The Guthrie Test for Early Diagnosis of Phenylketonuria [1]

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The Guthrie test, also called the PKU test, is a diagnostic tool to test infants for phenylketonuria a few days after birth. To administer the Guthrie test, doctors use Guthrie cards to collect capillary blood from an infant's heel, and the cards are saved for later testing. Robert Guthrie invented the test in 1962 in Buffalo, New York. Phenylketonuria (PKU) is a congenital birth abnormality in which toxic levels of the amino acid phenylalanine build up in the blood, a process that affects the brains in untreated infants. Guthrie's test detects phenylalanine in the blood of newborns, enabling for early diagnosis of PKU. Early diagnoses of PKU prevent the development of mental disabilities in the thousands of individuals affected each year.

PKU is an inherited metabolic disease in which the body does not produce the enzyme phenylalanine hydroxylase. Phenylalanine hydroxylase breaks down the amino acid phenylalanine in the blood. Individuals with PKU cannot digest high concentrations of the amino acid phenylalanine. High concentrations of phenylalanine are present in protein-rich foods, such as meat and fish [3], as well as in the sweetener aspartame. When individuals with PKU fail to digest the phenylalanine in foods they consume, high levels of phenylalanine build up in the blood, causing brain damage. Therefore, individuals with PKU must eat severely restricted diets to avoid ingesting foods with high concentrations of phenylalanine. Untreated PKU can lead to mental disabilities, brain damage, and seizures. PKU affects approximately one in 15,000 people in the US annually.

Prior to the creation of the Guthrie test, doctors tested infants for PKU with a less reliable ferric chloride urine test. The ferric chloride urine test detected the presence of phenols, including phenylpyruvate, in urine. The presence of the phenol phenylpyruvate in urine indicated that the individual failed to digest phenylalanine because phenylpyruvate is a derivative of phenylalanine. To conduct a ferric chloride urine test, a doctor dissolved a urine sample in a solution of water, ethanol, and a few drops of ferric chloride. If phenylpyruvate was present in the sample, it formed a complex with the ferric chloride and produced an intense blue-green color, indicating that the patient had PKU. Doctors noted several problems with the ferric chloride urine test, included that it failed to produce a positive test result with phenylalanine levels below 20 milligrams per 100 milliliters. Such a test had limited sensitivity and practitioners could easily miss lower concentrations of phenylalanine levels in individuals with PKU. As a consequence, doctors struggles to diagnose and treat those individuals with PKU whose urine samples contained low levels of phenylalanine, leading to irreversible brain damages. Many infants tested with the ferric chloride urine test failed to test positively for PKU in the first six to eight weeks after birth. However, evidence indicated that phenylalanine levels in the blood increase rapidly in the first few days of life, possibly leading to neurological damage during that period of time. Due to the inaccuracy of the ferric chloride urine test, many infants with PKU went undiagnosed and developed symptoms that persisted throughout their lives.

In 1957, physician Robert Warner at the Children's Rehabilitation Center in Buffalo, New York, asked Guthrie to develop a more reliable and rapid method of detecting PKU than the one available at the time. Warner knew about Guthrie's PKU research through their joint affiliation with the Erie County National Association of Retarded Children in Buffalo. He asked Guthrie to create a more convenient way to test for PKU because at the time, to confirm a positive ferric chloride test, physicians had to send fifty to twenty cubic centimeters (cc) of venous blood to a testing site in California to test each patient. Lab technicians at the testing site then performed a blood test to confirm or disconfirm the presence of high levels of phenylalanine in the blood. Guthrie developed a new test for Warner a few days after the initial request. The test Guthrie developed used a bacterial inhibition assay to detect phenylalanine.

