# Tilapia Heat Shock Protein: Molecular Cloning and Characterization

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## Abstract

A new member of heat-shock protein family (HSP 9) identified in Tilapia *Oreochromis niloticus* immunized by killed *Flavobacterium columnarae*. Suppressive subtractive hybridization (SSH) was utilized to construct a cDNA library and a semi-quantitative RT-PCR analysis used to examine HSP9 immune gene regulation. *O. niloticus* heat shock protein (ONHSP9) cDNA composed of 1228 bps with a 1167 bps open reading frame, the predicted gene product is 389 amino acid with molecular weight of 42.1 kDa, it shares 92% similarity with that of *Danio rerio*. Compared to ß-actin, the semi-quantitative RT-PCR revealed that ONHSP9 expressed in tissues of stimulated fish as up-regulated gene suggesting that this member of heat-shock genes is probably involved in the general immune response against the pathogenic bacteria. [Nature and Science. 2009;7(2):46-57]. (ISSN: 1545-0740).

*Keywords*: Tilapia, cDNA library, Immune genes, Heat-shock protein.

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### *Introduction*

Heat shock proteins (HSPs) are widely distributed in nature, and are highly conserved proteins among prokaryotes and eukaryotes (Lindquist, *et al*., 1986). They are known to be involved in immune responses to many bacterial and parasitic pathogens (Kaufmann *et al*., 1990, 1994), and in the pathogenesis of some types of autoimmune diseases (Mo¨ller, 1991). HSPs have long been suspected to act as danger signals (Young *et al*., 1990 and Van Eden *et al*., 1988). Initially described as a family of intracellular proteins essential for various vital cell functions (Raulet, 1989 and Ohga *et al*, 1990), HSPs have later been demonstrated to stimulate potently immune responses. HSPs are attractive as danger signals because they are evolutionarily highly conserved, constitutively present in virtually all body cells and upregulated and released in response to cellular stress. In infections, this release could support induction of immunity. HSPs release, however, is not specific to infections but also occurs in response to physical stimuli, such as heat or irradiation, and in noninfectious inflammatory diseases (Ohga *et al*, 1990); so, members of the HSPs family are candidate molecules that potentially signal tissue damage or cellular stress to the immune system. The expression of HSPs is up-regulated rapidly during several forms of cellular stress and HSPs can be released from damaged tissue. Thus, in recent years, substantial interest has focused on the interplay of HSPs with the immune system (Wallin, *et al*., 2002).

Bacterial infection induces changes in the expression of host cell genes. A global knowledge of these modifications should help to better understand the bacteria / host cell interactions. The host response to bacterial stimulation represents a complex coordination of gene products, which are precisely turned to activate or inactivate specific pathways and finally counteract the effects of the bacterial antigen. *Flavobacterium columnare* has been recognized as a worldwide pathogen of freshwater fish, it is the etiological agent of columnaris disease, characterized by gill necrosis, greyish white spots on the body, skin erosion, and fin rot (Annemie ***et al.***, 1997).

With the use of large-scale screening of mRNA changes, it is possible to define changes in gene expression that underlie the host response to bacterial pathogens and to gain specific insights into the molecular nature of the host pathways that govern bacterial pathogenesis. Subtractive cDNA hybridization has been a powerful approach to identify and isolate cDNAs of differentially expressed genes (Tsoi *et al*., 2004, Chang *et al*., 2005). Numerous cDNA subtraction methods have been reported in general, they involve hybridization of cDNA from one population (tester) to excess of cDNA from other population (driver) and then separation of the unhybridized fraction (target) from hybridized common sequences. SSH is used to selectively amplify target cDNA fragments (differentially expressed) and simultaneously suppress non target DNA amplification. In this regard, to obtain a more comprehensive view of the tilapia response and to identify the immune genes and their expression in response to *Flavobacterium columnarae*, a model of stimulation, we used the subtractive suppressive hybridization and the semiquantitative RT-PCR methods.

***Materials and methods***

Tilapia *Oreochromis niloticus* were obtained in September 2007 in Wuhan, Hubei province, China. The fish were acclimatized in an aerated freshwater tank at room temperature under natural photoperiod for a week. They were stimulated for four days each with intraperitoneal injection of $10^7$ *Flavobacterium columnari*, killed using 0.5% formaldehyde in PBS. A healthy fish was injected with PBS and used as a control. Two stimulated tilapia, 24.5 cm and 27 cm in fork length were selected with the gills, liver; spleen, head kidney and intestine were dissected out for the construction of the infected group subtracted library. One healthy fish 23.7 cm in fork length was used also for the subtracted library with the mentioned organs dissected out. All samples were stored in -70°C for further use

**Total RNA and poly $A^+$ RNA isolation**

Total RNA was prepared for RT-PCR detection using Trizol Reagent (Invetrogen, USA). About 1.5 g of mixed tissues containing approximately equal amount of different formally mentioned tissues from both infected and uninfected fish were used as tester and driver samples, respectively. The mRNA was then isolated from total RNA using the poly Atract Isolation System (Promega). Concentration of mRNA was determined using a spectrophotometer.

