Chapter I: Introduction

Snakes are a group of amazing reptiles which have fascinated human being since beginning of mankind. This elongated, cylindrical, legless vertebrates belonging to the order serpents under the class reptilia. Its body is covered by scales and has no shoulder girdle and limbs **(Hsiang, Field et al. 2015)**. Snakes are an important parameter of our ecosystem and have tremendous importance in biopharmaceutical research, that been ignored for proper recording possibly due to the danger and panic of handling of this mysterious reptiles. There are about 3000 different snake species described on earth, of these, only about 350 are regarded as poisonous and dangerous for humans. Most snakes are non-poisonous and harmless to human being **(Roly, Hakim et al. 2015)**. Bangladesh, being located in the humid tropical region is very rich in species diversity both for flora and fauna. According to the report funded by UNDP, IUCN has reported that the country has about 125 species of reptiles and 22 species of amphibians **(Khan 1987)**. There are about 90 species of snakes in Bangladesh among which only one fourth are venomous. Poisonous species of snakes have some kinds of poison or venom glands and poison fangs. Also they must have the mechanism to pour or push the poison from the venom gland through the fangs into the body of a prey or a victim **(Lu, Clemetson et al. 2005)**. Amid of venomous snakes cobras are most dangerous and commonly found in Bangladesh. Two species of cobra, the spectacled/binocellate cobra (khoia or khodom paia gokhra), *Naja naja* and monocled/monocellate cobra (gokhra), *Naja kaouthia* occur in Bangladesh. Cobras belonging to the family Elapidae and *Naja naja* were first described by Swedish physician, zoologist, and botanist Carl Linnaeus in 1758 **(Bonato and Minelli 2014)**. The cobra is a moderately sized, heavy bodied species. This cobra species can easily be identified by its relatively large and quite impressive hood, which it expands when threatened. This species has a head which is elliptical, depressed, and very slightly distinct from neck. The snout is short and rounded with large nostrils. The eyes are medium in size and the pupils are round. The majority of adult specimens range from

1 to 1.5 meters (3.3 to 4.9 ft) in length **(Pandey 2012)**. Cobras are now a threatened species in Bangladesh. The monocellate cobra is particularly vulnerable, and the binocellate cobra and the king cobra are endangered snakes **(Bangladesh 2000)**. It is estimated that about 1600 people die every year of snakebite in the country. Cobra bites are associated with neuromuscular paralysis; necrosis of soft tissues; and secondary bacterial complication has also been reported.

