Part IV
Discussion, Summary and Acknowledgments
General discussion
A substantial number of patients, all over the world, suffer from one or multiple wounds. These wounds can have a significant impact on the quality of life of patients, especially when there is a delay or failure of the wound healing process. As a consequence, a major part of healthcare resources is devoted to the care for wounds. The impact of wounds on both patients and the healthcare system is even increased in case of wound infection. Wound infection is a major complication for patients with one or more wounds, as it causes a further delay of the wound healing process. The ability to detect wound infection in an accurate and timely manner is essential to prevent further complications, such as severe damage to surrounding tissues and spreading of the infection to the bloodstream. In addition, the ability to accurately rule out infection in wounds can prevent unnecessary use of antibiotic treatment. Therefore, the aim of this Thesis was to assess traditional and novel diagnostic techniques that potentially fulfill this need for accurate and timely detection of wound infection. In this chapter we will discuss and reflect upon our findings. We will conclude with practical implications and recommendations for future research.

TRADITIONAL TECHNIQUES TO ASSESS WOUND INFECTION

Wound infection is often defined as a state in which pathogens, that have invaded the wound bed, outcompete the immune response. However, in clinical practice, it seems difficult to translate this definition into an accurate (diagnostic) measure. Usually, the diagnosis wound infection is based on two traditional methods; on the one hand clinicians assess clinical signs and symptoms of wound infection to determine whether there is an (excessive) immune response and on the other hand, microbiological culture is used to determine whether certain pathogens are present in the wound. In addition, culture results can indicate to what extent the cultured microorganisms are susceptible to available antibiotics.

The two most frequently used sampling techniques for microbiological culture are wound biopsy and wound swab. Traditionally, wound biopsy samples are believed to yield the most accurate results as it includes actual collection of wound tissue. In clinical practice, this invasive sampling method is rarely used and clinicians rather turn to wound swabs. The extent to which these wound swabs yield similar results as to wound biopsy has, however, not been properly investigated in clinical practice. Therefore, we have conducted a study in which we compared microbiological culture results from wound samples taken through wound biopsy and wound swab (chapter 3).
We found that culture results from wound samples taken from the same location in the wound through wound biopsy or wound swab do not significantly differ. The differences we did observe were mainly due to the fact that we cultured normal human skin flora more frequently from wound swabs. ‘Contamination’ with skin flora is inherent to the superficial character of wound swabs. This superficial character is commonly advocated as major disadvantage of wound swabs, as it is expected that only superficial microorganisms are captured rather than microorganisms that have invaded wound tissue. We, however, demonstrated that the capture of superficial skin flora by wound swabs did not significantly influence the recovery rate of other possible pathogenic microorganisms. Moreover, our exploratory analyses demonstrated that remaining differences between swab and biopsy did not have significant implications for the choice of antibiotic treatment; antibiotic susceptibility reports were the same in 96% of all wounds. Therefore, we recommend clinicians to initially choose wound swabs as sampling method for microbiological culture as they pose a lower burden on the patient and on clinical logistics than wound biopsies.

Microbiological culture results are often believed to be an accurate and objective measure for wound infection. However, we know that the growth of a specific microorganism on culture plates is not necessarily related to infection as it does not include the immune response; e.g. a specific microorganism might be present in abundance in a wound, but it might not actually outcompete the immune system. The pathogenic effect of a microorganism depends also strongly on the presence of other microorganisms, as they can exacerbate or mitigate pathogenic effects. Moreover, microbiological culture results do not always accurately represent all microorganisms present in a wound. Standard microbiological culture methods often focus on the detection of a limited range of species, with the risk of not identifying all possible pathogens. Furthermore, the ability of a microorganism to grow on standard culture plates also depends on the technique used to collect a sample from the wound. For example, anaerobic microorganisms are, on average, difficult to culture as strict anaerobic conditions to protect the organisms from oxygen are often not applied in clinical practice. Such conditions include the use of anaerobic transport media, storage of samples at room temperature instead of in a refrigerator, and transportation to the laboratory within 2 hours. In clinical practice, samples are usually stored in standard sample containers that are not oxygen-free and do not contain anaerobic transport media. Furthermore, it is not always logistically possible to transport wound samples within 2 hours. Our results in chapter 3 also demonstrate a low recovery rate (1-2%) of
anaerobic microorganisms. Therefore, we believe that microbiological culture results always have to be interpreted in combination with other relevant (clinical) information.