**Driver and tester Preparation (SMART cDNA synthesis)**

Driver cDNA was synthesized from 2 μg. each of different tilapia poly (A) RNA using the Great Lengths manufacture's instructions. First- and second-strand cDNA synthesis and blunt-ending of DNA ends by T4 DNA polymerase were carried out according to the manufacturer's protocol. The resulting cDNA pellet was dissolved in 50 μl of deionized water and digested by *Rsa*I in a 50 μl reaction mixture containing 15 units of the enzyme for 6 h. at 37°C. The cDNAs were then extracted, precipitated, by column using the DNA binding and washing buffers, and resuspended in 5.5 μl of deionized water.

Tester was Prepared as described above for the driver. Digested tester cDNA (1 μl) was diluted in 5 μl of H2O. The diluted tester cDNA (2 μl) was then ligated to 2 ml of adapter 1 and adapter 2 (10 mM) and 6ml ligation mixture (2μl 5x ligation buffer, 1μl T4 enzyme ligase, 3μl H2o) in separate ligation reactions in a total volume of 10 μl at 16°C overnight, using 0.5 units of T4 DNA ligase (Life Technologies) in the buffer supplied from the manufacturer. After ligation, 1 μl of 0.2 M EDTA was added and the samples were heated at 70°C for 5 min to inactivate the ligase and stored at 20°C.

**Analysis of ligation:**

Ligation efficiency was examined using beta-actin forward primer (1μl) and reverse (1μl) for tester 1 (ligated adaptor 1) and tester 2 (ligated adaptor 2) in a PCR mix. Of 22 μl. to verify that a proper quantity of the cDNAs has adaptors on the both ends.

**Suppression Subtractive Hybridization and PCR Amplification**

1.5 microliters of Rsa 1 digested driver dscDNA was added to each of two tubes containing 2μl of adaptor 1- and adapter 2-ligated tester cDNA. The samples were mixed and resuspended in 1.0 μl of 4X hybridization buffer (CLONTECH). The solution was overlaid with mineral oil, the DNAs were denatured (1.5 min, 98°C), and then allowed to anneal for 10 h at 68°C. After this first hybridization, the two samples were combined in a second hybridization and a fresh portion of heat-denatured driver (1μl) in 1.0 ml of hybridization buffer in a total volume of 4 μl was added. The sample was allowed to hybridize overnight at 68°C. The final hybridization was then diluted in 200 ml of dilution buffer (CLONTECH).

For each subtraction, we performed two PCR amplifications. The primary PCR was conducted in 25 ml. It contained 1 μl of diluted, subtracted cDNA, 1 μl of PCR primer P1 (10 μ M), and 24 ml of PCR master mixture prepared using the PCR-select $^{TM}$ cDNA subtraction kit (CLONTECH). PCR was performed with the following parameters: 94°C for 5min; 30 cycles

at (94°C for 30 sec; 66°C for 30 sec; 72°C for 1.5 min); and a final extension at 72°C for 10min. The amplified products were diluted 9-fold in deionized water. Some of the product (1 μl) was then used as a template in secondary PCR using the nested PCR primer PN1 and PN2 and performed with the following conditions, 94°C for 5min; 13 cycles at (94°C for 30 sec; 68°C for 30 sec; 72°C for 1.5 min); and a final extension at 72°C .

**PCR analysis of subtraction efficiency**

Beta-actin was used as a positive control to confirm the reduced relative abundance of SSH libraries following the PCR selection procedure. The beta-actin gene primers were designed according to the conseved domains in Tilapia nilotica, forward: CGAGGGTTATGCCTTGCC and reverse: TGTAGGTGGTTTCGTGGATT. PCR condition was 94°C for 5min; 35 cycles at (94°C for 30 sec; 56°C for 30 sec; 72°C for 40 sec); and a final extension at 72°C . 5 μl PCR product from each reaction for 15, 20, 25, 30, and 35 cycles were represented on 2% agarose/EtBr gel.

**Cloning and dot blot hybridization:**

Products from the secondary PCRs were inserted directly into pGEM-T using a pGEM-T Easy Vector System ( Promega), which was then transformed into Escherichia coli and screened by the the colonieswhich were selected and amplified with nested PCR primer 1 (5'-TCGAGCGGCCGCCCGGGCAGGT-3') and nested PCR primer 2 (5'- AGGGTGGTCGCGGCCGAGGT-3'). 3 μl of PCR product were denatured in 3 ml of 0.5 M NaOH. Two identical H-bond $N^+$ nylon membranes were prepared by loading 1 μl of denatured PCR product of each clone on the same location, after 5 min. neutralization in 0.5 M Tris-Hcl (pH7.5), the membranes were baked for 30 min at 120 °C to cross link the cDNAs.

