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Original Article

Production of Antibody Raised Against Lipopolysaccharide (LPS) of *Vibrio cholerae* Non-O1

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Abstract

Background: Cholera, an infectious disease caused by *Vibrio cholerae*, is primarily transmitted by ingestion of contaminated food or water. In severe cases, cholera may lead to severe dehydration, metabolic acidosis, and ultimately, hypovolemic shock and death.

Methods: In this study *V.cholerae* non-O1 was cultured in suitable media. LPS was extracted from the surface of bacteria by hot phenol-water method and then purified by high-speed centrifugation. For production of specific antibody against LPS, white newzeland rabbits were first immunized by whole cell bacteria and then immunized with highly purified LPS. The titre of the antiserum was determined by ELISA for each serogroup.

Results: Results presented in this study indicate that serum anti-LPS antibodies raised against purified LPS of *V.cholerae* non-O1 can detect *V.cholerae* non-O1.

Conclusion: This antibody had low cross reactivity with *V.cholerae* O1, serotype Inaba or Ogawa. So, this antibody can be used for for detection of *V. cholerae* non-O1.

Keywords: *Immunoglobulin, Lipopolysaccharide,* Vibrio cholerae *non-O1, ELISA*

Introduction

The facultative human pathogen *Vibrio cholerae* that is a permanent threat to human populations (1) exists as natural inhabitants of aquatic ecosystem. It can be transmitted by ingestion of contaminated food or water, causing health problems for human. More than 200 serogroups of these bacteria including O1, non-O1 and O139 has an important role in its spreading.

Regarding the spread of non-o1 strains all over the world and its isolation from sewage water, contaminated surface water, estuarine, salty water, see foods and animals, its recognition is of high importance (2-6).

Non-O1 *V.cholerae* can cause severe gastroentritis dehydration (cholerae like) whose common symptom is abdominal cramp, fever and 25% of the infected patient show bloody diarrhea that in the case of no apppropriate treatment it would be comprehensively critical, although resulting diarrhea from non-O1 is milder than that of O1 (7).

Since the organism is isolated from extraintestinal infections, using infected water for bathing can also lead to infection of eyes, ear, bladder and body wounds in susceptible individuals (8, 9).

A new serogroup of the bacterium named Bangal O139, has been responsible for recent cholera epidemy in Bangladesh (10) and because the immunization against *V.cholerae* O1 gives no protection to human it indicates that formerly cholera contracted people are also at risk. This bacterium is derived from Eltor biotype, is much resistance to environment in contrast with Eltor and classic biotypes, and can be rapidly spread in environment. One of the best ways to control the cholera disease that needs an epidemiological study is its origin recognition.

Two major biotype and serotype characteristics are remarkable in epidemiological study of *V. cholerae* and according to its biotype and serotype the repertoire, spreading and its contagion would be identified. Hence regarding the defini-

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tion of its serotype by O antigen (polysaccharide portion of LPS), antibody production against lipopolysaccharide is very important in epidemiological study.

Studies indicate that aquatic environment is the most important source of *V.cholerae* non-O1. Reportedly sea water and sea food products are the main cause of infection (11). In contrast to *V. cholerae* O1, non-O1 strains has been isolated from blood, ears and respiratory organs (12). Several virulance agents can be produced by *V.cholerae* each of which, lipopolysaccharide for example, can be the causetive of disease (13).

The aim of this study was isolation and purification of endotoxin of *V.cholerae* non-O1 and production of antibody against it.

Materials and Methods

V.cholerae Ogava and Inaba serotypes and *E.coli* O157 obtained from Iranian Reference Laboratory has been used in this study as controls. Antiserum against *V.cholerae* O1 antigen (Difco) was used for serogrouping. *V.cholerae* non-O1 obtained from reference laboratory was a patient isolated strain in Iran. Biochemical tests such as growing on TCBS agar medium, gram staining, oxidase and catalase test and carbohydrate consumption had been used to define the bacterium. Confirmed non –O1 strain had also been used for other studies**.**

Bacterial cell body preparation Non –O1 V*ibrio cholera*e was transferred to yeast Extract tryptone media (TY-2) and grown in appropriate aeration (150 RPM) at 37° C overnight. Supernatant was centrifuged for 10 min at 6000 RPM and pellet was extracted for lipopolysaccharide. Distilled water was added in addition to equal volume of 90% phenol. To isolate lipopolysaccharide from proteins, the material was at 68° C for 15 min, thereafter it was centrifuged at 100000 g for 4 h, to purify LPS. The precipitate was resolved in DW, dialyzed, and lyophilized.

