

CLINICAL MANIFESTATIONS AND DIAGNOSIS OF CELIAC DISEASE IN CHILDREN

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ABSTRACT

Celiac disease (CD) is defined as a permanent intolerance to gluten with clinical manifestations and abnormal small intestinal mucosa. A case series study was performed to obtain information about clinical manifestations, age of presentation and diagnosis of CD. For this purpose, hospital records of patients who were admitted to the gastrointestinal ward in the Children's Medical Center from 1986 to 1996 with suspected diagnosis of CD and discharged with such a diagnosis were reviewed. Thirty-five patients (17 male, 18 female) with diagnostic criteria for CD were selected and followed. The average age of onset was 3.07 years but mean age of diagnosis (the first small intestinal biopsy) was 7.7 years. The most common symptom was chronic diarrhea. The interval between initial symptoms and diagnosis was surprisingly long. During this period these subjects had multiple medical visits at different hospitals. With recognition of this treatable disorder and by available diagnostic procedures and obtaining small intestinal biopsy specimens, we can reduce the mentioned interval and diagnose this entity earlier in Iranian children.

MJIRI, Vol. 13, No. 1, 33-36, 1999

Keywords: Celiac disease, Gluten, Children.

INTRODUCTION

Celiac disease (CD) is defined as intolerance to gluten and abnormal proximal intestinal mucosa in genetically predisposed patients. Elimination of gluten (wheat, rye, barley) from the diet has been proven to improve clinical manifestations and prevent villus atrophy in intestinal mucosa.¹⁻⁵

Incidence is variable in different areas, from 1 in 250 to 1 in 4000 live births. Incidence in the USA is lower than Europe and there are some reports from Asia and the Middle East.^{3,6-9}

The immunologic response to gluten ingestion results in villus atrophy, crypt hyperplasia and damage to the surface epithelium of the small bowel and it has an important role in the pathogenesis of the disease. The greatest injury is in the proximal small bowel and extends distally for a variable distance.⁹ On the basis of a positive family history of 8.7% in first degree relatives, 70% concordance rate in identical twins and associated HLA (DRW₂, DR₇, DQW₂, B₈), a genetic predisposing factor is advocated.¹⁰ Environmental factors such as antibiotics, surgery, pregnancy and serotype 12 adenovirus infection have been reported to play a role.^{5,8,11-13}

Although CD may appear in any age, presentation is usually between 6 months and 2 years when the diet contains gluten. Clinical manifestations vary and span from chronic diarrhea and failure to thrive (Fig. 1) to constipation and short stature.⁹ We may only notice mucosal damage without

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Celiac Disease in Children

any clinical manifestation in silent forms in the patients, siblings.^{3,13,14}

Delay in diagnosis may cause major complications associated with growth retardation. In order to recognize CD and age of presentation in children of Iran, we made a case series study of the patients admitted and followed at the gastrointestinal ward of the Children's Medical Center.

PATIENTS AND METHODS

All hospital records of patients suspected of CD who were admitted to the gastrointestinal ward of the Children's Medical Center from 1986 to 1996 were reviewed. A complete work-up including clinical presentation, physical examination and laboratory data (CBC; serum albumin, globulin, electrolytes; D-xylose test; serum cholesterol and triglycerides; serum carotene; stool examination for pH, reducing substances, fat and parasites; sweat iontophoresis, and radiologic study) were performed.

Thirty-five patients who met the disease criteria and responded to gluten-free diet and had follow up visits, were selected. Some of these patients had undergone repeated small intestinal biopsy.

All 35 patients had a proximal small intestinal biopsy, obtained through a pediatric flexible fiberoptic upper endoscope after admission (Fig. 2). Diagnosis was based on criteria introduced by ESPGAN* in 1990,^{3,9,11,15} in which two essential conditions consist of pathological damage (villus atrophy, crypt hyperplasia, increased crypt depth and cellular infiltration) and clinical improvement after gluten elimination from the diet.