Forward-subtracted cDNAs were digested with Rsa1 and labeled as probes with digoxigenin using a DIG High Prime system (Boehringer Mannheim) by following the manufacture's instruction. Positive clones were sequenced using the dedoxy chain sequencer (ABI Applied Biosystems Model 337).

**Sequence homology, alignment and phylogenetic analysis**

The sequences were compared with the sequences in the database using the BLASTX program at the web server of the National biotechnology information. Protein prediction was performed using software at the ExPASY Molecular Biology Sever (http://expasy.pku.edu.cn) and

SAPS program (Statistical Analysis of Protein Sequences). The open reading frame of *Oreochromis niloticus* HSP9 was searched using the NCBI server ORF finder. Sequences were aligned, employing the distance matrix; a neighbor-joining tree was constructed using Clustal W (version 1.83).

**Tissue specific expression of HSP9 by RT-PCR**

Total RNA from different mixed tissues was treated with DNase, 2 μg RNA was reverse transcribed with M-MLV reverse transcriptase using hexanucleotides (Promega) to prim the reaction**.** The first strand cDNA was used as templates for RT-PCR with a pair of HSP specific primers designed forward 5'- ACAGTGCGGCGGATAAGG-3' and
reverse: 5'ACGGCTGAACAAGACCAGAATA3' .
The PCR cycling parameters were one cycle of 94 ° C for 5 min, 35 cycles of 94° C for 30 s, 58° C for 30 s and 72 ° C for 1 min, with a final extenssion step of 72° C for 10 min. The RT-PCR products were analyzed by electrophoresis on 1.5% agarose gel with PCR products derived from beta actin of the infected and non infected tilapia as controls.

***Results***

The electrophoresis analysis of ligation efficiency showed that tester and β-actin are about the same intensity (Fig. 1)

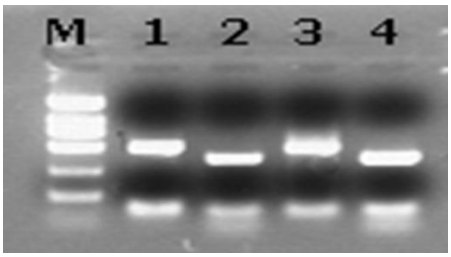


Fig.(1): ligation analysis efficiency (Lane 1, 3: Tester; lane 2, 4 :β-actin and M: marker)

**Subtracted cDNA library**

Subtracted cDNA library specific for immunized tilapia fish were evaluated by PCR analysis using β actin gene after the subtractive hybridization was performed ( Fig.2). The subtraction efficiency was noticed as shown in fig. 2. The β actin product could be observed at 15 cycles for the unsubtracted cDNAs, while the amplified product was seen at 30 cycles in the subtracted cDNAs.

The abundance of β actin was theoretically calculated as $2^{15}$ between the unsubtracted and subtracted cDNAs, this indicates the enrichment of cdNAs specific for tilapia immunized by *F. columnarae* about $2^{15}$ times by SSH.

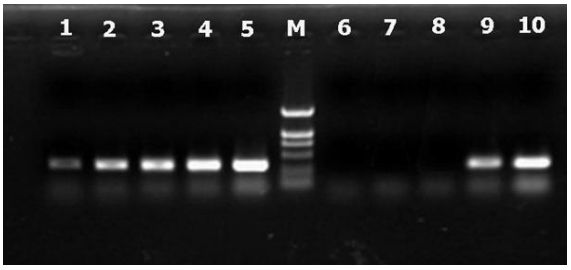


Fig.(2): Reduction of β actin abundance by SSH, PCR analysis was performed on secondary PCR product, 15 cycles( Lane 1,6); 20 cycles ( lane 2,7); 25 cycles ( Lane 3,8); 30 cycles( Lane 4,9); 35 cycles ( Lane 5,10); M=DNA marker.

**Isolation of differentially expressed clones**

The comparison of pooled RNA samples from *Oreochromis niloticus* immunized by *Flavobacterium columnarae* against unimmunized healthy *Oreochromis niloticus* yielded multiple differentially expressed clones as revealed by dot blot analysis (Fig.3).

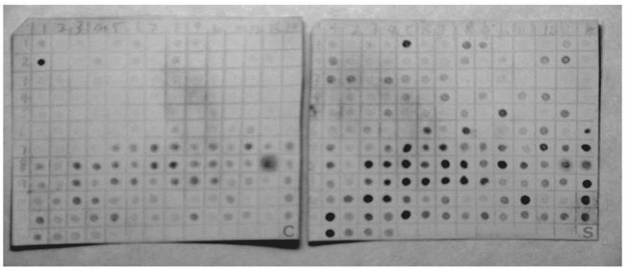

**Fig.3**: Screening of colonies with differential cDNA fragments from subtractive cDNA library by dot blot analysis. Two identical nylon membranes in which each colony was dotted in the same place were hybridized with subtractive cDNA library probd(s) and control cDNA library probs(c), respectively.

