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COMPARISON OF EXTRACTION METHOD FOR MIRNA AS A BIOMARKER FOR THE DIAGNOSIS OF SEPSIS

Future diagnostics of sepsis

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Abstract

Sepsis is a severe condition caused by a dysregulated host response to an infection that may lead to organ failure and death. Early diagnostic to be able to provide correct treatment is crucial for survival. Performing a blood culture is the most common way in the diagnostic of sepsis, a time-consuming process with many false negative results. MicroRNA has been suggested as a potential biomarker for sepsis due to the stability of the microRNA and the possibility to diagnose more complex diseases. Blood was donated from self-assessed healthy individuals and the plasma was used for extractions of microRNA. Two different isolation kits were used in extractions with a starting plasma volume of 100 µl and 200 µl to determine which of the kits that provide the highest amount of miRNA concentration with the highest quality, the kits were then compared to each other. The two kits used were miRNeasy Serum/Plasma Advanced kit (Qiagen) and Total RNA Purification kit (Norgen). The result from the extractions were analyzed in SPSS and showed a statistically significant difference of microRNA concentration. The miRNeasy kit had a higher miRNA concentration in the extractions than the Norgen kit while the quality of the extractions did not show any significant difference between the two kits. The miRNeasy kit show a possibility of providing extractions with a high microRNA concentration using both a starting plasma volume of 100 µl and 200 µl and could be a possible kit to use in further extractions in the research of sepsis.

List of abbreviations

CRP	C-reactive protein
PCT	Procalcitonin
miRNA	Micro Ribonucleic acid
EDTA	Ethylenediaminetetraacetic Acid
qPCR	Quantitative polymerase chain reaction
RT-qPCR	Quantitative reverse transcription polymerase chain reaction
NGS	Next-Generation Sequencing

Table of contents

Introduction **1**

Materials and method **3**

Preparation of blood plasma *3*

Thawing of plasma *3*

miRNeasy® Serum/Plasma Advanced kit (Qiagen) *3*

Total RNA purification kit (Norgen) *3*

Quantification of miRNA *4*

Statistical test *4*

Ethical considerations *4*

Result **5**

Analyze of the miRNeasy® Serum/Plasma Advanced kit *5*

Analyze of the Total RNA Purification kit *8*

Comparison of miRNeasy® Serum/Plasma Advanced kit and Total RNA Purification kit *11*

Discussion **12**

miRNA quantity *12*

miRNA quality *14*

Downstream applications *15*

Conclusion *15*

Acknowledgments **16**

References **17**

Appendices **21**

Appendix A *21*

Appendix B *22*

Introduction

Sepsis is a severe condition that can lead to death if not treated correctly and early diagnostic is crucial for survival (Ljungström et al., 2017). Sepsis is the one of the 10 most common causes of death with a mortality rate between 25 % and 70 % depending on the severity. In the United States there are 750 000 cases of sepsis every year (Lever & Mackenzie, 2007). It occurs when there is a dysregulated host response to the infection, causing an organ dysfunction that may lead to organ failure. Sepsis may arise from an infection caused by bacteria, virus, fungi or other infectious pathogens (Ljungström et al., 2017; Singer et al., 2016). Earlier diagnostic of the type of organism that has caused the infection can provide the patient to get the right treatment and reduce the risk of antibiotic resistant and misuse (Ljungström et al., 2017). Most common causes of sepsis are pneumonia, intrabdominal infections and urinary tract infections. The most common gram-positive bacteria that are the cause of sepsis are the *Staphylococcus aureus* and *Streptococcus pneumonaie,* which causes pneumonia. *Escherichia coli* and *Pseudomonas aeruginosa* which may cause urinary tract infections are the most common gram-negative bacteria that could result in sepsis (Angus & van der Poll, 2013).

Early goal-directed therapy has been studied and show a lower mortality rate and better short-term and long-term benefits in the treatment of sepsis provided the treatment start in the early stage of sepsis (Rivers et al., 2001). Unfortunately, it is difficult to distinguish between sepsis and other non-infectious diseases, an increased release of substances used in the detection of sepsis may be caused by other reasons than infection, e.g. trauma or surgery (Castelli et al., 2004). This reduce the possibility to provide the right treatment from the beginning. The gold standard method in the diagnostic of sepsis is performing a blood culture to determine the infectious pathogen. The method of performing cultures are very time-consuming, it can take up to 72 hours for the culture to grow. It may not always show a correct result, and can many times result in a false negative result (Benz, Roy, Trautwein, Roderburg & Luedde, 2016; Schwarzenbacher et al., 2019). Most of the blood cultures that are performed on patient that are suspected of sepsis only display a positive result in one out of three cases (Angus & van der Poll, 2013). Another challenge with blood culturing is the possibility of a false-positive result, this could happen when there is a lack of antiseptic procedure when collecting the sample from patients and resulting in a contaminated blood culture. The result from blood culture may often be incomplete or too late and therefore it is crucial to find a more sensitive and less time-consuming method for the diagnosis of sepsis (Sinha et al., 2018).

Biomarkers are used in the process of diagnosing different diseases. There are many definitions of what a biomarker is, but most commonly stated is that a biomarker is a measurable characteristic that demonstrate interactions between biological processes and possible threats (Strimbu & Tavel, 2010). Biomarkers play a key role in the diagnosis due to the ability to indicate if there is a presence of sepsis, what kind of pathogen that causes it and the severity (Pierrakos & Vincent, 2010). Several biomarkers have been suggested for detection of sepsis, although C-reactive protein (CRP) and procalcitonin (PCT) are the most common biomarker in diagnosis of bacterial sepsis (Kibe, Adams & Barlow, 2011; Ljungström et al., 2017). However, both of these biomarkers have a limited ability in the accuracy of the diagnostic (Kibe et al., 2011). Lactate is also commonly used in clinical practice when diagnosing sepsis, however, many other conditions may also show a raise in lactate level that are not connected to sepsis which demonstrate that the use of lactate as a biomarker do not provide a high specificity

(Huang et al., 2014; Ljungström et al., 2017). CRP and PCT have a difficulty to distinguish between sepsis and other non-infectious diseases, a raise in PCT may indicate sepsis but it could also be an indication of other cases where an inflammation response is activated, these could be trauma and also surgery. CRP and PCT lack the sensitivity and the specificity needed for a fast diagnosis of sepsis that is crucial to reduce the mortality rate (Pierrakos & Vincent, 2010). These singleplex biomarkers, that are used one at a time, do not provide a good enough result. By using multiplex biomarkers, which is a combination of different biomarkers, could improve the diagnosis of sepsis due to the possibility of diagnosing more complex diseases like sepsis (Ljungström et al., 2017; Backes, Meese & Keller, 2016). Future diagnostics of sepsis are in research for an earlier and more accurate way of diagnosing sepsis. One key attribute is the multi marker panel, which would allow to monitor a combination of biomarkers directly from the blood of the patient contributing to an earlier diagnostic (Helldin, Pernestig & Tilevik, 2017).

MicroRNAs (miRNAs) are non-coding RNAs and function as negatively regulators in the regulation of gene expression. They help regulate the production of inflammatory pro-cytokines, which could cause organ damaged due to an excessive secretion during the initial acute phase and the transient phase of sepsis. They also regulate organ dysfunction during the later stage of sepsis along with regulation of apoptosis and immunosuppression (Kingsley & Bath, 2017). Due to the stability of the miRNAs, they have been suggested as a biomarker considering the potential contribution to diagnosis of more complex diseases and also in the diagnosis of sepsis. miRNA is released from cells in the body and then circulate in the extracellular environments. The miRNAs in these places are found to be very stable and can be detected in serum, plasma and other body fluids (Benz et al,. 2016; Correia et al,. 2017). However, miRNA is only a small portion of the total RNA in body fluids and could therefore be challenging to work with (Correia et al,. 2017). Due to the stability of the miRNAs they can resist extreme temperature. They also have a resistance against exogenous and endogenous RNAses providing an easy detection of the miRNAs in the blood (Vasilescu et al., 2017). There are many studies with miRNAs as biomarkers for sepsis, and an altered level of the miRNA may indicate sepsis (Pogribny, 2018). Wang et al identified six potential miRNA biomarkers for sepsis using solexa sequencing. miR-15a, miR-16, miR-193b, miR-483-5p, miR-122 and miR-223 showed a significant difference in levels between non-survivors and survivors (Wang et al., 2012). With further studies concerning miRNAs role and expression pattern in sepsis could result in a faster and more accurate way in the diagnostics of sepsis, providing the opportunity to start the correct treatment earlier, reducing the use of antibiotics and death prognosis while increasing the possibility for the patient to have a better recovery time and life after sepsis (Kingsley & Bath, 2017).

