Zoological Society of Orissa

Brief History

Pranikee, the annual journal of Zoological Society of Orissa, publishes original research articles on Zoology.

The Society was founded in 1958 in order to promote effective communication between Zoologists through its publications, seminars and annual meetings.

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The emblem of Pranikee

The emblem "***NABAGUNJARA***" is a chimeric animal and a common motif of Odishan art and literature. It literally means "Nine form". This form has been described by poet Sarala Das in the Odia version of the epic Mahabharata. Apparently, Lord Krishna appeared in Nabagunjara form consisting of the body of an elephant, a leg each of a horse, a deer and a tiger respectively; throat of a peacock, tail in the form of a serpent, waist of a lion, hump of a bull and head of a cock, to fool his friend Arjuna. The Chimera was holding a lotus flower in a human hand. Arjuna had never seen such a creature in his life and guessed that this could not be a real animal but a form assumed by Lord Krishna and immediately bowed down at his feet. It is said that the human hand with the lotus provided the clue. In the paintings and sculptures however, the lotus is often replaced by a "Chakra" or the "stylized discus" of Lord Krishna. Chimeric forms are encountered in literature and art all over the world. However, a chimera of nine animals is uniquely Odishan. Therefore, it was considered to be an appropriate emblem for the Journal of Zoological Society of Odisha.

Padma Shri Prof. Priyambada Mohanty-Hejmadi
Former Editor

From the Editor's desk

An insight that is still not acknowledged in many expositions of Zoological sciences, is that some central questions pertaining to life forms like what makes the biodiversity such a marvelous dynamic process?, what are the principles of birth, growth, and death?, have no simple answers. Insteed, the mechanisms, processes, and the (diverse) constituents are there to be discerned, layer upon layer, from levels below the membrane architecture to levels above organizational complexity. The Zoologists of the state of Odisha and outside incessantly pursue their goal to unzip the secrets of life and life processes.

"PRANIKEE", the journal of the Zoological Society of Orissa, is emboldened as always by publishing some exciting research findings in the field of current Zoology. The present edition of the Journal (Volume XXVIII) is ready for circulation which carries one review and six research articles. The review article deals with the application of molecular markers in fish genetic improvement. Research articles cover different aspects of Zoology including taxonomy of a snake inhabiting northeast India, diarrhoeagenic *Escherichia coli*, intestinal bacterial flora of the common Asiatic toad, quantification of *Drosophila* genome, DNA fragmentation facilitated cell death in the tail of an anuran tadpole and blood profile of indigenous breeds of chicken. It is my credo that this volume will not go unnoticed in the spectrum of explosively expanding knowledge in life sciences.

P K Mahapatra

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APPLICATION OF TRADITIONAL AND MOLECULAR MARKERS FOR STOCK IDENTIFICATION

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ABSTRACT

During the last few decades, molecular markers have entered the scene of genetic improvement in different fields of agricultural research. The ease and simplicity of markers whether belong to traditional or molecular can make it ideal for genetic improvement programs, and stock identification, with particular utility in the field of population genetics. This review summarizes the use and application of morphometrics and molecular markers particularly, microsatellite and mitochondrial markers in fish research.

Key Words: Population, Genetic stocks, Marker, Microsatellite, Mitochondrial DNA.

INTRODUCTION

India possesses vast inland water resources in the form of rivers, estuaries, natural and manmade lakes. The inland water bodies have been divided into five major riverine systems and their tributaries extending to a length of about 29,000 km in the country – Indus, Ganges, Brahmaputra, East flowing riverine system, and West riverine system. The major river systems of India on the basis of drainage can be divided broadly into two major river systems namely (i) Himalayan river system (Ganga, Indus and Brahmaputra) and (ii) Peninsular river system (East coast and West coast river system). The peninsular rivers flow towards the east into the Bay of Bengal is the east coast river system. It comprises of the Mahanadi, Godavari, Krishna and Kaveri rivers. The length of east coast river system is 6,437 km. Mahanadi is the

largest river of Odisha. Godavari and Krishna are the largest of the east coast river system, found in Maharashtra and Andhra Pradesh, respectively. The delta regions of these rivers are very abundant in fishes, but the percentage of major carp spawn is only 20.3 in the Godavari at Rajahmundry. The upper regions of the Kaveri, being fast-flowing and sufficiently cool, are unsuitable for carp fishery, the middle and lower reaches harbour a fairly good fishery of major carps. The major rivers of the west coast are Narmada and Tapti but river Mahi which is found in Madhya Pradesh, Maharashtra, and Gujarat also serves as a major river in west coast, length of the river system is 3,380 km. All these rivers, their tributaries, canals, and irrigation channels have an area of roughly 13,000 km. These water bodies harbor the original germplasm of one of the richest and diversified fish fauna of the world comprising of 930 fish species belonging to 326 genera.

Fishes are cold blooded aquatic vertebrates, possessing gills and fins. There are 24,600 species of known fishes (Nelson, 1994), which comprise almost half the number of total vertebrates. About 95% of fishes are teleost. The largest family of freshwater fishes, the Cyprinid, which are commonly known as minnows or carps, comprises of 210 genera and 2,010 species (Liu and Chen, 2003). Approximately 1270 species are native to Eurasia, 475 species in 23 genera are native to Africa while about 270 of Cyprinid species in 50 genera are endemic to North America. Of this, the greatest diversity and number of species occurs in China and Southeast Asia (Wu, 1981; Nelson, 1994). The major carps of India fall under three genera like *Catla, Labeo,* and *Cirrhinus*. Due to their fast growing nature and taste, Indian major carps enjoy a prime position in the Indian aquaculture scenario. The Indian major carp species Catla (*Catla catla*), Rohu (*Labeo rohita*), and Mrigal (*Cirrhinus mrigala*) are considered to be the best suitable carp species for their cultivable qualities. Without doubt the carps are the most largely cultivated species throughout the peninsular region.

Conservation of Natural resources

Overfishing, when particularly directed towards certain sizes or age classes, may reduce population size to levels at which inbreeding and loss of genetic diversity may become serious problems or result in extinction of local population or population segments (Lakra et al., 2007). Habitat destruction is another reason that leads to the depletion of these natural resources. There are many factors, such as deforestation, watershed erosion, silting, agricultural runoffs, pesticides, fertilizers, sewage and chemical pollutants which destroy the breeding habitats or add additional stresses to many aquatic species. Attempts to promote aquaculture practices in the area using transplanted Indian major carps and other exotic species have led to further deterioration in the situation. Ponniah and Gopalakrishnan (2000) reported an alarming rate of depletion of the fish diversity in gangetic region due to over-exploitation and clandestine export.

Stock Identification

Stock identification is an interdisciplinary field that involves the recognition of self-sustaining components within natural population and is a central theme in fisheries science and management. The obvious role of stock identification is as a prerequisite for the tasks of stock assessment and population dynamics, because most population models assume that the group of individuals has homogeneous vital rates (e.g., growth, maturity, and mortality) and a closed life cycle in which young fish in the group were produced by previous generations in the same group. Because stock structure and delineation are uncertain, the reliability of stock assessments, and therefore, the effectiveness of fishery management, is severely limited for many fishery resources. There are also various roles for stock identification in fishery science that may be equally important. Assessment of the genetic composition and variability of fish stocks is important for the scientific and judicious exploitation or management of fishery, conservation and rejuvenation of endangered species and improvement of stocks of cultivable species. Population of most species are composed of subpopulation, also called genetic

stocks, between which limited gene flow occurs. These subpopulations maintain their genetic makeup or characteristics distinct from other subpopulation of the same species due to genetic variation within the species. A strategy, thus, developed for the scientific management of these resources is to identify the natural units, the 'stocks' of the fishery resources under exploitation (Altukhov, 1981). Stock identification can be viewed as a prerequisite for any fishery analysis, just as population structure is considered a basic element of conservation biology (Thorpe et al., 1995). This can be undertaken by morphometrics, biological analysis, and molecular genetics of the population.

Morphometrics

The morphometric technique based on the truss network method is an important technique that is frequently applied to detect species and population differences not detected using traditional morphological technique (Strauss and Bookstein, 1982; Cavalcanti et al., 1999; Jayasankar et al., 2004; Rutaisire et al., 2005). It involves measuring distances between homologous landmarks over the entire length on the surface of the fish. It is a reconstruction of the original shape of the organism. It is able to detect differences in overall shape and size of individuals among species as well as population. Both factors are known to be influenced genetically and environmentally. The new landmark-based technique of geometric morphometric or known as truss network morphometrics poses no restriction on the directions of variation and localization of shape changes, and are much more effective in capturing information about the shape of an organism (Schweigert, 1990; Turan, 1999; Cavalcanti et al., 1999; Cadrin, 2000; Palma et al., 2002; Kassam et al., 2002; Jayasankar et al., 2004). There are many well documented studies on population or stock structure in fishes using morphological traits. Jayasankar et al. (2004) observe morphological homogeneity in mackerels from Indian coasts. Significant morphological heterogeneity was reported in silver perch (*Leiopotherapon plumbeus*) populations in the Philippines by Quilang et al. (2007). Morphometric analysis revealed a high degree of dissimilarity of the anchovy (*Engraulis encrasicolus*) in the Black, Aegean and Northeastern Mediterranean Seas (Turan et al., 2004).

The study of Nowak et al. (2008) was confined to common gudgeon (*Gobio gobio*). Hossain et al. (2010) studied the morphometric difference in *Labeo calbasu* populations whereas Sen et al. (2011) focused on *Decapterus russelli*; Sajina et al. (2011) in *Megalaspis cordyla* and Ujjania et al. (2012) in *Catla catla*. Moneva et al. (2012) describe sexual dimorphism of *Vivipara angularis* by geometric morphometric analysis.

Biological Analysis

Length Weight Relationship

The history of length-weight relationships and condition factor is intertwined. In the beginning, there was the square cube law of *Galileo Galilei* (1564–1642), who apparently was the first to state that volume increases as the cube of linear dimensions, whereas strength such as the diameter of legs increases only as the square. Later, Galileo's law was redefined as follows: In similarly-shaped bodies the masses, and therefore, the weights, vary as the cubes of the dimensions. This subsequently became known as the cube law. Accordingly, a fish which doubles its length increases by eight times in weight. Fulton (1904) applied the cube law to 5675 specimens of 19 fish species of the Scottish North Sea and found that it does not apply with precision to fishes. He concluded that most species increase in weight more than the increase in length would, according to the law, imply. Fulton also noticed how greatly the weight for a given length differs in different species. Within species he found that the ratio varies somewhat at different places and at certain times of the year, Length and weight data are useful standard results of fish sampling programs (Morato et al., 2001).

Length and weight of Indian major carps in relation to growth parameters have been studied by Jhingran (1952), Johal et al. (1992), Zafar et al. (1992), Ahmed et al. (1996) and Saxena et al. (2009). Choudhari et al. (1982) studied the condition factor for four Indian major carps from the river Brahmaputra and found that value of relative condition factor in *C. catla* was

high during August and low during January. Rajbanshi et al. (1984) observed an isometric growth pattern at juvenile stage of the *C. mrigala* from Roopsagar Pond (Southern Rajasthan). Kartha et al. (1990) in a similar study from the Gandhi Sagar (Madhya Pradesh) reported an isometric growth pattern in *C. catla.* Sarkar et al. (1998) while performing a similar study on *C. mrigala*, observed that fish spawned in Bundh and a hatchery reared stock indicated an allometric growth from both these environments. These authors have opined that it is mainly dependent upon the growth, maturity of gonads and length of fish.

Age determination

Age studies of fishes form an important aspect of their biology and relationship with their environment. Hubert (1981) observed, that it aids in the productivity, longevity, periods of maturity, recruitment of various year classes and determination of potential yield of fish stock. Information obtained on age could contribute to the optimal, or at least a rational exploitation of a fishery.

Scale pattern analysis has advantages and disadvantages relative to other indirect methods of determining stock composition ("indirect" refers to methods based on statistical analysis of biological data and stock composition estimation; "direct "methods are based on proof of origin, e.g., tagging and detection of region-specific parasites). Scales are relatively inexpensive and easy to collect, store, measure, and analyze, and large samples can be obtained without killing or mutilating fish. The disadvantage of inter annual variability of data is offset by the ready availability of scale samples, because scales are commonly collected by many agencies for other purposes.

In the 1990s, scale pattern analysis was used to estimate origin of salmon in mixed-stock ocean catches. Japanese research vessel samples were used to identify origins of chum, coho, and chinook salmon migrating in the area of the former Japanese high-seas salmon driftnet fisheries (Davis, 1990). Scales were sampled directly from the catch of the land based driftnet salmon fishery for the first time in 1990 (Fisheries Agency of Japan 1990); and stock

proportions and estimates of catch of Russian and Alaskan sockeye, Coho, and Chinook salmon in 1990 were determined (Walker, 1992). Walker (1992) estimated origin of salmon in confiscated Taiwanese catches and origin of Coho salmon caught in the Asian high-seas squid driftnet fisheries. Temnykh (1994) used morphometrics (including scale measurements) to differentiate populations of pink salmon in the Sea of Okhotsk.

Molecular Markers

A lack of knowledge about the genetic structure of many populations may result in the differential harvest of the populations that will ultimately have a drastic and long-term effect. To overcome this, there is always a need for investigations encompassing the genetic variations at the intra and inter-population levels as well as at the intra and inter- specific levels of the fish and shellfish resources of any nation (Allendorf and Utter, 1979). The development of DNA-based genetic markers has had a revolutionary impact on animal genetics. It is theoretically possible to observe and exploit genetic variation in the entire genome of organisms with DNA markers.

The classical molecular technique for studying genetic variation at co-dominant Mendelian inherited loci is allozyme electrophoresis. The technique was developed in the 1960s and was dominating until the early 1990s. In the early 1980s the first population genetic studies based on analysis of mitochondrial DNA emerged (Avise et al., 1994). Later, with the advent of the PCR a number of different techniques emerged, ranging from sequencing of the DNA of interest to methods analysing length polymorphisms, such as microsatellites. Today, many molecular methods are available for studying fish populations but they are basically categorized under two types of markers, protein and DNA. There are three general classes of genetic markers that are routinely used in population genetic and phylogenetic studies: (1) allozymes, (2) mitochondrial DNA, and (3) nuclear DNA. They have been subject to a number of recent reviews (Avise, 1994).

Microsatellite Markers

Microsatellites are the most versatile ones among these molecular markers and are co-dominant in nature. Although identifying microsatellite in a species of interest can be time consuming, the techniques employed are well established and reliable. Microsatellite markers are being increasingly applied in fisheries and aquaculture (Liu and Cordes, 2004). A few pairs of highly polymorphic microsatellites can be enough if the objective is to address genetic diversity related questions. Very high levels of variability associated with microsatellites, the speed of processing and the potential to isolate large number of loci provides a marker system capable of detecting differences among closely related populations. As popular marker system they have been used in higher vertebrates such as human (Weber, 1990), cows (Beckman and Soller, 1990), sheep (Crawford et al., 1991), horses (Murkund et al., 1994) and plant species (Lagercrantz et al., 1993). They have also been used as genetic markers for seabass (Garcia de Leon et al., 1995), salmon (Slettan et al., 1993) and European flat oyster (Naciri et al., 1995). Various authors have reported microsatellite polymorphism and sequences in several marine and freshwater fish and shell fish species for the population or stock analysis (Ghiasi et al., 2009; Ma et al., 2012).

Mitochondrial DNA Markers

The mitochondrial genome is typically between 16,000 and 18,000 base pairs in length, haploid and is, in general, maternally inherited. Its rapid rate of evolution, clonal inheritance, and lack of recombination has made it a valuable source for studies ranging from intraspecific phylogeography and gene flow to delineation of species boundaries, and phylogeny reconstruction. The mtDNA molecule has a circular structure. The vertebrate mitochondrial genome is composed of about 15 to 20 kb in different organisms, coding for 37 genes responsible for 2 ribosomal RNAs, 22 transfer RNAs, and 13 proteins essential in respiration (Ferris and Berg, 1987; Hartl and Clark, 1997). It also has a non-coding region (+ 1000bp) responsible for replication, known as the “control region” or “D-loop”, that evolves 4 – 5 times faster than the entire mtDNA molecule which itself evolves 5 to 10 times faster than

nuclear DNA (Brown et al., 1979), mainly because the mitochondria do not have repair enzymes for errors in the replication, nor for the damages of the DNA (Clayton, 1982). MtDNA is a haploid and maternally inherited molecule that is highly informative for describing historical process that has influenced population structure. Each individual receives one copy of its maternal mtDNA during replication of DNA.

