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Pathology and Molecular Characterization of *Eimeria tenella* Isolated from Clinically Infected Broiler Chickens in District Lahore, Pakistan

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Coccidiosis, Phylogenetic analysis, Haematology, Serum biochemistry, Histopathology.

ABSTRACT

Coccidiosis caused by *Eimeria tenella* is a parasitic disease affecting chickens. In Pakistan, there has been no previously published report on phylogenetic analysis of *Eimeria tenella.* In this retrospective study, tissue samples were collected from a flock of clinically infected chicken followed by haematology, serum biochemistry, and histopathology. Species specific PCR based on polymorphic site of the ITS1 gene was developed and used to identify the organism. Haematological examination of the blood demonstrated a decrease in total erythrocytes, packed cell volume, haemoglobin concentration, and red blood cell indices. Differential leukocyte analysis revealed leukocytosis, heterophilia, eosinophilia, monocytosis, and lymphocytosis. Serum biochemistry showed marked elevation in aspartate transaminase, alkaline phosphatase and creatinine and a significant decline in alanine transaminase, total protein, total albumin, globulin, triglycerides and cholesterol values. Histopathological examination demonstrated degenerative changes, necrosis haemorrhages, and sloughing off epithelial cells of broad folds of caeca, mild lymphoplasmacytic infiltration in the periportal area of the liver and mild depletion of lymphocyte in the bursa of *Fabricius*. The seven clades of avian *Eimeria* species strongly support that *E. necatrix* and *E. tenella* were closely associated and placed in the same sister clade with high bootstrap support (98%). Our two isolates RSI and RSII showed a homology index of 99.82% (nucleotide level) and 99.47% (amino acid level) with each other. The maximum similarity percentage indicated that RSI and RSII isolates were closely related to strains reported from India and China. This study is the first report on molecular characterization of *E. tenella* in Pakistan highlighting the pathological potential and distribution of *E. tenella*.

INTRODUCTION

Avian coccidiosis is a parasitic disease caused by genra *Eimeria* and *Isospora*, which have a complex life cycle and belong to the phylum *Apicomplexa*. Members of *Apicomplexa* mainly affect the intestine of mammals and birds (Murakami *et al*., 2014). This disease has great economic significance mostly in the chicken industry as birds at the farm level are reared together in high densities where chances of a disease outbreak are prominent. The economic importance of coccidiosis is attributed to a decrease in production, the cost involved in the treatment and control of disease, and a high mortality rate. Worldwide, the annual loss inflicted by coccidiosis to the poultry industry has been estimated at USD 3 billion (Jatau *et al*., 2014). The most common mode of transmission is mechanical, by a person who moves through the pens, houses, and farms. Infection is usually self-limiting and depends on the number of oocysts ingested and the immune status of the birds. According to surveys, coccidia have been found in litter samples of all farms in America, Europe, and Asia (Fornace *et al*., 2013).

So far about 1800 species of genus *Eimeria* are reported that affect the intestinal mucosa of birds and animals (Haug *et al*., 2008). Nine species of coccidia (*E. brunetti E. maxima, E. necatrix, E. tenella*, *E. acervulina, E. mitis*, *E. mivati*, *E. praecox,* and *E. hagani*) have been isolated from chickens, each with specific tissue tropism and pathogenicity (Jadhav *et al*., 2011; Morgan *et al*., 2009; Nematollahi *et al*., 2008). In Pakistan, the prevalence of *E. maxima* (22.42-34.10%), *E. tenella* (27.04-30.62%), *E. acervulina* (19.89%), *E. mitis* (13.95%) and *E. necatrix*,

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(4.02-7.75%) has been documented (Awais *et al*., 2012; Khan *et al*., 2006) but the most commonly found pathogenic species accounting for high mortality in the poultry industry of Pakistan is *E. tenella*. The highest prevalence of coccidiosis is evident in September (73.33%) while the lowest is during April (42.86%) (Bachaya *et al*., 2012).

