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The Hemolytic Properties of Clinical Isolates of *Morganella morganii.*

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ABSTRACT

Morganella morganii is a gram-negative bacterium from the *Enterobacteriaceae* family which causes a wide range of clinical infections sometimes with fatal consequences. It is known that more than 50% of isolates of *M. morganii* from clinical specimens have hemolytic activity that increase their virulence. Pore-forming toxins (PFT) represent the most common group of cytotoxic proteins which contribute the delivering of the bacterial proteins into host cells, loss of nutrients and ions by eukaryotic cells, as well as the exit of bacteria from phagosome into cytosol. In this study we investigated the hemolytic activity of two *M. morganii* strains. It has been shown that hemolytic activity for strain of *M. morganii* 4 is 3 times higher than for strain of *M. morganii* 1. The maximum hemolytic activity is observed in LB medium but synthesis of hemolysins is higher in synthetic urine. Finally, the PCR-analysis of 5 hypothetical hemolysin genes has shown that strain *M. morganii* 1 does not contain homologous of α-hemolysin from *E. coli* that may explain the observed differences in hemolytic activity of the investigated strains.

Keywords: *M. morganii,* opportunistic infections, hemolysis, bioinformatic analysis.

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INTRODUCTION

Morganella morganii is a gram-negative bacterium which belongs to the *Enterobacteriaceae* family [1]. Normally, these bacteria are found in the environment and intestinal tracts of humans and other mammals as a normal microflore [2, 3]. However, it is also an important opportunistic pathogen that causes a wide range of clinical infections, sometimes with fatal consequences [4, 3]. In general, *M. morganii* causes urinary tract infections, catheter-associated bacteriuria, wound infections and septicemia [5, 4]. *M. morganii* infections are most often encountered in postoperative patients and people with a weakened immune system. It is known that septicemia caused by *M. morganii* can be associated with a mortality rate of 22-38% [6, 2].

Hemolysins belong to the pore-forming toxins (PFT) which represent the most common group of cytotoxic proteins [7]. Majority of PFT are representatives of RTX family (repeat-in-toxin) which also includes the well studied α-hemolysin (HlyA) from *E. coli* [8]. Hemolysins form the pores in the membrane of red blood cells, contributing to their lysis, and also can cause the loss of ATP by granulocytes which leads to their death [2]. In addition, some hemolysins capable to affect renal tubule cells, some of them can be a powerful leukocidin [9] and can induce apoptosis of target cells [10]. Mutation on the PFT genes leads to partial or complete loss of virulence, making them an attractive target for antimicrobial therapy [7].

There are some dates about *M. morganii* hemolysin which is functionally similar to the α-hemolysin of *E. coli* [9]. The aim of this study was the investigation of the extracellular hemolytic activity of clinical isolates of *M. morganii* depending on various factors, and conduct bioinformatic analysis, identification and sequencing of the hemolysin genes, including orthologous genes of hlyA from *E. coli*.

MATERIALS AND METHODS

Bacterial strains

Clinical isolates of *M. morganii* 1 and *M. morganii* 4 were provided by the clinical diagnostic laboratory Biomed of Kazan city. Identification of strain was performed based on microbiological tests and mass spectrometry on the MALDI BioTyper (Bruker Daltonik).

Media and growth conditions

Bacteria were grown at 37 °C with aeration (shaker Braun, Germany). The optical density of the cultures was measured at 590 nm (microplate spectrophotometer BioRad XMarkTM, Singapore). As inoculum was used the overnight culture grown in LB medium (1% triptone, 0.5% yeast extract, 0.5% NaCl, pH 8.5).

The composition of synthetic urine was represented by the following components: 0.1% peptone, 0.005% yeast extract, 0.01% lactic acid, 0.04% citric acid, 1% urea, 0.037% $CaCl_2 \times 2H_2O$, 0.52% NaCl, 0.0012% $FeSO_4$, 0.049% $MgSO_4 \times 7H_2O$, 0.32% $Na_2SO_4 \times 10H_2O$, 0.095% KH_2PO_4, 0.12% K_2HPO_4, and 0.13% NH_4Cl.

Hemolysis assay

To determine the hemolytic activity of strains the blood agar containing 5% human erythrocytes was used.

For quantitative analysis of hemolytic activity was used the optimized method proposed by Senior and Hughes [11]. Fresh Luria broth or synthetic urine was inoculated by overnight culture to get 1% bacterial culture. Flasks were incubated with shaking at 37 °C. At specific intervals of time small amounts of culture were taken to precipitate the bacteria and get the culture fluid.

100 µl of culture fluid was mixed with 900 µl 2% erythrocyte suspension in 0.85% NaCl containing 20 mM $CaCl_2$. Samples were incubated for 30 min at 37°C. After incubation samples were centrifugated for precipitate the cell debrises. The hemolytic activity was determined by measure the optic density at 540 nm.