**Identification of cDNA sequence of ONHSP9**

Blast queries of the sequenced nucleotides showed that some of the sequenced genes were immune- related genes, HSP gene was identified as acute- phase reactant. The deduced amino acid sequence of ONHSP9 (Fig.4) with the genebank accession no. GH159106 contains 1228 nucleotide bps with 1020 bps open reading frame encoding a protein of 389 aa and molecular weight 41.2 kda. A signal peptide was predicted using Signal P 3.0, the signal peptide probability = 0.026, signal anchor probability = 0.126 and the Max cleavage site probability: 0.017 between residues 44 and 45.

```
  1 SVVAAEVLLS ILDHVLNVIF TEASLDHNLP LAACAFVLGR HMDDAVGVDV ECDLDLRDSA
 61 RGWRDSYQSK LTQQLVVCCH LSLTLAHFDL HLSLSISCCG EHLALLGGNC GVPVDELGKG
121 TTQSLDTQRQ WSHIQKQHIG YIASQNTTLD GCSNSDSFIR VHRLAGGSAK QILDCLLNLG
181 HACHASHQHL SDVSLGHFSI LHGLLARSHS AADKVSHNTF KLSTGLHVKM FGTGGVHSQV
241 GEVDVSLQRG QLTLCLLSSL SDSLKSHVVL HHVYTRLSLE LLDNVSERCS KSSPPGSVTI
301 GGLHLKTPFC ISRMRCQTYL HLDHIQHYLI LVLFSRRPAT TVSLSTQSSN STAGGARNSR
361 IPVRLESIMA YGSVKLRQSN QGAVNLGSL
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Fig.4: Deduced amino acid sequences of ONHSP9.

**Alignment analysis and phylogenetic tree construction**

A Basic Genebee ClustalW 1.83 was performed on a variety of HSP genes from *Homo sapiens, Pongo abilii, Bos Taurus,* Equus caballus and *Danio rerio*, GeneBank accession nos. AAH00478, NP_001126860, NP_001029696, XP_001502580, AAH44175 and GH159106 (Fig. 5). A phylogenetic tree was constructed based on six aligned amino acids sequences using Clustal W program (Fig. 6).

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Homo            MISASRAAAAR---LVGAAASRGPTAARHQDSWNGLSHEAFRLVSRRDYASEAIKGAVVG
Pongo           MISASRAVAAR---LVGAAASRGPTAARYQDGWNGLSHEAFRIVSRRDYASEAIKGAVVG
Bos             MISASRAAVSR---FVGTAASRGPTAARHQDGWNGLSHEAFRIVSRRDYASEAIKGAVVG
Equus           MISVSRAAAAR---LVGAAASRGPTAARHKDGWNGLSHEAFRIVSRRDYASEAIKGAVVG
Danio           MLSVSRTARLVRNVSCSQKTSSGVSDLIKKACLNGWTQKTLQTAARRHYASEAIRGAVIG
Oreochromis     ------------------------------------------------------------
Homo            IDLGTTNSCVAVMEGKRAKVLENAEGARTTPSVVAFTADGERLVGMPAKRQAVTNPNNTF
Pongoab         IDLGTTNSCVAVMEGKQAKVLENAEGARTTPSVVAFTADGERLVGMPAKRQAVTNPNNTF
Bos             IDLGTTNSCVAVMEGKQAKVLENAEGARTTPSVVAFTADGERLVGMPAKRQAVTNPNNTF
Equus           IDLGTTNSCVAVMEGKQAKVLENAEGARTTPSVVAFTADGERLVGMPAKRQAVTNPNNTF
Danio           IDLGTTNSCVAVMDGKNAKVLENAEGARTTPSVVAFTSDGERLVGMPAKRQAVTNPNNTL
Oreochromis     -----SVVAAEVLLSILDHVLN----------VIFT------------EASLDHNLPL
                     :  .. *: .   :**:          * **             :*  : * .:
Homo            YATKRLIGRRYDDPEVQKDIKNVPFKIVRASNGDAWVEAHGKLYSPSQIGAFVLMKMKET
Pongo           YATKRLIGRRYDDPEVQKDIKNVPFKIVRASNGDAWVEAHGKLYSPSQIGAFVLMKMKET
Bos             YATKRLIGRRYDDPEVQKDIKNVPFKIVRASNGDAWVEAHGKLYSPSQIGAFVLMKMKET
Equus           YATKRLIGRRYDDPEVQKDIKNVPFKIVRASNGDAWVEAHGKLYSPSQIGAFVLMKMKET
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Danio                YATKRLIGRRFDDAEVQKDLKNVPYKIVRASNGDAWLEVHGKMYSPSQAGAFILIKMKET
Oreochromis          AACAFVLGRHMDDAVG----VDVECDLDLRDSARGWRDSY--------------------