In chapter 4 we investigated whether there would be a difference in the assessment of wound infection between wound biopsy and wound swab if we would provide wound experts with such a combination of information. When we combined the assessments of 6 experts, we found that in 88% of all wounds the same assessment was provided for both methods. Agreement between the two methods for individual experts was also fairly high. Remarkably, we did see many differences in wound infection assessment between individual experts. The clinicians in our expert panel assessed substantially more wounds as infected than the microbiologists did. A possible explanation for this difference might be that clinicians often have less knowledge about the clinical meaning of specific microorganisms. During the evaluation of the study, clinicians in our expert panel mentioned that they often were not familiar with specific microorganisms. Therefore, they might have had a tendency to assess wounds as infected merely due to the presence of microorganisms instead of assessing microorganisms in the light of their possible clinical impact. In chapter 4, we explored whether the availability of culture results would influence the assessment of wound infection for the whole expert panel. When we differentiated between clinicians and microbiologists within this exploratory analysis, we noticed that clinicians assessed a substantial higher number of wounds as infected when microbiological culture results are available; 29 wounds were assessed as infected when solely clinical information is available versus 80-82 wounds when microbiological culture results are available (table 9.1). Therefore, there seems to be an indication that the availability of microbiological culture results does have a significant impact on the assessment of wound infection by clinicians. However, these results are limited by the fact that both assessments (with and without culture results) were not performed by the same experts.
Table 9.1. Assessment of wound infection solely based on clinical information versus the combination of clinical information with microbiological culture results; differences between clinicians and microbiologists.

<table>
<thead>
<tr>
<th>Assessment solely based on clinical information*</th>
<th>Infection</th>
<th>No infection</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clinician’s assessment based on clinical information and biopsy culture results</td>
<td>Infection</td>
<td>26</td>
<td>56</td>
</tr>
<tr>
<td></td>
<td>No infection</td>
<td>3</td>
<td>95</td>
</tr>
<tr>
<td>Microbiologist’s assessment based on clinical information and biopsy culture results</td>
<td>Infection</td>
<td>16</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>No infection</td>
<td>13</td>
<td>137</td>
</tr>
<tr>
<td>Clinician’s assessment based on clinical information and swab culture results</td>
<td>Infection</td>
<td>22</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>No infection</td>
<td>7</td>
<td>93</td>
</tr>
<tr>
<td>Microbiologist’s assessment based on clinical information and swab culture results</td>
<td>Infection</td>
<td>17</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>No infection</td>
<td>12</td>
<td>140</td>
</tr>
<tr>
<td>Total</td>
<td>29</td>
<td>151</td>
<td></td>
</tr>
</tbody>
</table>

* Assessments solely based on clinical information were made at the time of inclusion by one wound care nurse, nurse practitioner or physician.

We did not only observe differences in the assessment of wound infection between microbiologists and clinicians but also between different individual experts within these groups. However, our study was primarily designed to assess differences between wound biopsy and wound swab. For a better understanding about the differences in the assessment of wound infection by different experts, we would recommend a study which eliminates some of the limitations we encountered. For instance, we were not able to provide experts the opportunity to use their own senses to assess and interpret clinical signs and symptoms of wound infection as it would complicate the comparison between wound biopsy and wound swab. In clinical practice, an expert usually has the ability to assess clinical signs and symptoms of infection visually, or for example, based on smell (wound odor) or touch (warmth, edema). In addition, we would recommend to include a wider variety of experts (e.g. wound care nurses, physician assistants) to explore the influence of level of expertise on wound infection assessment.

The findings in our study do underpin that traditional methods for wound infection detection, i.e. clinical signs and symptoms and/or microbiological culture results, require subjective interpretation and their accuracy therefore might vary strongly between clinicians and other wound experts. A failure to accurately detect wound infection in time
might cause a further delay of the healing process or more severe complications (e.g. sepsis), while inappropriate initiation of antibiotic therapy in non-infected wounds favors the development of antibiotic resistance. Moreover, disagreement in the assessment of wound infection, in for example multidisciplinary wound care teams, might lead to a delay in the initiation of the appropriate treatment for a wound. Including microbiological culture results in the assessment of wound infection delays the decision process as well, since it takes 3-5 days before culture results are available.

NOVEL TECHNIQUES TO ASSESS WOUND INFECTION
Over the past years, several diagnostic techniques have been developed to meet the need for a fast and accurate method to detect wound infection. In this Thesis, we have assessed three different novel techniques that potentially meet this need; enzyme assays, pH measurement and headspace analyses by an electronic nose.

