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Optimization of DNA Extraction from Human Milk

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To the Dean of the Graduate School:

We are submitting a thesis written by Cassandra Z. Rutherford entitled OPTIMIZATION OF DNA EXTRACTION FROM HUMAN MILK

We recommend acceptance in partial fulfillment of the requirements for the degree of Master of Science.

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OPTIMIZATION OF DNA EXTRACTION FROM HUMAN MILK

A Thesis

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In Partial Fulfillment

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Of

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By

Cassandra Z. Rutherford

Abstract:

Background: Techniques are emerging for determining the best and most cost-effective way to test for human milk adulteration. Currently, the literature is focused on the use of qPCR testing, a technique used to isolate and amplify pieces of DNA for analysis. However, no recommendation currently exists on the best DNA extraction kit to use to achieve optimal DNA yield or purity from human milk samples for downstream qPCR use. Thus, the objective of this study is to assess and compare two DNA extraction kits for use with human milk samples for future DNA-based analysis in the testing for bovine milk adulteration in human milk.

Methods: Forty mothers pumped human milk samples under the observation of a researcher using a brand-new hand pump. Eight unadulterated samples were then randomly chosen for DNA-extraction. The eight samples were thawed, pooled, and DNA was isolated using the Omega Bio-Tek's E.Z.N.A.® Blood DNA Mini Spin Kit and the Norgen Biotek Corporation Plasma/Serum Circulating DNA Purification Mini Kit (Slurry Format) per the protocols included in the kits on arrival. An overnight incubation modification was also added to both kits to try to obtain optimal yield and purity. UV/VIS spectroscopy was used to determine DNA yield and purity using the ThermoFisher Scientific NanoDrop 2000TM 260/280 ratio, and a cost comparison was done between kits.

Results: The Norgen kit with no modification provided 143% more DNA than the E.Z.N.A kit with no modifications. Similarly, the average nucleic acid yield was 134% greater when comparing the Norgen and E.Z.N.A kit with an overnight incubation. The Norgen kit provided a 17.0% greater 260/280 ratio and an 11.4% greater 260/280 ratio than the

E.Z.N.A. kit, with and without modifications, respectively. The Norgen kit costs $2.37 more per extraction than the E.Z.N.A kit. Modifying both DNA extraction kits with an overnight incubation decreased the average nucleic acid yield and purity of the resulting DNA.

Conclusion: From these results, the Norgen kit without overnight incubation is a better extraction kit for DNA extraction from raw human milk for both nucleic acid yield and purity. However, the EZNA kit costs less per extraction at $1.45 vs. $3.82. For extraction purposes, purity should be prioritized over nucleic acid yield because contaminants can compromise results and shorten the shelf-life of samples.

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To all of the women in STEM:
"Young girls are told you have to be the delicate princess. Hermione taught them that you can be the warrior." -Emma Watson

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Introduction

The term breastfeeding can encompass many definitions. It not only includes the feeding of infants via the breast of ones' mother, but it also includes the cross- or shared feeding and the use of human milk in general[1]. Human milk provides a variety of benefits to both preterm and term infants containing many protective antibodies and nutrients that aid in the infant's physiological and psychological development. According to the World Health Organization (WHO) and the American Academy of Pediatrics (AAP), breastfeeding is one of the most effective ways to ensure child health and survival, and it is recommended that mothers exclusively breastfeed their infant for at least the first six months for optimal development[2,3].

The definition of exclusive breastfeeding is the consumption of human milk with no other solids, water, or liquids, unless medically indicated,[2] and while many infants do receive human milk in some capacity, The Center for Disease Control's Breastfeeding Report Card reports that in 2015 the percentage of infants breastfed at all through six months was 57.6%, and 24.9% of infants were breastfed exclusively for the recommended duration of at least 6 months nationally[4]. In South Carolina alone in 2015, only 45.1% of infants were breastfed at all in the first 6 months of life, and 24.4% of infants were breastfed exclusively, which is below the national average for both categories[4]. There are many reasons as to why infants are not exclusively breastfed for the recommended duration of at least six months, some of which include delayed milk production, inadequate supply, difficulty with transfer, and maternal or infant medical conditions[5]. If mother's own milk

(MOM) is unavailable, other options are necessary to meet both the infant's nutritional needs and the mother's breastfeeding goals.

Chapter 1: The History of Breastfeeding

Women have been breastfeeding for a very long time, with records dating back to 2000 BC in Israel[6]. Breastfeeding was the only way mothers were able to feed their infants, and without it, they were at a greater risk for illness or death due to the lack of other safe feeding methods available at that time [6,7]. Throughout history, breastfeeding has been considered the preferred method of infant feeding among healthcare professionals because of the ease in absorption and digestibility and due to the changing nutrient composition based on an infant's developmental needs[6]. However, when mothers were unable to breastfeed due to lactation failure, illness, separation, or death, shared lactation or wet nursing was the common alternative; or the sharing of expressed milk to another parents' infant[1].

Shared Lactation & Wet Nursing

For many early cultures, specifically hunter-gatherer cultures, shared lactation was a common practice, ensuring nourishment for all children within a family[1]. As cultures became more socially structured throughout both Europe and America in the 1700s, wet nursing was a safe feeding alternative, and became a social status for women of a higher social strata[1]. Breastfeeding at this time was considered socially unacceptable for those of higher class, and so the job proceeded to be carried out by mothers in poorer communities[1,6]. This allowed wealthier women to focus their time on aristocratic and social efforts instead of breastfeeding[6,7]. During the early 1800s, rural-slave owners often assigned slaves to nurse their infant. Allowing slaves to breastfeed their infants had its benefits to slaveowners, because it meant continued maximization of labor[6]. Many times,

the slaveowners infants were breastfed to the detriment of the slave's own children. It wasn't until the 19th century when artificial feeding was developed that overall breastfeeding and wet nursing rates began to decline.

Alternative Feeding Methods

Animal Milks

In the 19th century, agricultural societies began to develop. The close proximity of farm animals like cows, goats, and donkeys made it possible for infants to be taken directly to the animals' teat for feeding[1]. Goats and donkeys were most often used because it was thought that their milk was most similar to that of human milk[1]. However, as cultures continued to evolve and families moved further and further away from the country and into the developing cities, the use of dairy milk in a bottle emerged[1]. Bottle feeding allowed women to work away from home while also providing adequate nutrition for their children[1]. However, the consumption of unpasteurized, contaminated dairy caused an increase in infant morbidity and mortality due to inadequate milk storage and sterilization[1]. Many infants and children became ill as a result of the contamination, with 18% of infants dying before their first birthday due to gastrointestinal infections or diarrhea[1]. Before the development of refrigerated cars, cow's milk was often shipped via railway cars resulting in high amounts of bacteria during hot summer months[6]. It was also common for dairymen to dilute the cow and donkey milk they were supplying with water, and when the milk began to look gray they would adulterate it with chalk to make it look more white and creamy. It wasn't until several campaigns and decades later that testing began on how to best seal, bottle, pasteurize and ship dairy products safely[1].

