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Detection and antibiotic sensitivity of bacteria isolated from tiger and lion in Dhaka Zoo

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ABSTRACT

The present study was conducted to isolate, identify and characterize the bacterial pathogens present in feces of tiger and lion of Dhaka Zoo and their antimicrobial activities during the period from July to December 2013. 42 (twice from 6 tiger and 15 lion) faecal samples were collected from the cages of animals just after defecation and brought to the Microbiology laboratory of the Department of Microbiology, Hajee Mohammad Danesh Science and Technology Universiy, Dinajpur for bacteriological examination. Isolation and identification of the microorganisms were performed by their morphology on different cultural media, staining characteristics and biochemical tests. Then, the isolated bacteria were characterized by antimicrobial susceptibility analyzed by the online software ABIS Encyclopedis/tgw1916, 2014 and detected by polymerase chain reaction (PCR) for molecular detection of *E. coli*, *Salmonella and Staphylococcus* sp. Out of 42 samples 17 (40.47%) samples were positive for *E. coli*, 15 (35.71%) samples were positive for *Salmonella* sp, 18 (42.85%) samples were positive for *Staphylococcus* sp. The antibiogram study revealed that most of the *E. coli*, *Salmonella* spp. and *Staphylococcus* spp. were resistant to Doxycycline, Norfloxacin, Ciprofloxacin, Neomycin, Enrofloxacin, Ciprofloxacin, Amoxicillin, Levofloxacin and Tetracycline. However, most of the *E. coli*, *Salmonella* sp. and *Staphylococcus* sp. were susceptible to Neomycin, Gentamicin and Colistine sulphate which indicate that the use of these antimicrobial may be chosen in clinical control of *Salmonella*, *E. coli*, and *Staphylococcus* sp infection. PCR results confirmed 7 (3 *E. coli* and 4 *Salmonella*) isolates out of 11 in tiger and lion. Most of the isolates have zoonotic importance and confirmed the presence of either commensal or pathogenic for tiger and lion in zoo.

Introduction

Zoo provides opportunities to see and interact with animals' experience and exposure to animals can be beneficial as educating the public about animals, pondering over an understanding of the human-animal bond, conserve and save the animals. The tiger and lions are the most count upon extinction-conservation topics such as saving the Royal Bengal tigers also lion has susceptible potentials for transmission of infectious diseases such as outbreaks of diseases like colibacilosis, salmonellosis staphylococcosis, cryptosporidiosis, and dermatomycosis (ringworm), etc. Most of the infections of concern associated with petting zoos spread via the fecal-oral route, meaning the bacteria or parasites are shed in the feces (stool or manure) of an animal and transmitted to people (or other animals) who swallow them. This usually happens when people get fecal contamination on their hands, which is then easily transferred to the mouth (Taylor et al., 2001). Tiger and lions are often suffered from many bacterial diseases with often involvement of normal flora or environmental pathogens in response to stress and immunosuppression. *Escherichia coli* and *Salmonella sp* is a facultative anaerobic, non-spore forming rods both are motile and Gram negative in Family Enterobactereaceae. *Staphylococcus sp* and *Streptococcus sp* is a Gram-positive Cocci in Chains or Pairs, Catalase positive. Most of these bacteria have the antimicrobial resistance because of repeated exposure due to unhygienic condition and lack of proper knowledge of health monitoring of caged animal as well as feeding with meat may also found due to improper uses of antibiotics to zoo animals are primary causes of the increase drug-resistant bacteria. As a result, the bacteria are growing resistant to antibiotic owing to improper use. It is, therefore, important that sensitivity of different bacteria isolated from diarrhoeic tiger, lions needs to be studied from time to time in order to formulate appropriate therapeutic measures (Kaura et al., 1988). Subsequently more powerful and/or new antibiotic is being needed to deal with the altered bacterial population. In this way the bacteria become more and more resistant to antibiotics and new generation of antibiotics are being developed.

