarii, E. arabatum, Exiguobacteriumsp, Jeotgali bacillusalimentarius, Bacillus Flexus*and *Bacillus sp*. are diverged in the same node and further diverged in different branches due to course of evolution. From the overall phylogenetic analysis reveals the *Pseudomonas aeruginosa, Ferrimonas futtsuensis, Vibrio alginolyticus*are shown the distantly related with other selected species of bacteria involved in biofouling activities.

Figure-1: Effect of antifouling coating on Microbes- Steel

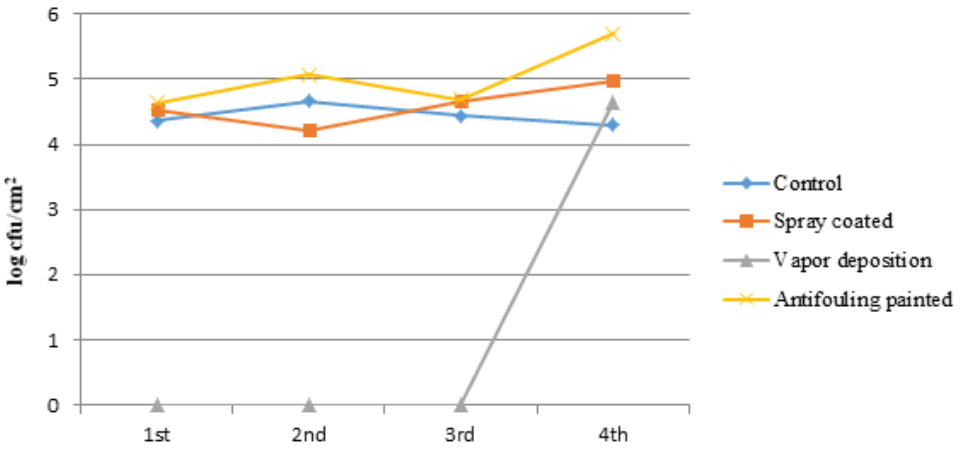


Fig 2.Ethidium bromide stained 2% agarose gel showing results of electrophoretic analysis of amplified 18S rRNA gene eukaryotic specific primer obtained from different bacterial species. Lane M- 1200 bp DNA marker; Lane A- *Pseudomonas aeruginosa*; Lane B – *Ferrimonasfuttsuensis*; Lane C-*Vibrio alginolyticus*

