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RESEARCH ARTICLE

Detection of hydrolases of different subclasses in cell-free fractions of *Vibrio cholerae* O1 and O139 serogroups using radial enzymatic diffusion in agarose gel

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ABSTRACT

Cholera remains an actual infection worldwide, which dictates the need for a comprehensive study of its pathogens and, in particular, their hydrolytic enzymes that interact with the cells of the macroorganism. We determined the presence of hydrolases from different subclasses in preparations of cell-free fractions obtained from 58 strains of *Vibrio cholerae* O1 and O139 serogroups of different epidemic significance and origins using radial enzyme diffusion in agarose gels with various substrates. The analysis revealed statistically significant differences in enzyme activity depending on the origin and epidemiological significance of the original strain. We found that preparations obtained from non-toxigenic strains had increased activity of proteases and chitinolytic enzymes, while those from toxigenic strains showed high activity of mucinases, lipolytic enzymes, and nucleases. These data can be applied in the microbiological diagnosis of cholera as an additional biochemical characterization of *Vibrio cholerae* strains, as well as contribute to the understanding of the role of enzymes in the pathogenesis and adaptation of cholera pathogens.

Keywords: hydrolase, radial enzyme diffusion, *Vibrio cholerae*, agarose gel

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INTRODUCTION

Fighting cholera remains a relevant global task due to the ongoing spread of this infection in most countries worldwide. This situation dictates the need for a comprehensive study of the cholera causative agents and, in particular, the hydrolytic enzymes since they interact with the cells of the macroorganism. The hydrolases of *Vibrio cholerae* have not been sufficiently studied and characterized. Furthermore, there is no information in the literature about the predominance of the activity of certain hydrolytic enzymes in *Vibrio cholerae* strains of O1 and O139 serogroups, depending on their epidemiological significance and origins. It is known that the presence of some hydrolytic enzymes can ensure persistence of these strains in the environment [1, 2, 3]. Currently, there is great demand for reliable, rapid, and inexpensive methods for the detection of hydrolytic

enzymes in various biological samples, including pathogenic bacteria. A rapid and pictorial method that does not require preliminary preparative isolation of enzymes is the radial enzyme diffusion (RED) assay [4]. This is a non-electrophoretic version of zymographic methods; therefore, it is widely used to determine the presence and assess the total activity of hydrolytic enzymes in the biopreparations analyzed. Usually, the RED is performed by placing a sample containing the enzyme in direct contact with a substrate dissolved or suspended in agar or agarose gels. An enzymatic reaction occurs when the enzyme diffuses from the wells into the gel. The reaction rate is assessed by the appearance of hydrolysis zones (clearing, turbidity, or colored zones, etc.), indicating the consumption of the substrate or the emergence of enzymatic reaction products. In some cases (e.g., when using suspensions), the agarose gel containing the substrate is relatively opaque; therefore, the clearing zone can be observed without staining. Alternatively, enzyme activity can be assessed after acid precipitation or addition of a dye specific to the substrate. The emergence and size of these zones of clearing, turbidity, or colored, can be used to detect the action of a particular enzyme, and evaluate its activity. Theoretically, the RED can be used to determine the activity of any enzyme, provided that the enzyme-substrate reaction can be visually monitored. However, in laboratory practice, this method is mainly used for the analysis of hydrolytic enzymes [1]. RED is a convenient semiquantitative method that does not require expensive equipment and is suitable for studying small volumes of enzyme solutions. It can be used to analyze crude enzyme samples and is not affected by either the turbidity or viscosity of the enzyme solutions [1]. Available literature does not provide sufficient information on the activity of hydrolases of different subclasses in strains of *Vibrio cholerae* O1 and O139 serogroups of different epidemiological significance determined using the RED method.

Our goal was to study the preparations of cell-free fractions of *Vibrio cholerae* strains of O1 and O139 serogroups of different epidemic significance and origins for the presence of hydrolases of different subclasses by means of RED assay in agarose gels containing appropriate substrates.