As pointed out by Lansman et al. (1983) the level of diversity among mtDNAs appears to be uncoupled from that of morphological or nuclear gene diversity. Mutation rates appear to be often relatively constant although different genes appear to evolve at different rates. Thus, an analysis of mtDNA provides a high resolution of evolutionary processes among species. Furthermore, a number of independent studies can share the published fragment data for the estimation of genetic diversity. In addition to the sequence analysis of specific haplotypes on the mitochondrial DNA (Ellsworth et al., 1993; Saitoh et al., 1995) can also be performed. In the present study mtDNA sequencing is done to delineate genetic variation.

Mitochondrial DNA (mtDNA) has proven to be a useful marker in population genetic studies and fisheries management and also to define pattern of population differentiation in highly migratory pelagic fishes such as skipjack tuna (*Katsuwonus pelamis*) (Menezes et al., 2006), bigeye tuna (*Thunnus obesus*) (Alvarado Bremer et al., 1998), bluefin tuna (*Thunnus thynnus*) (Alvarado Bremer et al., 1997), swordfish (*Xiphias gladius*), round scale spearfish (*Tetrapturus georgii*) and silky shark (*Carcharhinus falciformis*) (Reeb et al., 2000). Nucleotide sequence analysis of this DNA region is, therefore, useful for evaluating genetic, demographic and phylogeographic pattern in population suggested by Graves et al. (1984) and Hoolihan et al. (2004). Bagda et al. (2012) depicted lower genetic structuring in mitochondrial DNA than nuclear DNA among the nesting colonies of green turtles (*Chelonia mydas*) in the Mediterranean. Garcia et al. (2000) utilized 324 base pairs of the mitochondrial cytochrome *b* gene to examine the phylogenetic relationships in 14 species of the genus *Cynolebias* (Cyprinodontiformes, Rivulidae) while, Ghorashi et al. (2008) carried out the

phylogenetic analysis of anemone fishes of the Persian Gulf. Phylogeography of *Glyptothorax fokiensis* and *Glyptothorax hainanensis* in Asia (Chen et al., 2007); Neotropical catfish *Pimelodus albicans* (Siluriformes: Pimelodidae) from South America (Vergara et al., 2008); and an endangered fish *Zoogoneticus quitzeoensis* (Cyprinodontiformes: Goodeidae) (Dominguez et al., 2008) are also well studied based on mtDNA cyt *b* sequence variations. Goswami et al. (2008) has used mtDNA cyt *b* region to study the genetic stock structure of two seahorse species (*Hippocampus kuda* and *Hippocampus trimaculatus*) from the south east and south west coasts of India. Perdices et al. (2002) studied the evolutionary history of the genus *Rhamdia* (Teleostei: Pimelodidae) in Central America whereas Perdices et al. (2005) inferred the phylogenetic relationships of *Synbranchus marmoratus* and *Ophisternon aenigmaticum* from 45 drainages throughout South, Central America and Cuba, based on mitochondrial genes cytochrome *b* and ATPase 8 or 6. John et al. (2013) studied the population structure of Denison's barb, *Puntius denisonii* from Western Ghats of India.

A synthesis of classical techniques with recent morphometric analysis and modern molecular techniques examining specific portions of the fish mtDNA and Microsatellite markers may provide a solution to the stock identification of the fish populations. The present review provides useful insights into genetic diversity and population structure of fishes using mtDNA markers as well as microsatellites. Information gathered on rate of evolution of three mtDNA genes along with the taxonomic status of Indian major carps will facilitate future investigations dealing with phylogeography, taxonomy, conservation, and co-evolution of this important group of fish.

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TAXONOMY AND NATURAL HISTORY OF SNAKES OF THE GENUS *OLIGODON* (FITZINGER, 1826) FROM NORTHEAST INDIA

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ABSTRACT

We present here an account of nine species of colubrid snakes belonging to genus *Oligodon* Fitzinger 1826 from Northeast India. This taxonomic study is based on morphological observations and includes morphometry, coloration, lepidosis, distribution and natural history of multiple specimens in each species. The study also includes description of type material for *Oligodon erythrorhachis* Wall, 1910 and *Oligodon melanozonatus* Wall, 1922. An account of rare and endemic *Oligodon kheriensis* Acharji and Ray, 1936 with new distribution records are provided in this study.

Key Words: Colubrid snakes, *Oligodon*, Northeast India

INTRODUCTION

North-eastern India comprising the states of Arunachal Pradesh, Assam, Meghalaya, Nagaland, Manipur, Mizoram, and Tripura extends from 88° E - 97°E and 22° N - 29°30' N with a geographical area of approximately 255083sq. km. The area spans across the Himalaya and Indo-Burma biodiversity hotspots (Mittermeier et al., 2004). The region is broadly differentiated into the Eastern Himalaya to the north, the North-east Hills (Meghalaya and Mizoram-Manipur-Kachin forest zones) to the south, and the Brahmaputra plain (the Brahmaputra valley forest

zone) in between (Mani, 1974; Olson and Dinerstein, 2002). North-eastern India encompasses wide physiognomic range, e.g., an altitude ranging from approximately 20 m to >6000 m above sea level and habitat diversity from tropical to alpine (Champion and Seth, 1968; Olson and Dinerstein, 2002). Distribution data across multiple plant and animal groups indicate that the region's biological affinities closely resemble those of South-east Asia (Mani, 1974).

Ophidian fauna of northeastern India is poorly known. This is evident from the fact that many new species and new range records have been reported from this region (David et al., 2001; Slowinski et al., 2001; Ahmed and Das, 2006).

So far, 102 species of snakes have been reported from northeastern India of which nine species belong to genus *Oligodon* (Ahmed et al., 2009). The present contribution is based on original field observation, museum specimens and data gathered from literature.

MATERIALS AND METHODS

The survey was carried out in the following habitat types: tropical evergreen forests (hill slopes, lowlands), grasslands, caves, grassy swamps and ponds, man made structures (roads, culvert, huts, and buildings), plantations (teak, tea, beetle-vine, agricultural field, jhum cultivation areas and mixed type). Opportunistic sightings include specimens found during field survey, collected or killed by local indigenous people, road kill, specimens in zoo or rescued individual. Taxonomic data were also gathered from the study of museum specimens, college and university collections or from personal collections made by other herpetologists of the region. We took measurements with the help of a digital Mitutoyo dial caliper (to the nearest 0.01mm) and a meter tape.

Morphometric characters

Following characters were followed for morphometric studies. Snout to vent length (SVL) from the tip of the snout to the posterior end of the anal plate; tail length (TL) from the posterior end of the anal plate to the tail tip; head length (HL) from the joint of the upper and lower jaws to the snout tip; head width (HW) at the joint of the upper and lower jaws; head depth (HD) at the angle of the jaw; horizontal eye diameter; eye to nostril (E-Ns) from the anterior tip of the eye to the posterior edge of the nostril opening; eye to snout (E-Sn) from the anterior side of the eye to the tip of the snout; nostril to snout (Ns-Sn): from the anterior end of the nostril to the tip of the snout; median inter-orbital distance: the distance across the eyes at the mid-horizontal line. Different parts of the body described in the text include: Rostral (R); Internasal (IN); Pre-frontal (PF); Frontal (F); Infralabial (IL); Supralabial (SL); Temporal (T); Anal (A); Dorasl Scale Row (DSR); Ventral (V) and Subcaudal (SC).

Measurements of head scales have been given in length by width format. In the case of the frontal shield, its height has been compared with the distance from the anterior end with the tip of the snout. In case of the rostral shield, width at the base and width at tip was noted.

Study locations

Coordinates were recorded as latitude and longitude in decimal degrees. The geo-coordinates of some old literature records are either collected personally or are derived from a digitized toposheet map of North-east India. The locations were converted into a digital database and imported into geospatial environment using ArcGIS*v*. 10.3. Location for species *Oligodon catenatus* could not be verified. Thus, it has not been mapped.

Other abbreviations used in the text are as follows: pers. obs. = personal observation; pers. comm = personal communication; NP: National Park; Wls: Wildlife Sanctuary; RF: Reserved Forest.

Museum abbreviations described in the text are as follows: AVCM: Arya Vidyapeeth College Museum, Guwahati, India; BMNH: Museum Natural History, London, U.K.; ZSIC: Zoological Survey of India, Calcutta, India; Personal collections are abbreviated as: AD: Abhijit Das; AD/AS: Abhijit Das/Assam; AD/BR: Abhijit Das/Barail Series; AD/NL: Abhijit Das/Nagaland Series; AD/MZ: Abhijit Das/Mizoram Series; AD/AR: Abhijit Das/Arunachal Pradesh Series; HTS:HT Lalremsanga Collection; MFA: Mohammad Firoz Ahmed Collection.

RESULTS

SPECIES ACCOUNTS

***Oligodon* Fitzinger, 1826**

Oligodon Fitzinger: 1826: 30 and 56. – Type of genus: *Oligodon bitorquatum* Boie, 1827.

Diagnosis: Short to medium snake, head slightly distinct from neck; Rostrum prominent, head often with ornamentation; posterior maxillary teeth enlarged and compressed, ventrals often with black squaring spots; tail pointed.

Oligodon albocinctus (Cantor, 1839)

[Type Locality Cherrapunjee, Assam (=Meghalaya)]

Description of species based AD/MZ 09 (♂), Shimmui, Mizoram: A medium growing species; body cylindrical; head slightly distinct from neck; head length (15.4) is 1.2 times of the head width (11.91); eyes small with a round pupil; eye diameter (3.15) is 20% of the head length; eye to snout distance (5.88) is twice that of eye to nostril distance (3.03); parietal height (6.12/5.05) is slightly higher than its width; frontal (5.39/4.58) higher than wide and its height is slightly more than parietal width; prefrontal (3.00/3.49) wider than high, its height is equal to eye to nostril distance; internasal (1.95/2.68) 1.3 times wider than high, distinctly visible from above;

supraocular (4.16/2.70) height is 1.5 times that of its width, its height is less than frontal height; height of anterior chin shield (4.91/2.86) 1.7 times of its width and is higher and wider than posterior chin shield (2.17/2.17); inter-narial space is 4.03. Supralabials 7/7, 3rd and 4th touch eye, 5th broadest, 6th highest; Infralabials 9/9, first 4 touch anterior genial, 4th and 5th touch posterior genial; Preocular 1/1 (Large); Post ocular 2/2; Loreal slightly wider than high; T: 1+2, anterior temporal 190 much larger than posterior temporals; Dorsal scale Rows: 19:19:17, smooth; Ventral: 183; Subcaudals: 61; Anal: 1, SVL 620 mm; TL 160 mm.

Live Colouration: Head with typical pattern namely, a dark edged stripe across the forehead running from the upper lips through the eyes, a dark edged, V-shaped stripe running from angle of mouth and join the head stripe across the parietals. Small black bar extend from below eye. Dorsally, reddish brown above with 24 black edged cream on body and 7 on tail (known range 19-27 in numbers on the back and 4-8 on tail). Posterior part of ventrum with squarish black spots densely arranged, anteriorly the squarish black spots arrange sparsely only at the outermost edges of ventral scales. Schleich and Kästle (2002) reported that the ventral colour whitish, yellowish or may be coral red with squarish alternating black spots best marked posteriorly.

Additional specimen studied: AD/AR 18 (♀), Koronu, Arunachal Pradesh: HL 10.96; HD: 5.40; ED: 2.22; E-Sn: 4.69; E-Ns: 2.30; Ns-Sn: 1.83; P: 4.66/3.53; F: 4.29/3.62, F height is more than the distant from Snout, PF: 1.74/2.75, connects loreal; IN: 1.01/2.14; R: 2.17/2.64, distinctly visible from above; Supraocular: 3.46/1.96; 1st Chin 3.18/1.63; 2nd Chin: 1.69/1.40; SL: 7/7, 3rd and 4th touch eye; 6th highest; IL: 8/8, 2nd IL is very small, 4 touch anterior genial. Mental is pointed. Infralabial edged with black; Post ocular: 2/2; Preocular: 1/1; Loreal slightly higher than broad; T: 1+2, anterior temporal large. DSR: 19:19:17; V: 190; SC: 49; A:1; SVL 275 mm TL 50 mm. 25 narrow black edged cream coloured dorsal bands on whole body. Bands are at 8-9 scale wide gap. Ventral bands are whitish anteriorly and pinkish posteriorly. Each ventral scale is with

alternate squarish black spot at its outer edge. Subcaudals also with similar pattern; tail tip pointed. Head with typical markings- black edged cream coloured mark from middle of frontal to the two side of the neck; other half of the mark from frontal junction pass along back of eye to labial. Small black bars from below eye.

Locality records: ASSAM: Hengrabari, Veterinary college campus (Guwahati), Barail Wildlife Sanctuary [this work]. Dilcosh (ZSIC 11360), Goalpara, Assam. (ZSIC 7145); ARUNACHAL PRADESH: Mehao Wildlife Sanctuary [this work], Chimpu, Dejoo (Boulenger, 1913), Doimara of Eaglenest Wildlife Sanctuary (Athreya, 2006); MEGHALAYA: Shillong Khasi and Tura Garo Hills (Sclater, 1891); MIZORAM: Tamdil, Aizawl, Saiha [this work]; MANIPUR: 8 miles south of Imphal (Acharji and Kripalini, 1950), Tamenglong (Singh, 1995); NAGALAND: Pungro [this work]; Medziphema (Ao et al., 2004), Samagooting (Sclater, 1891).

Altitudinal Distribution: In Assam, from 57-80 m elevation. In Mizoram, 455-860 m and Nagaland from 1490 m. Athreya (2006) record from 450 m. Schleich and Kastle (2002) reports from 1500 m. Orlov et al. (2000) report from 900-1100 m in Tam Dao Mountain. Whitaker and Captain (2004) report its occurrence up to 1980 m.

Natural History Note: One individual was found under loose rocks of a landside area. Another was among prop root of a tree at 0.6 m above in a forested hill slope. The individual tried to escape into the thick bamboo leaf litter. Individuals also recorded from human habitation close to moist deciduous forest, semi-evergreen forest and tea gardens. Activity recorded as nocturnal. Whitaker and Captain (2004) regard this species as chiefly hill form. Acharji and Kripalini (1950) mentioned that this is essentially a hill form. However, during this study the species was obtained from Assam plain. In Arunachal Pradesh, hatchlings obtained in the month of June. Snake and Lizard eggs, mice are recorded in its diet. Wall (1926) recorded eggs of *Rhabdophis*

subminiatus from its stomach having developed embryos. Coil its tail and expose the subcaudals when threatened.

Oligodon cinereus (Günther, 1864)

(Type Locality: Cambodia)

Description of species based on AVCM S 067 (♀), Amsoi, Assam: Head ovoid, short, thick, barely distinct from neck; snout projecting beyond the lower jaw, long, rounded; head length (15.04) is 1.19 times of head width (12.58); eyes small with round pupil; eye diameter (2.61) is 17.3% of the head length; eye to snout distance (6.09) is 2.33 times of the eye diameter; eye diameter is smaller than eye to nostril distance (2.94) but slightly larger than nostril to snout distance(2.43); parietal (5.04/4.35) higher than wide, its height is less than the eye to snout distance, parietal height equal to its distance from snout; frontal (5.30/4.53) higher and wider than parietal, its height is more than the anterior end of frontal to the tip of snout; prefrontal (2.66/3.25) wider than long, connected to loreal; loreal slightly wider than long; internasal wider than high, Rostral (3.03/3.86) thick, slightly wider than high, curved onto the upper snout surface, well visible from above; internarial space (4.73) is 2.6 times of the height of internasal; supraocular (4.41/2.94) longer than wide, its height is almost equal to nostril to snout distance, smaller than frontal; nasal divided; tail thick and tapering. First chin (4.27/2.18) is higher and slightly wider than second chin shield (2.11/2.11). Supralabials 8/8, 4 and 5 touch eye, 6th highest; Infralabials: 9/9, first 4 touches anterior genial, 4 and 5 touch posterior genial; Post ocular: 2/2, Preocular: 1/1; asubocular below preocular; Ventrals: 173; Subcaudals: 33; Anal: 1; Dorsal Scale Rows: 17:17:15; SVL 500 mm TL 60 mm.

Live colouration: Head shield finely spotted with black, no typical head marking. Anteriorly ventral spotted with squarish black spots on outer edge. Posteriorly ventral almost dark;

subcaudals also heavily spotted with black; 46 narrow irregular black crossbars on dorsum that develops from the dorsal scales edged with black; 3 bands on top of tail.

