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## Development of a New Quantitative Bacterial Micro-Assay for Rapid Detection of Galactosemia: Application in Galactosemia Screening

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**Abstract:** In the present study, a new economic, rapid and inexpensive bacterial micro-assay for simultaneous detection and quantitative measurement of serum galactose was developed. Quantitative measurement of galactose in the serum of galactosemia patients is necessary to confirm the disease. Analysis of the standard curve showed a broad linearity range for galactose from 2 to 180 mg dL<sup>-1</sup> with a regression equation of  $Y = 0.013-0.083$ ;  $R^2 = 0.962$ . The advantage of the method is its ability to measure serum galactose quantitatively. The cost per sample is about 20-50 cents, which is much less than HPLC and enzymatic commercial kits. The method can be automated which is suitable for galactosemia neonatal and mass screening especially in developing countries in which funding is a limiting factor.

**Key words:** Bacterial micro-assay, galactose, galactosemia, screening

### INTRODUCTION

Galactosemia (OMIM 230400), a life-threatening disorder with severe symptoms in the neonatal period, is caused by deficiency in the enzyme galactose 1-phosphate uridyl transferase (GALT3; EC 2.7.7.12), which is an important key enzyme in the Leloir pathway of galactose metabolism (Wang *et al.*, 1998; Elsas and Lai, 1998; Holdena *et al.*, 2004).

The disease is inherited as an autosomal recessive manner which occurs with a frequency of about 1 in 62,000 (Wang *et al.*, 1998). The affected infants usually born normal, but symptoms start developing after milk feeding (Elsas and Lai, 1998). In this disorder, ingestion of milk causes accumulation of galactose in the blood and urine, leading to high intracellular concentrations of galactose 1-phosphate (gal-1-P). Gal-1-P is considered toxic for several tissues, especially the liver, brain and renal tubules (Wang *et al.*, 1998). Clinical manifestations of the disease appear shortly after ingestion of milk, predominantly as gastrointestinal, hepatic, neurological symptoms and lethargy. Patients become comatose and if treatment is not initiated early, death often occurs during the first week of life as a result of gram-negative sepsis, hepatic or renal failure (Holden *et al.*, 2003; Segal, 2004; Gitzelmann and Steinmann, 1984). Early treatment with a dietary of galactose/lactose restriction causes regression of symptoms and signs within 1 or 2 weeks (Bosch *et al.*, 2004; Novelli and Reichardt, 2000).

Unfortunately, despite dietary restriction, long-term complications may involve growth retardation, speech defects, ovarian failure, learning disabilities and other neurological and psychological abnormalities (Lee *et al.*, 2003; Waggoner *et al.*, 1990).

Rapid and in time diagnosis of galactosemia in affected infants can help preventing the progression of mental and developmental disorders associated with the disease. There are currently a number of methods for the quantitative and qualitative determination of galactose in the blood. The most common methods used are enzymatic colorimetric, Paigen, a biological inhibition assay and Beutler, a fluorescent method (Jensen *et al.*, 1966; Baker *et al.*, 1966; Hongl *et al.*, 2001; Berry *et al.*, 1997, 2004; Beutler, 1973; Jakobs *et al.*, 1998; Paigen *et al.*, 1982). Prospective screening of newborns with galactosemia is widely accepted procedure throughout the world. However, in most of the developing countries, especially those with limited health budget, neonatal mass screening programs such as galactosemia are basically delayed or ignored.

Quantitative measurement of galactose is necessary in positive cases to confirm the disease and to omit the false positive results as well as to determine the type of galactosemia (Classic form or Duarte form). This is very important for both molecular analysis of the disease and selection of the therapy procedures. HPLC and enzymatic assays which are usually used after Thin Layer Chromatography, TLC, or Gatheri bacterial

inhibition assay, GBIA, for quantitative measurement of galactose, are excellent approaches, but are very expensive and not suitable for mass population screening. Therefore, an economic, simple and rapid method is always favored. GBIA has been a widely accepted inexpensive method for analysis of serum galactose. However, usually a second method is required for quantitative measurement of galactose and to rule out the false positive results which naturally occur using the GBIA. Taking advantage of the GBIA method, we have developed a new economic, rapid and inexpensive bacterial micro-assay for simultaneous detection and quantitative measurement of serum galactose.