Eventually, an infant food was developed by a man by the name of Justus von Liebig in 1865. He was a chemist and his "formula" consisted of cow's milk, wheat and malt flour, and potassium bicarbonate. Food preservation continued to advance with the development of sealed containers and eventually evaporated milk was patented. Many different evaporated formularies were created, and after the first initial development of Liebig's formula with cow's milk, other commercial companies began to experiment in the creation of other infant foods that came in a variety of forms from liquids to powders.

Unfortunately, infant formulas cannot change in composition as breast milk does, based on the needs of the infant, nor did they have the same nutrient density when they were first created[6]. Additionally, these new formulas consisted of added carbohydrates, were high in fat, and contained few vitamins or minerals. Micronutrients were eventually added; however, as with previous efforts, the development of these infant foods and formulas resulted in increased infant mortality due to inadequate milk storage resulting in gastrointestinal upset[8]. To solve these issues, industry developed easy-to-clean bottles and the in-home iceboxes. It wasn't until the 1920s that nonmilk-based formulas were developed due to infant allergy and intolerance to cow's milk. Again, these formulas lacked essential nutrients and had to be fortified. During this period, what physicians suggested was held in high regard by the public, and so formula companies began targeting their marketing campaigns towards physicians. Sales representatives of these formula companies would often pretend to be medical professionals and integrate themselves into the healthcare social society giving gifts in exchange for hearing their sale’s pitch. The relationship between physicians and formula companies resulted in the removal of the

instructions on formula cans replacing them with a message to consult their physician on what is best. As a result of popular demand for formula and the support from physicians in its safety, breastfeeding rates declined[6].

In the late 1970s, many infants were becoming ill because women were diluting their infant formulas to make them last longer due to inadequate income. Infants in underdeveloped countries were also becoming ill due to using contaminated water in their formula preparation. As a result, physician attitude regarding formula began to change, and breastfeeding rates began to rise[8]. This caused formula manufacturers to target consumers to ensure the success of their products. In 1974 the World Health Organization (WHO) put out a resolution stating that breastfeeding was the most appropriate nutritional solution for infants. The resolution also noted that the decline in breastfeeding was related to misleading advertisements by formula companies on the quality of their products, ultimately contributing to infant mortality in the developing world[1]. In 1990 the American Academy of Pediatrics put out a statement opposing formula advertisements, bringing to light to the impact they were having on breastfeeding rates and infant nutrition[6]. According to the CDC's most recent breastfeeding report card (2015) 83.2% of infants started out breastfeeding at birth, providing evidence that most mothers want to breastfeed their infants[4].

Donor Human Milk Banks

In the early 1900s, physicians began to recognize that feeding human milk increased survival rates of premature infants of earlier gestational ages and infants with more complex illnesses, and as a result of technological and medical advances, donor milk

banks were established. Donor milk banks provide milk to premature or critically ill infants whose mothers may not have an adequate supply yet due to pre-term birth and dyad separation. The first milk bank was established in Vienna in 1909, and then two more in 1910; one in the United States and the other in Germany[1]. Through education and support for other institutions, milk banking continued to increase in both Canada and the United States. By the 1940s, the AAP had developed guidelines for donor milk banking and by the early 1980s, there were 30 milk banks in the United States providing for primarily premature or critically ill infants[1]. The Human Milk Banking Association of North America (HMBANA), anon-profit organization that was founded in 1985, was established to standardize donor milk baking operations[1]. Mothers in the U.S. and Canada can donate their milk to HMBANA milk banks to support the provision of human milk to fragile and premature infants.

In the mid-1980s, the potential transmission of two viruses through human milk raised concerns. Cytomegalovirus (CMV) and Human Immunodeficiency Virus (HIV), both of which have detrimental effects on infants[1]. Many milk banks closed as a result of decreased milk orders due to fear of transmission. To address the concerns, requirements for both screening and heat processing of all donor milk were suggested. Unfortunately, several milk banks closed due to a lack of funding for these additional processing steps. The development of specialty formulas for preterm infants at this time also caused a detrimental drop in the number of milk banks in North America, reaching an all-time low in 1990[1].

However, when younger-gestational-age infants continued to survive as a result of continued medical advances and because of research on appropriate nutrition for preterm

infants, there was a revival in both awareness of human milk and the impact that donor human milk can have on preterm infant morbidity and mortality[1]. Today, 29 nonprofit milk banks exist in North America with more than 500 banks operating worldwide[1,9]. In 2019, 7.4 million ounces of pasteurized donor human milk was donated to fragile babies in both Canada and the United States, representing a one-million-ounce increase over 2018. Of this 7.4 million ounces, over five million ounces were being received in hospital NICUs and over one million ounces at home[10].

As milk banking became more popular, for-profit milk banks began to emerge. As of 2019, there are two major for-profit human milk banks that exist in the United States and provide human milk to NICUs: Prolacta® and Medolac®. Unlike HMBANA, both Prolacta and Medolac compensate mothers for their milk in order to obtain the necessary volumes to produce their commercial products.

Chapter 2: Nutritional Benefits of Human Milk

Human milk is similar to that of other living tissues in the body. Just like blood, it aids in the transport of nutrients, enhances immunity, affects biological systems, and additionally, it influences circadian rhythms and modulates gene expression.[1] All of these capabilities of human milk aid in the physiological and psychological development of the infant. Human milk is extremely specific, as all of its components are based on the needs of the infant as they grow, and contains on average 65-90 kcals/dL[1]. Human milk provides macronutrients including lipids, proteins, and carbohydrates, all of which are necessary for regular growth and development[7]. Additionally, infants can receive 100% of their micronutrient needs from human milk if the mother is well nourished[7].

The overall composition of human milk changes throughout the different stages of lactation and based on many other factors like gestational age, feeding frequency, and stage of the feeding[1]. Three stages of human milk occur after a baby is born. Colostrum is the first stage and is usually only produced for the first three to five days postpartum in small volume[7]. The production of this milk is occurring during Lactogenesis I, which means that it is dependent on endocrine or hormonal control that is developed during pregnancy and activated after the delivery of the placenta. Colostrum, is high in protein, fat-soluble vitamins, and contains many immunological components[7]. The onset of transitional milk, the next stage of human milk production, is called Lactogenesis II. Transitional milk usually lasts for about two weeks and is also under autocrine control and the more frequent that nipple stimulation and milk removal are occurring, the more milk is produced[7]. Transitional milk is of larger volume than colostrum, and contains higher levels of water-soluble vitamins. The last stage of human milk is mature milk, and this production occurs

during Lactogenesis III. This milk is considered the "maintenance milk" and is highly dependent on supply and demand (autocrine control). The more often the breast is emptied the more frequently the body is signaled to produce milk[7]. Throughout a feed, mature milk shifts in its composition, with the milk at the beginning of a feed containing more lactose and water (foremilk) and the milk at the end of the feeding containing more fat (hindmilk). Due to the higher lactose and water levels of more milk, it contains many water-soluble vitamins[7]. Conversely, the high-fat nature of the hind milk means that it contains higher levels of fat-soluble vitamins and nutrients[7]. Complete milk removal is necessary for the growth and development of the infant to ensure that they are receiving a balanced intake of water- and fat-soluble nutrients.