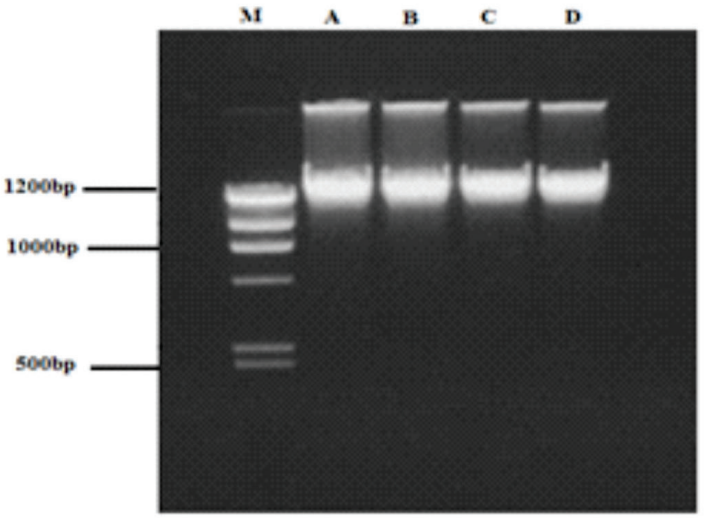
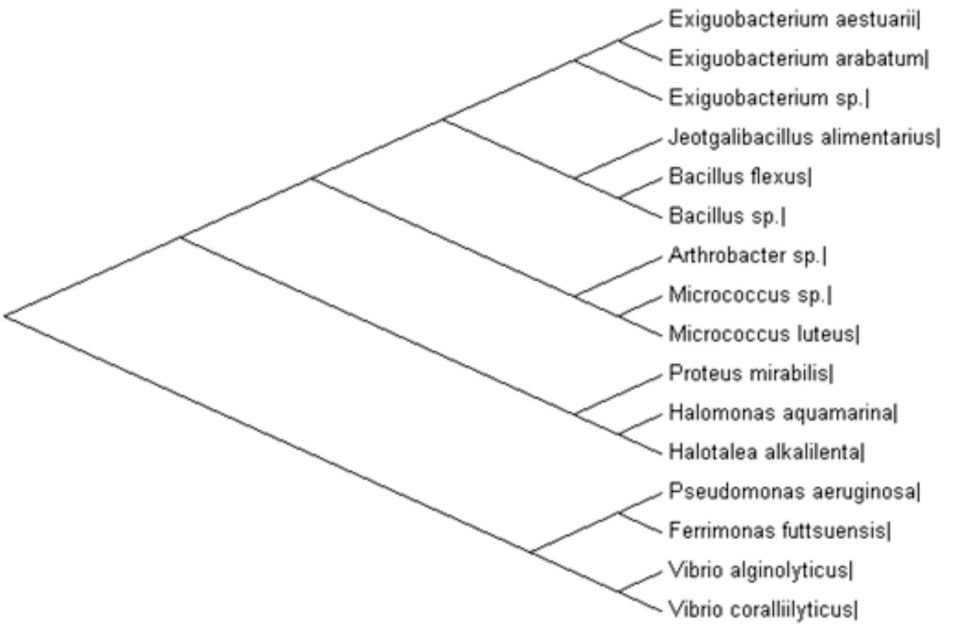


Fig-3: Phylogenic tree constructed using the Minimum Evolution Method



Sequencing of the amplified 16S rDNA gene region

The bacteria isolated identified based on the amplification 16S rDNA fragments and sequencing are presented in Table 2. All the three speciesviz., *Pseudomonas aeruginosa, Ferrimoasfuttsuensis, Vibrio alginolyticus,* showed more than 99% homogeneity based on the entries available in the NCBI databases.

Effect of antifouling coating on biofilm forming bacteria on various test panels

Deposition of microorganisms on surfaces and biofilm formation is an important bacterial survival strategy. Biofilms occur spontaneously on both inert and living systems, being of concern to a wide range of scientific disciplines. In industry, biofilms can have a detrimental impact because of accumulation at interfaces (Characklis,1983; Cooksey, 1983). Competition for living space is more intense in themarine environment; hence all submerged surfaces in the marine environment are rapidly colonized by bacteria and they form the important component in the development of a fouling community (Mitchell and Kirchman, 1984). Biofilms are formed by microbial cells embedded in an exopolymeric matrix. The extracellular matrix is mainly composed of polysaccharides and proteins, along with compounds such as DNA and humic substances (Nielsen *et al.,* 1997; Jahn*et al.,* 1999).

In the present study, when the panels coated with copper following three different

methods were exposed to seawater in order to determine their potential quality and recruitment of biofilm bacteria formed over the, biofilm bacterial load (TVC) was found to increase gradually i.e., from 4.3 up to 4.66,4.22 to 4.98, 0 to 4.65 and 4.64 to 5.7 log cfu/ cm^2in the control mild steel, spray coated, nano-coated and antifouling coated, respectively from 24 to 72h intervals. The assessment of bacterial population in the mild steel inferred theslow rate of succession of thebacterial load with respect to thetime interval. This may be due to corrosive nature and changes in ionic charges of the substrates.