Additional specimens studied:

1. HT/MZ 164, Tamdil, Mizoram: HL: 16.74; HD: 9.73; HW: 13.90; ED: 2.80; E-Ns: 2.95; Ns-Sn: 2.46; P: 5.71/4.91; F: 5.67/5.09, height equal to its distance from snout. PF: 4.02 /3.22, connected to loreal; IN: 2.06/3.29; Rostral: 3.43/4.39, visible from above; first chin: 4.80/2.33; second chin: 3.21/2.19; internarial: 5.00; supraocular: 4.31/2.70. T: 1+2, Preocular: 1/1; Post ocular: 2/2, upper one slightly reaching top of head; SL: 8/8, 4 and 5 touch eye, 7 largest; IL: 8/8, 4 touch anterior genial, only five touch posterior genial and is largest. Loreal slightly wider than long; one small presubocular, that separate fourth labial from eye. V: 181; SC: 42, pointed; A: 1; DSR: 17:17:15; SVL: 710 mm TL 105 mm. Snout slightly projecting, dorsum with small irregular bars middorsally and dorso-laterally, head marks indistinct. Ventrals were with large squarish distinct spots most distinct from mid-ventral and on subcaudals.

2. HT/MZ, R 163, Tamdil, Mizoram: HL: 16.15 (barely distinct from neck); HD: 10.59; HW: 11.93; ED: 2.92; E-Ns: 3.38; Ns P: 5.96/4.42; F: 5.17/4.44, height equal to its distance from snout. PF: 2.83/3.59, connected to loreal; IN: 1.47/2.99; Rostral: 3.30/4.284, visible from above; 1st chin: 4.54/2.07; 2nd chin: 3.51/1.73; supraocular: 3.74/2.46; T: 1+2, Preocular: 1/1; Post ocular: 2/2, upper one slightly reaching top of head; SL: 8/8, 4 and 5 touch eye, 7 largest; IL: 8/8, 4 touch anterior genial, 4 and 5 touch posterior genial; Loreal slightly wider than long; 1 small presubocular, that separate 4th labial from eye; V: 180; SC: 41, pointed; A: 1; DSR: 17:17:15; SVL: 620 mm TL 90 mm. Ventral heavily spotted with black squarish spots more pronounced towards posterior and caudals. Body with 23 narrow irregular bands dorsally, they disappear dorsolaterally. Top of tail was without any bands.

3. HT/MZ 164, Saiha, Mizoram: HL: 17.75 (barely distinct from neck); HD: 11.48; HW: 14.55; ED: 3.07; E-Ns: 3.83; Ns-Sn: 2.78; P: 5.86/5.73; F: 5.95/4.64, height equal to its distance from snout. PF: 3.12/4.59, connected to loreal; IN: 2.22/3.47; Rostral: 3.81/4.55, visible from above; 1st chin: 4.29/2.70; 2nd chin: 2.82/2.39; internarial: 5.55; T: 1+2, Preocular: 1/1; Post ocular: 2/2; SL: 8/8, 4 and 5 touch eye, 7 largest; Loreal slightly wider than long; IL: 8/8, 4 touch anterior genial, only 5 touch posterior genial and is largest; V: 178; SC: 36, blunt may be missing; A: 1; DSR: 17:17:15; SVL: 740 mm TL 95 mm.

4. ZSIC 23529, Jamduar, Goalpara: HL: 11.59; HD: 5.58; HW: 9.78; ED: 2.34/1.85; E-Ns: 1.82; Ns-Sn: 1.40; Parietal: 4.45/3.77; Frontal: 4.56/3.64; PreFrontal: 1.89/2.56; Internasal: 0.81/2.24; Rostral: 2.88/2.50; Supraocular: 3.55/2.29; Interorbital: 2.92; Supralabial: 7, 3 and 4 touch eye Infralabial: 9, first four touch anterior genial; V: 171; SC: 37, tail pointed end up in a spiny projection; A: 1; Post ocular: 2; Preocular:1; T: 2+3; DSR: 17:17:15. SVL: 33.5 cm; TL: 5 cm Head unpatterned, dorsum with indistinct black bands. The scales at band area edged with black. Dorsal bands run up to tail. Ventrals were with squarish brownspots on two sides.

Variation: *Oligodon cinereus* known to represent remarkable variation across its range. Smith (1943) recognized four-colour form. HT/MZ 165 is reddish brown in colour, half of ventral without any markings; faint black spots develop on two sides of ventral scales from mid-ventral increases posteriorly. Subcaudals are with distinct black spots. HT/MZ R 165 and HT/MZ 163 showed conspicuous black squarish marking on the ventral scales. ZSIC 23529 showed 7/7 supralabials (vs 8/8) and 9/9 infralabials (vs 8/8). Smith (1943) assigned the Northeast Indian population to “form III” defined as dorsum with distinct cross bars, alternating with one or two indistinct ones, belly heavily spotted at the outer margin of ventral. Based on pattern and colouration, Taylor (1965) described two forms from Thailand- *Oligodon cinereus swinhonis* and

Oligodon cinereus multifasciatus. However, the dorsal pattern of AVCM S 067 and ZSIC 23529 corresponds to variety *swinhonis* (Smith, 1943; Taylor, 1965).

Locality records: ASSAM: Amsoi near Kolaghat Reserved forest [this work]; ARUNACHAL PRADESH: Chimpu, Doimara of Eaglenest Wildlife Sanctuary (Borang et al., 2005; Athreya, 2006); MIZORAM: Tamdil, Saih.

Range: India (Northeast India); Myanmar, Southern China. Hong Kong, Indochina and Malay Peninsula**.**

Altitudinal Distribution: The field records are from 120 m- 890 m elevation. Athreya (2006) reported the species from 450 m in Eaglenest Wildlife Sanctuary; Orlov et al., (2000) from 700-900m.

Natural History Note: One individual was caught from near a rocky stream at 2030 h. Mizoram individuals were obtained from near forest streams as well as while road construction beside forested areas. Orlov et al., (2000) mentioned that the species lives primarily in mountain forest.

Oligodon cyclurus (Cantor, 1839)

[**Type Locality:** Not Known].

Note: Zhao and Adler (1993), opined that the type locality is presumably the Indian subcontinent, based on the title of Cantor's paper (1839) and other information in the article.

Description of species based on AD/AS 33, (♂), Assam State Zoo Campus, Guwahati, Assam: Head barely distinct from neck; head length (15.38) is 1.3 times of the head width (11.58); eyes rather small with round pupil; eye diameter (2.86) is18.5% of the head length; eye diameter is slightly less than eye to nostril distance (2.92); nostril to snout (2.50) distance is slightly less than the eye to nostril distance; parietal (6.10/5.40) 1.12 times higher than wide, touch upper post ocular; frontal (5.34/4.56) higher than wide, its height slightly more than its distance from snout;

prefrontal (2.33/3.73) wider than high, its width is more than eye to nostril distance, connected to loreal and preocular; internasal (1.75/2.72) wider than high, its width is almost equal to eye diameter; supraocular (3.82/2.44) higher than wide, its height is less than the frontal height; rostral (3.09/4.35) thick slightly wider than high, distinctly visible from above; internarial distance is 4.87; anterior chin shield (3.89/2.35) is higher but less wider than the posterior chin shield (2.40/2.42).T: 1+2, Preocular: 1/1; Post ocular: 2/2; loreal slightly wider than high; Supralabials:7/7, 3, 4 touch eye, 6 largest; Infralabials: 9/9, 4 touch anterior genial, only 5 touch posterior genial; Ventrals: 180; Subcaudals: 60; Anal: 1; DSR: 19:17:15,smooth; SVL: 550 mm TL 130 mm.

Live colouration: Dorsally brownish cream, grayish brown or yellowish brown, speckled with black; back with dark edged crossbars; a light brown stripe can be seen on tail; interscale colour white; head with dark band across prefrontal and frontal that pass across eyes, one V- shaped dark mark runs from neck up to frontal, another two bar by its side up to angle of jaw; transverse markings; tongue pinkish; anterior part of ventral unpatterned gradually small black ventral spots develop that increases towards posterior. Posterior part of ventral is almost heavily spotted. Subcaudals were with widely separated black spots. Top of tail was with an indistinct light brown line dorsolaterally from neck to vent.

Additional specimens studied HT/MZ R 165, Saiha, Mizoram: HL: 16.41; HD: 9.30; HW: 11.79; ED: 3.23; E-Ns: 3.23; Ns-Sn: 2.42; P: 6.03/4.98, touch upper post ocular; F: 5.81/4.64, height slightly more than its distance from snout. PF: 2.78/3.64, connected to loreal and preocular; IN: 1.64/2.40; supraocular: 3.90/2.50; Rostral: 2.85/4.15, visible from above; 1stchin: 3.86/2.41; 2nd chin: 2.71/2.35; internarial: 4.48, T: 1+2, Preocular: 1/1; Post ocular: 2/2, lower one very small; loreal slightly wider than high; SL: 7/7, 3, 4 touch eye, 6 largest; IL: 9/9, 4 touch anterior genial, only 5 touch posterior genial; V: 185; SC: 60; A: 1; DSR: 19:19:17:15 SVL: 590 mm TL 150

mm. Head mark very distinct, 1 black bar from 4 and 5 SL pass across eyes and cover prefrontal and half frontal on top. Two dark edged bar from parietal runs diagonally towards angle of mouth and beyond. One large V- shaped mark from two side of nape join each other at parietal and move to frontal. Dorsal series of rough edged black incomplete bars. Anterior ventrals with black spots on two sides, gradually increased posteriorly. Subcaudals anteriorly spotted with black spots, posteriorly unpatterned. 20 bands were present dorsum up to anus and 5 indistinct bands on tail.

Locality records: ASSAM: Hengrabari Reserved forest; MIZORAM: Aizwal, Saiha.

Range: India (Eastern and Northeast India), Nepal, Southern China, Myanmar, Thailand, Laos, Cambodia, Vietnam.

Altitudinal Distribution: Present records are from 70m (Assam) and 860 m (Mizoram). Orlov et al., (2000) 200-1500 m in Tam Dao.

Natural History Note: The snake was recorded from leaf litter of teak plantation area and another was found inside an albino mice culture house. Schleich and Kastle (2002) recorded its habitat as open forest, garden, cultivated areas and report this species as terrestrial, crepuscular and nocturnal small rodents, snakes, lizards are taken as food. Schultz (1988) mentioned 3-6 eggs per clutch; Grossmann (1992) reports up to 11- 26 eggs under terrarium condition. Eggs measured 23×16 mm to 31.5×18.5 mm. It coils up its tail when threatened. Schmidt (1997) reported this species as quiet and docile.

Oligodon dorsalis (Gray and Hardwicke, 1834)

[Type Locality: Chittagong]

Description of species based on AD/NL 28 (♂) Khonoma, Nagaland: A small growing species, head slightly distinct from neck; Head length (10.80) is 1.5 times of the head width (6.91) and

2.3 times of the head depth (4.67); eyes rather small and cryptically placed; eye diameter (1.90) is 17.5% of the head length; eye to snout distance (4.41) is twice that of the eye to nostril distance (2.14) and 2.7 times of the nostril to snout distance (1.62); parietal (5.02/3.27) height is 1.5 times of its width, its height is more than head depth; frontal (3.76/3.32) slightly higher than wide, its height is more than the distance from snout; prefrontal (1.62/2.52) wider than high, move downward and touches loreal; internasal (1.63/1.65) almost equal in height and width, its height is same as nostril to snout distance; rostral (2.00/3.21) 1.6 times wider than high, visible from above; internarial space (2.68) is slightly more than prefrontal width; supraocular (2.60/1.65) height is 1.5 times of its width, its height is smaller than frontal. Temporal: 1+2; Post ocular: 1; preocular: 1; Supralabial: 7/7, 3rd and 4th touch eye; Dorsal Scale Rows: 15:15:13; Ventral: 164; Subcaudal: 45; Anal:2. SVL: 410 mm; TL: 90 mm.

Live colouration: Dorsum purplish in colour; with a distinct reddish one scale row, wide mid dorsal line running from back of parietal up to tail tip and is bordered by black lines. Head with indistinct typical markings. White spots on rostrum, just before eye and at angle of jaw up to neck. Black lines run from neck to tail on two sides of the body; Ventral with squarish black spots and many ventrals of belly region are completely black. Subcaudals unpatterned and coral red in life.

Hemipenis: Extending to the 18th caudal plate, forked, with large spines at the base, spines smaller at tip.

Additional specimen studied: AD/NL 29 (♀), Khonoma Nature conservation and Tragopan Sanctuary, Nagaland: HL 12.06; HD: 5.35; HW: 7.87; ED: 1.96; E-Sn: 4.68; E-Ns: 2.23; Ns-Sn: 1.84; P: 4.85/3.46; F: 3.96/3.59, its height is more than the distance from snout; PF and IN joint is not complete; R: 2.20/3.12, visible from above; SO: 2.88/1.97; T: 1+2; Post ocular: 1; Preocular: 1; SL: 7/7, 3rd and 4th touch eye; 1st chin bigger than 2nd chin shield; IL: R7/6L, 4

touch anterior genial; DSR: 15:15:13; V: 173; SC: 17(Missing); A:2; SVL: 440 mm; TL: 40 mm (missing).

Locality records: NAGALAND: Khonoma, Dzuleke, Puilwa [this work], Kohima (Ao et al., 2004); MANIPUR: Kakching, Churachandpur, Nambol (Singh, 1995); MEGHALAYA: Garo Hills and Shillong (Wall, 1908).

Range: India (Northern West Bengal, Northeast India), Bhutan (Das and Palden, 2000); Bangladesh, Northern Myanmar (Smith, 1943).

Altitudinal Distribution: The field records are from 1500 m to 2675 m elevation. Das and Palden (2000) reported it from around 270 m in Bhutan.

Natural History Note: One individual was found under loose rocks of a landslide area. Another was collected from under matted grass of a forest trail along a slope during day. One example was collected on a gravel road by forest/cultivated side. The recorded habitat is subtropical forest areas at upper elevation of Barail range. Das and Palden (2000) reported this species from edge of a forest path, besides moss covered boulders in a shade. The snake never attempted to bite while handling. Threat display includes lifting and coiling the tail in to aspiral and showing the contrasting black–white colour of posterior ventrals and red colour of subcaudals. It hides its head under body coil while display.

Oligodon erythrorhachis Wall, 1910

[**Type locality**: Namsang, Jaipur, Assam].

Description of species based on BMNH 1910.12.31.4 (♀, Type), Namsang, Jaipur, [presently in Tirap District, Arunachal Pradesh]: Head length (10.18) is 1.3 times of the head width (7.39) and twice that of head depth (4.96); eye diameter (2.15) is 21% of the head length; eye to snout distance (4.05) is 1.8 times of the eye diameter;eye to nostril distance (2.70) is twice that of

nostril to snout distance (1.35); parietal (4.40/ 3.02) higher than wide, its height is slightly less than head depth; frontal (3.94/.3.20) shorter but wider than parietal, twice the height of prefrontal; prefrontal (1.93/2.78) wider than long; internasal (1.20/1.77) height is almost equal to eyediameter; supraocular height is twice that of its width; average interband distance:8.98; rostral (1.96/2.11) slightly wider than high, distinctly visible from above; tailpointed. Supralabial: 7/7, 3rd and 4th touch eye, 5th largest; Infralabials: 7; lorealabsent; T: 1+2; Post ocular: 2; Preocular: 1; Anal: 2; Ventral: 154, not angulate laterally; Caudal: 46; DSR 15 rows, smooth; SVL: 312 mm; TL: 61 mm.

Coloration in alcohol: Dorsum brown with light vertebral stripe. Dorsum with 29 narrow black cross bars that disappear laterally, top of tail with 7 small cross bars that reduce to mere black spots posteriorly. Ventrum is with squarish black spots.

Locality record: ASSAM: Jeypore (Wall, 1910); ARUNACHAL PRADESH: Namsang [type locality], Chessa, Papumpare District (Bhatt et al., 1998).

Altitudinal Distribution: The two reported localities come within an elevation range of 120-340m.

Natural History Note: Nothing is known about the natural history of the species. The known locality of the species falls within the tropical region of Northeast and is characterized by evergreen forest.

Oligodon melanozonatus Wall, 1922

[**Type Locality:** Upper Rotung Valley, Abor Hill].

Description based on ZSIC 16799 (♀, syntype); Upper Rotung valley, Abor hills: Head barely distinct from ill-defined neck; head length (12.68) is 1.3 times of the head width (9.56); eyes rather small with round pupil; eye diameter (2.37/2.32) is 18.6% of the head length; rostral

(2.78/3.68) visible from above, and it touches six head shields, its contact with nasal is shorter than its connection with internasal; parietal (5.80/3.77) 1.5 times higher than wide; frontal (4.89/3.98) height is twice that of eye diameter, slightly shorter than parietal but width of frontal is slightly wider than parietal; prefrontal (2.92/2.24) slightly higher than wide; internasal (1.69/2.16) wider than high; supraocular almost equal to its distance to the mid-internasal; nasal entire; loreal absent; preocular one; Post ocular two; banding: 15.30 mm apart; anterior chin shield bigger than posterior chin shield. Supralabial: 6, 3rd and 4th touch eye, 5th largest; Ventral: 169; Subcaudal: 43; Anal: 2; Temporal: 1+2 (Left) and 2+2 (right); Dorsal Scale Row: 17:17:15. SVL: 490 mm; TL: 100 mm.

