Bovine Coronavirus Infections in Turkey: Molecular Analysis of the Full-Length Spike Gene Sequences of Viruses From Digestive and Respiratory Infections

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Abstract

BCoV can be spread by animal activity. Despite the widespread cattle farming in the region, limited studies exist in Turkey about BCoV. The aim of this study was to reveal the latest aspects in Turkey about BCoV and evaluate the data. This is the first study reporting the full-length sequences of S genes of BCoV in Turkey.

In this study samples were collected from 119 cattle, presenting clinical signs of respiratory (n=78) or digestive tract infection (n=41) in distinctive farms located in widely different provinces of Turkey. Samples were screened for bovine coronavirus (BCoV) by a RT-nested PCR targeting the N gene. BCoV was found positive in 35 samples (9 faeces and 26 nasal discharge) and RT-PCR analysis of the S gene was performed. The partial/full-length S gene sequences were able to be obtained from 11 samples (8 faeces and 3 nasal discharge). Phylogenetic tree of the S gene sequences was analyzed for the genetic relationships among BCoVs from Turkey and other countries. Results showed that many different amino acid changes exist in local strains and these changes were persistent in faeces and nasal discharge origin. Likewise, some nucleotide and amino acid changes were compatible with previous studies which have critical importance for tropism. These results could represent a novelty in the current literature adding new data on BCoV from Turkey and these data could have potentially positive effects on the vaccination approach and control strategies due to lack of information.

Introduction

The coronaviruses cause infections in many species, including humans. In the last decade, they have gained importance among zoonotic viruses after SARS-coronavirus (SARS-CoV) and MERS-CoV infections and recently COVID-19 pandemic caused by a novel coronavirus named SARS-coronavirus-2 (SARS-CoV-2) [1–3]. In animals, coronavirus is etiologically associated with enteric and respiratory disease across a wide range of mammalian and avian species. Bovine coronavirus (BCoV) is mainly recognized as a cause of severe neonatal calf diarrhea. It has also emerged as the reasons that the winter dysentery in adult cattle and the respiratory tract illnesses in calves and adult cattle, leads to economic losses to the livestock industry [4–6].

BCoV belongs to the species *Betacoronavirus 1*, which was recently assigned by the International Committee on Taxonomy of Viruses (ICTV) to the order *Nidovirales*, suborder *Cornidovirineae*, family *Coronaviridae*, subfamily *Orthocoronavirinae*, genus *Betacoronavirus*, and subgenus *Embecovirus* [7]. The genome is 31,028 base length, contains linear and single-stranded RNA [8]. BCoV genome consists of five structural genes: the phosphorylated nucleocapsid (N), the integral membrane (M), and small membrane (E), the hemagglutinin/esterase (HE), spike (S) genes [9]. S genes that synthesized the S protein has an important function in viral infectivity. The S glycoprotein is cleaved into S1 (N terminus) and S2 (C terminus) subunits by an intracellular protease [10]. The S glycoprotein has two main biological functions: the attachment to target cells and the fusion of viral and cellular membranes [11–13]. S1 is responsible for attaching the large receptor binding domain of S protein to cell receptors and induces neutralizing antibodies and hemagglutination activity. In the meantime, the S2 protein mediates the fusion of viral and cellular membranes by forming a "spear-like" stem. Besides, the S2 subunit is highly conserved and the S1 subunit is more variable among BCoVs [6, 13–15]. It is known that the mutational changes in amino acid composition in cleavage sites may have a significant effect on altered tissue and cell tropism and pathogenicity.

Although there have been reports about the presence of BCoV in Turkey until 2011, the first detailed molecular study was conducted by Alkan et al. [16]. The partial sequence of the S gene of 2 faeces samples was obtained in their study. Seroprevalence studies have been continued for many years, but detailed molecular studies have not been conducted.

The first aim of this research was to unveiled the full-length S gene of Bovine coronaviruses in Turkey. Relatedly that, the research could lead to novelty in the current literature adding new data on BCoV from Turkey and these data could have

potentially positive effects on the vaccination approach and control strategies. Another aim of the study was to present the possible interaction of digestive and respiratory agents of the BCoVs.

