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Designing primers potentially specific to *Entamoeba gingivalis* genes

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ABSTRACT

Entamoeba gingivalis normally exists in the human oral cavity, namely in the gums, and brings about some specific diseases. However, it can also trigger some more serious illnesses. Among these are infections of the genital tract, acute osteomyelitis of the mandible and pulmonary abscess. *Entamoeba gingivalis* identification by light microscopy is difficult, hence polymerase chain reaction (PCR) is used. The contemporary primers for PCR are complement to 18S rRNA. This article informs the reader of the process that was involved in designing new primers for three genes which were thought to be present on the *Entamoeba gingivalis* genome, but their sequences were unknown. The newly obtained sequences of primers have better properties for identification purposes, compared to these which are currently used.

INTRODUCTION

Entamoeba gingivalis is a protozoan which can be found in the gums and around the teeth. It exists solely as a trophozoite and does not produce any cysts [3]. Currently, researchers are unsure whether it is a parasite, but its occurrence is connected with diseased gingival pockets [10]. Humans can be infected as a result of direct oral contact or by sharing dishes or cutlery. *Entamoeba gingivalis* has been also found in cases of more serious nature such as acute osteomyelitis of the mandible [1] and in pulmonary abscess [6] in the elderly. It is also found in the genital tract [5]. *Entamoeba gingivalis* can be identified by light microscopy, but it bears high resemblance to other *Entamoeba* species. Polimerase Chain Reaction (PCR) is a more specific and sensitive method. The *Entamoeba gingivalis* genome has not been fully sequenced yet, and the 18S rRNA gene is the only gene of this protozoa with known sequence, and therefore, it is used in PCR [10]. Primers to other genes of *Entamoeba gingivalis* can be designed on the basis of sequence resemblance to closely related species, such as pathogenic *Entamoeba histolytica*, non-pathogenic amoebae *Entamoeba dispar* and *Entamoeba moshkovskii,* as well as more distant species of amoeba, such as *Acanthamoeba castellanii*. Candidate genes for this case are cysteine proteinase, actin and 5.8SrRNA, because sequences of the genes from different amoebae species are available in the GenBank Database.

The cysteine proteinases present in amoebae are connected with invasion and host tissue penetration because they are involved in cleaving the extracellular matrix. They also interfere with the immune system of the host, e.g. by complement and antibodies IgA and IgG degradation. *Entamoeba histolytica* produces a large amount of different cysteine proteinases which *correlate* with the invasion. *Entamoeba dispar* which is a very similar, though non-pathogenic amoeba, also produces some cysteine proteinases, but in smaller amounts [8]. Actin, which is a protein present in most eukaryotic cells, is highly conserved among the species, and takes part in maintaining cell shape [4], whereas, 5.8 S rRNA is a type of non-coding rRNA which is a component of the large subunit of the eukaryotic ribosome, and so plays a role in protein translation [2].

MATERIALS AND METHODS

Primer design. In order to design sets of primers for genes such as cysteine proteinase, actin and 5.8S rRNA, the GenBank sequences of these genes from closely related protozoa were used. In this work, sequences of each gene were aligned along one another using Clustal X. Subsequently, the potential primers were designed using Primer 3 program to be complement to the most conservative regions of each gene [9]. The primers specificity was then tested

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using the Primer-BLAST program. Next, the OligoAnalyser program was used to determine primer properties such as the possibility of hairpin, self-dimer and hetero-dimer formation. On this basis, one pair of the best primers for each gene was chosen.