Coloration in Alcohol: Dorsally light brown, light mottling with black more pronounced towards posterior part of dorsum; dorsum with twenty dark brown rough edged cross bars; a black edged spot on the nape; head with a roundish cream stripe that runs from frontal through back of eye to angle of jaw; another V-shaped cream coloured dark edged mark runs from side of neck and joins at frontal; ventrals and subcaudals whitish with squarish black spot often clumped together showing a banding appearance.

Locality record: ARUNACHAL PRADESH: Upper Rotung Valley, Abor Hill. (Wall, 1922). Only known from Abor Hill, Arunachal Pradesh, India**.**

Altitudinal Distribution: Recorded from 2000 ft (~ 606 m elevation).

Oligodon catenatus (Blyth, 1854)

[**Type locality**: Assam: Type lost]

Oligodon herberti Boulenger, 1905

[**Type locality**: Mogok, Burma]

Description of species based on literature: Nasal undivided, no internasal; Frontal almost as large as parietals; rostral in contact with and just separating the anterior end of the prefrontals which are very large; loreal absent; prefrontal in contact with the second labial; 6 supralabials, 3rd and 4th touch eye; three or four lower labial in contact with anterior chin shield; anterior chin shield longer than posterior chin shield; 1 anterior temporal; Post ocular one; Scales in 13 rows. Ventral ♂ 186-196; ♀ 179-212, not angulate laterally; Subcaudal: 34-43, anal divided.

Coloration: Purplish grey or brown above, with four dark brown longitudinal stripes, the median pair is separated by a yellowish brown vertebral stripe, the outer pair on scale row 2 and 3. Yellowish below (red in life), ventrals with alternating pattern of black squarish spots. Tail is immaculate (*fide*, Smith, 1943).

Range: India, Myanmar, Southern China, Cambodia**.**

Altitudinal Distribution: Distributed above 4000 ft (=1220 m) (Wall, 1925).

Natural History Note: Very little information is available Wall (1925) reported egg yolk in its stomach. One specimen killed between July and October contained 5 eggs in the oviduct about 18 mm long. He remarked the snake as a hill species.

Oligodon theobaldi (Günther, 1868)

[Type locality: Pegu]

Description of species based on BMNH: 1946.1.4.9 (♀): Head length (10.83) is 1.2 times of head width (8.51); head width is slightly more than the head depth (6.62); eyes rather small with round pupil; eye diameter (2.18) is 20% of the head length; eye to snout (3.75) distance is 1.5 times that of distance between eyes to nostril (2.38); eye to nostril distance is more than the nostril to snout (1.37) distance; parietal (4.65/ 3.44) higher than wide, its width is same as height of frontal; frontal (3.44/2.73) higher than wide and its height is almost same as internarial space

(3.42); prefrontal (1.86/2.01) slightly wider than high; internasal (2.22/0.97) 2.2 times higher than its width. Rostral were large and distinctly visible from above. Supralabial: 8, 4 and 5 touch eye; Infralabial: 9, 4 touch anterior genial; Loreal: 1, longer than high; Post ocular: 2; Preocular: 1; Temporal: 1+2; Ventral: 177; Subcaudal: 33: Anal: 2; Dorsal Scale Rows: 17:17:15; SVL 380 mm; TL: 50mm

Coloration in alcohol: Dorsally light brown with closely set rough edged cross bars; neck with wide arrow head mark; head markings distinct; ventrals with black spots sparsely arranged. Spots are more prominent before vent. No spots under tail.

Locality records: MEGHALAYA: Tura, Garo Hills (Prater, 1920); NAGALAND: Sitikhima by Ao et al. (2004).

Range: India (Northeast India), Myanmar.

Altitudinal Distribution: 250 m Ao et al. (2004).

Natural History Note: Little information is available. Wall (1925) found *Gecko* and a Cricket in the stomach. Egg bound females are obtained between August and October.

Oligodon kheriensis Acharji and Ray 1936

[Type locality: Lakhimpur, Kheri]

Description based on the female specimen from Palpara, Bongaigaon town, Assam: Head short, broad, hardly distinct from the thick neck. Head length (21.18 mm) 1.46 times of head width (14.45 mm) and almost 3 times of eye to snout distance (7.14 mm).Distinct eye with round pupil.Eye to nostril distance (4.12 mm) larger than eye diameter (3.90 mm).Parietal higher than wide (6.63/4.19 mm), its height slightly less than eye to snout distance. Frontal higher than wide (5.70/5.36 mm), its height remained larger than eye to nostril distance and less than eye to snout distance. Pre-frontal remained higher than wide (5.25/ 2.56 mm). Supra ocular was higher than

wide (5.12/1.79 mm). Internasal higher than wide (4.75/2.11 mm). Nasal was vertically divided, posterior part distinctly smaller than anterior one; nostril piercing middle of nasal just in front of the division; portion of the rostral seen from above almost as long as its distance from the frontal. Portion of the rostral (6.13/5.80 mm) seen from above almost as long as its distance from the frontal. Anterior chin shields (4.78/3.04 mm) higher than posterior chin (3.45/2.58 mm). Body scalation: two head lengths behind the head 19, middle of the body 19, two head lengths before the vent 17; all scales small and smooth. Ventrals: 196; Anal plate entire; subcaudals 38, all paired. Supralabials 7+7; 1st smallest, 2nd and 3rd in contact with loreal, 4th and 5th touching eye; 6th and 7th largest; infralabials 8+8; preoculars 1+1; Post oculars 2+2; temporals 1+1/1+1, anterior slightly broad ones and elongated compare to posterior ones. Snout vent length: 994 mm; tail length: 105 mm.

Coloration in life: uniform coral red dorsally and ventral scales including subcaudals faint pinkish; unlike most of the species from the genus *Oligodon* the typical chevron shape marking on the head is absent in this species.

Range: India and Nepal**.**

Locality records

India: The type was reported from a forest camp near Kheri Division, Lakhimpur-Kheri district, State of Uttar Pradesh (Acharji and Ray, 1936); from Katerniaghat Wild life Sactuary, Uttar Pradesh; Sirshtala, Rajbaripara, College Para, Mohitnagar in Jalpaiguri, West Bengal (Pandey et al., 2016); ASSAM: near Kachugaon Reserve Forest, Kokrajhar District (Sutradhar and Nath, 2013) and Palpara, Bongaigaon District, Assam.

In Nepal, the species was recorded from Siddhanath Science Campus, Mahendranagar in western Nepal (Schleich and Kästle, 2002); Municipality of Damak in Jhapa, south-east Nepal;

Saktikhor, Sauraha area and Sauraha village and Pithuwa village, Chitwan District, south-central Nepal (Pandey, 2012; Pandey et al., 2016).

Natural History

All individuals of this species were recorded either in the Terai or at the human habitation nearby edge of Terai region in the Himalays, altitudes ranging from 54 m to 414 m a.s.l (Sutradhar and Nath, 2013). The species was observed in both forested as well as human habitation (mostly areas surrounded by crop fields) throughout its range. Individuals recorded in Damak and Shaktikhor were found under the soil surface when exposed by an excavator. Subsequently, most of the individuals tried to dig themselves into the soil for escape (Pandey et al., 2016).

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Table 1 Locality records of snakes of the genus *Oligodon* spp. from Northeast India

Sl No	Locality	GPS Coordinates	
		Latitude	**Longitude**
	Assam		
1	Near Kachugaon RF, Kokarajhar	26.50534	90.02229
2	Palpara, Bongaigaon	26.48418	90.56629
3	Goalpara	26.16986	90.62022
4	Hengrabari Reserved forest; Veterinary College Campus, Guwahati	26.15115	91.79514
5	Amsoi near Kolaghat Reserved forest Assam	26.13754	92.43203
6	Diju, Lakhimpur	27.28333	94.05
7	Jeypore	27.18662	95.36441
8	Barail Wildlife Sanctuary	24.9925	92.807
9	Dilcosh	24.77649	93.02001

Cont.

Sl No	Locality	GPS Coordinates	
		Latitude	Longitude
	Arunachal Pradesh		
10	Doimara of Eaglenest Wildlife Sanctuary	26.97506	92.40484
11	Chimpu	27.07115	93.61481
12	Chessa, Papumpare	27.0115	93.71441
13	Upper Rotung Valley, Abor Hill	28.62366	95.03647
14	Mehao Wildlife Sanctuary	29.19163	95.96976
15	Namsang	27.10667	95.51099
	Manipur		
16	Tamenglong	24.94425	93.50314
17	Nambol	24.71284	93.83826
18	Kakching	24.49691	93.98303
19	Churachandpur	24.34234	93.69818
	Meghalaya		
20	Tura, Garo Hills	25.50005	90.33333
21	Shillong, Khasi Hills	25.57356	91.8936

Cont.

Sl No	Locality	GPS Coordinates	
		Latitude	Longitude
	Mizoram		
22	Aizwal city	23.72711	92.71763
23	Tamdil	23.73808	92.95386
24	Saiha	22.48667	92.99028
	Nagaland		
25	Samagooting	25.92327	93.72903
26	Sitikhima	25.78696	93.79769
27	Medziphema	25.75659	93.86808
28	Puilwa	25.4432	93.77803
29	Dzuleke	25.6171	93.93951
30	Khonoma	25.65234	94.02298
31	Kohima	25.65832	94.10533
32	Pungro	25.6096	94.86206

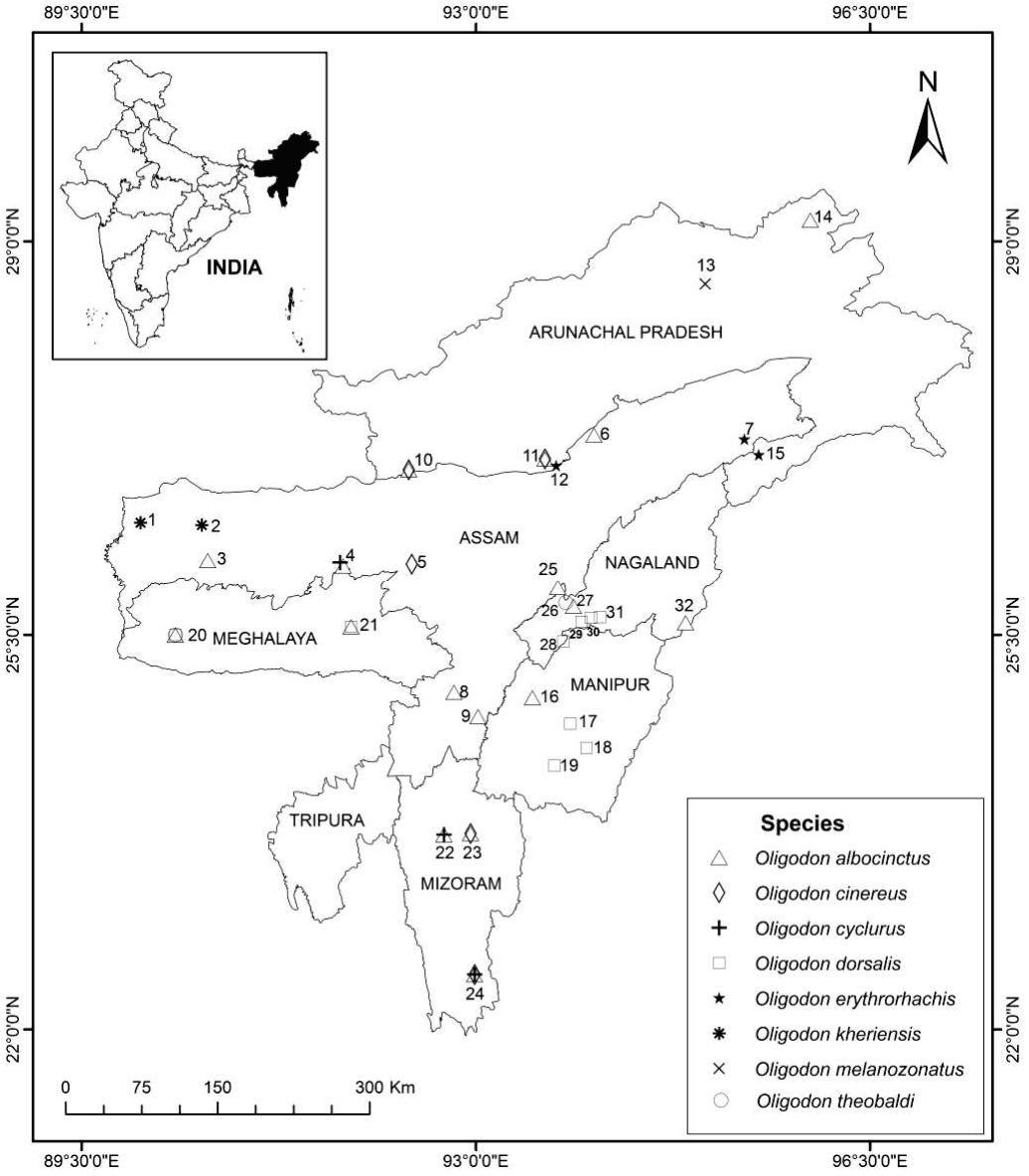

Fig.1 Map of Northeast India showing distribution localities of the *Oligodon* spp.

Fig. 2 *Oligodon albocinctus*

Fig. 3 *Oligodon dorsalis*

Fig. 4 *Oligodon cinereus*

Fig. 5 *Oligodon cyclurus*

Fig. 5 *Oligodon erythrorachis*

Fig. 6 *Oligodon melanozotus*

Fig. 7 *Oligodon kheriensis*

Fig. 8 *Oligodon theobaldi*

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DETECTION AND CHARACTERIZATION OF DIARRHOEAGENIC *ESCHERICHIA COLI* AMONG HOSPITALIZED CHILDREN WITH ACUTE DIARRHOEA IN BHUBANESWAR, INDIA

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ABSTRACT

Childhood diarrheal illness caused by diarrheagenic *Escherichia coli* (DEC) is one of the major concerns in resource-limited countries. The objective of this study was to determine the incidence of DEC in stool specimens from children with acute diarrhea using Multiplex PCR assay and antibiogram of isolated DEC. Rectal swab samples were collected from hospitalized 110 children with acute diarrhea in Bhubaneswar, eastern India. Multiplex PCR assay was employed for amplification of *etl, est, eae, bfpA*, CVD432 genes for detection of enterotoxigenic *E. coli* (ETEC), enteropathogenic (EPEC) and enteroaggragative *E. coli* (EAEC). During the 24 month study period of the 110 rectal swab samples, DEC was detected in 15 (13.6%) patients. Among the DEC, ETEC was 6 (5.4%) and EPEC 9 (8. %) and no other DEC was isolated from any of the samples. Both typical and atypical EPEC were detected with almost equal proportion. Antibiogram pattern showed > 90% resistant to at least one commonly prescribed antibiotic. Strains were commonly resistant to first-line antibiotics such as ampicillin, co-trimoxazole and sulfamethoxazole-trimethoprim. This study suggests that among the DEC, the atypical EPEC are the newly emerging group in Bhubaneswar in eastern Indian region. Further studies are suggested to evaluate the epidemiology and virulence properties of atypical EPEC strains.