To use miRNA as biomarkers, the miRNA need to be extracted that could be a challenging process due to both technical and non-technical factors that could affect the concentration of the circulating RNA in the body fluids (Lee, Baxter, Lee, Scherler & Wang, 2017). The amount of miRNA circulating in total RNA can be estimated to 0.01% in a sample, however, this can vary depending on the tissue or body fluid that is used (Peltier & Latham, 2008). Today there are many different kits on the market, and they all work differently, and the amount of miRNA extracted varies (Lee et al., 2017).

In this report the miRNeasy Serum/Plasma Advanced kit (Qiagen) and Total RNA Purification kit (Norgen) were used for extraction with a starting plasma volume of 100 µl and 200 µl. The kits are then compared with each other to determine which of the kit provide the highest miRNA concentration in the extractions and could be used in future research in the area of sepsis.

Materials and method

Preparation of blood plasma

Blood was withdrawn from self-assessed healthy donors into 6 ml EDTA tubes (Greiner Bio-One). The blood was then centrifuged in a Scan Speed 1580R Multipurpose centrifuge at 1790 rpm for 15 minutes and 4°C according to A-K. Pernestig (personal communication, February 19, 2020). The plasma was then transferred into 1.5 ml Eppendorf tubes with a volume of 800 µl and 400 µl, the tubes were then stored in -80°C to be used later for isolation of total RNA including miRNA.

Thawing of plasma

The plasma sample was thawed according to S. Jurcevic (personal communication, February 24, 2020), 10 minutes on ice and then placed in room temperature for 10 minutes.

miRNeasy® Serum/Plasma Advanced kit (Qiagen)

miRNA extraction from plasma was performed by following the protocol of the miRNeasy® Serum/Plasma Advanced kit (Qiagen). The volume of the starting plasma was both 100 µl (n=13) and 200 µl (n=13).

The protocol was followed accordingly except for the fourth step in the procedure, there was no spike-in control added to the samples. When performing extractions using a volume of 100 µl of starting plasma, the volume of the buffer RPL and buffer RPP was adjusted to 30 µl and 10 µl respectively according to the protocol.

Total RNA purification kit (Norgen)

The Total RNA purification kit (Norgen) was used to extract miRNA that can be used for biomarkers using the plasma volume of 200 µl (n=10) and 100 µl (n=10). In total there was 20 samples extracted using this protocol.

The protocol was followed, starting with the lysate preparation. The steps were followed accordingly, however, the optional step in the lysate preparation was not performed. After the lysate preparation the protocol was followed accordingly including all the additional centrifuge steps. Between the binding to column step and the column wash step, an additional step following the RNase-Free DNase I kit from Norgen was performed following all the steps as the protocol stated. To perform the steps in the RNase Free DNase I kit a mixture of Enzyme Incubation Buffer and DNase I was prepared while the plasma was thawing so the miRNA would not degrade due to a delay in the extraction. The procedure was then continued according to the Total RNA Purification protocol except for a small change in the wash step. In the RNase Free DNase I kit at step 7 it was said to proceed to the second wash step, this was not followed, all the samples went through three washes. Every sample went through the elution steps twice to attain maximum RNA recovery, the spin column was moved to a new Eppendorf tube after the first elution and the procedure was then repeated according to the elution steps in the protocol.

Quantification of miRNA

After the extractions, all the samples were measured using the Qubit® microRNA Assay kit (Thermo Fisher Scientific) and the Qubit® 3.0 Fluorometer (Thermo Fisher Scientific) for the miRNA concentration and the NanodropTM 2000 Spectrophotometer (Thermo Fisher Scientific) for the quality of the samples.

Statistical test

For the all statistical test the program SPSS (IBM SPSS, Version 25, 2017) was used . A test of normality was first performed on the result that was attained from the Qubit and the Nanodrop with a p value threshold set to 0.05. The result of the normality test determined which test was best suitable to continue with depending on the distribution of the concentration of the samples. The result from all the extractions from using the two different kits were then compared to each other using an independent t-test/Mann-Whitney U test.

Ethical considerations

For this experiment the blood used have been donated voluntarily by self-assessed healthy donors involved in the University of Skövde, students and teachers. No paper of consent had to be signed since the donation was voluntarily and an active choice from the person who made the donation. The voluntarily donation gave the students the possibility to perform their thesis work. The blood was used for extractions of miRNA and analysis of the extractions. The blood not used for this experiment was saved to be used in other areas of the research in sepsis.

However, in other areas of the sepsis research, patients at Skaraborgs Hospital that are believed to suffer from sepsis have been asked to provide sample of their blood for the research. All of the patients have signed a consent paper approving the use of their blood in the sepsis research.

Result

The extraction performed with the two kits were first analyzed separately to determine if there was any statistical significance in the use of starting plasma volume. The kits were then compared with each other to determine if there was any difference in miRNA concentration depending on the kit.

Analyze of the miRNeasy® Serum/Plasma Advanced kit

The Qubit® microRNA Assay kit and Qubit® 3.0 Fluorometer was used to obtain result from the samples extracted with the miRNeasy® Serum/Plasma Advanced kit to perform a statistical analysis. A Shapiro-Wilk test of normality was performed on the result obtained from the Qubit® 3.0 Fluorometer (Appendix A) to determine if the samples had a normal distribution of miRNA concentration. The test showed a normal distribution of miRNA concentration between the samples in both the extractions with 200 µl starting plasma ($p=0.06$) and 100 µl of starting plasma ($p=0.58$) . The test of homogeneity of variance stated that there was an unequal variance between the two starting plasma volumes ($p=0.03$)

Table 1. Mean miRNA concentration extracted with the miRNeasy® Serum/Plasma Advanced kit.

Starting plasma volume (µl)	Mean value of miRNA concentration (ng/µl) (±1 SD)
200 (n=13)	1.36 (±0.55)
100 (n=13)	0.92 (±0.19)

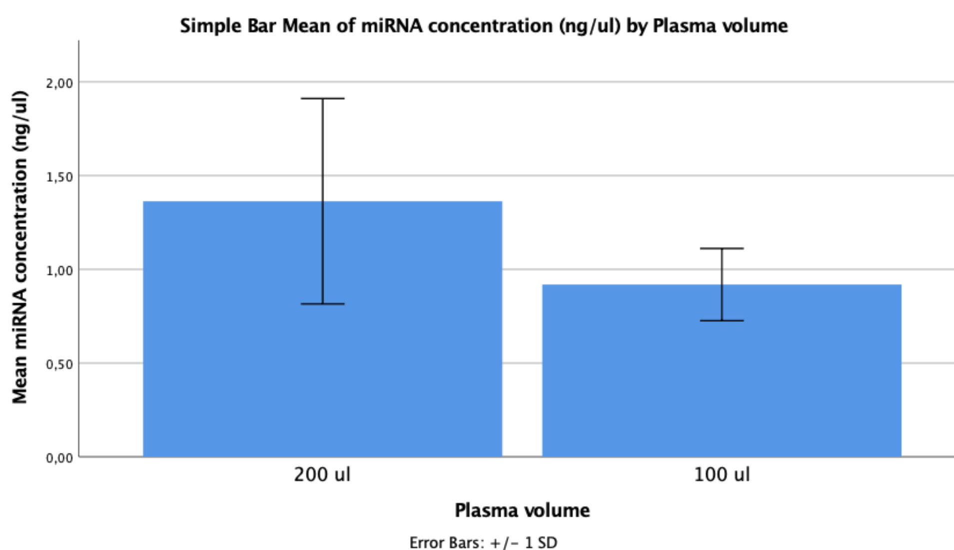

Figure 1. Bar chart with mean value and error bars of standard deviation for the extractions performed with the miRNeasy Serum/Plasma kit and a starting plasma volume of 100 µl and 200 µl .