Control biopsies after gluten-free diet were only needed for those who had partial improvement or those who had no symptom at the onset (discovered in family study). In this regard, 3 cases in our study had control biopsies. Circulating IgA antibodies against gliadin, reticulins and endomysium at the time of diagnosis and their disappearance on gluten-free diet adds weight to the diagnosis^{1,4,16} but were not available at that time.

Gluten challenge test (GCT) was needed when:

1. A patient started a gluten-free diet without initial small intestinal biopsy;
2. The first intestinal biopsy specimen was atypical or inadequate;
3. Initial symptoms were atypical;
4. To rule out entities such as cow's milk allergy that may have identical symptoms and are common before 2 years of age.
5. An adolescent patient began eating gluten-containing foods.

In this regard 3 cases in our study had GCT performed.

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Fig. 1. A 5.5 year old boy with active celiac disease. Note the loose skin folds, marked proximal muscle wasting, and full abdomen. The child looks ill.

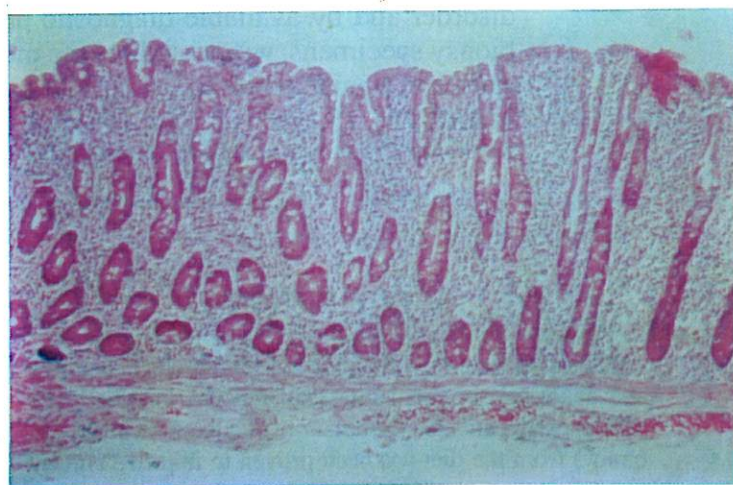


Fig. 2. Complete atrophy of villi, with deep crypts.

RESULTS

From 35 patients (18 female and 17 male) with a mean age of 7.7 years (17 months to 12 years) at the time of admission and first intestinal biopsy, the average age of onset of disease was 3.07 years (4 months to 8 years) [Figs.

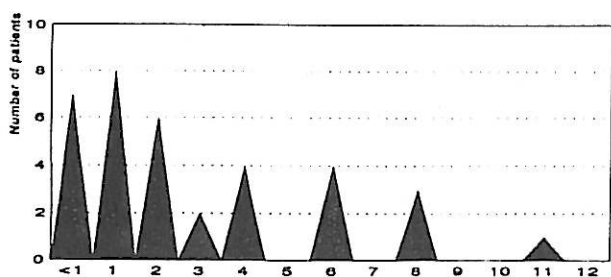


Fig. 3. Age of presentation in 35 children with celiac disease.

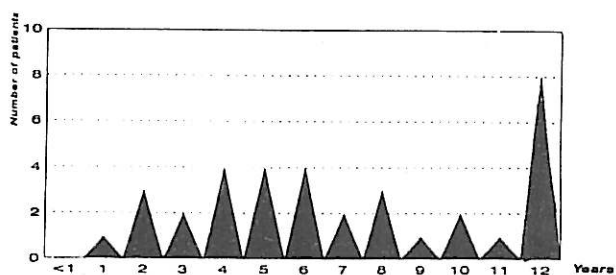


Fig. 4. Age at first biopsy in 35 children with celiac disease.