Keywords: Atypical, diarrhea, enteropathogenic, *Escherichia coli,* polymerase chain reaction

INTRODUCTION

Diarrhea and other gastrointestinal disorders are important cause of morbidity and mortality in infants and young children, resulting in considerable public health burden in both developed and developing countries (Liu et al., 2012). It is estimated, diarrhea is responsible for 2.5 million infant deaths per year with annual mortality rate of 4.9 per 1,000 children and incidence of 3.2 episodes per child per year. Of the 4.879 million global death of children below five years of age due to infectious diseases, diarrhea alone has caused 0.801 million deaths in 2010. During the same period, diarrheal death in India was 0.212 million (Liu et al., 2012). Acute diarrhea is caused by a wide range of enteric bacteria, viruses, protozoa and helminths (Elliott, 2007). The major bacterial pathogens include *Vibrio cholerae*, *V. parahaemolyticus*, diarrheagenic *Escherichia coli*, *Shigella* spp., *Salmonella* spp., *Aeromonas* spp, *Campylobactor* spp. etc. Different diarrheagenic *E.coli* (DEC) represent a leading bacterial cause of childhood diarrhea in developing countries (Nataro and Kaper, 1998). The DEC that cause diarrhea have been classified into six groups based on their specific virulence factors and phenotypic traits and include enteroaggregative *E.coli* (EAEC),enteropathogenic *E.coli* (EPEC),enterotoxigenic *E.coli* (ETEC, enteroinvasive *E.coli* (EIEC), diffusely adherent *E. coli* (DAEC) and enterohemorrhagic *E. coli* (EHEC) and shiga-like toxin (STEC) (Kaper et al., 2004). It is reported, among these DEC, ETEC and EPEC share major contribution for the cause of infantile diarrhea. ETEC strains are characterized by the production of colonization factors (CFs) and at least one of two enterotoxins: heat stable (ST) and/or heat-labile (LT) enterotoxins (Isidean et al., 2001). ETEC represents one of the most common causes of diarrhea in children in developing countries and in travelers to these regions. EPEC are those *E. coli* strains having the ability to cause diarrhea, to produce a histopathology on the intestinal epithelium known as the attaching and effacing (AE) lesion, and they are encoded on a chromosomal pathogenicity island known as the locus for

enterocyte effacement (Kaper et al., 2004). Typical EPEC (tEPEC) strains also possess the EAF plasmid, which encodes for the bundle-forming pili (*bfp*A gene) and the *per*ABC genes that regulate *eae* expression. Atypical EPEC (aEPEC) strains lack the EAF plasmid (Johnson and Nolan, 2009) and can affect children in both developed and developing countries and together with EHEC and EAEC strains, are considered an emerging pathogen (Trabulsi et al., 2002; Huang et al., 2004). DEC is the main source of diarrhea and therefore, of public health relevance, these strains are not routinely sought as enteric pathogens in clinical laboratories worldwide. Therefore, prevalence of diarrhea caused by DEC strains is generally unknown, particularly in areas where DEC strains are believed endemic.

Odisha as an eastern Indian state has been recognized to be worst affected by diarrheal disorder leading to high morbidity and mortality followed by monsoon break, cyclone, and flood. Although a good number of studies have acknowledged the prevalence of *V. cholerae*, the potential causative agents of diarrhea, limited knowledge is available about the different pathotypes of *E. coli* causing diarrhea among children. Given the lack of adequate information on ETEC and EPEC causing diarrhea in Odisha children, a hospital based study was conducted to determine their incidence, antibiogram, and virulence pattern.

MATERIALS AND METHOD

Sample collection

From February to June 2016 a total of 110 rectal swab samples were collected from hospitalized children with acute diarrhea in pediatric ward of Capital hospital, a major government hospital in Bhubaneswar, the capital city of Odisha. Pediatric patients with acute diarrhea came to the diarrhea ward where they were evaluated by physicians who confirmed that patients had the

passage of three or more loose or liquid stools per day or more frequent passage than is normal. Rectal swabs were collected from the children with acute diarrhea by standard procedures and transported in Cary-Blair to Microbiology Department of Regional Medical Research Center (RMRC) for bacteriological analysis.

Isolation and Identification of *E. coli* Strains

Soon after reaching the laboratory, the samples were processed usually within 2 h. to isolate *E, coli* and other enteropathogens such as *V. cholerae*, *Shigella* spp, *Salmonella* spp, and *Aeromonas* spp by standard technique (Chhotray et al., 2002). To isolate and identify the *E. coli* strains, stool swabs in Cary–Blair transport medium were directly inoculated onto MacConkey agar medium and incubated at 37°C over night. At least three lactose fermenting pink colored colonies from the MacConkey agar plate were tested individually by biochemical tests to check the Indole production and glucose and lactose fermentation with gas in Triple sugar Iron Agar medium. *E. coli* strains thus presumably identified by biochemical tests were genetically confirmed by Multiplex PCR assay to ETEC, EAEC and EPEC with the detection of specific marker genes.

Preparation of Template DNA and Multiplex PCR to Identify ETEC and EPEC Strains

At least three *E. coli* colonies per sample were analyzed to detect the presence of genes *elt* and *est* for (enterotoxin gene of ETEC), for CVD432 (the nucleotide sequence of the EcoRI PstIDNA fragment of pCVD432 of EAEC) and *eae* and *bfpA* (structural genes for EPEC) using specific primer pairs as described earlier (Nguyen et al., 2005; Panchalingam et al., 2012). The presumptive *E. coli* strains were grown in 3 ml of LB broth for 18 h, pelleted by centrifugation at 10,000 × *g* for 10 min, and then re-suspended in 0.3 ml of distilled water. Pellets were boiled at 100°C for 10 min, vortexed for 10s, and centrifuged again at 12,000 × *g* for 3 min. DNA-

containing supernatants were transferred to 0.5 ml eppendorf tubes and stored at -20°C until used. An aliquot of the DNA prep was used as a template in multiplex PCR reaction for the characterization of DEC strains as previously detailed (Dutta et al; 2013). PCR amplification was done with appropriate volume of 10x amplification buffer (500mM KCL, 100mM Tris-HCl,15mm MgCl2,PH-8.3), 2.5mM each deoxynucleoside triphosphate, 10Pmol of each primer, 1.25 unit of Taq DNA Polymerase (Bangalore Genei, India) and 5µl template DNA. The reaction volume was adjusted to 25 µl using sterile triple distilled water. PCR assay was performed in an automated thermocycler (G-Storm, England) for 30 cycles using conditions as described earlier (Dutta et al., 2013). Aliquots of PCR product was analyzed by Agarose (1.8% wt/vol) gel electrophoresis in Tris-borate EDTA buffer stained in ethidium bromide and visualized under UV in Alpha Imager (Alpha Infotech Corporation, USA).

Antimicrobial Agent Susceptibility Testing

Antimicrobial susceptibility test of the DEC was done following the modified Kirby-Bauer disk diffusion technique (Bauer et al**.,** 1966) and with commercially available discs (Difco, USA) ampicillin (10µg), chloramphenicol (30µg), co-trimoxazole (25µg), ciprofloxacin (5µg), furazolidone (100µg), gentamicin (10µg), neomycin (30µg), nalidixicacid (30µg), norfloxacin (10µg), streptomycin (10µg), tetracycline (30µg) and trimethoprim-sulfomethoxazole (23.75 µg). Characterization of strains as being susceptible or resistant was based on the size of inhibition zone around each disc according to manufacturer's instruction, which matched interpretive criteria, recommended as Clinical Laboratory Standard Institute (CLSI) (Anonymous, 2011). The protocol was performed as follows fresh cultures were inoculated into LB broth and incubated until they reached a turbidity of 0.5 using the McFarland standard. Then, Mueller-Hinton agar plates were swabbed with these cultures and antibiotic disks (BD BBL, Franklin Lakes, NJ, USA) were placed onto inoculated plates under a sterile environment. The plates were incubated

at 37°C for 18–20 h. Diameters (in millimeters) of clear zones of growth inhibition around the antimicrobial agent disks was measured.

RESULTS

Analysis of 110 rectal swab samples collected during the study period between February- June 2016 revealed 65 (59%) were positive for *E. coli* followed by other enteropathogens. Multiplex PCR analysis revealed that 13.6% (15/110) were DEC harboring different virulent genes. Among the DEC, ETEC 40% (6/15) and EPEC 60% (9/15) were genetically confirmed with the detection of *elt, est* and *eae, bfpA* genes respectively (Fig1). The detection rate of ETEC and EPEC was 6 (5.4%) and 9 (8. 1%) respectively.The other pathogens such as vibrios, salmonellea, shigellae, campylobacters and aeromonas, were detected in 12 (11%) swab specimens whereas in 23 (21%) samples there were no known etiological agents.

In majority of the ETEC infected cases, strains harboring the virulence genes *est* or with *est* and *elt* (2 and 3 cases, respectively) were more compared to the strains that harbors *elt* alone (single case). Of the nine EPECs, five were identified with *bfpA* and *eae* and four cases were with *eae* gene alone. This indicates, out of nine EPEC, five and four were identified as tEPEC and aEPEC, respectively. Among the total DEC cases, only one case was found with mix infection of EPEC and ETEC carrying *elt, est* and *eae* virulent genes.

Antibiotic resistance of DEC strains to twelve antibiotics was evaluated. This study demonstrates that >65% of DEC strains were resistant to ampicillin, Trimethoprim-sulphomethaxozole, co-trimoxazole and chloramphenicol. A lower prevalence (<35%) of resistance was demonstrated for nalidixic acid (33.1%), chloramphenicol (23.6%), ciprofloxacin (21.1%), and gentamicin (19.8%).The DEC were commonly susceptible to ofloxacin, norfloxacilin, azithromycin, doxycycline and tetracycline.

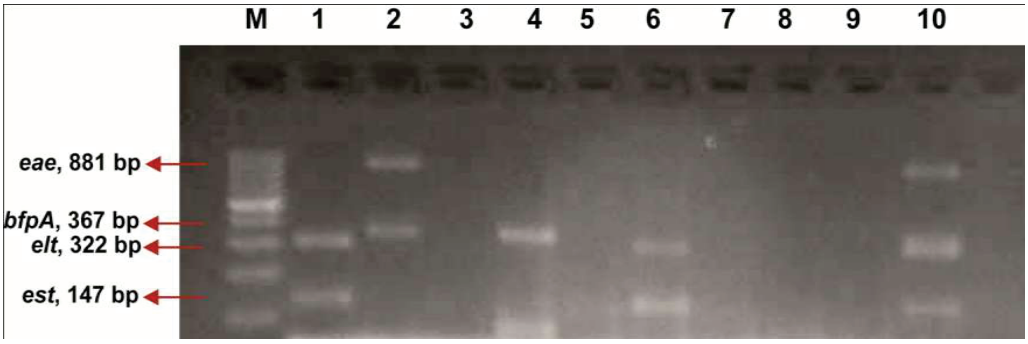

Fig.1 Multiplex PCR assay for detection of toxigenic *E. coli* from the children with diarrhea.

PCR assay detected lane1: positive for *elt* and *est* (ETEC), lane2: *eae* and *bfpA* (EPEC), lane3: negative, lane4: *bfpA* (EPEC), lane5: *est* (ETEC), lane6: *elt* and *est* (ETEC), lane7, 8, 9: negative and lane10: *est*, *elt* and *eae* (ETEC and EPEC)

DISCUSSION

DEC has been reported as the major etiological agent next to rotavirus causing dirrhea among children in developing countries. It is important to notice the association of particular DEC, antbiogram and genotype for monitoring and control measures. Till date very limited knowledge is available regarding the involvement of DEC causing diarrhea among children in Odisha.

In this study, DEC such as ETEC and EPEC as a whole was isolated in 13.6% of pediatric diarrhea cases while other DEC was not detected by PCR analysis. This incidence number of DEC was more than the other reports from developing countries (Estrada-Garcia et al., 2009; Ochoa et al., 2009), less than the south India (Shetty et al., 2012) and very closely consistent

with the report in Kolkata (Dutta et al., 2013). The present study detected the distribution of ETEC and EPEC differently in all ages between 2 to 14 years in Odisha.

In the present study the incidence rate of ETEC was higher than the reports in other Indian states (Dutta et al., 2013) and elsewhere in globe (Regua-Mangia et al., 2004; Jafari et al., 2009). Considering the age and infection, this study revealed ETEC as a sole pathogen causing diarrhea in age groups from 2 to 14 years while several other studies reported the occurrence of ETEC in younger children causing diarrhea (Ochoa, et al., 2009; Estarda-Garcia et al., 2009; Dutta et al., 2013). The frequency of ETEC with *est* (50%) was more common compared to the ETEC carrying *elt* (16.7%) alone and in combination of both *elt* and *est* (33.3%) gene. It is well understood, *est* is the source of important virulent heat stable enterotoxin causing diarrhea in the globe (Binsztein et al., 1991; Rao et al., 2003).

Different studies reported differently the prevalence of EPEC as typical EPEC or/and atypical EPEC in developing and developed countries. The rate of isolation of the EPEC was much higher in Chile (38.3%) and Brazil (34.0%) whereas the frequency was lowest in Somalia (4.0%) and Thailand (5.5%) (Casalino et al., 1988; Echeverria et al., 1991; Chen and Frankel, 2005). Studies in India demonstrated the prevalence of EPEC 1.8% in Kolkata (Dutta et al; 2013), 7.6% in Kashmir (Wani et al., 2006) and 10.4% in Mangalore (Shetty et al., 2012). However, the result of our study remained intermediately with 8.2% EPEC compared to the above studies.

After understanding the distinction between typical EPEC and atypical EPEC, strains causing acute and chronic watery childhood diarrhea in developing countries, major attention has been diverted to find out their epidemiology and genetic mechanisms. A significant association of typical EPEC strains (eae^{+} $bfpA^{+}$) with diarrhea was reported previously from developing countries (Bischoff et al., 2005). In recent years, several studies have shown that atypical EPEC strains (eae^{+}, $bfpA^{-}$) are more prevalent than typical EPEC strains in developed countries as well

as in resource-limited countries including Mexico, Nicaragua, Vietnam, and Mozambique. Contrast to the above reports, we reported here in our study, higher detection of tEPEC compared to aEPEC. However, tEPEC was marginally higher than the aEPEC. Studies conducted in many countries indicated that tEPEC was more in infants, 6 months old (Chen and Frankel, 2005). In other age groups, aEPEC predominated. Consistent with these reports we detected tEPEC in <2 year children and aEPEC in 5 Year age group children. Looking into the recent global scenario of frequent occurrence causing diarrhea, aEPEC has encountered as a major emerging pathogen in developing and developed countries.

Emergence of resistance to a series of antibiotics regarded as the major concern in recent years globally. Parallel to this view, most of our strains we found resistant to commonly susceptible antibiotics those were empirically used by general population such as, ampicillin, TMSX, co-trimoxazole and chloramphenicol. These drugs are highly prescribed in some countries as a treatment for enteric infections caused by Gram-negative bacteria (Livermore et al., 2002; Yang et al., 2009). This trend has been observed in other developing countries, such as Iran, where DEC strains isolated from adults were resistant for ampicillin, co-trimoxazole, and tetracycline with 67% of DEC strains being multidrug-resistant (Alikhani et al., 2013). Accordingly, strains isolated from Iranian children with diarrhea were resistant to ampicillin and tetracycline (89 and 83%, respectively) with all of DEC isolated from this particular population presenting the MDR phenotype (Heidary et al., 2014). In Mexico, (Estrada-Garcia, et al., 2005) found in a study carried out in children that 70% of DEC strains were resistant to ampicillin and SMTX; whereas most strains were sensitive to ciprofloxacin and cefotaxime. Our study hypothesizes, probably, irrigational and disproportionate use of these drugs could be the key factor for development of resistance in DEC.

The central theme of this study suggests DEC as one of the major pathogens causing childhood diarrhea. Surveillance program should be in continuous process to acknowledge its etiology for control measures. As aEPEC is an emerging pathogen for the cause of a large number of diarrheal cases, further studies should focus to document its epidemiology and virulence properties.

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QUANTIFICATION OF SINGLE *DROSOPHILA* FLY GENOMIC DNA USING UV SPECTROPHOTOMETRY, NANODROP AND QUBIT FLUOROMETRY

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ABSTRACT

Efficient use of DNA samples in high-throughput genotype and sequence analysis demands high quality DNA isolation, storage and accuracy in estimation of DNA concentration. Contribution of *Drosophila* as a model for unravelling the basic information especially in the field of genetics, genomics, and evolution is admiring but executing advanced molecular biology techniques in a single fly remains challenging in the field of *Drosophila*. In this paper, we have estimated the genomic DNA (gDNA) extracted from a single *Drosophila* fly by using three different methods, e.g., UV spectrophotometer, NanoDrop, Qubit Fluorometery, and the result is presented.

Keywords: *Drosophila*, gDNA, UV Spectrophotometer, NanoDrop, Qubit Fluorometery.