RESULTS

Primer design. GenBank cysteine proteinase gene sequences from *Entamoeba histolytica* (gi|1246522) and *Entamoeba invadens* (gi|881587*),* as well as mRNA from *Entamoeba dispar* (gi|1246518) were aligned.

```
ed_cpr          AAAGATTGGAGAGCTGAAGGTAAAGTTACTCCAGTTAGAGATCAAGGAAATTGTGGATCA
eh_cp           AAAGATTGGAGAGCTGAAGGTAAAGTTACTCCAGTTAGAGACCAAGGAAATTGTGGATCA
ei_cp           GTTGATTGGAGAAAAGAAGGAAAAGTAACTCCAATTAGAGATCAAGCACAATGCGGATCA
                   *********   ***** ***** ****** ******* **** * * ** ******

ed_cpr          TGTTATTCATTCTCATCACTTGCTGTCCTTGAATCAAGATTATTAATTGCTGGAAGCAAA
eh_cp           TGCTATTCATTCTCATCACTTGCTGTTCTTGAATCAAGATTATTAATTGCTGGAAGCAAA
ei_cp           TGTTATACATTTGGTTCACTTGCAGCTCTTGAAGGAAGATTATTAATTGA-AAAAGGAGG
                ** *** ****    ******** *  ******  **************    *** *

ed_cpr          TACAACCAAAACAATCAAGATCTTTCAGAACAACAAATTGTTGACTGTA------GTACT
eh_cp           TACAACCAAAACAACCTTGATCTTTCTGAACAACAAATTGTTGATTGTA------GTGCT
ei_cp           TGATGCTAATACA--CTCGATCTT-CAGAAGAACATATGGTTCAATGCACAAGAGATAAT
                *    * ** ***  *  ****** * *** **** ** *** * ** *       *  *

ed_cpr          GCTAATAATGGATGTAATGGAGGATCTCTCTCTGCTACTTATCTTTATGTTAA-AAATAA
eh_cp           GCTAATAATGGATGTAATGGAGGATCTCTTTCTGCTACTTATCTTTATGTTAA-AAATAA
ei_cp           GGAAATAATGGATGTAATGGAGGACTTGGATCAAATGTCTATGATTACATTATTGAACAC
                *  ********************  *   **   *   ***  ***  ***   ** *

ed_cpr          TGGTGTTACTGATGAAGCTTCATACCCATACACAGCTACTAAGGGAACTTGCAAAGCTT-
eh_cp           AGGTGTTACTGATGAAGCCTCATATCCATACACTGCTACTAAGGGAACTTGTAAAGCTT-
ei_cp           GAGTGTCA---AAGAAAGTGATTATCCATACACTGGAAATGATTCTACATGCAAAACTAA
                  **** *   * ***      ** ******** *  * * *    ** ** *** **

ed_cpr          --TCACTCCAAAAGTTCAAACTACTGGTTTAACTCATGTCACTCCAAATGAAGATGCT--
eh_cp           --TCACTCCAAAAGTTCAAACTACTGGTTTAACTCATGTTACTCCAACTGAAGAAGCT--
ei_cp           TGTAAAATCATTTGCTAAAATTACTGGATATACTAAAGTCCCAAGAAACAATGAAGCTGA
                  * *   **   * * *** ****** *  *** * **  *   **   * ** ***

ed_cpr          -TTGACTTCAGCTCTTGAACAAGGACCAGTTGCTGTTTGTATTGATGCTGGTAAAGCTTC
eh_cp           -TTAACTGCTGCTCTTGCAGAAGGACCAGTTGCTGTTTGTATTGATGCTGGTAAAGCTTC
ei_cp           ACTTAAAGCTGCACTTTCACAAGGTCTTATTGATGAAACAAATGATGCATCATCTGCTAA
                  * *   * ** ***  * **** *   *** **     * ******       ***

ed_cpr          ATTCCAATTATATAAATCTGGAGTTTATGATGAACCAAAATGTAGTAA-AACTGTT----
eh_cp           ATTCCAATTATATAAAAATGGAGTTTATGATGAACCAAAATGCAGCAA-AACTGTT----
ei_cp           ATTCCAATTATACAAGAGCGGAGCTTATACTGATACTAAATGCAAGAATAACTACTTTGC
                ************ **    **** ****  ***  * ***** *  ** ****  *

ed_cpr          ----AACCATGGTGTCGCCGCTGTTGGATATGGTACTCAAGATGGTAAAGACTATTATAT
eh_cp           ----AACCATGGTGTTGCCGCTGTAGGATATGGATCTCAAGATGGTCAAGACTATTACAT
ei_cp           TTTGAATCACGAAGTTTGTGCTGTTGGATATTGTGTTGTTGATGGAAAAGAATGTTGGAT
                    ** ** *  **    ***** ****** *   *   *****  **** * **  **

ed_cpr          TGTTAAGAACTCATGGGGAACTTCATGGGGAGACAAAGGTTATATTTTAATGTCAAGAAA
eh_cp           TGTTAAGAACTCATGGGGAACTTCATGGGGAGATAAAGGTTATATTTTAATGTCAAGAAA
ei_cp           AGTTAGAAACTCATGGGGAACATCATGGGGA----------------------------
                 ****  ************** *********
```