Macronutrients in Human Milk

Lipids

Lipids provide the greatest source of calories in human milk, making up roughly 50% of an infant's daily needs[1]. The total amount of milk fat does not change, but the amount of lipid transferred increases throughout an infant feeding. Human milk contains triglycerides, phospholipids, cholesterol, and more than 200 fatty acid structures, including linoleic and alpha-linoleic acid[11]. Triglycerides specifically make up 98% of lipids in milk and support the transport of fat-soluble vitamins A, D, E, and K. Long-chain polyunsaturated fatty acids (LC-PUFAs) make up 88% of the lipids in human milk[1]. LC-PUFAs are the most variable component of human milk, and if the maternal diet is supplemented with omega-3 LC-PUFAs, we see that they are preferentially incorporated into the milk fat[1]. The fat in human milk is extremely important for the development of the

infant's brain; docosahexaenoic acid (DHA) and arachidonic acid (AA) account for 20% of the fatty acid content of the infant's brain. DHA and AA also aid in increasing visual acuity, cognitive ability, neurological growth and repair, and nerve myelination[1].

Proteins

Another important macronutrient in human milk is protein, with over 400 different types available for transfer[1]. The protein in human milk aids in infant survival by playing many roles in the infant's body, including immune function, endocrine function, structural support, and buffering[1]. Protein also functions to increase the bioavailability of other nutrients present in human milk. Casein and whey are the primary proteins present in human milk, with their concentrations decreasing as lactation progresses. Casein is incomplete and while not easily digestible, it inhibits microbial adhesion. Whey protein, in contrast, is easily digestible. They whey-to-casein ratio is about 9:1 when lactation begins and levels out to 1:1 in late lactation[1]. Colostrum contains high levels of whey proteins, including alpha-lactalbumin, serum albumin and lactoferrin, as well as enzymes, immunoglobulins, and bioactive peptides[1].

Immunoglobulins are a class of proteins that act as antibodies in the immune system. There are several different types of immunoglobulins in the human body, however, circulating levels of secretory immunoglobulin A (sIgA) are low when infants are born[1]. Human milk allows for the transfer of secretory IgA, which is important because it blocks the adhesion of pathogenic microorganisms to the intestinal epithelium and in doing so, protects the infant from infection and illness[7]. The frequent feeding between mother and infant is essential to transfer sIgA.

Another protein of importance in human milk is lactoferrin. Lactoferrin has many roles, with the absorption of iron being one of them, which is necessary for adequate bone growth in infants. Lactoferrin attracts iron and binds to it, preventing pathogenic bacteria from binding, and thus, reducing the risk for bacterial growth[1].

Lysozyme is another important protein found in human milk. Lysozyme is an anti-microbial enzyme primarily found in human mucus (gastrointestinal tract and nasal cavity), tears, and saliva that functions to prevent the human body from harmful bacteria. The presence of lysozyme in human milk protects the infant's gastrointestinal tract by breaking down the cell walls of gram-positive bacteria[1].

Carbohydrates

The carbohydrate fraction in human milk is made up of complex and simple carbohydrates. The primary carbohydrate found in human milk is lactose[1,7]. Lactose enhances calcium, magnesium, and manganese absorption and functions as an energy source to the infant's growing brain. Lactose is a disaccharide made up of two monosaccharide subunits called glucose and galactose. Glucose is the primary energy source for infants and so the breakdown of lactose into glucose is essential for glycolysis. Glycolysis yields adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide (NADPH), the body's two high energy molecules[12].

Human milk also contains over 200 specific sugars known as human milk oligosaccharides (HMOs). HMOs are the third most abundant component in human milk after lactose and fat. These sugars are indigestible by the infant and act as prebiotic substrates supporting the colonization of intestinal flora in the gastrointestinal tract[11,

[13,13,14]. HMOs also protect the infant from pathogens by acting as a soluble glycan decoy, blocking the pathogen's attachment to epithelial cell surface receptors preventing infection and disease development in the gut, urinary and respiratory tracts[15,15]. By inhibiting inflammatory gene expression and reducing the platelet-neutrophil complex which develops during inflammation, HMOs have also been shown to have an anti-inflammatory effect on an infant's immune system[15,13].

Table 1: Concentration of Macronutrients in Term Human Milk.

Macronutrient	Average g/mL
Lipids	0.4-5.2g/dL
Protein	0.8-0.9g/dL
Carbohydrates (Lactose)	7.0g/dL

Water

Human milk is 87.5% water and provides infants what they need to stay hydrated during the first 6 months of life[7]. As water sources in many developing countries are not sterile, maintain breastfeeding and the use of human milk protects against gastrointestinal disease from contaminated drinking water. Preparing infant formula requires the use of water and so in a developing country, it would be dangerous. In these countries, formula preparation is discouraged due to inadequate and contaminated drinking water.

Micronutrients

Vitamins

Human milk is a great source of vitamins and minerals, satisfying most full-term infant needs. As lactation advances, the number of water-soluble vitamins in milk increases and the level of fat-soluble vitamins decrease as a result of developed infant stores from maternal-infant dietary exchanges throughout early lactation[1]. Vitamin A specifically reaches its highest levels within the first week after birth at 200 IU/dL. It presents as mostly Retinol and has shown to be essential for infant eye health and development[1]. Vitamin A deficiencies can result in the development of xerophthalmia, and if left untreated, blindness[1]. As Vitamin A deficiency is the leading cause of preventable childhood blindness worldwide, breastfeeding is encouraged to protect against deficiencies. Colostrum is rich in vitamin E or tocopherol[1]. Vitamin E is an antioxidant and protects against reactive oxygen species (ROS) which at elevated levels causes oxidative stress in the body that cause damage to proteins, lipids, and DNA. Preterm and term mothers have similar levels of vitamin E (3IU/100kcal) and carotenoid levels[1].