## MATERIALS AND METHODS

**Bacterial strains, media and reagents:** *Bacillus cereus* and *Proteous mirabilis*, facultative anaerobic bacteria were used as the bacterial strains through the present study. These strains were obtained from Microbiology Department of University of Isfahan, Isfahan, I.R. Iran. Both bacterial strains were negative for galactose utilization which was confirmed using the galactose utilization test as described by Boone and Castenholz (2001).

The culture medium for maintaining the bacteria contained yeast extract (3 g L<sup>-1</sup>), NaCl (5 g L<sup>-1</sup>) and Na<sub>2</sub>HPO<sub>4</sub> (1 g L<sup>-1</sup>). Agar plates were prepared by the addition of 1.5% Bacto agar (Difco, Detroit, MI, USA) to the culture medium before autoclaving. To prepare MSB (Mineral Base Salt) Medium, KH<sub>2</sub>PO<sub>4</sub> (1 g L<sup>-1</sup>), KHPO<sub>4</sub> (1 g L<sup>-1</sup>) and (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (1 g L<sup>-1</sup>) were autoclaved, then added to autoclaved stock solutions of FeCl<sub>3</sub> (0.001 g L<sup>-1</sup>) and MgSO<sub>4</sub> (0.002 g L<sup>-1</sup>).

The medium contains the basic minerals that are required for bacterial growth but lack any carbon resources. Also the medium provides an isotonic environment for bacteria to survive.

Di-Nitro Salicylic Acid (DNS) reagent 1% was made up DNS (10 g L<sup>-1</sup>), NaOH (16 g L<sup>-1</sup>) and KNaC<sub>4</sub>H<sub>4</sub>O<sub>6</sub> (sodium potassium tartarate) (250 g L<sup>-1</sup>). This is a specific reagent which reacts specifically with only reduced sugars such as glucose and galactose. For preparing of the 1% galactose medium, 1 g galactose were added to 100 mL MSB medium and then autoclaved. This medium were used for making different concentrations of galactose. Analytical grade chemicals from Merck (Germany) were used in this study.

**Bacterial culture:** The bacteria were streaked on agar plates and incubated at 37°C overnight to produce single colonies. A single colony was inoculated into 2 mL MSB

medium and incubated at 37°C and agitated at 150 x g in a thermo mixture (Eppendorf, Hamburg, Germany) for 2 h. This bacteria suspension was then used to assay serum galactose.

**Serum samples preparation:** Control healthy blood was obtained from blood transfusion centre of Isfahan. After incubating for 30 min at 25°C, the blood samples were centrifuged at 1800 xg for 5 min to get clear solution of serum. Working galactose standard solutions containing 2, 4, 8, 10, 16, 20, 32, 40, 60, 120 and 180 mg dL<sup>-1</sup> were prepared immediately before the assay by diluting a stock solution of 600 mg dL<sup>-1</sup> galactose. The galactose concentrations were then added to normal serum samples, to prepare different serums with galactose.

**Experimental design:** *B. cereus*, *P. mirabilis* and mixture of the suspension of these bacteria in 1:1 (v/v), were applied for assaying the galactose in the serum samples. Different concentrations of the galactose in MSB medium were prepared as mentioned above in 100 µL MSB medium, then 100 µL of normal serum was added. Finally, 50 µL of the suspension of each bacterial culture and the mixture of suspension of both bacteria were added separately in 1.5 mL eppendorf tubes. The tubes were incubated for 5 h at 37°C in 150 g shaking incubators (Eppendorf, Hamburg, Germany). After incubation the contents of the tubes were centrifuged at 3000 g for 5 min to sediment the bacterial cells. Then, 200 µL of the clear supernatant was transferred into new micro-tubes containing 50 µL fresh DNS 1% reagent. The contents of the tubes were briefly mixed by flicking and were then placed for 5 min at 100°C water bath. The presence of the galactose in the medium causes the color to change to brown, which can be measured spectrophotometrically at 540 nm. The different concentrations of galactose samples were plotted against the corresponding optical density and the linearity of the standard curve was analyzed. The standard curve was calculated by a linear regression or a weighted linear regression function.