E. tenella is the most recorded coccidia in poultry known for its recognizable lesions and remarkable losses in young broilers and layer pullets (Zaman *et al*., 2012). It inhabits the caeca and causes a severe disease which is characterized by intestinal bleeding, high morbidity, high mortality, a decrease in weight gain with emaciation, loss of skin pigmentation, and bloody cores that accompany clusters of large schizonts and oocysts in the caecum (Habibi *et al*., 2016). The diagnosis and differential diagnosis between other species are usually carried out by observing the clinical signs of the disease, the morphology of the parasites, location of development of oocytes within the intestine and their natural appearance in faeces, endogenous stages in the caecal and intestinal mucosa, postmortem analysis of birds (Carvalho *et al*., 2011).

Recently, coccidian parasites of both humans and mammals have been differentiated through the amplification of DNA. An ideal genomic DNA target for polymerase chain reaction (PCR) is the internal transcribed spacer 1 (ITS-1) gene of ribosomal DNA (rDNA) (Lew *et al*., 2003). This spacer gene separates the 3' of 16S-ribosomal RNA from 5' of 5.8S-ribosomal RNA within individual rDNA. Due to base sequences and heterology of the ITS-1 gene, it tends to be suitable to design specific primers and hence provides multiple copies of potential PCR targets (Kumar *et al*., 2015).

In this study, we investigated the pathological changes induced in broiler chickens naturally infected with *E. tenella*. The infection was confirmed using a molecular technique (PCR) targeting the ITS-1 gene of rDNA. No previous information has been reported on genetic diversity in the ITS-1 gene of *E. tenella* infection in Pakistan. Therefore, nucleotide sequences of field isolates were generated to investigate the ITS-1 based phylogenetic relationship of *E. tenella* prevailing in Pakistan with those reported in various geographical areas of the world.

MATERIALS AND METHODS

Sample collection

Blood and tissue samples (caeca, bursa of *Fabricius* and liver) of apparently healthy birds (n=25) and those (n=25) showing clinical signs of bloody diarrhoea, retarded growth, dullness, and reluctance to move were collected from a flock of approximately 3,000 three-week-old broilers suspected to have caecal coccidiosis in the farm located in the surroundings of Lahore district. The day-old-birds were vaccinated against Newcastle disease, infectious bursal disease, and infectious bronchitis. The farm had a previous history of impaired conditions and clinical symptoms compatible with caecal coccidiosis, accompanied by an increase in mortality rate reaching 0.3% per day, with a cumulative mortality rate of 14.7%. The economic losses due to retarded growth were evident for over a week. The blood sample was collected in a five mL syringe and shifted equally into ethylenediaminetetraacetic acid (EDTA) and gel containing vacutainers. Then, birds were euthanized through cervical dislocation to examine the postmortem changes in caeca, small intestine, and large intestines. For histopathological examinations, tissue samples of 4×4 mm were fixed in 10% neutral buffered formalin.

Isolation and sporulation of oocysts of E. tenella

Caeca samples were rinsed with normal saline, homogenized, and transferred to 35% sodium chloride solution to induce the floatation of oocysts. The supernatant was separated to collect the oocysts and observed under a light microscope. The oocysts were treated with 2.5% potassium dichromate for sporulation (Ogedengbe *et al*., 2011).

Haematological analysis

Haematological parameters, including total erythrocytes (TE) and leukocytes, differential leukocyte count (DLC), packed cell volume (PCV) and haemoglobin concentration (Hb) were measured according to methodology as described by Akhtar *et al*. (2015). The red blood cell indices [mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentrations (MCHC)], were calculated from RBC, PCV, and Hb percentage, respectively (Adamu *et al*., 2013).

Serum biochemistry profile

The serum biochemistry was analyzed in a spectrometer (Pharmacia Biotech, Sweden). Blood samples in a gel containing vacutainer were centrifuged for 10 min at 3,000 *g* to separate the serum samples. The concentration of liver enzymes [alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP)], total protein (TP), total albumin (TA), globulin, cholesterol, triglycerides (TG), and creatinine was evaluated using specific kits (Roche Diagnostics, Switzerland) as described by Dar *et al*. (2014) and Mohammed (2012).