Vitamin D, which is essential for adequate bone development and growth, and vitamin K, which is essential for regular blood coagulation, are both present only in small amounts in human require supplementation[1,7, 16]. Without supplementation, vitamin D deficiency in infants can often result in rickets, and a deficiency in vitamin K can lead to issues with blood clotting and hemorrhage. The AAP currently recommends supplementing 400 IU per day of birth for Vitamin D, and a 1-mg oral dose of vitamin K administered at birth, at 1-2 weeks, and 4-6 weeks for breastfed infants[1,7].

Water-soluble vitamins like ascorbic acid, nicotinic acid, B_{12}, riboflavin, and B_6, are influenced by maternal diet, but supplementation is typically not needed if the mother is well nourished. Of all of the B-vitamins, folate is especially important for dietary consumption as levels in human milk are maintained even if it adversely impacts maternal stores of folate. Folate remains at the same levels in human milk throughout lactation, and maternal stores diminish slightly from 3-6 months to maintain milk folate levels[1].

Minerals

Most minerals are found in consistent amounts in human milk. Mineral levels tend to reach their highest concentrations in human milk within the first few days after birth and decrease with little variation as lactation advances. The regulation of minerals comes from maternal body stores[1]. Sodium is typically elevated in early colostrum but falls by 3rd day postpartum and levels of zinc rise on the 2nd day postpartum and then eventually decline[1]. Zinc is more abundant in colostrum than in mature milk and the bioavailability in human milk is due to the low-molecular-weight zinc-binding ligand that acts as a cofactor for zinc absorption[1]. Calcium is observed in small quantities in human milk (20-34mg/dL) but absorbed at 67% when compared to that of cow's milk at 25%. Hypocalcemia can be seen in formula-fed infants due to the higher concentration of phosphorus, which leads to decreased absorption and increased excretion of calcium[1].

Small amounts of iron are present in human milk(0.5-1.0mg/L), but newborns are rarely iron deficient. Most infants are sustained by iron stores they obtain in utero. Additionally, the lactose and vitamin C levels in human milk assist in iron absorption.

Copper is also present at high levels in human milk, and also aids in the absorption of iron[1]. Selenium is also higher in human milk than in infant formula, which helps protect against oxidative stress. Small amounts of aluminum, iodine, chromium, and fluorine are also present in human milk[1].

Chapter 3: Feeding Options after Early Breastfeeding Cessation

In 2013, it was found that 60% of mothers do not breastfeed for as long as they would like to[17]. This is influenced by many factors, some of which include: issues with latch, low milk supply, concerns about infant nutrition and weight, infant or maternal illness, concerns with medication use, and problems with breast pump usage[17]. The duration of breastfeeding has also been shown to be impacted by maternal workplace characteristics. Mothers who are supported in the work place and are provided private pumping spaces and utilities have higher breastfeeding initiation and duration rates[18]. Despite high rates of early cessation, it is still recommended that mothers exclusively breastfeed their infant for at least the first six months and to continue to breastfeed while introducing complementary foods until one year of age[19]. Glucose homeostasis is a requirement for the initiation of lactation and so if a mother has Type 1 Diabetes Mellitus, she is also at risk for delayed lactation[7]. Being primipara or having retained placental fragments also puts mothers at an increased risk of delayed milk production[7]. Lactogenesis III is also heavily influenced by supply and demand. If an infant isn't feeding due an aversion, illness, or tongue-tie the mother may not be adequately emptying her breasts, resulting in decreased milk production. All of these reasons are why if MOM is unavailable, other options for maintaining an exclusively human milk diet are necessary to meet global recommendations, and support the infant's nutritional needs and the mother's breastfeeding goals.

Donor Human Milk

Donor human milk (DHM) is breast milk that has been donated for the use in hospital and outpatient facilities for mothers of infants, specifically preterm infants, who are unable to produce an adequate milk supply. In this way, the infants are still able to receive the benefits of human milk for healthy development, regardless of maternal supply. There are many reasons as to why mothers donate their excess breast milk. Some women do it because it is practical [20]. The mother may have an oversupply or need to free up space in her freezer[20]. Others donate for altruistic reasons and enjoy feeling as though they are helping other families and babies[20]. There also can be personal and social benefits resulting from the donation of human milk[20]. A mother may donate due to the recent loss of an infant and chooses to donate as part of the grieving process and to decrease postpartum discomfort[20,21]. The use of DHM has also shown to increase breastfeeding rates at discharge among neonatal intensive care units (NICU)[22,23].

Both the WHO and AAP reference DHM in their infant-feeding statements as alternatives to when a mother's own milk is not available for use[1].

Medically, maintaining an exclusively human milk diet has been shown to decrease the risk of infants developing certain diseases like necrotizing enterocolitis (NEC). NEC is a condition where the intestines of an infant become filled with bacteria resulting in inflammation and infection. If left untreated, this can cause destruction of the bowel wall and intestinal perforation, which results in stool leakage into the abdomen and death. Premature or fragile infants who consume donor human milk have shown to have decreased rates of NEC[22,24] , and when using in combination with human milk-based fortifiers (HMF)

for extremely premature infants, DHM has shown to decrease the incidence of NEC by 50%, and surgical NEC by 90% when compared to bovine-based human milk fortifiers[25].

Non-Profit Milk Banks

Non-profit milk banks are a way for mothers to donate their excess human milk safely and effectively to infants whose mothers cannot provide their own milk. There are currently 29 non-profit milk banks located throughout the United States and Canada[10]. Strict standards are in place by The Human Milk Banking Association of North America (HMBANA) for the handling and distribution of DHM, to ensure the safety of the DHM provided. All donor milk processing can be found on the HMBANA website[26].

Briefly, for the handling and distribution of DHM to ensure the safety of the DHM provided, milk donors must complete a medical and lifestyle history questionnaire and undergo several blood tests for conditions such as HIV, syphilis, and hepatitis B; all of which can be transferred via breast milk. Once received, the donor milk is transferred into a glass flask for pasteurization. Each pool of milk includes the milk from three to five donors and is mixed to ensure an even distribution of nutrients. The milk is then pasteurized using Holder pasteurization to eliminate any harmful bacteria as well as other infectious agents that could be present in the human milk. The milk is pasteurized at 62.5° C for 30 minutes and then quick-cooled. The pasteurized milk is then frozen and stored at -20° C until it is ready to be distributed to outpatient facilities and hospitals[26]. Following pasteurization, the milk is receives repeated biological bacteriological testing to ensure that there is not any growth of bacteria after heat processing. The main recipients of DHM from HMBANA milk banks are premature or critically ill infants and so these screening and

heating processes are in place to ensure the safety and protection of these infants from the effects of harmful bacteria, disease and contraindicated drugs, medications, and alcohol[26].