MATERIALS AND METHODS

In this study, we used cell-free fractions (preparations from urea extracts, outer membranes, and culture fluid supernatants) obtained from the cells of 58 genetically heterogeneous strains of cholera germs. These germs contained 23 toxigenic strains, isolated from cholera patients and the environment during cholera outbreaks, and 35 non-toxigenic strains isolated from the water bodies during the period free from cholera epidemics. Of these, 11 – clinical toxigenic strains of the O1 serogroup (from patients), 1 – a classical biovar, and 12 – non-toxigenic strains. Of the O139 serogroup 5 strains were studied, of which 3 – clinical toxigenic and 2 – aquatic non-toxigenic strains. The bacteria were cultured on Hottinger's agar (pH 7.6). A daily culture of each cholera germ grown at 37°C was washed off with buffered saline solution (BSS). The bacterial mass (at a concentration of 10^9 microbial cells (m. c.) in 1.0 ml) was treated with a sterile 9 M urea solution at a 1:1 ratio for 24 h at room temperature for lysis and disinfection. Bacteriological control of the specific sterility of the resulting material was carried out in accordance with the Instructions for the control of specific sterility of experimental preparations from cultures of plague or cholera microbes (Saratov, 1982). The urea extracts (UE) and outer membranes (OM) were isolated by differential centrifugation of the resulting lysates, followed by dialysis and freeze-drying [5]. To obtain culture fluid supernatant (CFS) preparations, cholera germs were initially grown on alkaline meat-peptone agar (pH 7.6) at 37°C for 24 h. Then, the daily culture was washed off with physiological solution and inoculated into bottles with meat-peptone broth (pH 7.6) at a concentration of 10^8 m. c./ml. After 2 h of incubation at room temperature, sodium merthiolate (0.01%) was added to the bottles to sterilize the inoculated culture, and the bacterial suspension was kept in cold for 48 h. After control and confirmation of specific sterility, the suspension was centrifuged at 10,000 rpm. The resulting cell-free supernatant was dialyzed and freeze-dried. Samples for zymographic studies were obtained by suspending lyophilized preparations of subcellular fractions and SCF in BSS at a protein concentration of 4 mg/ml.

The detection of hydrolases was carried out using the RED in a 1-1.5% agarose gel containing different substrates for the detection of the corresponding enzymes: gelatin, bovine serum albumin (BSA), and G-class human immunoglobulins for the detection of proteases; Tween-20 and Triton X-305 (Sigma-Aldrich, USA) for the detection of lipolytic enzymes; glycolchitosan, colloidal chitin, and peptidoglycan for the detection of chitinolytic enzymes. Yeast RNA, commercial mucin preparation, and egg lecithin were used to detect nucleases, mucinases, and lecithinases, respectively. Sodium alginate and sodium dodecyl sulfate at a final concentration of 0.5% were used to detect the sulfatases.

After a 24-h incubation, agarose gels were treated with a 10% solution of trichloroacetic acid (in the case

of using protein substrates). In the case of using non-protein substrates, the gels were stained with an aqueous Lugol's solution containing 1% iodine and 2% potassium iodide, a 5% solution of sulfuric acid, 5% solution of calcium chloride in 70% ethanol or 0.02% methylene blue solution. The hydrolytic activity of the enzymes was assessed by the formation of clearing zones around the wells on agarose gels. Diluted enzyme solutions were used as positive controls, and distilled water, saline, and solutions of inert proteins (bovine serum, egg albumin, and protamine sulfate) were used as negative controls. Lipolytic activity was assessed by the emergence of matte halos, zones of substrate lipolysis in the form of rings of Saturn around the wells. The degree of hydrolysis (zones of clearing and turbidity) was determined by the size of the hydrolysis zone from the outer edge of the well to the outer edge of the circumference of the clearing (turbidity) zone in millimeters. Each experiment was performed in triplicates.

Enzyme activity was assessed according to the size of the hydrolysis zones using a four-cross system, where + corresponds to the hydrolysis zone of 1-2 mm, ++ – to a zone of 3-4 mm, +++ – to a zone of 5-6 mm, and ++++ corresponds to a hydrolysis zone of 7-8 mm and more. Statistical processing of the data included calculations of the mean and the standard error of the mean values in Statistica 7.0, program for Windows 10. The difference was considered significant if the probability of the error did not exceed 0.05 ($p<0.05$) versus the controls (positive and negative controls).

RESULTS

All the studied cell-free fractions of the cholera germ strains showed various degrees of protease activity in RED tests in agarose gels containing gelatin, BSA and human immunoglobulin G. Preparations from the non-toxigenic strains showed higher proteolytic activity than those from the toxigenic strains. The average size of non-toxigenic hydrolysis zones was 7.50±0.02 mm, while toxigenic strains had zones averaging 2.60±0.03 mm (Fig. 1A). Therefore, all preparations from the nontoxigenic strains had greater proteolytic activity than those from the toxigenic ($p<0.05$).