Escherichia coli (*E. coli*) is a Gram-negative, rod-shaped bacterium that is usually found in the lower intestine of warm-blooded organisms (Singleton,

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1999; Ali and Sultana, 2013). Most *E. coli* strains are harmless, but some serotypes can cause enterohaemorrhagic, enteroadhessive disorders, serious food poisoning in humans, and are occasionally responsible for product recalls due to food contamination (Vogt et al., 2005). The harmless strains of the bacterium are part of the normal flora of the gut, and can benefit their hosts by producing vitamin K2, and by preventing the establishment of pathogenic bacteria within the intestine (Bentley et al., 1982). Gastrointestinal upsets should be investigated as being caused by diet, infectious agents (Salmonella spp., Shigella spp, E.coli, Clostridium), or concurrent kidney failure. Systemic bacterial diseases have been seen in captive tigers such as Colisepticemia (Sathyanarayana et. al., 1983), Escheriasis (Hasina, 2006), Shigella flexneri (Zaki, 1980), Salmonella spp. (Kloss and Lang, 1976).Considering the above points the present study was undertaken to isolate and identify the bacterial pathogens from fecal sample of Zoo animal (Tiger and Lions) and their antibacterial sensitivity.

Materials and Methods

Collection and transportation of sample: A total number of 42(6 tigers and 15 lions) fecal samples were aseptically collected from tigers and lions from Dhaka Zoo for isolation, identification and molecular characterization of bacterial pathogens and carried to the Microbiology laboratory of the Department of Microbiology, Hajee Mohammad Danesh Science and Technology Universiy, Dinajpur. Isolation and identification of bacterial pathogens were performed as per procedures described by Marchant and Packer (1967) and Cowan (1985). Feces samples were collected on 1% buffered peptone water then carried with proper cool chain maintenance. Just after arrival of sample pre-enrichment was done at 37-41 ºC for 4 hours then cultivated at specific media further needed for exact isolation and identification of all suspected bacterial pathogens.

Isolation of bacteria

After collection, the samples of tigers and lions were grown in the recently prepared nutrient broth at 37^0C for 24 hours. Then overnight bacterial broths were streaked on SS agar, Brilliant Green Agar (BGA) (for *Salmonella*), EMB (for *E. coli*), Mannitol salt agar (for *Staphylococcus*) which were incubated at 37^0C for 24 hours.

Identification of bacteria

Identification of bacteria was performed on the basis of colony morphology, Gram's staining technique, Indole Urease (MIU) test, Motility test, Carbohydrate fermentation test (e.g. dextrose, sucrose, maltose, lactose and mannitol), Reaction of the organisms in TSI agar slant, Simmons citrate agar utilization test, indole test Voges-Proskauer test, Methyl Red (MR) test, oxidase and catalase.

Molecular Detection of bacteria: Fresh and pure colonies were selected for the purpose of extraction of bacterial DNA components and for characterization by PCR (Table 1 & 2).

Table 1: Primer used for amplification of target sequence of *E. coli* isolated from tiger and lion

Primer	Sequence (5`to 3`)	Target size	
Fw ECO-1	GACCTCGGTTTAGTTCACAGA	585 bp	Wang et al., (1996)
Rv ECO-2	CACACGCTGACGCTGACCA	585 bp	Wang et al. (1996)

Table 2: Primer used for amplification of target sequence of **Salmonella** isolated from tiger and lion

Primers	Sequence 5' to 3'	Target size	Reference
Fw -oligonucleotides	ACTGGCGTTATCCCTTTCTCTGGTG	496 bp	Noah et al (1993)
Rv- oligonucleotides	ATGTTGTCCTGCCCCTGGTAAGAGA	496 bp	Noah et al (1993)

Use of antibacterial discs

Fourteen different antibacterial discs (CARTIDGE DISC), manufactered by MAST DIAGNOSTICS, Merseyside, U.K, (Lot Ch. B-111888) were selected for the antibacterial sensitivity study against isolated *E.coli*, *Salmonella* and *Staphylococcus spp*. The antibacterial agents were- (Doxycycline, Amoxycillin, Colistin sulphate, Pefloxacin, Neomycin, Cephalexin, Gentamicin, Ciprofloxacin, Norfloxacin, Tetracycline, Levofloxacillin, Enrofloxacin, Ampicillin) (Table 3). These are

commonly used in the field condition for the treatment of Salmonellosis and Colibacillosis.