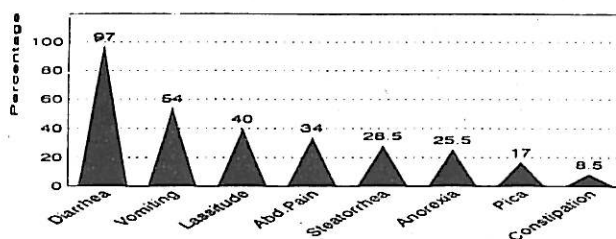


Fig. 5. Symptoms in 35 children with celiac disease.

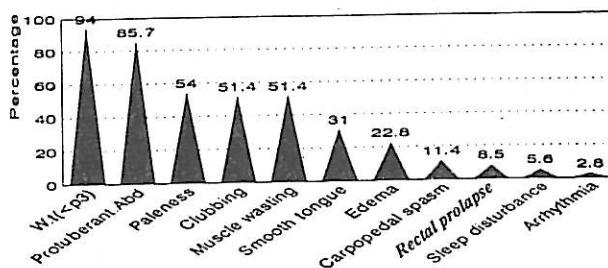


Fig. 6. Signs in 35 children with celiac disease.

3 and 4]. Chronic diarrhea was the most common symptom at onset in 18 patients (51.4%), followed by vomiting in 6 (17.1%), failure to thrive in 5 (14.3%), abdominal distension in 4 (11.4%), and anorexia in 1 (2.9%). The most common presenting symptom at the time of admission was diarrhea in 34, followed by weight under 3rd percentile in 33 and abdominal distension in 30. Other symptoms and signs are shown in Figs. 5 and 6.

Laboratory tests revealed abnormal D-xylose test in 83%, anemia 77%, hypoalbuminemia 60%, hypocalcemia 51.4% and hypokalemia in 40%. Associated disorders, except for liver cirrhosis (in one case) were not detected in any patient. All patients had pathologic findings in their proximal intestinal biopsy specific for CD. All of them showed an increased velocity of weight gain and clinical responses after elimination of gluten, in their follow up visits months and years later.

DISCUSSION

As mentioned before, there were 35 patients in a 10 year period. It seems that the number of patients is less than what is expected. This may have two reasons: first, in Iran, this disease is not common. Second, some of the patients die before they refer to the clinic for diagnosis. In this study there was no significant sex difference, while some studies state that the female to male ratio is as high as two.¹³ Mean age of onset was 3.07 years in our study, while in other studies it was 8-24 months.¹¹ Reasons may include delay in introduction of gluten into the diet, longer duration of breast

feeding,^{2,17,18} genetic factors and parental delay on seeking medical advice. Mean age at the time of diagnosis was about 7.7 years. The interval between onset and diagnosis is more than 4.6 years which is quite long. In other studies this interval was 2-4 years.³ The reasons for this delay are multifactorial, and include inadequate diagnostic procedures in other hospitals, common disorders such as malnutrition that can simulate CD, lack of information about this disease in the general population and referring the patients in late stages of the disease to special centers.

In the majority of other studies, clinical and paraclinical manifestations are the same as in our study, such as diarrhea that has been reported as the most common symptom. But pica and arrhythmia (due to electrolyte imbalance) were present in 6 and one of our patients, respectively. The latter symptoms did not appear in other studies. In the hospital records of our patients we did not notice any mood changes in our patients and their mothers, while other workers insisted on irritability and mood changes as one of the most common symptoms in CD.^{3,8,11,12}

It would have been better to investigate the antibodies against gliadin, reticulin and endomysium in these patients, but unfortunately the facilities for this kind of investigation were not available in Iran at that time.

Malignancy is a rare childhood complication. We had no cases of malignancy although we followed our patients up to 9 years.

By improving the methods of recognition of CD, obtaining further information about the various manifestations in children of our country, and educating

Celiac Disease in Children

parents to refer the patient to medical centers on time, we can achieve earlier diagnosis of this treatable disorder.