Histopathological examination

For histopathological examination, tissue samples fixed in 10% neutral buffered formalin were hydrated, dehydrated in ethanol in ascending order, cleared with xylene, embedded in paraffin, and sectioned off 4 µm with a microtome (SLEE, Germany). Paraffin tissue sections were then deparaffinized using xylene and hydrated in descending concentration of alcohol. Tissue sections were observed under a light microscope (Olympus, Japan) after staining with hematoxylin and eosin (H and E) dye.

DNA extraction of oocysts of E. tenella

For extraction of DNA, the purified sporulated oocysts were centrifuged at 12,000 *g* for 5 min (four times) with autoclaved phosphate-buffered saline (PBS) solution to remove the debris. The pellet was suspended in 100 µL of 5.75% hypochlorite after an incubation of 30 min on ice. The suspension was further diluted in 500 µL of double distilled water and pelleted after centrifugation. The pellet was also washed (four times) with PBS and diluted in 500 µL of double-distilled water. The oocysts were disrupted through speed sonication using the ultra-sonicator (Thomas Scientific, United States). The genome was extracted from the rigid oocysts and analyzed by agarose gel electrophoresis (Patra *et al*., 2010).

ITS-1 gene amplification and polymerase chain reaction

For ITS-1 gene amplification, previously reported oligonucleotide primer sequences for *E. tenella*; forward (5'- CCGCCCAAACCAGGTGTCACG -3') and reverse (5'- CCGCCCAAACATGCAAGATGGC -3') were used for amplification of ITS-1 gene with a predicted amplicon size of 539 bp (Gadelhaq *et al*., 2015). Each 40 µL reaction mixture consisted of 20 µL master mix (PrimeSTAR Max DNA polymerase, catalogue number: R045A), 1.5 µL primer (forward and reverse each), one µL DNA template and 16 µL nuclease-free water. The tubes were then placed in Veriti™ 96-Well Thermal Cycler (Applied Biosystems™, United States) with following reaction conditions; a single cycle of initial denaturation (95°C, 5 min), and 35 cycles of each [denaturation (94°C, 30 s), annealing (57.5°C, 30 s) extension (72°C, 90 s)], and a final extension (72°C, 10 min). The PCR product was separated on agarose gel (1.5% w/v) stained with 0.5 µg/mL ethidium bromide, run in gel electrophoresis at 110V, 230mA for 20 min and then visualized in a gel documentation system (Bio-Rad Laboratories, United States).

The PCR products were submitted for DNA sequencing to Comate Bioscience Co., Ltd., China. The oligonucleotide sequences were aligned using ClustalW with BioEdit software. The phylogenetic tree was constructed and inferred through the Maximum likelihood-method with statistical analysis based on 1,000 bootstrap replicates performed on MEGA-X software. Nucleotide and amino acid percentage identity was compared with the Geneious prime software. Nucleotide sequences of other *Eimeria* species were retrieved from the NCBI-GenBank database to conduct phylogenetic analysis.

The sequences were submitted to the NCBI GenBank database and are available under the accession numbers MN883392.1 [*Eimeria tenella* isolate PAK-UVAS-PATH-RSI (RSI)] and MN883393.1 [*Eimeria tenella* isolate PAK-UVAS-PATH-RSII (RSII)].

RESULTS AND DISCUSSION

Haematological analysis depicted a significant decrease ($P<0.05$) in TE (10^6/µL), PCV (%), Hb concentration, MCV (fL), MCH (pg), and MCHC (g/L) in infected birds. A significant increase ($P<0.05$) in leukocytes 10^3/µL, heterophils, eosinophils, and lymphocytes were observed in diseased birds (Table I). A decline in red blood cell indices is in concurrence with the reports of many researchers (Bogado *et al*., 2010; Jatau *et al*., 2014; Singh *et al*., 2013). The decrease in TE and Hb could be due to haemorrhages in caeca. The reduction in MCV, MCH and MCHC can be associated with microcytic hypochromic anaemia (Adamu *et al*., 2013). The activated macrophages release several pro-inflammatory cytokines that mediate the alterations in blood glucocorticoids level (Krams *et al*., 2012). Therefore, an increased level of corticosterone

Table I.- Haematological analysis of *E. tenella* infected and non-infected broiler chickens.