Enzyme assays
Enzyme assays were developed as diagnostic technique for wound infection as they potentially have the ability to measure the immune response to infection locally from wound fluid. It is well-known that the excessive stimulation of neutrophils during infection leads to the release of proteolytic and antimicrobial enzymes to mediate in the clearance of bacteria and destructed tissue. Heinzle et al. have shown that the activity of such enzymes can be measured from wound fluid and the activity of specific enzymes (myeloperoxidase (MPO), lysozyme, human neutrophil elastase (HNE) and cathepsine G (catG)) is elevated in wound fluid from infected wounds.\textsuperscript{8-11} We conducted two subsequent studies to assess the accuracy of using enzyme assays as a diagnostic technique in clinical practice. To enable fast and easy interpretation of results, the original enzyme assays were converted into a system that shows a change in transparency or development of color when the activity of the targeted enzyme is elevated; a change from colorless to red for elevated activity of MPO, a change from colorless to yellow for HNE and CatG, and a change from opaque to transparent for lysozyme. In the first study (chapter 5) we found that combining the enzyme assays for lysozyme, HNE and MPO into one result leads to better diagnostic properties then when separate enzyme assays are used. However, there was a desire to improve the enzyme assays to enable even faster (≤ 20 minutes) and more accurate detection of wound infection. Therefore, the enzyme assays were adjusted and tested in a second study (chapter 6). In this second study the enzyme assay, indeed, provided faster results (within 20 minutes).
However, diagnostic properties of both the separate and combined enzyme assays were lower than we would have expected based on the results from our first study. These differences might be explained by the use of slightly different enzyme assays. As mentioned before, the original assays used in the first study were adjusted to provide faster results in the second study. The substrates used in both enzyme assays were the same, i.e. peptidoglycan for lysozyme, N-Methoxysuccinyl-Ala-Ala-Pro-Val-p-nitroanilide for HNE and guaiacol (+ hydrogen peroxide) for MPO. However, in the second study, the substrates were produced in solid tablets for use in a diagnostic prototype. These tablets were made by hand, and therefore it might be possible that the amount of substrate available in the tablets varied between tablets and differed from the composition used in the liquid assays. A difference in the amount of substrate available in an assay might have influenced the enzyme activity measured. As mentioned in chapters 5 and 6, enzyme activity in both assays was measured by assessing the presence and strength of transparency and color changes. These transparency/color changes are a product of the reaction between the specific enzyme and its substrate (figure 9.1).

![Figure 9.1](image)

**Figure 9.1.** Schematic representation of the reaction between an enzyme (orange) and its substrate (blue), causing the formation of a product (yellow) that can be measured to determine enzyme activity.

In theory, the amount of product formed during an enzyme-substrate reaction (i.e. strength in transparency/color change) increases with an increasing amount of substrate available. At a certain concentration of substrate, the enzymes become fully saturated and a higher amount of substrate does not make a difference in enzyme activity measured. However, at low substrate concentrations, enzymes are not fully saturated and measured activity therefore strongly depends on the amount of substrate available. Figure 9.2 demonstrates the theoretical effect of different substrate concentrations on enzyme activity measured. However, given the precision with which the tablets were formed, we would only expect minor differences in measured enzyme activities between solid and liquid assays, which does completely explain the differences in diagnostic accuracy.
Table 9.2: The theoretical effect of different substrate concentrations on measured enzyme activity.

<table>
<thead>
<tr>
<th>Tablet with higher substrate concentration</th>
<th>Measured activity:</th>
<th>Measured activity:</th>
<th>Higher measured activity:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tablet with lower substrate concentration</td>
<td>Measured activity:</td>
<td>Measured activity:</td>
<td>Lower measured activity:</td>
</tr>
<tr>
<td>Liquid assay</td>
<td>Measured activity:</td>
<td>Measured activity:</td>
<td>Measured activity:</td>
</tr>
<tr>
<td>Low level of enzymes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Medium level of enzymes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High level of enzymes</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Another explanation might be found in the fact that the two studies differed with regard to the reference standard used to determine whether a wound was infected or not. In the first study, wounds were assumed to be infected when microbiological culture results of wound swabs showed an abundant growth of possible pathogenic microorganisms (PPM’s) in comparison to general flora (or growth of any PPM’s with no general flora). In the second study, we used a reference standard based on expert panel assessment of the combination of clinical information and microbiological culture results from wound biopsy. Based on the findings in chapter 4, we would not expect large differences in microbiological culture results from wound swabs (first study) versus wound biopsy (second study). However, the addition of clinical information to culture results and the use of an expert panel to assess wounds instead of using a pre-defined working definition might have resulted in a different assessment of wounds. In chapter 6, we made an attempt to demonstrate the effect of the use of a differently constructed reference standard on the diagnostic properties of the enzyme assays. We did observe differences in diagnostic properties between the reference standards (tables 6.2 and 6.3 versus table 6.4), indicating that the use of different reference standards in the first and second study might explain the differences in diagnostic properties of the enzyme assays.

