Improved Screening Efficiency for Phenylketonuria using a Modified Bacterial Inhibition Assay Protocol – Autoclaving the Bloodspot

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ABSTRACT

The Guthrie bacterial inhibition assay (BIA) tests for elevated phenylalanine (PHE) by measuring *B. subtilis* growth zone density in an agar medium. Dried blood spots with elevated PHE on initial BIA screening undergo repeat BIA testing and thin-layer chromatography (TLC). Specimens with elevated PHE by TLC or BIA on second-tier testing require recall. To streamline PKU screening and reduce the recall rate, we tested a modified BIA protocol incorporating autoclaving of dried blood spots. Autoclaving improves growth zone appearance and has been previously reported to reduce the number of specimen requiring repeat testing. From June to October 2006, dried blood spot samples with initially elevated PHE were autoclaved at 110°C for 5 min, then retested by BIA. Samples with still-elevated PHE were analyzed by TLC. 1078 of 37,268 samples (2.89%) had initially elevated PHE. After autoclaving, 1036 no longer exhibited elevated PHE decreasing to 42 (0.11%) the number requiring TLC. By comparison, the unmodified algorithm resulted in 3.14% of samples received from July – December 2006 requiring both repeat BIA and TLC testing. We have since modified our PKU screening algorithm to require repeat BIA testing from autoclaved samples prior to TLC analysis. This translates to a significant reduction in time and resources for second-tier testing and follow-up, and prevents stress for the parents of a newborn who would have been recalled unnecessarily.

Introduction

Phenylketonuria (PKU) is an autosomal recessive genetic condition associated with abnormally high levels of phenylalanine (PHE) in the body. Affected individuals are unable to metabolize phenylalanine, usually due to a deficiency of the enzyme phenylalanine hydroxylase, which converts phenylalanine to tyrosine.[1] The resulting buildup of phenylalanine and closely-related substances impairs normal brain development. The most common clinical manifestation in untreated PKU patients is developmental delay followed by mental retardation. Affected individuals may also develop microcephaly, delayed or absent speech, seizures, eczema, and behavioral abnormalities.[2] Early detection and diagnosis in the newborn period is important because dietary restriction of phenylalanine intake can prevent the onset of symptoms and long-term sequelae.[3-5] Children with PKU who are treated appropriately after diagnosis usually show normal intelligence[2]. There is an inverse correlation between the age at start of treatment and IQ, even in early treated PKU [6].

In our laboratory, the bacterial inhibition assay (BIA) developed by Guthrie[7] has been previously used for PKU screening. In this test, phenylalanine-free minimal agar is seeded with *B. subtilis* spores, and an inhibitor, β-2-thienylalanine, is added to suppress growth of the bacteria. The inhibitor's action is blocked by high phenylalanine levels, thereby allowing *B. subtilis* to grow. To verify elevated PHE concentrations, a repeat BIA is performed. In addition, a second-tier test, thin-layer chromatography (TLC), which gives a semi-quantitative estimate of the levels of various amino acids, is performed for all initially abnormal samples. Specimens exhibiting elevated PHE by either TLC or repeat BIA are presumed to be at increased risk for PKU and the newborn is recalled for further tests. This testing algorithm is illustrated in Figure 1.

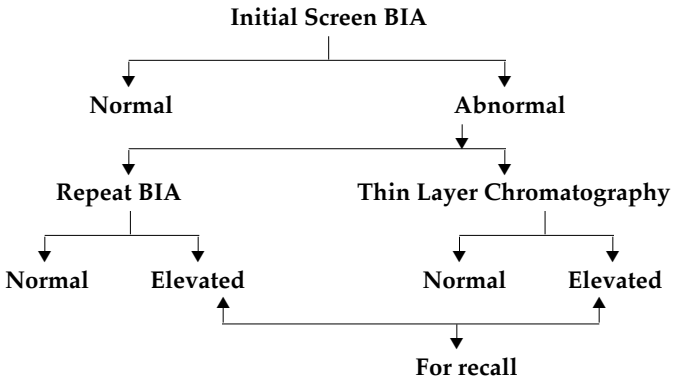

Figure 1. Original algorithm for PKU testing used in our laboratory, in which samples with initially elevated PHE are reanalyzed using repeat BIA and thin layer chromatography, performed in parallel. Neonates with elevated PHE for either or both tests will be recalled for further tests.

