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The Correlation between Threshold Cycle Values and Magnetic Beads Carried Over During DNA Extraction of Mastitis Milk Samples

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A B S T R A C T

Compared to conventional bacterial culture methods, PCR technology drastically alters disease diagnostics, and the use of magnetic beads in nucleic acid extraction and purification sparked a technological revolution in biological research. Therefore, this study aims to investigate the relationship between the cycle threshold (Ct) values of the real-time PCR reaction, which are employed as indicators of DNA concentration, and the magnetic beads that are carried over during the DNA extraction of mastitis pathogens from dairy cow milk samples. The Mag MAX™ CORE Nucleic Acid Purification Kit, one of the rabid and simple extraction methods that uses magnetic separation technique, was used to extract DNA, while applied Biosystems™ 7500 PCR equipment and applied Biosystems Vet MAX™ MastiType multiplex qPCR kits are utilized in DNA amplification. Eighty-five milk samples from cows with mastitis were obtained; of these, 53 samples (62.4%) had a normal DNA yield retrieved in which 79 microorganisms were identified; in contrast, 32 samples (37.6%) had a magnetic bead carried over from which there were 34 identified microorganisms. On the other hand, there were two samples (6.3%), and supernatants were unable to be obtained. To investigate the relation between cycle threshold and magnetic beads carried over during extraction, all data were analyzed by a T-test of independence, and it showed a significant difference in DNA concentration between each other (T-test =.066). The same data was retested by the Chi Square test, which showed the same significant result (Chi square =.054). This result highlighted the relationship between DNA concentration and magnetic bead carryover and its impact on DNA yield.

Introduction

All dairy businesses worldwide are impacted by mastitis, one of the most prevalent and costly types of intramammary inflamation (IMI) that is brought on by bacterial infection, trauma, or injury to the udder (Anita, 2014). Even though modern dairy farmers make a concerted effort to limit its impact, intramammary

inflammation, the most prevalent disease affecting dairy cows, has well-established implications for animal welfare and the profitability of dairy farms (Ruegg, 2017).

Due to the disease's urgent nature, lactating animals get infected, which results in significant economic losses for all affected countries (Romero *et al.,* 2018). In milk samples from nursing animals, about 140 pathogen species, subspecies, and serovars have been found. These pathogens have been categorized as infectious, environmental, and opportunistic mastitogens (Patterson, 2017).

The very sensitive method known as polymerase chain reaction drastically alters the way diseases are diagnosed. While nucleic acid extraction is the first step in every molecular biology investigation and is therefore regarded as the cure process, the PCR technique can be run directly utilizing milk samples and takes less time than methods of conventional bacterial culture (Amin *et al.,* 2011).

Real-time PCR is a highly specific quantitative technology that can simultaneously detect and quantify the pathogenic bacteria in milk samples. In addition, because it can effectively identify any nucleic acid, even at micro concentrations, it can exceed the concentration limit of conventional PCRs. It can also determine the kind and stage of the infection. Because of this, it outperforms traditional PCRs in terms of sensitivity, specificity, and speed. Despite any known drawbacks, this makes it the method of choice for molecular characterization as well as laboratory diagnosis (Zakary *et al.,* 2011). However, rtPCR was employed to identify bacteria in samples of mastatic cow's milk as well as to find bacterial species such as Staphylococci spp., Streptococcus uberis, Streptococcus dysgalactiae, Corynebacterium bovis, Staphylococcus aureus, Escherichia coli, Enterococcus spp., and Cyanobacterium pyogenes that do not grow in conventional bacteria culture (Taponen *et al.,* 2009).

Magnetic separation-based methods for extracting nucleic acids have been around since the early 1990s. Originally, they were employed to extract plasmid DNA from bacterial cell lysates (Hawkins *et al.,* 1994). Eventually, a novel procedure for the extraction of genomic DNA from whole blood samples was designed and verified, utilizing naked magnetic nanoparticles (Saiyed *et al.,* 2006 and 2008).

Magnetic separation-based methods are used to bind DNA to the surface of magnetic nanoparticles because they have a strong and specific affinity for DNA since they are coated with a polymer or antibody that binds DNA.

Generally speaking, the core of magnetic beads is made of magnetite or maghemite, and surface materials that can be used include silica and functional groups like sulphate and hydroxyl groups (Saiyed, 2007). By applying a magnetic field, such as that produced by a permanent magnet, particles with a magnetic moment can be easily extracted.