Figure 1. CLUSTAL 2.1 multiple sequence alignment of the cysteine proteinase gene

```
PRIMER PICKING RESULTS FOR eh_cp gi|1246522|emb|X91642.1| E.histolytica DNA
encoding for cysteine proteinase (1800 bp)
No mispriming library specified
Using 1-based sequence positions
OLIGO            start  len      tm     gc%   any   3'  seq
LEFT PRIMER       1084   22   57.26   45.45  4.00  0.00 GATTGGAGAGCTGAAGGTAAAG
RIGHT PRIMER      1625   21   59.40   47.62  5.00  2.00 CATGAAGTTCCCCATGAGTTC
SEQUENCE SIZE: 1800
INCLUDED REGION SIZE: 1800
PRODUCT SIZE: 542, PAIR ANY COMPL: 5.00, PAIR 3' COMPL: 3.00

PRIMER PICKING RESULTS FOR eh_cp gi|1246522|emb|X91642.1| E.histolytica DNA
encoding for cysteine proteinase (1800 bp)
No mispriming library specified
Using 1-based sequence positions
OLIGO            start  len      tm     gc%   any   3'  seq
LEFT PRIMER       1084   22   57.26   45.45  4.00  0.00 GATTGGAGAGCTGAAGGTAAAG
RIGHT PRIMER      1278   24   58.49   37.50  3.00  2.00 TCCTCCATTACATCCATTATTAGC
SEQUENCE SIZE: 1800
INCLUDED REGION SIZE: 1800
PRODUCT SIZE: 195, PAIR ANY COMPL: 5.00, PAIR 3' COMPL: 1.00
```

Figure 2. Two primer pairs generated using the Primer 3 program

The specificity of the generated primers was confirmed using Primer-BLAST. The GC content of the second pair of primers is below 40%, hence, it is not taken into account.