Bacterial inhibition assays are tests in which scientists use bacteria to measure the concentration of a substance in a sample. β-2-Thienylalanine, an amino acid, inhibits the growth of the bacteria *Bacillus subtilis*. Guthrie found that phenylalanine, a chemical found in the blood of people with PKU, reversed β-2-Thienylalanine's inhibition of *B. subtilis* growth. Thus, to conduct a PKU inhibition bacterial assay, Guthrie coated a gel used to grow bacteria with β-2-Thienylalanine. Guthrie then placed blood samples, dried on thick filter paper, onto that gel. If the blood sample contained phenylalanine, *B. subtilis* grew around the blood sample and indicated that patient had PKU. If the sample of blood did not contain phenylalanine, *B. subtilis* did not grow around the blood sample, indicating that the patient did not have PKU. Guthrie noted that a single technician could test one to 200 blood samples in a single day and that each test required only a finger prick of capillary blood on a filter paper disc.

After developing the test and showing it to Warner, Guthrie tested the sensitivity of his bacterial inhibition assay using normal blood samples spiked with phenylalanine. Sensitivity is the ability of a test to detect all cases where the disease is present. Guthrie added 2, 4, 8, 12, and 20 milligrams of L-phenylalanine per 100 milligrams of blood serum to normal blood samples to mimic the levels of phenylalanine found in patients affected with PKU. He incubated those dried blood samples with the added L-phenylalanine overnight on the bacterial inhibition assays and found that bacteria had grown around the paper discs. He determined that bacterial growth spiked in the samples containing 12 and 20 milligrams of phenylalanine per 100 milligrams of serum, meaning that those samples indicated an individual possibly had PKU and should undergo further blood testing.

After evaluating his test in the lab, Guthrie compared the results of his test and of the ferric chloride urine test on 3,118 specimens from the Newark State School in Newark, New York. Then, Guthrie confirmed all positive cases determined by the Guthrie test and ferric chloride urine test with a quantitative blood assay. He compared the results of the Guthrie test to the results of the ferric chloride urine test and their sensitivities for detecting the disease accurately. Guthrie found that his test detected twenty-one positive cases of PKU that were also confirmed by the quantitative blood assay, and the ferric chloride urine test only detected seventeen of those cases, meaning that the ferric chloride urine test failed to detect four cases of PKU. According to Guthrie, those data suggested that his test could be used to detect new cases of PKU in populations already screened using the ferric chloride urine test.

Guthrie then refined his method by making the blood collection more convenient for doctors. If doctors diagnosed patients earlier, patients could implement a protein-restricted diet before the amino acid phenylalanine rose to toxic levels in the blood and caused irreversible neurological damage. After his test's initial success, Guthrie modified his bacterial inhibition assay so that infants could be tested a few days after birth. To do so, Guthrie determined whether or not a small collection of capillary blood from an infant's heel would work on the bacterial inhibition assay. He made cards, later called Guthrie cards, which were thick filter papers, each with a metal blade that punctured an infant's heel and collected the resulting blood. He let the blood dry directly on the card and saved it for testing. From the card, he then hole punched a round paper disc of the blood to be placed later on the agar gel culture. Guthrie found that the bacterial inhibition assay worked with infant's heel blood.

Following Guthrie's development of his PKU test, the National Association of Retarded Citizens in New York City, New York, began supporting the use of the Guthrie test and selected two sisters who had PKU as their poster children for a 1961 effort to publicize the test. The older sister, Sheila McGrath, had mental disabilities and was diagnosed with PKU later in life. The younger sister, Kammy McGrath, was diagnosed with PKU by the Guthrie test and treated early in her life, thus avoiding mental disabilities. In the fall of 1961, the National Association of Retarded Citizens publicized the Guthrie test at their press conference in San Francisco, California, and *Life Magazine* [4] published an article on Guthrie and his newborn screen the following year.

Also in 1961, the US Children's Bureau in Washington, D.C., funded Guthrie to test the Guthrie test in 400,000 infants. Guthrie and his team tested 404,568 infants for PKU using the Guthrie test and the ferric chloride urine test. Guthrie and his team diagnosed thirty-seven cases of PKU in two years. Guthrie's test identified 275 infants as presumptive positive for PKU, meaning they were likely to have PKU. Of those 275 infants presumed positive for PKU, thirty-seven cases of PKU were confirmed by repeating the Guthrie test and using a quantitative blood assay, meaning the test was effective.