All UE preparations showed various IgG-degrading activities in the RED with G-class human immunoglobulins used as substrates. The variability of IgG-degrading activity was proved by formation of different sizes of hydrolysis zones: for preparations from toxigenic strains – 3.00±0.03 mm and for those from non-toxigenic strains – 1.00±0.03 mm ($p<0.05$) (Fig. 1B). The absence of a hydrolysis zone for immunoglobulin

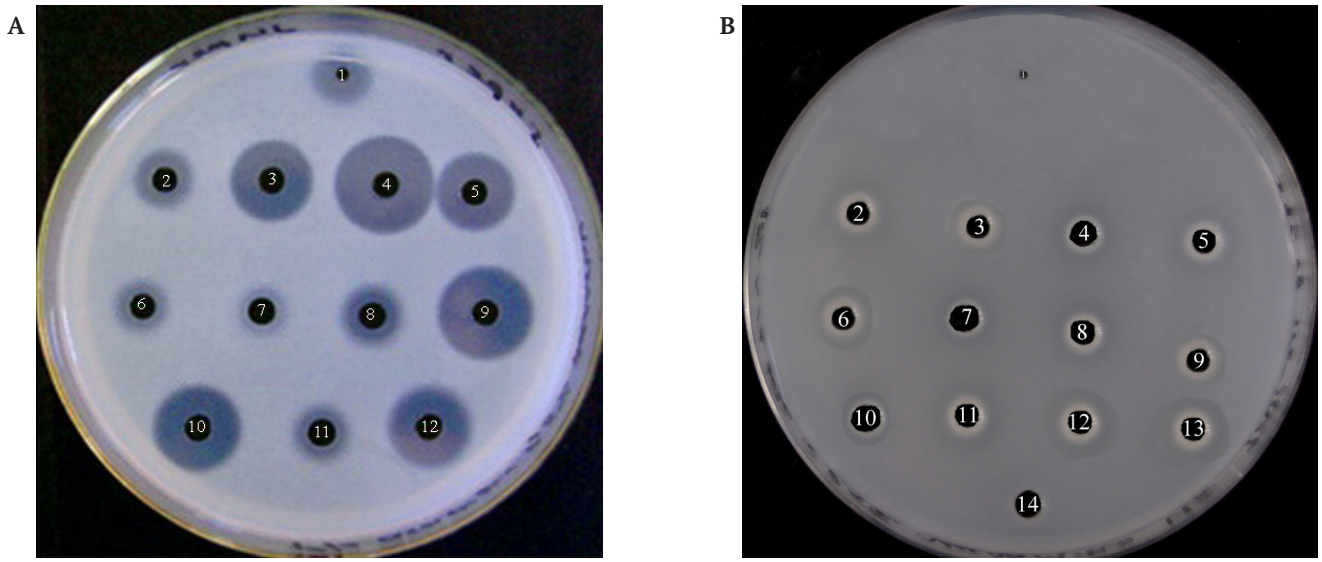

Fig. 1. RED of cell-free fractions O1 El Tor and O139 serogroups *V. cholerae* preparations in a 1% agarose gel. **A.** The gel contains 0.5% gelatin solution as a substrate: 1 – trypsin (K^+); 2 – SCF of O1 El Tor M-878 *V. cholerae* (*ctx*$^+$); 3 – SCF of O1 El Tor 129-05-B *V. cholerae* (*ctx*$^-$); 4 – SCF of O1 El Tor I-638 *V. cholerae* (*ctx*$^-$); 5 – SCF of O1 El Tor I-680 *V. cholerae* (*ctx*$^-$); 6 – SCF of O1 El Tor I-1263 *V. cholerae* (*ctx*$^+$); 7 – SCF of O1 El Tor I-1300 *V. cholerae* (*ctx*$^+$); 8 – SCF of O1 El Tor I-1334 *V. cholerae* (*ctx*$^+$); 9 – SCF of O1 El Tor I-1368 *V. cholerae* (*ctx*$^-$); 10 – SCF of O1 El Tor 2-01 *V. cholerae* (*ctx*$^-$); 11 – SCF of O139 I-12 *V. cholerae* (*ctx*$^+$); 12 – SCF of O139 I-16 *V. cholerae* (*ctx*$^-$). **B.** The gel contains IgG human immunoglobulins: 1 – trypsin (K^+); 2 – UE of O1 I-1263 *V. cholerae* (*ctx*$^+$); 3 – UE of O1 I-1368 *V. cholerae* (1 mg/ml) (*ctx*$^-$); 4 – UE of O1 1291 *V. cholerae* (*ctx*$^-$); 5 – UE of O1 129-05-B *V. cholerae* (*ctx*$^-$); 6 – UE of O139 I-16 *V. cholerae* (4 mg/ml) (*ctx*$^-$); 7 – UE of O139 I-16 *V. cholerae* (1 mg/ml) (*ctx*$^-$); 8 – UE of O1 M-878 *V. cholerae* (*ctx*$^+$); 9 – UE of O139 I-16 *V. cholerae* (1 mg/ml) (*ctx*$^-$); 10 – UE of O1 I-1368 *V. cholerae* (4 mg/ml) (*ctx*$^-$); 11 – UE of O1 I-1369 *V. cholerae* (*ctx*$^-$); 12 – UE of O1 2131 *V. cholerae* (*ctx*$^-$); 13 – UE of O1 2-01 *V. cholerae* (*ctx*$^-$); 14 – dH_2O (K^-).