In both studies, we tried to use the best available reference standard to determine whether a wound was infected or not. However, we know that both reference standards still are imperfect and might not have represented the actual infection status of the wound. Therefore, it might be possible that the enzyme assays might, in reality, have a higher diagnostic accuracy than we demonstrated in our studies. For instance, it is remarkable that in both studies the enzyme assays showed substantially more positive results than was expected based on clinical assessment of wound infection. This might be a case of ‘bad performance’, but it is also possible that the enzyme assays were positive before clinical signs and symptoms of wound infection could be observed. Assessment of clinical signs and symptoms is difficult in patients with comorbidities. Furthermore, it is possible that clinical signs and symptoms are observable in later stages of the infection, while the enzyme assays intent to measure the immediate response of the immune system to infection. Therefore, the relatively high number of false positive results for the enzyme assays might actually have been, to some extent, true positive results for (early) wound infection status. Unfortunately, we were not able to investigate this hypothesis in our earlier studies. In future studies, we intend to follow patients over time to determine whether positive results for the enzyme assays correlate with later
onset of clinically visible wound infection. If this is the case, the enzyme assays might serve as early indicator, or predictor, for wound infection.

**Wound pH measurement**

Measurement of pH might be a promising indicator for wound infection, especially since it is a simple technique and provides immediate results at low costs (approx. €0.01). In chapter 7, we demonstrated that the pH of wounds with a clinical visible infection tends to be higher than in wounds without clinical signs or symptoms of wound infection. Furthermore, the proportion of wounds assessed as infected based on clinical signs and symptoms rose with increasing pH. However, at the higher wound pH levels, still a substantial number of wounds were not assumed to be infected. Due to the lack of a perfect reference standard (i.e. gold standard), we are not sure whether these wounds were indeed not infected or whether there was an infection without observable signs and symptoms.

It remains difficult to fully contribute the observed differences in wound pH solely to the presence or absence of infection. For instance, it might be possible that the differences in pH were rather a representation of wound healing status. It is possible that wounds assessed as not infected by the clinician had a better healing status than wounds that showed a clinical visible infection. Moreover, in literature, wound pH has frequently been associated with wound healing. A slightly acidic wound environment has been reported to be optimal for wound healing, where wounds with impaired wound healing usually have a more alkaline wound environment. Several wound healing processes are known to be influenced by wound pH. For example, a reduction in wound pH from alkaline (pH >7) to a more acidic (pH < 7) environment is known to increase oxygen release, which is needed for appropriate wound healing. Although there is some evidence that a more alkaline environment is more favorable for some healing processes (e.g. uptake of a skin graft), the overall tendency is that the condition of a wound improves when pH is reduced.

To gain more insight in the potential for wound pH to serve as indicator for wound infection, we compared the results of wound pH measurements to the activity of the enzymes MPO, HNE and lysozyme in wound fluid. As explained in chapters 5 and 6, activity of these enzymes might serve as surrogate measure for the immune response to infection. Although there was some variation around the measured enzyme activity levels, there appears to be a trend for activity levels to increase with increasing wound pH.
Our findings are in accordance with research performed by Greener et al., who also demonstrated high activity levels for proteolytic enzymes, such as HNE, at pH > 7. Although all enzymes are stimulated by immune cells, the high activity of proteolytic enzymes might still rather be associated with the destruction of tissue components, and therefore deterioration of wound healing. However, high activity of antimicrobial enzymes might indicate that there is an increased effort to kill bacteria. The need for bacterial killing in alkaline wound environments might be explained by studies that have shown that microbial growth is rather seen in more alkaline wound environments. However, microorganisms are also able to promote an alkaline environment. For example, urease producing microorganisms can contribute to the alkalinity of a wound through the formation of ammonia.

Although the results in our study suggest that increasing wound pH is related to a higher risk of wound infection, further research is warranted to determine whether there might be alternative explanations for this relation. Up until then, we recommend to use wound pH in combination with other tools assessing wound infection.