This is a rapid, easy, and effective method of particle separation following the nucleic binding or elution stage (Watson and Blackwell, 2000). Additionally, automated devices for processing magnetic particles and liquids are addressed, as well as commercially accessible manual magnetic separators (Sonja, 2006). Separation may also be a component of an automated extraction system.

The very effective and targeted separation and purification of nucleic acids and other compounds using magnetic beads brought about a technological revolution in biological research. This method collects DNA considerably more quickly than other conventional procedures, which can take many hours (Abd El-Aal, 2010).

Because it doesn't rely on centrifugation, the magnetic bead nucleic acid extraction process is perfect for automation and requires minimal equipment to operate. Not using shear pressures, which could compromise the integrity of nucleic acids, is another benefit of this procedure versus centrifugation-based ones. But in contrast to other approaches, this methodology is not economical (Carpi *et al.,* 2011).

Additionally, according to Abdelhameed *et al.,* (2024), there is no discernible correlation between lactation, the frequency of mastitis, days in milk, gestation time, and magnetic beads carried over during DNA extraction.

Finding the quantity of a target sequence in a sample can be done easily and elegantly with real-time PCR, commonly known as quantitative PCR or qPCR. (Thermo Fisher, 2016). According to Corman *et al.,* (2020), a threshold cycle value (Ct value) is the number of amplification cycles needed to achieve a specific background fluorescence level at which the real-time

PCR result shifts from negative to positive. Stated differently, Thermo Fisher defined it as a relative measurement of the target concentration in the PCR process and the location where a threshold line and an amplification curve connect. The concentration of the target is not the only element influencing the absolute value of Ct. (Thermo Fisher, 2016). On the other hand, according to Thermo Fisher guidelines written by Elise Martin (2018), Ct readings >37 but <40 can suggest a low-level presence of the target and advise classifying ct values greater than 37 as negative.

On the other hand, pre-analytical, and post-analytical factors frequently have an impact on Ct levels in viral infections. The specimen age, the kind of specimen, the time the sample was obtained, the conditions under which it was transported and stored prior to testing, and the collection method are the pre-analytic factors (Abo-Youssef *et al.,* 2020; Nakatsu *et al.,* 2015). The effectiveness of nucleic acid extraction, the amount of viral RNA in the samples obtained, primer design, the type of target RNA, real-time PCR efficiency, and the technique for determining Ct value are among the analytical factors (Watson *et al.,* 2020). The interpretation and reporting of the results are examples of post-analytical factors (Watson *et al.,* 2020). Also, Rhoads *et al.,* (2020) reported that the amount of viral RNA that may be detectable by an assay depends on several factors, including the method and source of the specimen, the type and volume of the transport media, the time elapsed between specimen collection and analysis, and the number of days from infection to specimen collection. These factors are represented in the Ct values.

Threshold cycle values possess the potential to serve as a fundamental and easily accessible technique for forecasting and simulating epidemiological dynamics at the community level. Predictive models based on global databases and additional demographic studies are required (Abdulrahman *et al.,* 2020). All of these data imply that there might be a number of advantages to the Ct values' indications, as they might shed light on the infection's prognosis and effects (Rabaan *et al.,* 2021).

Materials and Methods

A total of 85 milk samples were aseptically collected from clinically confirmed mastitis cows during the first few months of 2019 from a herd of 9,000 Holstein milking cows. The cow's milk is produced three times a day, with an average milk yield of 37 litters. The milk line has a three-step cleaning in place system (CIP), which starts with a normal water rinse, then soda at 65-85 °C for seven to ten minutes, followed by normal water for ten more minutes. Then, rinse with VA4-nitric acid for five to ten minutes at 40–60 c°, followed by a normal water rinse and paracetic acid sanitization.

Ten milliliters of milk from the impacted quarter were taken out and put in sterile plastic tubes. They were then refrigerated at five degrees Celsius and sent straight to the lab.

For every sample that was gathered, the King Fisher™ Duo Prime Purification System, the Mag MAX ™ CORE Nucleic Acid Purification Kit, Applied Biosystems VetMAX™ MastiType multiplex qPCR kits and applied Biosystems™ 7500 PCR equipment, were used to extract DNA and perform the PCR experiment. Then 50 µl of Mag Max CORE Mastitis Panbacteria Solution was added to 200 µl of milk and stirred for 5 minutes at room temperature after pipetting up and down multiple times. 10 µl of Mag Max CORE proteinase K (20 mg/ml) were added, and the automated extraction technique protocol was run for 37 minutes. After loading the plate into the apparatus, launch the script "Mag MAX_ CORE_ DUO_ Mastitis." The script paused after about 8 minutes. At that point, the deep well plate was removed from the apparatus, and 720 µl of lysis-bending-Bead-Mix (Mag Max TM core lysis solution 350 µl + Mag Max TM core binding solution 350 µl + Mag Max TM core magnetic beads 20 µl) were added to each well after vortexing to allow for the digestion of the milk samples. The sample plate was then loaded back onto the apparatus to continue the run.