G in the experiment with trypsin (used as a positive control) could be explained by the natural resistance of immunoglobulin molecules to the action of proteolytic enzymes [5]. When comparing the total hydrolytic activity of cell-free fractions and SCF preparations towards protein substrates (gelatin, casein, protamine sulfate, IgG, BSA) the latter was observed to have a higher activity (9.00±0.04 mm, $p<0.05$). The highest activity was predominantly observed in preparations of non-toxigenic strains, which indicated a higher activity of secreted enzymes. Differences in proteolytic activity were observed between toxigenic strains of O1 as well as O139 serogroups. Thus, the gelatin hydrolysis zone of 8.00±0.03 mm was formed by the preparation ME *V. cholerae* El Tor O1 I-1263 (*ctx*$^+$), while in the case of ME *V. cholerae* El Tor O1 I-1337 (*ctx*$^+$) it was 2.50±0.03 mm. Also, in the preparation UE *V. cholerae* O139 I-12 (*ctx*$^+$) the gelatin hydrolysis zone was 3.00±0.03 mm, in contrast to UE *V. cholerae* O139 I-16 (*ctx*$^-$) (6.00±0.03 mm). The proteolytic activity of most OM preparations of *V. cholerae* of both serogroups, which formed hydrolysis zones of 1.50±0.03 mm on average, turned out to be lower than those of the UE and SCF preparations. The maximum lipolytic activity against Tween-20 was demonstrated by UE and OM nontoxigenic strains of the O1 serogroup, that formed hydrolysis zones with the halos size 7.00±0.04 mm and 2.50±0.03 mm, correspondingly. However, UE preparations of toxigenic strains of O1 and O139 *V. cholerae* serogroups showed high activity against Triton X-305: the hydrolysis zones were 8.00±0.03 mm and 5.00±0.03 mm ($p<0.05$) (Fig. 2A). When studying the action of chitinases, it was found that preparations of cell-free fractions and SCF from non-toxigenic strains have higher activity than those from clinical toxigenic strains: the hydrolysis zones of colloidal chitin were 6.00±0.04 mm (non-toxigenic) and 2.00±0.04 mm (toxigenic), $p<0.05$ (Fig. 2B).

The highest lecithinase activity was shown by OM and SCF preparations obtained from toxigenic strains with formation of 4.60±0.03 mm hydrolysis zones, while the preparations from non-toxigenic bacteria formed 1.00±0.04 mm zones on average.

The results of hydrolase activity evaluation for preparations from *V. cholerae* toxigenic and non-toxigenic strains against different substrates are presented in the Table, where ++++ corresponds to high activity, +++ – to medium activity, ++ – to moderate activity, and + – to low activity.

Preparations of subcellular fractions and SCF obtained from toxigenic strains showed clearing zones