The mean concentration in the 200 µl extractions (1.36±0.55) was higher than the 100 µl extractions (0.92±0.19) (Figure 1) but also had a higher biological variation than the 100 µl extractions (Table 1).

To determine if it would be possible to use a lower volume of starting plasma in future extractions, a comparison of the result that was obtained with the Qubit from both 200 µl plasma and 100 µl plasma was conducted. Both of the two volumes had a normal distribution but with unequal variance, therefore an independent t-test with a significant threshold set at 0.05 was performed (Table 2). The null hypothesis for the test were that there is no significant difference in miRNA concentration between the two starting plasma volumes. Since the significant number is lower than the set threshold of 0.05 (p=0.015), the null hypothesis is rejected and it is concluded that there is a statistically significant difference in miRNA concentration extracted, a higher starting plasma volume yield a higher miRNA concentration.

Table 2. Result from the independent t-test.

Independent Samples Test

		t-test for Equality of Means		
		t	**df**	**Sig. (2-tailed)**
miRNA concentration (ng/ul)	**Equal variances not assumed**	2.761	14.922	.015

The Nanodrop™ 2000 Spectrophotometer was used to analyze the quality of the extractions and the total RNA concentration (Table 3). The Shapiro-Wilks test of normality showed that the A_{260}/A_{280} had a normal distribution between the extractions with 100 µl plasma (p=0.35) and 200 µl plasma (p=0.42) with an equal variance (p=0.44) while the total RNA concentration in 100 µl plasma extractions (p=0.02) and 200 µl plasma extractions (p=0.01) did not have a normal distribution.

Table 3. Mean quality and total RNA concentration from extracted samples with the miRNeasy® Serum/Plasma Advanced kit.

Starting plasma volume (µl)	Mean value of A_{260}/A_{280} (nm) (±1 SD)	Median value of total RNA concentration (ng/µl) (±1 SD)
200 (n=13)	1.08 (±0.33)	7.50 (±35.4)
100 (n=13)	1.15 (±0.27)	11.20 (±14.01)

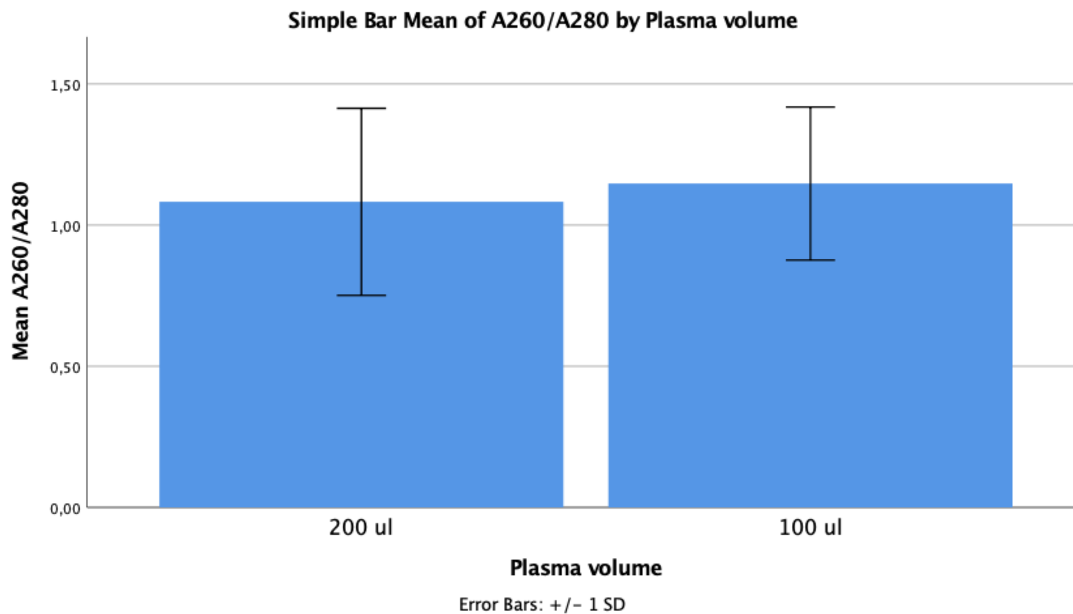

Figure 2. Bar chart with the mean of the quality of the samples with error bars displaying the standard deviation for the extractions performed with the miRNeasy Serum/Plasma Advanced kit (Qiagen) and a starting plasma of 200 μl and 100 μl.

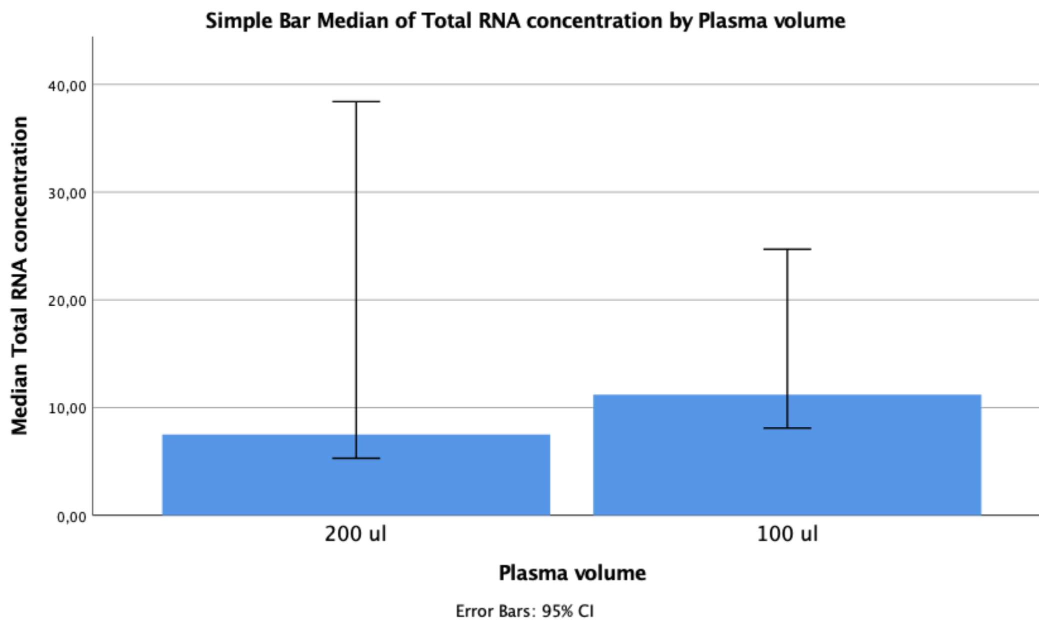

Figure 3. Bar chart of the median value of the total RNA concentration with a 95 % CI using the miRNeasy Serum/Plasma Advanced kit (Qiagen) with a starting plasma volume of 200 μl and 100 μl.

The extractions with 100 μl of starting plasma volume had a slightly higher mean value of the quality of the extractions and also a higher median value of the total RNA concentration in the samples with a lower biological variation (Figure 2 & 3).

An independent t-test was performed to determine if there was any significant difference in the quality of the extractions between the two starting plasma volumes. The significant number was

higher than the set threshold of 0.05 (p=0.59) and it could therefore be concluded that there was no difference in the quality between the two starting plasma volumes (Table 4).

Table 4. Independent t-test comparing the quality of the two starting volumes.

Independent Samples Test

		t-test for Equality of Means		
		t	**df**	**Sig. (2-tailed)**
A_{260}/A_{280} ratio	**Equal variances assumed**	-.544	24	.591

A Mann-Whitney U test was conducted to determine if there is a significant difference in the total RNA concentration between the extractions. The test stated that there was no statistically significant difference in the total RNA concentration (U=97, z=0.64, p=0.54) between the two starting plasma volumes.