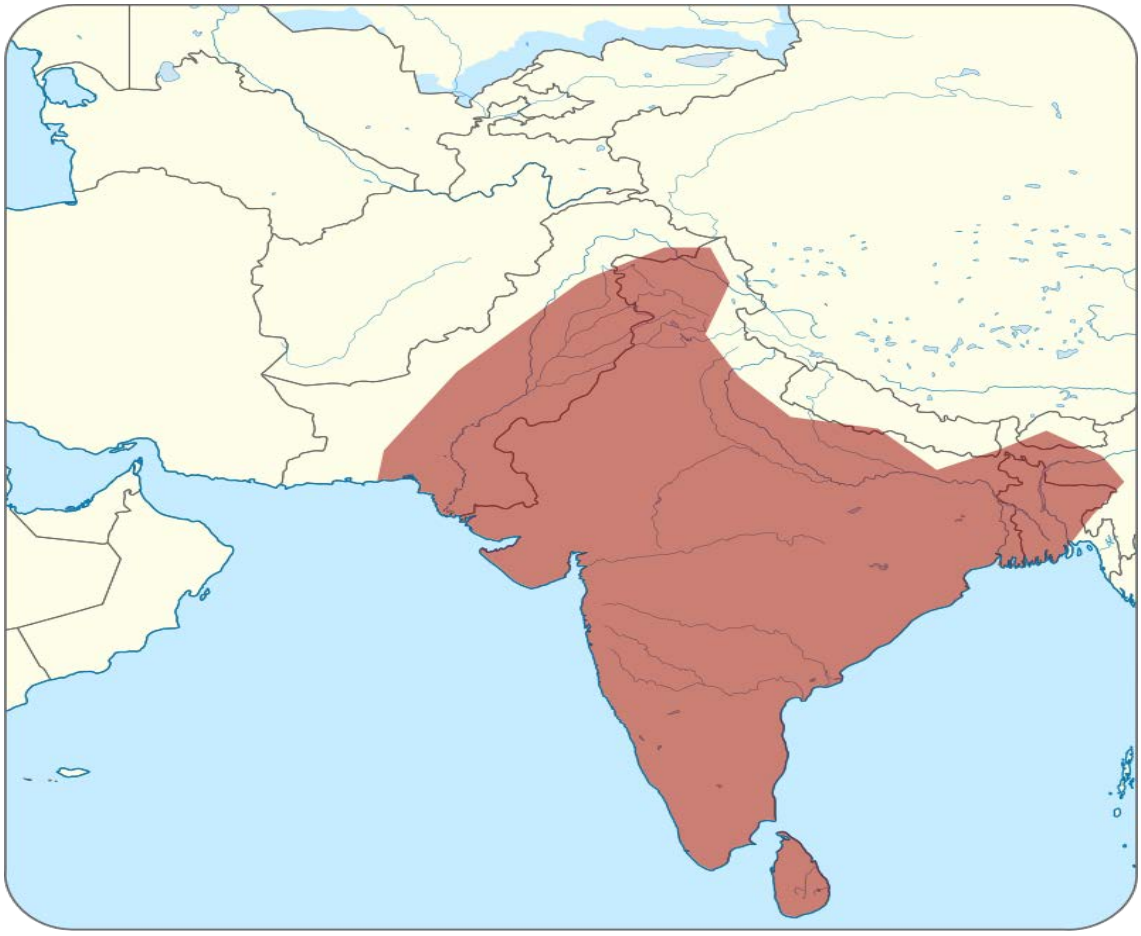


Fig: Distribution of *Naja Naja*

Recently studies using reptiles have increased in the areas of infectious disease, comparative anatomical physiology, the evaluation of phylogenic relations with birds and other vertebrates, stem cell experiments, and therapeutic drug development **(Weissman, Noctor et al. 2003, Handrigan, Leung et al. 2010)**. As medicinal applications of venom

increase, its risk to human health should be examined. Even though snake health can be affected by bacteria, there have not been many studies of the distribution of bacteria in snakes or the influence of predominant bacteria in snakes. Some bacteria not only affect snakes but also affect humans there have not been many studies of the distribution of bacteria in snakes or the influence of predominant bacteria in snakes **(Jho, Park et al. 2011)**. Venomous snakes may also harbor a wide range of bacteria in their oral cavity able to complicate the bite wounds **(Dipineto, Russo et al. 2014)** so the information on their micro flora should not be neglected. Secondary bacterial infections, such as subcutaneous abscess or tetanus, are possible complications of snake bites, either venomous or non-venomous snakes **(Habib 2003, Garg, Sujatha et al. 2009)**. In addition to bites, humans can be infected during manipulation of animals or via infected equipment **(Foster and Kerr 2005)**. The popularity of snakes as pets and the models of biological and veterinary research increased the risk for a public health due to the zoonotic potential of these animals **(Lukač, Horvatek Tomić et al. 2017)**.

Studies on oral of snakes are rare in Bangladesh. There is no information regarding micro flora and potential pathogens isolation from oral and cloaca of spectacled cobra (*Naja naja*) snakes. This present study was designated to isolate opportunistic *Salmonella spp.* and *E. coli* from oral and cloacal swab of binocellate cobras (*Naja naja*) that are reared by the snake charmer's. The main objective of this study was seen the relationship of positive cases with the collected sample area, age of and gender of the snake.