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The **hemolytic activity (U/ml)** was determined by the formula:

$$\mathbf{Absorption\ OD_{540}}/\left(\mathbf{0.01} \times \mathbf{Reaction\ time(min)} \times \mathbf{Volume\ of\ hemolysin(ml)}\right),$$

where 0.01 is a constant.

The **productivity of hemolysins (conventional units)** was determined as the ratio of hemolytic activity (U/ml) to optical density of bacterial culture at 590 nm.

Bioinformatics

The search of hemolysin genes was performed using the several databases and online programs like GenBank (http://www.ncbi.nlm.nih.gov/genbank/), Protein (http://www.ncbi.nlm.nih.gov/protein/), ASAP (https://asap.genetics.wisc.edu/asap/home.php), and BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). To design primers were used programs Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and Oligo Calc (http://basic.northwestern.edu/biotools/oligocalc.html).

For alignment of nucleotide sequences were used the programs BLAST and MEGA 4. Search of ORFs was carried out using the programs Clone Manager 7 and "Translation of nucleotide sequences" (http://molbiol.ru/scripts/01_13.html).

Genomic DNA extraction and PCR

For isolation of total genomic DNA was used the GeneJet™ DNA Purification Kit (Fermentas). PCR was performed on thermal cycler BioRad MJ Mini™ Gradient Thermal Cycler (Singapore) [12]. The oligonucleotides used to determine hemolysin genes represented in Table 2. Analysis of the products of the PCR amplification was carried out on horizontal DNA electrophoresis in agarose gel [13].

RESULTS AND DISCUSSION

Determination of the hemolytic properties of *M. morganii* isolates

M. morganii strains used in this study were isolated from community-acquired infections. To determine the hemolytic activity bacteria were grown on blood agar. *M. morganii* 4 strain has formed distinct clearness zone on this medium which is indicate the ability of this strain to β-hemolysis (true hemolysis). In contrast *M. morganii* 1 has caused the enlightenment of the whole medium but has not showed zones of hemolysis around its colonies which is indicate the ability of *M. morganii* 1 to α-hemolysis only (Fig. 1).

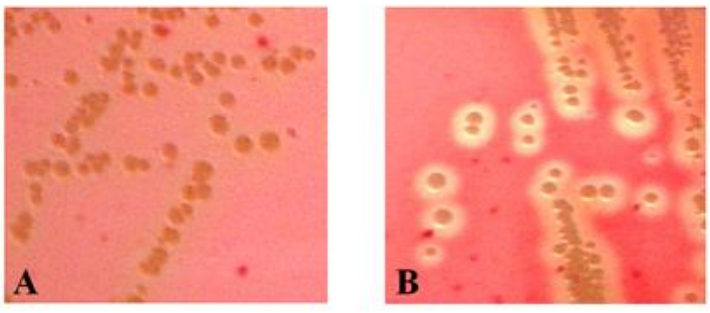

Figure 1: Determination of the hemolytic properties of *M. morganii* strains on blood agar. A - *M. morganii* 1, B - *M. morganii* 4.

We have investigated the hemolytic activity of culture fluid of *M. morganii* strains within 30 h of growth. It was shown that the highest value of hemolytic activity is observed at 6 h of growth (Fig. 2). The hemolytic activity for *M. morganii* strain 4 was 3 times more pronounced than for strain of *M. morganii* 1 that is consistent with experiments on blood agar. Therefore for further studies the strain of *M. morganii* 4 was selected.

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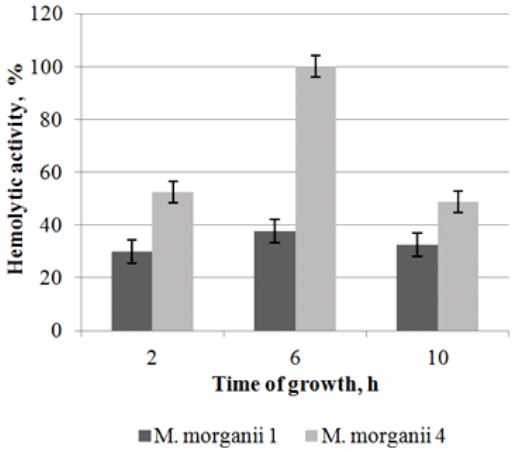

Figure 2: Comparative analysis of the hemolytic activity of clinical isolates of *M. morganii* at 10 h of growth. For 100% was taken the greatest hemolytic activity exhibited by the cultures.

