Bachelor Degree Project

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Suggestions for optimal biomarker miRNA extractions from plasma of sepsis patients

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Abstract

Sepsis is a life-threatening organ disfunction, which is caused by a dysfunctional immune response and develops when an infection overwhelms the body's defense mechanism and causes and uncontrolled inflammatory response. Biomarkers have a great impact on helping diagnosis and treatments of sepsis. The biomarkers, like miRNA, are needed for both more accurate and quicker diagnosis of sepsis in patients. The future diagnostics are looking at other types of biomarkers, e.g. miRNA, but low amounts of miRNA are present in biofluids and make it challenging to quantify. A new methodology is needed which is both accurate and does not require a lot of fluid. The aim of this project was to identify which kit of two kits and which of two volumes of plasma would lead to the highest concentration of miRNA and highest quality of miRNA extracted. This was quantified by using two different volumes, 100 µl and 200 µl, and extracting the two volumes with both exoRNeasy Serum/Plasma midi kit (Qiagen) and Total RNA Purification kit (Norgen). There was no statistical difference between median miRNA concentrations between the two volumes within the Qiagen kit. However, the mean miRNA concentration (0.833 ng/µl) obtained from the Norgen kit (100 µl plasma starting volume) was statistically higher than the mean miRNA concentration (0.570 ng/µl) obtained from the same kit with 200 µl, $p = 0.033$. The optimal kit and volume of this study is the Norgen kit with 100 µl. Further studies are needed to verify these results.

List of abbreviations

CRP	C-reactive protein
EDTA	Ethylenediaminetetraacetate
ICON	Intensive Care Over Nations
ICU	Intensive care unit
MiRNA	MicroRNA
PCT	Procalcitonin
PiwRNA	Piwi-interacting RNA
RT-qPCR	Real-time quantitative polymerase chain reaction
SiRNA	Small interfering RNA

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Introduction

Sepsis

Sepsis is a severe clinical problem that results due to the exaggerated host inflammatory response to infection (Kingsley & Bhat, 2017). The pathophysiology of sepsis involves the recognition of pathogen associated molecular patterns by pattern recognition receptors or by recognition of danger associated molecular patterns by the receptors (Taeb, Hooper & Marik, 2017). This results in the activation of signaling cascade in the macrophages and neutrophils with subsequent release of large amounts of cytokines, which in turn promote the inflammation.

Sepsis is surprisingly a global health care issue associated with increased rate of mortality worldwide (Napolitano, 2018). According to the study carried out by the Intensive Care Over Nations (ICON) confirmed that about 29.5% of patients had sepsis on hospitalization or during their stay in the intensive care unit (ICU). Moreover, 25.8% of mortality in the ICU was found to be with sepsis patient while hospital mortality is 35.3% (Napolitano, 2018). In 2016, the third international consensus defined sepsis as a life-threatening organ dysfunction resulting from dysregulated host responses to infection, while septic shock is a subset of sepsis with multiple organ failures (Cecconi, Evans, Levy & Rhodes, 2018). During the initial stages of sepsis, the signs and symptoms of the sepsis may likely not noticeable to the patients and health care professionals, therefore patients may appear generally unwell with fever or even without fever (Hunt, 2019).

There are several risk factors, which predispose to the development of sepsis. These include age, sex, immunosuppression, other co-morbidities etc. There is positive correlation with age and the incidence of sepsis. As the age of the individual increases there is a consequent decline in response and function of the immune system and this increases the susceptibility of the individual to infection. Comorbidities that increase the susceptibility of sepsis include the following, congestive heart failure, chronic obstructive lung disease, diabetes mellitus, malignancies and chronic liver failure (Rowe & McKoy, 2017). Patients with comorbidities have higher chance of infections and this will increase the risk of sepsis.

Furthermore, women who have passed menopause have high chance of developing infections due to the decrease in estrogen levels after menopause, which predisposes them to urinary tract infections, whereas males with prostatic hypertrophy are liable to urinary retention, which subsequently predisposes them to urinary tract infection (Rowe & McKoy, 2017). Other comorbidities, which can predispose an individual to sepsis include hospitalization, invasive procedures (like catheterization), and malnutrition (Rowe & McKoy, 2017).

Diagnosis of Sepsis

Currently sepsis is difficult to diagnose with high sensitivity and specificity because the pathological symptoms are similar to other inflammatory diseases (Singer, 2013). In addition, the mortality rate increases with late diagnosis or due to inappropriate antibiotic therapy; therefore a timely diagnosis is essential for the selection of the most effective therapy and improve the prognosis (Fabri-Faja et al., 2019). The traditional method used in the diagnosis of sepsis involves blood cultures. Other techniques include molecular diagnostic methods, microarray, polymerase chain reaction-based methods, mass spectrometry and fluorescent in-situ hybridization method.

Blood culture examinations are aimed at visualization and detection of the pathogen in the blood which is involved in the cause of the sepsis. This method is the gold standard for the conventional diagnosis of sepsis. Furthermore, there are certain pitfalls associated with this

method, firstly low sensitivity which simply means that not everyone with sepsis will show a positive test result. Secondly, the procedure is quite time consuming, it takes about 24-72 hours to get the results (Kumar, Tripathy, Jyoti & Singh, 2019). The molecular diagnostics methods are focused on the identification of pathogen, which was not identified by the blood culture examination and also this technique is faster for diagnosis as compared to blood cultures.