Editorial comment:

Several antibody tests are useful as adjuncts to small intestinal mucosal biopsy for establishing the diagnosis of celiac disease. These tests are also useful for screening selected target populations suspected of having celiac disease and in the follow up of patient compliance relative to adhering to a gluten-free diet.

In general, IgA anti gliadin antibody has greater specificity for the detection of celiac disease than IgG anti gliadin antibody.

A second antibody test for the diagnosis of celiac disease is the anti-endomysial antibody test, which measures an antibody that is directed against extracellular matrix components. Elevated titers of IgA anti-endomysial antibodies are present in 5% of patients with clinically active celiac disease who have subtotal or total villous atrophy.

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Basic Science In Medicine

MOLECULAR CHARACTERIZATION AND OPTIMIZATION OF VI-CAPSULAR POLYSACCHARIDE OF *SALMONELLA TYPHI* TY6S PRODUCTION IN BIOREACTOR

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ABSTRACT

The role of Vi-capsular polysaccharide (Vi-CPS) in human immunity against infection caused by *Salmonella typhi* is well known. The downstream process of purification generally causes depolymerization of Vi-CPS to a nonimmunogenic low molecular weight form. In the present study, a standard strain of *Sal. typhi* Ty6s was grown under submerged cultural conditions in a pilot-plant scale of 90 liter fermentor. At the late exponential growth phase, crude polysaccharide was obtained from fermentation broth by detergent-phenol extraction method and purified by ultracentrifuge differentiation technique. Analytical data reveals that by optimization of fermentation parameters, not only was the yield of production increased from 1.6 mg/L to 4.9 mg/L, but also the polysaccharide retained its native molecular stability and immunogenicity. Therefore, purified Vi-CPS can be regarded as a reliable immunogen to control typhoid fever in man.

MJIRI, Vol. 13, No. 1, 37-42, 1999

Keywords: *Salmonella typhi*, Vi-CPS, Polysaccharide vaccine.

INTRODUCTION

Typhoid fever still remains as one of the serious health problems in developing countries^{3,10} with 12.5 million cases annually (except China).^{3,23} In fact the final goal to control this infection is vaccination.^{2, 4, 18, 20, 21-23} Classical TAB-

typhoid vaccine⁴ and oral *Sal. typhi* Ty21a vaccine²² offer good protection in man^{2,22} but each of these suffers from some drawbacks as an ideal vaccine^{2,4,18,22} which makes this field open for further investigations.

Recently, it has been shown in two controlled field trials that Vi-CPS sub-unit vaccine could prevent typhoid fever. A single intra-muscular injection of this vaccine conferred about 65%¹² to 72%¹ protection in South Africa and Nepal, respectively.

The production rate of Vi-CPS in laboratory scale, under stationary cultural growth conditions, is low.⁵ Scaling

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Purification of *S. typhi* Vi-CPS

up the rate of production to a large scale is technically difficult and causes depolymerization of Vi-CPS. Besides, downstream processes are either patented or kept secret in multi-national companies marketing vaccines.

Our investigations were aimed at overcoming this problem by adaptation of *Sal. typhi* Ty6s R-mutant in submerged growth cultural conditions in five liters of bioreactor and optimized cultural conditions, then scaling the production rate up to ninety liters while retaining the native molecular and immunological identity of Vi-CPS after extraction and purification.

Analysis of obtained experimental data revealed that after adaptation to our procedure, Vi-CPS retains its native molecular status even in large scale production rates. Therefore Vi-CPS can be regarded as a safe and reliable immunogen for mass vaccination to control typhoid fever in man.

Further immunological investigations in volunteers are necessary to prove this claim in Iran.