In 1962, Guthrie entered a contract with Ames Company in Elkhart, Indiana, a division of Miles Laboratories, to produce and market Guthrie test kits to hospitals. However, Ames Company stipulated that they would only enter a contract if the test received a patent. Guthrie filed for a patent on the Guthrie test in his own name in 1962. He then signed an exclusive licensing agreement with Ames Company. The licensing agreement granted Guthrie no royalties, meaning that Guthrie would not make any money from the test kits sold. Five percent of the net proceeds from the test were divided among the National Association for Retarded Citizens Research Fund, the Association for Aid of Crippled Children in New York City, New York, and the University of Buffalo Foundation in Buffalo, New York. Guthrie did not stipulate what price the kits should be sold at in the contract.

After signing the contract, Ames Company struggled to produce enough test kits to meet demand. Guthrie helped produce the test kits and obtained financial support from the US Children's Bureau to produce and assemble kits to perform 500 tests in a rented house in Buffalo, New York. Guthrie's kits sold for $6 each. In 1963, Guthrie learned that Ames Company planned to charge $262 each for the same PKU test kit. According to biographer Jean Koch, Guthrie demanded the price be lowered. Guthrie asked Russell B. Long, a US Senator from Louisiana, to investigate the case to see if the Ames Company had full legal control over the price of the test kits.

Senator Long reported that the US Public Health Service, a division of the US Department of Health headquartered in Washington, DC, had not approved Guthrie's agreement with the private Ames Company. However, two voluntary health associations and the Children's Hospital of Buffalo had supported the agreement so the Ames Company had full control over the price. According to researcher Diane B. Paul, Guthrie deeply regretted not including a price provision in the contract. Ames Company then released a statement that no kits were sold for $262 and that thousands of dollars of proceeds were used to cover the expenses of building manufacturing facilities for the Guthrie test kits. The company sold test kits that could test for 325 infants for $67.71 each.

After finding a company to manufacture his tests, Guthrie struggled to get hospitals to implement the test and medical journals to publish his trial results. Guthrie and parents of children with PKU pushed legislators to mandate newborn screening of PKU in every state. In 1963, Massachusetts mandated newborn screening for PKU. The year, the Guthrie published some of his results in a peer-reviewed journal. Guthrie then campaigned across many states and countries, giving speeches and lectures at conferences to encourage governments to mandate the Guthrie test. By 1966, the Guthrie test was mandatory in most US states.

After the 1960s, researchers paid more attention to PKU. In 1990, scientists around the world convened to discuss issues and advancements in the diagnosis and treatment of PKU in Paris, France. Later that year, a group of researchers led by Yoshiyuki Okano in Houston, Texas, conducted a mutation analysis of the PAH gene, which is responsible for the production of the phenylalanine hydroxylase. That group of researchers also created a database for PAH gene mutations. The PAH database led to the documentation of more than 340 gene mutations that can cause PKU. Also in the 1990s, US researchers Donald Chase, David Millington, Naoto Terada, Stephen Kahler, Charles Roe, and Lindsay Hofman, developed a new technique to test for PKU using tandem mass spectrometry. Scientists use tandem mass spectrometry to separate ions based on their charge-to-mass ratio to detect PKU in blood samples. Tandem mass spectrometry, like the original Guthrie test, uses a hole-punch of dried blood. However, it can screen for a wider variety of congenital diseases using one sample.

By the twenty-first century, all fifty US states required hospitals to test newborns for PKU, and the Guthrie test was used in many places throughout the world. Doctors in less economically developed areas used the Guthrie test to diagnose infants with PKU. However, in the twenty-first century, many hospitals used tandem mass spectrometry, which can screen for a wider variety of congenital diseases than the Guthrie test. Both methods still use Guthrie cards to store the dried blood of infants for testing.

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Subject

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