For-Profit Milk Banks

For-profit milk banks provide compensation to the mothers who donate based on the volume of milk that they donate. These for-profit companies then pasteurize the human milk and sell it to the hospitals. The screening, handling, testing, and preparation of milk at for-profit milk banks are considered proprietary. Consequently, the processing methods are largely unknown.

Peer-to-Peer Milk Sharing

Peer-to-peer milk sharing is when mothers share milk with other mothers in need informally through social media outlets or milk sharing organizations. Two major milk sharing organizations in the U.S. are Eats on Feets and Human Milk 4 Human Babies. Options for milk sharing also occur among families or friends. While human milk contains several benefits over infant formula for preterm infants, some risks may accompany unscreened or untested human milk. These include, but are not limited to, the transmission of bacteria, viruses, medications, drugs, nicotine, and other contaminants due to maternal infection, inadequate handling, and/or inappropriate preparation[27]. Current health authorities including the Food and Drug Administration (FDA) as well as the American Academy of Pediatrics (AAP) caution against feeding infants milk from informal sources due to these risks.

Despite the recommendation from the FDA and AAP, mothers often share human milk informally for altruistic reasons. For example, in one study researchers found that 83% of recipients who had sought human milk informally had full-term babies and that the infant they were requesting milk for was on average 7.1 months old. The primary reason the mother was seeking donor milk was due to lactation insufficiency, and 42.2% reported that they had lactation insufficiency outside of an infant medical condition[5,17]. This sheds light on the fact that DHM is not just needed for critically ill or preterm infants, but also for completely healthy, full-term infants[5].

While it appears that mothers who receive milk informally are concerned about potential contamination and the harm it may cause their infants, the screening methods when mothers accept shared milk vary. Many mothers share milk with others who they know socially, so the perception of risk for contamination or adulteration may be lower[5]. Some professionals in the field have recently raised concern for the lack of screening and analyses occurring among informal milk sharers, specifically related to the risks related to adulteration with non-human milk (specifically bovine milk). In one study, eleven of 102 human milk samples purchased over the internet anonymously contained both human and bovine DNA, with ten being at high enough concentrations to rule out accidental adulteration[28]. This can be problematic for infants with underdeveloped GI tracts due to prematurity, as bovine milk intolerances or allergies and often can result in gastrointestinal distress. Human milk analyses are of importance for the safety of fragile, premature, or term infants due to the potential health risks that accompany adulterated milk.

Chapter 4: Human Milk Analysis

There have been several studies that focus on the analysis of animal milk. Caprine or goat, bovine, and buffalo are just a few of the animal milks that have been researched. Efforts have focused on determining the best method for detecting adulteration, or the act of making something of less pure quality by the addition or removal of another substance or material[29,30]. It is important to identify adulteration in any food item early on to prevent potential health risks due to adverse reactions or allergy. In India, due to its higher fat content buffalo milk is sold at a higher price[30]. Even though there are very few nutritional differences between buffalo and bovine milk, it has been reported that buffalo milks are being adulterated with bovine milk[30].

There has also been an increased demand for goat milk and goat milk-based products in other countries like Greece. Goat milk contains many vitamins and minerals and is rich in protein. Additionally, goat milk contains small fat molecules making it easier to digest[29]. Since goats produce a smaller quantity of milk, the price of the milk is higher when compared to bovine. Thus, adulteration of goat's milk with bovine milk has been observed[29]. In these situations, the detection of adulteration is key in preventing economic loss and potential public health hazards due to animal protein allergies in these countries.

PCR is a method primarily used in molecular biology for DNA-based analysis. In PCR, sequences of small, specific pieces of DNA that have been isolated from samples are amplified through a series of heat cycles. By creating millions of copies, researchers can analyze and study specific segments of DNA. To run PCR, DNA extraction of a sample must occur first. DNA extraction is a process of purification. First, the membrane of the cell is lysed. Then different elements like protein, fat, or RNA can be broken down by

enzymes. The solution is then centrifuged to precipitate these elements. The remaining solution, containing the DNA further then washed to remove these impurities or contaminants. As a result of the washes, you are left with a DNA solution for further analysis. UV/VIS spectroscopy is then used to determine the nucleic acid yield and purity. Nucleic acids in solution absorb maximally at a wavelength of 260 nm and proteins absorb maximally at a wavelength of 280 nm. The absorbance at 260 nm is used to quantify the amount of DNA present, and the ratio of absorbance at 260 nm to absorbance at 280 nm is used to determine the quality or purity of the sample. A ratio of at or above 1.8 is considered "pure" for DNA.

Several studies have examined and compared different DNA extraction methods using animal milks for the application of PCR. In one study using a food-based DNA extraction kit, it was discovered that when raw cow's milk was mixed with raw buffalo milk the presence of cow DNA could be detected at levels as low as 5%[30]. This study reported a range in DNA concentration from 15-18ng/uL and purity (260/280 ratio) of 1.85-1.88. In another study, using a different food-based DNA extraction kit and overnight incubation modification, it was found that when cow's milk was mixed with raw goat's milk, the presence of cow DNA could be seen at levels as low as 0.01%[29], with thirty-six of the forty samples containing the addition of cow's milk[29]. This means that 90% of the goat and goat milk-based products were adulterated with cow's milk. While both of these studies have shown that the extraction and analysis of DNA from raw animal milk samples can aid in the detection of adulteration, there have been few studies that have examined the impact of milk processing on DNA extraction or PCR use.

Milk is often pasteurized to destroy harmful bacteria, infectious agents, and to increase shelf-life. However, DNA is a heat-sensitive molecule and at high temperatures, DNA can denature. More simply, the double-stranded molecule can break and become to single-stranded molecules. Thus, it is important to better understand the impact that pasteurization can have on the extraction of DNA. In one study, different DNA extraction kits were compared for their effectiveness in isolating DNA from raw, pasteurized, retorted, and ultra-high temperature (UHT) processed dairy milk[31]. In retort processing, milk is placed in a large vat and heated to a high temperature ranging from 110- 120° C for 10-30 minutes. In ultra-high temperature processing, milk is placed in small, pressurized tubes and heated at higher temperature, from about 135-145° C for 10-15 seconds. This study utilized the differential centrifugation combined with Sodium dodecyl sulfate (SDS) method for extracting DNA. SDS is a detergent that aids in the solubility of lipids and proteins[31]. What they discovered was that while the mitochondrial DNA yields were appropriate for PCR analysis, the range of values was large, and all of the purity ratios obtained ranged from 1.10-1.30[31]. These results show that there could have been other molecules (such as free nucleotides, proteins, or other contaminants) present impacting the results. This study shows that the heat and pressure the dairy milk endures during processing may impact DNA-based analyses, specifically in the yield and purity of extraction.

