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Sheep health and hidden pathogens that can cause disease

Figure 1: Photography of a lamb in summer grazing field. Photo taken by Camilla Sobon.

Abstract

For the Norwegian culture, sheep play a major role for the culture, landscape as well for the economy. Meat, milk, and wool production are some of the incomes to Norway and is therefore important to have good health for the Norwegian sheep population. Bacterias and dangerous pathogens found inside the respiratory tract or from the environment is one of the threats to the development stock that will not lead to good meat quality. Some pathogens have been associated with pneumonia in caprinae, and other pathogens can cause other diseases. Fifty apparently healthy rams and ram-lambs were taken nasal swabs to see which bacteria that lived in the nasal cavities and what significance they have for sheep's health. After extracting the DNA from culturing the bacteria on blood agar, the samples went through a PCR amplification, followed by a gel electrophoresis to see if the right band length were obtained at 1500 kb, and then sent off to perform a sanger sequencing of the 16s rRNA gene. DNA sequences received back was analyzed and performed a blast search to find out which bacteria that were cultured. Five different bacteria were obtained from this project, and all of these were apparently normal findings that do not lead to infections or diseases on healthy sheep and lambs.

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The first thanks go to my supervisor Prof. Mark van der Giezen, for his support and guidance throughout this bachelor thesis. Your expertise in the research question has given me motivation and pressured me to sharpen my thinking to be able to submit a thesis at a higher level. Not to mention, you provided me with lots of useful feedback to improve the quality of the thesis.

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Thank you to NMBU Sandnes that prevented pre-maid blood agar plates for the project. You saved us time for having ready-made plates ready for use, and that they were of good quality so that we could get better bacteria growth when culturing.

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Small mention: iced mocha, the days writing this thesis would not be as good without it.

Contents

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List of abbreviations

°C → Celsius

µL → Microliter

µg → Microgram

BHI broth → Brain Heart Infusion broth

BLAST → Basic Local Alignment Tool

mg → Milligram

mL → Milliliter

bp → Base pair

g → Gram

rpm → Revolutions per minute

NWS → Norwegian White Sheep

PCR → Polymerase Chain Reaction

rcf → Relative centrifugal field

M → Molar

Introduction

1.1 – History

Humans breed domestic sheep for wide different reasons, primarily for meat, milk, and wool production, and some simply as a hobby or a means to introduce children to animal husbandry. Sheep is one of Norway's oldest domestic animals, it is assumed that the sheep came to Norway over 6000 years ago. Today, there are around 14 000 farms that breeds sheep all over the country [1]. Annually, it produces around 24 000 tones of meat that has been approved for food [2], and more than 4000 tones of wool in Norway. Most of the wool Norway produces is of a crossbred type, but there is also a good deal of wool from the spell-breed. Crossbred type makes up between 80 and 90% of the total amount of wool. Most of the crossbred type wool is form the Norwegian white sheep (NWS), but there are several other crossbred breeds in Norway [3].

Norway buys around 20-30% of high-quality wool, what we call class 1. It is especially autumn wool from crossbred that are sold to the Norwegian marked, but there are some demands for year-round wool, and a small amount of Spell-wool. Sorted wool from the belly, thigh and tail, class 2 wool, are exported in their entirety. Norway does not have an industry to utilize or have a demand for this type of wool as of today. The wool is exported to the UK, where they are exporting the wool further from there [4].

By having sheep for several thousands of years, grazing, mowing and other outfield use have helped to shape a varied and rich landscape. In Norway, it goes from fjords with open fields, to forests, to bare mountains. Although it is a very varied landscape, Norway has relatively small amount of agricultural land. Only 3.5% of the Norwegian land area is cultivated land. Of the cultivated area, ⅔ is best suited to be used in grass production [5]. In the summer, 2.4 million animals are released, of which 2.1 million are sheep, grazing in open fields. The sheep eat grass and other roughage and utilizes the resources that cannot be used directly as human food, to convert these resources into high-quality products such as meat and wool.

Rogaland is located in southwestern Norway and has a bit of everything when it comes to landscape. There are high mountains and deep fjords, agricultural land and forests, waterfalls, and calm waters, at the same time there are long sandy beaches and steep boulders. The heat form the Gulf Stream gives Rogaland a milder climate than what there is inland. With good climate conditions, this makes Rogaland one of the country's foremost agricultural and food counties [6]. Rogaland is the most important and largest sheep-meat-producing region and delivers more than 20% of the total amount of sheep approved for folk food in 2019 [2]

1.2 – Sheep production

From the time lambs are born until they are sent for slaughter, there are several controls that must be checked for things to be approved for use as food production. Before the farmer sends the lambs and sheep to slaughter, they check that the animals have no signs of disease. Sick animals will not be sent away but will be either treated and slaughtered later or euthanized on the farm and sent to an approved reception for carcasses. The healthy animals must have reached a certain weight class so that they can be sent for slaughter. Lambs that have not reached the weight are kept and fed so that they can slaughtered at the next collection. The animals are checked by a veterinarian at the slaughterhouse. Both the living and the dead animals are checked, which means both before and after the time of slaughter. The animals that cannot be used for human consumption are discarded and go to a company called Biosirk. Here can the discarded animals still be used in the value chain as other animal by-products. There is a small proportion who must be discarded, as there is little disease and good animal welfare in Norwegian sheep farming. Of all the sheep being slaughtered, there is an extremely small amount that cannot be used for folk food. From Fatland Oslo the percentage of lamb/sheep that got discarded was 0.039% in 2020, Fatland Jæren had 0.044% and Fatland Ølen had 0.025% [7].

Nortura and Fatland are two large slaughterhouses in Norway that produce a big part of the total amount of sheep meat in Norway. In 2020, Fatland slaughtered 177 000 lambs with an average slaughter weight of 18.1 kg, which gives 3202.7 tones of meat. 25 000 sheep/young sheep were also slaughtered, with an average weight of 31 kg, that will say 775 tones of meat. This is an increase from the previous year where 164 000 lambs and 21 000 sheep/young sheep were slaughtered [8]. For Nortura in 2020 they slaughtered 19 000 tones lambs and almost 5000 tones sheep which was an overproduction since the last year. They did also increase the wholesale price of lambs with 0.30 kr/kg [9].

In order for the economy to turn around, each party must receive its payment. For the farmers part in terms of price per lamb, the price is a somewhat complex calculation. The settlement price is set on the basis and this one follows the season. In addition, there are various bonuses and supplements that vary slightly between the competing slaughterhouses. One bonus is given according to which week the lamb is slaughtered and is deducted throughout the autumn. Week 34 and 35 is usually the weeks with the highest payment. Another bonus is given according to quality of the meat and how little fat there is on each lamb. On top of that there are also subsidies from the state on top of what the slaughterhouse pays. The payment of each lamb is not set on each different breed type, but more on the quality of the meat. The farmers are also paid for the wool, the farmer can shear the sheep himself, or let the

slaughterhouse do the work. If sheared himself, the wool must then be delivered afterwards. It's the slaughterhouse job to handle the inbound transport, slaughter and sales of raw materials [10], [11].

1.3 – Diseases in sheep

Sheep are fairly important domestic animals for the Norwegian economy. It is then important to have good and healthy sheep populations. Some diseases are easier to deal with than others, while others can be much more serious and fatal to the entire populations. Animal diseases are categorized into A- B- and C-diseases. A-Diseases are considered very serious, and an outbreak will lead to extensive control measure. B-diseases are considered serious, and systematic control is required to control the disease. C-diseases are diseases that are important that the Norwegian Food Safety Authority has an overview of. It can be relatively common diseases or more rare diseases [12].

There are several diseases that can affect the sheep, and many are often closely linked to the environment in which they live. Norway has a great variability in climate and geographical conditions, which means that farmers in the different parts of the country are affected by parasites differently [13]. The northern areas have an alpine climate while the southern and coastal lowland area have temperate rainy climate and mild winters. There is almost one million sheep in Norway [14], where ewes and lambs are normally put on fenced spring pastures one or more weeks after lambing. After a few weeks, they are then transferred to common rangeland pastures in the mountains or forests for summer grazing [13], [15]. When the sheep is released to the infield grazing in the spring, it may encounter parasites that may have been dormant in the pasture since last autumn, some such ailments can be different kinds of nematodes, liver fluke (*Fasciola hepatica*) and the disease myasis, which is an infestation of fly larvae, but can also get pneumonia from non-infectious substances that includes parasites, for example lungworms or aspirations from incorrect drenching.

Different species of nematodes occur to varying degrees in sheep in different parts of the country. This is due to significant differences in the ability of the various species free-living stages to develop and survive outside the host animal, primarily outdoors in the pastures. Some species nematodes, like for example *Nematodirus battus,* occurs across most of the country, and have a good ability to overwinter as eggs or larvae outside the host in the pastures. Other species of nematodes demand higher temperatures for the free-living stages to develop and overwinter in the pastures. These species have therefore mainly occurred in southern Norway where the climate is milder and warmer [16].

The three most important nematodes species, *Haemonchus contortus*, *Teladorsagia circumcinta* and *Nematodirus battus*, are associated with the development from infectious larva to early adult nematodes in the mucous membrane of the rumen or intestine. This leads to damage the glands in the duodenum

and small intestine, which will lead to the lamb/sheep won't have the optimal breakdown and absorption of nutrients. It will also cause mechanical damage and inflammatory reactions that leads to loss of protein to the intestinal lumen. The side effect of lambs with lot of roundworms means that they do not utilize their growth potential in the first grazing season and will have poorer growth than less parasitized lambs. *H. contortus* have the harmful effect with varying degrees of anemia due to the worms' blood suction, severe infection with *H. contortus* can lead to life-threatening blood loss [16].

Lungworms are found in many sheep herds but are in such small quantities that it has no particular significance for animal health and welfare [17]. The parasiticides given against roundworms in the stomach and intestines also affect the lungworm. Norway is trying to avoid unnecessary treatment for lungworms, as this will lead to an increased risk of developing resistance in the gastrointestinal parasites. Lungworm findings from the slaughterhouse do not necessarily mean treatment, there is only reason to consider treatment if the animals show clinical symptoms [17]. Another way to diagnose is to look at the clinical symptoms, grazing history and findings of eggs and larvae in the feces. *Dictyocaulus filaria* (the great lungworm) is the most pathogenic of the lungworms. The symptoms will vary after how great the infection is and is seen small to moderate infections and rarely clinical symptoms. *Protostrongylus rufescens* and *Muellerius capillaris* are other nematodes that are depended on other snail species as an intermediate host. These two give rarely clinical symptoms other than a little coughing but can lead to disposal of lungs at the slaughterhouse [18].

The large liverwort (*Fasciola hepatica*) is a parasite that thrives best in humid environments and mild weather and causes the loss of ruminants along the coast in Norway. The adult parasites live in the bile ducts of some mammals. When a large number of juvenile parasites migrate simultaneously in the liver tissue, the animal may show acute symptoms (acute fasciolosis) due to blood loss and hepatic strain. This in normally experienced in the period September to December [19]. In less acute fasciolosis, there are fewer larvae migrating at the same time, so that the symptoms appear somewhat later after ingestion of infection. The symptoms that occur are pale mucous membranes, which are due to blood loss, and the animals can lose weight quickly. This is most commonly seen in the periods October to January. Due to the climate in coastal areas, the disease hits Rogaland and Hordaland the hardest [19].

Pneumonia refers to inflammation of the lungs and may be caused by infectious substances such as bacteria, *Mannheimia haemolytica, Pasteurella multocida, Haemophilus* sp. *Chlamydia* sp., *Salmonella* sp., and *Mycoplasma* sp., and a few viruses. Pneumonia can also be caused by non-infectious substances as mentioned above. There have been studies where bacteria that cause pneumonia have been detected in apparently healthy sheep in Norway in the upper respiratory tract [20].