Chapter II: Materials and Methods

2.1. Study area and duration

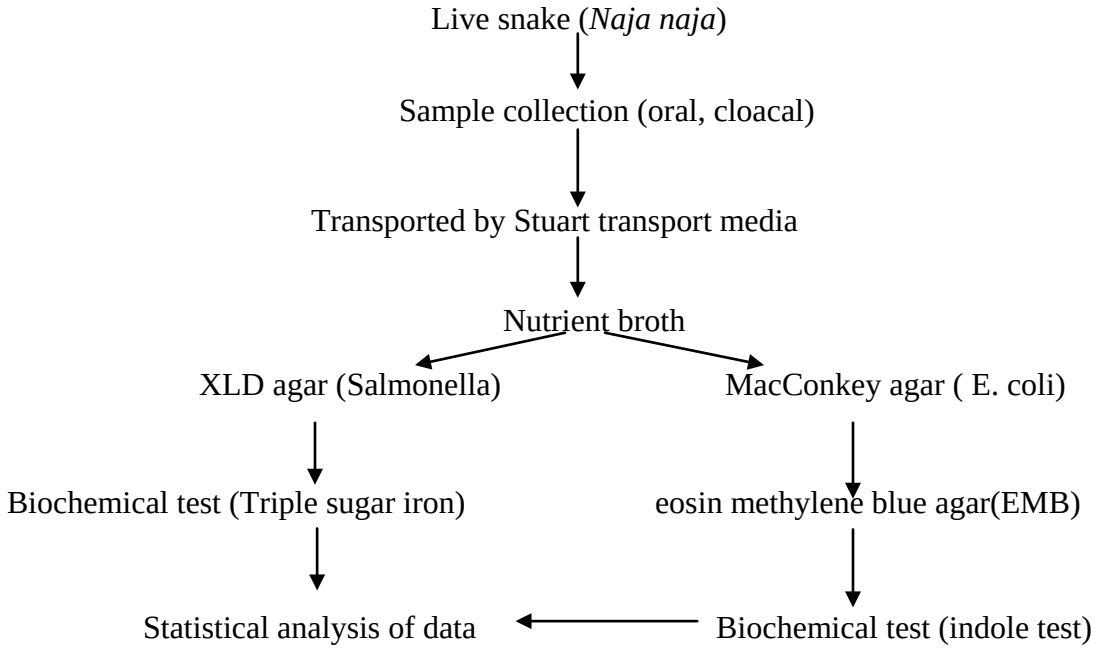
The study was conducted over Mymensingh and Sherpur district of Mymensingh division. The time period of study is march-2017 to june-2017 and it does involve from sample collection to laboratory isolation of *Salmonella* and *E. coli*

2.2. Study population

The selected population was those snakes which are reared by snake charmer for their earning purpose and for recreational purpose. They mainly reared various type of local snakes most of them are binocellate cobra (*Naja naja*). The total snake population is 26 of different body weight, length and age.

2.3. Study Design

This study was done by using the following study design:



2.4. Collection of sample

Swab sample were taken from oral cavity and cloaca of live snakes. Oral swab are mainly taken from around the flank region and upper jaw (figure 3). The cloacal swabs are directly collected from cloaca by inserting the cotton bar then suspending in 5 ml transport media within a falcon tube (figure 2). At the mean time a developed questionnaire was filled up for each and every sample regarding with information of body weight, length, age, gender, management system, clinical status etc. Those samples were brought to the laboratory for isolation of *Salmonella* and *E. coli.*

Figure-1: Collection of data

Figure-2: Collection of cloacal swab

Figure-3: Collection of oral swab

2.5. Sample collected area

Area	No of Samples
Mymensing	16
Sherpur	10
Total	26

Table-1: distribution of sample

2.6. Pre-enrichment of sample at nutrient broth medium

The collected sample were than subculture at nutrient broth which are prepared by dissolving 25gms powder in one litter distilled water and dissolve the medium completely by heating then sterilize it by autoclaving at 10 lbs pressure (1150C) or alternatively at 15 lb pressure(1210C)for 15 minutes or as per validated cycle. After sterilization pH of the medium should be 7.3±0.1.Normally each litter of nutrient broth medium contains 10gms peptone, 10gms beef extract and 5gms sodium chloride. Beef extract and peptone provide the necessary nitrogen compounds, carbon, vitamins and also some trace ingredients to the non-fastidious organism and sodium chloride maintain osmotic equilibrium of the medium. After giving subculture inoculation in nutrient broth, it was incubated at 35-37°C for 24 hours.