MATERIALS AND METHODS

Bacterial strains

Vi-CPS was produced from a standard rough strain of *Sal. typhi* Ty6s (CSBPI-B191) which is rich in Vi-polysaccharide. This strain was procured from Collection of Standard Bacteria, Department of Bacterial Vaccines, Pasteur Institute of Iran. Hyperimmune Vi-antiserum was prepared from *Citrobacter ballerup* (CSBPI-A124)

Culture

Sal. typhi Ty6s was grown on 90 liters of modified Frantz medium⁶ containing 5 g/L yeast extract-dialysate, 5 g/L glucose in classical batch and 20 g/L glucose in fed-batch processes in a NOVO-Pal Jas Bilthoven unit bioreactor. Fermentation parameters were adjusted at $36 \pm 1^\circ\text{C}$, $\text{pH}=7.6$, final speed of rotation 450 rpm and air flow 5 L/h.

Purification of Vi-CPS

At the late exponential growth phase (14 h), cells were harvested from fermentation broth by centrifugation at 4000 rpm at 4°C . The supernatant was treated with 1g/L of cetavlon (Fluka Chemical, AG.) with continuous shaking at 4°C for 30 minutes. The precipitate was collected by centrifugation at 4000 rpm at 4°C for 30 minutes. Crude Vi-CPS was obtained according to the method of Tacket et al.²⁰ The Vi-CPS was further purified by ultra-centrifuge differentiation technique⁷ using a Beckman Instruments Inc. LM 80 ultracentrifuge.

Purified and filter sterilized Vi-CPS was washed twice with alcohol, acetone and diethyl ether, then dried in a p205 desiccator at 37°C . Dried Vi-CPS was dispensed in 50 mL vials and kept at -20°C until used.

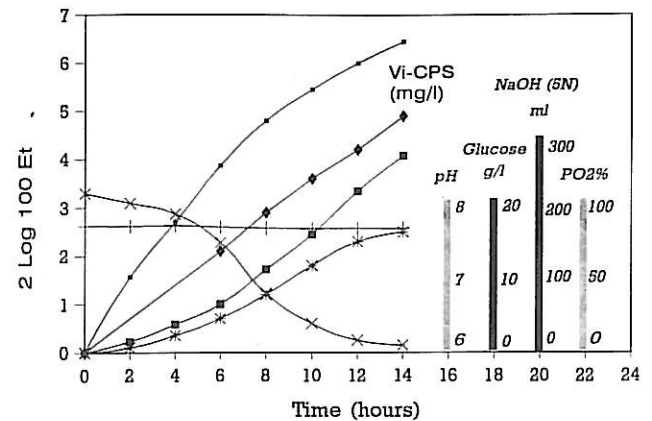


Fig. 1. Growth curve of *Salmonella typhi* in fermentor (fed batch culture).

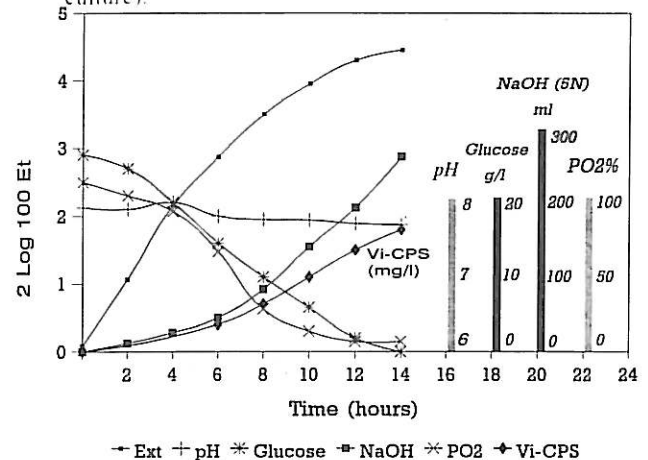


Fig. 2. Growth curve of *Salmonella typhi* in fermentor (batch culture).

Moisture content

The moisture content of the purified Vi-CPS was determined by drying under vacuum and P205 at 37°C until a constant weight was reached.

O-Acetyl content

O-acetyl content of purified Vi-CPS was determined according to Hestrin,⁸ using acetylcholine chloride as a reference.