We have studied the dynamics of growth and hemolytic activity of the *M. morganii* 4 strain (at 6 h of growth) at 28°C and 37°C using LB and synthetic urine. It has been shown that the growth of the strain was maximum in LB medium at any temperature (Fig. 3). The growth of bacteria in synthetic urine was about 4 times lower than in LB medium. The highest hemolytic activity was observed in LB medium at 37°C, and the smallest one in synthetic urine at 28°C. At the same temperature (37°C) hemolytic activity in synthetic urine was 1.5 times lower than in LB medium. At 28°C hemolytic activity was lower on about 18% and 7% for LB medium and synthetic urine, respectively. At the same time, the maximum production of hemolysins was observed in synthetic urine (data not shown).

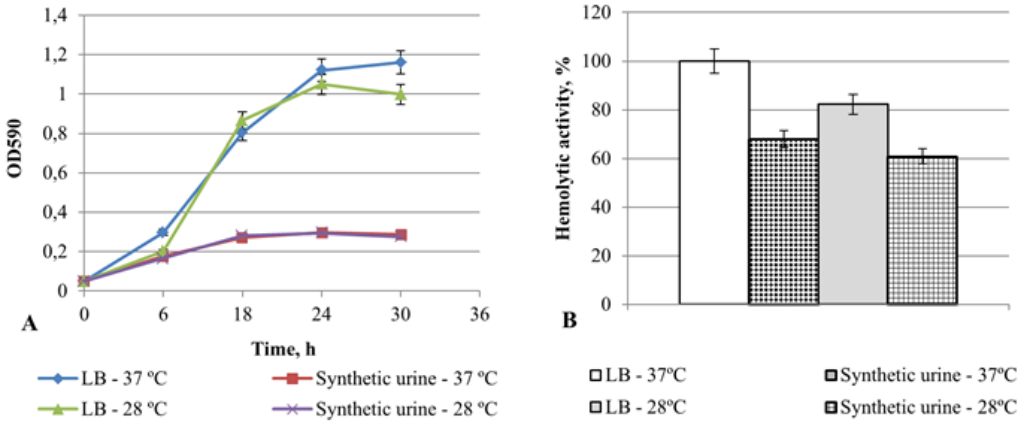

Figure 3: Growth curve (A) and hemolytic activity (B) of *M. morganii* 4 in different media at 28°C and 37°C. For 100% was taken the greatest hemolytic activity exhibited by the cultures.

Search of hemolysin genes in the sequenced genomes of *M. morganii*

Previously was shown that some strains of *M. morganii* synthesize hemolysins which genetic determinant has a pronounced but incomplete homology with α-hemolysin gene of *E. coli* [14].

To search the hemolysin genes in the genomes of *M. morganii* were used the nucleotide (AN NC_004431) and amino acid sequences (AN NP_755445.1) of *E. coli* CFT073 α-hemolysin (*hly*A) as the referent sequences. There are no homologous sequences were found in the genomes of *M. morganii* KT и *M. morganii* SC01 but they were found in the genomes of *M. morganii* FDA_MicroDB_63 (KGZ28812.1) and *Morganella sp.* EGD-HP17 (WP_036418382.1). The percent of homology of these genes with *hly*A was 77%. At the same time,

in the genome of *M. morganii* KT 4 hypothetical hemolysin genes have been found which are have not any homology with *hly*A, and 3 of them are also found in *M. morganii* SC01 (Table 1).

Table 1. Hypothetical hemolysin genes of *M. morganii*

Author's designation of the genes	Size, b.p.	Product	Genome	GenBank Accession Number
p_hem	573	hemolysin precursor	*M. morganii* KT	CP004345.1
hly	258	putative alpha-hemolysin	*M. morganii* KT	CP004345.1
*hem*1	1293	hemolysin	*M. morganii* KT	CP004345.1
*hem*2	4635	hemolysin	*M. morganii* KT	CP004345.1
ex (homologues of *hly*A)	3075	RTX toxin hemolysin A	*Morganella sp.* EGD-HP17	NZ_AZRH01000012.1

The analysis of these sequences using ASAP and blastx has shown that only a few gene products have conserved domains which are typical for hemolytic proteins. For example, it was found that product of gene *hly* has domain containing three conserved cysteine residues which has hemolytic activity and detected in short proteins. In addition *hly* is adjacent with gene encoding inner membrane protein of translocase component YidC.

The product of gene *hem*2 has conserved domains such as Haemagg_act which has carbohydrate-dependent hemagglutination activity, FhaB involved in heme utilization or adhesion, hemagluttinin repeat Fil_haemagg_2, and Pertactin-like passenger domains which is typical for autotransporter proteins belong to type V secretion system. Moreover, this gene forms operon with gene of channel-forming transporter/cytolysins activator of TpsB family which probably performs the function of hemolysin activator. Finally, only *ex* gene product has shown domains typical for RTX toxin family. They include RTX N-terminal domain which is found in cytotoxins and cytolysins, RTX C-terminal domain typical for bacterial leukotoxins and hemolysins, and Ca^{2}+-binding COG2931 domain.