                      *   ::**: **.       :*  .:   ... .* : :

Homo                 AENYLGRTAKNAVITVPAYFNDSQRQATKDAGQISGLNVLRVINEPTAAALAYGLDKSED
Pongo                AENYLGHTAKNAVITVPAYFNDSQRQATKDAGQISGLNVLRVINEPTAAALAYGLDKSED
Bos                  AENYLGHTAKNAVITVPAYFNDSQRQATKDAGQISGLNVLRVINEPTAAALAYGLDKSED
Equus                AENYLGHTAKNAVITVPAYFNDSQRQATKDAGQISGLNVLRVINEPTAAALAYGLDKSED
Danio                AESYLGQSVKNAVVTVPAYFNDSQRQATKDAGQIAGLNVLRVINEPTAAALAYGLDKTQD
Oreochromis          -----------------------------QSKLTQQLVVCCHLSLTLAHFDLHLSLSIS
                                                   .:::   *:    . * * :   *. : .

Homo                 KVIAVYDLGGGTFDISILEIQKGVFEVKSTNGDTFLGGEDFDQALLRHIVKEFKRETGVD
Pongo                KVIAVYDLGGGTFDISILEIQKGVFEVKSTNGDTFLGGEDFDQALLRHIVKEFKRETGVD
Bos                  KIIAVYDLGGGTFDISILEIQKGVFEVKSTNGDTFLGGEDFDQALLRHIVKEFKRETGVD
Equus                KIIAVYDLGG-TFDISILEIQKGVFEVKSTNGDTFLGGEDFDQALLRHIVKEFKRETGVD
Danio                KIIAVYDLGGGTFDISVLEIQKGVFEVKSTNGDTFLGGEGFDQHLLRHIVKEFKKESGVD
Oreochromis          CCGEHLALLGGNCGVPVDELGKGTTQSLDT-------QRQWSHIQKQHIGYIASQNTTLD
                           * * . .:.: *: **. :  .*         . :.:   :**    .::: :*

Homo                 LTKDNMALQRVREAAEKAKCELSSSVQTDINLPYLTMDSSGPKHLNMKLTRAQFEGIVTD
Pongo                LTKDNMALQRVREAAEKAKCELSSSVQTDINLPYLTMDSSGPKHLNMKLSRAQFEGIVTD
Bos                  LTKDNMALQRVREAAEKAKCELSSSVQTDINLPYLTMDASGPKHLNMKLTRAQFEGIVTD
Equus                LTKDNMALQRVREAAEKAKCELSSSVQTDINLPYLTMDASGPKHLNMKLTRAQFEGIVAD
Danio                LMKDNMALQRVREAAEKAKCELSSSLQTDINLPYLTMDASGPKHLNMKLTRSQFEGIVAD
Oreochromis          GCSNSDSFIRVHRLAGGSAKQILDCLLN--------------------------------
                       .:. :: **:. *  :  :: ..: .

Homo                 LIRRTIAPCQKAMQDAEVSKSDIGEVILVGGMTRMPKVQQTVQDLFGRAPSKAVNPDEAV
Pongo                LIRRTIAPCQKAMQDAEVSKSDIGEVILVGGMTRMPKVQQTVQDLFGRAPSKAVNPDEAV
Bos                  LIRRTIAPCQKAMQDAEVSKSDIGEVILVGGMTRMPKVQQTVQDLFGRAPSKAVNPDEAV
Equus                LIRRTIAPCQKAMQDAEVSKSDIGEVILVGGMTRMPKVQQTVQDLFGRAPSKAVNPDEAV
Danio                LIRRTVAPCQKAMQDAEVSKSDIGEVLLVGGMTRMPKVQQTVQDLFGRAPSKSVNPDEAV
Oreochromis          -LGHACHASHQHLSDVSLGHFSILHGLLARSHSAADKVSHNTFKLSTGLHVKMFGTGGVH
                      : ::  ..:: :.*..:.: .* . :*. . :   **.:.. .*      * .... .

Homo                 AIGAAIQGGVLAGDVTDVLLLDVTPLSLGIETLGGVFTKLINRNTTIPTKKSQVFSTAAD
Pongo                AIGAAIQGGVLAGDVTDVLLLDVTPLSLGIETLGGVFTKLINRNTTIPTKKSQVFSTAAD
Bos                  AIGAAIQGGVLAGDVTDVLLLDVTPLSLGIETLGGVFTKLINRNTTIPTKKSQVFSTAAD
Equus                AIGAAIQGGVLAGDVTDVLLLDVTPLSLGIETLGGVFTKLINRNTTIPTKKSQVFSTAAD
Danio                AIGAAIQGGVLAGDVTDVLLLDVTPLSLGIETLGGVFTKLINRNTTIPTKKSQVFSTAAD
Oreochromis          SQVGEVDVSLQRGQLTLCLLSSLSDSLKSHVVLHHVYTRLS--------------------
                           *  *  **           *  * * *
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Homo                 MISASRAAAAR---LVGAAASRGPTAARHQDSWNGLSHEAFRLVSRRDYASEAIKGAVVG
Pongo                MISASRAVAAR---LVGAAASRGPTAARYQDGWNGLSHEAFRIVSRRDYASEAIKGAVVG