INTRODUCTION

The genetic content, the DNA carried by an organism is its true identity that makes a distinction between different living beings. With this the concept of isolation and analysis of DNA of different organism arises. Extensive research has been carried out in the field of proteomics, genomics, and transcriptomics in order to come up with advanced techniques to assist the scientist in their research. The introduction of powerful genomic techniques, such as high throughput Next Generation Sequencing promises to unveil the link between phenotypic characters and genetic makeup of an organism. This high quality, expensive, time

consuming, and labor intensive process requires pure intact DNA for producing accurate results (Chakraborty et al., 2015). The inappropriately quantified samples can drastically have negative impact on entire sequencing resulting in erroneous results and higher costs. In order to achieve this goal, there is a need for consistent profiling methodology irrespective of its source and extraction protocol employed, to ensure reliable quantitation and qualification of DNA samples. The workflow for appropriate quantification consists of estimation of double strand DNA concentration and assessment of its suitability for various applications (Nielsen et al., 2006; Sah et al., 2013). *Drosophila* has emerged as an ideal model organism for understanding various biological processes including evolutionary and comparative genomics studies (Birney, 2007; Charlesworth, 2015). With rising concern on the use of animals in research by the animal lovers, the animal ethical committee has come up with stringent rules regarding the use of animals in research. Since there are no such issues while using fruit fly in the research more of the scientist now prefer to use *Drosophila* in such studies. Various researches are being carried out to study the molecular phylogeny of the fruit fly and whole of its genome has already been sequenced in twelve species (Ko et al., 2003; Crosby et al., 2007).The presence of lesser number of chromosomes along with the fact that 75% of disease causing genes in humans have a functional homolog in the fly making it a disease model for studying the molecular mechanism behind the pathogenesis of human diseases (Pandey et al., 2011). Although a number of studies have reported different DNA extraction methods of fly DNA, nowhere it is mentioned the quantification methods and total yield of the concentration of genomic DNA present in a single fly, which promoted to undertake this investigation.

In the present study, we have utilized three commonly used methods to assess the quantity and agarose gel electrophoresis method for quality of isolated genomic DNA from single fly. The UV spectrophotometry based assay for quantifying DNA includes conventional UV spectrophotometer (Manchester, 1996; Haque et al., 2003) and NanoDrop (Robertson, 2003; Desjardins et al., 2010) both relies on the principle of absorbance shown by aromatic rings of purines and pyrimidines at 260 nm. However UV spectrophotometer is more efficient in

estimating the bulk concentration of DNA present in the sample whereas NanoDrop being more sensitive works well with low quantity sample. The latter does not require cuvettes as it holds the sample in place due to surface tension and directly computes the concentration. The fluorometery based DNA quantification; Qubit assay is highly sensitive, selective and analytical in accurately determining the concentration of double stranded DNA. It is based on principle of fluorescence of the dyes that specifically binds to dsDNA ignoring the presence of any contaminants (Sah et al., 2013; Simbolo et al., 2013).

MATERIAL AND METHODS

Extraction of DNA

For each species, genomic DNA (gDNA) was extracted from single fly in replicates using standard DNA isolation (isopropanol-precipitation) protocol. The same solution used during DNA extraction in all *Drosophila* samples kept the same to avoid any biasness. A single fly was homogenized in 100µl of cell lysis buffer (NaCI, SDS, EDTA, Tris-HCI: pH 8.0) using microfuge tube pestle. Following incubation at 65°C for 15 min 0.5 µl RNaseA Solution (4 mg/ml) was added to the cell lysate. The lysate was incubated at 37°C for 45 min and then was cooled down to room temperature. Then 33µl of protein precipitation solution (8M potassium acetate) was added to that RNase A-treated cell lysate and tubes were centrifuged at 14,000 rpm for 10 min. The precipitated proteins form a tight pellet. The supernatant was transferred to a fresh 1.5 ml tube, followed by the addition of 100 µl 100% Isopropanol (^{2}-propanol). The tubes were centrifuged for 5 min. at 14,000 rpm. The supernatant was poured off and 100 µl 70% ethanol was added. The tubes were inverted several times to wash the DNA pellet and centrifuged at 13, 000-16,000 rpm for one minute. The ethanol was drained off and allowed to dry overnight at room temperature. Twenty µl nuclease-free water was added to the gDNA pellet and slightly vortexed. The gDNA was finally stored at -20°C till further step.

Qualitative and quantitative analysis of gDNA

The integrity of the isolated DNA was checked by running the sample on 0.8% agarose gel and visualizing under UV transilluminator. The concentration of isolated genomic DNA was estimated using UV spectrophotometry (Shimadzu Spectrophotometer UV-1800), NanoDrop (NanoDrop 1000) and Qubit assay (Qubit® 2.0 Fluorometer).

For estimation of DNA extracted from single fly in UV spectrophotometer, the instrument was calibrated by taking 300ul nuclease free water as blank in quartz curvette. The readings of all the samples were measured in accordance to total volume of 300 µl (as dilution of gDNA done with nuclease free water) and subsequently the dilution factor was calculated. The absorbance of diluted gDNA was measured both at 260nm and 280nm and the readings were noted down. The concentration of double stranded gDNA in the samples was calculated by multiplying the absorbance at 260nm by dilution factor and 50, assuming 1.0 A° = 50.0 ng/uL dsDNA. For quantification of gDNA using NanoDrop1000, 1µl of the sample was loaded on lower surface of the microvolume spectrophotometer sample retention system instrument. The software automatically calculated the nucleic acid concentration and purity ratios, i.e., 260/280 ratio and values were recorded down. In order to access the sample purity 260/280 ratios were analysed to check upon any contamination (Manchester, 1995).While estimating gDNA using Qubit Fluorometery assay, the Qubit™ working solution was prepared by diluting the Qubit™ reagent in Qubit™ buffer in appropriate ratio. The Qubit™ working solution was divided into two assay tubes each for standard and sample. The assay tubes were vortexed for 2-3 seconds and incubated at room temperature for 2-3 min. The tubes were inserted in the Qubit® 2.0 fluorometer and the readings were recorded.

RESULTS AND DISCUSSION

Genomic DNA yield from a single *Drosophila*

Extraction of gDNA from single *Drosophila* is of primary importance for studying the genotype diversity at individual level. In this report, we are providing the quality and quantity analysis of single fly gDNA to assure the purity and desired concentration for further use in the molecular biology. The total yield of single fly genomic DNA (gDNA) was calculated individually and on average from four different *Drosophila* species namely *D.nasuta, D. melangaster, D. kikkawai and D. ananassae* (Table 1 A, B) using three different assays, i.e., UV spectrophotometer, NanoDrop and Qubit Fluorometery. However, gDNA measured by these three methods shows remarkable variations amongst different *Drosophila* species studied. *D.nasuta* showed significantly higher gDNA concentration in all assays which could be a result of its large body size. Hence, it can be concluded that the variation in amount of the genetic content among the different *Drosophila* species can be correlated with its body size or weight. The average gDNA yield (total) was found to be 1890, 772 and 286 ng in the most commonly studied species, *D.melanogaster* while measured by UV spectrophotometer, NanoDrop and Qubit Fluorometer respectively. The mean value of gDNA yield from a single fly calculated from all species, were quantified to be 340, 803 and 2187.5 ng as measured using Qubit, NanoDrop and spectrophotometer. Our results show similar results in term of DNA yield, when compared to previous result (~150-150ng) (Colton and Clark, 2001).

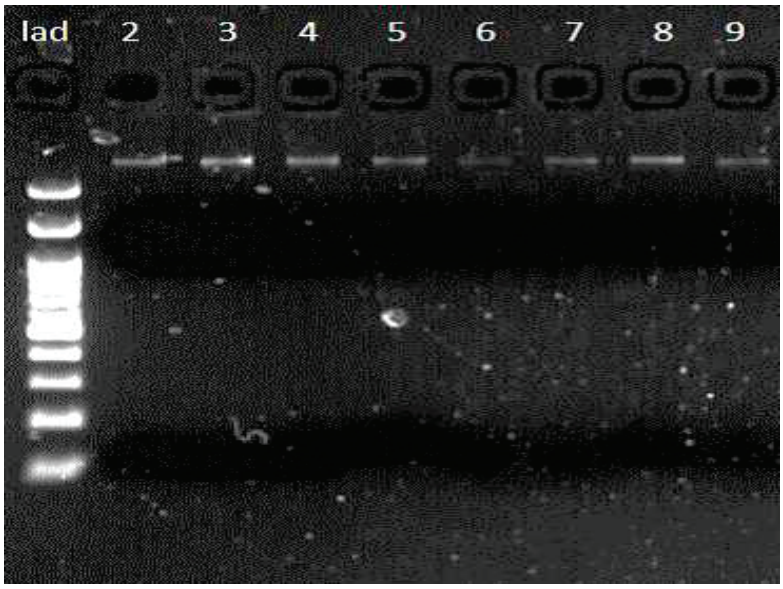

Fig.1 Agarose gel electrophoresis result showing bands of single fly g DNA. Lane 1: Ladder, lane 2, 3: replicates of gDNA isolated from *D. nasuta;* lane 4, 5: replicates of gDNA isolated from *D. melanogaster*; lane6, 7: replicates of gDNA isolated from *D. ananassae:* lane 8, 9: replicates of gDNA isolated from *D. kikkawai.*

Significance of purity ratio

The mean purity ratio (A260/A280) obtained using the NanoDrop and UV spectrophotometer for the extracted gDNA was 1.8 and 1.7 respectively (Table 2) which is indicative of pure DNA, and was almost consistent across all the samples. The integrity of gDNA was found be intact when samples were run on 0.8% agarose gel (Fig. 1).

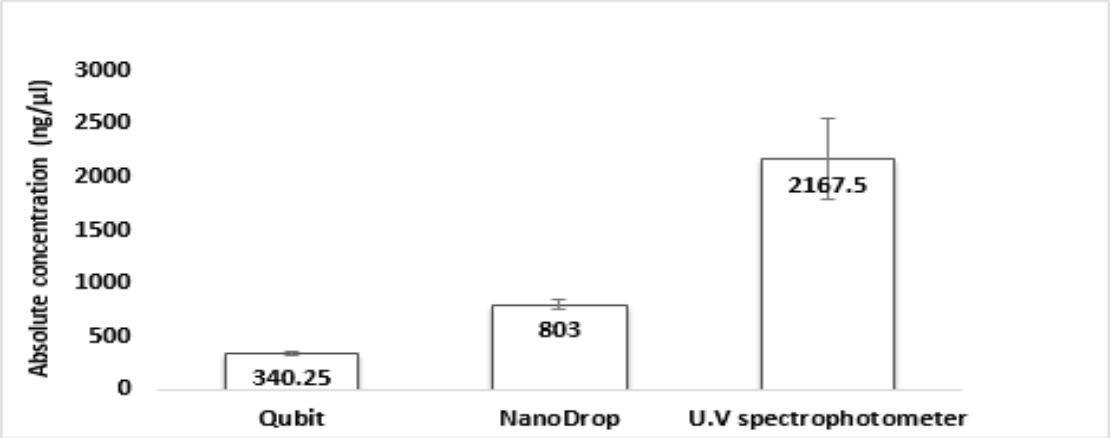

Fig.2 Comparison of absolute concentration of single fly gDNA using Qubit, NanoDrop and UV Spectrophotometer.

Comparative analysis of the outcome of three DNA quantification assays

The estimated mean yield of gDNA obtained from single fly including all species from UV spectrophotometer, NanoDrop and Qubit was observed to be 2087.2, 803 ng and 340.25 ng respectively (Table 1. A, B). Our result shows that the gDNA concentration estimated) by UV spectrophotometer was approximately six folds higher and NanoDrop yields was two folds higher than Qubit across DNA samples (Fig. 2). Comparative analysis of these assays shows that gDNA concentration values determined using UV spectrophotometry based assays (UV spectrophotometer and NanoDrop) were significantly higher than the fluorometery based assay (Qubit) (Simbolo et al., 2013). The most probable reason for this drastic variation could be the presence of negligible amounts of contaminants such as proteins, RNA and salts, which can increase the spectrophotometric based quantification of gDNA concentration.

Table 1 Quantification of single fly gDNA using different platforms: A. NanoDrop and Qubit Fluorometery assay; B.UV Spectrophotometer

(A)

S.No	Species	Replicates	NanoDrop			Qubit Fluorometer Assay		
			Conc. (ng/µl)	Total yield of DNA (single fly)	Mean	Conc. (ng/µl)	Total yield of DNA (single fly)	Mean
1	*D. nasuta*	R1	45.2	904	937	20	400	412
		R2	48.5	970		21.2	424	
2	*D. melanogaster*	R1	44.4	888	772	13.3	266	286
		R2	32.8	656		15.3	306	
3	*D. kikkawai*	R1	37.5	750	790	14.4	288	289
		R2	41.5	830		14.5	290	
4	*D. ananassae*	R1	32.6	652	713	19.2	384	374
		R2	38.7	774		18.2	364	
					803±40.99			**340.25±22.52**

(B)

S.No	Species	Replicates	Absorbance (260nm)	Conc. (ng/µl)	Total yield of DNA (single fly)	Mean
1	*D. nasuta*	R1	0.105	157.5	3150	3780
		R2	0.14	220.5	4410	
2	*D. melanogaster*	R1	0.059	88.5	1770	1890
		R2	0.067	100.5	2010	
3	*D. kikkawai*	R1	0.052	78	1560	1380
		R2	0.04	60	1200	
4	*D. ananassae*	R1	0.049	73.5	1470	1275
		R2	0.036	54	1080	
						2167.5±380.94

Table2. Comparison of absorbance ratios (260/280 nm) of DNA obtained using NanoDrop and UV Spectrophotometer assay

S. No	Species	Replicates	NanoDrop Absorbance ratio (260/280nm)	UV Spectrophotometer Absorbance ratio (260/280nm)
1	*D. nasuta*	R1	1.66	1.5
		R2	1.62	1.8
2	*D. melanogaster*	R1	1.73	1.4
		R2	1.58	2.03
3	*D. kikkawai*	R1	1.82	1.9
		R2	2.05	1.8
4	*D. ananassae*	R1	1.77	1.44
		R2	1.88	1.63

In addition, the UV spectrophotometry based assay cannot distinguish between single stranded DNA and double stranded DNA whereas in Qubit fluorometery method, flurochromes specifically binds to double stranded DNA ignoring the presence of any single stranded DNA or RNA contamination (Haque et al., 2003). However, the variation in the quantification results obtained from these three methods is misleading and the reason behind which still not understood. As given in the technical methods of the spectrophotometer and NanoDrop assay, our samples show free from contaminants as the purity ratio was found to be around 1.8. Finally, it can be concluded that estimated gDNA concentration results vary among different assays performed in present study and to correctly estimate DNA concentration, one should use combination of two assays preferably NanoDrop and Qubit. Therefore, the comparative analysis reflected in this paper may help the researcher in estimating the genetic content, the DNA of a single fly without any further confusion and proceed for other downstream processes using the estimated DNA.

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THE INTESTINAL BACTERIAL FLORA OF TADPOLES OF *DUTTAPHRYNUS MELANOSTICTUS* (SCHNEIDER, 1799)

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ABSTRACT

We examined the intestinal bacterial flora of the tadpoles of *Duttaphrynus melanostictus* collected from natural habitat. The bacteria from intestinal contents were grown aerobically using differential media, viz., nutrient Agar, XLD, Littman Oxgall Agar, MRS, and counted in terms of colony forming unit (CFU). The predominant bacteria were isolated and partially characterized by different biochemical tests. A total of 36 isolates were cultured. The prevalent isolated bacteria included *Citrobacter diversus, Enterobacter amnigenus, Escherichia coli, Aeromonas hydrophila, Streptococcus cohni, Citrobacter* sp., *Shigella* sp., *Micrococcus* sp., *Streptococcus* sp., *Staphlylococcus* sp., *Bacillus* sp., *Pseudomonas* sp., *Acetobacter* sp., *Cardiobacterium* sp. and *Alcaligens* species. Hindgut recorded higher bacterial population compared to that of foregut without any difference in bacterial diversity in both the regions. Majority of the isolated bacteria belong to families related to enteric bacteria. Among these, *Aeromonas hydrophila*, *Citrobacter* sp., *Staphylococcus* sp. and *Pseudomonas* sp. were reported as pathogens to frog. The differences in intestinal microflora may depend on the physiological conditions of host animals, environmental conditions, and feeding habits.

Keywords: *Duttaphrynus melanostictus,* tadpole intestine, microflora, biochemical tests.

INTRODUCTION

Vertebrates maintain a complex symbiotic relationship with a diverse community of microbes residing within their guts. Flora of gut primarily helps the host in getting more energy by the fermentation of undigested carbohydrates and subsequent production and absorption of short chain fatty acids. They also play an important role in synthesizing vitamin B and K, train the immune system to defend against pathogens causing diseases whilst stimulates cell growth (Guarner and Malagelada, 2003).The normal microbiota has profound significance on the life history of larval amphibians (Pryor, 2008). Among them, the enteric microflora plays an ecological role in anuran development and metamorphosis (Fedewa, 2006). Among vertebrates, it has been found that amphibian population around the world is declining due to numerous factors including habitat loss, deforestation, global climate change, competition from species introduced, pollution and infectious diseases (Kiesecker et al., 2001; Hayes et al., 2010). Several studies tried to identify the pathogens associated with specific amphibian decline; very few studies are conducted to identify the natural microflora present in amphibian digestive tract. Knowledge of the natural bacterial flora in alimentary canal of healthy individuals is highly significant for understanding disease outcome. The present report deals with the isolation and identification of bacteria from the alimentary tract of tadpoles of common Indian toad *Duttaphrynus melanostictus*.