Analyze of the Total RNA Purification kit

Most of the extractions did not give a miRNA concentration in the Qubit® 3.0 Fluorometer, many of the samples had a too low miRNA concentration to give a reading of the sample which can be seen in Appendix B. Therefore, the result with 100 µl are from both elution 1 and elution 2. The 200 µl did not have any measurable miRNA concentration from elution 2 and all the result are from the elution 1. However, all the samples had a measurable total RNA concentration when measured in Nanodrop™ 2000 Spectrophotometer (Appendix B).

A Shapiro-Wilks test of normality was conducted with the result from the extractions performed with the Total RNA Purification kit (Norgen). The result showed that there was not a normal distribution of miRNA concentration in both of the starting plasma volumes of 100 µl (p=0.02) and 200 µl (p=0.00), therefore the median result is presented below (Table 5).

Table 5. Median miRNA concentration using Total RNA Purification kit.

Starting plasma volume (µl)	**Median value of miRNA concentration (ng/µl) (±1 SD)**
200 (n=10)	0.47 (±0.23)
100 (n=10)	0.48 (±0.20)

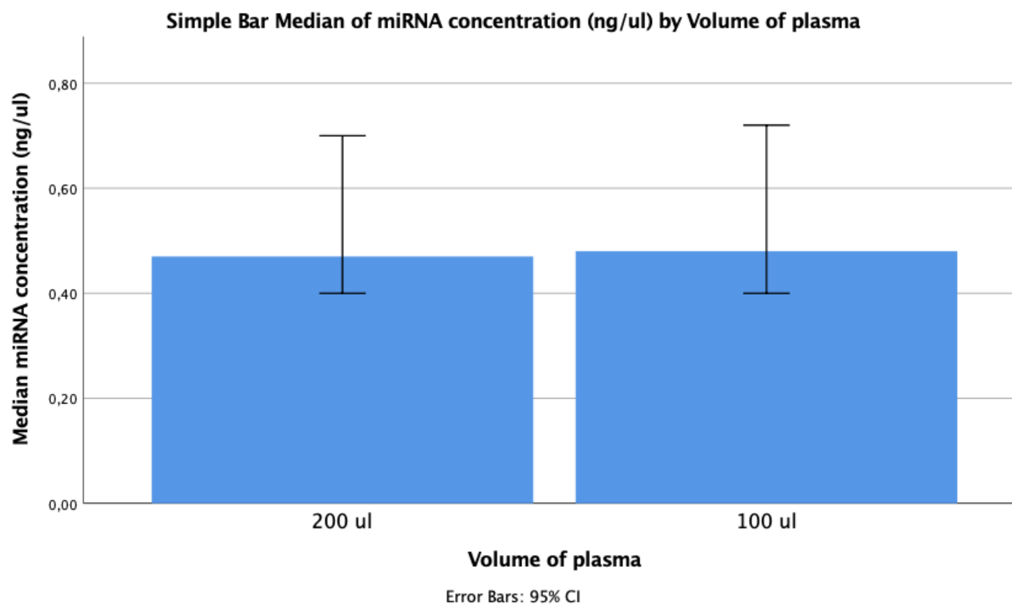

Figure 4. Median miRNA concentration with a 95% CI for the extractions performed with the Total RNA Purification kit (Norgen) and starting plasma volume of 200 µl and 100 µl.

The samples with 200 µl as starting plasma volume had a similar median value (0.47±0.23) as the samples using 100 µl as starting plasma volume (0.48±0.20) (Figure 4) and a slightly higher biological variation (Table 5).

To determine if it would be possible to use a lower starting plasma volume in future extractions, a comparison of the result obtain with the Qubit from both 200 µl plasma and 100 µl plasma was conducted. Due to the non-normal distribution of the miRNA concentration, a Mann-Whitney-U test was performed. The test showed that there was no statistically significant in miRNA concentration between the two starting plasma volumes ($U=52$, $z=0.162$, $p=0.912$).

The NanodropTM 2000 Spectrophotometer was used to analyze the quality and total RNA concentration of extracted samples (Table 6). The result was analyzed with a Shapiro-Wilks test of normality. The 200 µl starting plasma volume had a normal distribution for the quality of the samples ($p=0.06$) but not for the total RNA concentration ($p=0.03$). The 100 µl starting plasma volume had a normal distribution both for the quality of the samples (0.14) and for the total RNA concentration ($p=0.07$).

Table 6. Quality and total RNA concentration using the Total RNA Purification kit.

Starting plasma volume (µl)	**Mean value A_{260}/A_{280} (nm) (±1 SD)**	**Median value total RNA concentration (ng/µl) (±1 SD)**
200 µl (n=10)	1.25 (±0.18)	11.85 (±27.70)
100 µl (n=10)	1.28 (±0.18)	15.50 (±17.96)

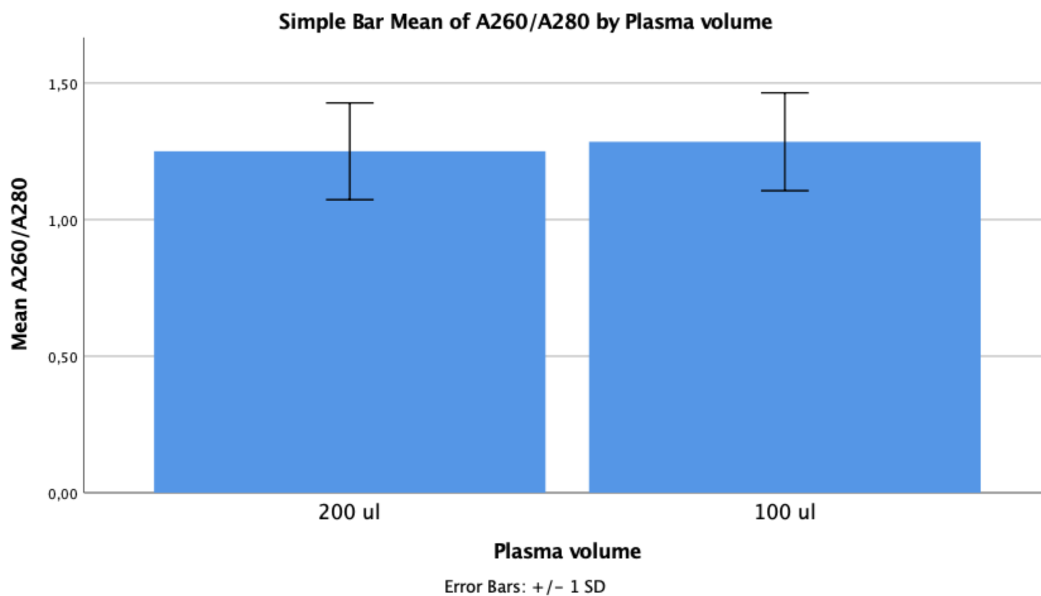

Figure 5. Bar chart with the mean value and error bars with the standard deviation of the quality of the samples performed with the Total RNA Purification kit (Norgen) and a starting plasma volume of 200 µl and 100 µl.

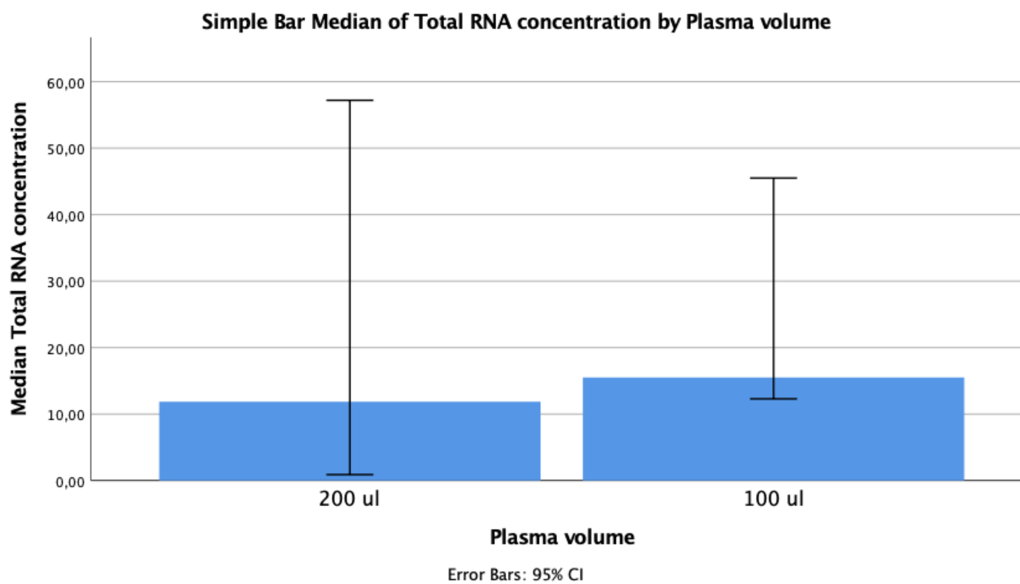

Figure 6. Bar chart with the median value of the total RNA concentration with error bars showing the 95% CI for the extractions performed with the Total RNA Purification kit (Norgen) and a starting plasma volume of 200 µl and 100 µl.