Bos                  MISASRAAVSR---FVGTAASRGPTAARHQDGWNGLSHEAFRIVSRRDYASEAIKGAVVG
Equus                MISVSRAAAAR---LVGAAASRGPTAARHKDGWNGLSHEAFRIVSRRDYASEAIKGAVVG
Danio                MLSVSRTARLVRNVSCSQKTSSGVSDLIKKACLNGWTQKTLQTAARRHYASEAIRGAVIG
Oreochromis          ------------------------------------------------------------
Homo                 IDLGTTNSCVAVMEGKRAKVLENAEGARTTPSVVAFTADGERLVGMPAKRQAVTNPNNTF
Pongo                IDLGTTNSCVAVMEGKQAKVLENAEGARTTPSVVAFTADGERLVGMPAKRQAVTNPNNTF
Bos                  IDLGTTNSCVAVMEGKQAKVLENAEGARTTPSVVAFTADGERLVGMPAKRQAVTNPNNTF
Equus                IDLGTTNSCVAVMEGKQAKVLENAEGARTTPSVVAFTADGERLVGMPAKRQAVTNPNNTF
Danio                IDLGTTNSCVAVMDGKNAKVLENAEGARTTPSVVAFTSDGERLVGMPAKRQAVTNPNNTL
Oreochromis          -----SVVAAEVLLSILDHVLN----------VIFT------------EASLDHNLPL
                          :  .. *: .   :**:           * **                :*  : * .:

Homosapiens          YATKRLIGRRYDDPEVQKDIKNVPFKIVRASNGDAWVEAHGKLYSPSQIGAFVLMKMKET
Pongoabelii          YATKRLIGRRYDDPEVQKDIKNVPFKIVRASNGDAWVEAHGKLYSPSQIGAFVLMKMKET
Bostaurus            YATKRLIGRRYDDPEVQKDIKNVPFKIVRASNGDAWVEAHGKLYSPSQIGAFVLMKMKET
Equuscaballus        YATKRLIGRRYDDPEVQKDIKNVPFKIVRASNGDAWVEAHGKLYSPSQIGAFVLMKMKET
Daniorerio           YATKRLIGRRFDDAEVQKDLKNVPYKIVRASNGDAWLEVHGKMYSPSQAGAFILIKMKET
Oreochromisniloticus AACAFVLGRHMDDAVG----VDVECDLDLRDSARGWRDSY--------------------
                       *   ::**: **.       :*  .:   ... .* : :

Homo                 AENYLGRTAKNAVITVPAYFNDSQRQATKDAGQISGLNVLRVINEPTAAALAYGLDKSED
Pongo                AENYLGHTAKNAVITVPAYFNDSQRQATKDAGQISGLNVLRVINEPTAAALAYGLDKSED
Bos                  AENYLGHTAKNAVITVPAYFNDSQRQATKDAGQISGLNVLRVINEPTAAALAYGLDKSED
Equus                AENYLGHTAKNAVITVPAYFNDSQRQATKDAGQISGLNVLRVINEPTAAALAYGLDKSED
Danio                AESYLGQSVKNAVVTVPAYFNDSQRQATKDAGQIAGLNVLRVINEPTAAALAYGLDKTQD
Oreochromis          -----------------------------QSKLTQQLVVCCHLSLTLAHFDLHLSLSIS
                                                   .:::   *:    . * * :   *. : .

Homo                 KVIAVYDLGGGTFDISILEIQKGVFEVKSTNGDTFLGGEDFDQALLRHIVKEFKRETGVD
Pongo                KVIAVYDLGGGTFDISILEIQKGVFEVKSTNGDTFLGGEDFDQALLRHIVKEFKRETGVD
Bos                  KIIAVYDLGGGTFDISILEIQKGVFEVKSTNGDTFLGGEDFDQALLRHIVKEFKRETGVD
Equus                KIIAVYDLGG-TFDISILEIQKGVFEVKSTNGDTFLGGEDFDQALLRHIVKEFKRETGVD
Danio                KIIAVYDLGGGTFDISVLEIQKGVFEVKSTNGDTFLGGEGFDQHLLRHIVKEFKKESGVD
Oreochromis          CCGEHLALLGGNCGVPVDELGKGTTQSLDT-------QRQWSHIQKQHIGYIASQNTTLD
                           * * . .:.: *: **. :  .*         . :.:   :**    .::: :*

Homo                 LTKDNMALQRVREAAEKAKCELSSSVQTDINLPYLTMDSSGPKHLNMKLTRAQFEGIVTD
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Pongo                LTKDNMALQRVREAAEKAKCELSSSVQTDINLPYLTMDSSGPKHLNMKLSRAQFEGIVTD
Bostaurus            LTKDNMALQRVREAAEKAKCELSSSVQTDINLPYLTMDASGPKHLNMKLTRAQFEGIVTD
Equus                LTKDNMALQRVREAAEKAKCELSSSVQTDINLPYLTMDASGPKHLNMKLTRAQFEGIVAD
Danio                LMKDNMALQRVREAAEKAKCELSSSLQTDINLPYLTMDASGPKHLNMKLTRSQFEGIVAD
Oreochromis          GCSNSDSFIRVHRLAGGSAKQILDCLLN--------------------------------
                        .:. :: **:. *  :  :: ..: .