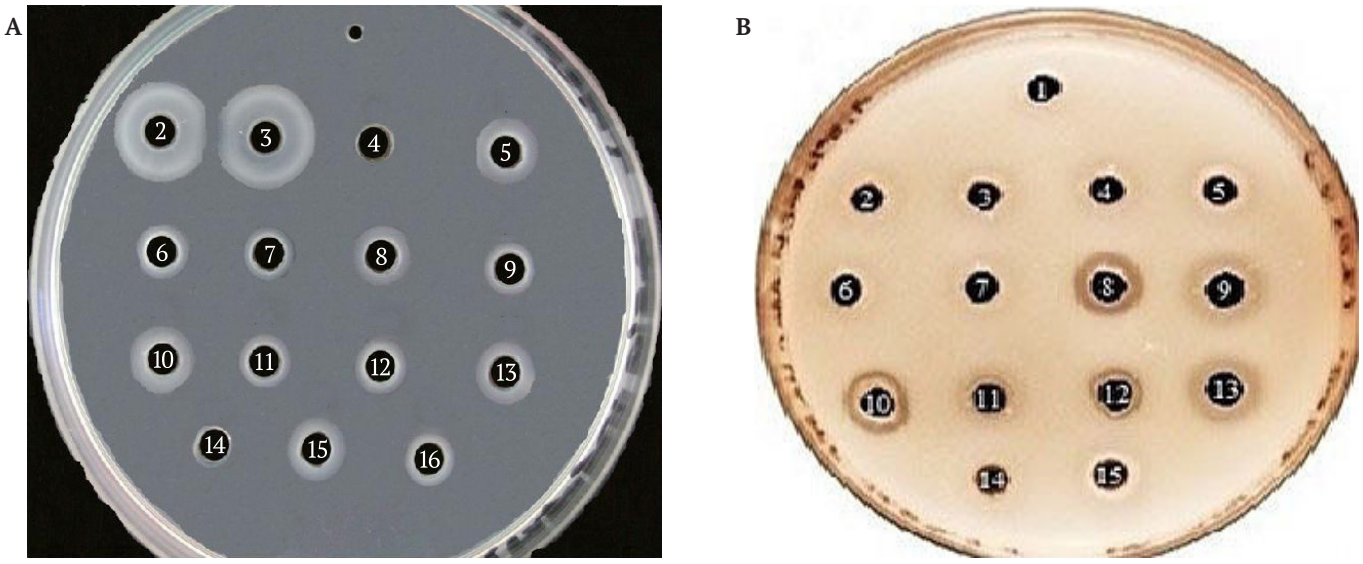

Fig. 2. RED of the preparations of subcellular fractions of *V. cholerae* O1 El Tor and O139 serogroups in a 1% agarose gel containing a 5% Triton X-305 solution. **A.** Response to lipolytic activity: 1 – BSS (K$^-$); 2 – UE of *V. cholerae* O1 I-1263 (*ctx*$^+$); 3 – UE of O1 M-878 *V. cholerae* (*ctx*$^+$); 4 – UE of *V. cholerae* O1 2-01 (*ctx*$^-$); 5 – UE of *V. cholerae* O1 I-1369 (*ctx*$^-$); 6 – UE of *V. cholerae* O1 2131 (*ctx*$^-$); 7 – UE of *V. cholerae* O1 1291 (*ctx*$^-$); 8 – UE of *V. cholerae* O1 I-1369 (*ctx*$^-$); 9 – OM of *V. cholerae* O1 I-1407 (*ctx*$^-$); 10 – UE of O139 I-12 *V. cholerae* (*ctx*$^+$); 11 – OM of *V. cholerae* O1 I-638 (*ctx*$^-$); 12 – OM of *V. cholerae* O139 I-16 (*ctx*$^-$); 13 – UE of *V. cholerae* O1 I-1361 (*ctx*$^-$); 14 – UE of *V. cholerae* O1 I-1299 (*ctx*$^-$); 15 – UE of *V. cholerae* O1 M-800 (*ctx*$^+$); 16 – UE of *V. cholerae* O1 I-1334 (*ctx*$^+$). **B.** Response of 0.5% colloidal chitin to chitinase activity: 1 – BSS (K$^-$); 2 – UE of *V. cholerae* O1 I-1337 (*ctx*$^+$); 3 – OM of *V. cholerae* O1 I-1337 (*ctx*$^+$); 4 – UE of *V. cholerae* O1 I-1334 (*ctx*$^+$); 5 – OM of *V. cholerae* O1 I-1263 (*ctx*$^+$); 6 – UE of *V. cholerae* O1 I-1298 (*ctx*$^+$); 7 – UE of *V. cholerae* O1 I-1300 (*ctx*$^+$); 8 – UE of *V. cholerae* O1 I-1369 (*ctx*$^-$); 9 – UE of *V. cholerae* O1 I-1368 (*ctx*$^-$); 10 – UE of *V. cholerae* O1 I-638 (*ctx*$^-$); 11 – UE of *V. cholerae* O1 I-1330 (*ctx*$^+$); 12 – UE of *V. cholerae* O1 129-05-B (*ctx*$^-$); 13 – UE of O1 *V. cholerae* 2-01 (*ctx*$^-$); 14 – Tris-HCl buffer (pH 7.6); 15 – dH_2O (K$^-$).

Table. Activity of preparations of cell-free fractions of *V. cholerae* O1 and O139 serogroups against different substrates

***V. cholerae* strains**	**Protease activity**	**Lipase activity (hydrolysis of Triton X-305)**	**Chitinase activity**	**Lecithinase activity**	**Mucinase activity**
Toxigenic strains	++	++++	+	++++	++++
Non-toxigenic strains	++++	+	++++	+	+

5.80±0.03 mm around the wells in RED tests for alginate lyase and sulfatase activities when agarose gels were developed with Lugol's solution. At the same time, in the case of preparations from the non-toxigenic strains, the radius of clearing zones was 3.00±0.04 mm on average when compared with negative controls (BSS and boiled samples), as well as with samples to which the aqueous solution of 5% $CuSO_4$ was added to inhibit hydrolases (no clearing zones) (Fig. 3A, B).