The two starting plasma volumes had a similar mean quality of the samples (Figure 5). The 100 µl starting plasma volume had a slightly higher median RNA concentration than the 200 µl starting plasma volume (Figure 6).

An independent t-test was performed to determine if there was any significant difference in the quality of the extractions between the two starting plasma volumes. The test stated that there was no significant difference between the quality of the two starting plasma volumes (p=0.66) (Table 7).

Table 7. Independent t-test to compare the quality between the two starting plasma volumes.

Independent Samples Test

		t-test for Equality of Means		
		t	**df**	**Sig. (2-tailed)**
A_{260}/A_{280} ratio	**Equal variances assumed**	-.439	18	.666

A Mann-Whitney U test was conducted to determine if there is any significant difference between the extractions with 200 µl plasma and 100 µl plasma for the RNA concentration. The test stated that there was no significant difference in the RNA concentration ($U=55.50$, $z=0.42$, $p=0.68$).

Comparison of miRNeasy® Serum/Plasma Advanced kit and Total RNA Purification kit

To determine if there is any difference in the amount of miRNA that is extracted using the miRNeasy® Serum/Plasma (Qiagen) advanced kit and the Total RNA Purification kit (Norgen) a Kruskal-Wallis test was conducted due to the non-normal distribution of concentration in the Norgen kit (Figure 7). The test showed a significant difference in both 100 µl ($p=0.04$) and in the 200 µl ($p=0.00$) of starting plasma volume.

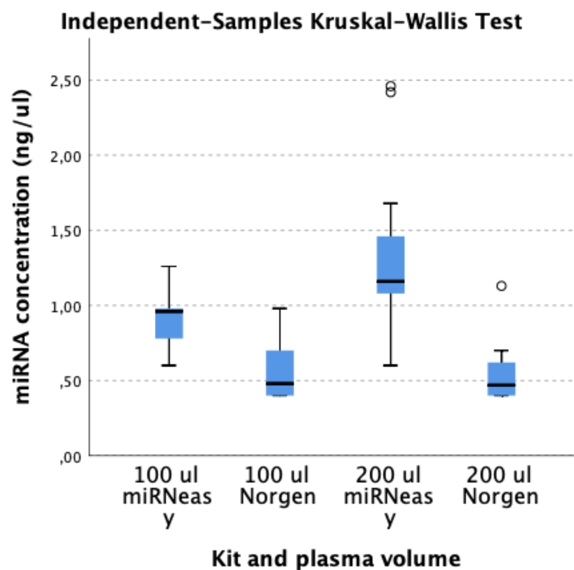

Figure 7 Kruskal-Wallis analyze of the two kits and the two different starting volume. The test showed a significant difference in both of the 100 µl ($p=0.04$) and the 200 µl ($p=0.00$) of starting plasma volume when the two kits were compared.

To determine if there was any significant difference in the quality of the extractions performed with the two kits, an ANOVA test was conducted. The test showed no significant difference in the quality of the extractions with the two different kits (p=0.23).

A Kruskal-Wallis test was conducted to determine if there was any significant difference in RNA concentration of the extractions performed with the two different kits (Figure 8). The test stated that there was no significant difference in any of the starting plasma volume (p=0.77).

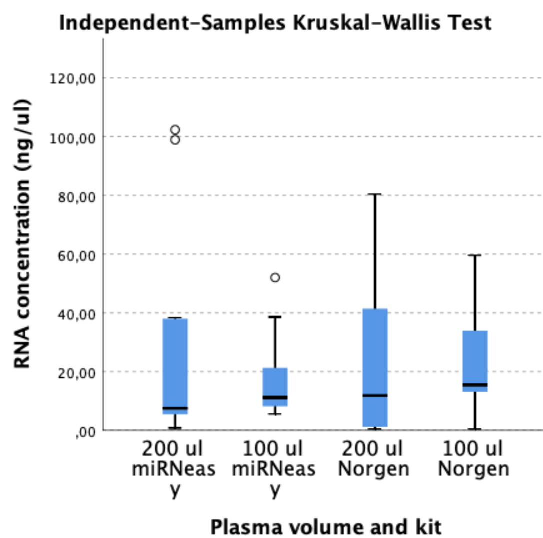

Figure 8. Kruskal-Wallis analyze of the RNA concentration. The test stated that there was no significant difference between the two starting plasma volume and the two kits (p=0.77).

Discussion

Many of the methods today in diagnosis of sepsis lack the specificity that is needed for an accurate diagnosis. Many researches are therefore in the process of finding another way to diagnose sepsis, with a simpler method with higher specificity. An earlier and more productive method to diagnose sepsis would reduce the unnecessary use of antibiotics preventing antibiotic resistant (Evans, 2018). Circulating miRNAs have been introduced as potential biomarkers in the diagnose of many diseases including sepsis (Benz et al., 2016; Dave et al., 2018). To be able to use miRNA as a biomarker the miRNA need to be extracted from blood or plasma. To do this there are many extractions kit on the market, however, it is important to find a kit which can extract a high amount of miRNA concentration and with a high quality of the miRNA. In this report the miRNeasy® Serum/Plasma Advanced kit (Qiagen) and the Total RNA Purification kit (Norgen) was used for extractions from plasma from healthy donors with the use of 200 µl and 100 µl of starting plasma volume.

miRNA quantity

The first extractions were performed with the miRNeasy kit with a volume of 200 µl and 100 µl as starting plasma. This was done to determine if it is possible to use the kit with a smaller starting plasma volume than recommended volume in the protocol to reduce the volume of blood needed to be withdrawn from patients with suspected sepsis. The amount of miRNA that was extracted with the use of the miRNeasy kit and a starting plasma volume of 200 µl (Table 1)

was similar to a previous study conducted by Wright et al. (2020) where they performed extractions on ovine plasma attained from sheep using different kits. The study includes the miRNeasy® Serum/Plasma Advanced kit (Qiagen) and the starting plasma that was used was 200 µl as the protocol says. In the study it is stated that the miRNeasy® Serum/Plasma Advanced kit performed well in detecting miRNA in fresh sample, however, it had a lower detection rate when using frozen samples. In the experiments performed during this thesis work, all the plasma samples used for the extractions were frozen and a higher miRNA concentration might have been detected if the plasma samples were fresh. The plasma used for the extractions was stored in -80°C freezer until needed. The plasma was first thawed by placing it on ice for 10 min and then in room temperature for another 10 min. This was done according to S. Jurcevic (Personal communication, February 24, 2020). During the plasma preparation the blood samples collected was centrifuged to separate the blood from the plasma. After the thawing, the plasma did not go through any centrifugation, however, studies show that plasma stored in -80°C and then re-centrifuged upon thawing may yield a higher miRNA concentration (Binderup, Houlind, Madsen & Brasen, 2016). During the freezing and thawing of the plasma, a process called hemolysis may occur. Hemolysis occur when the red blood cells die and release miRNA into surrounding fluid which may alter the original structure of the circulating miRNA, hence, causing an inaccuracy in the result (Nordén, 2020). Some miRNAs can be used for detection of hemolysis, a variation in the level of miR-16 and miR-451 may be an indication of hemolysis due to the presence of a large volume of this type of miRNA in red blood cells (Kirschner et al., 2011).

