

Full Length Research Paper

Study on differential display gene expression of *Eimeria tenella* multiple resistance strain isolated from Tangshan in chicken

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Total RNA of *Eimeria tenella* drug-resistant strain from Tangshan was extracted with Trizol. Differential-display reverse transcription- polymerase chain reaction (DDRT-PCR) was established by 3 anchored primers and 20 arbitrary primers. The products of polymerase chain reaction (PCR) were analyzed on the denaturing polyacrylamide gels by silver-staining. Ten differential bands were excised from the gels and reamplified with the same sets of primers. The products were purified and ligated with PMDTM18-T Vector, and then the dot-blot hybridization, sequence analysis and homology comparison. The results showed that through comparison of the nucleotide acid sequence, the similarity was 99% among the sequence S116 from mRNA of Tangshan multiple-resistant strain with the sequence 882 bp lengths in the first chromosome of *E.tenella* in Genebank and Sanger, which was an unknown protein. This study paved the way for cloning the full-length cDNAs (Complementary Deoxyribonucleic acid) and finding the molecular mechanism about the drug-resistance of *E.tenella*.

Key words: Chicken, *Eimeria tenella*, mRNA differential display polymerase chain reaction (PCR), denaturing polyacrylamide gel, gene cloning.

INTRODUCTION

Analysis of gene expression is a central aim in most studies in molecular and cell biology. Interests in tissue-specific gene expression and, in particular, the changes in the expression patterns occurring in response to either mutations or transfected genes promoted the search for proper methods to identify the actual differences between two situations (Bauer, 1994). At present, coccidiosis is controlled principally with drugs. However, because of

using coccidiostats for a long time, the drug resistances of *Eimeria tenella* (*E. tenella*) isolated from farms become serious, where resistant spectrum becomes wider and wider, and multiple resistances are very obvious, especially in developed areas of chicken production. It is an inevitable problem that *E. tenella* in poultry will produce drug resistances to commonly used coccidiostats. Since 1950s, Scientists in different countries had made a lot of investigations and researches on drug resistance mechanism. Chapman and Hacker (1994) reported that some *E. tenella* isolated from farms not only generated resistance to a single kind of drug, but also to two or more kinds. One of the reasons, the various characteristics of living organism come out is gene expression difference. Thus, the studies on differential expression genes are contributed to reveal pathogenesis and internal mechanism on differential expression of protein characters. DDRT-PCR was developed by Liang and Pardee (1992) and served as an effective method for identifying differentially expressed genes (Liang et al.,

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Abbreviations: DDRT-PCR, Differential-display reverse transcription- polymerase chain reaction; PCR, polymerase chain reaction; cDNA, complementary deoxyribonucleic acid; dNTP, deoxynucleotide triphosphate; M-MLV, reverse transcriptase; DTT, dithiothreitol; RNA, ribonucleic acid; DEPC, diethylpyrocarbonate; EST, eastern standard time; dT, delirium tremens.

1993; Bauer et al., 1993) where this method was shortcut, convenient, efficient, flexible and low cost. DDRT-PCR technology has a broad application and has made a very significant achievement for plant and animal research (Wang et al., 2009; Feng et al., 2009). Thus, in the experiment, the differentially expressed genes between multiple resistance strain of *E. tenella* isolated from Tangshan and sensitive strain were screened using DDRT-PCR, the aim was to determine the drug resistance mechanism of *E. tenella* at the molecular level.

MATERIALS AND METHODS

Materials

Sporulated oocysts of E. tenella

Sporulated oocysts of *E. tenella* multiple resistance strain isolated from Shijianzhuang (preserved by Key laboratory of preventive veterinary medicine of Hebei Province). Sporulated oocysts of *E. tenella* sensitive strain was kindly provided by Professor An Jian (Beijing Agricultural College).

Primers

Twenty 10 bp random primers were designed according to Han et al. (2006) report Anchor Primers: Oligo (dT) 12AG, Oligo (dT) 12CG and Oligo (dT) 12GG were purchased from Beijing Saibaisheng Genetics Company Limited. Deoxynucleotide triphosphate (dNTP) (250 μ M) was purchased from Sangon Biotech (Shanghai) Company Limited. Reverse Transcriptase (M-MLV) (200 U/ μ l) was produced by Promega Corporation. 5 \times RT Buffer (250 mM pH 8.3 Tris-HCl, 375 mM KCl, 15 MgCl₂, 50 mM Dithiothreitol (DTT) was produced by Promega Corporation.