According to the American College of Chest Physicians and Society of Critical Care Medicine Consensus Conference, both criteria are required to establish the diagnosis, these criteria for diagnosis of sepsis includes the following (Liu, Zhang, Guo, Li, & Su, 2016);

i. The presence of SIRS manifested by two or more of the following criteria: fever >38 degrees or hypothermia < 36-degree, tachycardia >90 beats per minute, tachypnoea >20 breaths per minute, hypocapnia PaCO2 < 32mmHg and leukocytosis or leukopenia which white blood cells count of >12,000 or < 4000/mm3 and
ii. A document source of infection.

Recent diagnosis of sepsis employs the use of serum biomarkers and the presence of microRNA in the blood of critically ill patients. For a few years, nucleic acid amplification technologies have promised to prevent the need for bacterial growth (Sinha et al., 2018). These technologies aim at creating multiple copies of DNA and RNA originating from the pathogen or host cells using biochemical reactions, hence amplifying the nucleotide sequences to a detectable level. New methods are needed for the diagnosis of sepsis because diagnostic certainty of sepsis needs to be increase and time for diagnosis of sepsis needs to be decreased (Sinha et al., 2018).

Biomarkers of Sepsis

Biomarkers are molecules or genes, which can be isolated and identified in a particular pathological or physiological process. Biomarkers can be used in the stratification of patients based on their risk profiles, assessment of prognosis and severity of sepsis and other diseases in general (Engelen, Wiersinga, Scicluna & Poll, 2018). Biomarkers of sepsis include protein biomarkers (like procalcitonin (PCT) and C-reactive protein (CRP), lactate, neutrophil-lymphocyte ratio and RNA based biomarkers. In addition to the aforementioned, another group of compounds that have been widely accessed as potential biomarkers are cytokines (Pierrakos & Vincent, 2010). CRP is an acute phase protein that can interact with the capsular polysaccharide *of Streptococcus pneumonia* (Cui, Zhang, Chen & Yu, 2019). It is produced in the liver in response to inflammatory cytokines produced by macrophages and it is the most often used biomarker for diagnosis of infection and inflammation in clinical practice (Kumar et al., 2019). It is regularly measured to monitor response to treatment in patients with chronic inflammatory diseases such as rheumatoid arthritis (Fan, Miller, Lee, & Remick, 2016). It is a very good biomarker for sepsis, but can also be elevated in other inflammatory conditions in the absence of sepsis, hence it is not a very specific and sensitive biomarker for the diagnosis of sepsis.

Under physiologic conditions, it is produced in the C cells of the parathyroid gland (Larsen & Petersen, 2017). It is the precursor for calcitonin hormone, which is found to be elevated following invasive bacterial infections (Fan et al., 2016). It is produced by many tissues including the local site of infections; therefore, it is an FDA approved biomarker for assessment of the risk of developing severe sepsis in critically ill patients (Fan et al., 2016). The rise in the PCT levels during the onset of infection in addition to its short half-life makes it a good marker of sepsis, but on the contrary the concentration of PCT does not correspond to the severity or mortality of the sepsis (Larsen & Petersen, 2017).

According to Sandquist and Wong (2014), biomarkers in sepsis include triggering receptor expressed on myeloid cells-1, soluble urokinase type plasminogen activator receptor, interleukin 27, neutrophil CD64, presepsin, cell free DNA and miRNAs. Presepsin is a fragment of CD14 which is a lipopolysaccharide binding protein complex receptor produced following a bacterial infection (Shozushima et al., 2011). It has lately attained a convincing consideration as a promising prognostic biomarker in sepsis and has been found to have superior diagnostic capacity compared to PCT. Compared to other serum biomarkers presepsin seem to have a better sensitivity and specificity (Zou, 2014), hence showing a great potential as a reliable biomarker in the diagnosis of sepsis. Lactate is another biomarker, which is used in the diagnosis of septic patients, but it is not a very specific type of biomarker (Ljungström et al., 2017). A single biomarker alone is not efficient for the diagnosis but a combination of biomarkers can improve the diagnosis of specific bacterial sepsis in critically ill patients (Ljungström et al., 2017).

MiRNAs are short sequences of endogenous RNAs that play a role in post transcriptional gene silencing and translational regulation of gene expression (Sandquist & Wong, 2014). They are capable of controlling gene expression by binding specifically to the 3' untranslated regions of complementary mRNAs and consequently inhibiting their translation (Reithmair et al., 2017). MiRNA have been detected in the blood and other body fluids and might serve as biomarkers in the diagnosis, prognosis and follow up of sepsis (Fan et al., 2016). Circulating miRNA found in the serum during several conditions show different expression patterns, resulting in their potential use in the diagnosis of an array of diseases (Kingsley & Bhat, 2017). Furthermore, circulating serum miRNA have shown a diagnostic potential for pulmonary tuberculosis, lung cancer, and pneumonia (Correia et al., 2017). Numerous studies have established that normal and pathological tissues can be distinguished by altered miRNA profiles (Pogribny, 2017). Kingsley and Bhat (2017) theorized that miRNAs could better the diagnosis of sepsis and help in the identification of various stages of sepsis.

Future diagnosis of Sepsis

Many of the traditional biomarkers of infection are notable for their inaccuracy (Singer, 2013), hence why there has to be a continuous development of more specific and sensitive markers for the diagnosis of sepsis. Due to limitations in the diagnostic value of the established sepsis protein biomarkers, enormous attempts have been proposed to identify novel markers in the context of sepsis with high sensitivity and specificity (Benz, Roy, Trautwein, Roderburg & Luedde, 2016). Up until today, no single biomarker with sufficient sensitivity and specificity could be identified. Therefore, circulating miRNAs might offer new perspectives due to the results from numerous field research on sepsis (Benz et al., 2016). In addition, circulating miRNAs might be superior to other serum biomarkers because they are relatively small in size, more stable, they do not undergo post-processing modifications and have a relatively less complex chemical structure (Benz et al, 2016).