1.4 – *Mycoplasma ovipneumoniea*

Mycoplasma is a genus of bacteria that lack cell wall around their cell membranes and have a low G+C content (23-40 mol%) of the genome [21], [22]. *Mycoplasma ovipneumonia* is a species of respiratory pathogen from *Mycoplasma* bacteria that lives on and effect caprinae, this includes domestic sheep, domestic goats, bighorn sheep and mountain goats [23].

M. ovipneumonia are labile organisms that are easily destroyed by contact with heat, dehydration, sunlight and common disinfectants, which means the bacterium does not survive long outside the host as they are completely dependent on the host for nutrition to be able to live [22]. *M. ovipneumoniae* causes atypical pneumonia and can further predispose to lung infections with other bacteria such as *Mannheimia hemolytic*. Infection from *M. ovipneumoniae* in domestic sheep is usually associated with mild disease, most often as a "cough syndrome" in lambs and young sheep under 6 months. It is less commonly seen that the bacterium is associated with severe or fatal pneumonia in lambs and adult sheep [23]. Bacteria from the *Mycoplasma* genus lack cell wall, which gives them natural resistance to penicillin [24]. They are resistant to β-lactams and cephalosporins, but are sensitive to erythromycin, tetracycline, aminoglycosides, rifampicin, chloramphenicol and quinolones [22]. To date, with many *Mycoplasma* diseases, no protective immune response has been achieved using vaccines, although antibodies can be obtained. Therefore, it requires antibiotics with wider range to treat the inflammation caused by the bacterium. Tetracycline is the first choice for the treatment of *M. ovipneumoniae* [25].

The bacterium can be found in the lungs, trachea, and nasal cavity of small ruminants. Detection of *M. ovipneumoniae* can be achieved by bacteriological culture and molecular diagnostics, so that the bacterium can be cultured and detected with DNA extraction, 16S PCR and gel electrophoresis. A disadvantage of culturing *Mycoplasma* is that they are highly fastidious and it typical takes several weeks to culture. Many serological tests are non-specific and insensitive. There are several *Mycoplasma* species, but to be able to detect one specific one there need to be a PCR that is developed for one single species, but there is none today [22].

Materials & Methods

2.1– Media, solutions, and primers

Brain Heart Infusion (BHI) broth

- 3.7 g BHI
- 100 mL Milli-Q water

Broth was stored at 4 ºC after autoclaving, until required.

Bacteria in BHI broth

- 2000 µL BHI broth
- One single bacteria colony

Bacteria solution was grown overnight at 37 ºC in a shaking incubator at 220 rpm.

80% Glycerol

- 80 mL glycerol
- 20 mL Milli-Q water

Glycerol was stored at room temperature after autoclaving, until required.

16% Glycerol Stock

- 200 µL 80% Glycerol
- 800 µL bacteria culture in BHI broth

Stored at -80 ºC.

Blood Agar plates

- 44 g Difco Columbia Blood Agar
- 15 mL Sodium citrate 6.5%
- 150 mL ovine blood
- Ion-exchanged water

The agar was poured carefully onto sterile 10 cm petri dishes in a flow hood cabinet after autoclaving and left to solidify. Stored at -4 ºC in sterile plastic bags until required.
Pre-prepared blood agar plates from NMBU Sandnes were also used.

Blood agar is a growth medium used for the most common bacteria cultivation. Bacteria can be grown, and hemolysis can be detected.

1xTAE buffer

- 20 mL 10xTAE buffer
- 80 mL Milli-Q water

Solution was made in a glass bottle and used immediately.

Agarose Gel

- 0.9 g Agarose
- 100 mL 1xTAE buffer

Agarose and buffer were poured in a 250 mL Erlenmeyer flask and heated in a microwave at full power with regular stirring. Solution was poured over in a casting frame and left to solidify. Used immediately.

Loading buffer

- 5x DNA Loading Buffer blue

Loading buffer were obtained from Bioline, Meridian Bioscience. Used in gel electrophoresis to monitor DNA migration on agarose gel.

PCR master mix

- DreamTac PCR master Mix (2x)

PCR master mix used in this study were obtained from Thermo Fisher Scientific and used for PCR amplification of 16s rRNA genes.

Primers

Primers used in this study were obtained from Thermo Fisher Scientific and used for PCR amplification of 16s rRNA genes and in the DNA extraction solution that were made ready and sent off to for Sanger sequencing in Germany.

Table 1: List of primers used in this study. Assigned names, primer sequences, references and purpose of use indicated.

Primer name	Sequence (5´-3´)	Reference	Purpose
27F	AGAGTTTGATCATGGCTCA	[26]	PCR amplification, DNA extraction
1492R	TACGGTTACCTTGTTACGACTT	[26]	PCR amplification, DNA extraction

Gel-Red

- Gel-Red Nucleic Acid Strain

Gel-Red was obtained from Biotium and diluted with Milli-Q water. Dilution stretched from 1:50 and up to 1:100. Most used dilution was 1:75, where the finest bands were obtained on the gel electrophorese.

Hyperladder

Hyperladder 1 kb were obtained from Meridian Bioscience and used for Gel-electrophoresis as molecular weight marker with higher intensity bands for easy orientation. The Hyperladder works on nucleic acids from 200 bp to 10 kb.

2.2– Animals and bacterial sampling

2.2.1 – Nasal swabs

During the month of October 2020, a total of 55 nasal swabs were collected from live, apparently healthy sheep belonging to different herds in Rogaland, Norway. The samples were obtained from randomly selected rams and ram-lambs during pre-mating flock health visit. Some of the sheep showed some signs of snout but had no other clinical signs of disease. The different farmers bred different breeds, some herds contained Texel sheep, other herds consisted of Rygja, and there were also some of the typical Norwegian White sheep (NWS). The herds where grassing on private grassing fields and had no relationship between each other.

The swabs were collected by a MSc under the supervision of a veterinarian, while the sheep were inside the barn for the winter season. Nasal samples were taken using sterile cotton swabs from each animal and placed inside a tube with Liquid Amies Elution Swab (eSwab) and stored in the freezer at -20 ºC.

2.2.2 – Culture of nasopharyngeal microorganisms

The swabs were then swabbed onto Columbia agar plates containing 5% ovine blood and incubated for 20-28 hours at 37 ºC until the required amount of bacterial growth was reached. After the incubation the plates were examined for different bacterial colonies and sub-cultured for another 20-28 hours at 37 ºC on blood agar plates. The bacterial colonies where then sub-sub-cultured using sterile wire needle and sterile technique and smeared with dilution to form single colonies. The blood-agar plates where then again incubated at 37 ºC for 20-28 hours. After incubation one single bacterial

colony were transferred for further culturing and DNA extraction, and the blood-agar plates containing bacteria were stored in the fridge at 4 ºC if needed for further testing.

2.2.3 – Bacterial culturing in Brain Heart Infusion broth

Brain hearth infusion (BHI) broth was made with mixing 70 mL Milli-Q water in a beaker with a magnetic stirrer under heating. Three and seven tenths BHI was poured into the beaker gradually. When all the broth was dissolved, the solution was transferred to a measuring cylinder and Milli-Q water was added until the solution was 100 mL. Then poured over a Schott bottle and autoclaved. The solution was then stored in the fridge at 4 ºC until ready for use.

Prior to DNA extraction, one single bacterial colony were mixed in 2000 µL BHI broth in glass tubes and incubated over night at 37 ºC in a shaking incubator at 220 rpm. The broth solutions were observed the next day for bacterial growth, if there were no visible growth, the mixture was then left for further incubation a few hours or until next day. Eight hundred µL of the broth solutions were then used to make 16% glycerol stock solutions that were stored at -80 ºC, and 1000 µL was used to extract the DNA. Sixteen precent glycerol stock was made by mixing 200 µL 80% glycerol in a tube and add 800 µL bacterial culture in BHI broth, mixed by pipetting up and down.

2.3 – DNA extraction

2.3.1 – DNA tissue extraction

One thousand µL of broth mixture with visible bacterial growth was transferred in two separate 1,5 mL Eppendorf tubes with 500 µL in each one. The tubes were then further processed with two different protocols targeting Gram-negative and Gram-positive bacteria, respectively (see attachment 1). Both tubes were centrifuged for one minute at 6000 rcf (Centrifuge 5418, Eppendorf), and the supernatant was discarded. DNA extraction was used using DNeasy Blood and tissue kit (Qiagen, Hilden Germany) with protocol 1 (see attachment 1).

The pellet in the Gram+ tube was resuspended in 180 µL enzymatic lysis buffer and incubated for at least 30 min until the pellet was completely lysed. Twenty-five µL Proteinase K and 200 µL Buffer AL was added, the mixture was mixed by vortex and spined. The mixture was incubated at 56 ºC for thirty minutes. Two hundred µL of 96-100% ethanol was added and mixed, and the solution was transferred into DNeasy Mini Spin Columns and centrifuged for one minute at 6000 rcf using an Eppendorf centrifuge (Centrifuge 5418, Eppendorf). The flow-through was discarded.

In the Gram- tube, the pellet was resuspended in 180 µL Buffer ATL and 20 µL Proteinase K were then added and mixed well. The mixture was incubated at 56 ºC until the pellet was completely lysed, with occasionally vortexing. Two hundred µL Buffer AL was added and the solution was mixed, then 200 µL of 96-100% ethanol was added and mixed thoroughly. The solution was transferred with a pipette into the DNeasy Mini Spin tubes that already contained the Gram+ solution and centrifuged for one minute at 6000 rcf. Flow-through and collection tube was discarded.

Columns were placed in new 2 mL collection tubes, 500 µL Buffer AW1 were added. Samples were centrifuged for one minute at 6000 rcf, (Centrifuge 5418, Eppendorf) the flow-through and collection tube was discarded. Columns were placed in new 2 mL collection tubes and 500 µL Buffer AW2 were added, followed by centrifugation for three minutes at >16 000 rcf (Centrifuge 5418, Eppendorf). Flow-through and collection tube were discarded, and the column was placed into clean 1.5 mL Eppendorf tubes. Twenty-five µL Buffer AE were pipetted directly onto the DNeasy membrane and incubated at room temperature for one minute and then centrifuged for one minute at 6000 rcf.

DNA concentration was measured using NanoDrop (NanoDrop One, Thermo Scientific). Blanking was made with the same solutions used to elute DNA, and one µL was used to measure the DNA concentration. Samples that did not get a good quality (around 1.80 for A260/A280 and around 2.00 for A260/230) or high enough DNA concentration from the measurement (30 ng/µL or lower) was set to do a DNA precipitation. The other samples that received the required amount of DNA concentration with well enough quality were transferred with no precipitation to perform PCR.

2.3.2 – DNA extraction from bacteria cultures on blood agar plates

One of the last times performing DNA extraction, another method was tried by scraping off a whole line of bacterial cultures from sub-sub-culture and then performed a DNA extraction without culturing it in BHI broth. The first two step was skipped (see attachment 1), as the bacteria were not in liquid solutions and could be directly dissolved in buffer ASL and ATL. There were no drastic improvements with this method on achieved DNA concentration and quality, which means that the samples were used for further testing.

2.3.3 – DNA precipitation

For the DNA precipitation, 40 µL of 10 M Ammonium Acetate and 101 µL ice-cold 99% ethanol were added to the samples and mixed well. Samples were then put in the freezer overnight at -20 ºC. Next day the samples were centrifuged at 4 ºC for 30 minutes at 15 000 rcf. (Centrifuge 5415 R, Eppendorf). The supernatant was carefully pipet out without disturbing the pellet. The pellet was washed twice with 200 µL 70% ethanol and centrifuged at 4 ºC for 30 minutes at 15 000 rcf. The pellet was air dried until all the ethanol was evaporated and then redissolved in 20 µL Buffer AE. The

mixture was warmed at 37-42 ºC for approximately 10 minutes and gently vortex. DNA concentration was then measured using NanoDrop.

