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RESEARCH ARTICLE

# Screening of Multiple Drug Resistant Genes of *Eimeria tenella* Infesting Chicken in China

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**ABSTRACT**

Total RNA of *Eimeria tenella* drug-resistant strains from Qinhuangdao, Tanshan and Shijiazhuang were extracted with Trizol. DDRT-PCR was established by 3 anchored primers and 20 arbitrary primers. The products of PCR were analyzed on the denaturing polyacrylamide gels by silver-straining. Twenty-six differential bands were excised from the gels and reamplified with the same sets of primers. The products were purified, and 12 differential bands (20□50ng) were recuperated. Twelve plasmid cloning identification differential bands were identified by the dot-blot hybridization. The results showed that 3 bands were positive. And then the positive cloning was sequenced and compared to homology. The results showed that through comparison of the nucleotide acid sequence, the similarity was 99% among the sequence S116 from mRNA of Shijiazhuang multiple-resistant strain with the sequence 882bp length in the first chromosome of *E. tenella* in Genbank and Sanger, which was an unknown protein. The sequence of T311 and Q19 from mRNA of Tangshan and Qinhuangdao multiple-resistant strains were unknown sequences. These novel cDNA fragments associated with drug resistance might be involved in the process of the drug resistances of *E. tenella.*



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## INTRODUCTION

At present, coccidiosis is controlled principally with drugs. However, because of using coccidiostats for a long time, the drug resistances of *E. tenella* isolated from farms become serious, where antimicrobial-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 (Akhtar *et al*., 2012; Abbas *et al*., 2012). 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. Why did the various characteristics of living organism come out, one of reasons 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. Differential display reverse transcription-polymerase chain reaction (DDRT-PCR) was developed (Liang and Pardee, 1992) and served as an effective method for identifying differentially expressed genes (Bauer *et al.,* 1993; Liang *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; Wang *et al.,* 2010; Wang *et al.,* 2011; Li *et al.,* 2012). Thus, in the experiment, the differentially expressed genes between multiple resistance strain of *E. tenella* isolated from Qinhuangdao, Tanshan and Shijiazhuang were screened using DDRT-PCR, the aim was to determine the drug resistance mechanism of *E. tenella* at the molecular level.

## MATERIALS AND METHODS

**Sporulated oocysts of *E. tenella*:** Sporulated oocysts of *E. tenella* multiple resistance strain were isolated from Qinhuangdao, Tanshan and Shijianzhuang (preserved by Key laboratory of Preventive Veterinary Medicine of Heibei Province).

**Primers and RNA extraction:** Twenty 10bp random primers and three anchor primers were designed (Han *et al.,* 2006) by Beijing Saibaisheng Genetics Co, Ltd,

China. Sporulated oocysts of *E. tenella* were purified (Han *et al.,* 2010). Total RNA was extracted from sporulated oocysts, of *E. tenella* using TRIzol (Invitrogen, Foster City, California) from Qinhuangdao, Tanshan, Shijianzhuang and sensitive strain. Total RNA was treated with DNase I (Invitrogen) to remove genomic DNA contamination. The process was performed by routine methods (An *et al.,* 2006).

**Reverse transcription of mRNA and PCR:** The cDNA templates were generated by M-MLV reverse transcriptase (Invitrogen) using anchor primer. cDNA was amplificed using random primers and anchor primer by PCR. The 100-ml amplification reaction mixtures contained 20.0μl production of reverse transcription, 8.0μl (10μM) Oligod T, 8.0μl (10μM) random primers, 8.0μl (2.5mM) dNTPS, 1.2μl Taq DNA polymerase, 8.5μl 10×buffer and 46.3μl sterile water. Reactions were carried out an initial denaturation at 95°C for 5 min, followed by 2 cycles of 94°C for 30 sec, 41°C for 2 min, 72°C for 1 min and then by 38 cycles at 95°C for 30 sec, 45°C for 2 min, 72°C for 1 min; and a final extension at 72°C for 7min then held at 4 C. PCR products were run on 1% agarose gel.

Six percent urea-polyacrylamide was put into vertical electrophoresis apparatus (DYY-III28A) for pre-running at 300V for 45min. Seven microliter (7μl) PCR products were added, and then electrophoresis at 300V for 3.5 hours. Then, the gel was set down for silver staining (Li *et al.,* 2012).