Newer biomarkers have their firm advocates, even though systematic reviews are less satisfactory (Singer, 2013). Procalcitonin is one of the examples of serum biomarkers that is been heavily promoted but has low diagnostic performance for the diagnosis of sepsis because it has average sensitivity and specificity of about 71% which is way below the ideal value of specific and sensitive biomarker (Singer, 2013).

Due to the lack of accuracy and sensitivity of single biomarkers, it has been proposed to use so-called multi-marker panels (Samraj, Zingarelli & Wong, 2013). When a multimarker panel is used several biomarkers are measured for diagnosis or prognosis of sepsis. There are over 100 biomarkers that could be used for multi-marker panels so further studies need to be done to

determine which biomarkers are optimal for inclusion in a sepsis multimarker panel (Casserly, Read & Levy, 2011).

Even though miRNA are promising biomarkers, there are some problems with the miRNA. First of all, miRNA is found in low concentrations in biofluids (Binderup et al., 2018). Furthermore, extraction methods can vary greatly due to different reasons, e.g. non-standardized techniques (Witwer et al., 2013) and difference in amount of starting materials (Brunet-Vega et al., 2015).

This project

To extract miRNA from different biofluids and for it to be used as a biomarker, testing and validation of different extraction kits is necessary. Additionally, this study will look at different starting plasma volumes because sometimes biofluids in the biobank can be limited, so it is essential to know if lower volumes of plasma can be used for biomarker miRNA extraction. This project is part of the research area 'Future diagnostics of sepsis' at the University of Skövde which aims to develop of multi-marker panel for fast and accurate diagnosis of sepsis.

In this study, the aim was to identify which kit of two kits and which of two volumes of plasma would lead to the highest concentration of miRNA and highest quality of miRNA extracted. This was quantified by using two different volumes, 100 µl and 200 µl, and extracting the two volumes with both exoRNeasy Serum/Plasma midi kit (Qiagen) and Total RNA Purification kit (Norgen).

Materials and methods

Ethical considerations

The blood samples were taken from self-assessed healthy donors for which no written consent was needed. The samples were taken on voluntary basis from University of Skövde students. Thus, no further ethical considerations needed to be considered.

Sample collection and storage

The laboratory work started with sample collection and storage of the blood samples. The blood donation from self-assessed healthy volunteers was drawn and collected in EDTA tubes (lavender tops, 6 ml), and after that preparation of plasma. The EDTA tubes were put into ScanSpeed 1580R centrifuge at 1790 rpm for 15 minutes to prepare the plasma according to a plasma preparation protocol (Life Technologies). The prepared plasma was aliquoted into Eppendorf tubes and then the Eppendorf tubes were stored in the freezer at -80 °C.

Thawing process and other preparations

The plasma for miRNA extractions with exoRNeasy Serum/Plasma midi kit (Qiagen) was thawed for 10 minutes on ice and 5 minutes at the room temperature. Afterwards, the plasma was centrifuged in the cold room at 4 °C for 12 minutes at 13000 RPM to remove larger particles than 0.8 µm (Qiagen).

The plasma for miRNA extractions with Total RNA Purification kit (Norgen) was thawed on ice for 10 minutes and then moved to room temperature for 15 minutes until the plasma was completely thawed.

miRNA extraction with exoRNeasy Serum/Plasma midi kit

Ten extractions were performed with 100 µl of plasma and ten extractions were performed with 200 µl of plasma. After thawing and large particles removal, the supernatant was transferred to a new tube and the miRNA extracted was extracted according to the protocol of exoRNeasy

Serum/Plasma midi kit (Qiagen). However, some changes were made to the protocol. These changes are described further in the text. The centrifugation at steps 3, 4 and 6 were all increased with 2 minutes because the centrifuge took some time to fully come to speed. After some steps, the samples were separated into 3 phases, colorless upper phase, thin white interphase, and lower red organic phase. The upper, colorless phase contained the RNA and was used for the subsequent steps. Finally, miRNA concentrations were measured with Qubit (Thermo Fisher Scientific) and purities and total RNA concentrations were measured with Nanodrop (Thermo Fisher Scientific). Only Qubit (Thermo Fisher Scientific) can measure the miRNA concentration. Nanodrop cannot measure the miRNA concentration. However, Nanodrop (Thermo Fisher Scientific) can measure the total RNA concentrations and the purities.

MiRNA extractions with Total RNA Purification kit

Total RNA Purification kit (Norgen) was used to perform ten extractions with 100 µl of plasma and ten extractions with 200 µl of plasma. All optional steps were also performed, except for adding 0.7 µl of 0.8 µg/µl MS2 RNA to the sample. Two elutions were performed. Finally, miRNA concentrations of both elutions were measured with Qubit (Thermo Fisher Scientific) and purities and total RNA concentrations were measured with Nanodrop (Thermo Fisher Scientific).