Each DNA sample is examined in a separate well, and the same well is used to specifically identify the DNAs of four pathogens and an internal amplification control (IAC).

In the mix preparation, first and foremost, prior to adding the 10% overage, we calculated the number of required reactions and scaled the reaction components depending on the quantities of each individual reaction. Second, using the master mix and primer mixes in microcentrifuge tubes of the right size, four different PCR reaction mixes were made, as shown in the table (1). All the steps are done following Thermo Fisher workflow guidelines.
Third, the tubes are sealed first to push the PCR reaction

mixtures to the bottom and get rid of any air bubbles. Then, the solutions are quickly centrifuged and mixed by vortexing.

In PCR plate preparation, 15µL of each PCR reaction mix is transferred to the appropriate wells of an optical reaction plate. Then both samples and controls were added to the well according to the following table.2.

At this point, the plate was sealed with optically clear caps, the contents were quickly centrifuged to remove any air bubbles and settle the contents to the bottom of the well, and the real-time PCR was conducted according to ThermoFisher workflow guidelines too.

Statistical analysis

The generated data from a cross-sectional study are subjected to both an independent T-test and a chi-square test. The data was analyzed using the SPSS program.

Results and Discussion

A total of 85 milk samples from cows with mastitis were obtained; of these, 53 samples (62.4%) had a normal DNA yield retrieved in which 79 microorganisms were identified, and 32 samples (37.6%) had a magnetic bead carried over in which there were 34 identified microorganisms too. On the other hand, there were two samples (6.3%) out of the 32 that were unable to obtain DNA.

The mean of the normal group threshold cycle value was 27.95±7.6. The minimum ct value was 10.13, while the maximum value was 37.02. On the other hand, the mean of the carried-over group threshold cycle value was 30.11±4.6. The minimum ct value was 18.51, while the maximum value was 36.99. There was a significant difference in the threshold cycle values of the normal and magnetic beads carried over samples (T. test =.066). (Table. 3)

The values of the threshold cycle (Ct) are classified into three classes as follows: The first one was less than 20 cycles, which had a frequency of 14, (17.7%) and 1, (2.9%), the second group, from 20 to less than 30, had a frequency of 22, (27.8%) and 15, (44.1%). The third group was more or equal to 30; this group had a frequency of 43 (54.4%) and 18, (52.9%) for the normally extracted and carried over samples respectively. (Table. 4). It can be seen that there was only one cycle threshold (Ct) in the normal group with a reading greater than 37; in contrast to that, no threshold cycle exceeded that number in the carried-over group (table 4 and 5).

When comparing the frequency of the ct values obtained from normally extracted and carried-over samples, it revealed a significant difference (P = 0.054) (Table. 6).

Since cycle threshold is used as an indicator of DNA concentration, the results demonstrated a strong and significant relationship between DNA concentration and the phenomenon of magnetic bead carryover. The pre-, analytical, and post-analytical factors that frequently have an impact on Ct values in viral infections (Abo-Youssef *et al.,* 2020 and Nakatsu *et al.,* 2015) can interpret this high concentration. The pre-analytic parameters include the specimen's age, kind, time of acquisition, transportation and storage conditions before testing, and mode of collection.

Also, it may be related to the effectiveness of nucleic acid extraction, the amount of viral RNA in the samples obtained, primer design, the type of target RNA, real-time PCR efficiency, and the technique for determining Ct value (Watson *et al.,* 2020). Furthermore, the interpretation and reporting of the results are examples of post-analytical factors that may also affect the DNA concentration result (Watson *et al.,* 2020). In addition, Rhoads *et al.,* (2020) reported that the amount of viral RNA that may be detectable by an assay depends on several factors, including the method and source of the specimen, the type and volume of the transport media, the time elapsed between specimen collection and analysis, and the number of days from infection to specimen collection.

Data in the results (tables 4 and 5) showed that within group percent of the first normal group was 17.7, while within group percent in the same group in the magnetic bead carried over was 2.9. According to statistical analysis, this significant difference demonstrates the impact of DNA concentration (cycle threshold) on both, the normal extracted one and the magnetic beads carried over.

Whereas both the second and third cycle threshold groups in both normal and magnetic beads carried over categories showed semi-similar results, indicating that the relationship comes from the first group, which include the very high concentration samples.