Table 3: Antibacterial agents and their discs concentrations

Antimicrobial agent (Discs)	Disc concentration
Doxycycline	30µg
Amoxycillin	10µg
Ampicillin	10µg
Pefloxacin	5 µg
Gentamicin	120µg
Enrofloxacin	5 µg
Colistine sulphate	10 µg
Ciprofloxacin	5 µg
Cephalexin	30 µg
Erythromycin	15 µg
Tetracycline	30 µg
Levofloxacin	5 µg
Neomycin	30µg
Norfloxacin	10µg

Antibacterial sensitivity pattern of the isolated *Salmonella spp* and *E. coli*

Susceptibility of isolates of the *Salmonella spp and E. coli* to different antimicrobial agents was performed to determine the drug sensitivity pattern and designated as highly sensitive ≤24, moderately sensitive≥20, less sensitive ≤15 and resistant ≥10. (Table 4).

Results

The result described the recovery of *E. coli*, *Staphylococcus and Salmonella sp* isolates of fecal samples collected from 6 Tigers and 15 Lions of Dhaka Zoo. *Escherichia coli* produce specific metallic sheen (greenish-black colonies) on EMB agar (Figure 3A,B). *Salmonella sp* produce yellowish colonies on XLD agar (Figure 1C,D) and Pinkish circular small colony on SS agar (Figure 2A). *Staphylococcus sp* produce smooth, circular, dew drop like yellowish- white colony on Mannitol Salt agar (Figure 1B).

Gram's staining

In Gram's staining and observation under compound light microscope the organism revealed Gram-negative character with red as well as the morphology small bacilli shaped arranged in single or paired which is the characteristics of *E. coli and Salmonella* gram negative very short plump rod. Gram positive character with violate colour as well as the morphology Cocci in change or pair which is the characteristics of *Staphylococcus* sp (Table 4).

Biochemical tests

Presumptively selected colonies were repeatedly streaked on the respective selected EMB agar media to check and confirm their purity. For identification, a series of biochemical tests especially for *E. coli, Salmonella and staphylococcus* as follows were performed (Table 5).

Sugar fermentation

Carbohydrate fermentation positive,dextrose, lactose and maltose of *E.coli, Salmonella, staphylococcus sp* .Some isolates produce of good amount of acid and gas while other hand small amount which was exhibited by colour change and gas production in Durhams tube. *E. coli* produce acid and gas and colour change from reddish to yellow and gas production noted gas bubble in inverted Durhams tube. *Salmonella* produce no acid or gas (Figure 4 A, B).

Other biochemical tests

Both types of the isolates *(E. coli and Salmonella spp)* were MR positive and VP negative. *E. coli* was indole positive and *Salmonella spp* were indole negative.Oxidase *E. coli* positive and *Salmonella* negative.Catalase positive *staphyloccus*. (Figure 4 B; 5A,B).

Table 4: Characterization of isolated bacterial pathogens from fecal sample of tiger and lion by Gram's staining technique

Gram's Staining			**Identification**
Shape	**Arrangement**	**Gram's staining reaction (+/-)**	
Short plump rods	Single, paired or in short chain	Gram negative	*E.coli*
Very short plump rods	Single	Gram negative	*Salmonella* spp
Cocci arranged	grape- like clusters	Gram-positive	*Staphylococcus* spp.

Table 5: Characterization of isolated bacterial pathogens by cultural properties

Name of Culture media used	***E. Coli***	***Salmonella* spp.**	***Staphyloccous spp.***
Nutrient agar	Smooth, circular, white to grayish colony with peculiar fetid odour	Small, round and smooth Colony	growth of circular, small smooth, convex, and golden yellowish colonies
Blood agar	Produce haemolysis	Produce haemolysis	Produce haemolysis
Staphyloccous agar	No growth (-)	No growth (-)	Yellowish color colony
Mac Conkey agar	Rose pink lactose fermenter colony	Colourless, pale, translucent colony.	No growth (-)
Eosin-Methylene Blue (EMB) agar	Moist circular colonies with dark centers yellow green metallic sheen	No growth (-)	No growth (-)
Salmonella- Shigella (SS) agar	Pink colour colony	Translucent colourless smooth Colony	No growth (-)

Table 6: Characterization by biochemical reactions of *E. coli*, *Salmonella* spp. and *Staphylococcus* spp.