By using a larger volume of starting plasma, a higher miRNA concentration can be detected. The extractions with 200 µl as a starting plasma volume hade a higher mean value (1.36±0.55 ng/µl) but also a higher biological variation than the extractions performed with a starting plasma volume of 100 µl (0.92±0.19 ng/µl) (Table 1). The result obtained from the Qubit from the extractions using the miRNeasy® Serum/Plasma Advanced kit showed a significant difference ($p=0.02$) between the amount of plasma used as a starting volume (Table 2). Since the biological variation is smaller in the 100 µl extractions, performing the extractions with the miRNeasy® Serum/Plasma Advanced kit and fresh plasma samples could yield a high enough miRNA concentration to be used in the diagnosis of sepsis. Using qPCR as a downstream application, 1.36 ng/µl and 0.92 ng/µl would be enough for detection of miRNA (Wright et al., 2020).

The Total RNA Purification kit (Norgen) showed a result that indicated that there was no significant difference ($p=0.91$) (Figure 4) between the two volumes of 100 µl (0.48±0.20 ng/µl) and 200 µl (0.47±0.23 ng/µl) starting plasma (Table 5). The extractions with 100 µl had a similar median value and a slightly lower biological variation (0.48±0.20 ng/µl) than the extractions performed with 200 µl (0.47±0.23 ng/µl) of starting plasma (Table 5). A smaller starting plasma volume could therefore be possible to use for future extractions using the Total RNA Purification kit according to the statistical tests. However, many of the extractions performed with the Total RNA Purification kit did not show a readable result in the Qubit due to too low miRNA concentration as can be seen in Appendix B. Other studies have similar result when it comes to extractions with Total RNA Purification kit (Nordén, 2020). The second elution when using 200 ul as starting plasma did not show any readable result, the Qubit only showed that the concentration was too low to give a reading. The first elution with 100 µl as a starting volume of plasma had three samples that gave a high enough amount of miRNA while the second elution of the same starting plasma volume had two samples that gave a reading, to provide for the statistical test to be performed these two were analyzed as one group. The Nanodrop could detect and quantify an RNA concentration and therefore there might be a miRNA concentration

but not high enough for the Qubit to detect, which has a detection rate of 0.05-100 ng/µl (Garcia-Elias et al., 2017). To perform the statistical test, the samples with a too low concentration in the Qubit was added with a miRNA concentration of 0.04 ng/µl since it is the closest concentration that falls out of the range that the Qubit can measure (Garcia-Elias et al., 2017). This was done to obtain a larger sample group size for a more accurate statistical analyze (Julious, 2005).

The miRNA concentration of the two kits were compared with each other using a Kruskal-Wallis test showing a significant difference between the two kits and the two starting plasma volumes of 100 µl (p=0.04) and 200 µl (p=0.00) (Figure 6). Both of the starting plasma volumes from the miRNeasy® Serum/Plasma Advanced kit yield a higher miRNA concentration than the ones from the Total RNA Purification kit. However, both of the kits had a small sample group size and with a larger group size the result would show a more accuracy (Julious, 2005). The elution volume was higher in the Norgen kit than in the miRNeasy® Serum/Plasma Advanced kit, 50 µl and 20 µl respectively. Studies show that a smaller elution volume does not necessary generate a higher concentration (McAlexander, Phillips & Witwer, 2013), which can also be stated for this report since the Total RNA Purification kit with a higher elution volume generated a higher RNA concentration. However, calculating the amount from the mean and median value from the two kits, the Total RNA Purification kit with 200 µl of starting plasma generated 23.5 ng in 50 µl of elution while the miRNeasy® Serum/Plasma Advanced kit generated 27.2 ng in 20 µl of elution. The Total RNA Purification kit with 100 µl of starting plasma generated 24 ng in 50 µl of elution while the miRNeasy® Serum/Plasma Advanced kit generated 18.4 ng in 20 µl of elution. However, it is not certain if a larger elution volume would generate a lower ng/µl concentration the same as it is not certain that a smaller volume generate a higher concentration (McAlexander et al., 2013). The concentration of miRNA may vary due to different conditions, i.e. a low RNA concentration or variation in the length of the miRNAs. It is important to make sure to use the right RNA isolation method and to store the samples correctly; the right isolation method may yield a higher miRNA concentration (Lee, Baxter, Lee, Scherler & Wang, 2017). The Norgen kit yield a higher RNA concentration (11.85 and 15.50 ng/µl) than the miRNeasy® Serum/Plasma Advanced kit (7.50 and 11.20 ng/µl). In a study by Monleau et al. (2014) where they compare the Total RNA Purification kit (Norgen) with two other kits, the Total RNA Purification kit yield a higher RNA concentration when extracting RNA from peripheral blood mononuclear cells and a slightly higher concentration when extracting RNA from serum. There was no significant difference between the kits in detecting miRNA, which was not the result in this thesis work. The miRNeasy kit yields a higher result in extracted miRNA concentration.

miRNA quality

The quality of the extractions was measured using a Nanodrop™ 2000 spectrophotometer. The median value of the quality of the extractions performed with the miRNeasy® Serum/Plasma Advanced kit (1.08 and 1.15 ng/µl) (Table 3) was lower than the median value of the extractions performed with the Total Purification kit (1.25 and 1.28 ng/µl) (Table 6). However, the ANOVA test showed that there was no significant difference in the quality of the extractions between the two kits (p=0.23). Neither of the kits gave a quality result that was between 1.9 and 2.0 which is classified as a standard for a pure sample (Nanodrop Technologies Inc, 2013). A low A_{260}/A_{280} ratio may indicate a contamination of proteins in the samples. In a previous study, extractions of miRNA also gave a lower quality ratio than 1.9-2.0 (Moret et al., 2013). Extraction miRNA can be challenging due to association with proteins or cellular fragments, which may contribute to different result when extracting miRNA (Moldovan et al., 2014).

Downstream applications

The concentration measured in the Qubit is a result of all the small RNA that can be detected in the samples, this may affect the result when measuring the miRNA concentration (Garcia-Elias et al., 2017). Therefore the kits may be further analyzed by using downstream applications e.g. reverse transcription quantitative PCR (RT-qPCR), Next-Generation Sequencing (NGS), and microarrays for a more accurate miRNA concentration (Tan, Khoo & Tan, 2015). The accuracy of these methods are determined by the quality and quantity of the extracted samples, the accuracy reduces if the quantity is at a low level while the reproducibility of the miRNA reduces if the quality of the samples are low due to contaminants (Pritchard, Cheng & Tewari, 2012). The RT-qPCR system is considered to be the gold standard in miRNA profiling due to the sensitivity of the detection rate. And by adapting the RT-qPCR system to a high-throughput platform the sensitivity and accuracy of the method increases (Tan et al., 2015). The quality of the sample is important when using RT-qPCR, contaminants in the samples may affect the result (Brown et al., 2018).

Downstream applications that can be used in the detection of the extracted miRNA biomarkers for the diagnosis of sepsis are two tailed RT-qPCR, however this was never conducted during this thesis work. The research group Future diagnostic of sepsis are currently optimizing the two tailed RT-qPCR and also running the Quantitative PCR with miRCURY LNA Serum/Plasma Focus PCR Panels (QIAGEN) for the detection of miRNA biomarkers for an earlier diagnosis of sepsis (Nordén, 2020).

Conclusion

This study was conducted to determine if there was any difference in the miRNA concentration extracted with two different kits and the possibility to use a lower starting plasma volume than according to the manufactures of the kits. The extractions performed with miRNeasy® Serum/Plasma Advanced kit showed a miRNA concentration in all of the extractions performed when measured in Qubit. Using 200 µl as a starting plasma volume yield a higher miRNA concentration and would be able to participate in downstream application for miRNA detection. However, using 100 µl starting plasma volume would contribute to a high enough miRNA concentration for further miRNA detection. The miRNeasy® Serum/Plasma Advanced kit have the potential to be used for extractions with a smaller starting plasma volume and contribute to an earlier diagnostic of sepsis.