Table.1 PCR Amplification Component

Component	Volume	
	1 Well	N (1) Wells
PCR Reaction Mix Multi-1		
2- Masti Type Master Mix	10 µL	N× 10 µL
3- Masti Type Multi Primer Mix 1	5 µL	N× 5 µL
PCR Reaction Mix Multi-2		
2- Masti Type Master Mix	10 µL	N× 10 µL
4- Masti Type Multi Primer Mix 2	5 µL	N× 5 µL
PCR Reaction Mix Multi-3		
2- Masti Type Master Mix	10 µL	N× 10 µL
5- Masti Type Multi Primer Mix 3	5 µL	N× 5 µL
PCR Reaction Mix Multi-4		
2- Masti Type Master Mix	10 µL	N× 10 µL
6- Masti Type Multi Primer Mix 4	5 µL	N× 5 µL

Table.2 Volumes of the Reaction

Sample Type	Component	Volume Per Reaction
Test Sample	Sample DNA	5 µL
PC	1 Masti Type Positive Control	5 µL
MNC	Nuclease Free Water	5 µL

Table.3 The comparison between cycle threshold values of the magnetic beads carried over and normally extracted DNA (T. test= .066)

Group	No. of Ct	Mean ± SD	Min. - Max.	P value
Normally extracted	79	27.95±7.6	10.13-37.02	.066
Carried over	34	30.11± 4.6	18.51-36.99	
Total	113	28.60 ± 6.9	10.13-37.02	

Table.4 Frequency of Ct values of the normally extracted DNA samples

Ct value	Frequency	Percent	Cumulative Percent
< 20	14	17.7	17.7
20, < 30	22	27.8	45.6
>= 30	43	54.4	100
Total	79	100	

Table.5 Frequency of the Ct values of the carried over samples

Ct value	Frequency	Percent	Cumulative Percent
< 20	1	2.9	2.9
20, < 30	15	44.1	47.1
>= 30	18	52.9	100
Total	34	100	

Table.6 The relation between Ct values of the normally extracted samples and the magnetic beads carried over samples (chi square = 5.85, P = .054)

Ct value	Description	Group		Total
		Normal	Carried over	
<20	Count	14	1	15
	% within group	17.7	2.9	13.3
20, <30	Count	22	15	37
	% within group	27.8	44.1	32.7
>=30	Count	43	18	61
	% within group	54.4	52.9	54.0
Total	Count	79	34	113
	% within group	100	100	100

It is noteworthy and significant to discuss that, in contrast to the results of the magnetic beads carried over group, where no threshold cycle exceeds 37 cycles, which reflect a high DNA concentration and reflect the relationship between the cycle threshold and magnetic beads carried over in a different way, there was only one cycle threshold (Ct) from the normal group with a reading greater than 37 cycles, indicating a very low DNA concentration and a high number of cycle thresholds. According to Abo-Youssef (2020); Nakatsu (2015); Watson *et al.,* (2020) and Rhoads *et al.,* (2020), there are numerous factors that affect cycle threshold values, so more research is necessary to identify the causes of this phenomenon. In particular, there are samples for which DNA cannot be obtained, which may result in financial losses.

However, using rtPCR in the current study was employed to co-relate the cycle threshold and magnetic beads carried over, but it also identified some bacteria in the mastatic cow's milk samples, which was also validated by Taponen (2009).

This study also demonstrates that, in terms of time management, DNA extraction and amplification are faster than other traditional techniques, as demonstrated by Abd El-Aal (2010) and Amin (2011).

Furthermore, the outcome resolves Abdelhameed's *et al.,* (2024) query and identifies one of the variables influencing the magnetic beads carryover phenomenon, which encouraged additional study of the theory. Consequently, this finding highlights the significance of the cycle threshold and demonstrates one of its benefits in terms of infection prediction, as stated by Rabaan *et al.,* (2021).

This finding demonstrated a substantial relationship between the cycle threshold and magnetic beads carry over, indicating how much DNA concentration can affect magnetic beads carry over and how far it can affect yielded DNA too.

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Author Contribution

Mohammed Farah Mohammed Abdelhameed: Investigation, formal analysis, writing—original draft. Ahmed Eltigani Almansoori: Validation, methodology, writing—reviewing. Rania Hassan Zaid:—Formal analysis, writing—review and editing. Mohamed T. Ibrahim: Investigation, writing—reviewing.

Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethical Approval: Not applicable.

Consent to Participate: Not applicable.

Consent to Publish: Not applicable.

Conflict of Interest: The authors declare no competing interests.

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