Parameters	Normal	Infected	P-value (P<0.05)
TE (10^6/µL)	2.94±0.19^b	1.72±0.25^a	<0.0001
PCV (%)	43.20±6.43^b	23.39±2.42^a	<0.0001
Hb (g/L)	10.83±1.33^b	8.80±0.96^a	0.0030
MCV (fL)	131.40±14.55^b	85.93±14.01^a	0.0001
MCH (pg)	43.72±4.28^b	33.75±6.73^a	0.0050
MCHC (g/L)	321.10±15.49^b	169.70±4.55^a	<0.0001
Leukocytes (10^3/µL)	13.69±1.49^a	20.35±1.66^b	<0.0001
Heterophils (10^3/µL)	4.22±0.32^a	6.63±0.70^b	<0.0001
Eosinophils (10^3/µL)	0.80±0.01^a	0.90±0.01^b	<0.0001
Lymphocytes (10^3/µL)	8.81±0.87^a	12.50±0.81^b	<0.0001
Monocytes (10^3/µL)	1.15±0.35	1.24±0.20	0.3169

TE, total erythrocytes; PCV, packed cell volume; Hb, haemoglobin; MCV, mean corpuscular volume; MCH, mean corpuscular haemoglobin; MCHC, mean corpuscular haemoglobin concentrations. ^{a,b}within a row, values having different superscripts are different significantly ($P<0.05$).