Both the starting plasma volume of the extractions with the Total RNA Purification kit yields a similar miRNA concentration. The miRNA concentration could be high enough for further downstream applications for miRNA detection according to other result from other thesis work (M. von Ehr, Personal communication, June 1, 2020). Since there was no difference in miRNA concentration between the two starting plasma volumes it would also be possible to use a smaller starting plasma volume in future extractions using the Total RNA Purification kit. However, since many of the extractions did not show a readable result in the Qubit, more extractions might have to be conducted to be able to use in downstream applications like RT-qPCR and more plasma have to be used, and larger blood samples need to be withdrawn from patients.

For future diagnostic of sepsis, the miRNeasy® Serum/Plasma Advanced kit could be a potential option, however, more studies should be conducted for a more accurate result due to low sampling number for the statistical analyze.

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References

Angus, D. C. & van der Poll, T. (2013). Severe Sepsis and Septic Shock. *The New England Journal of Medicine,* 369, 840-851. doi:10.1056/NEJMra1208623

Backes, C., Meese, E., & Keller, A. (2016). Specific miRNA Disease Biomarkers in Blood, Serum and Plasma: Challenges and Prospects. *Molecular Diagnosis & Therapy*, 20, 509–518. doi:10.1007/s40291-016-0221-4

Benz, F., Roy, S., Trautwein, C., Roderburg, C. & Luedde, T. (2016). Circulating microRNAs as biomarkers for sepsis. *International Journal of Molecular Sciences*, 17(1), 78. doi:10.3390/ijms17010078

Binderup, H. G., Houlind, K., Madsen, J. S., & Brasen, C. L. (2016). Pre-storage centrifugation conditions have significant impact on measured microRNA levels in biobanked EDTA plasma samples. Biochemistry and biophysics reports, 7, 195–200. doi:10.1016/j.bbrep.2016.06.005

Brown, R., Epis, M. R., Horsham, J. L., Kabir, T. D., Richardson, K. L., & Leedman, P. J. (2018). Total RNA extraction from tissues for microRNA and target gene expression analysis: not all kits are created equal. *BMC biotechnology*, 18(1), 16. doi:10.1186/s12896-018-0421-6

Castelli, G. P., Pognani, C., Meisner, M., Stuani, A., Bellomi, D., & Sgarbi, L. (2004). Procalcitonin and C-reactive protein during systemic inflammatory response syndrome, sepsis and organ dysfunction. *Critical care (London, England),* 8(4), R234–R242. doi:10.1186/cc2877

Correia, C. N., Nalpas, N. C., McLoughlin, K. E., Browne, J. A., Gordon, S. V., MacHugh, D. E. & Shaughnessy, R. G. (2017) Circulating microRNAs as Potential Biomarkers of Infectious Disease. *Front. Immunol*. 8(118). doi:10.3389/fimmu.2017.00118

Dave, V. P., Ngo, T. A., Pernestig, A-K., Tilevik, D., Kant, K., Nguyen, T., Wolff, A., & Bang, D. D. (2018). MicroRNA amplification and detection technologies: opportunities and challenges for point of care diagnostics. Laboratory Investigations, 99, 452-469. doi:10.1038/s41374-018-0143-3

Evans, T. (2018). Diagnosis and management of sepsis. *Clinical medicine (London, England)*, 18(2), 146–149. doi:10.7861/clinmedicine.18-2-146

Garcia-Elias, A., Alloza, L., Puigdecanet, E., Nonell, L., Tajes, M., Curado, J., Enjuanes, C., Diaz, O., Bruguera, J., Martí-Almor, J., Comín-Colet, J., & Benito, B. (2017). Defining quantification methods and optimizing protocols for microarray hybridization of circulation microRNAs. *Scientific Reports,* 7, 7775. doi: 10.1038/s41598-017-08134-3

Helldin, T., Pernestig, A-K., & Tilevik, D. (2017). Towards a Clinical Support System for the Early Diagnosis of sepsis. *Digital Human Modeling. Applications in Health, Safety, Ergonomics, and Risk Management: Health and Safety*, 10287, 23-35. doi:10.1007/978-3-319-58466-9_3

Huang, J., Sun, Z., Yan, W., Zhu, Y., Lin, Y., Chen, J., Shen, B. & Wang, J. (2014). Identification of MicroRNA as Sepsis Biomarker Based on miRNAs Regulatory Network Analysis. *BioMed Research International,* 2014. doi:10.1155/2014/594350

Julious, S. A. (2005). Sample size of 12 per group rule of thumb for a pilot study. *Pharmaceutical Statistics*, 4, 287-291. doi:10.1002/pst.185

Kibe, S., Adams, K., & Barlow, G. (2011). Diagnostic and prognostic biomarkers of sepsis in critical care. *Journal of Antimicrobial Chemotherapy*, 66(2), ii33-ii40. doi:10.1093/jac/dkq523

Kingsley, S. M. K., & Bhat, B. V. (2017). Role of microRNAs in sepsis. *Inflamm. Res.* 66(7), 553–569. doi:10.1007/s00011-017-1031-9

Kirschner, M. B., Kao, S. C., Edelman, J. J., Armstrong, N. J., Vallely, M. P., van Zandwijk, N., & Reid, G. (2011). Haemolysis during sample preparation alters microRNA content of plasma. *PloS one*, 6(9), e24145. doi:10.1371/journal.pone.0024145

Lee, I., Baxter, D., Lee, M. Y., Scherler, K., & Wang, K. (2017). The Importance of Standardization on Analyzing Circulating RNA. *Molecular diagnosis & therapy*, 21(3), 259–268. doi:10.1007/s40291-016-0251-y

Lever, A., & Mackenzie, I. (2007). Sepsis: definition, epidemiology , and diagnosis. *BMJ*, 335, 879-883. doi:10.1136/bmj.39346.495880.AE

Ljungström, L., Pernestig, A. K., Jacobsson, G., Andersson, R., Usener, B., & Tilevik, D. (2017) Diagnostic accuracy of procalcitonin, neutrophil- lymphocyte count ratio, C-reactive protein, and lactate in patients with suspected bacterial sepsis. *PLoS ONE*. 12(7). doi:10.1371/journal.pone.0181704

McAlexander, M. A., Phillips, M. J., & Witwer, K. W. (2013). Comparison of Methods for miRNA Extraction from Plasma and Quantitative Recovery of RNA from Cerebrospinal Fluid. *Frontiers in genetics*, 4, 83. doi:10.3389/fgene.2013.00083

Moldovan, L., Batte, K. E., Trgovcich, J., Wisler, J., Marsh, C. B., & Piper, M. (2014). Methodological challenges in utilizing miRNAs as circulating biomarkers. *Journal of cellular and molecular medicine*, 18(3), 371–390. doi:10.1111/jcmm.12236

Monleau, M., Bonnel, S., Gostan, T., Blanchard, D., Courgnaud, V., & Lecellier, C. H. (2014). Comparison of different extraction techniques to profile microRNAs from human sera and peripheral blood mononuclear cells. *BMC genomics*, 15(1), 395. doi:10.1186/1471-2164-15-395

Moret, I., Sánchez-Izquierdo, D., Iborra, M., Tortosa, L., Navarro-Puche, A., Nos, P., Cervera, J., & Beltrán, B. (2013). Assessing an improved protocol for plasma microRNA extraction. *PloS one*, 8(12), e82753. doi:10.1371/journal.pone.0082753

Nanodrop Technologies, Inc. (2013) 260/280 and 260/230 Ratios NanoDrop® ND-1000 and ND-8000 8-Sample Spectrophotometers. [collected March 15, 2020 at https://biosci-batzerlab.biology.lsu.edu/Genomics/documentation/3130_NanoDrop_tips.pdf]

Nordén, J. (2020). Assessment of methods for microRNA isolation, microRNA amplification, and development of normalization strategy for sepsis biomarker research. *(Dissertation).* Retrieved from http://urn.kb.se/resolve?urn=urn:nbn:se:his:diva-18442

Peltier, H. J., & Latham, G. J. (2008). Normalization of microRNA expression levels in quantitative RT-PCR assays: identification of suitable reference RNA targets in normal and cancerous human solid tissues. *RNA (New York, N.Y.),* 14(5), 844–852. doi:10.1261/rna.939908

Pierrakos, C., & Vincent, J. L. (2010). Sepsis biomarkers: a review. *Critical care (London, England),* 14(1), R15. doi:10.1186/cc8872