Homo                 LIRRTIAPCQKAMQDAEVSKSDIGEVILVGGMTRMPKVQQTVQDLFGRAPSKAVNPDEAV
Pongo                LIRRTIAPCQKAMQDAEVSKSDIGEVILVGGMTRMPKVQQTVQDLFGRAPSKAVNPDEAV
Bos                  LIRRTIAPCQKAMQDAEVSKSDIGEVILVGGMTRMPKVQQTVQDLFGRAPSKAVNPDEAV
Equus                LIRRTIAPCQKAMQDAEVSKSDIGEVILVGGMTRMPKVQQTVQDLFGRAPSKAVNPDEAV
Danio                LIRRTVAPCQKAMQDAEVSKSDIGEVLLVGGMTRMPKVQQTVQDLFGRAPSKSVNPDEAV
Oreochromis          -LGHACHASHQHLSDVSLGHFSILHGLLARSHSAADKVSHNTFKLSTGLHVKMFGTGGVH
                      : ::  ..:: :.*..:.: .* . :*. . :   **.:.. .*      * .... .
Homo                 AIGAAIQGGVLAGDVTDVLLLDVTPLSLGIETLGGVFTKLINRNTTIPTKKSQVFSTAAD
Pongo                AIGAAIQGGVLAGDVTDVLLLDVTPLSLGIETLGGVFTKLINRNTTIPTKKSQVFSTAAD
Bos                  AIGAAIQGGVLAGDVTDVLLLDVTPLSLGIETLGGVFTKLINRNTTIPTKKSQVFSTAAD
Equus                AIGAAIQGGVLAGDVTDVLLLDVTPLSLGIETLGGVFTKLINRNTTIPTKKSQVFSTAAD
Danio                AIGAAIQGGVLAGDVTDVLLLDVTPLSLGIETLGGVFTKLINRNTTIPTKKSQVFSTAAD
Oreochromis          SQVGEVDVSLQRGQLTLCLLSLSDSLKSHVVSLHHVYTRLS----------------------
                     :  . :: .:  *::*  ** .::    .  .*  *:*:*
Homo                 GQTQVEIKVCQGEREMAGDNKLLGQFTLIGIPP-APRGVPQIEVTFDIDANGIVHVSAKD
Pongo                GQTQVEIKVCQGEREMAGDNKLLGQFTLIGIPP-APRGVPQIEVTFDIDANGIVHVSAKD
Bos                  GQTQVEIKVCQGEREMAGDNKLLGQFTLIGIPP-APRGVPQIEVTFDIDANGIVHVSAKD
Equus                GQTQVEIKVCQGEREMAGDNKLLGQFTLIGIPP-APRGVPQIEVTFDIDANGIVHVSAKD
Danio                GQTQVEIKVCQGEREMATDNKVLGQFTLVGIPP-APRGV-QIEVTFDIDANGIVHVSAKD
Oreochromis          ------LELLDNVSERCSKSSPPGSVTIGGLHLKTPFCISRMRCQTYLHLDHIQHY------
                           ::: :.  * . ...  *..*: *:   :*  : ::.    :. : * *
Homo                 KGTGREQQIVIQSSGGLSKDDIENMVKNAEKYAEEDRRKKERVEAVNMAEGIIHDTETKM
Pongo                KGTGREQQIVIQSSGGLSKDDIENMVKNAEKYAEEDRRKKERVEAVNMAEGIIHDTETKM
Bos                  KGTGREQQIVIQSSGGLSKDDIENMVKNAEKYAEEDRRKKERVEAVNMAEGIIHDTETKM
Equus                KGTGREQQIVIQSSGGLSKDDIENMVKNAEKYAEEDRRKKERVEAVNMAEGIIHDTETKM
Danio                KGTGREQQIVIQSSGGLSKDDIENMVKNAEKYAEEDRRRKDRVEAVNMAEGIVHDTESKM
Oreochromis          ------LILVLFSRRPATTVSLSTQSSNSTAGGARNSRIPVRLESI--------------
                           :*: *    :. .:..  .*:   . .: *   *:*::
Homo                 EEFKDQLPADECNKLKEEISKMRELLARKDSETGENIRQAASSLQQASLKLFEMAYKKMA
Pongo                EEFKDQLPADECNKLKEEISKMRELLARKDSETGENIRQAASSLQQASLKLFEMAYKKMA
Bos                  EEFKDQLPADECNKLKEEISKMRELLARKDSETGENIRQAASSLQQASLKLFEMAYKKMA
Equus                EEFKDQLPADECNKLKEEISKMRELLARKDSETGENIRQAASSLQQASLKLFEMAYKKMA
Danio                EEFKDQLPADECNKLKEEISKVRELLSRKDTETGENIKQAATSLQQASLKLFEMAYKKMA
Oreochromis          ------------------------------------------------------MAYGSVK

                                                                          *** .:
Homo                 SEREGSGSSGTGEQKEDQKEEKQ
Pongo                SEREGSGSSGTGEQKEDQKEEKQ
Bos                  SEREGSGSSGTGEQKDNQKEEKQ
Equus                SEREGSGSSSTGEQKEDQKEEK-
Daniorerio           SEREGSSGSSSSGEAGEKKEGQQ
Oreochromis          LRQSNQGAVNLGSL---------
                      .:..... . .
```