2.7. Isolation of *Salmonella*

2.7.1. Isolation of *Salmonella* in xylose lysine deoxycholate (XLD) agar

For the isolation of Salmonella at first the subculture inoculums from nutrient broth are streaked in the xylose lysine deoxycholate agar which is composed of lactose 7.5gm, sucrose 7.5gm, sodium thiosulfate 6.8gm, l-lysine 5.0gm, sodium chloride 5.0gm, xylose 3.75gm, yeast extract 3.0gm, sodium deoxycholate 2.5gm, ferric ammonium citrate 0.8gm, phenol red 0.8gm, agar 15gm, per liter of deionized water. XLD Agar is both a selective and differential medium. It contains yeast extract as a source of nutrients and vitamins. It utilizes sodium deoxycholate as the selective agent and, therefore, is inhibitory to gram-positive micro-organisms. Xylose is incorporated into the medium

since it is fermented by practically all enteric except for the *Shigella* and this property enables the differentiation of *Shigella* species. Lysine is included to enable the Salmonella group to be differentiated from the non pathogens since without lysine, salmonellae rapidly would ferment the xylose and be indistinguishable from non-pathogenic species. After the salmonellae exhaust the supply of xylose, the lysine is attacked via the enzyme lysine decarboxylase, with reversion to an alkaline pH which mimics the *Shigella* reaction.

To prevent similar reversion by lysine positive coliforms, lactose and sucrose are added to produce acid in excess. Degradation of xylose, lactose and sucrose to acid causes phenol red indicator to change its color to yellow. Bacteria that decarboxylate lysine to cadaverine can be recognized by the appearance of a red coloration around the colonies due to an increase in pH. These reactions can proceed simultaneously or successively, and this may cause the pH indicator to exhibit various shades of color or it may change its color from yellow to red on prolonged incubation.

To add to the differentiating ability of the formulation, an H2S indicator system, consisting of sodium thiosulfate and ferric ammonium citrate, is included for the visualization of the hydrogen sulfide produced, resulting in the formation of colonies with black centers. The non pathogenic H2S producers do not decarboxylate lysine; therefore, the acid reaction produced by them prevents the blackening of the colonies which takes place only at neutral or alkaline pH. The suspected Salmonella colony then transferred to nutrient broth medium and incubated at 35-37°C for 24 hours.

2.7.2. Final identification of *Salmonella* through biochemical (Triple Super Iron) test

Finally the presence of Salmonella organisms in sample was confirmed by biochemical test in Triple Sugar Iron media. In *Salmonella* positive case the slants of the media become red, button become yellow and gas was formed, blackish appearance may indicate the production of hydrogen sulphide.

2.8. Isolation of *E. coli*

2.8.1. Isolation of *E. coli* in MacConkey agar

On the other hand for isolation of *E. coli* the initial pre-enriched nutrient broth inoculums are streaked into the MacConkey agar which is used for the isolation of gram-negative enteric bacteria and the differentiation of lactose fermenting from lactose non-fermenting gram-negative bacteria. It has also become common to use the media to differentiate bacteria by their abilities to ferment sugars other than lactose. MacConkey agar is a selective and differential media used for the isolation and differentiation of non-fastidious gram-negative rods, particularly members of the family Enterobacteriaceae. It contains Peptone 17.0gm, Lactose 10.0gm, Sodium Chloride 5.0gm, Proteose Peptone 3.0gm, Bile Salts 1.5gm, Neutral Red 30.0mg, Crystal Violet 1.0mg,Agar 13.5gm in per liter of deionized water. E. coli growing on the media is differentiated by their ability to ferment the sugar lactose. Fermentation of lactose cause the pH of the media to drop and the resultant change in pH is detected by neutral red, which is red in color at pH below 6.8. As the pH drops, neutral red is absorbed by the bacteria, which appear as bright pink to red colonies on the agar. Gram-negative bacteria that grow on MacConkey agar but do not ferment lactose appear colorless on the medium and the agar surrounding the bacteria remains relatively transparent. The suspected E. coli colonies then transferred to nutrient broth medium and incubated at 35-37°C for 24 hours. In this case there is no bright pint to red color colonies. So it indicates that there is absence of *E. coli* in the sample.