PCR method for the identification of bacteria using 16S rDNA gene

Suriya Murthy et *al.,* (2004) studied the biofilm control using plate heat exchangers of surface seawater from the open ocean for the OTEC power plant. Microbiological analysis of biofilms revealed that four distinct types of bacterial colonies were present, the pre dominant bacteria *Vibrio, Flexibacter*, *Pseudomonas and Aeromonas*, were the total viable bacteria in untreated controls were observed and tend to amplified counts with the age of the biofilms. There was a highly significant variation (P = 0.0001) between chlorinated and control biofilms. Feng*et al.* (2000) and Yamanaka *et al.* (2005) reported the mechanism of the inhibitory action of silver ions on microorganisms. They concluded that, when treated with silver ions, microbes their DNA replication ability, expression of ribosomal subunit proteins and other cellular proteins, and inactivation of enzymes essential for ATP production. It has also been hypothesized that Ag+ primarily affects the function of membrane-bound enzymes, such as those in the respiratory chain. However, the mechanism of bactericidal actions of silver nanoparticles is still not well understood.

In our study, the collected bacterial samples were investigated for the isolation, identification, sequencing, characterization bacterial species and their genetic makeup through advanced molecular biological studies including polymerase chain reaction, electrophoresis and sequencing techniques. This study has also helped to predict the mode of binding, genetic materials responsible for biofouling, etc. Polymerase chain reaction a molecular methods to identify the genetic makeup of identified bacterial DNA, which responsible for biofouling activities. Bacterial species, such as *Pseudomonas aeruginosa,* (Sonak and Bhosle, 1995) *Ferrimonas futtsuensis, Vibrio alginolyticus*(Muralidharan *et al.*, 2003) were identified. From the result, *Pseudomonas sp*was observed to be the most dominant bacteria.

The data presented here on the diversity of biofilm bacterial strains in the experimental panels indicated that most of the strain identified were gram-negative in nature. Among biofilm bacterial strains isolated, the most predominant bacterium recorded in all the experimental panels was *P. aeruginosa* (29 – 35%) and next dominant biofilm bacterial strains registered were *V.alginolyticus* with the percentage occurrence range of 17 – 19% respectively. The other biofilm bacterial strains were recorded in lesser proportion (1 – 10%).

From genetic profiling of identified bacterial samples this imperative research will focus and deliver the unknown information about bacterial species and genetic relationship among the different species. knowledge obtained from the bacterial sequence, similarity of this study, will help us to identify the target site of bacteria for the developmentof more potent antifouling materials in future.

CONCLUSION

Rougher surfaces accumulate and retain more colonization, by increasing the surface smoothness, which prevents the settlement of bacterial species which are considered to be effective fouling species and responsible for primary film formation. Copper nanoparticles in expand PVD shows more antifouling potential up to three weeks. This work integrates nanotechnology and bacteriology, leading to possible advances in the formulation of new types of bactericides or antifoulants. Resultsobtained from the present investigation demonstrated the anti microfouling potential of copper nanoparticles. Copper nanoparticles have a high potential to be may assembled or coated on marine industrialsurfaces and to study the possible biofouling control with furtherexperiments for a new anti microfouling material in future.

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Socio-Economic Profile and Management Practices Adopted by Sheep Farmers in Dhubri Distrct of Assam

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ABSTRACT

A study was carried out among sheep farmers in Dhubri district of Assam on socio-economic status and management practices adopt1ed by them. Altogether 120 numbers of farmers were selected randomly from two blocks of this district. The data were collected through personal interview method with the help of a well-structured, comprehensive and pre-tested interview schedule. Most of the sheep farmers were illiterate and belonged to middle age group. Majority (65.00%) of the respondents had agriculture as their main occupation. Majority (73.33%) of the respondents had low annual income followed by medium annual income (22.50%) and high annual income level (4.16%). The study revealed that sheep were sheltered during night only. There was no separate housing for the sheep alone. Sheep were let loose in the morning and returned back to the night shelter before evening when there was no crop in the field. During flood, the animals were shifted to an elevated area and were mostly fed with tree leaves such as jackfruit, mango, neem, guava, banana, and babool. During rainy season, when most of the fields were waterlogged, they even graze in knee deep condition in marshy land. Due to grazing in marshy land during rainy season, they were highly prone to parasitic infection particularly liver fluke infection. During flood, most of the sheep died due to severe starvation. Majority of the farmers reported two breeding seasons in sheep viz. June-August and January-March. None of the farmers vaccinated their sheep in the study area. Hence, there is an urgent need to adopt improved management practices to exploit the production potential of this local sheep. By adopting improved management practices, better growth could be achieved, which will ultimately increase the income of the rural poor.