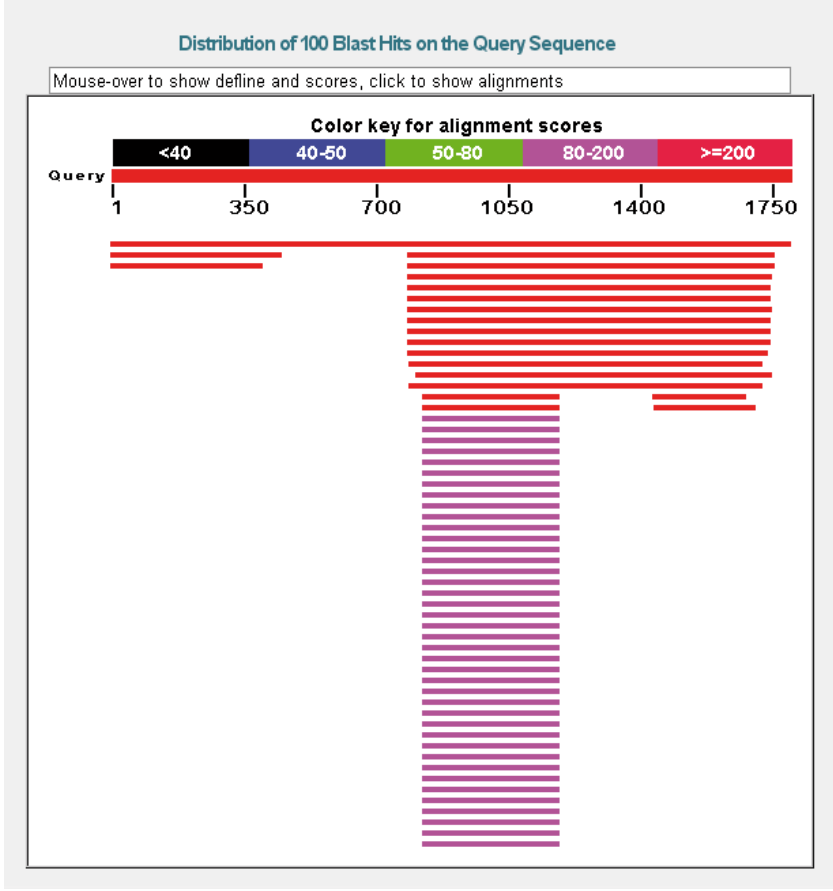

Figure 3. Alignment of Entamoeba histolytica cystein proteinase gene (gi 1246522) with other sequences of cystein proteinase (cds sequences) drawn from the database

On this basis, it can be assumed that there is a non-coding region between 472 and about 700 nt. However, a chosen pair of primers is complement to the fragment of the gene downstream to this site.

GenBank actine gene sequences from *Naegleria fowleri* (gi|1022820*), Entamoeba histolytica* (gi|118430606), *Acanthamoeba castellanii* (gi|5565) and *Dientamoeba fragilis* (gi|506956256) were aligned.

```
nf_a            TTGACTGAAGCTCCATTGAATCCAAAGGCTAACAGAGAAAAGATGACTCAAATCATGTTT
eh_a            TTAACTGAAGCCCCAATGAATCCAAAAGCTAACAGAGAAAAGATGACTCAAATTATGTTT
ac_a            CAGACTGAGGCCCCGCTCAACCCCAAGGCCAACCGCGAGAAGATGACGCAAATTATGTTC
df_a            CTTACAGAAGCTCCAATGAATCCAAAGGCTAACCGTGAAAAGATGATTCAACTTATGTTC
                   ** ** ** **  * ** ** ** ** *** * ** *******  *** * *****
nf_a            GAAACCTTCTCTGTTCCAGCCATGTATGTTGCCATTCAAGCTGTCTTGTCTTTGTATGCT
eh_a            GAAACATTCAACACCCCAGCTATGTATGTTGGAATTCAAGCTGTTCTTTCATTATATGCC
ac_a            GAGACCTTCAACACCCCCGCCATGTACGTCGCCATCCAGGCCGTGCTCTCGCTCTACGCC
df_a            GAAACATTCAACACACCAGCYTTYTATGTTGGTATCCAAGCYGTTCTTTCACTTTACGCA
                ** ** ***      ** **  * ** ** *  ** ** ** **  * **  * ** **
nf_a            TCTGGTCGTACCACTGGTATTGTTTTGGACTCTGGTGATGGTGTCTCTCACACTGTTCCA
eh_a            TCAGGTAGAACTACTGGTATTGTTATGGATTCAGGTGATGGAGTTTCACACACCGTCCCA
ac_a            TCGGGCCGTACCACTGGCATCGTGCTTGACTCGGGCGACGGCGTCACCCACACCGTGCCC
df_a            TCAGGTCGTACAACAGGTATTGTTTTCGATGCTGGTGATGGTGTTTCACACACAGTTCCA
                ** **  * ** ** ** ** **  * **  * ** ** ** **  * ***** ** **
nf_a            ATTTATGAAGGTTATGCTTTGCCTCATGCTATTTTGAGATTGGATTTGGCTGGTAGAGAT
eh_a            ATTTATGAAGGATTCTCACTTCCACATGCTATTCTTAGACTTGATCTTGCAGGACGTGAT
ac_a            ATCTACGAGGGTTATGCCCTGCCCCACGCCATCCTGCGTCTCGATCTCGCCGGTCGCGAT
df_a            ATTTATGAAGGTTATTCACTTCCACATGCTATCATGAGACTTAACCTTGCTGGTCGTGAT
                ** ** ** ** *   *  * ** ** ** **  *  *  *  *  * ** **  * ***
```