Steady increases in NBS specimens have resulted in a corresponding increase in specimens requiring repeat testing by BIA/TLC and recall for a second screen. The majority of these recalled newborns are eventually cleared

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as unaffected. Autoclaving blood spots before performing BIA has been reported to improve the appearance of bacterial growth zones because it denatures hemoglobin and other blood proteins and reduces antibiotic interference.[8-11] This study was conducted to assess whether a modified algorithm, in which samples with initially elevated PHE are autoclaved prior to repeat BIA, will decrease the number of specimens requiring second-tier TLC testing and the number of newborns requiring second specimen collection.

Methods

A modified algorithm for PKU screening incorporating repeat testing using autoclaved dried blood spots (DBS) was tested from June to October 2006 (Fig. 2). All dried blood spots with elevated PHE by the initial test were autoclaved at 110°C for 5 minutes, then retested by BIA. Samples that produced abnormal growth zones from autoclaved spots were analyzed by TLC. The final reportable outcomes of all samples were determined. The number of samples requiring TLC or a repeat card was determined and compared for both the modified algorithm and the original algorithm. Proficiency testing (PT) samples from the Centers for Disease Control and Prevention (CDC) were also analyzed to further evaluate potential differences between the two protocols.

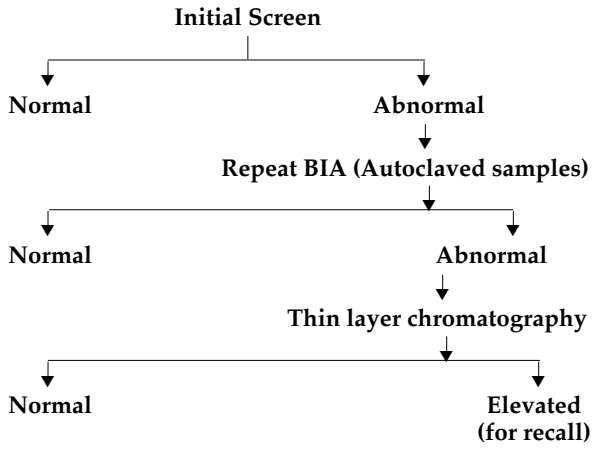

Figure 2. Revised algorithm for PKU testing, in which samples with initially elevated PHE are autoclaved before reanalyzing by repeat BIA. Abnormal samples are then assayed using TLC. Neonates with elevated PHE from TLC will be recalled.

Results

From July – December 2006, 84,165 samples were tested using the unautoclaved dried blood spots, and 2560 (3.14%) required second-tier TLC analysis. Of these, 2488 were determined to have normal PHE concentrations. Repeat screens were requested on 126 neonates and one was confirmed with PKU.

From June to October 2006, 37,268 NBS specimens from 609 contributing hospitals in Luzon were tested using the modified algorithm. Of these, 1,078 (2.89%) had elevated PHE on initial BIA testing. After autoclaving, 1036 (96.1%) were normal by BIA leaving only 42 (0.11%) for TLC testing. The growth zones from autoclaved samples were noticeably smaller and more defined compared to those of non-autoclaved samples (Figure 3), which made result interpretation easier. No false negatives were observed for the sample population tested, based on outcomes already known prior to performance of the modified screening protocol. This issue is important because incorrect autoclaving conditions also degrades PHE[13].

To further investigate our abilities to detect PKU with both protocols, we performed parallel studies using proficiency testing samples from the Centers for Disease Control and Prevention (CDC). The analyst was unaware of the correct interpretations for these samples until after testing results were scored. Using the non-autoclaved procedure, 10 specimens were identified with elevated PHE indicating possible PKU when only 8 were true cases (*i.e.*, 2 false positives). With the autoclaved procedure we identified all of the true PKU cases with no false positives.