Key words: Sheep**,** education, management practices, deworming.

INTRODUCTION

Sheep is a small, calm and versatile ruminant and is reared mainly for wool, meat and skin production in India. People of some regions are raising sheep as domestic animal for fulfilling family nutrition demand and business purpose from the ancient time. However, sheep rearing is not popular in India as in Australia and New Zealand. They have an excellent ability to survive over a prolonged period of drought and semi starvation and are less prone to extreme weather conditions, ecto-parasites as well as other diseases. Because of their hardiness and adaptability to dry conditions, the north-western and the Southern

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peninsular regions of the country have a large concentration of sheep. Assam has the sheep population of 5.18 lakhs the highest among the North-eastern states (BAHS, 2016), and more than 75 per cent of which are distributed in lower Assam. The Dhubri district possesses the maximum (22.81%) followed by Barpeta (18.85%) and Darrang (8.0%). Most of the landless, marginal and small farmers are rearing sheep and goat which provide substantial additional source of income particularly during agricultural lean period. The local sheep of Assam predominantly brown in colour, smaller in size produces low fat mutton, rough wool and good quality skin. The information on existing sheep husbandry practices are insufficient at present lacking which hinder the extension workers as well as policy makers to formulate an effective production strategies. Hence a study was undertaken with the objective of knowing the socio-economic status of sheep farmers, studying the existing sheep husbandry practices followed by the farmers and analyzing the disease occurrence in sheep.

MATERIALS AND METHODS

The Dhubri district of Assam was selected purposively for the study as it has the highest sheep population among the districts of State. Two blocks *viz.* Gauripur and Bilasipara were selected randomly for the study. In the next stage, five villages were selected from each of the block. Further twelve numbers of farmers were selected randomly from each of the selected villages. Thus a total of one hundred and twenty numbers of farmers were selected as respondents for the study. The farmers who had minimum 3 years of experience in rearing sheep were selected. The data were collected through personal interview method with the help of a well-structured, comprehensive and pre-tested interview schedule. The data on various parameters were collected, computed and analyzed as per standard statistical methods (Snedecor and Cochran,1994).

RESULTS AND DISCUSSIONS

Socio-Economic Status of Sheep Farmers

Education: Education plays a vital role in adopting improved management practices of sheep rearing. The study revealed that, majority (56.67%) of the sheep farmers were illiterate followed by 19.17, 13.33, 9.17 and 1.67 per cent of the respondents had primary, middle, high school and higher secondary level of education respectively (Table 1). The higher per cent of illiteracy might be due to lack of awareness and poverty among the farmers. The present findings were also similar Rajanna et al. (2012), who reported the higher per cent of illiterate (74.65%) sheep farmers in Telangana region of Andhra Pradesh.

Age: It was observed that almost three-fourth (74.17%) of the respondents belonged to middle age followed by young age (15.83%) and old age (10.00%) group (Table 1). Lower involvement of young people in traditional sheep farming might be due that most of young labourers in the study areas engaged in various construction works for their livelihood. The findings of present study also corroborated the findings of Rajanna et al. (2012).

