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Full Length Research Paper

Differential expression of ATP-dependent RNA helicase gene in viable but nonculturable *Salmonella pullorum*

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The purpose of this study was to screen new gene involved in viable but nonculturable (VBNC) *Salmonella* using messenger ribonucleic acid (mRNA) differential display. Strain cultured on liquid lysogeny broth (LB) medium at 4°C was induced for VBNC state; activated genes were detected using mRNA differential display and differential fragment of adenosine triphosphate (ATP)-dependent RNA helicase *rh1B* gene was verified. The fragment coded adenosine triphosphatase (ATPase) structure domain of DEAD-box helicase. The screened fragment which codes ATPase structure domain of DEAD-box helicase may be a candidate marker for VBNC *Salmonella* detection.

Key words: Viable but nonculturable, *Salmonella*, ATP-dependent RNA helicase.

INTRODUCTION

Salmonella is an important food-borne pathogen that influences public health greatly. Its outbreak is associated with consumption of contaminated fresh, minimally processed foods, poultry meat, contaminated water and undercooked eggs (Plym-Forshell and Wierup, 2006; Naugle et al., 2006; White et al., 2007; Mataragas et al., 2008). Reactive arthritis may occur after *Salmonella enterica* infection in United States (Townes, 2010).

Viable but nonculturable (VBNC) refers to a physiological state, under which bacteria cannot be detected by conventional culture methods due to no growing on routine media and they retain viability and capability of resuscitation under favorable conditions (Oliver, 2010). *Salmonella* enters into VBNC state when it is exposed to inalimental conditions or is cultured at low temperatures (Gupte et al., 2003; Oliver et al., 2005; Xu et al., 2008; Hayes and Low, 2009). To our anxiety, most VBNC pathogenic salmonella retain infection potential to threaten human health (Sun et al., 2008). VBNC state may be regulated by one or a group of genes which has low expression, even no expression as salmonella is in rapid growth or is not exposed to pressure, while these genes are expressed in natural environment and artificial uncomfortable environment (Dunaev et al., 2008; Cenciarini-Borde et al., 2009; Lai et al., 2009). Therefore, to explore genes related to VBNC state conduces to detecting pathogenic VBNC salmonella is used in order to prevent its infection. Jolivet-Gougeon et al. (2006) has targeted on virulence gene in normal and VBNC salmonella that virulence gene is inhibited in VBNC process. However, this strategy has to be based on known genes in *Salmonella*.

This study was directly to screen differentially expressed VBNC-associated genes in *Salmonella pullorum* (an indicator of inspection and quarantine) using messenger ribonucleic acid (mRNA) differential-display reverse transcription-polymerase chain reaction (DDRT-PCR). The screened gene might be a candidate marker for VBNC salmonella's detection.

MATERIALS AND METHODS

Stains and reagents

S. pullorum (CVCC578 reference strain) was purchased from China Institute of Veterinary Drug Control. Salmonella-Shigella (SS) medium was purchased from Japan RongYan Chemical

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Table 1. Sequences of primers for RT-PCR.

Anchor primers	Sequences (5' 3') →	Random primers	Sequences (5' 3') →
A1	TTTTATCCAGC	S1	GCTGGAAAAA
A2	ACTTTACGCAG	S2	GCTGCTGGCG
A3	TTTATCCAGCG	S3	GAAGTGCTGG
A4	TCAGCGTTTTA	S4	TGGCGGCGGC
A5	TTTCAGCGCCT	S5	AACTGGCGAA
A6	TTTTTTCAGCA	S6	ATGCGCTGGC
A7	TCTTTTTTACC	S7	TGCCGATGAA
A8	ATCATCCAGCA	S8	CTGGAAGAAG
A9	TTTTACCCAGC	S9	ATGGCGCTGG
A10	TTCAGCCAGCG	S10	ATGGCGATGA

Corporation; Live/Dead® Baclight™ Bacterial Viability Kit was from Molecular Probes. BioRT complementary deoxyribonucleic acid (cDNA) first strand synthesis kit was from Bloar Technology; AMV reverse transcriptase was from Promega Company (USA); DIG DNA Labeling and Detection Kit and DIG Easy Hyb was from Roche Company (Switzerland). Primers (Fislage et al., 1997) were completed by TaKaRa Company, Dalian, China (Table 1).