Methods

Extraction of total ribonucleic acid (RNA)

Sporulated oocysts of *E. tenella* were prepared and purified according to the method reported by Suo (2005) Sporulated oocysts of *E. tenella* (1×10^7 /ml), Trizol (1 ml) and Rnasin (1 μ l) were homogenated in free-glass homogenizer for 30 min, until to 90% oocyst walls had broken down. Total RNA of Sporulated oocysts of *E. tenella* isolated from Tangshan and sensitive strain was extracted and purified using TRIZOL. The integrality of total RNA extracted was analyzed on 1% agarose gels.

Eliminate, quantification and identification of deoxyribonucleic acid (DNA) in total ribonucleic acid (RNA)

Reactions were performed at 37°C for 30 min, and put on ice immediately. Isometric phenol/ chloroform (3:1) were added to the reaction, and gyrated vigorously for 30 sec, put in ice-bath for 10 min, and centrifuged at 14000 rpm at 4°C for 5 min. The supernatant was transferred into another RNase-free eppendorf tube. After 1/10 volume 3 M NaAc and 2.5 times volume absolute alcohol were added to reaction, and mixed, deposited for 30 min at 70°C, and centrifuged at 14000 rpm for 10 min and discarded supernatants,

washed the RNA precipitate with 600 ml 75% ethanol, removed

residual ethanol by centrifugation at 12000 rpm for 2 min, and then added 30 μ l Diethylpyrocarbonate (DEPC) water to re-suspend total RNA. The integrality of RNA was detected by electrophoresis. The absorbance values in OD₂₆₀ and OD₂₈₀ were determined by spectrophotometry to estimate the concentration and purity of total RNA.

Reverse transcription of messenger ribonucleic acid (mRNA)

15 μ l Total RNA, 9 μ l anchor primers and 9 μ l 10 mM dNTPs (2.5 mM) were put into a RNase-free Eppendorf tube, total reaction volume was 60 μ l. Reaction was carried out at 65°C for 5 min, 37°C for 10 min, ice bath for 5 min, and then 24 μ l 5 \times buffer (RT) and 1 μ l RNasin were added into. After 37°C warm bath for 10 min, 2 μ l 200 μ l M-MLV were put in and mixed, 37°C warm bath for 60 min, then M-MLV was inactivated at 70°C for 15 min.

PCR Amplification

PCR amplification was performed using 5.0 μ l production of reverse transcription, and total reaction volume was 25 μ l where 2.0 μ l (10 μ M) OligodT, 2.0 μ l (10 μ M) random primers, 2.0 μ l dNTPs (2.5 mM), 0.3 μ l Taq enzyme, 2.25 μ l 10 \times buffer and 16.45 μ l ddH₂O were put into a RNase-free eppendorf pipe. Reactions were carried out at 95°C for 5 min, 94°C for 30 s, 41°C for 2 min, 72°C for 1 min, for 2 cycles; followed by 38 cycles at 95°C for 30 s, 45°C for 2 min, 72°C for 1 min; and a final extension at 72°C for 7 min.

Denatured polyacrylamide gel electrophoresis and silver staining

6% urea-polyacrylamide was put into vertical electrophoresis apparatus (DYY-III28A) for pre-running at 300 V for 45 min. 7 μ l PCR products were put into an eppendorf tube and 7 μ l 2 \times formamide buffer was added, then was heated at 90°C for 3 min, the tube was put on ice immediately, samples were added, and then electrophoresis at 300 V for 3 to 5 h. Then, the gel was set down for silver staining. The gel was fixed with 500 ml 10% acetic acid for 30 min, rinsed twice with 300 ml ddH₂O and each time 2 min, dyed with 500 ml 0.1% silver nitrate for 30 min, rinsed with ddH₂O for one time, collocated with 500 ml 3% sodium carbonate, and cessation reaction with 10% acetic acid for 10 min.