Workflow

In order to benchmark two different volumes and two different kits, the quality and concentration parameters were compared. Figure 1 shows the overview of the experimental work. Ten extractions were performed with exoRNeasy Serum/Plasma midi kit (Qiagen) with a volume of 100 µl and ten miRNA extractions were performed with exoRNeasy Serum/Plasma midi kit (Qiagen) with a volume of 200 µl. Ten miRNA extractions were performed with Total RNA Purification kit (Norgen) with a volume of 100 µl and ten miRNA extractions were performed with Total RNA Purification kit (Norgen) with a volume of 200 µl.

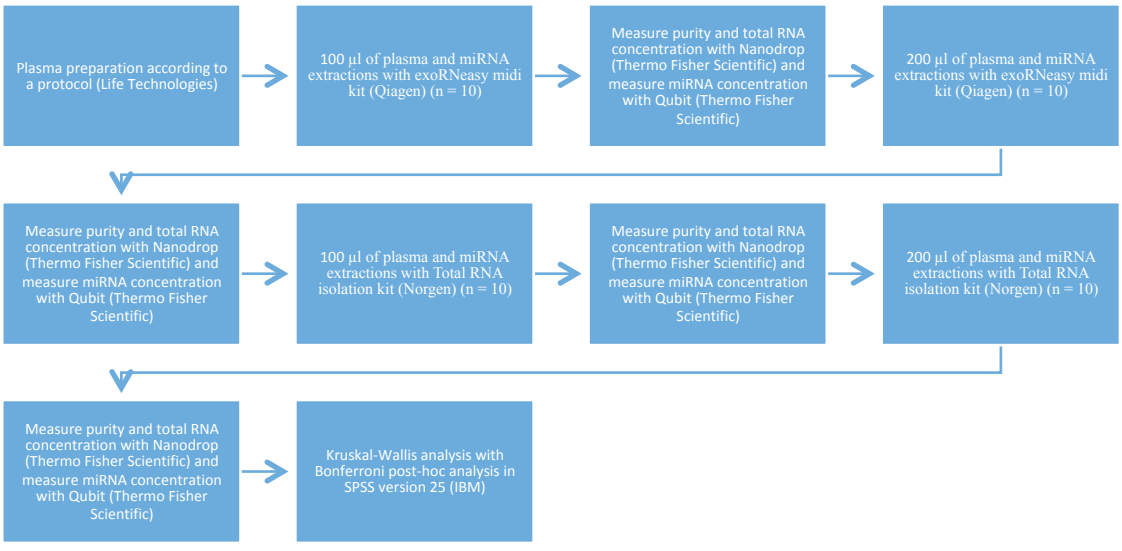

Figure 1. Overview of the experimental work.

Statistical analysis

A Shapiro-Wilk test was performed to see if the data in the four different groups were normally distributed or not. The test for normality was done to decide to either use a parametric test

(normally distributed data) or a non-parametric test (not normally distributed data). A Mann-Whitney test and an independent samples t-test were performed to test if there is a significant difference in miRNA concentrations between the two volumes, within one kit. Subsequently, a Kruskal-Wallis test was performed to test if there was a significant difference in the median miRNA concentrations between the four groups. The problem with multiple comparisons, like with the Kruskal-Wallis test, is that a significance that is found can be due to chance and not because there is a real difference (a false positive). A post-hoc analysis with Bonferroni corrections was also performed, which corrects for the false positives of multiple comparisons (McDonald, 2014). Significance level was 0.05 in all tests that were performed.

Results

The miRNA concentrations, purities and total RNA concentrations for the extractions with exoRNeasy Serum/Plasma kit (Qiagen) can be found in Table 1 (Qiagen 100 µl group) and Table 2 (Qiagen 200 µl group). The miRNA concentrations, purities and total RNA concentrations for the extractions with Total RNA Purification kit (Norgen) can be found in Table 3 (Norgen 100 µl group) and Table 4 (Norgen 200 µl group). Table 5 shows the number of samples, mean, median, standard deviation and standard error of the Qiagen 100 µl group, Qiagen 200 µl group, Norgen 100 µl group and Norgen 200 µl group. Only the descriptive statistics of the first elution of the Norgen 100 µl group and Norgen 200 µl group are shown.

Table 1. Qiagen 100 µl group - Sample numbers, volume of plasma, concentrations measured with Qubit and purities (260/280 nm and 260/230 nm) measured with Nanodrop.

Sample number	miRNA concentration (Qubit) (ng/µl)	Purity (260/280 nm) (Nanodrop)	Purity (260/230 nm) (Nanodrop)	Total RNA concentration (Nanodrop) (ng/µl)
1	0.56	1.48	0.45	28.80
2	1.20	1.54	0.36	41.10
3	0.50	1.53	0.13	21.50
4	1.02	1.41	0.50	22.80
5	0.58	1.08	0.23	88.00
6	0.56	0.79	0.07	9.900
7	0.57	0.86	0.27	16.20
8	n.m.*	1.34	2.36	89.50
9	0.64	1.57	0.64	38.50
10	0.58	1.35	0.14	40.50

* n.m. = concentration was not measurable

Table 2. Qiagen 200 µl group - Sample numbers, volume of plasma, concentrations measured with Qubit and purities (260/280 nm and 260/230 nm) measured with Nanodrop.

Sample number	miRNA concentration (Qubit) (ng/µl)	Purity (260/280 nm) (Nanodrop)	Purity (260/230 nm) (Nanodrop)	Total RNA concentration (Nanodrop) (ng/µl)
1	0.56	1.86	0.15	11.40
2	n.m.*	0.98	0.89	14.90
3	0.76	2.09	0.02	3.00
4	0.82	1.43	0.24	12.20
5	0.72	1.44	0.11	10.50
6	0.68	1.30	0.04	3.90
7	0.76	1.59	0.47	8.30
8	0.74	2.10	0.18	2.90

9	1.18	1.17	0.18	11.20
10	0.84	2.47	0.00	1.70

* n.m. = concentration was not measurable

Sample number 8 from Qiagen 100 µl group and sample number 2 from Qiagen 200 µl group were omitted from further analysis because there was no reading with Qubit.

