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Transcriptomic comparison of two *Entamoeba histolytica* strains with defined virulence phenotypes identifies new virulence factor candidates and key differences in the expression patterns of cysteine proteases, lectin light chains, and calmodulin

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Abstract

The availability of Rahman, and the virulent HM-1:IMSS strain of *E. histolytica*, provides a powerful tool for identifying virulence factors of *E. histolytica*. Here we report an attempt to identify potential virulence factors of *E. histolytica* by comparing the transcriptome of *E. histolytica* HM-1:IMSS and *E. histolytica* Rahman. With phenotypically defined strains, we compared the transcriptome of Rahman and HM-1:IMSS using a custom 70mer oligonucleotide based microarray that has essentially full representation of the *E. histolytica* HM-1:IMSS genome. We find extensive differences between the two strains, including distinct patterns of gene expression of cysteine proteinases, AIG family members, and lectin light chains.

Keywords: *E. histolytica*; HM-1:IMSS; Rahman; microarray; Strain comparison; Proteomics

Entamoeba histolytica infection is a constant threat to health in much of the world. *E. histolytica* trophozoites infect the colon, causing amebic dysentery, and can spread through the portal circulation to the liver, where they cause amebic liver abscess [1]. Interestingly, infection with *Entamoeba histolytica* does not always result in disease. One possible explanation for the varied outcome of infection may be differences in the underlying virulence of *E. histolytica* isolates. One strain known to exhibit reduced virulence is *E. histolytica* Rahman, which was isolated from an asymptomatic patient in England in 1972 [2]. The conserved nucleotide sequence of its of 5.8S rRNA indicates that Rahman is *E. histolytica*, yet in various in vitro assays it appears to be less virulent than the prototype *E. histolytica* HM-1:IMSS strain [3]. Recently, we have shown that Rahman exhibits defects in phagocytosis and shows significantly reduced virulence in a human intestinal xenograft model of amebic colitis [4].

Here we report an attempt to identify potential virulence factors of *E. histolytica* by comparing the transcriptome of *E. histolytica* HM-1:IMSS and *E. histolytica* Rahman. We used phenotypically defined laboratory strains that are known to differ in virulence, and compared the transcriptome of Rahman and HM-1:IMSS using a custom 70mer oligonucleotide based microarray that has essentially full representation of the *E. histolytica* HM-1:IMSS genome. Our data indicates that there are a number of potentially significant transcriptional differences between Rahman and HM-1:IMSS, including differences in the expression of genes linked to virulence, and new candidate virulence genes.

1. Materials and methods

1.1. Entamoeba strains

Entamoeba histolytica strain HM-1:IMSS was originally obtained as an uncloned line from Victor Tsutsumi at Cinvestav,

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and has been passaged through animal livers to maintain virulence [5]. Trophozoites used for mRNA had been passaged through mice approximately 30 days prior to use. Strain Rahman was obtained from ATCC, #50738. Both were maintained in culture medium BI-S-33 [6].

1.2. Microarray comparison

For microarray comparison, we designed a 70-base oligonucleotide array to analyze 6242 genes uniquely. The entire genome dataset available from the TIGR/Sanger *Entamoeba histolytica* sequencing project (http://www.tigr.org/tdb/e2k1/eha1/) in February 2004, with additional immune-related and "housekeeping" genes chosen from model organisms and minus highly repetitive sequences (mainly LINE/SINEs) was compiled and input into ArrayOligoSelector (http://arrayoligosel.sourceforge.net/) to generate 70mer oligonucleotides which have similar binding properties, and hybridize uniquely to one transcript. Oligonucleotides were manufactured by Illumina (San Diego, California) and were printed in triplicate on 100 Cel Associates Epoxy slides (Santa Clara, California) by the Washington University School of Medicine Microarray Core Facility. The average computed T_ms for all oligos was 80.8 °C, with a standard deviation of 2.73 (range 70.5–95.5 °C). Following the publication of the *E. histolytica* genome [7], printed oligo sequences were BLASTed against the database at NCBI (http://www.ncbi.nlm.nih.gov) and ascribed NCBI accession numbers. Additional annotations were computed for remaining genes representing hypothetical proteins using NCBI blastx against the nr database excluding *Entamoeba* genus with a cutoff of expectation value $<1 \times 10^{-10}$, and Interpro domain and feature characterizations. The microarray was tested for hybridization using labelled *E. histolytica* HM-1:IMSS genomic DNA, which detectably hybridized to 99.4% of the *Entamoeba* microarray elements.