MATERIALS AND METHODS

Tadpoles of *D. melanostictus*, collected during July, 2014 from different ponds around the campus of North Orissa University within the city limits of Baripada, were reared in plastic bowls at room temperature (35^{o}C) in the laboratory. They were fed with boiled spinach and

boiled egg yolk in alternate days throughout rearing. Water of rearing bowls also was changed in alternate days to maintain the water quality. All the tadpoles used in this study were in between 31 to 36 stages (Gosner, 1960). The intestines of both laboratory reared and collected from the wild were removed at stage 36 into sterile glass slides containing physiological buffered saline (PBS) in aseptic conditions under a laminar hood. The guts were measured and weighed and separated into foregut and hindgut and placed in sterile centrifuge tubes followed by homogenization using sterile Milli-Q water. The foregut homogenate was diluted 10 times and that of the hindgut 100 times as the later contained higher number of bacteria than the former. After dilution, 100µL of homogenate per plate were streaked onto four differential media, viz., nutrient agar (NA), xylose lysine deoxycholate (XLD), eosin-methylene blue (EMB) and de Man, Rogosa and (MRS) agar plates. Plates were incubated at 37°C for 48 h. Bacterial colonies exhibiting growth upon repeated subculturing on nutrient agar plates were selected and streaked onto nutrient agar plates in order to obtain pure cultures. These pure cultures were maintained in nutrient broth containing 60% glycerol and stored at −20°C until further analysis. These strains were subcultured on nutrient agar plates and identified. Characteristics of bacterial colony such as shape, margin, elevation, color, opacity, and consistency were recorded. All bacterial cultures were subjected to Gram staining for further characterisation. Certain specific biochemical tests were performed based on Gram stain character and morphological examination. Gram-positive bacteria cultures were tested for spore formation, catalase production and oxidase activity, whereas Gram-negative cultures were subjected to IMViC test by inoculating on citrate utilization medium and triple sugar iron (TSI) slants. A series of additional biochemical tests were performed following Berge's Manual of Determinative Bacteriology (Holt et al., 1994).

RESULTS AND DISCUSSION

A total of 94 bacterial strains were isolated from both the parts (foregut and hindgut) of the alimentary tract. There were no apparent differences in the type of bacteria among the two gut parts and tadpole collected from both the sources. However, they varied in terms of total bacterial number, i.e., hindgut recorded higher number of bacterial population compared to that of foregut (Table 1). Majority of the isolated bacteria belonged to families related to enteric bacteria. Thirty seven culturable bacterial strains were selected (basing on culture characters) for repeated culturing, while the remaining ones were either unidentifiable, could not be subcultured, or looked similar. Gram staining revealed that all the bacterial cultures were different:Gram-negative rods with catalase and oxidase positive, Gram-negative rods with catalase positive and oxidase negative, Gram-positive bacilli with catalase positive, Gram-positive cocci with catalase positive and Gram-positive cocci with catalase negative (Fig.1). Few strains were confirmed as *Pseudomonas* sp. based on odour: earthy smell with bluish green diffusible pigment. The isolates were Gram-negative rods, catalase and oxidase, citrate, arginine dihydrolysis positive and could tolerate temperature up to 40^{o}C. Isolates with Gram-negative rods or bacilli, catalase, indole, MR and citrate positive, H_2S and TSI slants were identified as *Citrobacter* species. *E. coli* was confirmed based on its Gram character such as negative, bacilli, catalase, Indole and MR positive, acid producing in TSI agar. It utilized glucose while negative to H_2S, VP and citrate. Isolates having Indole, MR (negative), and VP, citrate (positive) with Gram characters and sugar fermentation were identified as *Enterobacter* species. Isolates with Gram-positive bacilli, catalase, gelatinase, and amylase positive were identified as *Bacillus* species.

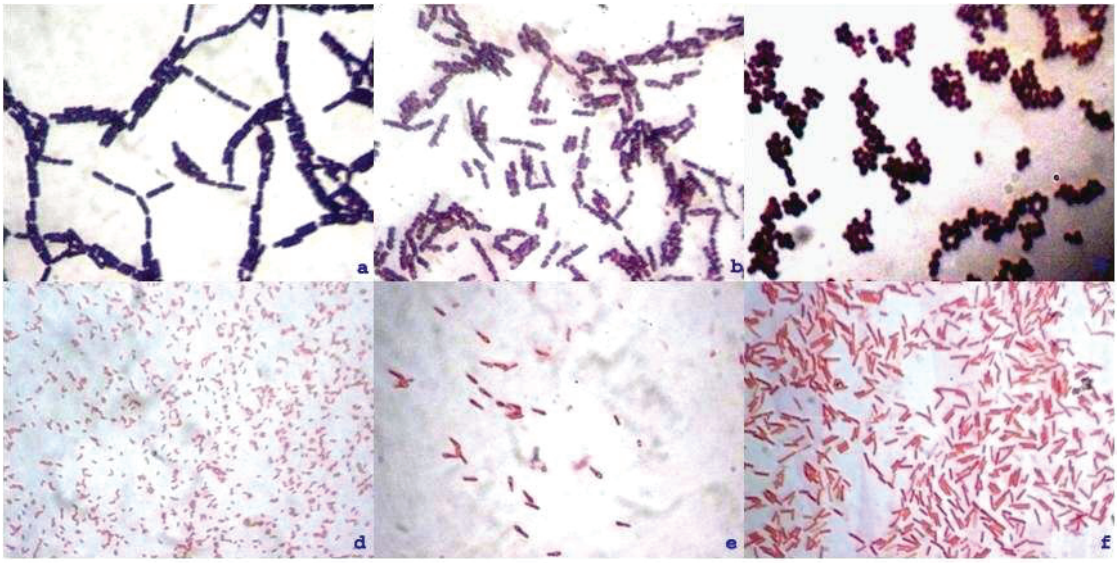

Fig.1 Gram characteristic of selected strains: **a**. Gram-positive bacilli (*Bacillus* sp.); **b**. Gram-positive coccus in chain (*Streptococcus* sp.); **c.** Gram-positive coccus in cluster (*Staphylococcus* sp.); **d**. Gram-negative rods with catalase positive and oxidase negative (*Escherichia coli*); **e.** Gram negative bacilli/rods with catalase and oxidase positive (*Aeromonas hydrophila*); **f.** Gram negative bacilli/rods with catalase and oxidase positive (*Pseudomonas* sp.).

Regardless of the taxonomic designation of bacterial strains isolated in this study, the enzymatic properties of these isolates are nonetheless insightful. Many of the isolates were positive for degradation of enzymes like amylase, protease and lipase. We presume these properties of bacteria provide advantage to the tadpoles for digestion of both protein (egg albumin) and cellulose (spinach) foods. Three strains were positive to degrade chitin, which seems

advantageous because reports are available that the foreguts of wild-caught tadpoles regularly contain the chitinous exoskeletons of aquatic invertebrates (Pryor, 2008). He concluded that chitinolytic bacteria retained through metamorphosis would confer obvious digestive advantages to the frogs. The ability of tadpole gut bacteria to hydrolyze sugars and starch in the fermentation tubes is also understandable considering the ease with which most animals digest these carbohydrates (Stevens and Hume, 1997).

Table 1 Bacterial density of foregut and hind gut of *Duttaphrynus melanostictus* tadpoles (N = 6)

Media		NA	XLD	EMB	MRS
Bacterial density	Foregut	11.3×10^3	7.7×10^3	4.0×10^3	1.8×10^3
	Hindgut	9.2×10^5	5.7×10^5	4.8×10^5	5.0×10^5

The comparative analysis of the gut flora isolated from *D. melanostictus* with other vertebrate gut flora revealed that members of the genera *Bacillus* sp., *Streptococcus* sp., *Alcaligens* sp., *Pseudomonas* sp. and *E. coli* are common inhabitants. It is interesting to note that some bacterial isolates identified in this study are known to infect humans especially *Aeromonas hydrophila* and *Staphylococcus cohnii*. Other bacteria such as *Citrobacter* and *Pseudomonas* species were also reported as pathogens to frog. All the isolates (37) fall under eleven genera and identified as *Alcaligens* sp., *Aeromonas hydrophila*, *Bacillus* sp., *Enterococcus* sp., *Citrobacter diversus*, *Citrobacter* sp., *Erwinia* sp., *Enterobacter amnigenus*, *Enterobacter* sp., *E. coli*, *Staphylococcus cohni, Pseudomonas diminuta, Pseudomonas* sp., *Staphylococcus* and *Streptococcus* species. However, the most dominant species were *Bacillus* sp., *Streptococcus* sp.,

Pseudomonas sp. and *E. coli.* Moreover, *Aeromonas hydrophila* is a heterotrophic Gram-negative, rod shaped bacterium found in warm climate and mostly associated with fish and humans as a disease causing agent. As the tadpoles were collected from ponds of different localities, where they might have been contaminated and not reported as indigenous flora. Similarly, presence of *Erwinia* sp., reported as a plant pathogen causing diseases in crops and vegetables, was also an exception. Spinach given to tadpoles as food might be the source of contamination. It is also difficult to identify this bacterium up to species level based on biochemical tests. Hence, further study is essential at molecular level for confirmation of the identification of this bacterium.

It is useful to study the roles of the gastrointestinal microbiota as they can have the ability to produce bioactive compounds which may lead to an important component of defense against pathogens. Besides, potential effects of aquatic contaminants on the gastrointestinal microbiota and thus, the digestive function of amphibians is another area of research. It can be concluded that the intestinal flora of *D. melanostictus* is similar to other vertebrates. The bacterial population of fore and hind guts were almost similar with a large difference in terms of population.

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A STUDY ON DNA FRAGMENTATION FACILITATED CELL DEATH DURING NORMAL AND VITAMIN A MEDIATED TAIL REGENERATION IN TADPOLES OF THE INDIAN TREE FROG

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ABSTRACT

DNA fragmentation is considered to be a hallmark event during apoptosis. Vitamin A inhibits tail regeneration and induces ectopic limb development in anuran tadpoles. In the backdrop of the existing fact, we have investigated the occurrence of such DNA fragments during both normal tail regeneration and Vitamin A mediated regeneration in the tadpoles of Indian tree frog, *Polypedates maculatus*. In the vitamin A treated tadpoles a ladder pattern was evident in the 12 hour regenerates only. However, such ladder was evident both in 12 and 24 hour regenerates of the control group. This is indicative of cell death to be an integral event at the onset of normal regeneration.

Key words: Apoptosis, DNA fragmentation, Regeneration, Vitamin A, *Polypedates maculatus*.

INTRODUCTION

Anuran tadpoles are known to regenerate their tail, including spinal cord, notochord, and muscle (Brockes, 1997). The process involves wound healing, blastema formation, and outgrowth (Stoick-Cooper et al., 2007). Regeneration of amphibian appendages, including the

tail, represents a valuable model system to analyze molecular mechanisms underlying cell growth, cell differentiation, morphogenesis, and pattern formation in epimorphic regeneration (Taniguchi et al., 2014). Programmed cell death (PCD) plays a key role in development and in maintenance of the steady state in continuously renewing tissues (Gavrieli et al., 1992). During tail regeneration in *Xenopus*, apoptosis, a form of programmed cell death has been reported to be crucial during the first 24 h post-amputation where endogenous inhibitory cells seemed to be destroyed by programmed cell death for regeneration to occur (Tseng et al., 2007). Moreover, in *Drosophila*, *Hydra*, planarians, zebrafish and mice, recent studies have been indicative of cell death opening paths to regeneration in adult animals (Vriz et al., 2014).

Apoptosis, is characterized by cytoplasmic condensation, plasma membrane blebbing and nuclear pycnosis, leading to nuclear DNA breakdown into multiples of ~200 bp oligonucleosomal size fragments (Ioannou and Chen, 1996). Its existence is inferred mainly from gel electrophoresis of DNA extract as PCD is known to be associated with DNA fragmentation (Gavrieli et al., 1992). The tadpoles of the Indian tree frog, *Polypedates maculatus* like other anuran larva can regrow their lost tail following an amputation within a span of 15 days. However, when the tadpoles are reared in 10 IU/ml vitamin A palmitate solution for 72 h abnormal regeneration takes place and ectopic limbs have been reported to develop at the tail region (Mohanty-Hejmadi et al., 1992). Aim of the present study was to have a comparative assessment of apoptosis in the normal and vitamin A treated tail regenerates. We have analysed the occurrence of DNA fragmentation visualized by agarose gel electrophoresis during normal and vitamin A mediated tail regeneration in the tadpoles of the Indian tree frog *Polypedates maculatus* as the appearance of the ladder of nucleosomal DNA fragments in agarose gel is the classic feature of apoptosis (Gavrieli et al., 1992).

MATERIALS AND METHODS

Staging of the embryos and tail amputation

Egg masses of the Indian tree frog, *Polypedates maculatus* were collected during monsoon from the campus of the Utkal University, Bhubaneswar, Odisha, India (20º 16' 35. 2" N 85º 50' 35. 0" E). Tadpoles were reared in the laboratory following the standardized procedure (Mohanty-Hejmadi, 1977) and were fed with boiled *Amaranthus* leaves *ad libitum*. For agarose gel electrophoresis, tadpoles of limb bud stage (Gosner stage 26 - 29) (Gosner, 1960) were selected.

Tail amputation was carried out by first anesthetizing the tadpoles with MS 222 (Tricaine methane sulphonate) obtained from Himedia laboratories Mumbai, Maharashtra, India. Tadpoles were positioned laterally on a presterilized porcelain plate while the tails were amputed from the middle with sharp and sterilized blades. Further, bloodloss was prevented by transferring the tadpoles immediately to the amphibian ringer solution. Tadpoles of the control group were transferred to conditioned tap water where as tail amputated tadpoles were treated with Vitamin A palmitate 10IU/ml for 72 hours in the treated group. All procedures were approved by the Animal Care Review Committee at the Utkal University, Odisha, India.

DNA Extraction

The tail tip tissues of less than 25 mg were cut into small pieces and collected in sterilized micro-centrifuge tubes for investigation. DNA was extracted as per the instructor's manual (DNeasy Blood and Tissue Kit, Qiagen). The isolated DNA samples were quantified using a spectrophotometer (Eppendorf Biospectrometer® kinetic).

Agarose Gel Electrophoresis

For the visualization of the ladder, isolated DNA samples six µl each were electrophoretically separated on 0.8% agarose gel containing ethidium bromide (0.5µg/ml). Four wells were selected for the analysis. DNA ladder of 1 kb (Nex-Gen, Puregene) was loaded in the first one while the subsequent wells were loaded with samples of the 12 h, 24 h and 48 h regenerates respectively. The same procedure was followed for the treated samples also. The power pack connected to the horizontal gel electrophoresis apparatus (Genei^{TM}, Sanmar Speciality Ltd., Bengaluru, India) was set at 80V and allowed to run for 90 minutes continuously. Gel was carefully removed and laddering was visualized under an UV gel documentation system (BIO-RAD, USA).

RESULTS AND DISCUSSION

Chromatin cleavage during tail regeneration

In the tadpoles of the control group tail regenerates were having a tapered distal end. In the treated group no such tapering was evident in the tail regenerates. In this group the regenerated tails showed formation of a pouch like structure.

A distinct ladder pattern with five bands having 1000, 750, 500, less than 500 and less than 250 base pairs were observed in the 12 h, 24 h regenerates (lanes A and B of the agarose gel of Fig. 1) of the tadpoles of the control group that were reared in conditioned water post amputation. No such ladder was formed in the normal tail regenerates after 48h of regeneration (Lane C of Fig.1). However, ladder like pattern was evident only in the well containing DNA sample of the 12 h tail regenerate of the tadpoles that were reared in 10 IU/ml vitamin A palmitate for 72 h following tail amputation (Lane D of Fig. 1). In the vitamin A treated tail

regenerates no DNA laddering was evident in the 24h and 48h tail regenerates (lane E and F of Fig.1).