**Optimization of bacterial incubation time:** One hundred microliter of *B. cereus* suspension culture was added to serum sample with 180 mg dL<sup>-1</sup> galactose concentration. Then the tubes were immediately incubated at 37°C and 150 g shaking in a thermo mixture. After different incubation time (from 1 to 10 h), the OD<sub>540 nm</sub> of the medium was measured every one hour as described above.

**Precision and sensitivity:** The precision of the assay was determined by analysis of intra-and inter-assay variations. Intra-assay variation was determined using 10 replicates

of three samples of 4, 60 and 180 mg dL<sup>-1</sup> galactose concentration. The inter-assay variation study involved analysis of the above samples in 10 different assays. The detection limit of the assay was determined on serum samples with different concentrations of galactose from 2-180 mg dL<sup>-1</sup>.

**Statistical analysis:** The results were analyzed using Students T-test and reported as this study were expressed as mean±SD. Regression lines were plotted for the data from three separate assays using SPSS 14.5 software (SPSS Inc., Chicago, IL, USA).

**RESULTS AND DISCUSSION**

This is the first study that introduces a new, rapid and inexpensive bacterial micro-assay test for simultaneous detection and quantitative measurement of galactose in the serum. The linearity, precision and sensitivity of the micro-assay were analyzed. The linearity of the assay was determined as described in the material and methods section. Analysis of standard curve for the serum galactose ranging from 2-180 mg dL<sup>-1</sup> showed linear as shown in Fig. 1. In this study bacterial strains named *B. cereus* and *P. mirabilis* and a mixture of the suspension of both were used separately. According to Fig. 1, the regression equations and R squares were  $Y = 0.013X - 0.083$ ;  $R^2 = 0.964$ ,  $Y = 0.01X - 0.014$ ;  $R^2 = 0.898$ ,  $Y = 0.009X - 0.155$ ;  $R^2 = 0.859$  for *B. cereus*, *P. mirabilis* and the mixture of them, respectively.

As shown in Fig. 1,  $R^2$  for *B. cereus* is about 0.962, which is higher than that of *P. mirabilis* and/or a mixture of both bacteria, resulting in a more linear curve. Therefore, *B. cereus* was selected for the micro-assay and used thought this study. According to the standard curves, the micro-assay is linear in 2-180 mg dL<sup>-1</sup> serum galactose. Since the normal concentration of the serum galactose in newborns is 0-20 mg dL<sup>-1</sup>, concentrations above 20 mg dL<sup>-1</sup> are considered to represent Duarte form or classical form of galactosemia, depending on the concentration of serum galactose. This micro-assay is capable of measuring normal and increased concentrations of the serum galactose, making it possible to classify the patients. Basically, the key concept that is involved in the present assay to determine the exact concentration of total galactose, is the use of galactose negative bacteria in order to remove all sugars except galactose and galactose-1-phosphate from serum, therefore it is possible to measure the total concentration of galactose spectrophotometrically by using of DNS reagent. Moreover, since this assay can measure both galactose and galactose-1-phosphate, this assumption could extend the application