**Extraction of differential bands, reamplification by PCR and cloning:** Differential cDNA were extracted from the gels at 70°C for 30 minutes and oscillated, then was incubated overnight at -20°C. After centrifugation, cDNA was extracted and amplified by PCR again using the same protocol. The PCR products were excised from the gel and purified using the QIA quick®Gel Extraction kit (Qiagen). Purified PCR products were ligated into pGEM-T-easy vector (Promega, Madison, Wisconsin) overnight at 4°C with T4 DNA ligase. Ligations were transformed into *E. coli* DH5α and plated on LB with ampicillin, X-gal, and IPTG. Selected white colonies were grown overnight in LB with ampicillin. Plasmid DNA integrity was confirmed by PCR using universal primers of PMD$^{TM}$18-T Vector and DNA sequencing.

**Northern Blot and sequence analysis:** The cDNA of sensitive and Shijiazhuang, Tangshan and Qinhuangdao 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). Fifty ng of cDNAs were denatured for 10 minutes 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). Then, the sequence of cloning product was verified respectively and homology was compared with EST database of GenBank and Sanger.

## RESULTS

**Quantification and identification of total RNA:** Total RNA was electrophoresed with 1% agarose gel for 10min at 170V, and then observed by the gel imager, the results showed that the appearances of 5S band, clear 28S and clear 18S, this suggested that RNA was complete. The OD value of total RNA in 260nm and 280nm were detected, the results showed that, the OD260 and OD280 of Qinhuangdao drug-resistant strain were 1.235 and 0.637, respectively, R = 1.939; Tangshan drug-resistant strain were 1.065 and 0.587, respectively, R = 1.814; Shijiazhuang drug-resistant strain were 1.109 and 0.608, respectively, R = 1.824; and sensitive strains were 1.135 and 0.602, respectively, R= 1.831. Because R valve 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 and silver staining of sporulated oocysts:** The products of PCR appeared different size and number bands with different random primers (Fig.1). The PCR products were reliable by gel electrophoresis. The majority gene fragments of mRNA were amplified, and bands were clear (Fig. 2).

**Recovery of differential bands by amplification PCR and cloning:** the recovered bands of the PCR products were single and had higher purity (Fig.3). The concentration was about 20~50 ng, and the products could be used for identification.

PCR products were detected using 1% agarose gel electrophoresis. The bands of the positive plasmid were clear, and the molecular weight met the requirements (Fig. 4). The fragments were amplified and identified by PCR using universal primers of PMD$^{TM}$18-T Vector. The identification results shown that the sizes of the cloned fragments were as the same as the insert and demonstrated that the PCR products were cloned into PMD$^{TM}$18-T (Fig. 5).

**Reverse Northern Dot-blotting and sequence analysis:** Twelve plasmid bands of cloning and identification were verified by reverse Northern Dot-blotting, three positive differential bands were found (Fig. 6 to 8), and then sequenced. Three differentially expressed sequences were sequenced in this experiment. To visit 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 NR database in Genbank and EST database in Sanger, and the function of sequence were predicted. The similar standards were set at 180bP overlapping region in order to obtain more reliable results in this experiment and sequence similarity ≥79% or E value ≤e-30. The differentially expressed fragments which had high similarity were analyzed on their structure and function, and speculated the possible relationship with drug resistance of *E. tenella*. By comparing and analysis, S116 sequence had 99% homology with 882bp sequence in the first chromosome of *E. tenella* which had been published, T311 and Q19 sequences were unknown sequence.