Standard LPS of *V.cholerae* serotype Inaba was obtained from Sigma Company.

Tricine SDS-PAGE gel electrophoresis Laemmli method was used for electrophoresis. For this, 15 µg of purified LPS was resolved in 3 µl SDS lysis buffer and then heated at 100° C for 10 min, and 6 µl of it was loaded in the well, and finally the gel was coomasie blue and silver stained.

Antibody preparation Killed bacteria were used to prepare antibody. For this purpose isolated *V.cholerae* was exposed to formalin (1.5%) at 4° C overnight. After washing and deformalization, it was heated at 65° C for 1 h to obtain killed vaccine. The vaccine was injected subcutaneously to white Neusland rabbit weighing about 2 kg. The mixture of injection contained 10^9 bacteria per 1 ml of physiological serum, 1 ml complete adjuvant (for first injection), and then homogenized from which 0.5 ml was injected under shoulder subcutaneously. Complete adjuvant had been used as booster on the days 0, 14, 28, 42. Bleeding was done in order to control antibody production before each injection.

To raise antibody against LPS, 100 µg of LPS with incomplete adjuvant had been used subcutaneously for second, third and fourth turn of immunization instead of whole cell killed bacteria, and after four injection which was done fortnightly final bleeding had been accomplished.

ELISA for bacterium and LPS The wells of flat-bottomed, microtitration plates (Dynatech Immulon™) were coated with either 5 x 10^7 bacteria per ml, grown in TSB medium, or 1 mg/ml of LPS both diluted in 0.05 M carbonate buffer, pH 9.6 in the proportion of 1 in 100. The plates were incubated at 4° C overnight in a humidified box then washed three times with washing buffer containing gelatin 0.5% w/v and Tween 20 (0.01% v/v in PBS), pH 7.4. To each well, 100 µl of rabbit polyclonal antibody raised against bacteria or LPS, at a dilution of 1 in 3000 in washing buffer, were added. The plates were incubated for 4 h at 37° C in a humidified box, washed, and 100 µl of anti-rabbit IgG-HRP conjugate diluted 1 in 3000 in washing buffer were added to each well and the plate was incubated 1 h at 37° C and washed as above. The enzyme reaction was initiated by the addition of

100 µl of 0-phenylenediamine (34 mg/ml; Sigma) and H_20_2,(20 µl) in 100 ml of 0.15 M citrate-phosphate buffer, pH 5.0 (freshly prepared). The plate was incubated for 30 min at room temperature in the dark for colour development. The reaction was stopped by addition of 50 µl of H_2SO_4 12.5% v/v to each well. Absorbance was measured at 492 nm in an Anthos 2001 ELISA reader.

Antibody purification IgG was purified using G protein affinity column. For this rabbit serum was obtaind from 20 ml blood. The column was prepared by rinsing with 100 mM Tris first and 10 mM Tris secondly, pH was adjusted to 8 by 1 mM Tris. After passing the sample, the column was washed with 100 mM and 10 mM Tris respectly to remove extra proteins. There after IgG was removed with 100 mM glycin, pH=3, and stored in microtubes containing 50 ml of 1M Tris.

Protein and polysaccharide assay One mg/ml concentration of LPS was prepared. Its polysaccharide was measured by phenol sulphuric acid method while its protein content was also measured by Bradford method using BSA as standard.

Results

A non-O1 *Vibrio cholerae* strain was isolated from water, identified by biochemical methods. Grown in alkaline peptone water medium at 37° C overnight, and was able to form yellow colony with the diameter of 2-3 mm, in TCBS agar at 37° C for 24 h.

Two serotypes, Ogava and Inaba obtained from Iranian Reference laboratory was used as control. The prepared non-O1 cholera was defined as albensis biotype.

Measurement of protein in LPS After harvesting the bacteria from TY-2 medium, LPS was purified and assayed for protein content. For this, 1 mg/ml of LPS was assayed by Brathford method (weve length 595 nm) resulting 0.01 absorbance, indicating of as low as zero protein.