2.4 – PCR, gel electrophoresis and sequencing

2.4.1 – PCR

PCR amplification was performed in 0.2 mL PCR tubes containing 25 µL DreamTac PCR master Mix (2x) (Thermo Fisher Scientific, USA), 2 µL of both forward and reverse primer (27F (5 µM) and 1492R (5 µM)), 11 µL Milli-Q water and 10 µL of 50-80 ng/µL DNA solution from broth solution. DNA solution was obtained by calculating wanted final DNA concentration and divide this on DNA concentration from the sample. The wanted amount of DNA was taken out and Milli-Q water were added so that the total amount was 10 µL. There were times in the beginning where one single bacterial colony were mixed in 10 µL of water, but by not getting good enough results, this method was no longer performed after a few trials and fails.

PCR reactions were run on Bio-Rad T100 Thermal Cycler. There were run five different steps to make sure there the selected primers and template together with the DNA polymerase would go through the correct steps of amplification. Negative control without template were also run to for each experiment to check for contamination of reagents. Initial denaturation was carried out 95 ºC for 7 minutes, followed by 31 PCR cycles: denaturation at 95 ºC for 30 seconds, annealing at 58 ºC for 30 seconds and extension at 72 ºC for 1 min, followed by a final extension at 72 ºC for 10 minutes before being held at 4 ºC.

Table 2: Settings used for PCR reaction on Bio-Rad T100 Thermal Cycler

Steps	**Temperature, ºC**	**Time**	**Number of cycles**
Initial denaturation	95	7 min	1
Denaturation	95	30 sec	31
Annealing	58	30 sec	
Extension	72	1 min	
Final extension	72	10 min	1

2.4.2 – Gel electrophoresis

Following PCR, a gel electrophoresis was performed on Bio-Rad PowerPac Basic, to analyze DNA fragments and check for PCR products according to the DNA band size. The gel was made by boiling 0.9 g agarose in 100 mL 1xTAE buffer before cooling. Two µL of DNA samples were mixed with 2 µL Gel-Red (1:75) (Biotium, San Francisco, USA), 2.4 µL Loading buffer (Bioline, Meridian Bioscience) and 3.6 µL Milli-Q water. Nine µL were loaded on the gel where one well was loaded with 5 µL Hyperladder 1kb (Meridian Bioscience, Ohio, USA) mixed with 2 µL Gel-red. Electrophoresis was run at 60-80 V for different times, 80-120 minutes, to get the best results from the fragments that were being analyzed. Gel was imaged on Bio-Rad ChemiDoc Touch Imaging (Bio-Rad, USA).

Table 3: The amount of solution added into the wells on the gel to perform a gel electrophoresis

Reagent	**Volume (µL)**
Gel-Red	2.0
5x DNA Loading buffer blue	2.4
DNA sample	2.0
Milli-Q water	3.6

2.4.3 – PCR purification

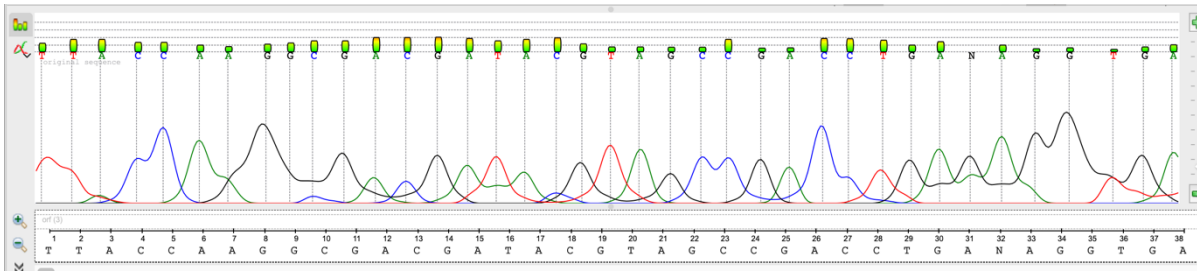
DNA fragments that with the right band size were purified using the QIAquick PCR Purification Kit (Qiagen), following the manufactures instructions (see attachment, protocol 5). The kit removed primers, primer dimers, dNTPs and restriction enzymes that all that was left was clean DNA with no contamination.

In tubes containing PCR samples, 500 µL of Buffer PB were added to the PCR reaction and mixed. A QIAquick column were placed in a provided 2 mL collection tube where the solution was transferred over and centrifuged for one minute at >16 000 rcf (Centrifuge 5418, Eppendorf). Flow-through were discarded and the QIAquick column were placed back into the same tube. Seven hundred fiftieth µL buffer PE were added to the column and then centrifuged twice with same time and speed. Flow-through were discarded. The QIAquick column were placed onto a clean 1.5 mL Eppendorf tube. Fifteen mL Buffer EB were added to the center of the membrane and incubated at RT for one minute, then centrifuged with same settings. Fifteen mL Buffer EB were added again to the center of the column, incubated and centrifuged. QIAquick columns were discarded, and DNA were measured on NanoDrop.

2.4.4 – Sequencing and analyzing DNA fragment

To check what type of bacteria colonies they originated from, DNA samples were then sent to Microsynth Seqlab GmbH in Göttingen Germany for Sanger sequencing. Samples were mixed with forward and reverse primers (27F (20 µM) and 1492R (20 µM)), concentration of DNA was 30 ng/µL in a final volume of 12 µL. Files received back from Microsynth Seqlab GmbH were analyzed the alignment computer software Ugene (Unipro, available at: www.ugene.net) to find the best sequence quality and then performed with a Blast search online (available at: www.blast.ncbi.nlm.nih.gov/Blast.cgi). DNA sequences where there were bad peaks that overlapped each other were removed and trimmed away that the best quality strands was left.

A



B

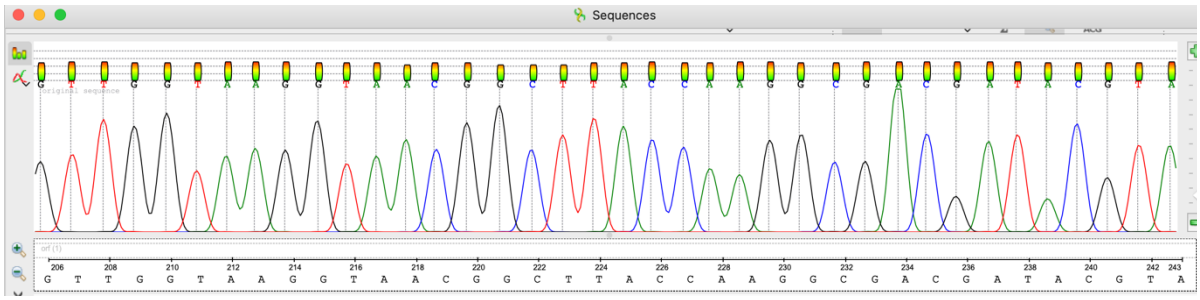
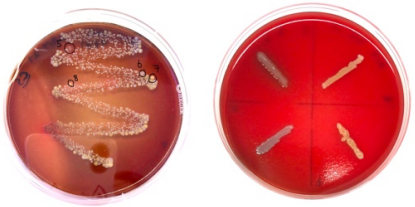


Figure 2: **Image showing quality of DNA strand when analyzed R19 C7 sample in Ugene.** A: Image showing bad quality strand where peaks are overlapping each other and there is hard to see which DNA base is most likely to be the correct one. B: Peaks showing good quality strands with high peaks with which DNA base pairs that is most likely to be the correct one.

Results

3.1 – Bacteria cultures on blood agar plates

There was a total of five different nasal swabs that were swabbed onto blood-agar plates and incubated for approximately 24 hours. From the first swab the plates were examined for different bacterial colonies, the R19 samples had a total of 4 different bacteria that were sub-cultured and then sub-sub-cultured. After the first incubation, the parafilm around the petri dish crack, which caused some of the blood agar to dry out. This did not affect bacteria colonies, and they could be used for a single bacteria culturing. The sub-culture with the 4 different colonies showed good growth and all of them were used for a sub-sub-culture. For the sub-sub-culture, the parafilm cracked for R19 C5 which led to the whole plate dried out and could not be used for further analyzing. The parafilm did also crack on R19 C6 and R19 C7 as well on R19 C8, but the blood agar did not dry out as much as on the first plate, which meant a single bacteria colony from all these 3 plates could be used for DNA extraction.



R19 swab R19 sub-culturing

A B C D

Figure 3: **Bacteria culture and sub-culture for R19 sample**. Figure showing first swab of R19 sample and sub-culture of R19 bacteria colonies. A: R19 C5 with dried out blood agar, B: R19 C6, C: R19 C7, D: R19 C8

Nasal sample R5 and R6 were swabbed onto the same plate, R6 sample showed a visible β-hemolysis around the whole swab, there were no visible hemolysis on the R5 sample. A total of 3 different bacteria colonies were observed from each swab. Each colony were transferred to new plates for single bacteria culture. Colony number one from R6 sample showed hemolysis on second culturing and had

a mixture of bacteria colonies showing β-hemolysis and bacteria colonies showing no hemolysis on the-sub-sub-culture. All of the other 5 bacterial colonies did not show any hemolysis (image not showed).

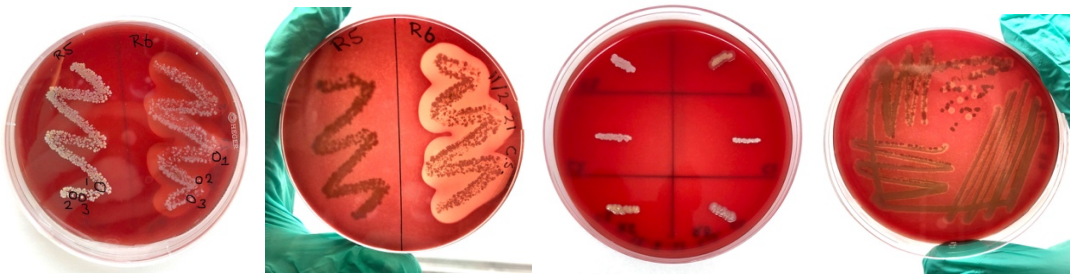


Figure 4: **R5 and R6 samples on blood agar plates**. First and second left image showing first swab of R5 and R6 sample. A visible beta-hemolysis can be seen on the R6 sample. Third image showing different single bacteria cultured from R5 and R6 samples. To the right: image showing sub-sub-culture of R6 C3 where there are places with hemolysis.

For the R27 and R28 nasal sample there were almost no bacterial growth from the first swab. For the R27 sample there were only one visible colony growing on the blood agar plate, for R28 sample there were a few more, but only two different colonies were observed. Of these three colonies, no one had visible hemolysis in the sub-culture. The single colony from R27 sample did not grow in one clean line, but a few single colonies did spread from the main colony swab. After sub-sub-culturing the colonies, there were good visible growth and single bacteria colonies were obtained that could be used for DNA extraction.

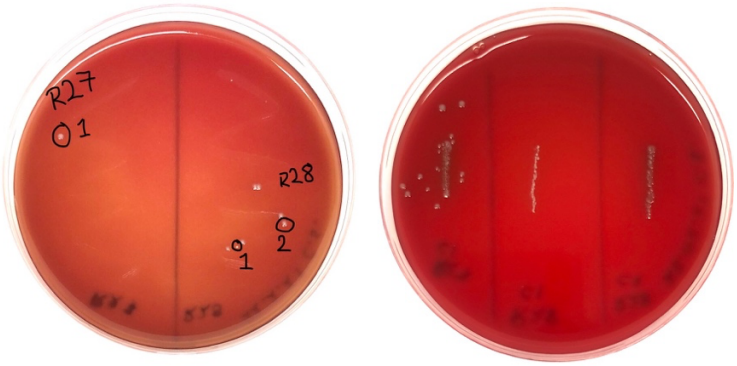


Figure 5: **R27 and R28 bacteria on blood agar plates.** Image to the left showing R27 and R28 swab with little bacterial growth. Image to the right showing sub-culture of the three colonies obtained from the R27 and R28 sample.