It should be noted that we observed rapid discoloration of the agarose gel and disappearance of clearing zones 10-15 min after staining the gels as well as the appearance of these zones on the agarose gel without any substrate at all. Clearing zones were formed with the studied samples when testing for the presence of alginase and sulfatase activity using a 0.02% solution of methylene blue. They also were formed in wells containing inert proteins, lysozyme, and BSA. At the same time, there were no clearing zones in the negative control samples – in the wells containing distilled water and pancreatic lipase. When a plate containing sodium alginate was developed with a 5% $CaCl_2$ solution, clearing zones appeared in the wells with preparations of cell-free fractions from toxigenic clinical strains and were absent in the wells with negative controls containing BSS, BSA, and protamine sulfate (Fig. 3B). A similar pattern (clearing zones) was observed without the addition of sodium alginate or sodium dodecyl sulfate to the agarose. To precipitate the material, 2N H_2SO_4 solution was added to the agar, both with and without sodium alginate. No visible changes on the agar surface were observed upon addition of this reagent, indicating the non-specificity of the occurring reactions.

However, when performing the RED in 1% agarose using L-cysteine, ovalbumin, and glutathione (Reanal, Hungary) that contain thiol (SH-) groups and are

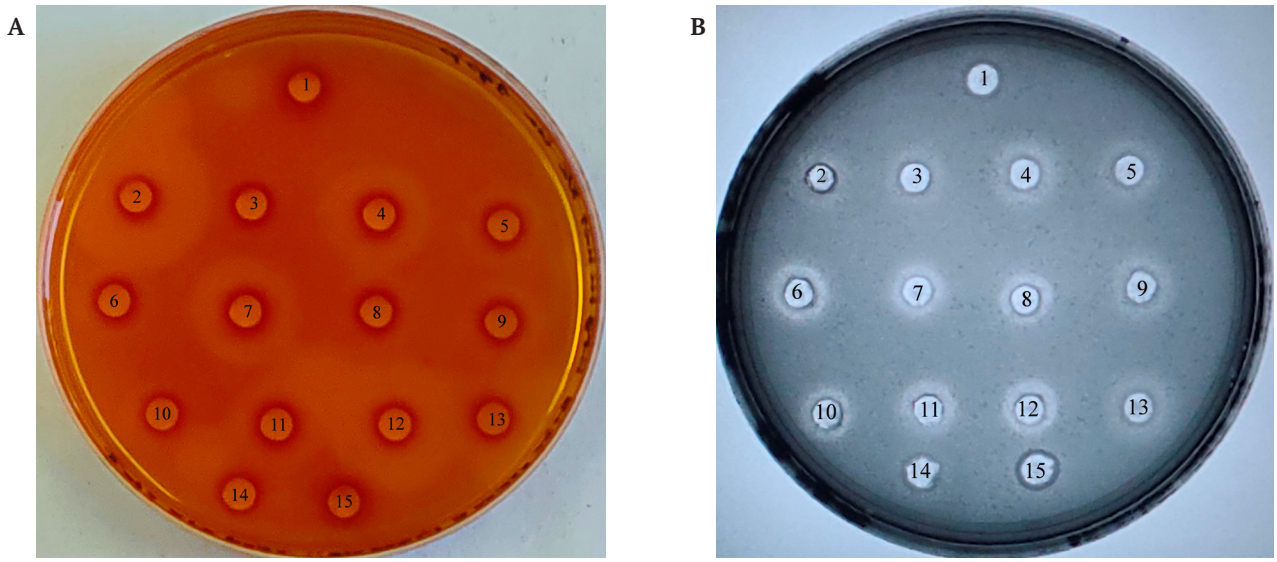

Fig. 3. RED of the preparations of cell-free fractions of *V. cholerae* O1 El Tor and O139 serogroups in a 1% agarose gel containing a 0.5% sodium alginate solution as a substrate. **A.