2.9. Data Analysis

All the data that were collected (categorical variables like area, age, sex etc.) were entered into MS excel (Microsoft office excel-2007, USA). Data management and data analysis were done by STATA version-12.1 (STATA Corporation, College Station, Texus, USA). Descriptive analysis was done by means of creating histogram, line graph . To identify the association between a categorical explanatory variable with the outcome, chi- square (χ^2 test) test was performed. An association was regarded as significant if the p value was <0.05

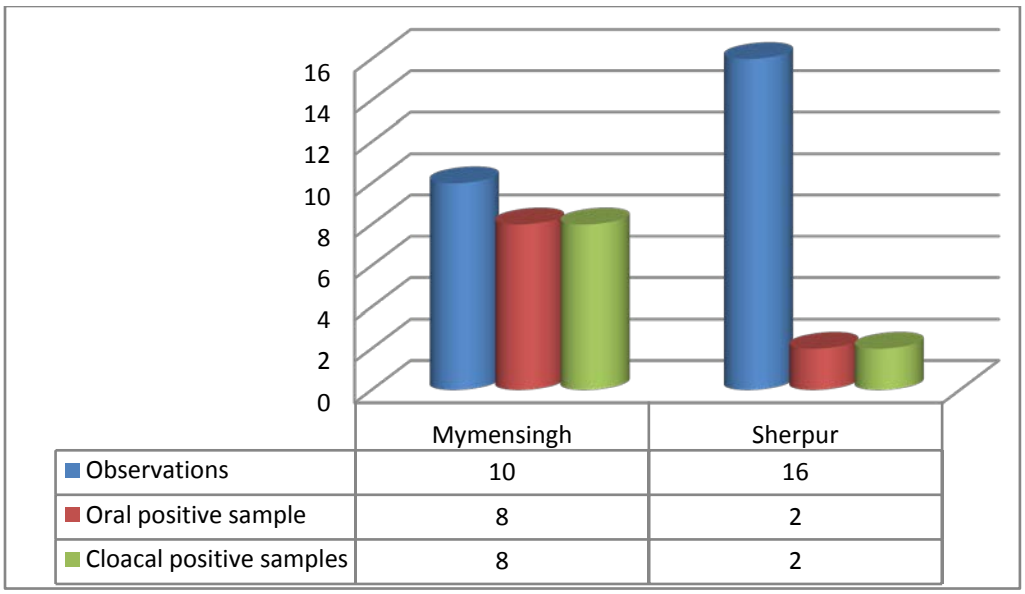
Chapter II: RESULTS

3.1. Proportion of *Salmonella* positive case in relation to the study area

Variables	Category	Number of observation (n)	Salmonella positive (n) (%)		Chi-square value	P-value
			Oral	Cloacal		
Area	Mymensingh	10	2 (20%)	2 (20%)	4.46	0.27
	Sherpur	16	8 (50%)	8 (50%)	4.46	0.27

Table-2: Proportion of *Salmonella* positive case in relation to the area of population

The study was designed to show the number of *Salmonella spp.* and *E. coli* positive samples taken from the Mymensingh and Sherpur district under Mymensing division. The result was in respect of area of sampling states that, among the 10 samples 20% cases found positive for *Salmonella spp.* both in oral and clocal swab that are collected from Mymensingh with the p- value of 0.27. On the other hand 8(50%) cases were found positive for *Salmonella spp.* both in oral and clocal swab with the p value of 0.27 in the samples of Sherpur district **Graph 1**.