Table 3. Norgen 100 µl group - Sample numbers, volume of plasma, concentrations measured with Qubit and purity (260/280 nm) measured with Nanodrop. Two elutions were performed.

Sample number	**miRNA concentration (Qubit) (ng/µl), 1st elution**	**miRNA concentration (Qubit) (ng/µl), 2nd elution**	**Purity (260/280 nm), 1st elution (Nanodrop)**	**Purity (260/280 nm), 2nd elution (Nanodrop)**	**Total RNA concentration (Nanodrop) (ng/µl), 1st elution**	**Total RNA concentration (Nanodrop), 2nd elution**
1	n.m.*	0.52	1.78	1.66	26.70	27.20
2	n.m.*	n.m.*	0.88	1.31	4.90	8.80
3	n.m.*	n.m.*	0.97	1.07	2.20	2.80
4	n.m.*	0.82	1.30	0.96	3.30	4.40
5	n.m.*	0.56	0.96	0.89	5.20	5.30
6	n.m.*	0.64	5.69	1.51	1.20	10.80
7	0.99	0.54	0.56	1.19	4.70	20.70
8	0.94	0.62	0.55	0.42	5.20	1.80
9	0.78	0.62	0.65	1.03	16.00	20.80
10	0.62	0.68	0.65	1.11	10.40	8.40

*n.m. = concentration was not measurable

Table 4. Norgen 200 µl group - Sample numbers, volume of plasma, concentrations measured with Qubit and purity (260/280 nm) measured with Nanodrop. Two elutions were performed.

Sample number	**miRNA concentration (Qubit) (ng/µl), 1st elution**	**miRNA concentration (Qubit) (ng/µl), 2nd elution**	**Purity (260/280 nm), 1st elution**	**Purity (260/280 nm), 2nd elution**	**Total RNA concentration (Nanodrop) (ng/µl), 1st elution**	**Total RNA concentration (Nanodrop) (ng/ul), 2nd elution**
1	n.m.*	n.m.	0.82	0.95	12.30	4.60
2	0.70	n.m.	0.86	0.93	13.90	7.00
3	n.m.*	n.m.	0.77	0.94	11.60	18.40
4	n.m.*	n.m.	0.24	0.20	15.80	8.10
5	n.m.*	n.m.	0.92	1.07	6.40	11.00
6	n.m.*	1.03	0.97	0.96	10.40	6.50
7	0.56	0.82	0.79	0.86	9.00	6.20
8	0.52	n.m.	0.95	0.97	5.10	10.30
9	0.50	n.m.	0.88	1.00	9.10	11.30
10	n.m.*	n.m.	0.74	1.05	9.80	13.60

*n.m. = concentration was not measurable

Table 5. Descriptive statistics of the four groups

Group	**N**	**Mean**	**Median**	**SD**	**SE**
Qiagen 100 µl	9	0.690	0.580	0.245	0.082

Qiagen 200 µl	9	0.784	0.760	0.169	0.056
Norgen 100 µl, 1st elution	4	0.833	0.860	0.168	0.084
Norgen 200 µl, 1st elution	4	0.570	0.540	0.090	0.045

Many samples from the extractions with Total RNA isolation kit (Norgen) had no miRNA readings from Qubit, especially when the miRNA was measured for the second elution for the Norgen 200 µl group. However, for the Norgen 100 µl group more of the second elutions gave measurable miRNA concentrations. The samples with no miRNA readings were omitted from further analysis. Only the first elution miRNA concentration measurements of the Norgen 200 µl group and the Norgen 100 µl were used for further analysis.

A Shapiro-Wilk test of normality (significance level = 0.05) was performed with SPSS version 25 to test if the different groups are normally distributed or not. The Shapiro-Wilk test showed that the data of the Qiagen 100 µl group ($W(9) = 0.696$, $p = 0.001$, $n = 9$) was not normally distributed. However, the Shapiro-Wilk test also showed that the data of the Qiagen 200 µl group ($W(9) = 0.838$, $p = 0.055$, $n = 9$), the Norgen 100 µl group ($W(4) = 0.936$, $p = 0.632$, $n = 4$) and the Norgen 200 µl group ($W(4) = 0.851$, $p = 0.230$, $n = 4$) were normally distributed. To analyze if there is a significant effect of the volume within the kit, Mann-Whitney U test was performed for Qiagen 100 µl group and Qiagen 200 µl group because the Qiagen 100 µl group was not normally distributed. However, an independent samples t-test was used to analyze if there is a significant effect of the volume within the kit for the Norgen 100 µl group and Norgen 200 µl group. The miRNA concentrations for the Qiagen 100 µl group (median = 0.580 ng/µl, n = 9) and the Qiagen 200 µl group (median = 0.760 ng/µl, n = 9) were not statistically different, $U = 22$, $z = -1.64$, $p = 0.113$. An independent samples t-test was performed to determine if there were differences in mean miRNA concentrations between the Norgen 100 µl group and the Norgen 200 µl group. The mean miRNA concentration of the Norgen 100 µl group (0.833 ng/µl ± 0.167 ng/µl, n = 4) was statistically significant higher than the Norgen 200 µl group (0.570 ng/µl ± 0.090 ng/µl, n = 4), $t_6 = 2.76$, $p = 0.033$.