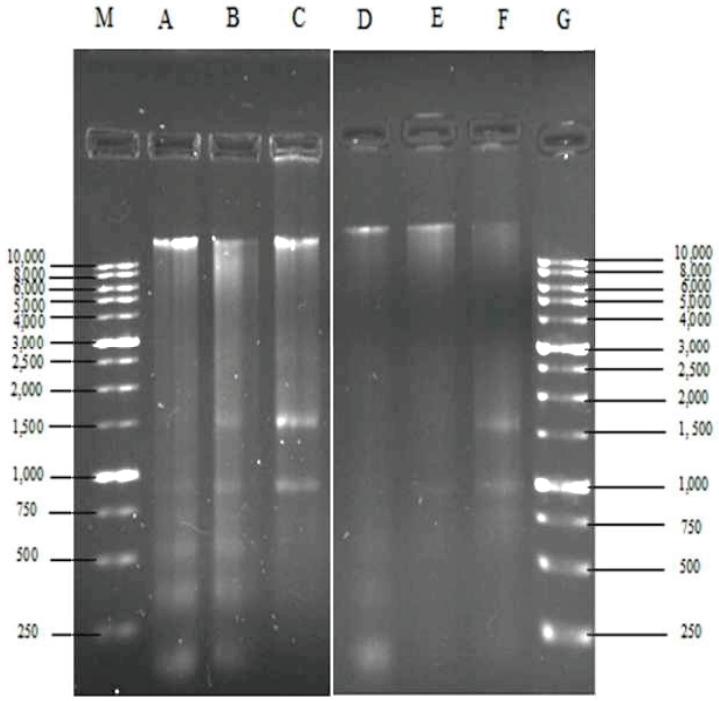

Fig. 1 DNA analysis by agarose gel. Lanes M and G stand for the molecular marker. Lanes A to C represents the DNA extracts from tails of 12 h, 24 h and 48 h tail regenerates of the control group, lanes D to F represents the DNA extracts from tails of 12 h, 24 h and 48 h tail regenerates treated with Vitamin A palmitate10IU/ml.

Apoptosis, a morphologically distinct form of programmed cell death plays significant role during the development. Interestingly, study on tail regeneration in *Xenopus* suggests apoptosis to be an early and obligate component of normal tail regeneration where certain endogenous inhibitory cells are destroyed by programmed cell death for regeneration process to occur (Tseng et al., 2007). In invertebrates like *Hydra,* apoptosis, has been reported to be necessary and sufficient for head regeneration (Bergmann and Stellar, 2010). Similary, in planarians and newts localized massive apoptosis has also been observed at the cut side after amputation (Bergmann and Stellar, 2010).

The novel role of apoptosis during regeneration has also been a subject of research in several vertebrate models also such as *Xenopus* and mice. In the *Xenopus* tadpole, a large number of apoptotic cells have been detected in the regeneration bud within 12 h of tail amputation (Tseng et al., 2007). Cleavage of chromosomal DNA into oligonucleosomal size fragments is a biochemical hallmark of apoptosis (Hua and Xu, 2000). In the present paper we have investigated the occurrence of DNA fragmentation, a classic feature of apoptosis during early stages of normal and also vitamin A mediated tail regeneration.

We found distinct ladder like pattern in the regenerates of the first 12 and 24 h post amputation in normal regeneration while only in the regenerates of 12 h post amputation in the vitamin A mediated regeneration. Ladder was absent in 48 h regenerates in both the cases suggesting a possible role of apoptosis in the early stages during the tail regeneration process. Further, evidence of no DNA laddering in the 24h tail regenerates of the vitamin A treated group, suggests possible role of vitamin A in preventing programmed cell death in these tail regenerates. Our finding based on the DNA fragmentation analysis by agarose gel electrophoresis is a preliminary attempt to study the role of apoptosis during tail regeneration in

anurans. However, further research shall be crucial for determining how apoptosis regulates appendage regeneration.

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HAEMO PROFILE OF TWO INDIGENOUS BREEDS OF CHICKEN ASEEL AND KADAKNATH (*GALLUS GALLUS DOMESTICUS*)

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ABSTRACT

Hematological analysis of birds reveals the health condition which may differ due to difference in sex, breed etc. The present study aims at the evaluation of hematological parameters of indigenous breeds of chicken namely Aseel and Kadaknath. Ten adult males and ten adult females from each breed were taken for this investigation. Hematological parameters such as Hemoglobin percent, Total Leukocyte Count, Total Erythrocyte Count, Packed Cell Volume, Mean Corpuscular Volume, Mean Corpuscular Hemoglobin, Mean Corpuscular Hemoglobin Concentration, Differential Leukocyte Count and morphometry of length of RBC and lymphocytes were taken into account. The data were subjected to PAST software for ANOVA and Tukey's pair wise comparison tests. The parameters were significantly different among and between the breeds at $p<0.05$ and $p<0.01$ with sexual dimorphism. The variations recorded may be due to difference in breed and sex.

Key words: Blood, Aseel, Kadaknath, Indian breed

INTRODUCTION

Chicken is a highly popular bird of delicacy worldwide having different breeds. The indigenous breeds (Indian breeds) of chicken, *Gallus gallus domesticus* considered for this study,

are Aseel and Kadaknath. Both these breeds originated in India. The literal meaning of Aseel is real or pure. It is well known for its pugnacity, high stamina and majestic gait and dogged fighting qualities. Andhra Pradesh is said to be the home of this important breed. The standard weight varies from 3 to 4 kg for cocks and 2 to 3 kg for hens. Sexual maturity is gained at 196 days. The annual egg production is 92eggs per year. The egg weight at 40 week is 50g (Anonymous, 1993-2009).

The original name of popularly known Kadaknath seems to be Kalamasi, meaning a fowl with black flesh (Anonymous, 2010). The eggs are light brown. The day-old chicks are bluish to black with irregular dark stripes over the back. The adult plumage varies from silver and gold-spangled to bluish-black without any spangling. The skin, beak, shanks, toes and soles of feet are slate like in colour. The comb, wattles and tongue are purple. Most of the internal organs show intense black color which is more in trachea, thoracic, abdominal air-sacs and gonads and at the base of the heart and mesentery. Varying degrees of black coloration are also seen in the skeletal muscles, tendons, nerves, meninges, brain etc. The blood is darker than normal blood. The black pigment has been due to the deposition of melanin. The meat and eggs are recorded to be a rich source of protein and iron. The body weight is 920 g at 20 weeks and attains sexual maturity at 180 days of age. The annual egg production is 105 in number and the egg weight at 40 week is 49 gram. The fertility rate is around 55 per cent (SA PPLPP, 2009).

Haematology is the branch of study concerned with blood, blood-forming organs and blood diseases. It includes the study of aetiology, diagnosis, treatment, and prognosis of blood diseases. Many birds do not express clinical symptoms until late in a disease process and the signs that they do exhibit may be subtle and non-specific. Consequently, the use of haematological assays may aid in the early recognition of disease, thus, facilitating the best opportunity for management and therapy to resolve the process (Clark et al., 2009).The

haematological parameters considered in this study include total erythrocyte count or total RBC count (TEC), total leukocyte count or total WBC count (TLC), differential leukocyte count (DLC), packed cell volume (PCV), haemoglobin percentage and erythrocyte indices.

Cytomorphometry of blood cells is undertaken to measure the size of the cells. It helps to bring out a comparative account on the basis of cell size between different taxon. Whether the cell is under functional or over functional can be diagnosed by morphometrical analysis. Cytomorphometry also explains facts regarding diseases. In this study, length of RBC and lymphocyte is measured in micron meters. Though haematological and cytomorphometrical studies on various breeds of chicken are available but comparison of these characteristics between two Indian breeds of chicken is rare and inadequate. Therefore, an attempt has made to report on this aspect of comparative study between two indigenous breeds of chicken of India.

MATERIALS AND METHODS

The investigation was conducted on indigenous breeds of chicken (*Gallus gallus domesticus*), i.e., Aseel and Kadaknath with reference to their sexual dimorphism. The blood samples were collected from 10 males and 10 females of each category of birds being maintained at Central Poultry Development Organisation (CPDO), Eastern Region (ER), Government of India, Bhubaneswar, Odisha, India under standard farm managemental practices. Samples were taken out with the help of sterile two ml syringes [Dispo Van Single Use Syringe, Hindustan Syringes and Medical Devices Ltd., Faridabad, India] and 25 gauge needles [Dispo Van Single Use Needle, Hindustan Syringes and Medical Devices Ltd., Faridabad, India] from the wing vein (ulnar vein) of the birds aseptically (Talebi et al., 2005) and collected in anticoagulant vials containing Ethylene Diamine Tetra-acetic Acid (EDTA) [K_3 EDTA, 2ml *13×75mm, Mfg By: HXS Tech Co., Ltd. PRC. For: Peerless Biotech Pvt. Ltd., Chennai, Tamil Nadu, India] and

labelled properly. This was performed during afternoon hours, i.e., between 2.00pm to 4.00pm. Blood smears were prepared at site on clean grease free slides [Blue Star Pic-2, Polar Industrial Corporation, Mumbai, Maharashtra, India], air dried and fixed in methanol [Qualigens Product No.34457, Thermo Fisher Scientific India Pvt. Ltd.,Mumbai, Maharashtra, India] for two minutes. Samples collected were kept in ice box and taken to laboratory for haematological analyses on the same day.

Sahli's acid haematin method (Sonia et al., 2012) was used to estimate Hb% with Sahli's haemometer [HiMedia GW 191-1NO, Plane haemometer (Square Type), HiMedia Laboratories Pvt. Ltd., Mumbai, Maharashtra, India] with the help of 0.1 N HCl [Merck CAS NO. 7647-01-0, Merck Specialities Pvt. Ltd., Mumbai, Maharashtra, India]. Haemocytometer having Neubauer's chamber was used to count TLC and TEC according to Dacie and Lewis (1991) and Turk's [HiMedia RO16-500ML, W.B.C. Diluting Fluid, HiMedia Laboratories Pvt. Ltd., Mumbai, Maharashtra, India] and Hayem's fluid [HiMedia RO13-500ML, R.B.C. Diluting Fluid (Hayemis), HiMedia Laboratories Pvt. Ltd., Mumbai, Maharashtra, India] respectively. By using centrifuge [REMI CENTRIFUGE, Catalogue No.C852 7/94, Serial No. GCLC-1632, REMI MOTORS, Bombay, Maharashtra, India], the Wintrobe haematocrit tube containing blood was centrifuged at 3,500 rpm for 15 minutes to estimate PCV. As per appropriate formula of Campbell (1988), MCV, MCH and MCHC were calculated from the values of PCV, TEC and Hb %. Blood smears made earlier were stained with Giemsa stain following Clark et al. (2009). Giemsa stain was prepared from Giemsa powder [Qualigens CAS NO. 51811-82-6 ProductNo. 39382, Thermo Fisher Scientific India Pvt. Ltd., Mumbai, Maharashtra, India] as per the standard protocol (Lillie, 1977). Slides were observed on subsequent days under Hunds Weltzar photomicroscope [MICROSCOPE H 600 WILOZYT PLAN, Serial No. 1024980, Helmut Hund GmbH, Wetzlar-Nauborn, Germany]. Differential leukocyte count (DLC) was evaluated by

following the method of Nowaczewski and Kontecka (2012). Length of erythrocytes and lymphocytes was measured in case of both groups with sexual dimorphism by the help of Microscope Eyepiece Digital Camera [CatCam130 – 1.3 Mega Pixel (MP), Code No. CC130, Catalyst Biotech, Maharashtra, India] attached to above mentioned microscope and computer. The entire data obtained were analysed by the statistical software Paleontological Statistics (PAST) Version 2.17 [Natural History Museum, University of Oslo] for One-Way Analysis of Variance (ANOVA) followed by post-hoc analysis (Tukey's pair wise comparison tests). Significant differences were tabulated and studied at $p< 0.01$ and $p<0.05$.

RESULTS AND DISCUSSION

Haemoglobin percentage, TEC, PCV, MCV, MCH, and percentage of lymphocytes in DLC differ among and between the groups at $p<0.01$. Percentage of heterophils in DLC differ between individuals at $p<0.05$. But MCHC, TLC, and percentages of monocytes, eosinophils, and basophils in DLC are not having any significant difference among males and females of both groups and between Aseel and Kadaknath as a whole (Table 1). Male Aseel have significantly different haemoglobin and PCV ($p<0.01$) from that of male Kadaknath. Also the female Aseels have significantly different Hb%, TEC, PCV, MCV and MCH from that of male and female Kadaknath at $p<0.01$. Even the male Aseel shows significantly different TEC at $p<0.01$ from that of male and female Kadaknath. MCV and MCH of male Aseel are significantly different with that of female Kadaknath ($p<0.01$). Lymphocyte percentage from DLC reflects difference at $p<0.01$ in between females of Aseel and Kadaknath breed. The haematological parameters are in accordance with our earlier report (Bhattacherjee et al., 2014). Hb%, TEC and PCV values are in accordance with that of indigenous chicken of Bangladesh (Sharmin and Myenuddin, 2004)

Table1 Haematological parameters of Indian breeds of chicken

SL NO	PARAMETERS	ASEEL		KADAKNATH		ANOVA (F-value)
		MALE (10)	FEMALE (10)	MALE (10)	FEMALE (10)	
1	Hb (%)	10.74±0.64[a]	9.26±0.48[b]	13.30±0.52[a,b]	11.43±0.40[b]	10.34**
2	TEC (millions/mm^3)	2.65±0.17[a]	2.13±0.19[b]	3.78±0.15[a,b]	3.31±0.12[a,b]	19.23**
3	PCV (%)	31.80±1.87[a]	27.15±1.26[b]	39.60±1.22[a,b]	34.20±1.42[b]	12.43**
4	MCV (μ^3)	120.79±3.14[a]	132.22±7.21[b]	105.40±2.34[b]	103.21±2.18[a,b]	10.35**
5	MCH (pg)	40.66±0.60[a]	45.10±2.71[b]	35.31±0.73[b]	34.56±0.74[a,b]	11.08**
6	MCHC (%)	33.79±0.68	34.11±0.68	33.51±0.32	33.50±0.34	0.28[NS]
7	TLC (thousands/mm^3)	8620.00± 2077.17	9116.00± 1950.53	5907.10± 1382.19	5758.90± 1242.28	1.07[NS]
8	Lymphocytes (%)	42.80±5.35	40.60±4.94[a]	58.00±3.85	59.40±4.11[a]	4.58**
9	Monocytes (%)	8.50±2.52	10.20±3.32	6.70±1.98	6.60±2.00	0.45[NS]
10	Eosinophils (%)	11.20±3.99	10.50±4.34	5.00±1.39	5.40±1.33	1.11[NS]
11	Heterophils (%)	36.40±5.18	35.20±4.64	25.50±1.48	24.20±1.69	3.02*
12	Basophils (%)	3.10±2.10	1.30±0.26	1.60±0.22	1.60±0.22	0.57[NS]

Figures in parentheses reflect number of observations; Mean ± SE bearing similar alphabetical superscripts differ in rows at p<0.01; p<0.05* and not significant[NS].**

which may be due to almost same climatic conditions prevailing in both countries. Except basophils, all other leukocyte percentages of Kadaknath and TLC of Aseel matches with that of Indigenous breed from Iran (Abdi-Hachesoo et al., 2011). Nigerian local chicken (Isidahomen et al., 2011) reflects similarity with this study with reference to TEC, MCV, MCH, and MCHC as

both are indigenous breeds belonging to tropical countries. Kadaknath and Aseel show similar haemoglobin, TEC, MCV, and MCH with that of haematological profile of Aseel and Kadaknath reported by Pandian et al. (2012). TEC of Assel reflects same results with that of Assel reared in Sylhet region of Bangladesh (Islam et al., 2004).

Table 2 Comparative dimensions of erythrocytes and lymphocytes of Indian breeds of chicken

SL NO	PARAMETERS	ASEEL		KADAKNATH		ANOVA (F-value)
		MALE (30)	FEMALE (30)	MALE (30)	FEMALE (30)	
1	Length of RBC (µm)	10.34 ± 0.16^{a}	9.84 ± 0.15^{b}	$10.63\pm0.14^{b,c}$	$9.52\pm0.13^{a,c}$	11.00**
2	Length of Lymphocyte (µm)	8.92 ± 0.96	8.26 ± 0.23^{a}	9.18 ± 0.27^{a}	9.08 ± 0.17	2.96*

Figures in parentheses reflect number of observations; Mean ± SE bearing similar alphabetical superscripts differ in rows at p<0.01 and p<0.05***

Length of erythrocytes differs significantly at $p<0.01$ between male Aseel and female Kadaknath, female Aseel and male Kadaknath and among hens and cocks of kadaknath breed. Similarly, length of lymphocytes reflects difference at $p<0.05$ between female Aseel and male Kadaknath (Table 2). The size of avian erythrocyte differs from species to species but they generally range between 10.7×6.1µm to 15.8×10.2µm (Sturkie and Griminger, 1986). The present study is in accordance with this range. Length of RBC matches with the work of Smith and Hattingh (1979). Lengths of lymphocytes of these adult birds are in accordance with the work of Tadjalli et al. (2003).

Present study focuses on haemocytomorphometry of two significant breeds of farmed chickens namely Aseel and Kadaknath reared in well managed farm conditions. Specific factors such as sexual dimorphism, age, breeding, egg laying and environment affect the blood profile.

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