3.2– Bacterial Morphology

Of all the different bacteria cultured, the first nasal swab on blood agar plates were examined to find four different bacterial colonies. Some of them looked similar, but most of them had different appearance. The first bacterial colony, R19 C5, got destroyed while incubating for sub-sub-culturing. A full bacterial morphology description is therefore not possible, but seeing the sub-culture, the agar under the colony appeared a bit green and had α-hemolysis, while the bacteria were shiny with bulky appearance.
For the R19 C6 there were no visible hemolysis, the colonies had a circular form with matte appearance. Colonies were single cocci with a raised elevation. Color of the bacteria were opaque grey. Bacterial colony number seven from R19 on blood agar plates had a circular form with a shiny and smooth appearance. They were opaque with a color shown as creamy yellow, and the elevation was raised. No visible hemolysis where displayed.
Colony number eight from R19, colonies were singular cocci with circular form with a raised elevation. There was no visible hemolysis. The bacteria were shiny and opaque while color was a mix of yellow and grey. See figure 1 for picture of the bacteria on blood agar plates.

The colonies from bacterial colony number one from R5 had circular form, and a raised elevation. The surface was shiny and a little rough, and the colonies were vague transparent with a grey-ish color, and no visible hemolysis. For the second colony, R5 C2, bacteria formed singular cocci and a flat elevation. Opacity of the colonies was translucent, with a white grey color. No hemolysis on the blood agar plates. The last colony from R5 sample, C3, had the same appearance as R19 C7 described above. They had circular form, raised elevation, shiny and smooth appearance, opaque and had a creamy yellow color. No hemolysis here either.

For the R6 samples, first bacterial colony had a mixture of two different bacteria. There was sign of β-hemolysis from some colonies, and others had no hemolysis. The colonies that showed hemolysis were transparent with no color, had and a raised elevation with circular shape. The other colonies that did not show hemolysis were singular circular cocci, with a raised elevation. They were matte with an ash yellow color. Second colony from R6 sample formed singular circular colonies. Flat elevation, with a matte appearance, and opaque opacity. Color was white grey, with no hemolysis shown on the blood agar plate. Last colony, C3, were circular single cocci, a bit bigger than R6 C2. Opacity was opaque with a grey color. No hemolysis.

R27 bacterial colony number one, colonies had a circular form with smooth and cloudy appearance. Color was grey and slightly transparent. No visible hemolysis.

First bacterial colony from R28 sample had singular circular cocci. A dull surface with creamy yellow color. The opacity was translucent, with no hemolysis on the plates.

Second and last bacterial colony, R28 C2, the colonies had a circular form, with a translucent and viscous appearance with a rough sticky surface. Elevation was flat, and there was no color on the bacteria or any hemolysis visible.

A B C D E

Figure 6: **Sub-sub-culture of different bacterial colonies on blood agar plates.** A: R19 C7, B: R5 C3, C: R5 C1, D: R27 C1, E: R28 C2

3.3– DNA extraction

DNA was extracted as mentioned in section 2.3.1. Nine of the total thirteen colonies were used for DNA extraction after being grown in BHI broth, while three colonies were extracted directly from the sub-sub-culture as mentioned in section 2.3.2.

Table 4: Overview of DNA concentration measured by bacterial colonies after DNA isolation.
*DNA extracted directly from a single bacteria colony from sub-sub-culture and not from BHI broth.

RAM Sample	**DNA concentration**		
	ng/µL	A260/A280	A260/230
R19 C5	-	-	-
R19 C6	17,1	2,04	0,41
R19 C7	29,3	2,12	0,70
R19 C8	10,8	2,09	0,27
R5 C1	36,6	1,88	0,64
R5 C2	43,1	1,85	0,90
R5 C3	57,8	1,54	0,74
R6 C1	126,1	2,03	0,64
R6 C2	29,2	2,04	1,25
R6 C3	110,0	1,94	1,81
R27 C1*	33,7	1,33	0,61
R28 C1*	52,8	1,84	1,39
R28 C2*	16,5	1,86	1,83

3.4– DNA precipitation and clean up

Five samples were selected to perform a DNA precipitation with Ammonium Acetate to increase the concentration of DNA (see Protocol 2). The samples were washed twice in ethanol, first time for 30 minutes, second for 15 minutes. Pellet for sample R5 C3 was visible, but not for the four other samples. After the DNA precipitation, DNA concentration was measured, and results can be seen in Table 5.

Table 5: Overview of DNA concentration measured after a DNA precipitation

RAM Sample	**DNA concentration**		
	ng/µL	A260/A280	A260/230
R5 C1	33,3	1,90	2,07
R5 C2	4,1	1,88	2,81
R5 C3	336,4	1,30	0,67
R6 C1	186,9	2,06	2,37
R6 C2	19,3	2,09	2,34

3.5– PCR, Gel electrophoresis and PCR purification

From the thirteen samples that got the DNA extracted from the bacteria colonies, a total of 35 PCR samples were prepared for PCR amplification of 16s rRNA genes. After the amplification, samples were run on 1% agarose gel to check if the correct DNA length were obtained, a length of around 1500 base pairs.

The first two gel electrophoresis performed on the three R19 samples, no band were visible on the gel. Third trial with new PCR amplification product from the R19 samples, a visible band were obtained on all three samples. There was also a visible band on the fourth trial from a new PCR product with the same samples (see figure 5 for image visualization). PCR samples where there was a visible band were taken out to perform a PCR purification on and then check for DNA concentration (see Table 6).

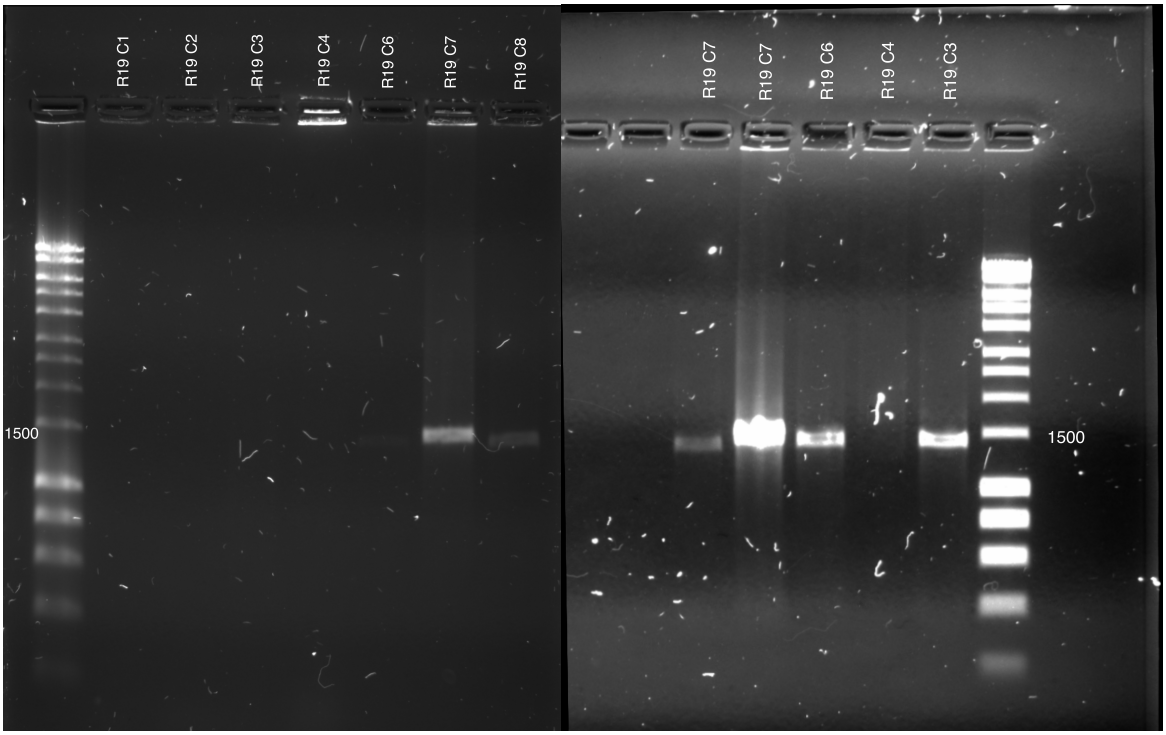


Figure 7: **Gel visualization of R19 samples with visible band at 1500 bp for PCR amplified DNA extract run with universal primers (27F and 1492R), run on 1% agarose gel**. Third trial to the left with almost a visible band on R19 C6 sample, a good visible band on R19 C7 sample, and some visible band on R19 C8 sample. Image to the right, fourth trial with good visible band on all three R19 samples.
Images taken on Bio-Rad ChemiDoc Touch Imaging System and edited with ImageLab software.

PCR amplification were done for the three colonies from R5 and the three colonies from R6, followed by a gel electrophorese (see Figure 6 for visualization). Band with correct base pair were obtained on all samples except R5 C2 and R6 C2. After the gel, samples with band were used to perform a PCR purification and DNA concentration was measured (see Table 6).

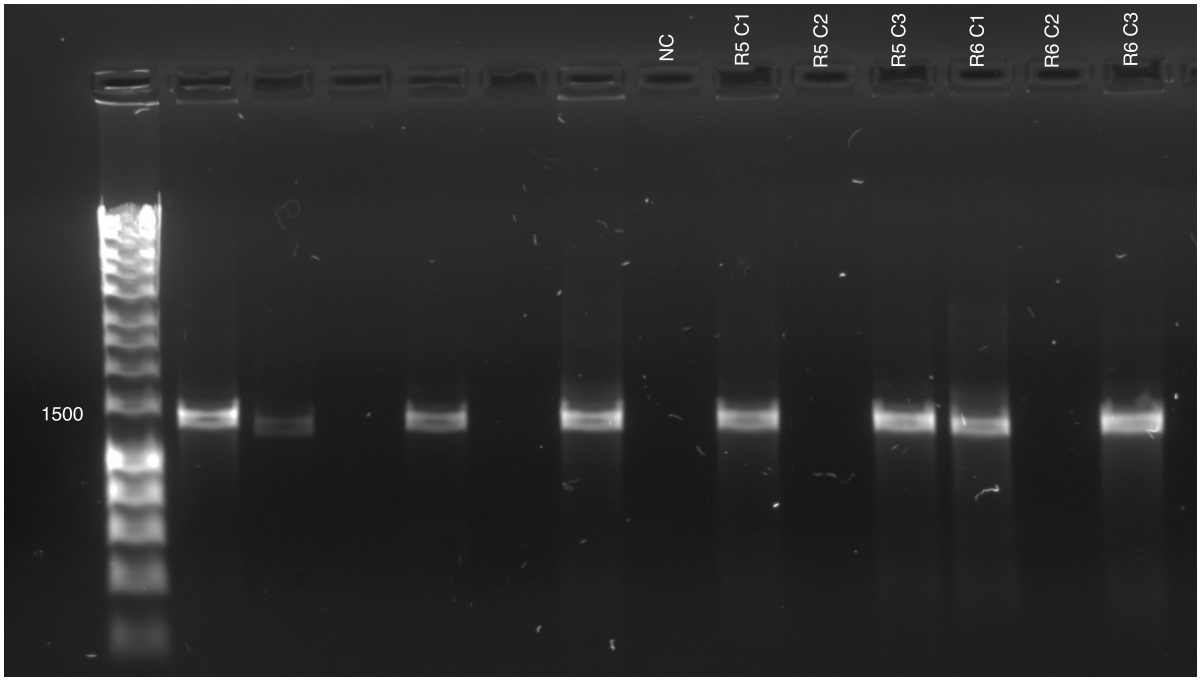


Figure 8: **Gel visualization of R5 and R6 samples with visible band at 1500 bp for PCR amplified DNA extract run with universal primers (27F and 1492R), run on 1% agarose gel.** Image showing good band at 1500 bp for PCR product R5 C1, C3 and PCR product R6 C1, C3. No obtained band on R5 C2 and R6 C2 PCR sample and no visible band on the negative control (NC).
Images taken on Bio-Rad ChemiDoc Touch Imaging System and edited with ImageLab software.