To identify which volume and which kit had the highest median miRNA concentrations, a Kruskal-Wallis test was performed, significance level = 0.05. There was a significant difference ($p = 0.044$) found between the median miRNA concentrations of Qiagen 100 µl group (median = 0.580 ng/µl), Qiagen 200 µl group (median = 0.760 ng/µl), Norgen 100 µl group (median = 0.860 ng/µl) and Norgen 200 µl group (median = 0.540 ng/µl), $\chi^2_2 = 8.12$, $p = 0.044$. Post-hoc analysis with Bonferroni correction was performed, which corrects for multiple comparisons. However, the post-hoc analysis with Bonferroni correction did not find any statistical difference between the four groups. Due to the discrepancy between the significant result from Kruskal-Wallis test and the non-significant results from the post-hoc analysis with Bonferroni corrections, a subsequent Mann-Whitney U test was performed between Qiagen 200 µl group and Norgen 200 µl group. Interestingly, there was a statistical difference found in median miRNA concentrations between the two groups, $U = 2.5$, $z = -2.40$, $p = 0.011$. Figure 2 shows the median miRNA concentrations of the different groups with error bars denoting 95% confidence interval. The median miRNA concentration of the Qiagen 200 µl group is significantly higher than the median miRNA concentration of the Norgen 200 µl group. Figure 3 shows the boxplots of the miRNA concentrations of the different groups. Table 6 shows the number of samples, medians, Interquartile Range (IQR), minimum values (min) and maximum values (max) of each group. Figure 6 also shows the two outliers in the Qiagen 100 µl group (marked with two stars) and the two outliers in the 200 µl group (one marked with a star and one marked with a circle).

Table 6. Number of samples, medians, IQR and range of the miRNA concentrations.

Group	N	Median	Min	Max	IQR
Qiagen 100 µl group	9	0.580	0.500	1.200	0.270
Qiagen 200 µl group	9	0.760	0.560	1.180	0.130
Norgen 100 µl group	4	0.860	0.620	0.990	0.318
Norgen 200 µl group	4	0.540	0.500	0.700	0.160

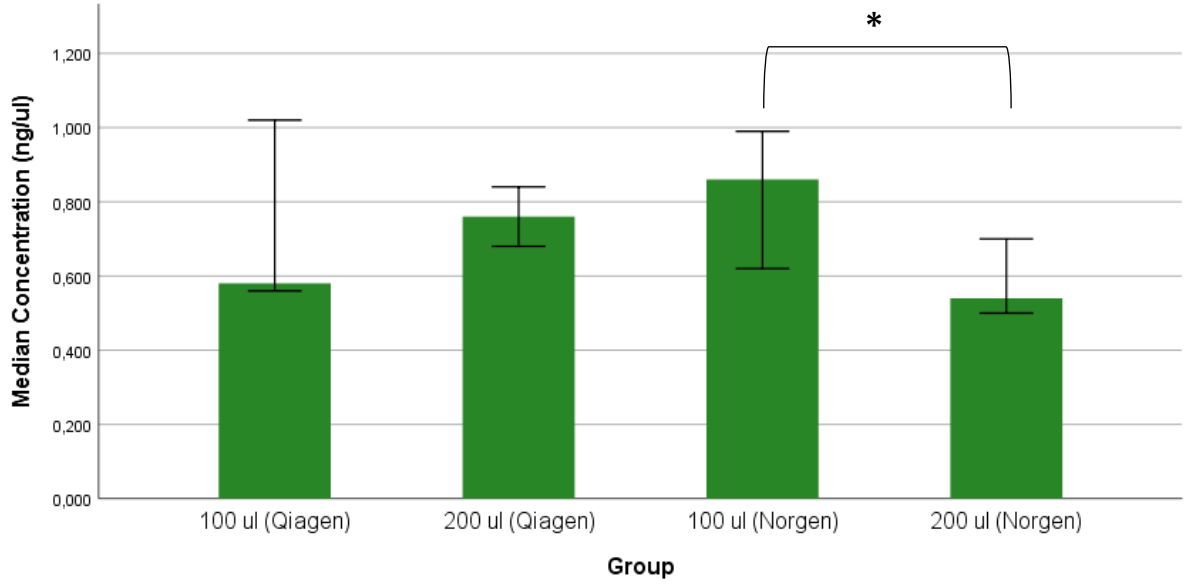

Figure 2. Median miRNA concentrations (ng/µl) of Qiagen 100 µl group (median = 0.580 ng/µl, n = 9), Qiagen 200 µl group (median = 0.760 ng/µl, n = 9), Norgen 100 µl group (median = 0.860 ng/µl, n = 4) and Norgen 200 µl group (median = 0.540 ng/µl, n = 4). Error bars denote 95% confidence interval. Asterisk shows $p < 0.05$.