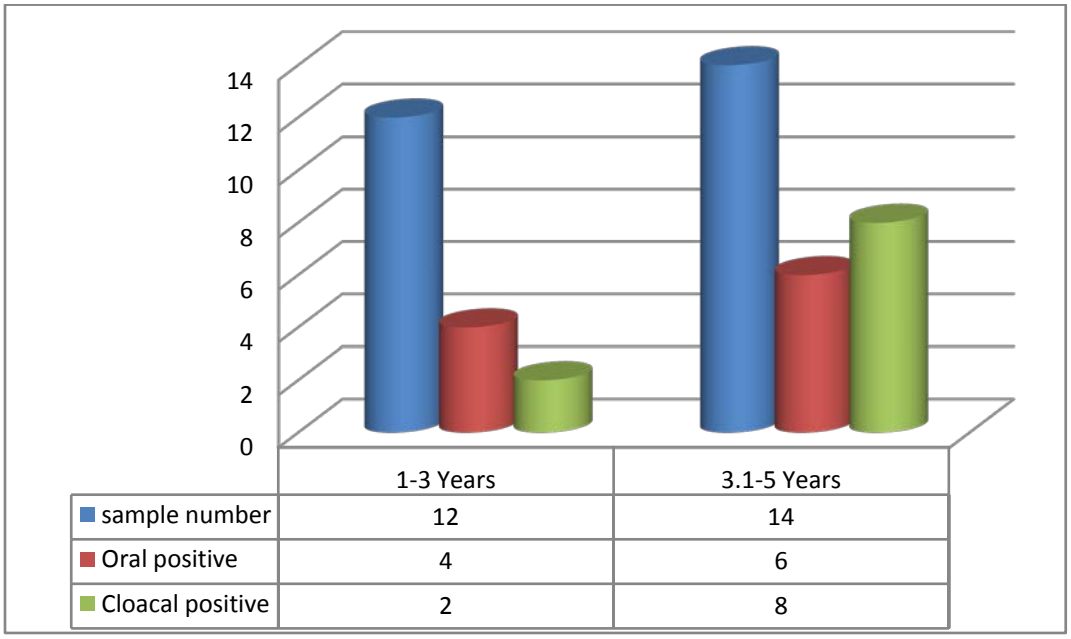
Graph 1: Proportion of *Salmonella* positive case in relation to the area of population

3.2. Proportion of *Salmonella* positive case in relation to age of population

Variables	Category	Number of observation (n)	Salmonella positive (n) (%)		Chi-square value	P-value
			Oral	Cloacal		
Age	(1-3) y	12	4(33.33%)	2(16.67%)	0.247	0.619
	(>3-5) y	14	6(42.86%)	8(57.14%)	4.47	0.03

Table-3: Proportion of *Salmonella* positive case in relation to the age of population

Oral and clocal samples taken from the snakes aged from (1-3) years and (>3-5) years of age. Total 12 samples collected from snakes aged between (1-3) from which 4(33.33%) oral and 2(16.67%) cloacal samples were positive for with the p-value of 0.619. Whereas 6(42.86%) oral and 8(57.14%) cloacal samples were found positive for with the statistically significant p-value of 0.03; among 14 samples that are collected from the snakes aged from (>3-5). Results are graphically represented below **Graph 2.**