Nucleic acid and protein contamination

Nucleic acid was determined by U.V. spectroscopy,²⁵ and protein was determined by Lowry's method¹⁴ using bovine serum albumin (BSA) as a reference.

Molecular size

The molecular size of purified Vi-CPS was estimated by gel filtration chromatography column (1.5x85) using sepharose 4B-CL.^{17, 25} The polysaccharide was eluted by 0.2M sodium chloride containing 2 mM Na_3N . The content of Vi-CPS in each fraction tube was measured by the

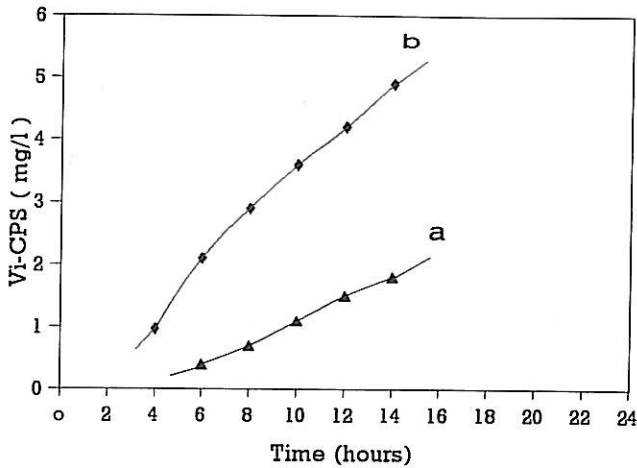


Fig. 3. Comparison of production rate of Vi-CPS a) before and b) after optimization of fermentation parameters.

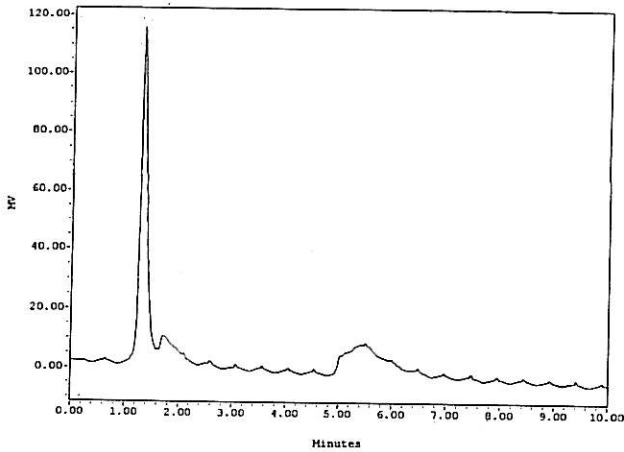


Fig. 4. HPLC profile of purified Vi-CPS, passed through a reverse phase column (p18, Delta pack, 5 micron, 100 Å, size 3.9x150mm) and R-410 differential refractometric detector.

Mv= Maximum velocity.

method of Hestrin.⁸

Sterility test

The purified Vi-CPS was tested for bacterial and mycotic sterility according to the requirements given in the revised WHO Requirements for Biological Substances.²⁶

Abnormal toxicity test

The purified Vi-CPS was tested for abnormal toxicity by intraperitoneal injection of 50 µg purified Vi-CPS into five mice (weighing 17-22g) and 250 µg purified Vi-CPS into two guinea-pigs (weighing 250-350 g). The test was considered satisfactory if the animals would survive for at least seven days without weight loss.

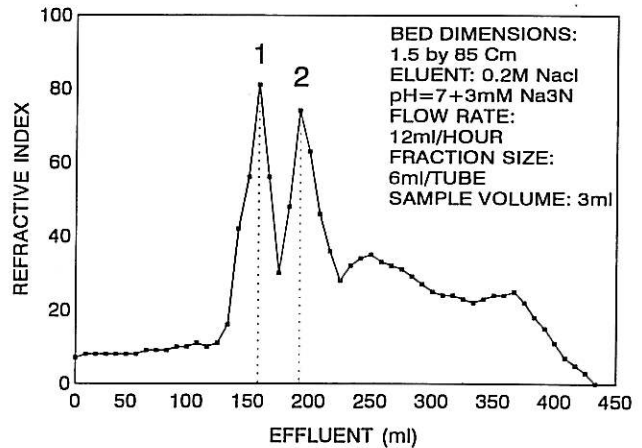


Fig. 5. Elution profile for *Salmonella typhi* Ty6s Vi-CPS on sepharose 4B-CL column.