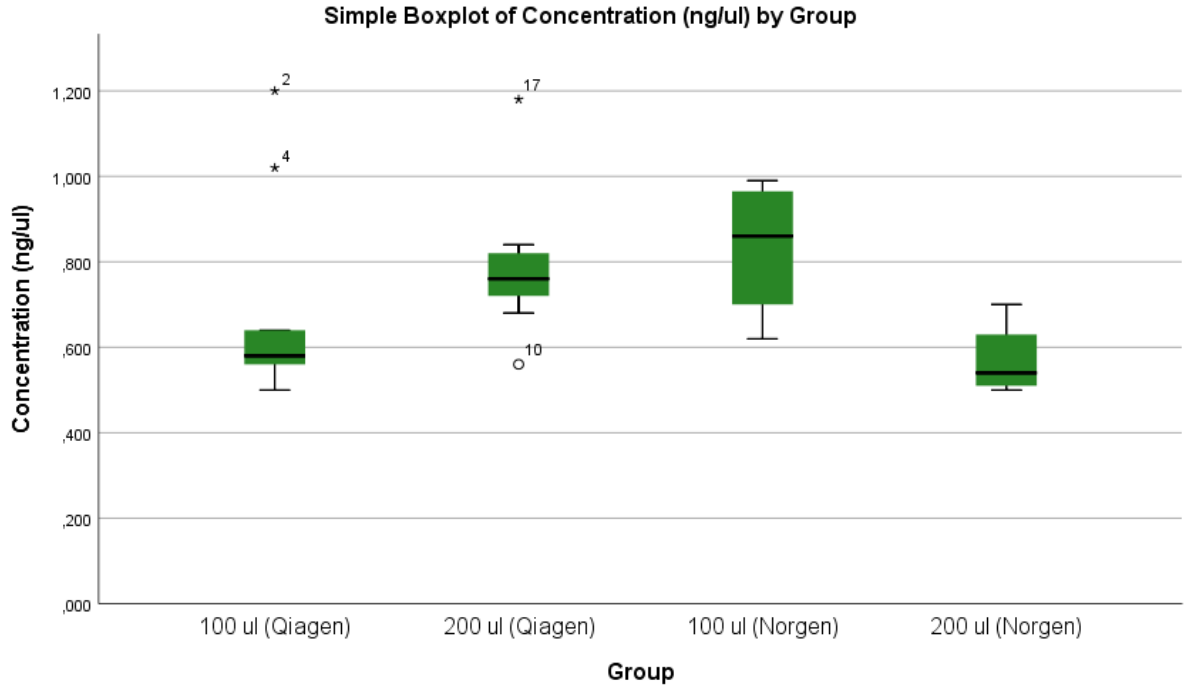

Figure 3. Boxplots of miRNA concentrations of Qiagen 100 µl group (median = 0.580, min = 0.500, max = 1.200, IQR = 0.270, n = 9), Qiagen 200 µl group (median = 0.760, min = 0.560, max = 1.180, IQR = 0.130, n

= 9), Norgen 100 µl group (median = 0.860, min = 0.520, max = 0.990, IQR = 0.318, n = 4) and Norgen 200 µl group (median = 0.540, min = 0.500, max = 0.700, IQR = 0.160, n = 4).

Discussion

In this study, the aim was to identify which kit of two kits and which of two volumes of plasma would lead to the highest concentration of miRNA and highest quality of miRNA extracted. This was quantified by using two different volumes, 100 µl and 200 µl, and extracting the two volumes with both exoRNeasy Serum/Plasma midi kit (Qiagen) and Total RNA Purification kit (Norgen). There is a difference in the type of RNA that is extracted by the two kits. ExoRNeasy Serum/Plasma midi kit (Qiagen) extracts total vesicular RNA (non-coding RNA, mRNA, miRNA and other small RNA) and Total RNA Purification kit (Norgen) extracts total RNA from the serum samples.

To analyse if the amount of the starting material affects the concentration of isolated miRNA, some samples isolated by the same kit and different starting volume, were compared. Additionally, several comparisons were made between samples that have been isolated with different kits but with the same starting volume to analyse if the two kits give the same miRNA concentration. There were no significant differences in median miRNA concentrations found between the Qiagen 100 µl group and Qiagen 200 µl group. However, the mean miRNA concentrations from the Norgen 100 µl group were statistically significantly higher than the mean miRNA concentrations from the Norgen 200 µl group. Possible reasons could be that the miRNA concentration can vary greatly (Norgen, 2018) and due to limited number of samples, the significant effect could still not reflect a true effect (Button et al., 2013). Mean miRNA concentrations from extractions with exoRNeasy Serum/Plasma midi kit (Qiagen) using 200 µl were statistically higher than extractions with Total RNA Purification kit (Norgen) using 200 µl. However, there was no significant difference in mean miRNA concentration between the Norgen 100 µl group and Qiagen 200 µl group.

Comparisons between kits and volumes

Aldosaky (2020) also used exoRNeasy Serum/Plasma midi kit (Qiagen) for miRNA extraction from human plasma. Aldosaky (2020) found significant higher miRNA concentration in miRNA extractions with 275 µl compared to miRNA extractions with 100 µl, both when the miRNA was isolated manually ($p = 0.003$) and automatically ($p = 0.021$). However, such significance was not found in this study. Possibly due to that the difference in plasma volume between 100 µl and 200 µl is not enough. Another possibility is that the number of samples was not enough to find a significant difference because Aldosaky (2020) had 10 samples in each group and this study used 9 samples each for both the Qiagen 100 µl group and Qiagen 200 µl group. Furthermore, Öberg (2020) found no significant difference in miRNA concentrations from miRNA extractions of 200 µl of plasma with the total RNA Purification kit (Norgen) compared to 100 µl of plasma with the same kit. This is not line with what was found in this study. In this case, Öberg used 10 samples in each group for comparison, while this study only had 4 samples in each group. This lowers the statistical power and thus reduces the chance of finding a true effect (Button et al., 2013).