Isolated organism	Indole production test	Methyl-red test	Voges-Poskauer reaction	Citrate utilization test	MIU test	TSI Test	Hydrogen sulphide
E.coli	+	+	_	_	All +	Butt-Y; Slant-Y	-
Salmonella spp.	_	+	_	_	+	Butt-Y; Slant-Y	+
Staphylococcus spp.	_	+	_	_	_	Butt-Y; Slant-Y	+

D = Differential biochemical types; + = Positive reaction; += Negative reaction

Table 7: Result of antibiotic sensitivity test of various isolates of *E. coli* and *salmonella spp*. from tiger and lion

Species	Name of organism	Total number of isolates	Antibiotic disc use									
			Do	AML	LEV	CIP	TE	NOR	N	CL	CEF	CN
Tiger	*E. coli*	5	+	+	-	-	-	-	+++	+++	+++	+++
	salmonella	4	+	-	+	-	-	-	-	+++	-	+++
Lion	*E. coli*	12	+	+	-	-	-	-	+++	+++	++	+++
	salmonella	11	+	-	-	-	-	+	-	+++	-	+++

Legends: DO= Doxycycline; AML= Amoxycillin; LEV= Levofloxacillin; CIP= Ciprofloxacin; TE= Tetracycline; NOR= Norfloxacin; N= Neomycine; CL= Clostine Sulphate; CEF= Cefixime; CN= Gentamicin
+ = Less sensitive; - = Resistance; ++ = Modarately sensitive; +++ = Highly sensitive

Table 8: Isolation, molecular characterization and antibiotic sensitivity-susceptibility of *E. coli*, *Salmonella* and *Staphylococcus* sp in tiger and lion of Dhaka Zoo

Bacteria /Animal	Isolation of Bacterial pathogen				No of PCR +ve	Antimicrobial resistant test	
	No of sample culture	% of isolation *E. coli*	% of isolation *Salmonella*	% of isolation *Staphylococcus*		% Resistant	% Susceptible
Tiger	12	41.67(5/12)	33.33(4/12)	50(6/12)	*E. coli*=1 *Salmonella*=2	Sal=7 E. coli=3 35.71(10/28)	Sal=2 *E. coli*=3 35.71(5/14)
Lion	30	40(12/30)	36.37(11/30)	40(12/30)	*E. coli*=2 *Salmonella*=2	Sal=*6 E. coli*=5 39.28(11/28)	Sal=2 *E. coli*=3 35.71(5/14)
Total	42	40.47(17/42)	35.71(15/42)	42.85(18/42)	63.63(7/11)	Sal=13/28 *E. coli*=8/28 37.5(21/56)	Sal=4/28 *E. coli*=6/28 35.71(10/28)

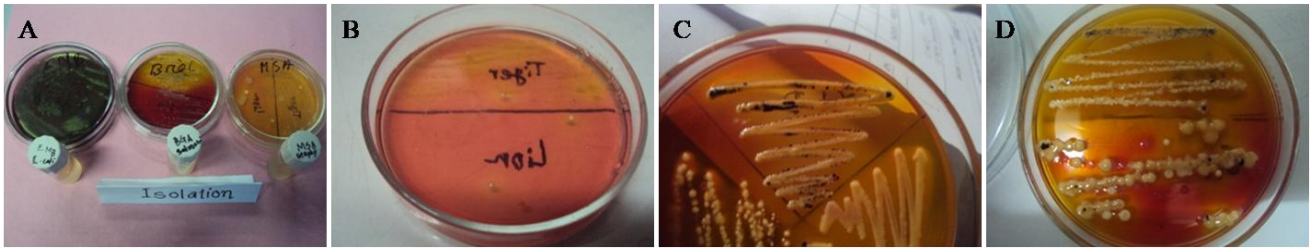

Figure 1: A. Isolation of *E.coli, Salmonella and Staphylococcus sp*, **B.** *Staphylococcus sp* on MSA (tiger and lion samples). **C** *Salmonella* on XLD agar (lion samples), (yellowish colonies**)** D. *Salmonella* on XLD agar (tiger samples).

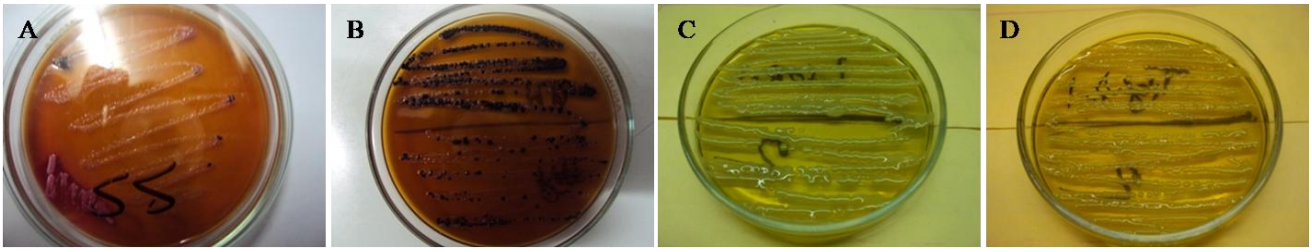

Figure 2: *A. Salmonella* on SS agar of lion (Pinkish colony) B: *Salmonella* on SS agar of tiger C: *Salmonella* on BGA of lion (dew drop colony) D: *Salmonella* on BGA (tiger)