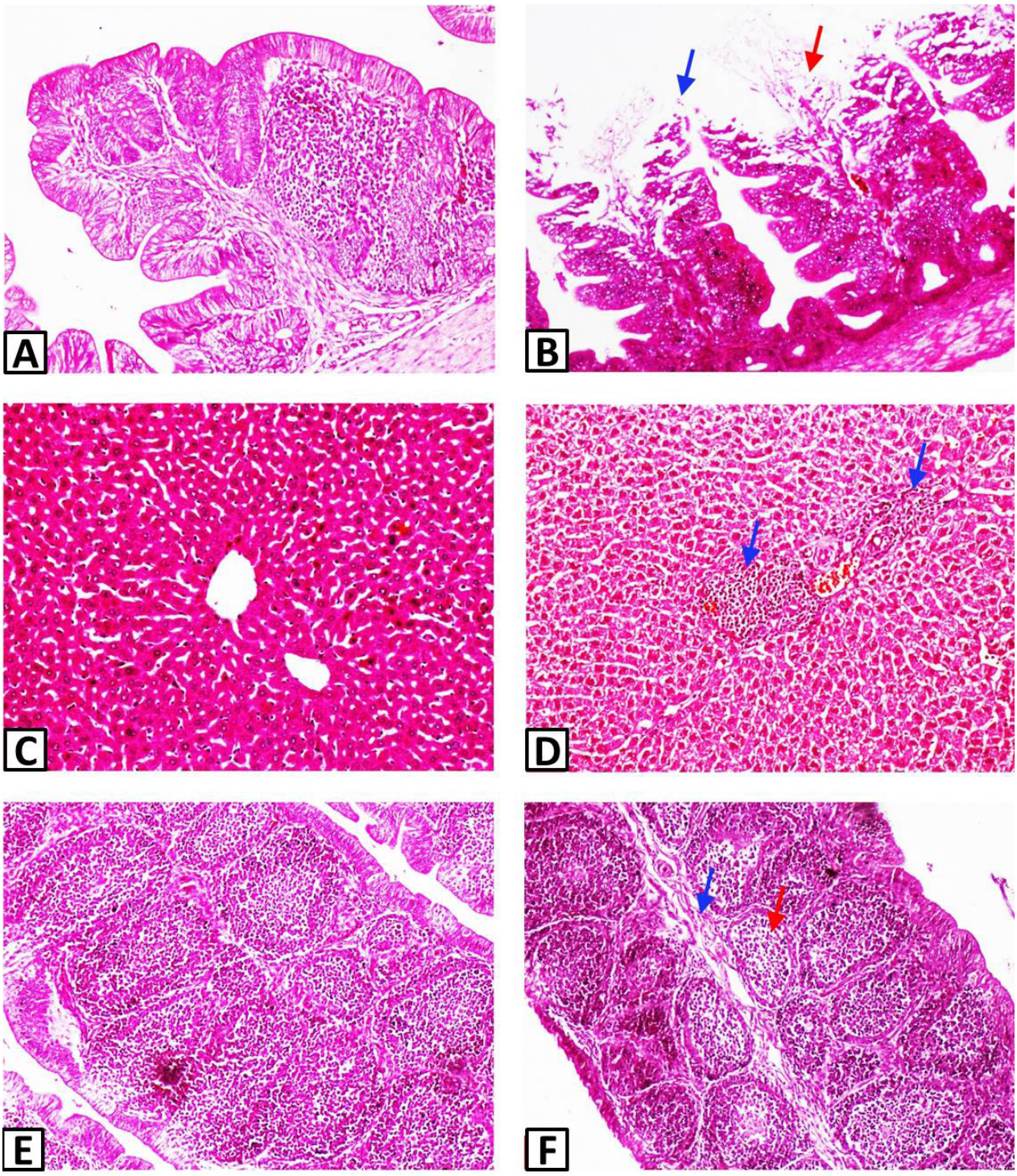

Fig. 1. Histological structure of parts of different organs of chicken infected with *E. tenella*. **A,** normal caeca with intact broad folds. **B,** necrotic debris in lamina propria (red arrow) and sloughing off epithelial cells of broad folds of caeca of *E. tenella* infected bird (blue arrow). **C,** normal liver with intact central vein, hepatocytes and hepatic cords. **D,** lymphoplasmacytic infiltration in periportal area of liver (blue arrow) of *E. tenella* infected bird. **E,** normal BF with intact bursal follicles. **F,** fibrous connective tissue accumulation (blue arrow) and depletion of lymphocytes in BF (red arrow) of *E. tenella* infected bird. Stain: H&E; Magnication: 10X.

in blood circulation can induce changes in haematological parameters (Duckworth *et al*., 2001; Shini *et al*., 2009). The significant increase (P<0.05) in heterophils, eosinophils and lymphocytes could be associated with the parasitic infestations that agree with the conclusions of Ahmed El-Shazly *et al*. (2020) and Khaligh *et al*. (2019). Heterophils are one of the granulocytic leukocytes and contribute as the first line of defense against pathogens. Heterophilia and monocytosis are often associated with acute and chronic inflammatory responses, respectively. Macrophages, monocytes, and dendritic cells are the essential hematopoietic cells and have a crucial rule in the defense system of the body. Eosinophilia is associated with the parasitic infestation and rarely reported in birds (Adamu *et al*., 2013).

Concerning the serum biochemistry profile of *E. tenella* infected birds, a marked elevation in AST, ALP, and creatinine was obtained which is in concurrence with the findings of many researchers (Ahmed El-Shazly *et al*., 2020; Mondal *et al*., 2011). A significant decrease (P<0.05) in serum ALT, TP, TA, globulin, TG and cholesterol agrees with the results of Ahmed El-Shazly *et al*. (2020) and Mondal *et al*. (2011). The higher values of serum AST and ALP may be attributed to protein malabsorption due to tissue damage produced by *E. tenella*. The inadequate feed intake and protein absorption resulted in elevated protein catabolism which subsequently leads to severe muscle degradation, and eventually elevated level of serum AST and ALP (Rajman *et al*., 2006). The increased level of ALP in serum might be due to metabolic alteration and damage of bone marrow due to blood loss or haemorrhages. Any injury to bone marrow may result in bone marrow hyperactivity eventually in elevated ALP level in serum (Adamu *et al*., 2013). Hypo-proteinemia, hypo-albuminemia, and hypo-globulinemia are may be due to acute stress resulted in the release of cortisol and protein catabolism (Mohammed, 2012). The haemorrhages and seepage of plasma protein into intestine due to tissue damage to caecum may cause the malabsorption of protein and other nutrients from feed (Williams, 2005). The decrease in serum TG and cholesterol may be associated with the anorexia, malabsorption of nutrients and severe tissue damage to the caecum by *E. tenella* infection (Fukata *et al*., 1997). In malnutrition, fat mobilization from fat depots, the disappearance of fatty acids, and hindering in lipogenesis due to disturbances in vitamin-B caused by coccidiosis are evident (Allen, 1988).