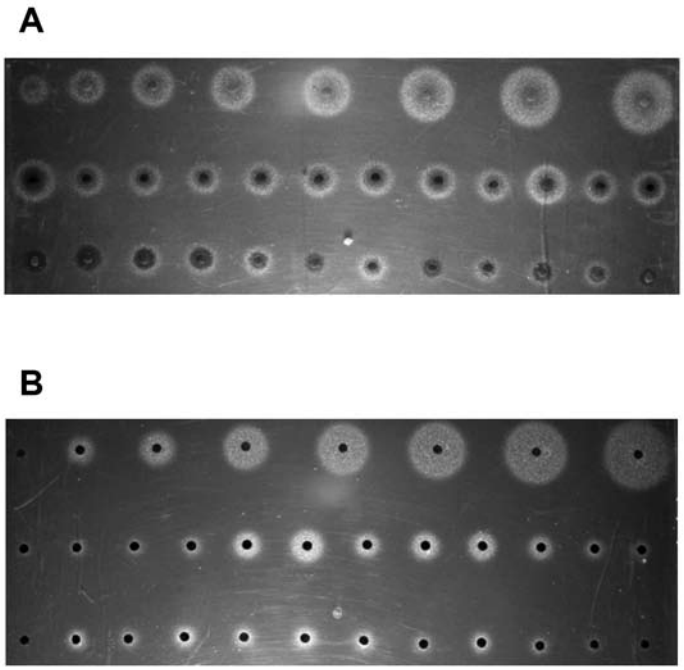

Figure 3. The growth zones from autoclaved dried blood spots (B) in the bacterial inhibition assay were smaller and more defined compared to the non-autoclaved samples (A).

Discussion

The BIA method for PHE screening is inexpensive, relatively simple, and reliable. However, it is semi-quantitative and subject to growth zone fluctuations related to use of the β-2-thienylalanine inhibitor.[12] It is also affected by the presence of antibiotics in the dried blood spot.[8,9] Autoclaving of dried blood spots denatures interfering proteins and antibiotics, and leads to improved growth zone

appearance, ease of test interpretation, and reduced growth unrelated to increased PHE. Because autoclaving can adversely affect other screening tests, only a portion of the original specimen is available for autoclaving. Therefore, it is logistically difficult to autoclave large numbers of samples prior to initial BIA testing, so specimens have routinely not been autoclaved. Instead, a second-tier test, TLC, has previously been used to confirm true PHE elevations and reduce the number of recalls. However, TLC is labor-intensive and time-consuming.

In the unautoclaved PKU screening algorithm, DBS samples with initially elevated PHE concentrations routinely undergo repeat BIA and TLC testing. Most of these samples are found subsequently to have normal PHE concentrations by TLC. Neonates with abnormal TLC results are recalled for further tests. To reduce the possibility of having a false-negative outcome, if a repeat PHE by BIA is elevated (despite a normal TLC result), the neonate is also recalled for further tests. Neonates with moderately elevated PHE (as measured by either TLC or repeat BIA) are asked for a repeat DBS sample for BIA and TLC retesting. However, neonates with significantly elevated PHE are required to immediately undergo confirmatory testing and shifted to a PHE-free diet.

Increased numbers of specimens necessarily results in increased numbers of samples requiring TLC, and higher recall. With the ultimate goal of decreasing both the number of samples needing TLC analysis and repeat specimen collection, we tested a modified algorithm in which DBSs with initially elevated PHEs were autoclaved and then retested by BIA. If an abnormal PHE was present after autoclaving, samples were tested using TLC. Neonates with elevated PHE from TLC were recalled for a second screen.

Because of the improved results when testing autoclaved dried blood spots, we adopted the revised algorithm for PKU screening (Figure 2). A review of 60,631 samples received from February to June, 2007 tested using this revised algorithm showed a significant drop in the number of samples for TLC. Since only samples that have elevated PHE by TLC were recalled, there was also a drop in the number of newborns requiring further testing. Only 7 of 118 samples analyzed by TLC needed recall; of these, there was one confirmed case of PKU. Using the old algorithm, which considers both repeat BIA and TLC results, the number of newborns recalled was 10-fold higher. Table 1 compares the sample number outcomes for the old and new algorithms with respect to TLC and repeat collection.

Autoclaving dried blood spots before repeat BIA testing significantly reduced the number of samples requiring TLC analysis. This resulted in a significant reduction in the number of newborns requiring repeat DBS collection or confirmatory testing. In laboratories that perform BIA as the method of choice for PKU screening, this second-tier algorithm change translates to significant savings in technician time and reagent expenditures in the laboratory, saves time and effort for follow-up personnel, and prevents unnecessary stress for the parents of a newborn who would have otherwise been recalled unnecessarily. These considerations are particularly important in economically developing countries.

Table 1. Comparison of outcomes for representative populations of newborns tested for PKU using the original and modified algorithms

	Original Algorithm (July – Dec. 2006)	Revised Algorithm (Feb. – June 2007)
No. of samples tested	84,165	60,361
No. of samples requiring TLC	2560 (3%)	118 (0.19%)
No. of samples for recall	126 (0.15%)	7 (0.012%)

The numbers of samples requiring TLC and repeat collection are significantly smaller in the revised algorithm.

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