**Electronic nose**

A foul wound odor has been used as indicator for wound infection since ancient times. However, recognition and interpretation of odors differs between humans. Electronic noses can use pattern recognition algorithms to differentiate between odors, based on measurement of volatile organic compounds (VOC’s). Disease classification based on electronic nose measurement is often based on the theory that VOC’s are released by metabolic processes in the human body (e.g. physiological or pathological) or by environmental factors such as bacteria and viruses. Over the past years, several clinical studies have demonstrated that electronic noses are indeed able to differentiate between several diseases, e.g. colorectal cancer, lung cancer and prostate cancer, by measuring VOC patterns from exhaled breath. We have demonstrated in a first pilot study (chapter 8) that an electronic nose can also be used to differentiate between VOC patterns from infected and non-infected wounds by measuring (the headspace of) wound fluid.

In our study, we decided to analyze wound fluid as it originates directly from the wound environment and therefore is believed to contain metabolic products that are specific to pathological processes in the wound. It is, however, important to notice that VOC’s are also secreted by microorganisms. An electronic nose does not differentiate between specific VOC’s, but rather focuses on the complete pattern. Therefore, we do
not know whether the difference in patterns observed in our study originates from actual wound infection or merely the presence of microorganisms. We did observe slightly better accuracy of our electronic nose, Aetholab, when we used microbiological culture results as reference standard. However, when our reference standard was based on clinical assessment rather than presence of organisms, Aetholab was still able to differentiate infected from non-infected wounds suggesting that this electronic nose is not solely sensitive to VOC’s produced by microorganisms. The advantage of using an electronic nose is that it uses objective algorithms instead of interpretation of clinical or microbiological information. Nevertheless, as we have discussed in chapter 8, the development of algorithms to differentiate between infected and non-infected wounds always depends on a reference standard. This reference standard provides the initial labelling of cases during the training phase, and therefore has a major influence on algorithm development.

The lack of a perfect reference standard (i.e. gold standard) for wound infection might have negatively influenced the developed algorithms and resulting diagnostic accuracy of Aetholab. Luckily, it is possible to initiate a new training phase for Aetholab when an (improved) reference standard becomes available. Furthermore, Aetholab is designed in such a way that it is possible to transfer newly developed algorithms to other Aetholabs to enable equivalent results between different devices.

**RECOMMENDATIONS FOR CLINICAL PRACTICE AND FUTURE RESEARCH**

Traditional techniques for the detection of wound infection include subjective assessment of clinical information and/or microbiological culture results, where novel diagnostic techniques have the potential to objectively detect infection in wounds. However, diagnostic properties are difficult to determine when a gold standard is missing.

The lack of a gold standard has resulted in the use of a variety of imperfect reference standards, which complicates the comparability of results between different studies assessing diagnostic techniques. Moreover, it is difficult to properly assess the accuracy of a diagnostic tool if the true infection status of the wound is not known. For instance, novel diagnostic techniques might be able to detect wound infection before clinical signs and symptoms become visible. We would therefore recommend future studies to include patient follow-up as it might help to determine whether a positive test result represents the development of a clinically visible infection later on.
The use of patient follow-up can still provide an inaccurate representation of true wound infection status at time of testing. A way to overcome this issue is to use clinical endpoints to evaluate the diagnostic technique. These clinical endpoints should represent the eventual clinical result that is aimed for with the diagnostic technique, e.g. wound healing. Novel diagnostic techniques for wound infection are usually designed to detect wound infection in a fast and accurate manner with the aim to enable timely treatment and thereby prevent further complications in the wound healing process. An example of a clinical endpoint for a novel technique for wound infection could therefore be ‘time to wound healing’, to determine whether the use of the diagnostic technique prevents major delays in wound healing. The use of this clinical endpoint might, however, be complicated in case specific treatment interventions are used to promote wound healing (e.g. revascularization). Another clinical endpoint might be ‘use of antibiotic treatment related to the wound’ as novel diagnostic techniques also aim to prevent the unnecessary use of antibiotic treatment by accurately ruling out infection. Moreover, early detection of infection enables the use of alternative treatments, such as antimicrobial dressings and antiseptics. We would, however, recommend researchers to always first gain some insight in the performance of a diagnostic technique, albeit using an imperfect reference standard, before conducting a study in which patients are actually treated upon the test results.

For clinical practice, the lack of a gold standard implies that there is no certainty about the accuracy of any technique used for the detection of wound infection. Although there is, as of yet, no solution, we hope our results have created awareness amongst clinicians about the imperfections that exist in diagnostic techniques they currently use. There is a variety of diagnostic techniques that might aid the detection of wound infection in clinical practice. Although there remains uncertainty about the diagnostic properties of these diagnostic techniques, it might be valuable to use them to support the assessment of wound infection in addition to clinical expertise and microbiological culturing.
REFERENCES


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