Figure 4. CLUSTAL 2.1 multiple sequence alignment of actine genes

The specificity of primers generated using Primer 3 was authenticated using Primer-BLAST, and the most specific primer pair with the best physical properties was chosen.

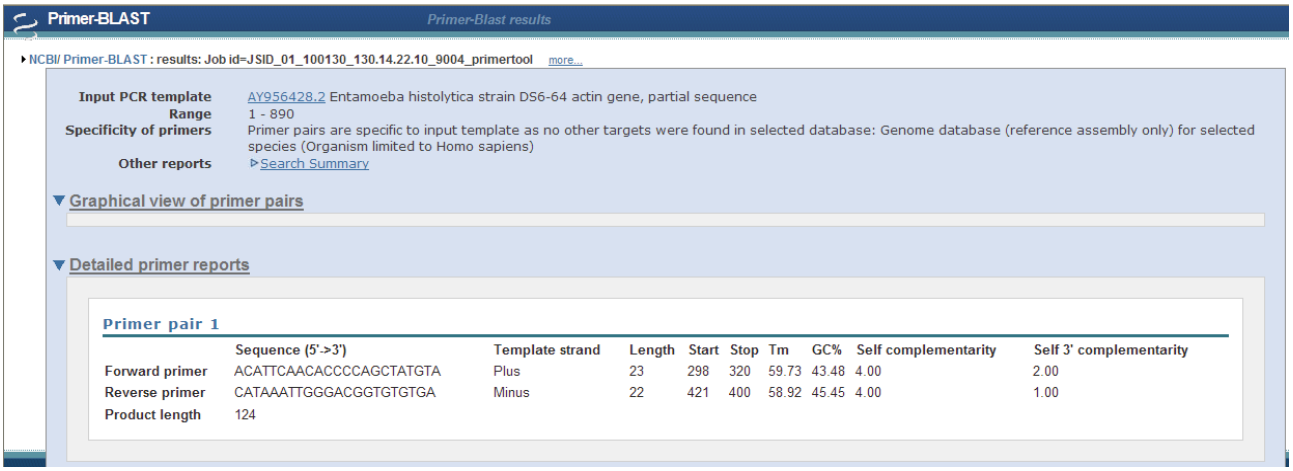

Figure 5. Primer-BLAST results for the generated primers

GenBank 5.8S rRNA gene sequences (without the internal transcribed spacer - ITS sequence) from *Entamoeba moshkovskii* (gi|908849), *Entamoeba dispar* (gi|1929041), *Entamoeba histolytica* (gi|1929043) and *Entamoeba invadens* were aligned.

```
em_dna58        ----------------------------AAAATATTACTCTGGATAGTTTAGTTTCTGGA
ed_dna58        ----------------------------AAAATATTACTTTGGATAGTTTAGTTTCCTGT
eh_dna58        GAATATAAAAATAAAGAAAAAGTATAATAAAATATTACTTTGGATAGTTTAGTTTCCTGG
ei_dna58        ----------------------------AAGAAAATGATTTTGAATCAATGAGTTTCTCGG
                                            * ** ** * * ** **   * ******  *
em_dna58        CGAAGAAGAACGCAAGGAATTGCG-ATAAGTAATAGGAACAATAGAACGTGAATATTTAA
ed_dna58        GCGATGAAGAACGCAATGAATTGC-GATAAGTGATAGGAACAATAAATGTGGATATCCAA
eh_dna58        GCGATGAAGAACGCAATGAATTGCGATAAGTGATAGGAACAATAAAATGTGAATATCCAA
ei_dna58        ACGAAGAAGACCGCAATACGTGC---ATAATTGCTCTGAAAATAGAA-GTGAAAGAGCGT
                   *  *  *    *     *       *         *  *   ** *** *
em_dna58        GATTTGAATGCATGACACTCCGCTTCCTGAACTTCAGATGCGTATGTAAGTTTCAATATC
ed_dna58        ACTTTGAATGCTTGAAAGTATACTTATGAACTTCAAGGTATATATGATATTCAATATCC-
eh_dna58        AATTTGAATGCTTGAAAGTATACTTATGAACTTCAAGGTATATATGATATTCAATATCCA
ei_dna58        TTGTTGAATGCGTGACC-------------------------------------------
                   ******** ***
```