**S116 Sequence and comparative results:** S116 Sequence from Shijiazhuang field of multi-drug resistant strains was 503bp and was analyzed by Blast and it had high homology with 882bp sequence of the first chromosome of

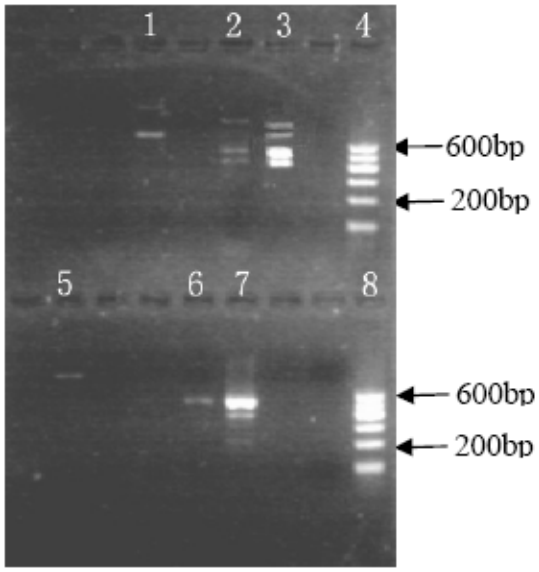


**Fig. 1:** The electrophoresis results after with anchor primers for reverse transcription, RT-PCR was carried out for mRNA using total RNA as a template; 4,8 . 600bp standard molecular weight marker; 1,2 . The products of Shijiazhuang strain by RT-PCR; 3. The products of sensitive strain by RT-PCR; 5. The products of Tangshan strain by RT-PCR.

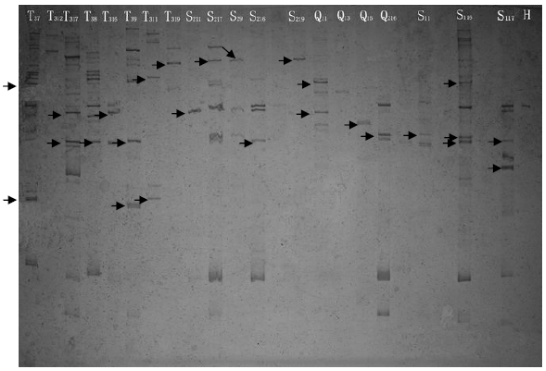

**Fig. 2:** Differential display results of mRNA by silver staining; H. Differential fragments of sensitive strain of *E. tenella;* S. Differential fragments of Shijiazhuang strain of *E. tenella;* Q. Differential fragments of Qinhuangdao strain of *E. tenella;* T. Differential fragments of Tangshan strain of *E. tenella;* ➔ Arrows referred to the differential bands cut; No marked lanes were blank.

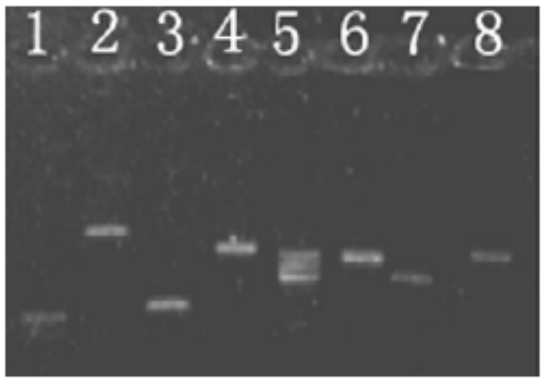


**Fig. 3**: The reamplifiction PCR products of differential bands; 5. DNA standard molecular weight (600, 500, 400, 300, 200, 100); 1-4, 6-8. $S_{219}$, $S_{29}$, $Q_{13}$, $S_{216}$, $T_{311}$, $Q_{19}$, $Q_{15}$ bands by reamplifiction PCR.

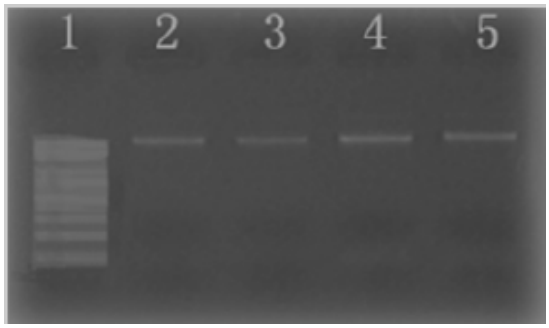


**Fig. 4:** The recovery of recombinant plasmid; 1. Maker of 100-3000bp Ladder-k DNA molecular weight; 2-5. Recovered plasmid.