Recuperation of differential bands and reamplification by PCR

Differential bands were excised from the gels and put into eppendorf tubes. 100 μ l ddH₂O was added into each tube. After these tubes were centrifuged and supernatant was discarded, 30 μ l ddH₂O was added into tubes, and then the gels were crushed, and heated at 70°C for 30 min and oscillated. The sediment was incubated overnight at 20°C, and centrifuged. Amplification reaction by PCR was performed using 5 μ l products. The procedure was at 95°C for 3 min, 94°C for 30 s, 42°C for 2 min and 72°C for 1 min for 30 cycles, followed by 20 cycles at 94°C for 30 s, 52°C for 2 min, and 72°C for 1 min, and a final extension at 72°C for 7 min.

Cloning and identification of reamplified product of the differential fragments

PCR products that were purified from 1% agarose gels according to the manufacturer's specifications of kit (Beijing Tiangen Biotech

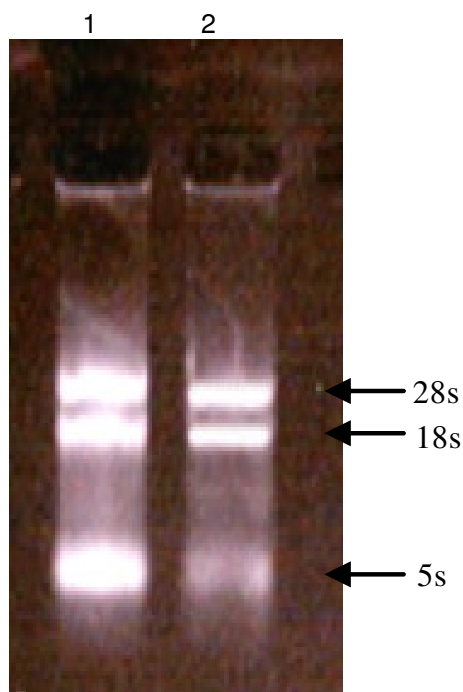


Figure 1. The results of agarose gel electrophoresis of total RNA.

1. Total RNA of sensitive strains of *E. tenella*
2. Total RNA of Tangshan strains of *E. tenella*.

Corporation) using the standard protocol. Then, 15 μ l linked products Transformed into chemically competent DH5 α *Escherichia coli* (Beijing Tiangen Biotech Company Limited.), transformants were selected for 100 μ g/mL ampicillin resistance on Luria-Bertani agar with X-gal/LB/IPTG (60 μ l/plate) Plasmid DNA integrity was confirmed by PCR using universal primers of PMDTM18-T Vector and DNA sequencing. The sequence of cloning product was then verified and homology was compared with Eastern Standard Time (EST) database of GeneBank and Sanger.

Northern blot and sequence analysis

The cDNA of sensitive and Tangshan strains of *E. tenella* were radiolabeled using digoxin, and the effects were detected according to the specifications of kit (Wuhan Boster Bio-Engineering Limited Company). 50 ng cDNAs were denatured for 10 min at 95°C. The samples were transferred to a nylon membrane and then hybridized according to the manufacturer's specifications of kit (Beijing Meilaiobo Hyb efficient hybridization kit).

RESULTS

The extraction, quantification and identification of total RNA

Total RNA were extracted from Tangshan field drug-resistant strain and sensitive strain of *E. tenella* by Trizol. The integrity of total RNA digested was tested with electrophoresis on 1% agarose gel for 10 min at 170 V

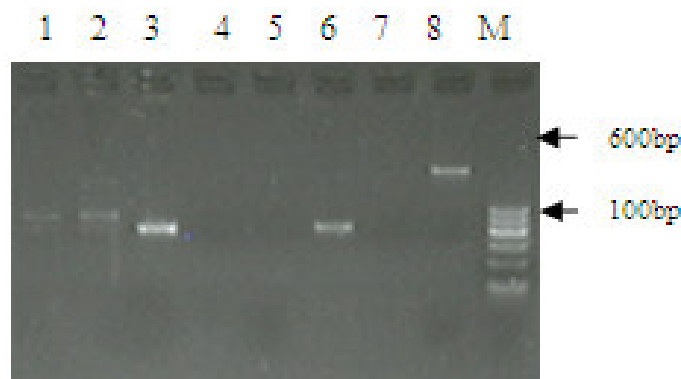


Figure 2. The electrophoresis results of mRNA RT-PCR, M. 600 bp standard molecular weight marker; 1,2,3,6. The products of Shijiazhuang strain by RT-PCR. 8. The products of sensitive strain by RT-PCR; The rest lanes were blank.

results showed that the appearances of 5S band, clear 28S and clear 18S suggested that RNA was complete. The OD value of total RNA in 260 nm and 280 nm were detected, the results showed that, the OD₂₆₀ and OD₂₈₀ of Tangshan drug-resistant strain were 1.065 and 0.587, respectively, R = 1.814; and sensitive strains were 1.135 and 0.602, respectively, R= 1.831. Due to the fact that the R value was from 1.8 to 2.0, the quality of the total RNA extracted and purified was better and could be used for differential display PCR.