Materials And Methods

Sampling

A total of 119 samples from cattle suspected of BCoV infection breed in the different farms and widely distributed provinces of Turkey between 2001 and 2019 were used (Fig. 1). Faecal samples (n = 41) were obtained from animals ages 4 days to 15 months and nasal discharge (n = 78) was obtained from animals aged 7 days to 5 ≥ years. Details of sample information are summarized in Table 1.

Samples were diluted 1:10 (v/v and w/v for nasal discharge and faeces, respectively) in phosphate-buffered saline solution and centrifuged at 3,000 x rpm for 20 minutes at 4 °C. Then, supernatants were collected and stored at -80 °C.

RNA Extraction and Reverse Transcription

Viral RNA was extracted using TRIzol ® LS Reagent (Thermo Fisher Scientific), following the manufacturer's instructions. Reverse transcription (RT) was carried out using Geneall ® HyperScript™ First-Strand Synthesis Kit according to the manufacturer's protocol. All RNAs and cDNAs were stored at -80 °C.

Polymerase Chain Reaction For N And S Gene Regions of Local BCoVs

To investigate the presence of BCoV, a nested PCR assay targeting the N gene region was carried out using the primers and methods previously described [17]. All PCR reactions were performed using Thermo Scientific DreamTaq DNA Polymerase with the use of Biometra (Germany) Thermal Cycler.

Briefly, for the first round of nested PCR for N gene amplification, 2.5 µl cDNA was added to the mixture of 2.5 µl of 10X DreamTaq buffer, 0.5 µl of 10 mM dNTP mix, 1 µl of 10 µM primers (BCoV N-F/BCoV N-R), 0.25 µl of DreamTaq DNA polymerase (5 U / µl) and 17.25 µl of nuclease-free water per sample. The PCR was performed according to the following protocol: an initial step of 3 minutes at 95 °C, followed by 35 cycles of 95 °C for 30 seconds, 50 °C for 30 seconds, and 72 °C for 1 minute, and a final extension at 72 °C for 10 minutes. The second round was conducted with the same master mix, using primers nBCoV N-F/nBCoV N-R, and under the same thermal profile with a different annealing temperature (58 °C). Thereafter, the samples which show positive results for the N gene were used for the S gene amplification (4,112 bp) with various primer pairs as previously reported [1, 6, 18] (Supplement 1).

For the PCRs for S gene region amplification, with separate master mixes each consisting 2.5 µl cDNA was added to the mixture of 10X Dream DreamTaq buffer 2.5 µl, 10 mM dNTP mix 0.5 µl, 1 µl of 10 µM primers (Supplement 1), 0.25 µl of Dream Taq DNA polymerase (5 U / µl) and 17.25 µl of nuclease-free water per sample. The PCR was performed according to the following protocol: an initial step of 3 minutes at 95 °C, followed by 35 cycles of 95 °C for 30 seconds, 51–58 °C for 30 seconds for the different reactions (Supplement 1), and 72 °C for 1 minute, and a final extension at 72 °C for 10 minutes. Each amplicon was analyzed on a 1% agarose gel stained with Safeview™ Classic (NextGen Life Sciences Private Limited) and using 1 µl of 100 bp marker (Fermentas, Lithuania) to determine the product size.

Sequence and Phylogenetic Analysis

Sequence analysis of PCR products was performed by a commercial company (BM Labosis, BM Lab. Schist. Ltd. Sti. Ankara, Turkey). Obtained sequences were identified using the BLAST (Basic Local Alignment Search Tool) provided by NCBI (National Center for Biotechnology Information) web page. Sequences were aligned using Aliview software [19], utilizing the references viral sequences extort from the GenBank [9, 20]. Following alignment, amino acid sequences and

sequence variation tables were braced MEGA X and Aliview software's based on nucleotide sequences. The sequences were submitted to the GenBank database under accession numbers MK787427-MK787439 and MK989614-MK989619 for the N gene and S gene sequences were shown in Table 2.

Nucleotide homology analyzes for the similarity comparisons equations were generated using the SIAS [21] web tool.

Phylogenetic relationships of S gene partial/full-length nucleotide and amino acid sequences among a number of other BCoV strains in the world (Fig. 2) (Supplement 2) analyzed using the neighbor-joining methodology with MEGA X software. Neighbour-joining trees were computed with p-distance parameters and confidence levels were estimated by 1000 bootstrap replicates.