Table 6: Overview of DNA concentration after a PCR purification, first trial

RAM Sample	**DNA concentration**		
	ng/µL	A260/A280	A260/230
R19 C6	11,3	1,72	1,08
R19 C7	24,9	1,76	1,59
R19 C8	17,2	1,76	1,40
R5 C1	23, 1	1,86	1,08
R5 C2	1,4	3,45	0,01
R5 C3	20,3	1,95	0,14
R6 C1	41,3	1,93	1,26
R6 C2	3,1	2,43	0,02
R6 C3	44,6	1,91	0,17

One last PCR amplification were performed on all DNA samples, except R6 C2 and C3. This was followed by a gel electrophoresis (see Figure 7 for visualization). Ten out of fourteen samples showed the correct band length on the gel and were then used to perform a PCR purification to remove unwanted enzymes, salts and other impurities from the PCR mix followed by a DNA measuring (see Table 7).

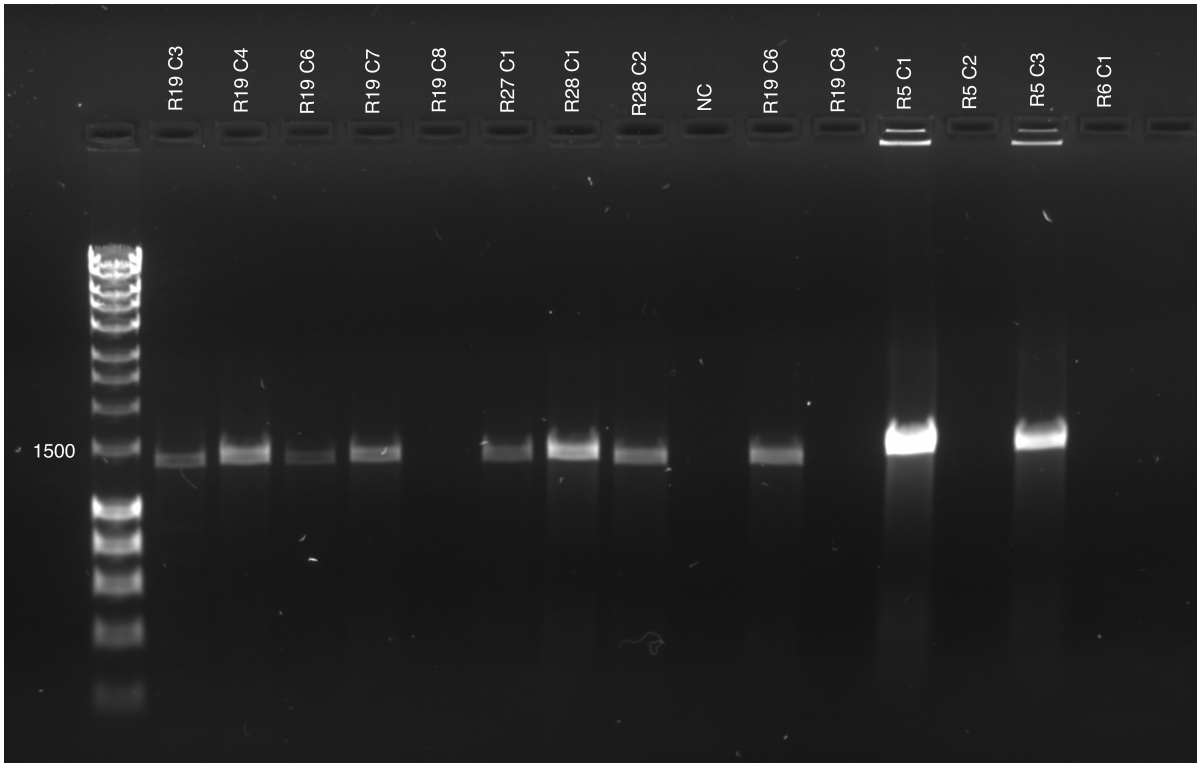


Figure 9: **Gel visualization on all DNA samples with visible band at 1500 bp for PCR amplified DNA extract run with universal primers (27F and 1492R), run on 1% agarose gel.** Image showing good band at 1500 bp for all PCR product except R19 C8, R5 C2 and R6 C1. No visible band on the negative control (NC). Images taken on Bio-Rad ChemiDoc Touch Imaging System and edited with ImageLab software.

Table 7: Overview of DNA concentration after PCR purification, second trial

RAM Sample	**DNA concentration**		
	ng/µL	A260/A280	A260/230
R19 C6	58,6	1,86	2,10
R19 C7	48,7	1,83	1,88
R19 C8	-	-	-
R27 C1	47,2	1,84	1,84
R28 C1	91,1	1,85	2,09
R28 C2	71,3	1,87	1,91
R5 C1	86,9	1,85	2,12
R5 C2	-	-	-
R5 C3	107,4	1,85	2,15
R6 C1	-	-	-

3.6– Sanger sequencing and Blastn search

From the total of 13 ram samples that got DNA extracted, performed PCR, Gel electrophoreses and PCR purification, 6 samples were selected to be sent to Germany for a Sanger sequencing. Samples needed to have 40 ng/µL DNA with a volume of 26 µL. See table 8 for which RAM samples that got selected with amount of DNA product taken out and mixed with amount of Milli-Q water. Each sample were placed in two separate Eppendorf tubes, where one contained forward primer (27F), and the other one contained a reverse primer (1492R). Both tubes contained 3 µL primer where the concentration of the primers were 20 µL.

Table 8: Overview of samples with amount of DNA after PCR product and Milli-Q water that got sent for Sanger sequencing

RAM sample	ng/µL	26 µL 40 ng/µL	µL PCR product	µL H_2O
R19 C3	53,6	1040/53,6	19,4	6,6
R19 C7	48,7	1040/48,7	19	5
R5 C1	36,9	1040/36,9	12,0	14,0
R5 C3	107,4	1040/107,4	9,7	16,3
R27 C1	91,1	1040/91,1	11,4	14,6
R28 C2	71,3	1040/71,3	14,6	11,4

Microsynth Seqlab GmbH in Göttingen Germany was the company who performed sanger sequencing on the six samples after DNA extraction, PCR amplification and PCR purification. Results for each colony were received as FASTA and ab files containing the nucleotide sequence together with chromatograms. After aligning and editing the sequences, a Blastn search was performed, followed by a Silva search to double check the species. Table 9 shows what bacteria species is from what ram sample.

Table 9: Overview of ram samples with detected bacteria species.
* Only forward strand search, no sequence on reverse strand.

RAM sample	**Bacteria**
R19 C3	*Citrobacter freundii*
R19 C7	*Staphylococcus xylosus*
R5 C1*	*Staphylococcus succinus*
R5 C3	*Staphylococcus xylosus*
R27 C1	*Enterococcus faecalis*
R28 C2	*Moraxella osloensis*

Discussion

The aim for this project was to see if *Mycoplasma ovipneumoniea* is present in local sheep herds in Rogaland Norway and what typical bacteria are found in the nose cavity of sheep and what significance they have for the physiology.

In this project, five different bacteria were cultured and harvested from the nose cavity of apparently healthy rams and ram-lambs. Bacteria that were obtained was: *Citrobacter freundii*, *Staphylococcus xylosus, Staphylococcus succinus, Enterococcus faecalis* and *Moraxella osloensis*. *Mycoplasma* spp. was not obtained or found in this project.

At the time incubating all the different bacteria, there were not used a CO_2 incubator, which may have affected the results. Other reports and studies with similar goals have deliberately used CO_2 incubators to be able to cultivate fastidious and picky bacteria [20], [25], [27], [28]. With a CO_2 incubator, the bacteria would have the optimum temperature, moisture, and pH in a sterile environment. If such an incubator had been available, there could have been obtained other bacteria beside those already found, and maybe there would be cases where *Mycoplasma* could have been cultured and found.

For the last nasal swabs, there were not as many bacteria colonies obtained, as from the first nasal swabs. A possible reason could have been that the blood agar plates were a bit old. After incubation, the blood agar turned lighter and lost its bright red coloration, plus there were not as many colonies seen on the plates as in the beginning. This would mean that the optimal growth medium was not as optimal as it could have been. Having new fresh blood agar plates would most likely have given other and better results with more bacteria growth.

For the DNA extraction and precipitation, most of the samples turned up with a low DNA concentration. Under DNA extraction process, there could have been reasons that may have affected the results. As seen under protocol 1, some of the bacteria cells from the broth mixture should maybe have been incubated for a longer time to achieve bigger growth, this could maybe give a higher DNA concentration. Some of the pellet could have been discarded or there could have been an old kit with old buffers. Or maybe it was not good enough sterile working technique that affected the results. When washed the DNA samples in ethanol, more of the samples should have had higher DNA concentration and better quality after the precipitation. When washing the bacterial pellet, only one side were washed, and not the other side that sticked to the wall of the Eppendorf tube. The pellet was also extremely small and hard to see where it was placed inside the tube. Without good enough pipette technique, maybe the pellet did not detach from the wall, meaning it would not have been completely lysed. Or it could have been pipette out by a mistake.

After the PCR and gel electrophoresis, the PCR products needed a PCR purification to remove unwanted salts, enzymes, and primers. As seen in table 6 and 7, there is a significant improvement in the DNA concentration as well in the quality. The first trials, different DNA concentrations of the PCR were used to see what concentration would give the best results on the gel and finest band. Samples that showed of band on the gel were used to perform a PCR purification. In the beginning, all the samples did not get a good quality, and lost a lot of DNA concentration after the purification. There was made several trials, help from other to see if it was the technique. None of these attempts help to get better results. Another possible solution could be that the kit from Qiagen was old and that the buffers did not work as they should do. With an old kit and solutions, the buffers could remove more than just the primers, enzymes, and impurities. A new PCR purification kit from Qiagen was ordered and the result did improve right away on the first trial. DNA concentration got higher (>40 ng/µL) and the quality improved significant. As seen in table 7, A260/A280 and A260/A230 had values around 2.0 which was where they should be, this meant that the samples could be prepared to be sent off for a sanger sequencing.

After receiving the files back from Germany, all samples got a good quality sequence, except the reverse strand for R5 C1 sample. The reverse strand did not get a sequence at all. When preparing the samples, the R5 C1 needed to have 26 µL to obtain right amount of DNA in correct volume. This sample did not have that a little bit more than enough solution for the preparation, but only the exact volume. A good pipette technique for transferring solutions was crucial. A possible solution that the reverse strand did not get a sequence may be because of the pipette technique. It could also be contaminated by some other solutions, or that it was just not high enough DNA in the sample to run the sequence. For the Blast search, only the forward strand was used to perform a search, and the result cannot be trusted 100%. If the reverse strand could also be used for a Blast search to find the species, the result could be more trustworthy.