While previous studies have demonstrated the effectiveness of using PCR in the detection of bovine milk, they have only demonstrated the presence, but have not quantified the level of adulteration. Quantitative polymerase chain reaction (qPCR) is a molecular method that uses the same principles of PCR, but rather than documenting the amount of

amplification after the reaction, qPCR monitors amplification during the reaction. This allows for quantification in a more specific, measurable manner.

There seems to be variation amongst extraction kits used for downstream PCR and qPCR analysis of animal milk. One study compared five DNA extraction approaches in regards to their impact on DNA yield and quality when assessing the human breast milk microbiome[32]. The study showed that the extraction method greatly influenced the DNA yield and purity ($p<0.001$)[32]. Another study compared eleven different DNA extraction kits for downstream qPCR use when examining bacterial spores in dairy samples[33]. For the processing of buttermilk and whole milk samples specifically, the milk protein and cream fractions were separated before DNA extraction. It was concluded that the use of the QIAamp DNA mini kit is appropriate for DNA extraction for a variety of dairy products (Buttermilk, Milk, Cream cheese) with minor modifications to the included protocol[33]. Interestingly, when the DNA extracted from the milk cream fraction was analyzed in the qPCR machine, no data was recorded[33]. Previous studies have suggested that the lipid content of certain foods like cooking cream, butter, and cheese interfere with the solutions and reagents in DNA extraction, and potentially inhibit of qPCR assays[34]. These results suggest that the cream fraction or higher fat content of milk inhibits qPCR amplification.

When it comes to human milk analysis, the literature is lacking. Previous studies have examined the use of PCR for the detection of bovine milk, but have only been able to determine if it is present in the sample, not to what degree. One study published in 2015 examined the concentration of adulterated human milk samples purchased online using qPCR[28]. What they discovered was that 11 of 102 of the randomly purchased human milk samples collected contained both human and bovine DNA, roughly 11%[28]. Ten of the

eleven samples collected also contained higher contamination amounts ruling out accidental contamination.

While both PCR and qPCR have shown to be promising in the analysis of animal milk, there is not a standard protocol in place for human milk DNA-based analysis. Most milk-based analyses have only included raw milk, and when processed milk has been studied, there appears to be a variation in the DNA yield and purity obtained. There also seems to be variation amongst extraction kits used and modifications made to achieve optimal DNA yield, purity, and PCR/qPCR amplification.

Social Implications for Human Milk Adulteration

Future human milk-based analyses are necessary to improve the quality and safety of human milk exchanges. The literature has demonstrated that informal milk sharing is occurring between mothers in an effort to support infant feeding. However, without adequate commercial testing methods, infants receiving donated milk may be at risk for consuming adulterated milk. The lack of information available on the processing methods occurring in for-profit milk banks also raises safety concerns. Fragile and critically ill infants are unable to adequately metabolize animal milks and so the consumption of these milks often result in detrimental health complications. If affordable human-milk based analyses are not developed, social and long-term health implications will continue to rise.

Chapter 5: Optimization of DNA Extraction from Human Milk

Introduction

According to the World Health Organization, breastfeeding is one of the most effective ways to ensure child health and survival. Human milk provides a variety of benefits to both preterm and term infants and contains protective antibodies and nutrients that aid in the infant's physiological and psychological development. Unfortunately, many infants are not exclusively breastfed for the recommended duration of at least six months.

There are many reasons as to why infants are not exclusively breastfed for the recommended duration of six months, some of which include delayed milk production, inadequate supply, difficulty with transfer, and maternal or infant medical conditions[5]. To better support these mothers and infants, women with excess breast milk can donate it to milk banks. In the United States, there are for-profit and non-profit milk banks. For-profit milk banks provide compensation to the mother for her donation based on the volume of milk she is providing.

Professionals in the field are concerned that with the increased need for human milk, there may be an increased rate of adulteration of human milk prior to donation[28]. This can be detrimental to newborns, due to the differing levels of protein and minerals in other animal-based milk. This high level of protein and minerals can stress the infant's kidneys, leading to severe illness. Currently, it is reported that one for-profit company screens donor milk for bovine adulteration; however, methods for screening are considered proprietary and no published protocols exist.

Efforts are ongoing to determine the best and most cost-effective way to test for human milk adulteration, with previous studies examining the use of qPCR testing, a

technique used to isolate and amplify pieces of DNA for analysis. Despite these efforts, the most effective approach for isolating DNA from human milk at high concentrations and sufficient purities remain unknown. The objective of this study is to assess and compare two DNA extraction kits for use with human milk for future DNA-based analysis in the testing for bovine milk adulteration in human milk samples.

Methods

Sample Preparations

All participants provided informed consent and procedures were followed in accordance with and approved by the Institutional Review Board at the University of North Carolina, Greensboro (UNCG; Greensboro, NC). Forty mothers pumped human milk samples under the observation of a researcher using a brand-new hand pump at the UNCG campus and then were shipped to Winthrop University (Rock Hill, SC). All human milk samples were then de-identified and aliquoted into 2mL microcentrifuge tubes and stored at -80° C until analysis. One mL of human milk from 8 unadulterated samples were randomly chosen and pooled together in preparation of DNA extraction.

DNA Extraction

DNA was isolated using the Omega Bio-Tek's E.Z.N.A.® Blood DNA Mini Spin Kit or the Norgen Biotek Corporation Plasma/Serum Circulating DNA Purification Mini Kit (Slurry Format). For both kits 200uL of pooled human milk was utilized to perform DNA extraction per the manufacturer’s instructions with no modifications in triplicate. Then DNA extraction was completed for each kit with the addition of a 65° C overnight

incubation period in triplicate (Figure 1). Isolated DNA was then stored at 4°C in the refrigerator.

DNA Yield and Purity Analysis

UV/VIS spectroscopy was used to determine DNA yield and purity using the ThermoFisher Scientific NanoDrop 2000™. For each extracted DNA sample, 1 uL was pipetted onto the Nanodrop pedestal and nucleic acid concentrations (ng/uL) and 260/280nm absorption readings for purity were documented.