Results

Detection of BCoV

35 samples out of 119, were found positive for the N gene fragments (407 bp). Gel electrophoresis results of some of the amplicons were presented in Supplement 3. The positivity rates were 21.9% (9/41) and 33.3% (26/78) for the faecal and nasal discharge samples, respectively.

Molecular Characterization of the S Gene of BCOVs and Phylogenetic Analysis

S gene PCR results were evaluated for 35 the BCoV positive samples, however not all samples were found to be amplicon positive. Nonetheless, as presented in Table 2, the full-length S gene was obtained from 5 samples (3 faecal and 2 nasal discharge). Further, the partial S gene was obtained from 6 samples (4 faecal and 2 nasal discharge). As it is expected, the amplicons from various regions of the S gene were not always in the same region. Solely, 488 bp S1 region amplified by S1 primers (Supplement 1) that plays a critical role in tropism and antigenicity was amplicon positive in total 11 samples (5 from full-length) and that region was used for the nucleotide and amino acid comparisons and phylogenetic analyses. Gel electrophoresis results of some of the amplicons were presented in Supplement 4.

The alignment of the deduced amino acid sequences from the mentioned gene fragment sequences of 11 BCOVs S1 region amplified by S1 primers samples (488 bp) with that of the Mebus strain demonstrated some changes. Notably, all samples with one exception (BCoV / Turkey / 1N / 2019, Acc. no: MK989625) has great numbers of amino acid differences (Table 3). Among mutations, most were synonymous mutations with the mutation detected in other local sequences (GQ259978 and GQ259979) previously reported [16]. No frameshift mutations, deletions, insertions or recombinations have been found in that study. In addition, no relationship was found between the nucleotide or amino acid changes and the type of material from which the virus originated (Table 3).

Comparison of the nucleotide and amino acid similarities of faeces (n = 3) samples between each other were 97.6–99.0% and 96.9–98.2% and nasal discharge (n = 2) samples between each other were 97.8% and 97.7%, respectively. The full-length S genes results (n = 5) showed that; nucleotide similarities was 97.4–99.0% and amino acid similarities was 96.9–98.2% between these sequences. Comparison of the nucleotide similarities of full-length S genes sequences (n = 5) with Mebus strain from faeces and LSU strain from nasal discharge, were 96.9–97.5% and 97.3–97.9%, respectively. Identity rates are also calculated by SIAS for the partial sequences 488 (bp) from local strains. The partial S1 gene sequences (n = 11) showed 90.92–100% nucleotide identity and 87.4–100% amino acid identity to each other. They have 94.78–99.11% and 96.23–97.72% nucleotide identity to Mebus and LSU strains, respectively. According to the similarity analysis of partial sequences (488 bp) made considering the sample types, the nucleotide and amino acid similarities are as follows: 90.92–100% nucleotide and 90.5–100% amino acid identities from the faeces (n = 7) samples between each other and were 95.13–98.75% nucleotide and 90–96.8% amino acid identities from nasal discharge (n = 4) samples between each other.

The phylogenetic tree is constructed from the local full-length (4,112) S gene samples with other randomly selected BCoV S gene sequences around the world (Supplement 2), shown in Fig. 2-A and Fig. 2-C. The phylogenetic tree constructed from the local partially sequenced S1 region amplified by S1 primers (488 bp) with other local sequences [16] and more randomly selected BCoV S gene partial S1 sequences around the world (Supplement 2), shown in Fig. 2-B and Fig. 2-D. Fig. A and B were constructed with amino acid sequences, while fig. C and D were created via nucleotide sequences.

Discussion

In this study; BCoV suspected faeces and nasal discharge samples were collected from all around Turkey (n = 119) and positive samples (n = 35) were scanned with molecular analyzes for the S gene region. 21.9% (9/41) of the faeces and 33.3% (26/78) of the nasal discharge samples were found positive. In previous studies conducted in Turkey, BCoV was reported in significant numbers in cases of calf diarrhea, alongside with many other enteropathogens [22–28]. Besides, various faeces based serological studies shown the prevalence of the BCoV in cattle in Turkey [16, 26, 28–32]. Apart, two studies were present the prevalence of the nasal discharge associated BCoV in Turkey [29, 33]. The prevalence results of this study were found to be consistent with other studies conducted in Turkey. Also, the prevalence of the BCoV in divergent regions of the world is considerably similar with this study and Turkey [5, 34–40].