Table 1: Socio-economic parameters of sheep farmers in Dhubri district

Variables	Gauripur (n=60)	Bilasipara (n=60)	Total (n=120)
Education			
a) Primary school	11 (18.33)	12 (20.00)	23 (19.17)
b) Middle school	9 (15.00)	7 (11.67)	16 (13.33)
c) High school	6 (10.00)	5 (8.33)	11 (9.17)
d) Higher secondary	2 (3.33)	0 (0.00)	2 (1.67)
e) Illiterate	32 (53.33)	36 (60.00)	68 (56.67)
Age (In years)			
a) Young (<30 years)	10 (16.67)	9 (15.00)	19 (15.83)
b) Middle (31-50 years)	44 (73.33)	45 (75.00)	89 (74.17)
c) Old (>50 years)	6 (10.00)	6 (10.00)	12 (10.00)
Occupation			
a) Agriculture	37 (61.67)	41 (68.33)	78 (65.00)
b) Business/trade	4 (6.67)	3 (5.00)	7 (5.83)
c) Labourer	11 (18.33)	9 (15.00)	20 (16.67)
d) Animal Husbandry	8 (13.33)	7 (11.67)	15 (12.50)
Land holding (In Bigha)			
a) Landless	13 (21.67)	19 (31.67)	32 (26.67)
b) Up to 5 Bigha	41 (68.33)	38 (63.33)	79 (65.83)
c) 6-10 Bigha	4 (6.67)	3 (5.00)	7 (5.83)
d) Above 10 Bigha	2 (3.33)	0 (0.00)	2 (3.33)
Family size			
a) Small (2-5 members)	11 (18.33)	13 (21.67)	24 (20.00)
b) Medium (6-10 members)	39 (65.00)	41 (68.33)	80 (66.67)
c) Large (above 10 members)	10 (16.67)	6 (10.00)	16 (13.33)
Annual family income			
a) Low (Rs. 20,000-50,000)	47 (78.33)	41 (68.33)	88 (73.33)
b) Medium (Rs. 50,000-1,00,000)	12 (20.00)	15 (25.00)	27 (22.50)
c) High (Above Rs. 1,00,000)	1 (1.67)	4 (6.67)	5 (4.16)
Sheep farming experience (In years)			
a) Up to 5 years	28 (46.67)	37 (61.67)	65 (54.17)
b) 6-10 years	27 (45.00)	16 (26.67)	43 (35.83)
c) Above 10 years	5 (8.33)	7 (11.67)	12 (10.00)
Religion			
a) Hindu	8 (13.33)	11 (18.33)	19 (15.83)
b) Muslim	52 (86.67)	49 (81.67)	101 (84.17)

**Figures in the parenthesis indicate per cent.*

Occupation: Agriculture alone was the main occupation (65.00%) of the respondents in the study area (Table 1). The laboureres for agriculture and construction works accounted for 16.67 percent followed by animal husbandry alone activities (12.50%) and only 5.83 percent had business as main occupation. Ramesh *et al*. (2012) also reported that all the small ruminant farmers had both animal husbandry and agriculture as main occupation in Karnataka.

Land holding: In the present study, it was evident that the landless farmers contributed a significant portion (26.67%) of the respondents, for which animal husbandry activities could be the better option for their livelihood. However, most of the landless farmers were engaged as labourers in various agricultural and construction works in the study areas. Most (65.83%) of the farmers had only up to 5 bighas of land followed by landless (26.67%), 6-10 bighas of land (5.83%) and only 3.33 per cent had above 10 bighas of land in the study areas. Rajanna et al. (2012) also reported the similar findings in Telangana region of Andhra Pradesh.

Family size: Table 1 revealed that two-third of the respondents had medium size family followed by small (20.00%) and large size family (13.33%). The family was found to be directly related with the size of the farm or flock. These results also corroborated the findings of Thilakar and Krishnaraj (2010) and Rajanna et al. (2012).

Annual family income: It was observed that majority (73.33%) of the respondents had low annual income followed by medium annual income (22.50%) and high annual income level (4.16%) (Table 1). These findings were in accordance with the findings of Ramesh et al. (2012) in Karnataka. Higher percent of lower family income among the respondents depicted poor economic condition of the farmers in these areas. Hence the farmers opted for sheep farming as an additional source of income to improve their livelihood security.

Experience in sheep farming: Gaining experience would result in success of any livestock farming. The study revealed that majority (54.17%) of the farmers had an experience of 5 years in sheep farming followed by 35.83 and 10.00 per cent with an experience of 6-10 years and above 10 years respectively in sheep rearing (Table 1). However, Ramesh et al. (2012) reported a mean experience in small ruminant farming was 9.1 years in Karnataka.