Citrobacter freundii is a soil organism and a species of facultative anaerobic gram-negative bacteria. They have a long rod shape and most of them have several flagella used for movement, but there are also those who that do not have flagella and are therefore non-motile. *C. freundii* can also be found in water, sewage, food and the intestinal tract of animals and humans [29]. This bacteria is one of nitrogen-fixing bacteria, and have been reported and isolated from the gut of termites [30], they play an important role for reducing nitrate to nitrite in the environment, which is a crucial stage in the nitrogen cycle [29]. To discover this from the nose cavity of sheep is therefore not surprising. Sheep and lamb grass in the grassing field for most of the year around, and while having the nose and mouth down by the soil, this bacterium can travel from the soil with the grass, and up the respiratory tract. There have been reported a case where the bacterium have caused fatal septicemia and encephalitis in china [31]. In this report, 13 out of approximately 1300 sheep were found dead over a 6-day period. If there are sudden death in sheep with clinical signs of septicemia and encephalitis, it may be a good

idea to check for this bacterium. For humans, *C. freundii* can cause a wide spectrum of infections, such as infection in the urinary tract, respiratory tract, and pneumonia. Most common is the urinary tract, but also abdomen, skin/soft tissues and pneumonia [32]. From the samples collected from the sheep, this were apparently healthy and had no clinical signs of disease. This may not mean they were healthy but can mean that diseases have not been developed yet. For further investigation, it could be an idea to see if this bacterium can be a fatal pathogen in sheep and other animals in the future.

The second bacteria, *Moraxella osloensis*, is a member of the family *Moraxellacea*. These bacteria are coccobacillary gram-negative rods, and non-motile in liquid, but there are species that have a twitching mobility [33]. For *M. osloensis*, this bacterium is a saprophytic on the skin and mucosal surfaces, particularly the human respiratory tract, and it is a natural mutualistic symbiont of *Phasmarhabditis hermaphrodita*, which is a slug-parasitic nematode that can kill slugs and snails [34][35]. There have been reported cases where *M. osloensis* have caused certain diseases in humans, like meningitis, arthritis and osteomyelitis [33], but most cases are in cancer patients [36], [37]. With the way sheep lives most of the year around, it is not an unusual finding to cultivate *M. osloensis* from the nasal cavities of sheep. After grazing for several months, nematodes and bacteria can start to live inside the respiratory tracts. There have not been reported cases where *M. osloensis* have caused disease in sheep and lambs, but since it can cause infections in humans, there may be a risk that the bacterium can cause infections in sheep that already may be sick or have other underlying diseases. It might me an idea to research more on this bacterium. For human it may be a good idea to have good hygiene after handling and being around sheep and lambs, since the bacterium have caused infections for some people like for example bacteremia [38].

Enterococcus faecalis, is commonly found in the intestinal flora of humans and animals, in the mouth and vagina, and are a genus of gram-positive cook-shaped bacteria [39][40]. *E. faecalis* is a well-known cause of urinary tract infections and inflammation in surgical wounds, but has also caused sepsis, meningitis and endocarditis in individuals who have weakened immune system [39][40]. This bacterium are very resilient, which means they can survive in hot, salty or acidic environments, and are very common found in hospitals, which have given them a natural high resistance of antibiotics [39]–[41]. There have been reported a study where they checked if *Enterococcus* had vancomycin-resistance, a concern for human health, after isolated them from sheep feces. This study did not find any resistance in the samples they tested [42]. But there have been reported where *E. faecalis* have caused serious infections that have led to encephalitis and death of lambs [43], and also one report where there was an outbreak of mastitis caused by *E. faecalis* that was multi-drug resistant [44]. Due to that this bacterium led to infections in both humans and animals, and are highly resistant to several types of antibiotics, it may be an idea to take a sample of the bacteria and test to see which antibiotic works best against it. Since the bacterium is a part of the natural gastrointestinal tract flora, and are commonly found in soil, sewage, water and food samples resulting from fecal contamination [45], it

will therefore not be unusual to find them in the nasal cavities of sheep and lamb. Inside the barn for winter season, sheep have less space to walk around, and may therefore be contaminated with pathogens that can cause infections. If there are open wound, it should therefore be treated immediately to not cause serious infections.

The last two species cultured were both from the genus *Staphylococcus*, *S. xylosus*, and *S. succinus*. Both are gram-positive bacteria, *S.* xylosus forms clusters of cells while *S. succinus* forms rosettes where one cell is surrounded by two to five peripheral cells [46], [47]. Both are a form of coagulase-negative staphylococci, and are a part of the natural bacteria flora of the skin, skin glands and mucous membranes of various mammals and birds and as well in the environment [48], [49]. One major role of *S. xylosus* is used for meat fermentation, and there have been reported that a few strains can be potentially dangerous and are related to animal and human opportunistic infections [50]. Since the samples were taken on healthy rams and ram-lambs, these species are from the natural bacteria flora in the nasal cavities and may therefore not be a threat for the health.

All in all, these result show that for healthy sheep, there is a diversity of different bacteria species found in the nasal cavities and is a part of the natural flora needed to function normal. Some of the bacteria live as a natural part of the environment, and with a natural way of live, nematodes, parasites, bacteria can find their way into sheep and lambs, but for the most part, it will not be a threat for the health of sheep and lambs. If the lambs are sick, it can then be a threat, but with good monitoring, normal health care, proper feeding and separating the sick animals from the healthy ones, this will therefore lead to a better overall health for sheep and lambs, and therefore higher quality for meat, milk and wool production and general animal husbandry.

Summary & implications

General health and monitoring bacteria species that can lead to infections, diseases, and death of sheep, it is therefore important to see which bacteria species that lives inside the respiratory tract, and to treat this before they cause severe and fatal pneumonia or other fatal infections and diseases. This study had samples from apparently healthy rams and ram-lambs and can halfway conclude that they are healthy after the findings found in the nasal cavities. It might be that the findings did not find dangerous pathogens because of lacking equipment to grow fastidious bacteria, and for future research, it might be an idea to have the right tools to grow these pathogens. There were a few complications under the project, but all in all, it worked out at last, and there were good results that can be used to discuss how the general health for the sheep in Rogaland Norway is.

REFERENCES

[1] «Gardsbruk, jordbruksareal og husdyr», *ssb.no*. https://www.ssb.no/jord-skog-jakt-og-fiskeri/statistikker/stjord/aar-forelopige-tall/2021-01-07 (åpnet feb. 16, 2021).

[2] SSB, «Kjøttproduksjon årlig SSB», *ssb.no*. https://www.ssb.no/jord-skog-jakt-og-fiskeri/statistikker/slakt/aar/2020-03-19 (åpnet jan. 27, 2021).

[3] «Ull og ullklassifisering». https://www.animalia.no/no/Dyr/ull-og-ullklassifisering/ (åpnet mar. 29, 2021).

[4] Marion Tviland, «private communication», jan. 27, 2021.

[5] SSB, «Jordbruk», *SSB*. https://www.ssb.no/jord-skog-jakt-og-fiskeri/faktaside/jordbruk (åpnet jan. 27, 2021).

[6] Ø. Nesvåg, «Om Rogaland - Rogaland fylkeskommune». https://www.rogfk.no/om-fylkeskommunen/om-rogaland/ (åpnet mar. 29, 2021).

[7] Tore Oftedal, «Private communication», mar. 29, 2021.

[8] Tore Oftedal, «private communication», feb. 25, 2021.

[9] Kjell S. Rakkenes, «Nortura SA Årsmeling 2020». https://www.nortura.no/assets/documents/Nortura_aarsmelding_2020_print.pdf

[10] O. K. Stokka, «Informasjon om prisene på småfe for slaktesesongen 2020», *www.fatland.no*. https://www.fatland.no/bonde/smaafe/priser/informasjon-om-prisene-paa-smaafe-for-slaktesesongen-2020 (åpnet apr. 06, 2021).

[11] Finn Avdem, «Lammesesongen 2020». Nortura AS, jul. 02, 2020. Åpnet: apr. 06, 2021. [Online]. Tilgjengelig på: https://medlem.nortura.no/getfile.php/13383854-1593437239/Nortura%20Medlem/medlem.gilde.no/Bildearkiv/DYR/Sau/lammesesong%202020/lammesesongen_2020_web.pdf

[12] Mattilsynet, «Dyresykdommer | Mattilsynet», *Dyresykdommer*. https://www.mattilsynet.no/dyr_og_dyrehold/dyrehelse/dyresykdommer/ (åpnet jan. 27, 2021).

[13] A. V. Domke *mfl.*, «Worm control practice against gastro-intestinal parasites in Norwegian sheep and goat flocks», *Acta Vet. Scand.*, bd. 53, nr. 1, s. 29, des. 2011, doi: 10.1186/1751-0147-53-29.

[14] «Husdyrhald», *ssb.no*. https://www.ssb.no/jord-skog-jakt-og-fiskeri/statistikker/jordhus/aar/2020-05-28 (åpnet mar. 30, 2021).

[15] M. Bhatti *mfl.*, «Adapting Seasonal Sheep Production to Year-Round Fresh Meat and Halal Market in Norway», *Sustainability*, bd. 11, nr. 6, s. 1554, mar. 2019, doi: 10.3390/su11061554.

[16] Animalia, «Bærekraftig håndtering av rundorm hos sau». [Online]. Tilgjengelig på: https://legemiddelverket.no/Documents/Veterinærmedisin/Terapianbefalinger/Bærekraftig%20håndtering%20av%20rundorm%20hos%20sau.pdf

[17] «Lungeorm hos sau - for veterinærer». https://www.animalia.no/no/Dyr/sau/utvidet-sjukdomsregistrering-hos-sau--tiltak-ved-funn/lungeorm-hos-sau/ (åpnet apr. 13, 2021).

[18] «Sauehelsenett». https://www.animalia.no/no/Dyr/sauehelsenett/sjukdommer/luftveier/lungeorm/ (åpnet mai 14, 2021).

[19] Johanne Bergstrøm Sundell, «Den store leverikten (Fasciola hepatica)», *Den store leverikten (Fasciola hepatica)*. https://www.vetinst.no/sykdom-og-agens/den-stor-leverikten-fasciola-hepatica (åpnet apr. 06, 2021).

[20] L. L. Poulsen *mfl.*, «Occurrence of haemolytic Mannheimia spp. in apparently healthy sheep in Norway», *Acta Vet. Scand.*, bd. 48, nr. 1, s. 19, 2006, doi: 10.1186/1751-0147-48-19.

[21] D. Greenwood, *Medical microbiology: a guide to microbial infections : pathogenesis, immunity, laboratory diagnosis and control*, Eighteenth edition. Edinburgh, New York: Churchill Livingstone, 2012.

[22] R. Nicholas, R. Ayling, og L. McAuliffe, *Mycoplasma Diseases of Ruminants: Disease, Diagnosis and Control*. Wallingford, UNITED KINGDOM: CABI, 2008. Åpnet: apr. 13, 2021. [Online]. Tilgjengelig på: http://ebookcentral.proquest.com/lib/uisbib/detail.action?docID=369403

[23] Dr. James Evermann, «Mycoplasma ovipneumoniae Diagnostics in Domestic and Wild Sheep and Goats», *WADDL*. https://waddl.vetmed.wsu.edu/animal-disease-faq/mycoplasma-ovipneumoniae-diagnostics-in-domestic-and-wild-sheep-and-goats (åpnet apr. 13, 2021).

[24] I. Nikolaidis, S. Favini-Stabile, og A. Dessen, «Resistance to antibiotics targeted to the bacterial cell wall», *Protein Sci. Publ. Protein Soc.*, bd. 23, nr. 3, s. 243–259, mar. 2014, doi: 10.1002/pro.2414.

[25] F. A. Tauni, «Association of Mycoplasma ovipneumoniae Infection with Respiratory Disease in Swedish Sheep», s. 33.