Induction of VBNC state

The procedure was according to Gupte et al. (2003) with a little modification. Briefly, strains were inoculated into SS liquid medium overnight in 37°C ventilation with 200 rpm of swirling. When OD_{600nm} was about 0.7, the sample was precipitated by centrifugation at 6,000×g for 5 min. The pellet were washed twice by 0.85% sodium chloride (NaCl), then inoculated into 100 ml of liquid lysogeny broth (LB) medium at concentration of 10^8 CFU/ml and cultured at 4°C aerobic conditions. Culturable bacteria were counted by plating on LB solid medium every five days. Viable and death states were detected using Live/Dead kit. VBNC state bacteria were defined as viable state bacteria minus culturable bacteria. The total bacteria were sum of viable and death states bacteria.

mRNA differential display

VBNC bacteria and normal bacteria (20 ml respectively) were precipitated by centrifugation. Total RNA was extracted using RNAiso Reagent (Luo et al., 2004). Total RNA (5 µL) was incubated at 70°C for 5 min, and was immediately placed in ice bath and supplemented according to BioRT cDNA Kit manufacturer's instruction as following: 0.5 µL of ribonuclease (Rnase) inhibitor (40 U/µL), 2 µL of 5*RT-Buffer, 0.5 µL of AMV reverse transcriptase (5U/µL), 2 µL of dNTP (10 mmol/L), 0.5 µL of random hexamer primer (10 pmol/µL) and filling RNase-free water to total 20 µL. The complex system was incubated at 42 and 95°C and in ice bath for 45 and 5 min, respectively for cDNA synthesis.

PCR amplification was run using combined anchor and random primers (Table 1). Total 50 µL of PCR system contained 2 µL of cDNA template, 2 µL of deoxyribonucleotide triphosphate (dNTP) (25 mmol/L), 1.5 µL of magnesium chloride [$MgCl_2$ (25 mmol/L)], 2.5 µL of Taq buffer, 1.5 µL of anchor primer (10 pmol/µL), 2.5 µL of random primer (10 pmol/µL), 0.5 µL of ex-Taq enzyme (10 U/µL), and filling double distilled water to 50 µL. PCR program contained pre-denaturation at 96°C for 8 min; 30 cycles (96°C, 40s; 38°C, 50s; 72°C, 40s); 72°C, 10 min and 15°C, 10 min. PCR products were separated on 2% agarose gel.

Second PCR amplification

Reaction system components were referred to first PCR except using 2 µL of first PCR products as template, 1 µL of dNTP (25 mmol/L), 1 µL of anchor primers (10 pmol/µL), 1 µL of random primers (10 pmol/µL) and 0.5 µL of ex-Taq enzyme (10 U/µL). PCR programs were referred to first PCR except using a cycle parameter 96°C, 40s; 50°C, 50s and 72°C, 40s. Second PCR products were separated on 1% agarose gel for cloning and hybridization and products were extracted and ligated with pMD18-T vector, followed with gene transfer into *Escherichia coli* DH5α and sequencing.

Reverse northern blotting

According to DIG DNA Labeling and detection kit manufacturer's instructions, cDNA were labeled as probes. Labeled probes were incubated at 94°C for 5 min and were immediately added to hybrid liquid in ice bath. Second PCR amplified fragments (1 µL) were blotted on nylon membrane, followed with incubation at 80°C for 40 min and incubation with prewarmed DIG Easy Hyb at 45°C for 30 min. Membrane was immersed in hybrid liquid containing probes and was incubated at 45°C for 16 h, followed with washing, immunostaining, and color development. Chromogenic reaction was ceased by adding Tris- ethylene diamine tetra-acetic acid [EDTA (TE)] solution (pH8.0). PubMed data base was retrieved for sequence analysis.

RESULTS

Bacterial status

Bacteria entered into VBNC state at time point of 55 days, VBNC state was maintained during 55 to 180 days. Strains were transformed from VBNC state to death stage after 180 days (Figure 1).

mRNA differential amplification

Primer combinations A5/S3 and A7/S2 could amplify cDNA fragments of VBNC and normal bacteria. From VBNC bacteria, fragment were amplified using A5/S3