The mRNA RT-PCR of sporulated oocysts

The products of reverse transcription were paired with anchored primers and random primers for PCR amplification, the products of PCR were carried out with 1% agarose gel electrophoresis, and the results were observed and photographed by the imager. The products of PCR appeared with different sizes and number bands with different random primers (Figure 2). The PCR products were deemed reliable by gel electrophoresis.

Polyacrylamide gel electrophoresis and silver staining

The majority gene fragment of mRNA was amplified, and bands were clear (Figure 3).

Recuperation of differential fragments and reamplification by PCR

The differential bands were recovered, and then reamplified by PCR. The PCR products were detected by 1% agarose gel electrophoresis (Figure 4). The reamplification products of PCR with a single band were

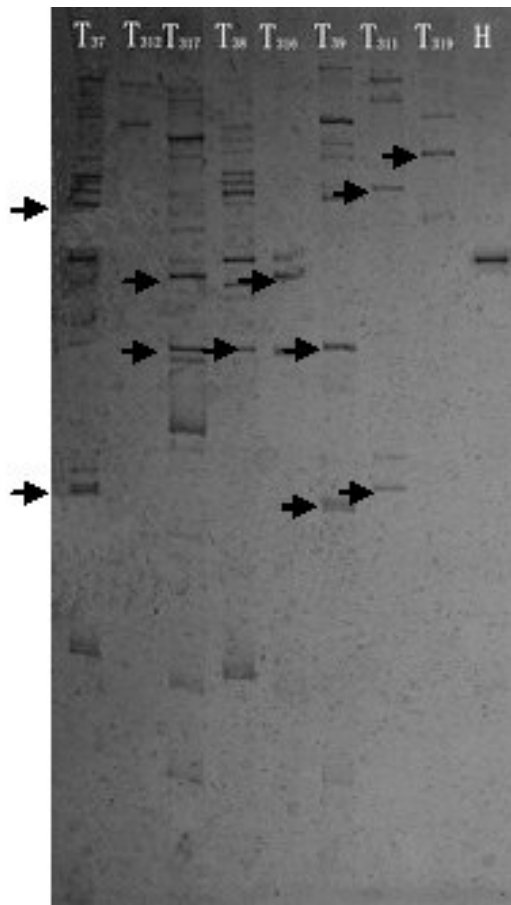


Figure 3. Differential display results of mRNA by silver staining, H. Differential fragments of sensitive strain of *E. tenella*, T. Differential fragments of Tangshan strain of *E. tenella*; ➔ Arrows referred to the differential bands cut; No marked lanes were blank.



Figure 4. The identified results of the differential bands by PCR, M.600bpDNA standard molecular weight; 1-11. The PCR amplification bands of Tangshan strain.

The recovery of differential bands by second PCR

The PCR products were recovered and purified using agarose gel recovery kit (Tiangen Company), and the

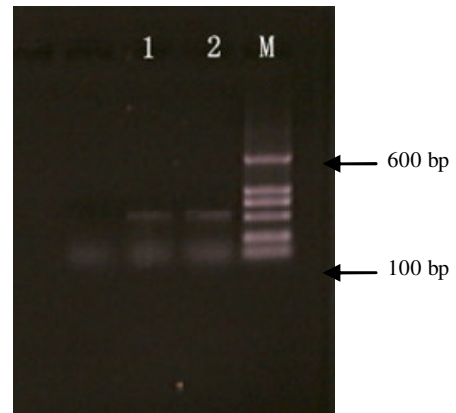


Figure 5. The reamplification PCR products of differential bands, 1-2. Recovered fragments by reamplification PCR; M. DNA standard molecular weight (600, 500, 400, 300, 200 and 100).