Pyrogenicity test

The purified Vi-CPS was tested for pyrogenic activity by intravenous injection into rabbits (New Zealand White rabbits weighing 2 to 2.5 kg each). Three healthy rabbits were used in each test. The polysaccharide was reconstituted in special diluent. Further dilution was done in pyrogen-free physiological saline. Each rabbit received 0.025 micrograms of purified Vi-CPS per kg of rabbit weight.¹⁹

Immunogenicity studies

In this study, five New Zealand White rabbits (2 to 2.5 kg each) were used. Each rabbit received 50 micrograms of purified Vi-CPS emulsified in 50%(vol/vol) complete Freund's adjuvant intramuscularly. Bleeding for serological analysis was performed at days 0, 15, 30, 45, 180, and 360. The titer of serum against Vi-CPS of each sample was determined by passive hemagglutination (PHA) technique.^{13,15}

Identity test

The identity of the Vi-CPS was estimated by Ouchterlony gel double diffusion technique¹⁶ using hyperimmune serum prepared from multiple injections of a standard culture of *Citrobacter ballerup* Vi+ (CSBPI-A124). The immunoglobulin was purified and concentrated against 45% saturated ammonium sulphate.

High pressure liquid chromatography (HPLC)

10 µg of Vi-CPS was injected to a HPLC Waters-4000, Reverse phase column (P18, Delta pack, 5 micron, 100 Å, size 3.9 x150 mm) and R-410 differential refractometric detector. Diluent was 80% methanol with flow rate of one mL/min.

Purification of *S. typhi* Vi-CPS

Table I. Yield of Vi-CPS production before and after optimization*(mg/L).

	Time (hour)							
	0	2	4	6	8	10	12	14
Before opt.	-	-	0	0.4	0.7	1.1	1.5	1.8
After opt.	-	-	0.97	2.1	2.9	3.6	4.2	4.9

* Increasing yeast extract from 3g/L to 5 g/L giving glucose in fed-batch process in fermentation broth and extracting Vi-CPS from live culture.

Table II. Determination of titer of pooled anti-Vi sera by passive hemagglutination.

Antigen	Antigen dilution					
	0	1/2	1/4	1/8	1/16	1/32
Vi-CPS*	+	+	+	+	+	+
Vi-CPS**	+	+	+	+	+	+
<i>N. meningitidis</i> A polysaccharide	-	-	-	-	-	-

* Before optimization
 ** After optimization
 +Positive hemagglutination
 - Negative hemagglutination

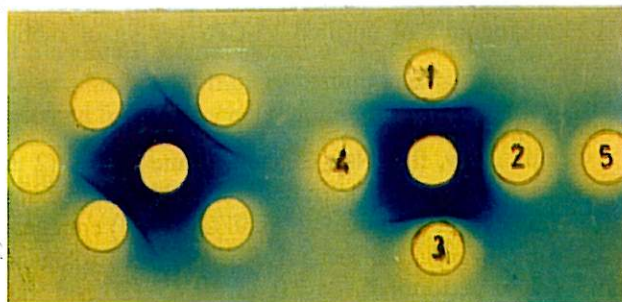


Fig. 6. Immunodiffusion analysis of native purified Vi-CPS. Wells 1, 2 and 3, purified Vi-CPS; well 4, *N. meningitidis* A polysaccharide as antigen control. Well 5, monospecific anti-LPS antiserum. *Sal. typhi* as negative control. Center well, monospecific purified Vi-antiserum.