Polysaccharide assay of LPS One mg/ml of LPS was assayed for polysaccharide using phenol sulphuric acid method, resulting to 250 µg/ml of polysaccharide. The extracted LPS was lyophilized for further usage.

LPS extraction One mg of extracted LPS by hot phenol water was resolved in 1 ml DW and electrophoresed on Tricine SDS-PAGE. It was then silver stained. In this experiment, LPS of Inaba serotype and, *E.coli* O157 and non-O1 *V.cholerae* were electrophoresed on 18% SDS-PAGE. Brown bands had been demonstrated after Periodic silver staining (Fig. 1).

Standard Inaba LPS (sigma), LO385 had shown two bands of 8 and 16 kDa (Lane 2), and *E. coli* O157 used as control, also revealed two bands of 8 and 18 kDa (Lane 3), and *V.cholerae* non-O1 has only shown one band of 8 kDa (Lane.4), Marker used in this study contained 17000 Da and 14200 Da bands (Lane 1).

ELISA Antibody against LPS of *V.cholerae* non-O1 serotype was raised in this study, and after purification, it was administered to rabbit. There after, bleeding through marginal vein was performed and obtained serum was stored at-70° C. The result of ELISA after first and booster injections is depicted in Fig 2 Considerable Ab was raised after first injection of non-O1 bacteria emulsified with complete adjuvant. After second injection of LPS instead of whole bacteria, increase in antibody was observed. The increasing trend continued after second and third boosters, and the titer of antibody for non-O1 recognition reached to 1/12800.

Cross reactivity of non-O1, Inaba and Ogava was studied by ELISA (Fig. 3) using antibody against LPS of non-O1 *V.cholera*e. Antibody cross reactivity with NAG bacteria was shown at concentrations of 1/200, 1/400, 1/800, 1/1600, 1/3200, 1/6400 and 1/128000.

The antibody could not recognize *V.cholerae* Inaba and Ogava serotypes at the concentration of 1/1600, although little cross reactivity was observed at 1/12800 concentration (Fig. 3). Antibody purification against LPS was done in order to increase its recognition ability. As depicted in Fig. 4, antibody titer of 1/16000 could recognize non-O1 LPS while unpurified antibody could

recognize non-O1 serotype at 1/1600 concentration (Fig. 3), expressing 10 times of recognition ability. Purified antibody was used for cross reactivity between non-O1 and, Inaba and Ogava bacteria (Fig. 4). For this reason, the serum was purified by affinity column after bleeding and serum isolation. Cross reactivity between produced antibody against NAG Lipopolysacharide and NAG, Inaba and Ogava bacteria was identified. The results indicate that purified antibody had less cross reactivity with Inaba and Ogava and its titer with NAG bacterium was 1/16000 (Fig. 5).