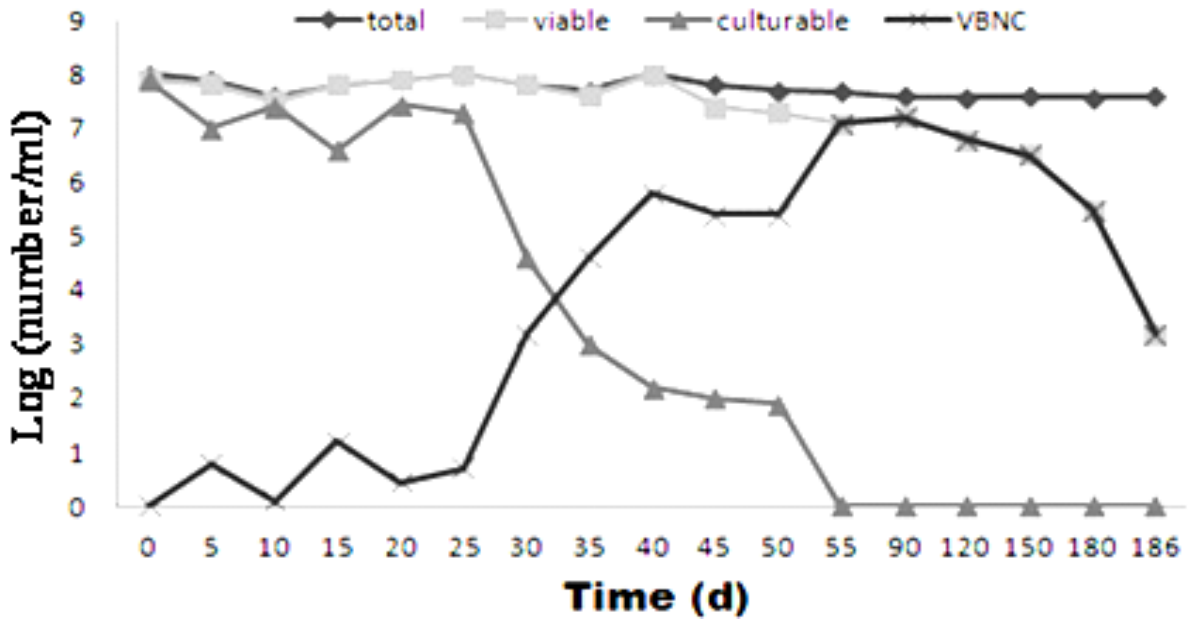

Figure 1. The curve of bacteria count and time.

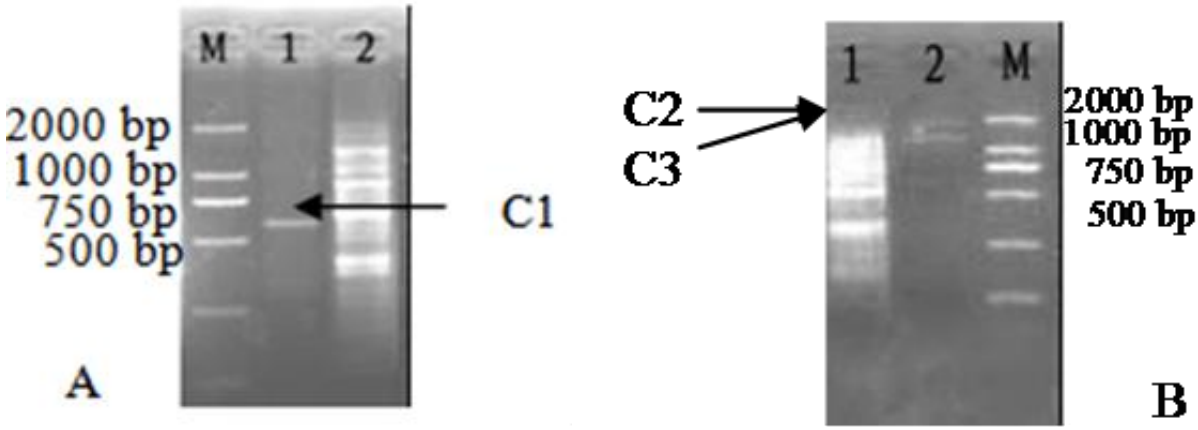

Figure 2. a) RT-PCR amplification, lane 1, VBNC strain, primer A5/S3; lane 2, normal strain, primer A5/S3; M, DL2000; C1, about 600 bp; b). Lane 1, normal strain, primer A7/S2; lane 2, VBNC strain, primer A7/S2; M, DL2000; C2, 2,000 bp; C3, 1,500 bp.

primers (C1; Figure 2a, lane 1) about 600 bp. 2,000 bp (C2) and 1,500 bp (C3) fragments were amplified using A7/S2 primers (Figure 2b, lane 2). These fragments were not present in normal bacterial gel and were specific fragments in VBNC bacterial gel. Thus C1 to C3 fragments were considered to be differential fragments. PCR amplification was repeated on cDNA template of VBNC and normal bacteria using two pairs of primers combinations above mentioned. A5/S3 primers amplified consistent mRNA differential display images indicated that the repeatability of fragments was high, and the detecting positive rate was higher than 86%. But A7/S2 primers indicated the repeatability of fragments was low, that was just 48% positive detecting rate.