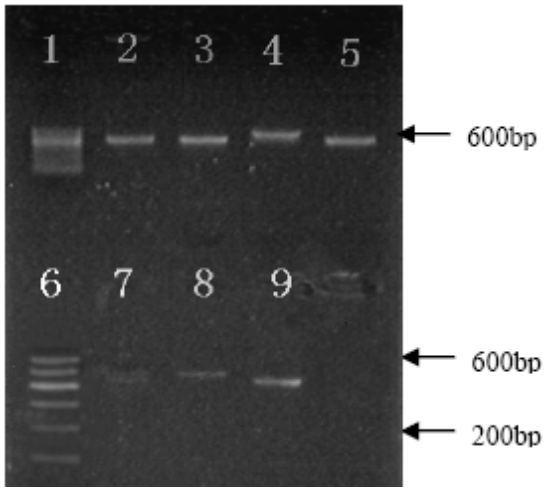


**Fig. 5:** Amplification and identification of the plasmid by PCR; 1,6. Maker of 600bp DNA molecular weight; 2-5, 7-9. Amplification and identification results by PCR.

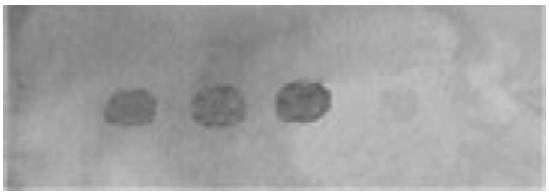

**Fig. 6:** Verified results by Northern Dot-blotting; Black dots were the positive hybridization results of S116.

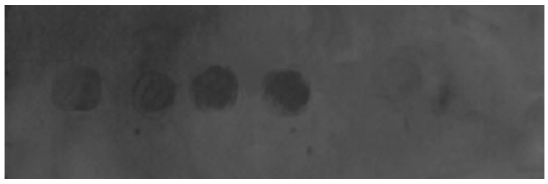

**Fig. 7:** Verified results by Northern Dot-blotting; Black dots were the positive hybridization results of T311.

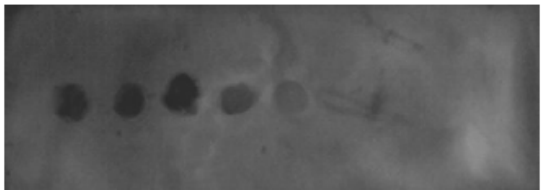

**Fig. 8:** Verified results by Northern Dot-blotting; Black dots were the positive hybridization results of Q19.

*E. tenella*. The results showed that E=5.6e-106 and the value<e-30, the overlapping length of fragment was 500(Identities=497/500), Similarity of the two sequences was 99 and >79%. Thus, the protein was an unknown which may be related with the drug resistance of *E. tenella.* Sequence of S116 was:

**GCTACATTGG**GGAATGGCTGGCGGCCTGCGCTT
TGCGCATGCACTGTTGGCACGCTTCAAAACCGCC
CTCTAGAATTTTGTTCGCCTATTTGACTTCATGGA
CAACTTTAGTAGCTTAGCCTATATGTCTTGGAGC
CTCAGACGATCGCACTGACAGAGTTTCTGTTGTA
GGGAAACGGTACACTAATTCTGAGCCTTTGTCTG
CTTGCACACTCACCGTTTTCGTTACTACTTAGTTC
TCTCTGCCGGCTGAAGGAACCTTGATGATGGTGC
GGGGCAATCCATGCACAGCGTCAGCCCTTTCAGC
CCCTCGCAATTGTTCCCGCAAGGTGCAGTTTGGT
GACTCGGCTGCAGCTGCTGCTCGCGTTTATGTGG
GGCGCTGTACGGCCTGCGGGTTAGAGCGCCTACT
CTCAGCCCAGGCTGGAACCAAGAAACTACTTGAG
GGCAGTGGCCTTCTCCACAACTCCGGCAAAGAGC
AGAAACCTATATGTGCCAATGCAGCA

**Sequence of T311 and comparative results:** T311 sequence from Tangshan field of multi-drug resistant strains was 286bp and 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 and >79%. E values didn't match the range and overlapping areas only 22 bases, much less than 179 base pairs, so the sequence was the new sequence and may have contact with the resistance of coccidia. Sequence of T311 was:

**TGGATTGGTC**GACGAATTTTCTGAGCTGAAACT
CAAACCGCTGTAAGTGGTCATGAGCTACGTTAGT
TCGAGCAAGCGCCTTGTCCTCTTCGCCGTTGATG
AACAAAGAGGTGAGTTGTCCATATTCGACGACGA
GAACAGACTGGTCGCCCGAGATGCCGGCAAGGG
CCAGACTCTCATCGAACGCATCCTCGGGGAAATC
GGTGAGGTCAGTATTGAGACCACGTTCAACGTAC
AAACTGTGCCGTGCAGCGACACTTTCGCCGATCC
CATTGGACCAATCCAA

**Sequence of Q19 and comparative results:** Q19 sequence from Qinhuangdao field of multi-drug resistant strains was 435bp, and 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 didn't match the range and overlapping areas only 22 bases, much less than 179 base pairs, so the sequence was the new sequence and may have contact with the resistance of coccidia. Sequence of Q19 was:

**GCTCATGGTC**GCCAAAGAAAACTTTGCGCATCA
TTGCATATATGCCATGCATGCTATCGGTGCATGA
TAGTGTAAAGGAGTTTCAATTTATGTACAGCGAA
GCATGGTTCTGCATTAGCGTCTGTGTCTGCGATG
GGTCGGCAATTTTCGTTGTCGGTGAAGTTGCATT
ATCTGCAGGAAAAACGAACAGTGTTGAAATTTTT
CAAGGCTATTTACAAGCCTTTAGCTCCACGCTTA
TGTTTTGTTTGCTCATTTGAGGTGCCAAGAAAGC
AGAATGCCACAACAAGCCCCAATGCGTGGCAAC
CATTCCATGTGGCTGAAAAATCGTATGCCCGCCG
TTTTAGCCGTACGGTTCCCCTAATCAAGGTTACC
ATTAGTGTGTTTAAAAACTGGACTAGACCATGAG
CAATCTCTAGAGGTCCCCGGTACCGAGCT

## DISCUSSION

Because of the overwhelming majority eukaryotic cell's mRNA with Poly (A+) tail, mRNA of total RNA is only transcribed, when primer Oligo (dT) pairs bond with total RNA. Han *et al.* (2010) 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* were extracted, mRNA were transcripted reversely into cDNA using Oligo (dT) as primer by RT-PCR technology, and then proceeded PCR amplification for the aim genes, but the mRNA were 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 reamplification PCR was ideal, and 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 distinguish 50~100 differential band (Han *et al.,* 2006; An *et al.,* 2006). Han *et al.* (2010) 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-blotting (Hao *et al.,* 2002). In this test, one cDNA differentially expressed fragment of Shijiazhuang resistant strain of *E. tenella* was screened and might be related with the drug resistance of *E. tenella* by preliminary identification.

Three differentially expressed sequences were sequenced in this experiment. Visiting to the United States National Center for Biotechnology Information and Sanger, the homology of the S116, T311and Q19 were compared to the known sequences from NR database in

Genbank and EST database in Sanger by blast software. In order to obtain more reliable results in this experiment, the similar standards were set at 180bP overlapping region and sequence similarity ≥79% or E value ≤e-30 (Dong *et al.*, 2011). The differential expressed fragments which had high similarity were analyzed on the structure and function, and speculated the possible relationship with drug resistance of *E. tenella*. S116 sequence had high homology with the *E. Tenella* which had been deposited on the Genbank and Sanger, T311 and Q19 sequence were unknown sequence and may have some contact resistance of coccidia.

S116 sequence from the mRNA which was extracted from Shijiazhuang field of multi-drug resistant strain had high homology with 882bp sequence in the first chromosome of *E. tenella*, and the similarity reached 99%. Thus, the protein was an unknown protein which might be related with the drug resistance of *E. tenella*. However, it needs to be studied further on the relationship with drug resistance of some anticoccidial drugs, and its function in the genome.

T311 and Q19 sequences were unknown sequence from the mRNA which was extracted from Tangshan and Qinhuangdao field of multi-drug resistant strain respectively. The two sequences which might be related with the drug resistance of *E. tenella*. However, T311 and Q19 need to be studied further on the relationship with drug resistance of some anticoccidial drugs, and its function in the genome is required.

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