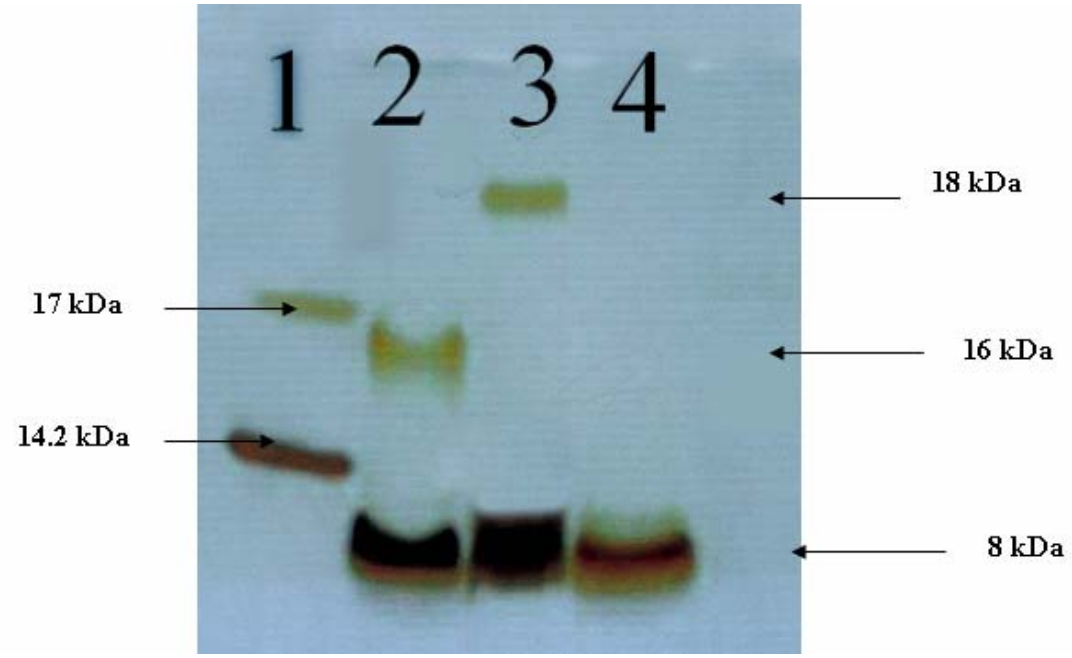

Fig 1: Tricine SDS-PAGE

Shown are lipopolysaccharides of *Vibrio cholerae* Inaba and, *E. coli* O157 and *Vibrio cholerae* NAG electrophoresed in SDS-PAGE 18%
1- 17000 and 14200 Marker
2- Inaba *Vibrio cholerae* LPS
3- LPS of *E. coli* O157 as control
4- non-O1 *Vibrio cholerae* LPS

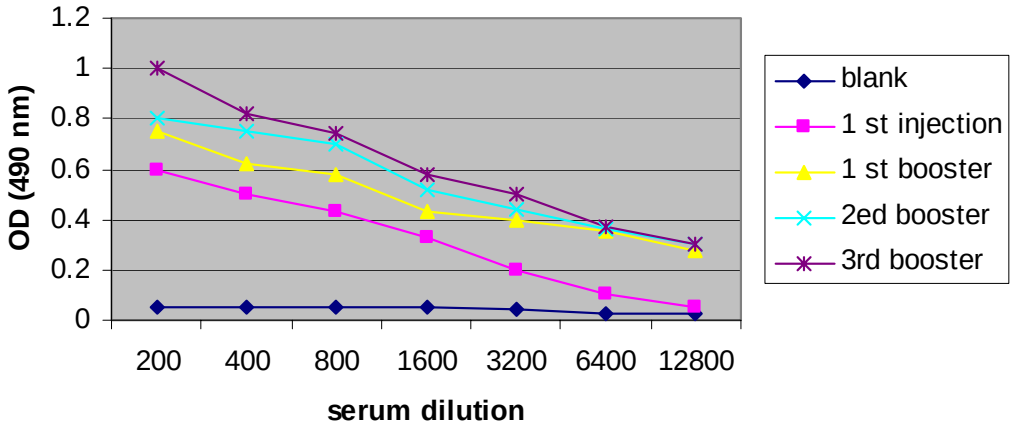

Fig. 2: Antibody production after 1st injection and next 1st and 3rd booster

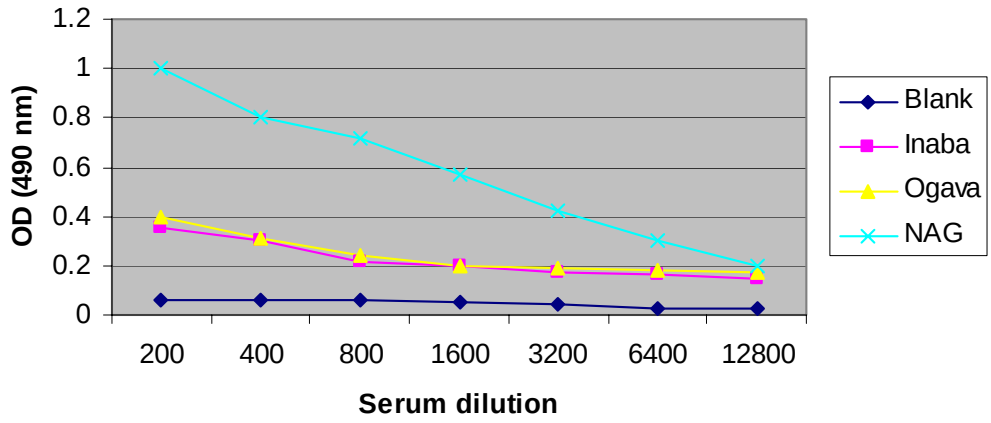

Fig. 3: Cross reactivity assay between non-O1 and Inaba, Ogava, using produced antibody against *Vibrio cholerae* non-O1. Results indicate low cross reactivity.