Histopathological examination demonstrated inflammatory cell aggregation in mucosa and submucosa of caeca. Caecal mucosa was destroyed with necrosis and disintegration of epithelial cells. Put together, broad folds in the mucosal layer were degenerated and sloughed off, and submucosa was exposed. The lumen of caeca was filled with enterocytes, the debris of epithelial layer cells, and remnants of lamina propria (Fig. 1B). The infiltration of heterophils, macrophages, and lymphocytes in mucosa indicates the acute and chronic inflammatory response which is caused by concurrent lodging of *E. tenella*, and this is in concurrence with the results of Zhang *et al*. (2012). The histopathological findings of the current study are similar to those reported by Adamu *et al*. (2013). They demonstrated that most pathogenic second-generation schizonts of *E. tenella* induce tissue damage, haemorrhages, and degenerative changes in the mucosa, and muscularis of caeca. There was focal lymphoplasmacytic infiltration in the liver (Fig. 1D) and marked lymphocytic depletion in BF (Fig. 1F) as outlined by Ogbe *et al*. (2010). No specific microscopic lesions were observed in caeca (Fig. 1A), liver (Fig. 1C) and BF (Fig. 1E) of normal birds.

Table II.- Serum biochemistry profile of *E. tenella* infected and non-infected broiler chickens.

Parameters	Normal	Infected	P value (P<0.05)
ALT (U/L)	15.66±2.66^{b}	6.24±1.62^{a}	<0.0001
AST (U/L)	122.40±21.65	130.50±18.36	0.4392
ALP (U/L)	77.58±12.83^{a}	99.93±11.92^{b}	0.0006
Creatinine (mg/dL)	0.29±0.04^{a}	0.49±0.06^{b}	<0.0001
TP (g/dL)	4.81±0.26^{b}	4.30±0.47^{a}	0.0123
TA (g/dL)	2.05±0.21^{b}	1.76±0.15^{a}	0.0035
Globulin (g/dL)	2.83±0.43^{b}	2.29±0.37^{a}	0.0267
TG (mg/dL)	78.53±1.32^{b}	69.10±0.67^{a}	<0.0001
Cholesterol (mg/dL)	142.70±4.98^{b}	93.96±3.08^{a}	<0.0001

ALT, alanine transaminase; AST, aspartate transaminase; ALP, alkaline phosphatase; TP, total protein; TA, total albumin; TG, triglycerides. ^{a,b} within a row, values having different superscripts are different significantly (P< 0.05).

After the ITS-1 gene sequence alignment, all 25 sequences were grouped into two isolates; RSI and RSII. The deduced nucleotide and amino acid sequences of the ITS-1 gene for isolate RSI and RSII were aligned with other strains reported in different geographical regions to calculate the percentage homology. Despite the high similarities, minor alterations in the ITS-1 gene of Pakistan isolate were noted. Isolate RSI showed similarity with RSII at the nucleotide level (99.82%) and the amino acid level (99.47%) (Table III). The identity range of RSI and RSII isolates was 94.17 to 99.82% at the nucleotide level and 57.89 to 99.47 at the amino acid level when compared with the sequences available in the public domain of NCBI GenBank database (Table III). Sequencing demonstrated high levels of identity in nucleotides and amino acids of our isolates with the strains reported in India, China, USA,