Identification of hypothetical hemolysin genes from clinical isolates of *M. morganii*

Discovered sequences were used to design primers for identification of homologous genes in the genomes of *M. morganii* 1 and 4 strains (Table 2).

Table 2. Primers used to determine hemolysin genes in isolates of *M. morganii*

Gene	Primer	Sequence (5'→3')	Annealing temperature, ºC	PCR product, b.p.
p_hem	**P_hemF**	**GAAATCCGCGTACCCTCACA**	**53.8**	**734**
	P_hemR	**AAGTCAGGTTTCTTATCGGT**	**47.7**	**734**
hly	**hlyF**	**CAAACGGTTAGCGCGTGAAT**	**59.8**	**493**
	hlyR	**GTAGCCGCGTTAAGTGCTTG**	**59.9**	**493**
*hem*1	**Hem1F**	**TAACGCCCTTTTCCGCTCAT**	**51.8**	**1595**
	Hem1R	**AGAAGCACATCAGATTGCAA**	**47.7**	**1595**
*hem*2	**Hem2F**	**GGTTAATGTCAGTTACGGCG**	**51.8**	**1647**
	Hem2R	**CCGGCATTCAGTCCCAGATT**	**53.8**	**1647**
	Hem2F_2	ATCTCAGTGCGGATGCCAAA	51.8	2131
	Hem2R_2	TTAGCGGTCAGGTCGTTTCC	53.8	2131
	Hem2F_3	GGGGCGAAGGTAAATGTGGA	53.8	1439
	Hem2R_3	TAAACCACGCCGGATTGTCA	51.8	1439
ex	ex_F2	AGTATCTGAATATCACGGAG	47.7	2058
	ex_R2	ACATCATGCCCCTTACCTGC	53.8	2058

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	ex_F	**ACCCGCAGCAGAAGACAATC**	**53.8**	**1609**
	ex_R	**AAATCCAGGCCCTTACCATCA**	**52.4**	**1609**

*primers used to sequencing of PCR products are indicated in bold font

DNA electrophoresis of PCR products showed that *M. morganii* 4 strain has sequences which are homologous to all investigated genes of the hemolysins. Whereas in the genome of *M. morganii* 1 there are no sites which are homologous to the exotoxin gene (Fig. 4). So, it can be assumed that *M. morganii* 4 strain synthesizes homologous of α-hemolysin.

The sequencing of PCR products has shown that all hypothetical hemolysins' genes of *M. morganii* 4 except *ex* gene have 100% homology with sequences from GenBank. It has 99% homology with exotoxin gene from *Morganella* sp. EGD-HP17 and 76% with site encoding chromosomal C, A, B, and D hemolysins of *E. coli* J96. Sequences from *M. morganii* 1 have homology of 99% with *p_hem*, 100% with *hly*, 98% with *hem*1, and 97% with *hem*2.

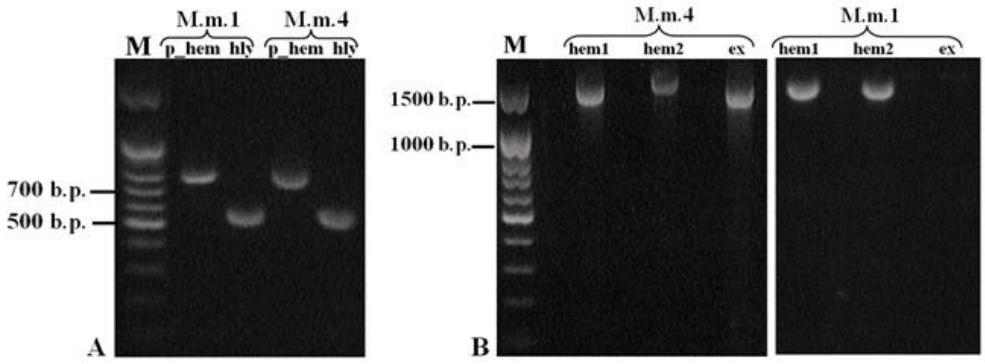

Figure 4: DNA electrophoresis of PCR products from genomic DNA of clinical isolates of *M. morganii*. M – DNA markers, M.m. – *Morganella morganii*. p_hem, hly, hem1, hem2, ex – designation of investigated genes.

CONCLUSION

Thus, the comparative analysis of hemolytic activity of investigated strains of *M. morganii* has showed that synthesis of hemolysins depends on medium composition and temperature, and production of hemolysins is stimulated by urea.

In addition, sequences with a high degree of homology about hypothetical hemolysin genes from annotated strains of *M. morganii* were detected in the genomes of clinical isolates *M. morganii* with various hemolytic activities. It can be assumed that differences in hemolytic activity of *M. morganii* 1 and 4 strains are associated with expression of exotoxin (*ex*) gene which has a homology towards *E. coli hly*A.

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