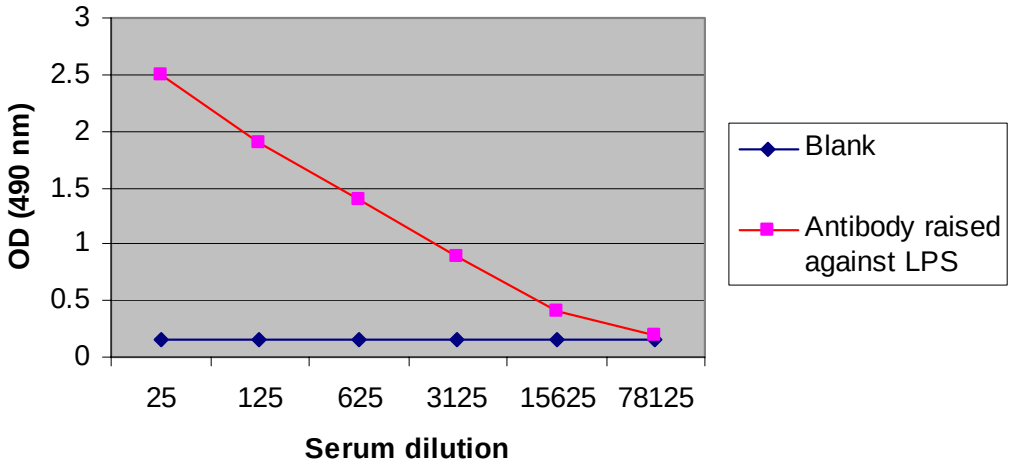

Fig . 4: The titer of purified antibody against LPS of non-O1 *Vibrio cholerae*.

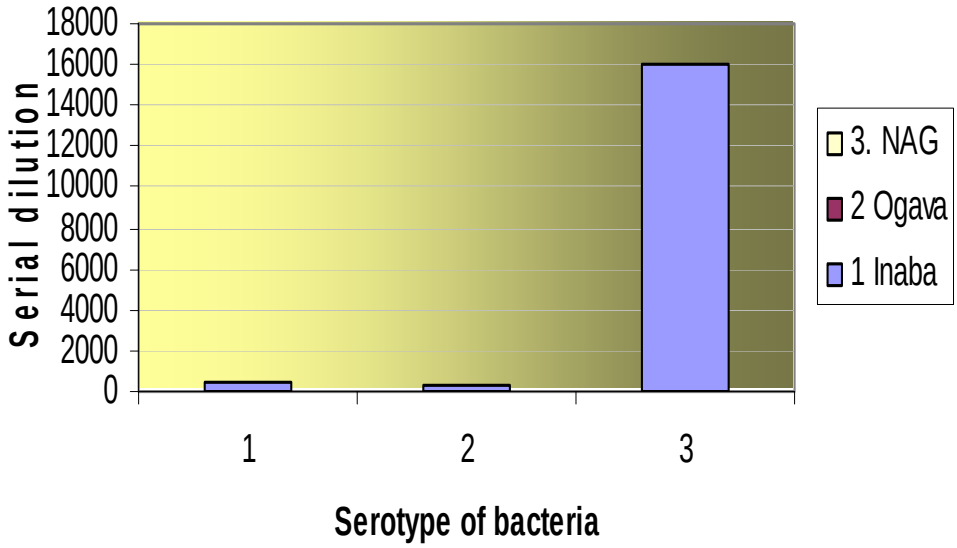

Fig. 5: Titer of 1/16000 could recoginze non-O1 *Vibrio cholerae*

Discussion

V.cholerae human pathogen bacteria can be transmitted through infected water and food. These bacteria are classified to more than 200 serogroups according to "O" antigen (LPS) (14), containing O1, non-O1 and O139. Cholera is usually caused by O1 and O139 serotypes, however, reportedly non-O1 serotype was also patient isolated (15, 16).