Pogribny, I. P. (2018). MicroRNAs as biomarkers for clinical studies. *Experimental Biology and Medicine*, 243(3), 283–290. doi:10.1177/1535370217731291

Pritchard, C. C., Cheng, H. H., & Tewari, M. (2012). MicroRNA profiling: approaches and considerations. *Nature reviews. Genetics*, 13(5), 358–369. doi:10.1038/nrg3198

Rivers, E., Nguyen, B., Havstad, S., Ressler, J., Muzzin, A., Knoblich, B., Peterson, E., & Tomlanovich, M. (2001). Early Goal-Directed Therapy in the Treatment of Severe Sepsis and Septic Shock. *The New England Journal of Medicine*, 345, 1368-1377. doi:10.1056/NEJMoa010307

Singer, M., Deutschman, C. S., Seymour, C. W., Shankar-Hari, M., Annane, D., Bauer, M., … Angus, D. C. (2016). The Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3). *JAMA,* 315(8), 801–810. doi:10.1001/jama.2016.0287

Sinha, M., Jupe, J., Mack, H., Coleman, T. P., Lawrence, S. M., & Fraley, S. I. (2018). Emerging Technologies for Molecular Diagnosis of Sepsis. *Clinical microbiology reviews*, 31(2), e00089-17. doi:10.1128/CMR.00089-17

Strimbu, K., & Tavel, J. A. (2010). What are biomarkers?. *Current opinion in HIV and AIDS*, 5(6), 463–466. doi:10.1097/COH.0b013e32833ed177

Schwarzenbacher, J., Kuhn, S-O., Vollmer, M., Scheer, C., Fuchs, C., Rehberg, S., Balau, V., Hahnenkamp, K., Bohnert, J. A., & Gründling, M. (2019). On-site blood culture incubation shortens the time to knowledge of positivity and microbiological results in septic patients. *PloS one*, 14(12) doi:10.1371/journal.pone.0225999

Tan, G. W., Khoo, A. S. B., & Tan, L. P. (2015). Evaluation of extraction kits and RT-qPCR systems adapted to high-throughput platform for circulating miRNAs. *Scientific Reports,* 5, 9430. doi:10.1038/srep09430

Vasilescu, C., Dragomir, M., Tanase, M., Giza, D., Purnichescu-Purtan, R., Chen, M., … Calin, G. A. (2017). Circulating miRNAs in sepsis-A network under attack: An in-silico prediction of the potential existence of miRNA sponges in sepsis. *PloS one*, 12(8), e0183334. doi:10.1371/journal.pone.0183334

Wang, H., Zhang, P., Chen, W., Feng, D., Jia, Y., & Xie, L. (2012). Serum microRNA signatures identified by Solexa sequencing predict sepsis patients' mortality: a prospective observational study. *PloS one*, 7(6), e38885. doi:10.1371/journal.pone.0038885

Wright, K., de Silva, K., Purdie, A. C., & Plain, K. M. (2020). Comparison of methods for miRNA isolation and quantification from ovine plasma. *Scientific reports*, 10(825). doi:10.1038/s41598-020-57659-7

Appendices

Appendix A

Result of the miRNeasy kit obtained from the Qubit and Nanodrop.

Table 1. Qubit and Nanodrop result from the miRNA easy serum/advanced kit with a starting plasma volume of 200 µl.

Tube name	miRNA concentration (ng/µl)	A_{260}/A_{280} (nm)	Total RNA concentration (ng/µl)
S1 200µl	1.32	0.75	6.20
S2 200µl	2.46	1.38	98.90
S3 200µl	2.42	0.42	0.90
S5 200µl	1.46	1.46	102.30
S6 200µl	0.86	1.25	27.70
S7 200µl	1.12	0.95	6.60
S9 200µl	1.16	1.00	5.50
S10 200µl	1.68	1.05	7.50
S11 200µl	1.37	1.49	38.00
S13 200µl	0.60	1.30	38.40
S14 200µl	1.16	0.64	0.80
S24 200µl	1.08	1.35	16.40
S26 200µl	1.03	1.03	5.30

Table 2. Qubit and Nanodrop result from the miRNA easy serum/advanced kit with a starting plasma volume of 100 µl.

Tube name	miRNA concentration (ng/µl)	A_{260}/A_{280} (nm)	Total RNA concentration (ng/µl)
S8 100µl	0.96	0.98	11.20
S12 100µl	0.98	1.46	11.50
S15 100µl	0.78	1.28	38.60
S16 100µl	0.96	1.28	21.30
S17 100µl	0.90	1.07	8.10
S18 100µl	1.01	0.97	10.10
S19 100µl	0.76	1.35	52.00
S20 100µl	1.21	0.92	8.20
S21 100µl	1.26	1.03	7.10
S22 100µl	0.64	1.33	12.40
S23 100µl	0.60	1.51	8.70
S25 100µl	0.98	1.22	24.70
S27 100µl	0.90	0.51	5.60

Appendix B

Result of the Total RNA Purification kit(Norgen) obtained from the Qubit and Nanodrop.

Table 1. Qubit and Nanodrop result from the Total RNA Purification kit with a starting plasma volume of 200 µl, first elution.

Tube name	miRNA concentration (ng/µl)	A_{260}/A_{280} (nm)	Total RNA concentration (ng/µl)
S1 200µl E_1	0.62	1.35	17.40
S2 200µl E_1	0.56	1.42	57.20
S6 200µl E_1	1.13	1.40	80.40
S7 200µl E_1	0.70	1.00	6.30
S10 200µl	TL	1.20	0.90
S11 200µl	0.54	1.43	41.40
S12 200µl	TL	1.45	21.80
S18 200µl	TL	0.50	0.50
S19 200µl	TL	1.08	5.00
S20 200µl	TL	1.09	1.20

TL= Too low concentration

Table 2. Qubit and Nanodrop result from the Total RNA Purification kit with a starting plasma volume of 200 µl, second elution.

Tube name	miRNA concentration (ng/µl)	A_{260}/A_{280} (nm)	Total RNA concentration (ng/µl)
S1 200µl E_2	TL	1.40	36.20
S2 200µl E_2	TL	0.90	2.70
S6 200µl E_2	TL	1.49	3.30
S7 200µl E_2	TL	1.48	20.90
S10 200µl E_2	TL	1.23	2.50
S11 200µl E_2	TL	1.20	15.30
S12 200µl E_2	TL	1.37	49.50
S18 200µl E_2	TL	1.41	13.00
S19 200µl E_2	TL	1.01	3.20
S20 200µl E_2	TL	1.32	16.50

Table 3. Qubit and Nanodrop result from the Total RNA Purification kit with a starting plasma volume of 100 µl, first elution.

Tube name	miRNA concentration (ng/µl)	A_{260}/A_{280} (nm)	Total RNA concentration (ng/µl)
S3 100µl E_1	0.98	1.01	15.40
S4 100µl E_1	TL	1.47	33.90
S5 100µl E_1	0.72	1.38	45.50

S8 100µl E_1	TL	1.16	13.40
S9 100µl E_1	0.56	1.38	59.60
S13 100µl E_1	TL	2.70	0.80
S14 100µl E_1	TL	1.34	13.10
S15 100µl E_1	TL	1.10	0.50
S16 100µl E_1	TL	1.37	25.00
S17 100µl E_1	TL	1.49	15.60

Table 4. Qubit and Nanodrop result from the Total RNA Purification kit with a starting plasma volume of 100 µl, second elution.

Tube name	miRNA concentration (ng/µl)	A_{260}/A_{280} (nm)	Total RNA concentration (ng/µl)
S3 100µl E_2	TL	1.42	119.30
S4 100µl E_2	0.70	1.44	18.70
S5 100µl E_2	TL	1.39	9.30
S8 100µl E_2	TL	1.26	10.50
S9 100µl E_2	TL	1.31	30.50
S13 100µl E_2	TL	1.09	1.80
S14 100µl E_2	TL	1.24	10.20
S15 100µl E_2	TL	2.30	6.90
S16 100µl E_2	TL	1.28	21.50
S17 100µl E_2	0.56	1.08	12.30