Second PCR amplification

Second PCR amplification using A5/S3 primers repeatedly produced differential fragment C1 without noise as first PCR amplification.

Reverse northern blotting

Second PCR products containing differential fragment

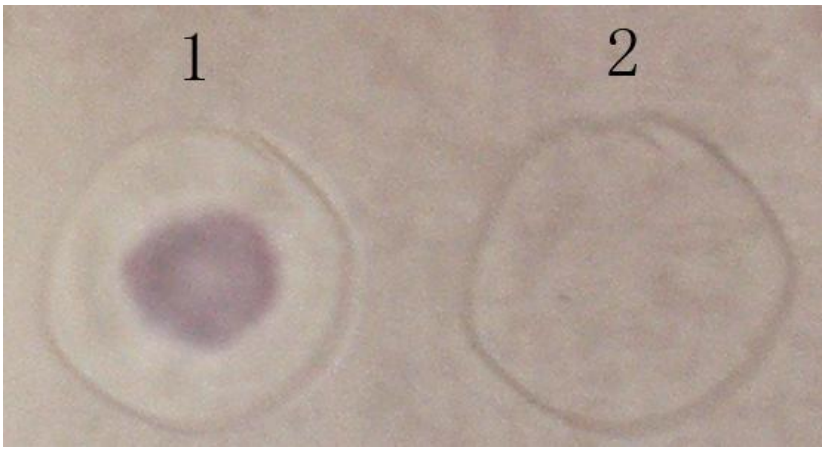

Figure 3. The hybridization of C1 differential fragment with VBNC (1) and Normal (2) cDNA probes by reverse northern blotting.

were blotted on nylon membrane, followed with hybridization to cDNA probes of VBNC and normal bacteria respectively (Figure 3). Second PCR purification products of C1 differential fragments were positive to VBNC bacterial cDNA probes, negative to normal bacterial cDNA probes. This result confirmed that the fragment was the major differential gene expressed by VBNC salmonella.

Sequence analysis

Blast showed that 573bp cDNA sequence of C1 differential fragment (EMBL Database: HM037252) coded 191 amino acids. This cDNA sequence had a homology of 95 to 100% to the nucleotide of adenosine triphosphate (ATP)-dependent RNA helicase *rh1B* gene in different salmonella and the amino acid homology was more than 98%; but the homology was less than 88% to the nucleotide in *Enterobacteriaceae* such as *E. coli, Shigella* sp. *and Yersinia* sp. Cordin et al. (2006) provided the amino acid sequence of DEAD-box helicase. Alignment analysis showed that the amino acid sequence encoded by C1 differential fragment contained four conservative motifs of DEAD-box helicase, *Q-motif*, *moti I*, *moti Ia* and *moti II* (Figure 4). The four motifs are highly conservative during evolution and are the main components of helicase structural domain I (namely ATPase). C1 differential fragment was assigned to ATP-dependent RNA helicase *rh1B* gene in *Salmonella.* The differential gene was extensively present in different strains of *Salmonella* and was a sort of highly conservative species genes in *Salmonella*.

DISCUSSION

The fragment which codes ATPase structure domain of DEAD-box helicase was verified using mRNA differential display in VBNC salmonella. This gene is promising to be a candidate marker for *Salmonella's* VBNC state. By detecting this gene, VBNC salmonella can be determined to avoid possible salmonella infection.

Currently, the widely accepted criteria for bacterial VBNC originates from the VBNC concept proposed by Xu et al. (1982). Bacteria in adverse environment contract into spherical shape and can not proliferate, but are still living when cultured using the conventional method (plate method, the maximum possible approximation method). This is a kind of special form that bacteria can recover culturable state in appropriate conditions. Gupte et al. (2003), has extensively described VBNC state to help us understand it deeply. In fact, the conventional method fails to detect VBNC state. Thereby VBNC state puts animal products or food at risk because of the potential pathogenicity.