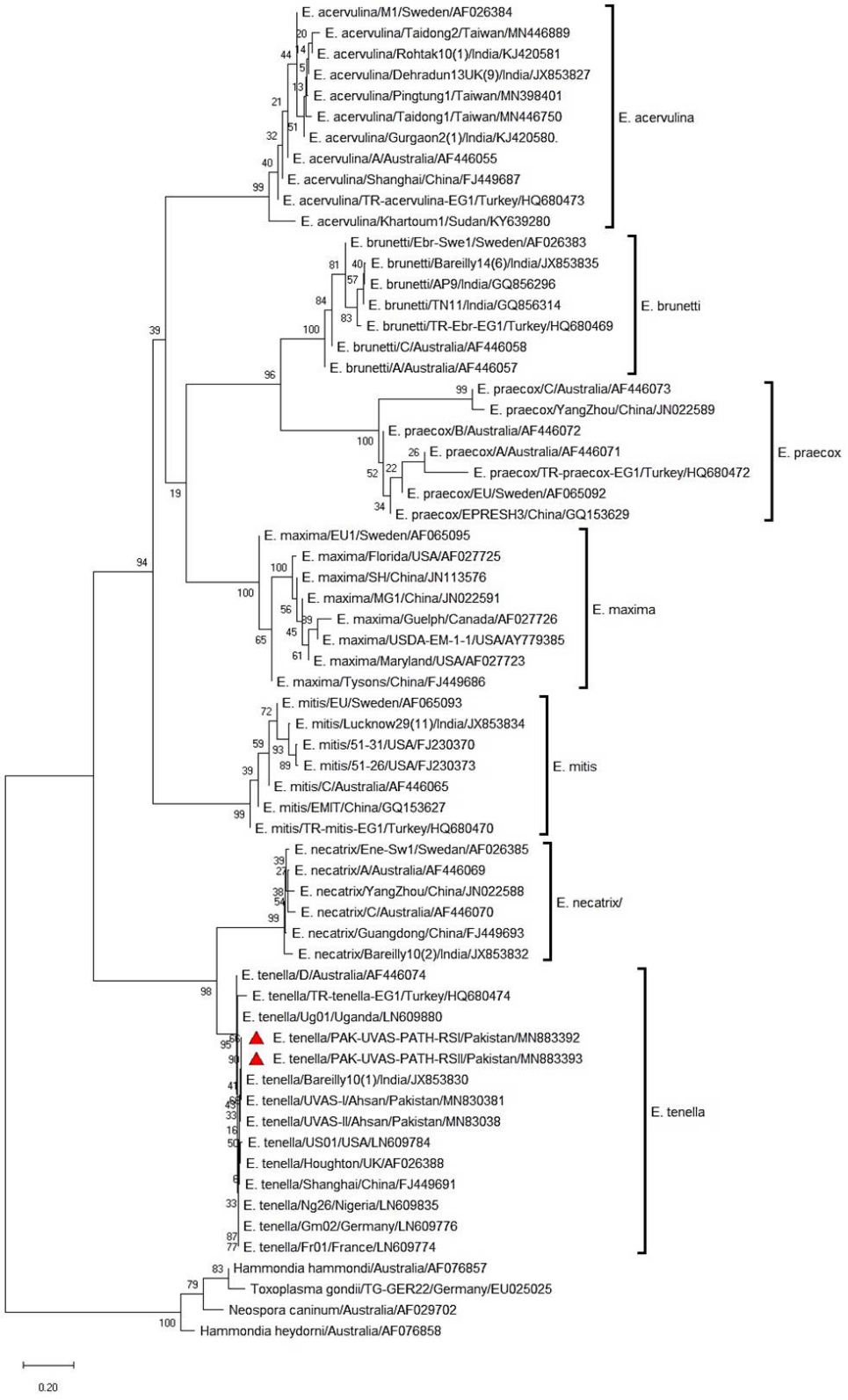

Fig. 2. Phylogenetic tree based on ITS-1 gene of *Eimeria* species using Maximum Likelihood method.

Table III.- The nucleotide and deduced amino acid identities of RSI and RSII isolates.