In this study there were some miRNA concentrations that could not be detected. One sample of the Qiagen 100 µl group, one sample of the Qiagen 200 µl group, six samples of the Norgen 100 µl group and six samples of the Norgen 200 µl group had undetectable readings with Qubit (Thermo Fisher Scientific). This has also been the case in other research studies (Nordén, 2020). This could have several reasons. Firstly, the total RNA yield from plasma can vary greatly, i.e. between 0.01 to 1 ng/µl (Norgen, 2018). The amount of miRNA could have also been

exceptionally low so that Qubit cannot detect the low concentrations of miRNA. Lastly, it could also be that hemolysis significantly lowered the detectable miRNA levels (Kirschner et al., 2013).

There are many studies and thus many kits compared for total RNA and/or miRNA extractions from different biofluids, including plasma. Many of the kits tested in the studies were different kits from Qiagen and Norgen (e.g. Brown et al., 2018; Ge et al., 2015). For example, extractions with miRCURY (Qiagen) gave the purest RNA and extractions done by miRNeasy (Qiagen) could be detected the easiest (El-Khoury, Pierson, Kaoma, Bernardin & Berchem, 2016). Another study found that exoRNeasy Serum/Plasma midi kit (Qiagen) gave the highest miRNA concentration in plasma and highest purity compared to other kits (Xu et al., 2018). Another study also found that the Qiagen kit performed better than the Norgen kit in terms of miRNA concentration (Meerson & Ploug, 2016). However, in this study, exoRNeasy Serum/Plasma midi kit (Qiagen) did not give the highest miRNA concentrations. Monleau and colleagues (2014) found that plasma miRNA extraction kits from Qiagen, Norgen and Machinery-Nagel performed similar to each other, while in this study the Norgen kit (with a starting volume of 100 µL) gave higher miRNA concentrations than the Qiagen kit. Thus, from the comparisons with previous literature, the Qiagen and Norgen kits either perform similar or sometimes the Qiagen kit seems to provide higher miRNA concentrations. Something to note is that the studies did not look at different plasma starting volumes.

Quality

The purities (260/280 nm) from most samples that were extracted, were considerably lower than what is considered pure, i.e. approximately 2.0 (Thermo Scientific, 2012). This has also been found in other studies (Bergallo et al., 2015; Monleau et al., 2014; Ramón-Núñez et al., 2017). Low purity (260/280 nm) values can be due to reagents used in the protocols, chemicals like phenol and small changes in acidity (Wilfinger, Mackey & Chomczynski, 1997). However, the protocol (Norgen, 2018) of Total RNA Purification kit (Norgen) indicates that 'unpure' samples can still be used effectively in downstream applications, e.g. RT-qPCR. Unpure samples were also found to be useful and provide good results in downstream applications, e.g. RT-qPCR in studies done by El-Khoury, Pierson, Kaoma, Bernardin and Berchem (2016).

Weaknesses of methods used

One of the weaknesses of this study was that the Qubit was used to measure miRNA concentrations. In fact, Qubit measures all small RNA, which includes small interfering RNA (siRNA), Piwi-interacting RNA (piRNA) and miRNA (Zhang, 2009). Small RNA consists only approximately 50% of miRNA (Garcia-Elias et al., 2017), thus the results of this study are higher than the true miRNA concentration values. Other ways to compare kits would be to evaluate the kits with high-throughput RT-qPCR, which increases accuracy, reproducibility and sensitivity (Tan, Khoo & Tan, 2015). Furthermore, the amount of samples that were used were limited, so suggestions for further studies would be to use more samples to validate the results that were obtained.

Ethical aspects and impact on society

Research in how to diagnose sepsis faster, has a positive impact on society. The development of a multi-marker panel based on these results and results from other research, can significantly reduce diagnosis time and thus increase healthy outcomes for sepsis patients. Moreover, the use of less starting material can also be positive for the biobank because of the limited amount of biofluids available. Faster and more reliable diagnosis of sepsis will also save money because less time and expenditures are needed, e.g. nursing costs (Chalupka & Talmor, 2012). Thus,

there is also a positive economic impact on society. More importantly, early detection of sepsis and appropriate treatment can increase the survival rate of sepsis patients (Kim & Park, 2019).

Conclusion and future perspectives

In conclusion, this study provides some evidence for the a slight preference of the use of Total RNA Purification kit (Norgen) over the exoRNeasy Serum/Plasma midi kit (Qiagen) because of the significant higher miRNA concentration obtained from 100 µl of starting plasma than from 200 µl of starting plasma. Thus, interestingly, the optimal volume that should be used is 100 µl of plasma. Additionally, the quality measured was lower than what is considered pure but could still be useful for downstream applications, like RT-qPCR. Therefore, this kit and this volume can be one step in the development of a gold standard procedure for miRNA isolation. Currently the future sepsis diagnosis is also optimizing the methods for detection of the isolated miRNA. All these steps are important for the aim to develop a multi-panel biomarker tool for sepsis diagnosis. However, these results need to be verified first. Further studies could include more samples, different volumes and even a machine that can accurately measure the miRNA concentration. Moreover, future research could make use of RT-qPCR results as a validation for miRNA concentration results. The development of a multi-marker tool, with the help of research like this study, can lead to an application that is both accurate and fast. This will benefit the diagnosis of sepsis and subsequent treatment of sepsis. For validation of these results further research is needed. A suggestion would be to increase the number of samples for comparison. Furthermore, other kits can also be considered for inclusion in the multi-marker panel tool.

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