** Development of the gel with Lugol's solution: 1 – BSS (K-); 2 – SCF of *V. cholerae* O1 I-1263 (*ctx*$^+$); 3 – SCF of *V. cholerae* O1 I-1299 (*ctx*$^-$); 4 – SCF of O139 59 Din *V. cholerae* (*ctx*$^+$); 5 – SCF of O1 *V. cholerae* O1 1296 (*ctx*$^-$); 6 – UE of *V. cholerae* O1 I-1407 (*ctx*-); 7 – UE of *V. cholerae* O1 1298 (*ctx*$^+$); 8 – UE of *V. cholerae* O1 I-638 (*ctx*$^-$); 9 – UE of *V. cholerae* O1 I-1337 (*ctx*$^+$); 10 – UE of *V. cholerae* O1 2-01 (*ctx*$^-$); 11 – UE of *V. cholerae* O1 I-1330 (*ctx*$^+$); 12 – UE of *V. cholerae* O1 M-878 (*ctx*$^+$); 13 – UE of *V. cholerae* O1 I-1334 (*ctx*$^+$); 14 – BSA (K$^-$); 15 – protamine sulfate solution (K$^-$). **B.** Subsequent development with 5% $CaCl_2$ solution: 1 – BSS (K$^-$); 2 – UE of *V. cholerae* O1 I-1337 (*ctx*$^+$); 3 – OM of *V. cholerae* O1 1298 (*ctx*$^+$); 4 – UE of *V. cholerae* O1 I-1300 (*ctx*$^+$); 5 – UE of *V. cholerae* O1 217-N-16 (*ctx*$^-$); 6 – UE of *V. cholerae* O1 1298 (*ctx*$^+$); 7 – UE of *V. cholerae* O1 2-01 (*ctx*$^-$); 8 – OM of *V. cholerae* O1 2-01 (*ctx*$^-$); 9 – UE of *V. cholerae* O1 I-680 (*ctx*$^-$); 10 – UE of *V. cholerae* O1 I-1369 (*ctx*$^-$); 11 – UE of *V. cholerae* O1 1291 (*ctx*$^-$); 12 – UE of *V. cholerae* O1 2131 (*ctx*$^-$); 13 – UE of *V. cholerae* O139 I-16 (*ctx*$^-$); 14 – BSA (K$^-$); 15 – protamine sulfate solution (K$^-$).

hydrogen donors as control samples, and subsequent staining of the dishes with Lugol's solution, clearing zones were observed in comparison with the negative control (buffer) wells. This indicated the occurrence of redox reactions that caused discoloration of the area around the wells (Fig. 4). These results demonstrate that Lugol's solution cannot be used for the detection of agarase, alginase and sulfatase activities.

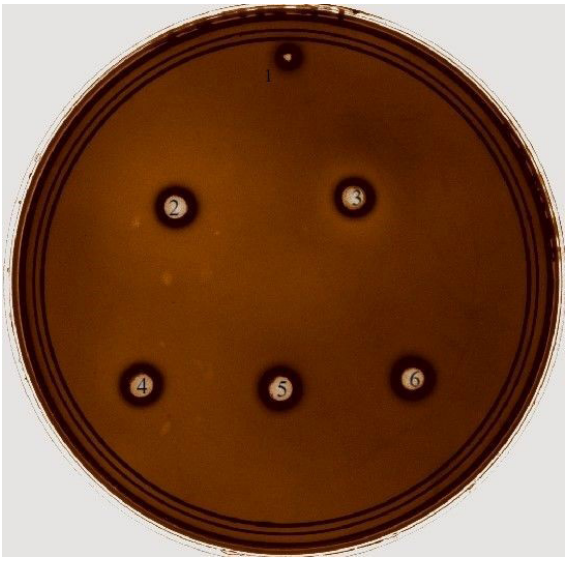

Fig. 4. RED of inert proteins and preparation UEs of *V. cholerae* O1 I-1263 on 1% agarose. Agarose gel was developed using Lugol's solution. 1 – 0.05 M Tris phosphate buffer solution, pH 7.6 (K-); 2 – L-cysteine solution (initial); 3 – ovalbumin solution; 4 – glutathione solution; 5 – BSA solution; 6 – UE of *V. cholerae* O1 I-1263.