With the help of molecular biological technology, specific gene expression in VBNC can be revealed. The *rh1B* gene which codes ATPase-dependent DEAD-box RNA helicase can unfold bacterial double-stranded RNA and is associated with the basic cellular process (Cordin et al., 2006). Recent studies demonstrated that *rh1B* gene can make bacterial survive in cold shock conditions and grow at low temperatures, perhaps it is because it plays an important role in removing the cold-stable subprime structure in mRNA molecules (Cordin et al., 2006). In the present study, differential fragment of *rh1B* gene, which will not be expressed in normal condition, was verified to mark VBNC salmonella. This finding conduces to the detection of VBNC *Salmonella* at molecular level to avoid infection of potential pathogensis. In addition, the detection shall be performed by experienced operators in laboratory.

Random primers used in this study are designed according to repeating sequence of high frequency in *E. coli* genomes; these random primers exhibit high

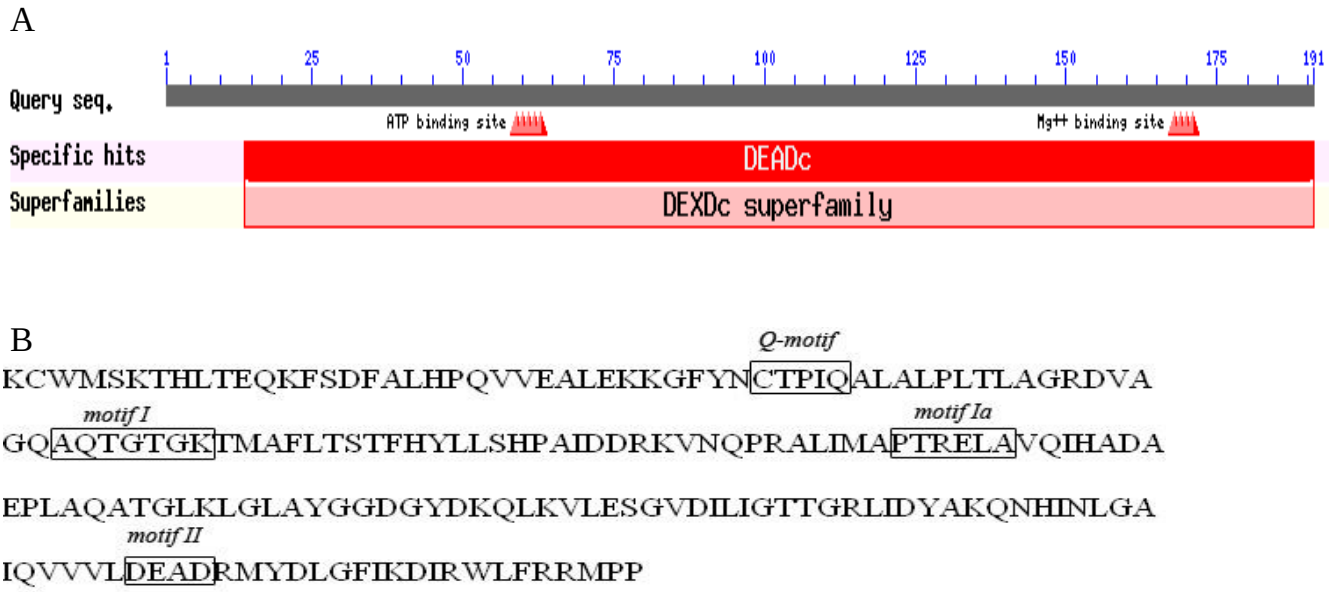

Figure 4. NCBI conserved domain search result of deduced amino acids sequence of differential fragments. a) Conserved domain search result of C1; b) DEAD-box RNA helicases's conserved motifs in deduced amino acids sequence of C1. Conserved motifs of DEAD-box RNA helicases are underlined. RNA, Ribonucleic acid.

amplification efficiency in salmonella (Fislage et al., 1997) and thus are likely to amplify inducible genes which are expressed in VBNC *Salmonella*. As results, DDRT-PCR produced numerous random-distributed amplification fragments in normal salmonella and little fragments in VBNC salmonella. The random primers seem to be VBNC "specific". This finding suggests that salmonella promotes new specific genes, which will not be expressed in normal condition, to resist adverse condition.

Whether *rh1B* of VBNC chicken diarrhea salmonella are specific to all bacterial VBNC? Can gene be expressed in other VBNC salmonella? If confirmed, as pathogens to endanger human health, the genetic detection of VBNC *Salmonella* will be feasible, and the underlying mechanism of *Salmonella's* VBNC state can also be comprehensively clarified. Therefore, the specificity and extension of *rh1B* in other VBNC salmonella will be studied in the future.

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