Numerous studies based on partial or full-length S gene sequences have been conducted to determine the biological, antigenic and genetic characteristics of BCoV strains derived from respiratory system infection and enteric infections [1, 18, 41–42]. Previous studies have been determined that the virus affecting both systems is identical and has dual tropism [5, 17–18]. Examination of the amino acid similarity results of the BCoV S gene region partial / full-length sequences in this study shown that; high similarity rates were found between these studies results between each other (87.4–100%) and with reference strains (94.78–99.11%). Withal, the phylogenetic trees (Fig. 2) constructed using BCoV S gene region partial / full-length sequences in the present study shown that; respiratory or digestive system origin does not make a significant difference in phylogenetic tree placement and amino acid similarity rates were found, which is akin to the other studies worldwide [9, 20, 34–35, 40–41].

The S1 subunit of the S protein is responsible for the binding of the virus to host cell receptors, stimulation of neutralizing antibody synthesis and hemagglutinin activity [12, 15, 43]. Changes in this subunit could be conspicuously effective in terms of antigenicity and pathogenicity [44]. In the present study, BCoV S gene region partial / full-length nucleotide and amino acid variations (Table 3) and phylogenetic trees shown that the S1 subunit is more susceptible to genetic variability than the S2 subunit, as previously reported in Turkey [16] and in the world [18, 45–46].

Recognizedly, mutations ensure significant contributions to the survival of infectious agents in nature. Further, while many mutations would not cause any notable differentiations in the structure of the virus, some could even possibly to lead cross-species transmission or increased contagiousness. One of the most recent and striking examples is that the SARS-CoV-2 S1 region 614 (D → G) mutation cause to increase in its infectivity of the virus [47]. Another, six amino acid deletion (526–531) of the Brazilian BCoV strains have the same amino acid sequence as Human coronavirus (HCoV) OC43 [1]. In this study, multiple changes were observed in the local BCoV S gene amino acid sequences. Moreover, as it is seen on the reference strain Mebus based amino acid variation table, this studies sequences and two previously reported local viruses (GQ259978 and GQ259979) contains some persistent changes on full-length S gene. Additionally, similar persistent changes also apprehensible for different countries (Table 3). Exceptionally, in one sample (BCoV/Turkey/IN/2019 with acc. Number MK989625) these changes were not seen, and it was seen that it has the same sequences as Mebus. Hence, the sample which has the closest position to Mebus in the phylogenetic tree was this sample. The possible reason could be considered to be the vaccination shortly before the sampling but no evaluation could not be doable because no information was obtained on this matter.

Vaccination has a crucial role to protection from infections. Among the factors affecting achieved vaccination, the level of antigenic affinity between the vaccine strain and local strains is exclusively important. The increase in antigenic differences may occur due to serotype differences and variations resulting from spontaneous mutations. Previous studies reported that one amino acid differentiation of BCoV 528 (A→V) may cause resistance to neutralization [12]. In the present study, no 528 (A → V) mutations were found in local BCoVs. Consequently; based on these study's data, present vaccines are protective at a certain level, however, further studies should be conducted on whether amino acid changes in the antigenic domains of local viruses would lead to resistance occurrence or not.

Conclusions

As a result, the full-length BCoV S gene is presented for the first time in Turkey. In evaluating the sequence results, it has been demonstrated once again that the strains of BCoVs affecting both the digestive and respiratory systems are the same. Sequence differences of the local strains and the strains in the previous works of literature were found greatly similar. Moreover, sequence differences of the local strains and the strains used for vaccination were found insignificant. No sample has shown 528 (A → V) mutation which is important for the neutralization and vaccination. Also, no frameshift mutations, deletions, insertions or recombinations have been found. Consequently; this study has shown, BCoV still continues to exist widely in Turkey and present vaccines are protective at a certain level.

Declarations

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Conflict of Interest

All Authors declare that there are no financial or other relationships that might lead to a conflict of interest. All authors have seen and approved the manuscript and have contributed significantly to the work.