Figure 6. CLUSTAL 2.1 multiple sequence alignment without internal transcribed spacers (its)

```
WARNING: Left primer is unacceptable: Tm too low/High 3' stability; Right primer
is unacceptable: Tm too low/High end self complementarity
OLIGO            start  len      tm      gc%   any   3'  seq
LEFT PRIMER        159   21   53.73   38.10  4.00  3.00 TTGGATAGTTTAGTTTCCTGG
RIGHT PRIMER       254   20   56.80   30.00  7.00  7.00 TCAAGCATTCAAATTTTGGA
SEQUENCE SIZE: 383
INCLUDED REGION SIZE: 383
PRODUCT SIZE: 96, PAIR ANY COMPL: 4.00, PAIR 3' COMPL: 1.00
    1 AGGTGAACCTGCGGAAGGATCATTAAAAGAAAAGAAATAATCTTTTAAAATAAAACGAGA

   61 AATTTATAGAATAAATAATCTACAAAGAAAATAATAATAAGTAAGAATAAAAAGAATTAG

  121 AATATAAAAATAAAGAAAAAGTATAATAAAATATTACTTTGGATAGTTTAGTTTCCTGGG
                                                >>>>>>>>>>>>>>>>>>>>
  181 CGATGAAGAACGCAATGAATTGCGATAAGTGATAGGAACAATAAAATGTGAATATCCAAA
                                                              <<<<<<
  241 ATTTGAATGCTTGAAAGTATACTTATGAACTTCAAGGTATATATGATATTCAATATCCAA
      <<<<<<<<<<<<<<
  301 AATAAGAGAATATATTAAAATCCAATGCAAGTACAACAGAGAAGAAGTAGCTAGTAGATA

  361 AATGAGAGAAGAAGTAAAGAGCT
```

Figure 7. Primer 3 results for primers to 5.8S rRNAThe melting temperature of both primers is considered too low, because of a too low GC content. It is thus, deemed impossible to design primers for the 5.8S rRNA gene with the appropriate parameters

DISCUSSION

The best pair for cysteine proteinase gene and actine gene identification was chosen after a comparison of sequences and the designing of primers for the most conserved regions. The sequences of primers for cysteine proteinase are as follows: forward – GATTGGAGAGCTGAAGGTAAAGT, reverse – CATGAAGTTCCCCATGAGTTCTTA while that for actin are: forward – ACATTCAACACCCCAGCTATGTA, reverse – CATAAATTGGGACGGTGTGTGA. The primers are specific to the appropriate gene sequence of *Entamoeba histolytica*. However, they should be examined experimentally in order to be considered suitable for the amplification of relevant *Entamoeba gingivalis* genes. As the primers may not be completely complementary to *E. gingivalis* genes, a modification of conditions (such as providing annealing temperatures) should be applied.

The primers complement to SSU rDNA and currently used to identify the *Entamoeba gingivalis* are called EGO-1, EGO-2 [5,7,10]. They are characterized by having high self-complementarity and 3' end complementarity. This results in a high probability of hairpin and homo and heterodimer formation, as well as difficulties in their usage. Finding primers with good properties would be considered to be important for *both* epidemiological studies and for diagnostic purposes. Such primers would contribute to greater efficiency of the method.

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