DISCUSSION

We have shown the presence of activity as well as statistically significant differences in the activity of hydrolases of different subclasses by RED tests in preparations of cell-free fractions (UE, OM, SCF) obtained from *V. cholerae* strains of O1 and O139 serogroups of different epidemic significance and origins – proteases, chitinases (predominant in non-toxigenic strains), and lecithinases, mucinases, and lipases (predominant in toxigenic strains). Using the same detection methods, we found statistically significant differences in the activity of hydrolases (determined by the size of the substrate hydrolysis zones) in samples from different strains. Numerous studies have shown that the pathogenicity of cholera germ and its ability to persist in EOs includes several factors and their combinations, and not all these factors have been well studied to date. One such factor is the action of hydrolytic enzymes such as mucinases and hemagglutinin/proteases. They participate in the destruction of mucin in the epithelium of the small intestine, promoting the spread of cholera germ and increasing the binding of cholera toxins to the intestinal epithelium. They also hydrolyze antimicrobial proteins such as fibronectin, lactoferrin, ovomucin, and secretory immunoglobulin sIgA, which are factors of the local human immunity [6]. The hemagglutinin/protease inactivates a temperate filamentous phage of *V. cholerae* carrying cholera toxin *ctxAB* genes (CTXφ), preventing phage infection of the cholera germ. This allows the cholera germ cells to survive in different ecosystems. The destruction of the glycoprotein matrix of chironomid eggs, which serves as a reservoir of *V. cholerae* of different serogroups, allows them to persist in larvae, which explains the absence of cholera outbreaks during the interepidemic period (in unfavorable conditions). The presence of chitinases allows the cholera germ to adhere to chitin-containing substrates in the environment, using them as a source of carbon and energy, which is also important for survival. The detected increased chitinolytic activity of non-toxigenic strains compared with toxigenic strains indicates the contribution of chitinases to the adaptive potential of non-toxigenic isolates obtained from the environment. The most pronounced ability of preparations from non-toxigenic strains of *V. cholerae* El Tor O1 and toxigenic strains of the cholera germ O139 serogroup to hydrolyze substrates containing short-chain fatty acids (Tween-20) may be due to the predominance of esterases in these samples. The higher activity of UE preparations from toxigenic strains against Triton X-305, which contains long-chain fatty acids, can be associated with the action of lipases. The observed differences in lipolytic activity of preparations from toxigenic and non-toxigenic strains against the studied substrates reflect the individual properties of the corresponding strains. These differences are also associated with the composition of lipolytic enzymes, and the specific activity of the preparations obtained from the bacterial strains. All these factors, including sensitivity to environmental conditions, demonstrate the intensity of lipid metabolism in microbial cells. In addition, the higher activities of lipases, lecithinases, and mucinases in preparations from toxigenic strains may indicate their contribution to the pathogenic potential of the original strains. We also observed the dependence of hydrolase activity in RED tests on the toxigenicity of the original strains, which can be used for additional phenotypic characterization of strains in relation to their potential epidemiological significance and persistence potential. Thus, the RED is a suitable method for screening cell-free fraction preparations of cholera germ for the presence of hydrolytic enzymes of different subclasses. When using development solutions containing iodine or methylene blue for the detection of

alginate lyase and sulfatase activity, the occurrence of artifacts such as the rapid appearance and disappearance of clear zones, their appearance on an agarose gel without a substrate, and their formation with some inert proteins and amino acids (BSA, protamine sulfate, L-cysteine) can be explained by the redox reactions of molecular iodine, which is one of the components of Lugol's solution, and methylene blue with the SH-groups of the proteins contained in the analyzed preparations [7, 8]. Oxidation of thiol groups of amino acids cysteine and methionine, glutathione tripeptide, and numerous proteins leads to discoloration of the iodine and methylene blue solutions and, correspondingly, to nonspecific reactions, which can lead to incorrect interpretation of the obtained results (false-positive results). Iodine solutions are widely used for the detection of agarases, cellulases, and xylanases in agar gels, both with and without substrates, as it is believed that the application of these solutions is a faster method than other methods that use organic dyes [8, 9, 10, 11]. It was believed that some agarolytic bacteria can change the double helical structure of agar without liquefying it, so that it does not darken when stained with iodine solution [12]. However, our data prove that redox reactions make iodine solutions unsuitable for the detection of agarase and cellulase activities in RED tests. The same assertion was previously made by J. A. O'Hair about the unsuitability of using iodine solution for the detection of cellulase activity in Gram's iodine assay [13].

CONCLUSION

We detected and assessed the activity of hydrolytic enzymes of different subclasses in preparations of cell-free fractions of cholera germ O1 and O139 serogroups using the RED assay in agarose. The results of our study showed statistically significant differences in their activity and revealed the following pattern: non-toxigenic strains have increased production of proteases and chitinases, while toxigenic strains produce more lipases, lecithinases, and mucinases. We showed that iodine and methylene blue solutions are not suitable for staining agarose gels in RED tests assessing alginate lyase, sulfatase, and agarase activities because it can lead to false-positive results. Based on our results, the RED proved to be a reliable and promising method for determining the presence of hydrolases, although in some cases careful interpretation of the obtained results is required. However, further significant modifications to this method are necessary. The results of this study can be used in the microbiological diagnostics of cholera for the differential biochemical characteristics of cholera germ strains.

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