Ethics Approval

The study accepted of ethical committee of Ankara University with the document number 2017-15-127.

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Tables

Table 1
Information about the samples used in this study.

City	SamplingDate	Number of samples	Ages of Animals	Samples Species	Clinical Signs
Ankara	2001	2		Faeces	Diarrhea
	2014	1	7 d	Faeces	Diarrhea
	2017	3	2–4 m	Nasal discharge	Nasal discharge, cough
	2018	9	15 d	Nasal discharge	Nasal discharge, cough
	2018	4	8 m	Nasal discharge	Nasal discharge, cough
	2019	5		Nasal discharge	Nasal discharge, dyspnea
	2018	4	8 m	Nasal discharge	Nasal discharge, cough
	2019	7	15 d	Nasal discharge	Nasal discharge, cough, depression
Aksaray	2005	3		Faeces	Diarrhea, dehydration
	2006	2	11–14 d	Faeces	Diarrhea
	2008	2	5–15 d	Faeces	Diarrhea
Eskisehir	2006	3	15–20 d	Faeces	Diarrhea
	2006	1	15 d	Nasaldischarge	Nasal discharge, cough
Kars	2006	3		Faeces	Diarrhea, dehydration
Van	2006	2		Faeces	Diarrhea
Kirklareli	2011	1	70 d	Faeces	Diarrhea, anorexia
Erzurum	2014	2	2 y	Nasal discharge	Nasal discharge, cough
	2017	3	1 m	Faeces	Diarrhea
S. Urfa	2014	3	8–10 d	Faeces	Diarrhea, dehydration
Amasya	2015	2	4 d	Faeces	Diarrhea
Cankiri	2016	1	5 d	Faeces	Diarrhea, dehydration
Izmir	2013	1	14 d	Faeces	Diarrhea, dehydration
	2015	2	15 d	Faeces	Diarrhea
	2017	4	4 y	Nasal discharge	Nasal discharge
	2018	1	15 d	Faeces	Diarrhea

d:day; m:month; y: year

City	SamplingDate	Number of samples	Ages of Animals	Samples Species	Clinical Signs
	2018	3	5 ≥ y	Nasal discharge	Nasal discharge
	2019	2	15 d	Nasal discharge	Nasal discharge, cough
Denizli	2017	5	7–30 d	Nasal discharge	Nasal discharge, cough, anorexia
	2017	9	7 d- 15 m	Faeces	Diarrhea, anorexia, dehydration
Burdur	2019	5	1,5–9 m	Nasal discharge	Nasal discharge
	2019	5	1–1,5 y	Nasal discharge	Nasal discharge, cough
Hatay	2019	4		Nasal discharge	Nasal discharge, cough, dyspnea
Tokat	2018	5	15 d-8 m	Nasal discharge	Nasal discharge, cough
	2019	10	45–90 d	Nasal discharge	Nasal discharge, cough
d:day; m:month; y: year					

Table 2

The accession numbers of the partial gene fragment and full-length sequences of the S gene of local BCoV.

BCoV isolate name	Primer Pair								
	SA	SB	SC	SD	SE	SF	SG	SH	S1
BCoV/Turkey/4950/2015 *	MK989620								+
BCoV/Turkey/4945/2015 *	MK989621								+
BCoV/Turkey/U1/2014 *	MK989622								+
BCoV/Turkey/S8/2017	MK989624								+
BCoV/Turkey/T5/2018	MK989623								+
BCoV/Turkey/G76/2015 *	MK989628				-	MK989629			+
BCoV/Turkey/G83/2015 *	MK989630		MK989631	-	-	MK989632		+	
BCoV/Turkey/H1/2013 *	-	-	MK989633	-	-	-	MK989634		+
BCoV/Turkey/79/2018	MK989627			-	-	-	-	-	+
BCoV/Turkey/8553/2015 *	-	-	-	-	-	-	-	-	MK989626
BCoV/Turkey/1N/2019	-	-	-	-	-	-	-	-	MK989625
S1 partially sequenced region (488 bp) was located in the region amplified with the SC and SD primers, thus, additional accession number was not taken.									
Faeces samples are marked with an asterisk (*).									