RESULTS

The biochemical and some of the immunological properties of purified Vi-CPS prepared in a semi-industrial scale of 90 liter bioreactor were studied by different methods.

Sal. typhi Ty6s was grown under controlled-submerged cultural conditions and Vi-CPS was extracted from fermentation broth. The product was highly purified containing more than 4.7 mMol O-acetyl and less than 10 mg protein and 20 mg nucleic acid per gram polysaccharide as contaminants. Moisture content was less than 2.5% of dry polysaccharide weight.

As observed in Table I and Figs. 1, 2 and 3, after

optimizing the fermentation parameters, the yield of production was increased by 3.1 mg/L.

HPLC

To determine the purity of the product and apparent molecular size, high pressure liquid chromatography was performed and only one peak with a retention time of one minute and 10 seconds was observed (Fig. 4).

Chromatography

As observed in Fig. 5, the Vi-CPS was eluted as a high molecular weight aggregate form with two major adjacent peaks at tubes No. 25-29 and 30-40, respectively.

Gel-Precipitation reaction

This method was employed to detect the identity of the product. Fig. 6 illustrates a single precipitation line of identity exhibited by purified mono-specific polyclonal Vi-antibodies in the central well and wells containing highly purified Vi-CPS. However, the well containing the capsular polysaccharide of *Neisseria meningitidis* type A shows no cross-reaction with Vi-antiserum. In addition, anti-LPS antibodies of *Sal. typhi* Ty2 did not exhibit any precipitation line of reaction with Vi-CPS.

Determination of anti-Vi-CPS antisera titer

The antiserum titers of immunized rabbits were determined by passive hemagglutination method. From Table I, it can be inferred that antiserum titers for Vi-CPS were 1/32.

DISCUSSION

Being a homopolymer of (1-4) linked N-acetyl alpha D-galactosaminuronic acid acetylated at 0-3,⁹ the immunogenicity of Vi-CPS is directly related to molecular size and configuration.^{11,16} The downstream process of purification generally causes depolymerization of Vi-CPS to a non-immunogenic low molecular weight form.²⁰

The present investigation was aimed to produce Vi-CPS in semi-industrial scale while retaining its molecular configuration, as native as possible, to be used in mass vaccination programs against typhoid fever in Iran.

As illustrated in Fig. 3 and Table I, the yield of the production was increased from 1.8 mg/L to 4.9 mg/L (total increase by 3.1 mg/L), when *Sal. typhi* Ty6s was adapted in submerged growth cultural conditions in modified Frantz fermentation broth, whereas the production rate of Vi-CPS reported by Elaine et al.⁵ was 1.6 mg/L. The extraction was done from live cells, and the product was highly purified, having more than 4.7 mMol O-acetyl, less than 10 mg protein and less than 20 mg nucleic acid as contaminants.

The elution profile of Vi-CPS in HPLC illustrates only one peak of high molecular weight (Fig.4) with a retention time of 11 minutes, which proves the purity of the product. Moreover, the Vi-CPS was eluted in high molecular weight aggregated in Sepharose 4B-CL. At least more than 50% of the recovered Vi-CPS was eluted before a KD value of 0.25.

There was no decrease in weight of guinea pigs and mice when the abnormal toxicity test was performed. Moreover, pyrogenic response tests of the rabbits were done and no rise in body temperature observed when 10, 20, 50 and 100 µg of purified Vi-CPS was injected intravenously.

Concerning the observation of the present study, Vi-CPS can be produced in semi-industrial scale while retaining its native configuration and immunogenic status. Therefore, Vi-CPS can be regarded as a safe, nontoxic and reliable immunogen to control typhoid fever in man.

ACKNOWLEDGEMENT

With due respect, we acknowledge the Biotechnology Committee of the Iranian Presidential Office for approving the project, and Dr. M. Azarnoosh, Director of Pasteur Institute of Iran for his financial support to produce Vi-CPS vaccine in semi-industrial scale.

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