Accession No.	Country	Nucleotide identity (%)		Amino acid identity (%)	
		RSI	RSII	RSI	RSII
MN830381	Pakistan	99.82	99.65	99.47	98.94
MN830382	Pakistan	99.11	98.94	98.40	97.87
GQ856310	India	98.76	98.58	97.87	97.34
GQ856299	India	98.23	98.05	96.28	95.74
GQ856300	India	97.52	97.34	95.74	95.21
JX853830	India	99.82	99.65	99.47	98.94
JX853825	India	99.29	99.11	98.94	98.40
KM382066	India	99.29	99.11	98.94	98.40
GQ856308	India	98.94	98.76	98.40	97.87
JX853831	India	98.94	98.76	98.94	98.40
GQ856297	India	97.70	97.52	95.74	95.21
LN609975	India	98.58	98.40	97.34	96.81
LN609974	India	99.29	99.11	98.94	98.40
FJ449691	China	98.94	98.76	99.47	98.94
GQ153633	China	98.76	98.58	97.87	97.34
GQ153630	China	96.64	96.46	78.42	77.89
GQ153632	China	96.64	96.46	78.42	77.89
GQ153636	China	97.87	97.70	96.28	95.74
GQ153634	China	98.23	98.06	67.02	66.49
LN609835	Nigeria	98.76	98.58	97.87	97.34
LN609828	Nigeria	98.76	98.58	97.87	97.34
LN609784	USA	98.40	98.23	97.87	97.34
AY779514	USA	98.94	98.76	98.94	98.40
AF026388	UK	98.40	98.23	57.89	57.89
LN609785	UK	98.05	97.87	95.74	95.21
LN609774	France	98.76	98.58	97.87	97.34
LN609773	France	96.99	96.81	78.95	78.42
LN609946	Libya	98.40	98.23	57.89	57.89
LN609948	Libya	99.29	99.11	99.47	98.94
LN609952	Libya	96.64	96.46	77.89	77.37
LN609888	Uganda	99.29	99.11	99.47	98.94
LN609903	Uganda	98.94	98.76	98.94	98.40
LN609880	Uganda	98.58	98.40	97.87	97.34
LN609776	Germany	98.76	98.58	97.87	97.34
LN609777	Germany	96.03	95.85	95.34	94.82
AF446074	Australia	98.23	98.05	96.81	96.28
HQ680474	Turkey	94.35	94.17	73.54	73.02
LN609809	Japan	98.58	98.40	97.87	97.34

UK, Germany, France, Japan, Australia, Nigeria, Libya, and Uganda (Table III).

The phylogenetic tree was constructed through the Maximum Likelihood method (Fig. 2). The two nucleotide sequences produced in this study and fifty-eight sequences of *Eimeria* available in NCBI GenBank, reported in Asia, Europe, Africa, Australia, and America were used for phylogenetic analysis. The nucleotide sequences of *E. acervuline, E. brunetti, E. praecox, E. maxima, E. mitis, E. necatrix* and *E. tenella* were clustered separately irrespective of their geographical distribution. Our two isolates RSI and RSII were located in a clade formed by ITS-1 gene sequences of *E. tenella* (Fig. 2). *E. necatrix* and *E. tenella* were closely associated and placed in the same sister clade with high bootstrap support (98%). Likewise, *E. praecox* and *E. brunetti* were closely linked and placed in the same sister clade with high bootstrap support (96%). The maximum likelihood tree revealed that our isolates are grouped with other Pakistani, Indian and Chinese strains which are in concurrence with Kumar *et al*. (2015).

CONCLUSION

In conclusion, the results of the current study depicted that haematological parameters and serum biochemistry profile were adversely altered in broiler chickens naturally infected with *E. tenella.* Moreover, genetic diversity among our isolates undoubtedly throws some light on the genetic makeup of *E. tenella*. However, the immunological diversity of *E. tenella* in Pakistan requires to be further investigated to manufacture an anticoccidial vaccine that can prove equally effective against the genetically diverse population of *E. tenella* around the globe.

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Statement of conflict of interest

The authors have declared no conflict of interests.

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