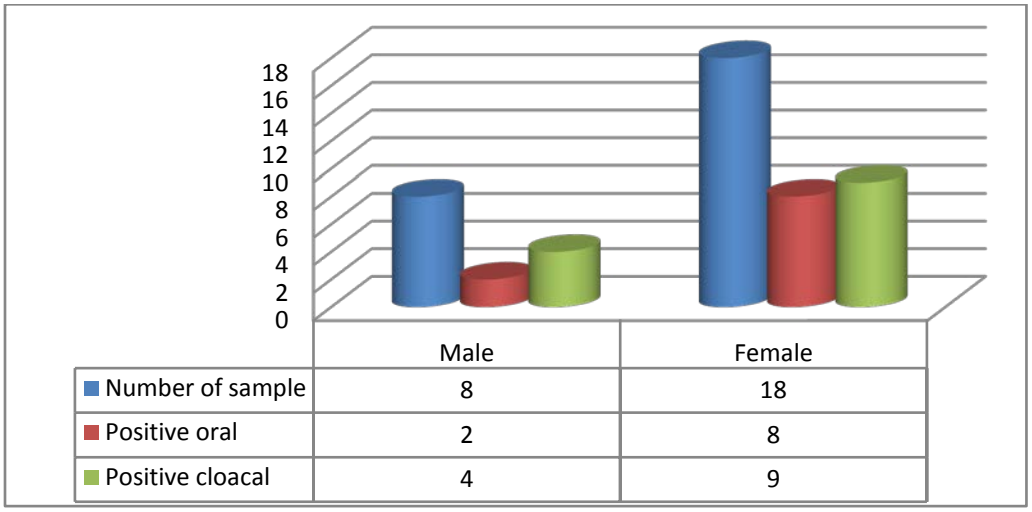
Graph 2: Proportion of *Salmonella* positive case in relation to the age of population

3.3. Proportion of *Salmonella* positive case in relation to gender of population

Variables	Category	Number of observation (n)	Salmonella positive (n) (%)		Chi square value	P-value
			oral	cloacal		
Length	Male	8	2(25%)	4(50%)	0.884	0.347
	Female	18	8(44.4 %)	9(33.3%)	0.650	0.420

Table-4: Proportion of *Salmonella* positive case in relation to the gender of population

Total samples (n=26) were collected from both the sex (male: 8 and female: 18). The result was in respect of gender of snake's shows that; oral 2(25%) and cloacal 4(50%) samples from male were positive for *Salmonella spp* with the p-value of 0.347. In female 8(44.4%) oral and 9(33.3%) cloacal sampes were found *Salmonella spp* positive with p-value of 0.420. The results are explained in the **Graph 3**



Graph 3: Proportion of *Salmonella* positive case in relation to the gender of population

Oral and cloacal samples collected from different area, age and sex were also culture in the differential media for isolation and identification of *E. coli*. But *E. coli* was negative in all the samples.

Chapter IV: Discussion

The binocellate cobras (*Naja naja*) are one of the most dangerous venomous snakes that are found in Bangladesh. In Bangladesh binocellate snakes are now endangered **(Bangladesh 2000)**. Large number of binocellate cobra reared by the snake charmer's of Mymensing and Sherpur district.

The present study was planned to isolate the *Salmonella spp.* and *E. coli* from oral and cloacal sample that are taken from the snakes of Mymensing and Sherpur district of Mymensing division. From total 26 snakes, bacteria were isolated from 10 oral and 10 cloacal swabs. In all the positive samples *Salmonella spp.* was isolated but *E. coli* were not found in any case. This findings is not coincides with the result of Lukač, Horvatek Tomić et al. 2017; who isolated many gram negative organisms except *Salmonella spp.* from the oral and cloacal sample of snake.

No significant differences between two districts in the number or type of bacteria are noted. This gram negative opportunistic micro flora has already been described as a potential cause of disease in animals and human **(Harris and Rogers 2001, Chen, Wu et al. 2011)**.

Samples were collected from two different age groups (1-<3) and (>3-5) years. Significant variation (p=0.03) were observed among these two age group. All the samples both oral and cloacal are positive for Salmonella that were collected from the snakes older than 3 years where 50% samples found positive for *Salmonella spp*. that are collected from younger than 3 years of old. No study was found relating the age group of isolation of bacteria.

Salmonella spp. were isolated from oral and cloacal swabs of both male 17(n=18) and female 6(n=8) samples. No significant variation was noted regarding the isolation of bacteria among the male and female snakes. Authentic study was not found relating to variation of the bacterial isolation with gender.