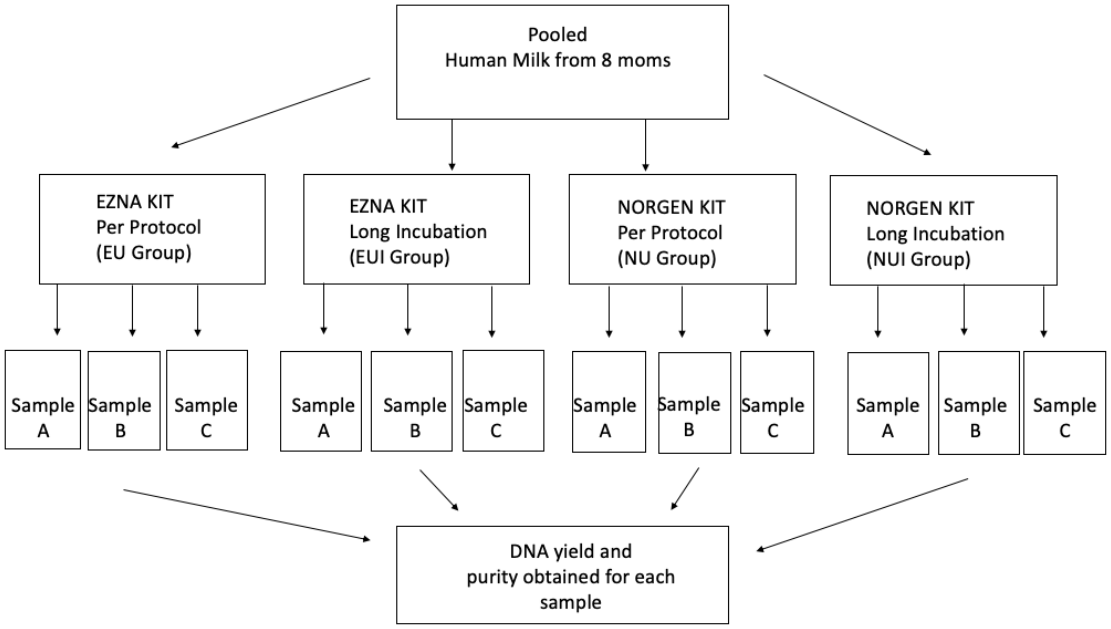

Figure 1. Methods overview.

Cost Analysis

The E.Z.N.A.® Blood DNA Mini Kit and the Norgen Biotek's Plasma/Serum Circulating DNA Purification Mini Kit (Slurry Format) costs per DNA extraction was

calculated using total kit cost (Winthrop University purchase price) divided by the number of extractions possible per kit.

Statistical Analysis

Descriptive statistics and box-and-whisker plots were completed using Microsoft Excel 2016 (Redmond, WA).

Results

Nucleic Acid Yield

Average nucleic acid yields and 260/280 ratios for the four extraction methods are reported in Table 1. Descriptive statistics are represented visually in Figure 2. Modifying both DNA extraction kits with an overnight incubation decreased the average nucleic acid yield with 3.73% decrease and 19.1% decrease in nucleic acid yield for the E.Z.N.A kit and the Norgen kit, respectively (Table 2 and Table 3). Based on average nucleic acid yield, the Norgen kit with no modifications provided 143% more DNA than the E.Z.N.A kit with no modifications (Table 2). Similarly, the average DNA yield was 134% greater when comparing average nucleic acid yield when using an overnight incubation (Table 3).

Purity

Descriptive statistics for purity are visualized in Figure 3. Similar to nucleic acid yield, adding the overnight incubation decreased the purity of the resulting DNA; with a 5.92% decrease in the 260/280 ratio and a 10.9% decrease in the 260/280 ratio for the E.Z.N.A kit and the Norgen kit, respectively (Table 2 and Table 3). Additionally, the

Norgen kit provided a 17.0% greater 260/280 ratio and an 11.4% greater 260/280 ratio than the E.Z.N.A kit, with and without modifications, respectively (Table 2 and Table 3).

Table 2. Descriptive statistics for samples extracted using the E.Z.N.A. Blood DNA Mini Kit (EU) and the Norgen Biotek DNA Mini Kit (NU) when using standard kit protocol.

Variable	**Kit**	**Mean**	**StDev**	**Minimum**	**Median**	**Maximum**
DNA Yield (ng/uL)	EU	8.033	1.050	7.000	8.000	9.100
DNA Yield (ng/uL)	NU	48.63	3.38	44.90	49.50	41.50
260/280 Ratio	EU	14633	3.38	14200	1.4700	1.5000
260/280 Ratio	NU	1.7333	0.0252	1.7100	1.7300	1.7600

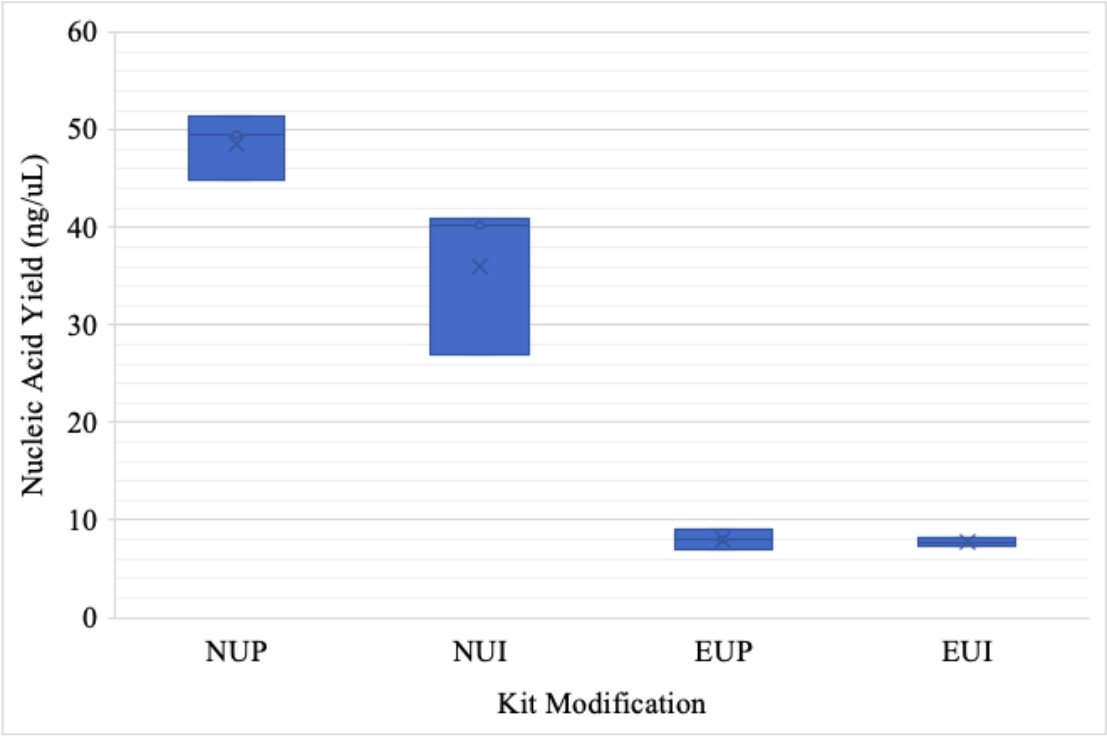

Figure 2. DNA yield of samples extracted using the E.Z.N.A. Blood DNA Mini Kit (EU) and the Norgen Biotek DNA Mini Kit (NU) when using standard kit protocol (P) or modified by adding an overnight incubation (I).