[26] J. A. Frank, C. I. Reich, S. Sharma, J. S. Weisbaum, B. A. Wilson, og G. J. Olsen, «Critical Evaluation of Two Primers Commonly Used for Amplification of Bacterial 16S rRNA Genes», *Appl. Environ. Microbiol.*, bd. 74, nr. 8, s. 2461–2470, apr. 2008, doi: 10.1128/AEM.02272-07.

[27] B. Tesfaye, T. Sisay Tessema, og G. Tefera, «Diversity of bacterial species in the nasal cavity of sheep in the highlands of Ethiopia and first report of Histophilus somni in the country», *Trop. Anim. Health Prod.*, bd. 45, nr. 5, s. 1243–1249, jun. 2013, doi: 10.1007/s11250-013-0355-x.

[28] A. Sakmanoglu, Z. Sayin, U. S. Ucan, Y. Pinarkara, A. Uslu, og O. Erganis, «Comparison of five methods for isolation of DNA from Mycoplasma cynos», *J. Microbiol. Methods*, bd. 140, s. 70–73, sep. 2017, doi: 10.1016/j.mimet.2017.07.003.

[29] «*Citrobacter freundii*», *Wikipedia*. feb. 24, 2021. Åpnet: mai 14, 2021. [Online]. Tilgjengelig på: https://en.wikipedia.org/w/index.php?title=Citrobacter_freundii&oldid=1008636646

[30] M. Ohkuma, S. Noda, og T. Kudo, «Phylogenetic Diversity of Nitrogen Fixation Genes in the Symbiotic Microbial Community in the Gut of Diverse Termites», *Appl. Environ. Microbiol.*, bd. 65, nr. 11, s. 4926–4934, nov. 1999.

[31] H. Liu, Z. Zhao, Y. Xue, K. Ding, og Q. Xue, «Fatal cases of Citrobacter freundii septicemia and encephalitis in sheep», *J. Vet. Diagn. Invest.*, bd. 30, nr. 2, s. 245–248, mar. 2018, doi: 10.1177/1040638717731090.

[32] C. V. Marecos, M. Ferreira, M. M. Ferreira, og M. R. Barroso, «Sepsis, meningitis and cerebral abscesses caused by Citrobacter koseri», *Case Rep.*, bd. 2012, nr. jan23 1, s. bcr1020114941–bcr1020114941, jan. 2012, doi: 10.1136/bcr.10.2011.4941.

[33] «Moraxella spp», *MSDSonline*. https://www.msdsonline.com/resources/sds-resources/free-safety-data-sheet-index/moraxella-spp/ (åpnet mai 18, 2021).

[34] «Phasmarhabditis hermaphrodita». https://biocontrol.entomology.cornell.edu/pathogens/phasmarhabditis.php (åpnet mai 18, 2021).

[35] abrickmicrobiologicscom, «Environmental Isolate Case File: Moraxella osloensis», *Microbiologics Blog*, nov. 08, 2018. https://blog.microbiologics.com/environmental-isolate-case-file-moraxella-osloensis/ (åpnet mai 18, 2021).

[36] Y. Hadano, K. Ito, J. Suzuki, I. Kawamura, H. Kurai, og K. Ohkusu, «Moraxella osloensis: an unusual cause of central venous catheter infection in a cancer patient», *Int. J. Gen. Med.*, bd. 5, s. 875–877, okt. 2012, doi: 10.2147/IJGM.S36919.

[37] X. Y. Han og J. J. Tarrand, «Moraxella osloensis blood and catheter infections during anticancer chemotherapy: clinical and microbiologic studies of 10 cases», *Am. J. Clin. Pathol.*, bd. 121, nr. 4, s. 581–587, apr. 2004, doi: 10.1309/QBB3-AVCM-GWA3-K1XK.

[38] Y. Maruyama, T. Shigemura, K. Aoyama, N. Nagano, og Y. Nakazawa, «Bacteremia due to Moraxella osloensis: a case report and literature review», *Braz. J. Infect. Dis.*, bd. 22, nr. 1, s. 60–62, jan. 2018, doi: 10.1016/j.bjid.2017.10.008.

[39] «Enterococcus Faecalis: Causes, Symptoms, and Treatments», *Healthline*, sep. 26, 2017. https://www.healthline.com/health/enterococcus-faecalis (åpnet mai 18, 2021).

[40] K. Bøvre og T. Tønjum, «enterokokker», *Store medisinske leksikon*. jun. 23, 2020. Åpnet: mai 18, 2021. [Online]. Tilgjengelig på: http://sml.snl.no/enterokokker

[41] «*Enterococcus faecalis*», *Wikipedia*. mai 06, 2021. Åpnet: mai 18, 2021. [Online]. Tilgjengelig på: https://en.wikipedia.org/w/index.php?title=Enterococcus_faecalis&oldid=1021762366

[42] «Sheep11_is_Entero.pdf». Åpnet: mai 18, 2021. [Online]. Tilgjengelig på: https://www.aphis.usda.gov/animal_health/nahms/sheep/downloads/sheep11/Sheep11_is_Entero.pdf

[43] X. Zhou, X. Wang, B. Guo, og X. Wang, «Isolation and identification of Enterococcus faecalis and detection of its virulence factor genes in lambs presenting with encephalitis in Xinjiang province, China», *Afr. J. Microbiol. Res.*, bd. 7, nr. 20, s. 2238–2244, mai 2013, doi: 10.5897/AJMR12.912.

[44] G. Sanciu, G. Marogna, B. Paglietti, P. Cappuccinelli, G. Leori, og P. Rappelli, «Outbreak of mastitis in sheep caused by multi-drug resistant Enterococcus faecalis in Sardinia, Italy», *Epidemiol. Infect.*, bd. 141, nr. 3, s. 582–584, mar. 2013, doi: 10.1017/S0950268812000647.

[45] M. N. Byappanahalli, M. B. Nevers, A. Korajkic, Z. R. Staley, og V. J. Harwood, «Enterococci in the Environment», *Microbiol. Mol. Biol. Rev. MMBR*, bd. 76, nr. 4, s. 685–706, des. 2012, doi: 10.1128/MMBR.00023-12.

[46] «*Staphylococcus xylosus*», *Wikipedia*. feb. 24, 2021. Åpnet: mai 18, 2021. [Online]. Tilgjengelig på: https://en.wikipedia.org/w/index.php?title=Staphylococcus_xylosus&oldid=1008673651

[47] «*Staphylococcus succinus*», *Wikipedia*. feb. 24, 2021. Åpnet: mai 18, 2021. [Online]. Tilgjengelig på: https://en.wikipedia.org/w/index.php?title=Staphylococcus_succinus&oldid=1008673573

[48] «Staphylococcus equorum and Staphylococcus succinus isolated from human clinical

specimens», s. 523–528, mai 2006, doi: 10.1099/jmm.0.46246-0.

[49] N. Nagase *mfl.*, «Isolation and Species Distribution of Staphylococci from Animal and Human Skin», *J. Vet. Med. Sci.*, bd. 64, nr. 3, s. 245–250, 2002, doi: 10.1292/jvms.64.245.

[50] E. Dordet-Frisoni, G. Dorchies, C. De Araujo, R. Talon, og S. Leroy, «Genomic Diversity in Staphylococcus xylosus», *Appl. Environ. Microbiol.*, bd. 73, nr. 22, s. 7199–7209, nov. 2007, doi: 10.1128/AEM.01629-07.

Attachment 1 – Protocols

Protocol 1

DNeasy Gram ± Bacteria

Gram + Bacteria

1. Preheat a heating block to 37 ºC
2. From the broth-mixture, pipette 500 µL into Eppendorf tubes and centrifuge at 1 min at 6000 rcf. Discard the supernatant.
3. Resuspend bacterial pellet in 180 µL enzymatic lysis buffer (Buffer ASL)
4. Incubate for at least 30 min at 37 ºC
5. Add 25 µL proteinase K and 200 µL Buffer AL. Mix by vortexing and spin the tubes.
6. Incubate at 56 ºC for 30 min
7. Add 200 µL ethanol (96-100%), mix thoroughly by vortexing
8. Pipet the mixture into DNeasy Mini Spin column. Centrifuge at 6000 rcf for 1 min. Discard flow-through
9. Pipet the Gram – mixture to the spin column and centrifuge again. Discard flow-through and collection tube.

Gram - Bacteria

1. Preheat a heating block to 56 ºC
2. From the broth-mixture, pipette 500 µL into Eppendorf tubes and centrifuge at 1 min at 6000 rcf. Discard supernatant
3. Resuspend bacterial pellet in 180 µL Buffet ATL
4. Add 20 µL proteinase K. Mix thoroughly by vortexing and incubate at 56 ºC until the tissue is completely lysed. Vortex occasionally during incubation
5. Vortex for 15 sec. Add 200 µL Buffer AL and mix thoroughly by vortexing
6. Add 200 µL ethanol (96-100%) and mix thoroughly by vortexing
7. Pipet the mixture to the spin column containing Gram + mixture

8. Place the column in a new 2 mL collection tube, add 500 µL Buffer AW1, centrifuge for 1 min at 6000 rcf. Discard flow-through and collection tube
9. Place the column in a new 2 ml collection tube, add 500 µL Buffer AW2, centrifuge for 3 min at 16 873 rcf. Discard flow-through and collection tube
10. Place the column in a clean 1,5 mL Eppendorf tube, pipet 25 µL Buffer AE directly onto the DNeasy membrane. Incubate at RT for 1 min, centrifuge then for 1 min at 6000 rcf to elute.
11. Measure the DNA concentration on NanoDrop.

Protocol 2

DNA Precipitation (24 µL sample)

Day 1:

- Add 23 µL 10 M ammonium Acetate to the samples
- Add 52,8 µL ice cold 99% ethanol and mix well
  → Let the ethanol stand in the freezer for 30 min
- Put the samples in the freezer overnight at -20 ºC

Day 2:

- Set the centrifuge at 4 ºC, it takes approximately 15 min
- Centrifuge the samples at 4 ºC for 30 min at 15 000 rcf
- Pipette the supernatant carefully out without disturbing the pellet
- Wash the DNA pellet by adding 200 µL 70% ethanol
- Centrifuge the samples at 4 ºC for 30 min at 15 000 rcf
- Pipette the supernatant carefully out without disturbing the pellet
- Repeat step 3-5, but centrifuge only 1for 5 min at 15 000 rcf
- Air-dry the pellet for 5-20 min until all the ethanol is gone. Close the lid when the ethanol is gone
- Redissolve the DNA in 20 µL Buffer AE
- Watm the mixture at 37-42 ºC for approximately 10 min
- Gently vortex the mixture and spin the tubes
- Measure the DNA concentration on NanoDrop

Protocol 3

PCR

Gently vortex and centrifuge DreamTaq PCR Master Mix (2x) after thawing

Place a tube on ice and add the following components for each 50 µL reaction:

DreamTaq PCR Master Mix (2x)	25 µL	$25\ \mu L + 10\% \times x\ samples$
Forward primer	2 µL	$2\ \mu L + 10\% \times x\ samples$
Reverse primer	2 µL	$2\ \mu L + 10\% \times x\ samples$
Water, Milli-Q	11 µL	$11\ \mu L + 10\% \times x\ samples$
TOTAL		X µL

Samples + 1 negative control = x

Place the PCR tubes on ice and add 40 µL of the master mix to each tube

Calculate the amount of DNA we need to take out and add the rest amount off Milli-Q water to have a total of 10 µL

50 µL / DNA concentration = amount DNA solution

Take the PCR tubes into a laminar flow hood and add the 10 µL DNA to the PCR reaction solution. Spin the tubes