Fig. 5. The alignment of deduced amino acid sequences of HSP gene from human, Pongo abilii, Bos Taurus and Danio rerio, GeneBank (accession nos. AAH00478, NP_001126860, NP_001029696, XP_001502580 , AAH44175 and GH159106), identical amino acids are indicated with an asterisk, similar amino acids are shaded. Dashes are gaps generated by alignment.

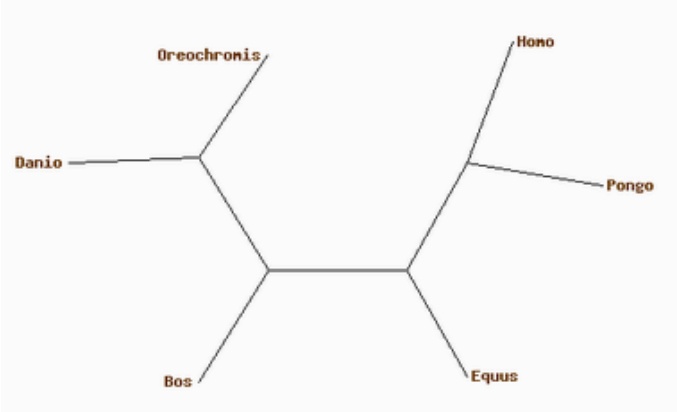

Fig.6: phylogenetic tree showing the relationship among the HSPs from different vertebrates

**Expression of HSP9 in *Oreochromis niloticus***

ONHSP9 expression studied by RT-PCR in mixed tissues from infected and uninfected control fish was detected only in the infected tilapia (Fig.7).

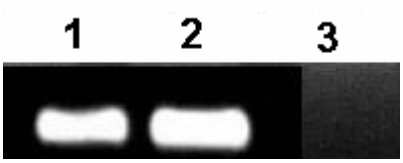


Fig.7: Tissue specific expression revealed by RT-PCR in *Oreochromis niloticus*; 1=βactin, 2=immunized fish, 3= unimmunized fish.

***Discussion***

Suppression subtractive hybridization (SSH) has led to the enrichment of specific expression genes as the non-specific expression of beta actin was reduced to about $2^{15}$ time lower after subtraction, suggesting that non-specific expressed genes have been avoided and immunization- specific genes have been enriched efficiently in this study. The identification of new genes involved in the bacterial immunization provides the foundation for further research on the immunoilogical interaction between host and the bacteria, In this study, cDNA library construction in case of *O. niloticus* immunized by *F. columnarae* introduced some new immune genes, one of them was Heat shock protein 9. HSP9 is a member of HSPs family which are intracellular proteins that can be released in various forms of cellular stress (Andreas *et al*., 2005)**.** HSPs, which are expressed constitutively in all cells, are also essential for several, important cellular processes, such as protein folding, protection of proteins from denaturation or aggregation, and facilitation of protein transport through membrane channels (Hartl *et al*., 1996). A wide variety of stressful stimuli, such as heat shock, ultraviolet radiation, and viral or bacterial infections, induce an increase in the intracellular synthesis of HSPs, clearly, HSPs play an important role in antimicrobial, as well as autoimmune, responses and have potent effects in inducing antigen-specific immunity to bound material upon immunization of animals (Wallin**,** *et al*., 2002), thus it is important to identify and characterize these genes which may help best understanding the process of host-pathogen relationship. ONHSP9

identified and characterized as it is composed of 1228 bps with a 73bps 5'UTR, a 135 3'UTR and 1020 bps open reading frame, the predicted gene product is 389 amino acid with molecular weight of 42.1 kDa. A signal peptide was predicted and suggested that HSP9 is a non secreatory protein. ClustalW alignment performed on variety of HSPs, ONHSP shares overall identity of 62.6% - 92% with other known vertebrates. Using Clustal W, a phylogenetic tree was constructed to demonstrate the distance similarity. Tissue expression of ONHSP9 was examined by RT-PCR and demonstrated that it is constitutively expressed in the immunized fish in contrast to unimmunized fish, thus it is suggested to be included in the immune response against *F. columnare*.

It could be concluded that killed *F. columnarae* did stimulate the immune response in the host fish, tilapia, some immune genes could be identified and characterized, one of these genes is HSP9, which may have a role and included in the immune response to bacterial infection, thus the present study provides some information for further research on the structure of HSP9 gene as an immune gene in relation to bacterial infection.

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