Table 3. Descriptive statistics for samples extracted using the E.Z.N.A. Blood DNA Mini Kit (EU) and the Norgen Biotek DNA Mini Kit (NU) when standard kit protocol was modified by adding an overnight incubation.

Variable	Kit	Mean	StDev	Minimum	Median	Maximum
DNA Yield (ng/uL)	EU	7.733	0.451	7.300	7.700	8.200
DNA Yield (ng/uL)	NU	39.37	2.08	37.00	40.20	40.90
260/280 Ratio	EU	1.3767	0.0929	1.3000	1.3500	1.4800
260/280 Ratio	NU	1.5433	0.0153	1.5300	1.5400	1.5600

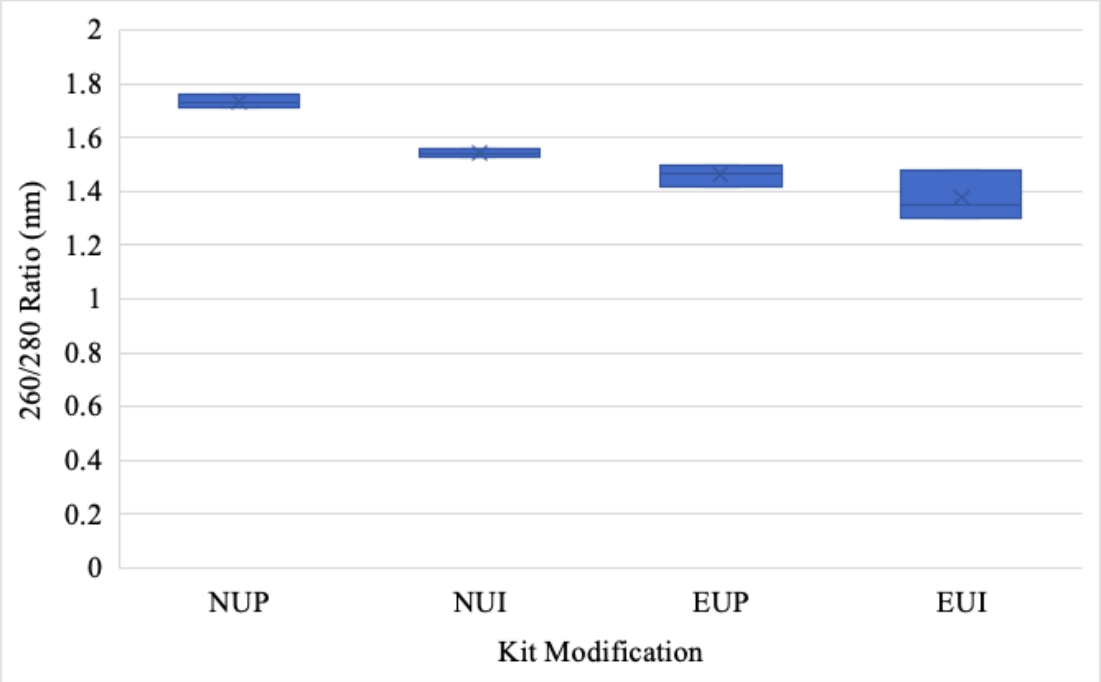

Figure 3. 260:280 ratios of samples extracted using the E.Z.N.A. Blood DNA Mini Kit (EU) and the Norgen Biotek DNA Mini Kit (NU) when using standard kit protocol (P) or modified by adding an overnight incubation (I).

Cost Comparison

The Norgen Biotek's Plasma/Serum Circulating DNA Purification Mini Kit cost $2.37 more than the E.Z.N.A. Blood DNA Mini kit, at $3.82 per DNA extraction, a 62% increase (The E.Z.N.A.® Blood DNA Mini kit costs $1.45 per DNA extraction).

Discussion

Human milk-based analyses are necessary to improve the quality and safety of informal human milk exchanges. Previous studies have examined the use of different DNA extraction kits for the analyses of animal milk. Keim et al. (2018) reported that the use of the Norgen Biotek Plasma/Serum Circulating DNA Purification Mini Kit provided sufficient DNA quantity and purity for downstream qPCR analysis. Our results are in agreement with this publication; with the Norgen Kit groups yielding 143% and 134% (with and without modification, respectively) greater nucleic acid concentrations and 17% and 11.5% (with and without modification, respectively) greater purity when compared with the E.Z.N.A. Blood DNA Mini Kit.

Previous studies have demonstrated that longer incubation periods can be utilized to increase the total concentration of nucleic acid and increased purity, improving the opportunity for downstream qPCR use[29]. The results presented in this paper are not in agreement with these studies. The overnight incubation resulted in decreases in total nucleic acid yield for the Norgen and E.Z.N.A. kits, respectively. Additionally, the overnight incubation resulted in decreased DNA purity for the Norgen and E.Z.N.A. kits, respectively.

In order for genomic DNA to be considered pure, the ThermoFisher Scientific NanoDrop 2000™ protocol reported that a 260/280 nm absorbance ratio of greater than 1.80 is needed. Our 260/280 ratios ranged from 1.3 to 1.76 thus demonstrating that neither of these extraction kits produced DNA with purity ratios acceptable for downstream qPCR use. However, the Norgen kit with and without a longer incubation provided a greater increase in purity when compared to the E.Z.N.A kit.

This study is the first to investigate the effectiveness of different DNA extraction kits for the quantity and quality of DNA extracted from human milk samples.

A limitation of this study was that only two DNA extraction kits were evaluated. Additionally, this study contained a small sample size, and the research team was only able to evaluate the impact of a single modification: overnight incubation. Specific to this project, the COVID-19 pandemic reduced the amount of in-person laboratory time available for continued analysis. Future research needs to evaluate these kits using larger sample sizes, different extraction kits, and additional modifications to achieve optimal DNA yield, purity, and PCR/qPCR amplification.

Conclusion

From these results, the Norgen kit without incubation appears to be the most effective extraction kit for DNA extraction from raw human milk for both nucleic acid quantity and 260/280 purity. However, the EZNA kit is more cost effective per extraction at $1.45 (the Norgen kit is $3.82 per sample). For extraction purposes, purity should be prioritized over DNA yield because contaminants can compromise results and shorten shelf-life of samples. Future studies of DNA extraction kits should include the use of food-

based kits seen they have been previously used and successful in other raw animal milk-based extractions. One modification that could be investigated is the removal of both lipid and protein layers prior to extraction, as human milk has more overall fat than whole milk and this may be impacting the purity of the DNA extraction. Additionally, the use of a clean-up kit to re-purify and improve lower than desired 260/280 ratios. Lastly, analysis should be done on both pasteurized bovine and human milk in order to see how processing further impacts the quality of genomic DNA.

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