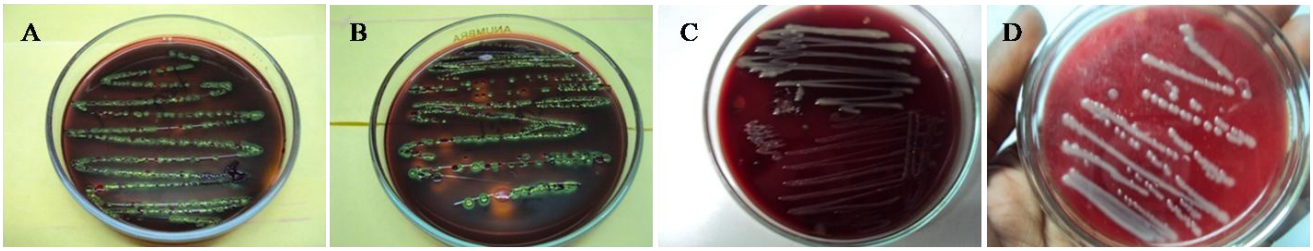

Figure 3: A. *E. coli* in EMB of tiger (metallic sheen); **B.** *E. coli* in EMB of lion. **C.** *E. coli* in blood agar (hemolysis) **D.** *Salmonella* on blood agar

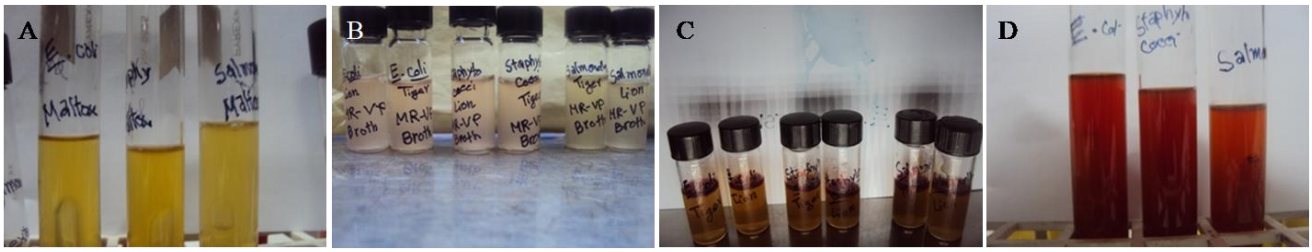

Figure 4: A. Carbohydrate Fermentation positive: Dextrose, Lactose and Maltose of *E.coli, Salmonella, staphylococcus sp* (Left to Right); **B.C.**: MR (Left) and VP (Right) test negative *E coli*, *Salmonella and Staphylococcus sp. D*: Acid gas production on McConkey broth *E coli* (positive*), Salmonella* (Negative) and *Staphylococcus sp* (Positive)

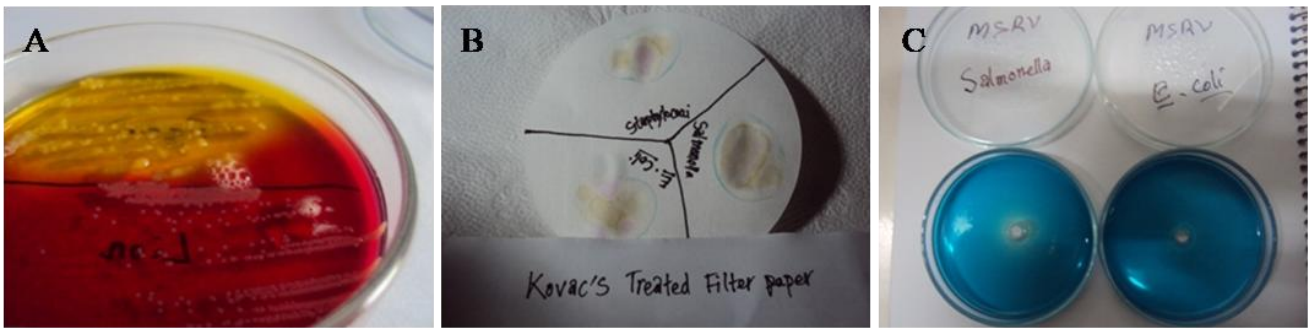

Figure 5: A. Oxidase *E coli* positive and *Salmonella* negative, B. Catalase positive *staphylococcus*; C. *Salmonella* and *E.coli* on MRSV medium