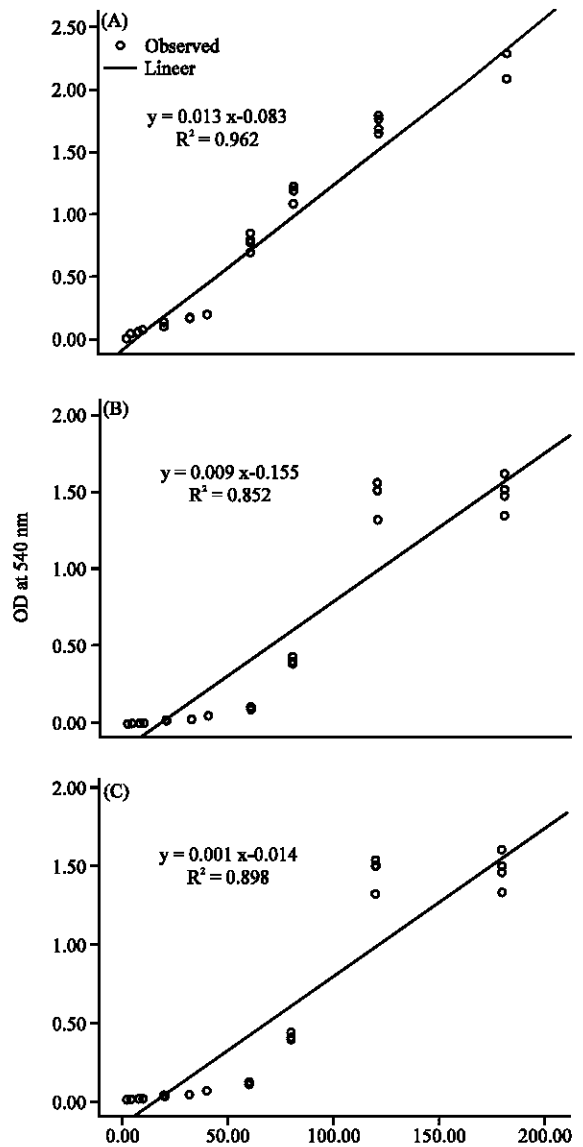


Fig. 1: Standard curves for serum galactose quantitative measurement. (A) *B. cereus*, (B) *P. mirabilis* and (C) suspensions of both *B. cereus* and *P. mirabilis*. The curves were plotted based on spectrophotometric measurements of culture of at 540 nm

of the introduced micro-assay to other galactose metabolic disorders besides classical galactosemia. However, this micro-assay could not distinguish which enzyme is defected in the galactose metabolic cycle.

The precision and sensitivity of this assay was determined by analysis of intra-and inter-assay variations. As shown in Table 1 the micro-assay introduced in the present study is capable of measuring a relatively wide

spectrum of possible concentrations of serum galactose, from normal to highly elevated levels, with suitable accuracy and precision.

In order to investigate the capability of the micro-assay to be used for long-term studies, ambient storage and automation, the bacterial strains were lyophilized and then the lyophilized powder was utilized for assaying the serum galactose. The data demonstrated that the lyophilized bacteria also produced similar results as compared to the bacterial suspensions. Therefore, the lyophilized form of the bacteria can be stored at ambient condition and be used with no limitation for the micro-assay with the advantage of having the potential to be easily automated as a quantitative micro-assay kit.

The optimum time of bacterial incubation in respect to the speed of diagnosis of galactosemia by the micro-assay was examined. The OD<sub>540 nm</sub> of the culture media containing 180 mg dL<sup>-1</sup> serum galactose was measured every 1 h after the time of bacterial incubation for 12 h. The culture media were incubated in a thermo mixture incubator at 37 °C and 150 xg. As shown in Fig. 2 OD<sub>540 nm</sub> was decreased as the time of incubation was increased. However, after about 5 h incubation, no decrease in the amount of the OD<sub>540 nm</sub> of the culture medium was observed, indicating that the only reduced sugar in the media culture was galactose. This could be explained as the bacteria could not consume galactose; therefore, it took at least 5 h in the optimized condition for

the bacteria to remove other sugars except galactose from the culture media. Therefore, it was possible to detect quantitatively the total concentration of galactose in the serum after 5 h of incubation of bacteria.

Using the micro-assay, it was possible to determine the exact concentrations of the galactose in the serum samples, which facilitates classification of the samples as normal, duarte and classical forms of galactosemia. The rapid nature of the micro-assay could provide the chance for early diagnosis of the galactosemia in emergency cases to avoid subsequent clinical problems. Since, often patients will be diagnosed at a stage of medical emergency that may lead to sequel or death and some cases will be misinterpreted as gram-negative sepsis, a frequent complication of galactosemia (Jensen *et al.*, 2001; Walter *et al.*, 1999). Therefore, always a rapid and careful diagnosis of galactosemic newborns is incredibly helpful.