All the samples are collected from the snakes that are reared by the snake charmer's. They often handle their snake without caution. This may increase the danger of contracting zoonotic disease responsible for bacterial infection **(Tu, Zeitlin et al. 2004)**. In addition to secondary infection arising from bacteria coming from the snake's mouth inoculated at the time of a bite. Bacteria are also responsible for snake bite associated wound infection **(Garg, Sujatha et al. 2009)**

4.1. Limitation

The study has some constraints. The sample size was not sufficient to do the appropriate statistical analysis. The *Salmonella spp.* was not confirmed by molecular technique like polymer chain reaction (PCR).

Chapter V: Conclusion

In conclusion, the results of this study indicate that binocellate cobras from Mymensingh and Sherpur district of Mymensingh division harbor gram negative opportunistic *Samonella spp.* organism. This organism has already been described as causes of infection in both reptiles and humans. To the author's knowledge this is the first survey of oral cavity and cloacal micro flora of binocellate cobras in Bangladesh.

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Author

October-2017

Biography

This is Ajoy dev nath, son of Sudher deb nath and Sabita devi. I am the dweller of Cox's Bazaar. I completed S.S.C in 2009 and H.S.C in 2011. I got admitted in doctor of veterinary medicine course under Chittagong veterinary and animal sciences university in 2011-2012 sessions. During internship program, I got the opportunity to make a clinical report on the isolation of salmonella and e. coli from the oral and cloacal swab of binocellate cobra under supervision of lecturer Dr. Pranab Paul, Dept of Medicine and Surgery. I am enthusiastic to be a researcher on microbiology and want to be a a skilled wildlife in future.

PICTURE GALLERY

FIGURE: BINOCELLATE COBRA

FIGURE: HANDELING OF SNAKE

FIGURE: BINOCELLATE MARK IN THE HOOD

FIGURE: salmonella on TSI

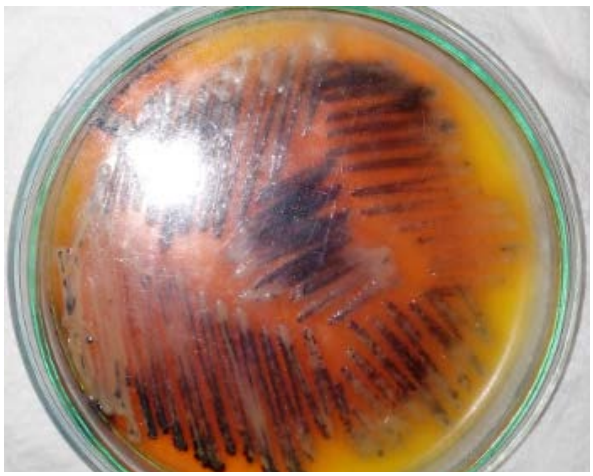

FIGURE: salmonella on XLD

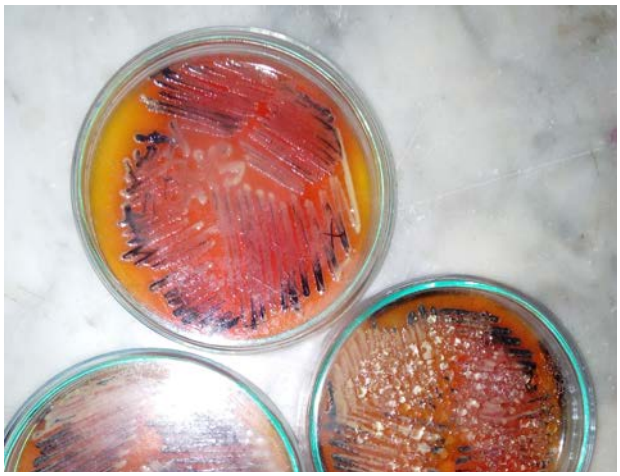

FIGURE: salmonella on XLD