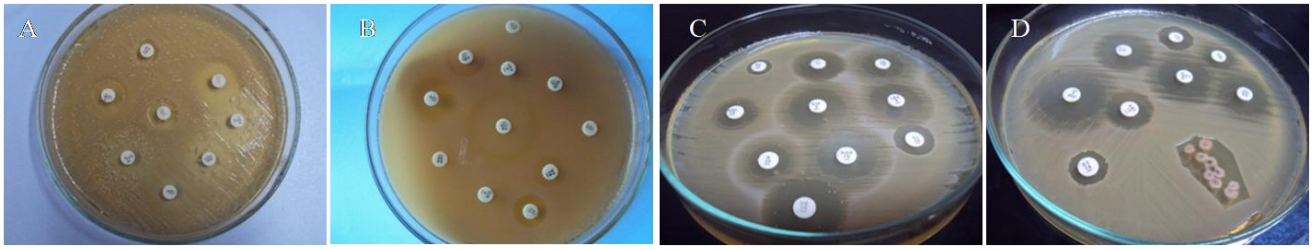

Figure 6: A. Antibiogram of lion *E.coli* B. Antibiogram of lion *Salmonella*, C Antibiogram of tiger *E.coli*, D. Antibiogram of tiger *Salmonella*

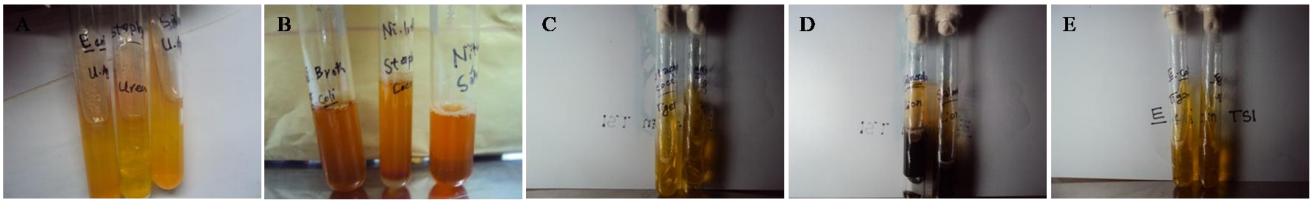

Figure 7: A. Urease negative *E. coli*, *Staphylococcus* spp. and *Salmonella* spp ; B. Nitrate Reduction before Zn powder addition negative. C. *Staphylococcus* spp. on TSI agar negative, D. *Salmonella* spp. on TSI positive; E. *E. coli* on TSI slant agar negative

Molecular detecton of the isolates

PCR results confirmed 7 (3 *E. coli* and 4 *Salmonella*) isolates out of 11 in tiger and lion (Figure 8 & 9). Most of the isolates have zoonotic importance and confirmed the presence of either commensal or pathogenic for tiger and lion in zoo.

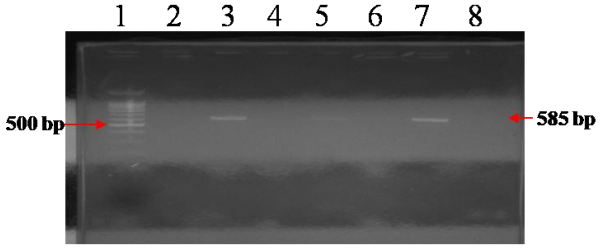

Figure 8: PCR image of *E. coli*, Lane 1: 100 bp DNA ladder, Lane: 3,5,7 representing the isolated positive *E. coli*, Lane 8 : Negative control. (Electrophoresis was done on 1.5% Agarose gel stained with ethedium bromide) of tiger and lion.