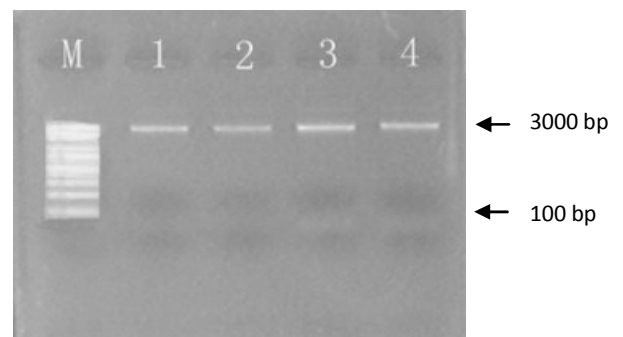


Figure 6. The recovery of recombinant plasmid, M. Maker of 100 to 3000 bp ladder-k DNA molecular weight; 1 to 4.recovered plasmid.

(Figure 5). To compare with the brightness of Marker, the concentration was about 20~50 ng, and the products could be used for identification.

Cloning and identification

PCR products that were purified from agarose gels were cloned into pMDTM18-T Vector, and then the ligated products were transformed into competent cells of DH5α *E. coli*. The plasmids were extracted and then products of 1% agarose gel electrophoresis were identified by blue-white screening. The bands of the positive plasmid were clear and the molecular weight met the requirements (Figure 6). The fragments were amplified and identified by PCR using universal primers of PMDTM18-T Vector. The identification results shown that the sizes of the cloned fragments were as the same as the insert and demonstrated that the PCR products were cloned into

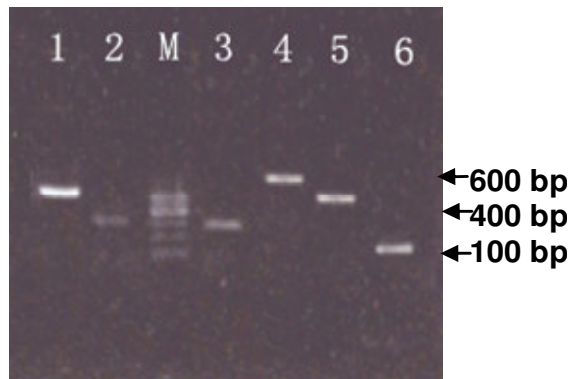


Figure 7. Amplification and identification of the plasmid by PCR, M. Marker of 600 bp DNA molecular weight, 1 to 6 amplification and identification results by PCR.

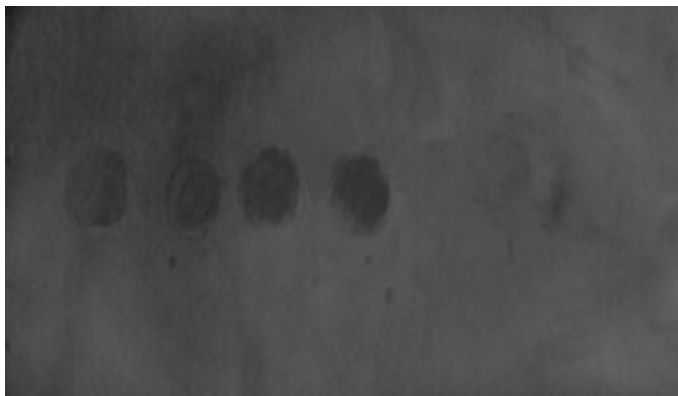


Figure 8. Verified results by northern dot-blotting, black dots were the positive hybridization results of T311.

Reverse northern dot-blotting and sequence analysis

Six plasmid bands of cloning and identification were verified by reverse northern dot-blotting, one positive differential band was found (Figure 8), and then sequenced. The differentially expressed sequence was sequenced in this experiment. Using the United States National Center for Biotechnology Information and Sanger, the homology of the sequence which measured by blast software compared to the known sequences from the NR database in Genbank and EST database in Sanger and the function of sequence was predicted. The similar standards were set at 180 bP overlapping region and sequence similarity $\geq 79\%$ or E value $\leq e^{-30}$ (An Jian, 2004). In order to obtain more reliable results in this experiment. The differentially expressed fragment which had high similarity was analyzed for their structure and function, and speculated the possible relationship with drug resistance of *E. tenella*. By comparison and analysis, T311 sequence had 99% homology with 882 bp sequence in the first chromosome of *E. tenella* which had