Non-O1 serotypes are isolated from contaminated sewage and surface water estuarine and salty water, see food products and other animals (17). In contrast with O1 serotypes, this serotype is able to grow in different conditions (18), having more chance of human infection.

Since the bacteria serogroup is of high epidemiological importance, more study has been done on it. O1 serogroup has formerly been studied in Imam Hosein University and antibody directed against its lipopolysaccharide was extracted (19), however no study was accomplished over non-o1 yet, in Iran.

Regarding cholera-infected countries neighboring to Iran, the risk of transmission and spreading of the disease is high and should be highly considered. Non-O1 recognition and identification is comprehensively important, while some non-O1 strains can produce heat-stable toxin and cholera toxin and cause cholera disease (20, 21). In past time, O1 *V.cholerae* was known to be the only agent of epidemic cholera, but emerging of Bangal O139 (one of non-O1 serogroups) in 1992, with vast epidemiology in Bangladesh and India and other neighboring countries has changed the viewpoint. In two studies in 1988 and 1995, the possibilities of changing non-O1 strains to O1 and vice versa were reported. It was shown that O139 serogroup is derived from O1 Eltor biotype and is identical to it in biochemical characteristics.

Studies by Rafael Montilla et al. have shown that under different environmental conditions and in stationary phase 10^5 out of 10^{10} non-O1 bacteria had been changed to O1 serotype (22), expressing the possibility of Eltro cholera disease by water containing non-O1 serotypes and its clinical importance.

Studies show that non-O1 *V.cholerae* is severely threatening to human population so they should be well informed for inhibition of its spread (12). One of the ways to prevent its spreading is rapid detection, which in gram-negative bacteria; LPS can be used for this purpose. Several methods have been used for LPS purification of different bacteria and in this study *V.cholerae* LPS was extracted and purified by westphal method and in contrast to trade sample (sigma), Inaba lipopolysaccharide 10385 had less protein. The protein content of trade sample was reported 1-10% (sigma catalogue 2000-2001), while the purified protein in this study was less than 1%. The LPS purified by Villeneuve in 1999, contained 1% protein (23). Low rate of protein seems to be an advantage because proteins are more immunogenic than lipids and polysaccharides and can interfere antiserum preparation.

Antibody production against Lipopolysaccharide Several reports cover antibody production against *V.cholerae* (24, 13, 25). Adams and his colleagues (1988) demonstrated cross reactivity between monoclonal antibody against O1 LPS and some non-O1 bacteria (26). Qadri et al., after monocloral antibody production against O139 *V.cholerae* announced that the antibody can recognize O91 cholera bacteria (27), however the antibody produced by Gustafsson (1984) showed no cross reactivity with non-O1 *V.cholerae* and *E.coli*, *Shigella sonnei* and *Salmonella* strains (28). The difference can be related to the clonies prepared by the researchers.

Because of low molecular weight of antibody against LPS, it cannot activate immune system lonely, leading to researchers' efforts in linking to a protein to stimulate immune system. Since 40% of the surface of bacterium is covered with LPS, a low molecular weight molecule, but cooperation with other surface molecules it can easily stimulate immune system, so the dead bacterial vaccine was used at the first injection and next as the first booster purified LPS had been used. In addition to detection of different strains

of *V.cholerae*, the produced antibody can be used to decrease bacterial adhesion to digestive system leading to protection against the disease (13, 29). Studies state that there is cross reactivity between different strains of *V.cholerae* (30) but produced antibody against O1 serotype did not have protection against non-O1 serotype (31).
The results of ELISA after first booster revealed that the immune system has been well stimulated by LPS, so at next booster purified LPS was used and the result of this study indicate that IgG titer against non-O1 LPS can not rise up as O1 strains and no out standing difference in antibody titer has been seen after third boosting, so the injection was not continued.
Produced antibody against *Vibrio cholerae* was tittered by ELISA showing its recognition ability. Because of some antigenic similarity between non-O1 and O1 strains, some cross reactivity with Inaba and Ogava strains has been observed, the fact that was reported by other researchers (2, 4). However, in this study the mentioned cross reactivity was at the lowest rate indicating the structural difference of LPS in these strains.
In conclusion, regarding high specificity of this antibody and its low cross reactivity it can be used for recognition of non-O1 strains.

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