Religion: Table 1 revealed that majority (84.13%) of the farmers were Muslims followed by Hindus (15.83%) in the study areas. But Kuldeep et al. (2006) reported in their study that Muslims formed the second category after Hindus in Western Rajasthan. Higher proportion of Muslim farmers might be due to the demographic distribution of population in the study area. As there was no any social taboo in rearing of sheep, irrespective of religion they have adopted sheep farming as a mean to improve their livelihood security.

EXISTING HUSBANDRY PRACTICES

Housing: From the present study, it was revealed that all the farmers followed extensive system of sheep rearing. When

there was no crop in the field the sheep were let loose in the morning and were provided with night shelter only. There was no separate housing for the sheep alone. Most (95.33%) of the farmers kept their sheep either with goat or cattle during night. Only few (4.16%) farmers kept their sheep in one corner of the cattle shed surrounded by bamboo sticks to protect them from other livestock. Majority (98.33%) of the farmers housed sheep in mud floor without any bedding material during night. However, during winter season, they were provided with some bedding materials such as old gunny bags, paddy straw etc. to protect them from cold weather and special care was taken for new born lambs during winter. Sireesha et al (2014) reported that majority (60.70%) of the farmers housed their sheep nearer to their dwellings and some (39.30%) were away from their houses in Guntur district of Andhra Pradesh. Smaller flock size might be the reason for not providing separate house for sheep. None of the house had adequate ventilation system. Most of the houses were made with locally available materials like bamboo, wooden planks, thatch etc. without any specific dimensions. All the farmers cleaned houses daily. Majority of the farmers reported that they housed the sheep to protect them from theft and predators. During monsoon, the sheep were shifted to an elevated area, where temporary housing arrangements were made with plastic papers, bamboo etc. to protect them from flood. Sometime, the sheep were kept in boats as a man shift arrangement during sudden flood in the river islands of the district.

Feeding practices: Mostly the marginal and landless labourers were engaged in sheep farming and maintained a small flock of sheep of 5-7 and raised them on open grazing field, fallow land and on the sides of the road. They were let loose in the morning and returned back to the night shelter before evening when there was no crop in the field. During cropping season, the sheep were tied with 4-5 meters length rope in the community grazing field for feeding. During flood, they were shifted to an elevated area and were mostly fed with tree leaves such as jackfruit, mango, neem, guava, banana and babool. During rainy season, they even graze in knee deep condition in waterlogged marshy land. Concentrate mixtures were not provided by farmers to their sheep. Mostly, women and children were involved in feeding of sheep. It was evident that none of the farmers followed any special feeding care to their pregnant, lactating ewes and breeding rams in the study area. After lambing, the lambs were also allowed to go along with their mother immediately after 1 day of lambing. The findings of the present study were in accordance with the findings of Sireesha et al. (2014), who also reported no special feeding to pregnant and lactating ewes in Andhra Pradesh.

Breeding practices: All the respondents, kept both males and females together during grazing and during night sheltering. Hence, there was random unplanned and flock mating and there was no restriction and rotation of ram on mating. The farmers allowed all the animals to be naturally bred. None of the farmers aware of proper ram to ewe ratio for breeding. This was contrary to the present findings, Rajanna et al. (2014) reported the mean ram and ewe sex ratio was 1:20 and 1:24 respectively in

Nellore and migratory Coimbatore breeds of sheep. Majority of the farmers reported that two breeding seasons in sheep *viz.* June-August and January-March in the study area. However, Rajanna et al. (2014) reported three breeding seasons in sheep in Andhra Pradesh. The age at sexual maturity ranged between 7-9 months and age at first lambing ranged between 12-14 months. The average gestation period was 150 days. They lamb twice in 18 months and gave multiple births mostly twins and triplets, even quadruplets at times.