Table 3

Amino acid variations BCoV S gene. Asterisk (*) represent amino acids identical to the reference sequence. The strains investigated in this study are indicated by black.

Strain / Isolate Name	458	465	470	484	499	501	509	525	531	543	571	608
BCoV/USA/Mebus/1971	F	V	H	S	N	P	N	H	N	S	Y	D
BCoV/Turkey/1N/2019	*	*	*	*	*	*	*	*	*	*	*	-
BCoV/Turkey/4950/2015	*	A	D	T	S	S	T	Y	D	A	H	G
BCoV/Turkey/4945/2015	*	A	D	T	S	S	T	Y	D	A	H	G
BCoV/Turkey/U1/2014	S	A	D	T	S	S	T	Y	D	A	H	G
BCoV/Turkey/G76/2015	S	A	D	T	S	S	T	Y	D	A	H	G
BCoV/Turkey/G83/2015	S	A	D	T	S	S	T	Y	D	A	H	-
BCoV/Turkey/H1/2013	*	A	D	T	S	S	*	Y	D	A	H	-
BCoV/Turkey/8553/2015	S	A	D	T	S	S	T	Y	D	A	H	-
BCoV/Turkey/T5/2018	S	A	D	T	S	S	T	Y	D	A	H	G
BCoV/Turkey/S8/2017	*	A	D	T	S	S	*	Y	D	A	H	G
BCoV/Turkey/79/2018	*	A	D	T	S	S	T	Y	D	A	H	-
BCoV/Turkey/AN4/2005	S	A	*	T	*	S	*	*	D	*	*	-
BCoV/Turkey/AN11/2005	S	A	*	T	S	S	*	Y	D	G	H	-
BCoV/USA/LY138/1965	S	*	D	T	S	S	*	*	*	*	*	*
BCoV/China/HLJ-14/2015	S	A	D	T	*	S	*	*	D	A	*	*
BCoV/Denmark/05 - 2/2005	S	A	D	T	S	F	*	*	D	A	H	G
BCoV/France/-/1979	S	*	D	T	*	*	*	*	*	*	*	*
BCoV/S.Korea/KCD10/2007	S	A	D	T	S	S	*	*	D	A	*	*
BCoV/Sweden/C-92/1992	S	A	D	T	T	*	T	*	D	*	H	G
BCoV/Italy/339-06/2006	S	A	*	T	T	S	*	Y	D	A	H	G
BCoV/Japan/Kakegawa/1976	S	*	D	T	*	*	*	*	D	*	*	*
BCoV/Vietnamese/PL84/2017	S	A	D	T	S	S	H	*	D	A	H	*
BCoV/USA/LSU/1994	S	A	D	T	S	S	*	*	G	A	*	*
BCoV/Germany/G95/1989	S	A	D	T	T	*	T	*	D	*	H	G
BCoV/Denmark/05 - 3/2005	S	A	D	T	S	F	*	*	D	A	H	G
BCoV/France/EPICaen01/2005	S	A	D	T	S	S	*	Y	D	A	H	G
BCoV/S.Korea/KWD1/2005	S	A	D	T	S	S	*	*	D	A	*	*
BCoV/Sweden/U-09-3/2009	S	A	D	T	S	S	*	Y	D	A	H	G
BCoV/Italy/438-06TN/2008	S	A	D	T	S	S	*	Y	D	A	H	G

Strain / Isolate Name	458	465	470	484	499	501	509	525	531	543	571	608
B CoV/Germany/V270/1983	S	*	*	T	*	*	*	*	D	*	*	*

Figures

Figure 1

The map showing the cities where the farms are located in this study. Grey color indicates the cities where the farms are located, and numbers indicate the sample numbers provided. Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.

Figure 2

The phylogenetic tree constructed using the partial (B and D) or full-length (A and C) S gene sequences of local BCoV from Turkey and reference BCoV strains from other countries. * Phylogenetic trees seen in A and B were prepared using amino acid sequences, those in C and D were prepared using nucleic acid sequences. The strains from faeces and nasal discharge samples in this study are indicated by black circle and square, respectively. The tree was constructed by the Neighbor-Joining method with bootstrap values calculated for 1000 replicates.

Supplementary Files

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