Sequence of T311 and comparative results

TGGATTGGTCGACGAATTTTCTGAGCTGAAACTCAAA
CCGCTGTAAGTGGTCATGAGCTACGTTAGTTTCGAGCA
AGCGCCTTGTCTCTTCGCCGTTGATGAACAAAGAG
GTGAGTTGTCCATATTTCGACGACGAGAACAGACTGGT
CGCCCGAGATGCCGGCAAGGGCCAGACTCTCATCG
AACGCATCCTCGGGGAAATCGGTGAGGTCAGTATTG
AGACCACGTTCAACGTACAAACTGTGCCGTGCAGCG
ACACTTTCGCCGATCCCATTGGACCAATCCAA. T311
sequence from the mRNA which was extracted from
Tangshan field of multi-drug resistant strains was 286 bp.
The T311 fragment was analyzed by Blast, the results
showed that $E=3.4 > e^{-30}$, the overlapping length of
fragment was 22 (Identities=22/22), Similarity of the two
sequences was determined to be 100%, $> 79\%$. E values
do not match the range and overlapping areas only 22
bases, much less than 179 base pairs, so the sequence
is the new sequence and may have some contact
resistance of coccidia.

DISCUSSION

Because of the overwhelming majority eukaryotic cells' mRNA with Poly (A+) tail, mRNA of total RNA is only transcribed, when primer Oligo Delirium tremens (dT) pairs bond with total RNA. Han (2007) reported that the detachment of mRNA demanded by purification column, but a part of mRNA was lost during through purification column each time and increased the risk of decomposition. In this study, after total RNA of *E. tenella* was extracted, mRNA was transcribed reversely into cDNA using Oligo dT as a primer by RT-PCR technology, and then preceded by PCR amplification for the aim genes, but the mRNA was not isolated and purified. The DDRT-PCR technique is carried out with cDNA of mRNA reverse transcription of treatment and the control groups as template, using efficient PCR amplification. Through rational design and combination to 5' and 3' terminal primers, the differential expression fragments of cDNA between treatment group and control group could be identified. Mou et al. (1994) discovered that the primer with at least one G was better than that with one C, however, the primer with A or T as ends was inefficient. Thus, in this experiment, 3 anchor primers in 3' terminal, including Oligo dT 12AG, Oligo dT 12CG, Oligo dT 12GG and 20 random primers (10 bp length) in 5' terminal were used. With sensitive strain of *E. tenella* as a control, 10 differential bands were amplified randomly, the effect of the second PCR amplification was ideal, furthermore, the response number was reduced. Consequently, the experimental processes were greatly simplified, and the efficiencies of reverse transcription and the amplification were improved.

At present, the differential display is used more widely, in which denature polyacrylamide gel has the ability to

An Jian et al., 2006). Han (2006) reported that the differential gene screening by the silver-staining mRNA differential display is a rapid, simple, low cost and very effective technique.

Thus, in this test, denature polyacrylamide gel was used to avoid the shortcomings or defects of non-denaturing polyacrylamide gel and agarose gel. The differential bands were displayed by silver staining and had shown successful results. In order to avoid false positive bands of differential display, the mRNA differential display bands by silver staining were cloned into vector and identified, and then verified by reverse northern dot blot (Hao et al., 2002). In this test, 1 cDNA differentially expressed fragment of Tangshan resistant strain of *E. tenella* was screened and might be related with the drug resistance of *E. tenella* by preliminary identification. One differentially expressed sequence was sequenced in this experiment. Using the United States National Center for Biotechnology Information and Sanger, the homology of the S116 was compared to the known sequences from NR database in GeneBank and EST database in Sanger by blast software. In order to obtain more reliable results in this experiment, the similar standards were set at 180 bP overlapping region and sequence similarity $\geq 79\%$ or E value $\leq e^{-30}$ (Wang et al., 2009). The differentially expressed fragments which had high similarity were analyzed on the structure and function, and speculated the possible relationship with drug resistance of *E. tenella*. T311 sequence is the new sequence and may have some contact resistance of coccidian. T311 sequence from the mRNA which was extracted from Tangshan field of multi-drug resistant strain was 286 bp. T311 sequence is the new sequence and may have some contact resistance of coccidia. However, further study on the relationship with drug resistance of some anticoccidial drugs and its function in the genome is required.

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