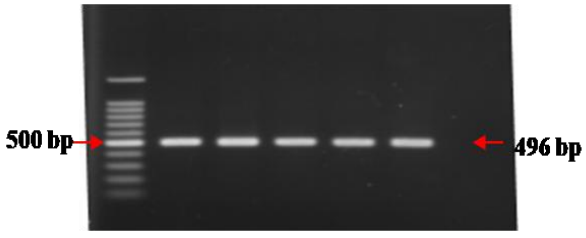

Figure 9: PCR image of *Salmonella*, Lane 1: 100 bp DNA ladder, Lane 2: Positive control, Lane 3, 4, 5, 6: Isolated positive *Salmonella*, Lane 7: Negative control. (Electrophoresis was done on 1.5% Agarose gel stained with ethedium bromide) of tiger and lion.

Motility test

On Microscopic test, straight forward movement belongs for *E. coli* due to flagella present on one side acts as tail, for *Salmonella* individual swirling of organism around the focus. On MSRV medium, migration of bacterial pathogen by forming a round circles around the inoculation point. In this case *E. coli* has no motility whereas *Salmonella* has motility capacity on this media (Figure 5C).

Antibiogram

The antibiogram study revealed that most of the *E. coli*, *Salmonella* spp. and *Staphylococcus* spp. were resistant to Doxycycline, Norfloxacin, Ciprofloxacin, Neomycin, Enrofloxacin, Ciprofloxacin, amoxicillin, Levofloxacin and Tetracycline (Table 7 & 8; Figure 6 A,B,C,D). However, most of the *E. coli*, *Salmonella* sp. and *Staphylococcus* sp. were susceptible to Neomycin, Gentamicin and Colistine sulphate this is to indicate that the use of these antimicrobial may be chosen in clinical control of Salmonella, *E. coli*, and Staphylococcus sp infection.

Discussion

In this study, colony chracteristics of *E. coli* observed in NA, EMB, MacConkey agar/broth, BG, XLD, MS and SS agar (table: 2) were similar to the findings of Buxton and Fraser (1977), Ali et al. (1998), Nazir et al., (2005), Hasina (2006) and Kali et al., (2012).In Gram's staining, the morphology of the isolated bacteria exhibited Gram positive cocci in chains or pairs *(Staphylococcus sp.)* and Gram negative character with short rod arranged in single or paired (*E. coli*, *Salmonella* sp.) that was supported by Merchant and Packer (1967), Buxton and Fraser (1977) and Freeman, (1979).

The isolates of *E. coli* show a complete fermentation of the five basic sugars such as dextrose, maltose, sucrose, lactose, manitol by producing both acid and gas that was supported by Beutin et al., (1991), Mckec et al., (1995), Sandhu (1996) and Thomas (1998).The isolates also revealed positive reaction in VP test (Buxton and Fraser, 1977 and Honda et al., 1982).

The antibiogram study was performed with the *E. coli* isolates to study their sensitivity and resistance pattern against the available and commonly used antibiotics in the market. Hussain et al., (1982) described that the high incidence of multi- drug resistant *E. coli* in this study region appeared to be analogous to what was predicted by many previous studies.

Antibiotic resistance is developed under constant selection pressure and when clinical isolates survive long term in the environment they are likely to loose the drug resistance determinant due to lack of this pressure. In this study, motility test were observed by hanging drop method under light microscope. Straight forward movement belongs for *E. coli* due to flagella present on one side acts as tail, for *Salmonella* individual swirling of organism around the focus.

On MSRV medium, migration of bacterial pathogen occurs by forming a round circles around the inoculation point. We observed **that** *E. coli* has no motility whereas *Salmonella* has motility capacity on this media. *E. coli strain was confirmed by targeting* 16S rRNA which is supported by the method applied by Wang et al. (1996) and for *Salmonella* 16S rRNA gene were detect using methods by Noah et. al. (1993). Out of 42 sample 32 were positive by cultural, morphological and biochemical test. Out of 32 representative samples *E. coli* (5) and *salmonella* (6) were subjected to molecular detection by PCR. In our present study out of 6 *E. coli* PCR sample 3 were positive and out of 5 *salmonella* PCR sample 4 were positive.

Conclusion

Form the study it was found that most of the isolates have zoonotic importance and on firmed the presence of either commensal or pathogenic for tiger and lion in zoo. The study will help to gain knowledge about the threat of the isolated bacteria for the water birds in captive condition. Further studies are needed for molecular characterization of the isolates for detection and analyzing antimicrobial resistant gene present in these isolates.

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