Moreover, prospective screening of newborns for galactosemia is a widely accepted procedure throughout the world. In many laboratories screening utilizes the Guthrie test to assay for total galactose (galactose plus galactose-1-phosphate) and/or activity of the GALT enzyme using the Beutler screening test (Beutler and Mitchell, 1968).

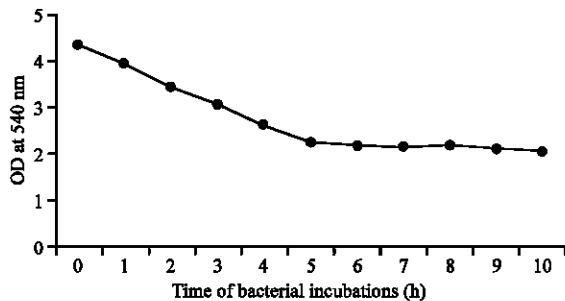
False positive results in newborn screening for galactosemia are frequent and represent a substantial problem for screening programs. A common observation is the adverse affects that environmental factors and sample handling procedures (practiced at the site of specimen collection or during specimen transport) may have on the GALT assay, resulting in low activity and false positives. The most notable environmental influences are heat and especially humidity. Specimens collected during hot, humid summer seasons or in climates where such conditions are persistent often present with reduced GALT activity. Also, since the activity of the GALT enzyme in a dried blood specimen deteriorates over time at room temperature, the practice of batching, where dried blood specimens are permitted to accumulate at the hospital before being mailed to the screening g Laboratory, may also adversely affect GALT activity.

All current methods such as HPLC and TLC, require special reagents with limited stability, involving multi-step sample preparation procedures and are expensive and time-consuming. Thus, an accurate, inexpensive and economical system for rapid diagnosis of galactosemia with less false positive results is favored in newborn screening programs. In the present study, a novel method of screening for galactosemia is introduced. The method uses inexpensive materials and reagents and could measure serum total galactose in a relatively short period of time with high accuracy and precision. One of the main

**Table 1: Analytical precision of the micro-assay**

	n	Galactose (mg dL <sup>-1</sup> )	Mean±SD	CV (%)
Within-run	10	4	0.045±0.005	10.40
	10	60	0.780±0.065	8.08
	10	180	2.196±0.105	4.80
Between-run	10	4	0.043±0.007	15.60
	10	60	0.730±0.083	11.40
	10	180	2.170±0.089	4.10

n: No/ of samples; SD: Standard Deviation; CV: Coefficient of variance. Mean for within-run is the average of 10 OD at 540 nm for each galactose concentration and for between-run is the average of 10 OD at 540 nm at different assays for each galactose concentration



**Fig. 2: Optimization of incubation time of bacterial culture for measurement of serum galactose, Standard galactose serum samples (mg dL<sup>-1</sup>)**

advantages of the method is the use of both galactose and galactose-1 phosphate as the target sugars in the serum samples. Therefore, total galactose is measured by this method. Moreover, due to the persistent nature of these sugars in the serum samples, old samples (up to one month) could be used. However using the enzymatic methods (kits), freshness of the sample is a key factor affecting the final results.

The speed and easy setup of the method, as well as the lack of false positive (i.e. a known normal serum Galactose level detected wrongly as an abnormal level) and false negative results (i.e. a known abnormal serum galactose level detected as a normal one) could make the method as an attractive option in situations where a unlimited number of samples are to be analyzed. This can be the case with selective screening procedures and in confirmatory testing of samples from galactosemia screening, where low GALT activity has been found. Furthermore it is noteworthy that before performing the test patients have to be under particular diet regiment in order to avoid galactose variations in serum and do not use any antibiotics that inhibit the bacterial growth. Also, the method lends itself to rapid follow-up analysis of routine neonatal screening samples with increased concentrations of galactose. Therefore, this test could provides suitable mean for mass screening galactosemia in developing countries, which have low financial funding for performing this kind of screening programs. We previously developed a new quantitative bacterial micro-assay for our PKU (Phenylketunoria Disease) screening program (Vallian and Moeini, 2006; Vallian *et al.*, 2003) and now with conjunction of this method, a dual micro-assay kit could be produced for simultaneous detection of serum phenylalanine and galactose.

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