Health management practices: Most of the farmers reported that parasitic infection and diarrhoea in the rainy season were the main health problems of sheep in the study area. The results of the present study was in accordance with the findings of Mandal et al. (2006), who also reported that parasitic infection and diarrhea were the main health problems of sheep in West Bengal. Due to grazing in marshy land during rainy season, they were highly prone to parasitic infection particularly liver fluke infection. During flood, most of the sheep died due to severe starvation. During winter season, they infected with some external parasites also. None of the farmers practiced deworming to treat parasitic infection. In villages, tick and lice infestation were treated mostly by sprinkling ash powder over the whole body. None of the farmers vaccinated their sheep in the study area. Majority (91.67%) of the respondents did not consult with any veterinary officer to treat their ailing sheep. The incidence of mortality was always higher in lambs during rainy season followed by winter and summer.

CONCLUSION

From the above study, it may be concluded that mostly illiterate, economically poor, under privileged rural people were involved in sheep farming in Dhubri district. The farmers were still practicing traditional system of rearing. Hence, there is an urgent need to adopt improved management practices to exploit the maximum production potential of this local sheep. During flood, special care has to be taken to prevent mortality in lambs, by adopting improved management practices, better growth could be achieved, which will ultimately increase the income of the rural poor sheep farmers.

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Short Communication

Report on the occurrence of *Octolasmis angulata* (Aurivillius, 1894) in *Portunus pelagicus* (Linnaeus, 1758) fromPulicat Lake, Tiruvallur district, Tamil Nadu, India

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Barnacle of the genus*Octolasmis* are commonly called goose-neck barnacle and are frequently found in infesting corals, echinoderms, molluscs, horseshoe crabs, lobsters, isopods, sea-snakes, stomatopods (Jeffries and Voris, 1996). They are found in shallow waters attached to the exoskeleton of the decapod crustacea, including crabs and few are found in depth greater than 1000 meters (Foster, 1987). A number of *Octolasmis* species live in the gill chambers of their hosts cemented to the gill lamellae rendering the space available for respiration leading to the death of the host (Gannonandand Wheatly, 1992). During a visit to Pazhaverkadu fish landing center located near Pulicat Lake, Tiruvallur district, a single individual of *Portunus pelagicus* (Linnaeus, 1758) infested with *Octolasmis*sp. (Gray, 1825) was encountered. Examination of the species based on the literature by Ihwan et al. (2014), revealed to be*Octolasmis angulata* due to the absence of tergum and carina. Till date, there is no report of *Octolasmis* sp. infesting crabs from Pulicat region of Pazhaverkadu, though there is a report of *Octolasmis angulata* fromPulicat Lake recorded by Sanjeeva Raj (2006). The individual of *O. Angulata*was found attached toboth the cheliped, posterior region of the carapace, carpus and near the eye. About 15–20 individuals were found cemented to the posterior region and about 4 nos. were found occupying the space between the eyes (Image1 A&B). Dissection of the host revealed that there was thinning of body muscle, which was indicated by very thin muscle fibers and lean muscle mass. It is reported that infestation of *Octolasmis*

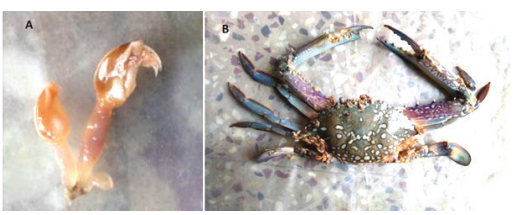

Image1. **A.***Octolasmis angulata* (Aurivillius, 1894) **B.** *Portunus pelagicus* (Linnaeus, 1758) infested with *Octolasmis angulata*.Ghani. R.A, Khattak NA, Saqlain M, Asad MJ, Khanum A, Naqui SMS and Raja GK

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sp. in the gills leads to suffocation and mortality, but in the present study thinning of body muscles were observed. *Octolasmis* infestation is a problem in mud crab and blue crab culture in the most Asian country like Thailand, Vietnam, and Philippines (Shelley and Lovatelli 2011). A further work on the effect of *Octolasmis* sp., and the correlation of its infestation with the thinning of muscle mass and histopathological changes must be studied.

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