RNA was isolated from approximately 5×10^6 pre-stationary phase *E. histolytica* HM-1:IMSS and Rahman each simultaneously grown in 15-ml glass flasks using the Qiagen RNeasy kit (Valencia, California) following the manufacturer's protocol, including the DNase treatment. RNA quantity and quality were obtained from an absorbance ratio at 260 and 280 nm. RNA quality was confirmed for each sample using an Agilent 2100 Bioanalyzer (Palo Alto, California) according to the manufacturer's instructions. Cy3 and Cy5 labelled cDNA was created using the Genispehere 3DNA array350 kit (Hatfield, Pennsylvania). Six samples (three Rahman and three HM-1:IMSS) were competitively hybridized on six individual chips. Each biological replicate was hybridized to two chips in which the Cy fluorescent channel was alternated in order to reduce dye-specific effects (dye swap). The primary hybridization was performed by adding 48 μl of sample to the microarray under a supported glass coverslip (Erie Scientific, Portsmouth, NH) at 70 °C for 16–20 h at high humidity in the dark. Prior to the secondary hybridization, slides were gently submerged into 2× SSC, 0.2% SDS (at 70 °C) for 11 min, transferred to 2× SSC (at room temperature) for 11 min, transferred to 0.2× SSC (at room temperature) for 11 min, and then spun dry by centrifugation. Secondary hybridization was carried out using the complimentary capture reagents provided in the 3DNA Array 350 kit. Slides were scanned on a ScanArray Express HT scanner (Perkin-Elmer, Boston, MA) to detect Cy3 and Cy5 fluorescence. Laser power was kept constant for Cy3/Cy5 scans and PMT was varied for each experiment based on optimal signal intensity with lowest possible background fluorescence. Gridding and analysis of images was performed using ScanArray v3.0 (Perkin-Elmer). Log_2 ratios of HM-1:IMSS versus Rahman samples were calculated, local background subtracted, and Loess normalized. Data was imported into a MySQL database (www.mysql.org) and data analysis was accomplished with custom SQL scripts and Spotfire DecisionSite software (Somerville, MA). Averages and standard deviation were calculated for each transcript, and transcripts considered significant showed a Student's *t*-test *p*-value of <0.01, two-fold or more increase or decrease from HM-1:IMSS, and a normalized standard deviation ratio (standard deviation/average) <1 to eliminate overly varying probes between biological and technical (dye-swapped and triplicately plated) replicates.

1.3. Real-time PCR

Real-time PCR assays were conducted by reverse transcribing one randomly chosen biological replicate and its microarray pair using Invitrogen (Carlsbad, CA, USA) Superscript III without RNase and Invitrogen oligo dT primers per manufacturer's instructions. cDNA was treated with Invitrogen RNase H to degrade remaining RNA. Treated cDNA was then diluted and amplified using SYBR Green Master Mix 2× reagent (Applied Biosystems) in an Applied Biosystems 7500 Real-time analyzer in a total of 25 μl per reaction, per manufacturer's instructions. All primer sets were run in triplicate, and primer dissociation curves were analyzed to ensure that the primers used were not amplifying multiple products. Up to four reference gene transcripts were measured from each sample and calculated using the Excel (Microsoft, Redmon, WA, USA) add-in geNorm (http://medgen.ugent.be/~jvdesomp/genorm/) to normalize overall transcript abundance between strains [8]. Reference genes were chosen from a list of transcripts which showed the lowest normalized variability from over 15 microarray experiments. Graphs were constructed using Microsoft Excel. Primers were designed with and obtained through PrimerQuest (www.idtdna.com) using sequence information from NCBI, and NCBI BLAST was used to confirm primer specificity against the current *Entamoeba* dataset. Primer sequences can be found in supplementary data, at http://stanleylab.wustl.edu/data/rahmanarray/.

2. Results and discussion

2.1. Comparing the transcriptome of E. histolytica Rahman and E. histolytica HM-1:IMSS

The goal of our work was to compare two *E. histolytica* strains with defined virulence phenotypes to identify potential virulence factors. *E. histolytica* strain HM-1:IMSS is the prototype viru-