Run the PCR

95°C → 7 min

95°C → 30 sec

58°C → 30 sec

} 31 cycles

72°C → 1 min

72°C → 10 min

Protocol 4

Gel electrophoresis

1. Make a 200 mL 1xTAE buffer from 20 mL 10xTAE and 180 mL Milli-Q water
2. Make the gel:

TAE concentration	Amount agarose in 100 ml water
0.8%	0.8 g
1%	1 g
1.5%	1.5 g
2%	2 g

   - For large gel: 0,9 g agarose in 100 mL 1xTAE
   - Small gel: Same amount, but don't pour all the gel in the tray
   - Add the agarose to an Erlenmeyer flask (250 mL) and pour the 1xTAE into the flask
   - Boil in a microwave at full power for 2 min. Stop every 30 sec to stir, then every 15 sec after 1 min
   - Pour the solution into a casting frame with a comb after it has cooled down a bit
   - Let it polymerize for 30 min
   - Transfer the gel to the electrophoresis machine
3. Prepare the samples for the gel:
   - For large gel, add these solutions, small gel: only half the amount
     - Gel-Red = 2,0 µL
     - Loading Buffer = 2,4 µL
     - DNA sample = 2,0 µL
     - Milli-Q water = 3,6 µL
     - TOTAL = 10,0 µL
   - Load 9 µL onto the gel, small gel: 4 µL
4. Run the gel electrophoresis
5. Take picture, ChemiDoc visualization
   - Choose the following:
     - Application: Nucleic acid + Gel-Red
     - Image size: small
     - Exposure: Intense bands

Protocol 5

PCR Purification - Qiagen

1. Add 5 volumes Buffer PB to 1 volume of the PCR reaction and mix
2. Place a QIAquick column in a provided 2 mL collection tube
3. To bind DNA, apply the sample to the QIAquick column and centrifuge for 1:15 minute at 16 873 rcf
4. Discard the flow-through and place the QIAquick column back into the same tube
5. To wash, add 750 µL Buffer PE to the column and centrifuge for 1:15 minute at 16 873 rcf
6. Discard the flow-through and place the QIAquick column back into the same tube
7. Centrifuge the column for an additional 1:15 minute at 16 873 rcf and discard the flow-thorugh
8. Place the QIAquick column in a clean 1.5 mL Eppendorf tube
9. To elute DNA, add 15 µL Buffer EB to the center of the QIAquick membrane and let it incubate in room temperature for 1 minute. Centrifuge for 1:15 minute at 16 873 rcf
10. Repeat step 9: Add 15 µL Buffer EB, incubate and centrifuge
11. Measure the DNA concentration on NanoDronp

Attachment 2 – DNA sequences

R5 C3 = *Staphylococcus xylosus*

GCTTGCTCCTTTGAAGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTACCTA
TAAGACTGGGATAACTTCGGGAAACCGGAGCTAATACCGGATAACATTTAGAACCGC
ATGGTTCTAAAGTGAAAGATGGTTTTGCTATCACTTATAGATGGACCCGCGCCGTAT
TAGCTAGTTGGTAAGGTAACGGCTTACCAAGGCGACGATACGTAGCCGACCTGAGAG
GGTGATCGGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAG
TAGGGAATCTTCCGCAATGGGCGAAAGCCTGACGGAGCAACGCCGCGTGAGTGATGA
AGGGTTTCGGCTCGTAAAACTCTGTTATTAGGGAAGAACAAATGTGTAAGTAACTGT
GCACATCTTGACGGTACCTAATCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGC
GGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGTAG
GCGGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAA
ACTGGGAAACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGTGAAATG
CGCAGAGATATGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCTGTAACTGACG
CTGATGTGCGAAAGCGTGGGGATCAAACAGGATTAGATACCCTGGTAGTCCACGCCG
TAAACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGCTAACGC
ATTAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACG
GGGACCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTT
ACCAAATCTTGACATCCTTTGAAAACTCTAGAGATAGAGCCTTCCCCTTCGGGGGAC
AAAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAA
GTCCCGCAACGAGCGCAACCCTTAAGCTTAGTTGCCATCATTAAGTTGGGCACTCTA
GGTTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCC
CCTTATGATTTGGGCTACACACGTGCTACAATGGACAATACAAAGGGCAGCTAAACC
GCGAGGTCATGCAAATCCCATAAAGTTGTTCTCAGTTCGGATTGTAGTCTGCAACTC
GACTACATGAAGCTGGAATCGCTAGTAATCGTAGATCAGCATGCTACGGTGAATACG
TTCCCGGGTCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAACACCCGAAGC
CGGTGGAGTAACCA

R19 C7 = ***Staphylococcus xylosus***

AGTCGAGCGAACGGATAAGGAGCTTGCTCCTTTGAAGTTAGCGGCGGACGGGTGAG
TAACACGTGGGTAACCTACCTATAAGACTGGGATAACTTCGGGAAACCGGAGCTAAT
ACCGGATAACATTTAGAACCGCATGGTTCTAAAGTGAAAGATGGTTTTGCTATCACTT
ATAGATGGACCCGCGCCGTATTAGCTAGTTGGTAAGGTAACGGCTTACCAAGGCGAC
GATACGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGAACTGAGACACGGTCCAG
ACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGGCGAAAGCCTGACGGA
GCAACGCCGCGTGAGTGATGAAGGGTTTCGGCTCGTAAAACTCTGTTATTAGGGAAG
AACAAATGTGTAAGTAACTGTGCACATCTTGACGGTACCTAATCAGAAAGCCACGGC
TAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTAT
TGGGCGTAAAGCGCGCGTAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCA
ACCGTGGAGGGTCATTGGAAACTGGGAAACTTGAGTGCAGAAGAGGAAAGTGGAATT
CCATGTGTAGCGGTGAAATGCGCAGAGATATGGAGGAACACCAGTGGCGAAGGCGAC
TTTCTGGTCTGTAACTGACGCTGATGTGCGAAAGCGTGGGGATCAAACAGGATTAGA
TACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCCC
CTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTT
GAAACTCAAAGGAATTGACGGGGACCCGCACAAGCGGTGGAGCATGTGGTTTAATTC
GAAGCAACGCGAAGAACCTTACCAAATCTTGACATCCTTTGAAAACTCTAGAGATAGA
GCCTTCCCCTTCGGGGGACAAAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTG
TCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTAAGCTTAGTTGCCAT
CATTAAGTTGGGCACTCTAGGTTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGA
TGACGTCAAATCATCATGCCCCTTATGATTTGGGCTACACACGTGCTACAATGG
ACAATACAAAGGGCAGCTAAACCGCGAGGTCATGCAAATCCCATAAAGTTGTTCTCA
GTTCGGATTGTAGTCTGCAACTCGACTACATGAAGCTGGAATCGCTAGTAATCGTAG
ATCAGCATGCTACGGTGAATACGTTCCCGGGTCTTGTACACACCGCCCGTCACACCA
CGAGAGTTTGTAACA

R5 C1 = *Staphylococcus succinus*

ACAATACAAAGGGCAGCTAAACCGCGAGGTCATGCAAATCCCATAAAGTTGTTCTCA
GTTCGGATTGTAGTCTGCAACTCGACTACATGAAGCTGGAATCGCTAGTAATCGTAG
ATCAGCATGCTACGGTGAATACGTTCCCGGGTCTTGTACACACCGCCCGTCACACCA
CGAGAGTTTGTAACA

R19 C3 = *CIitrobacter freundii*

GCTTGCTCCTTGGGTGACGAGTGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCC
GATGGAGGGGGATAACTACTGGAAACGGTAGCTAATACCGCATAACGTCGCAAGACC
AAAGAGGGGGACCTTCGGGCCTCTTGCCATCGGATGTGCCCAGATGGGATTAGCTA
GTAGGTGGGGTAACGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGA
CCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGG
AATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCC
TTCGGGTTGTAAAGTACTTTCAGCGAGGAGGAAGGTGTTGTGGTTAATAACYGCAGA
AATTGACGTTACTCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAA
TACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGT
CTGTCAAGTCGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATCCGAAACTGG
CAGGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTA
GAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTC
AGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAA
ACGATGTCGACTTGGAGGTTGTGCCCTTGAGGCGTGGCTTCCGGAGCTAACGCGTT
AAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGG
GCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACC
TACTCTTGACATCCAGA

R27 C1 = ***Enterococcus faecalis***

AATTGGAAAGAGGAGTGGCGGACGGGTGAGTAACACGTGGGTAACCTACCCATCAGA
GGGGGATAACACTTGGAAACAGGTGCTAATACCGCATAACAGTTTATGCCGCATGGC
ATAAGAGTGAAAGGCGCTTTCGGGTGTCGCTGATGGATGGACCCGCGGTGCATTAG
CTAGTTGGTGAGGTAACGGCTCACCAAGGCCACGATGCATAGCCGACCTGAGAGGGT
GATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAG
GGAATCTTCGGCAATGGACGAAAGTCTGACCGAGCAACGCCGCGTGAGTGAAGAAGG
TTTTCGGATCGTAAAACTCTGTTGTTAGAGAAGAACAAGGACGTTAGTAACTGAACG
TCCCCTGACGGTATCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGT
AATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCG
GTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACT
GGGAGACTTGAGTGCAGAAGAGGAGAGTGGAATTCCATGTGTAGCGGTGAAATGCG
TAGATATATGGAGGAACACCAGTGGCGAAGGCGGCTCTCTGGTCTGTAACTGACGCT
GAGGCTCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTA
AACGATGAGTGCTAAGTGTTGGAGGGTTTCCGCCCTTCAGTGCTGCAGCAAACGCAT
TAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGG
GGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTAC
CAGGTCTTGACATCCTTTGACCACTCTAGAGATAGAGCTTTCCCTTCGGGGACAAAG
TGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCC
CGCAACGAGCGCAACCCTTATTGTTAGTTGCCATCATTCAGTTGGGCACTCTAGCGA
GACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTT
ATGACCTGGGCTACACACGTGCTACAATGGGAAGTACAACGAGTCGCTAGACCGCGA
GGTCATGCAAATCTCTTAAAGCTTCTCTCAGTTCGGATTGCAGGCTGCAACTCGCCT
GCATGAAGCCGGAATCGCTAGTAATCGCGGATCAGCACGCCGCGGTGAATACGTTCC
CGGGCCTTGTACACACCGCCCGTCACACCACGAGAG

R28 C2 = *Moraxella osloensis*

GGACGGGTGAGTAACATTTAGGAATCTGCCTAGTAGTGGGGGATAGCTCGGGGAAA
CTCGAATTAATACCGCATACGACCTACGGGTGAAAGGGGGCGCAAGCTCTTGCTATT
AGATGAGCCTAAATCAGATTAGCTAGTTGGTGGGGTAAAGGCCCACCAAGGCGACGA
TCTGTAACTGGTCTGAGAGGATGATCAGTCACACCGGAACTGAGACACGGTCCGGAC
TCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGGGCAACCCTGATCCAGC
CATGCCGCGTGTGTGAAGAAGGCCTTTTGGTTGTAAAGCACTTTAAGCAGGGAGGAG
AGGCTAATGGTTAATACCCATTAGATTAGACGTTACCTGCAGAATAAGCACCGGCTAA
CTCTGTGCCAGCAGCCGCGGTAATACAGAGGGTGCGAGCGTTAATCGGAATTACTGG
GCGTAAAGCGAGTGTAGGTGGCTCATTAAGTCACATGTGAAATCCCCGGGCTTAACC
TGGGAACTGCATGTGATACTGGTGGTGCTAGAATATGTGAGAGGGAAGTAGAATTCC
AGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGATGGCGAAGGCAGCT
TCCTGGCATAATATTGACACTGAGATTCGAAAGCGTGGGTAGCAAACAGGATTAGAT
ACCCTGGTAGTCCACGCCGTAAACGATGTCTACTAGCCGTTGGGGTCCTTGAGACTT
TAGTGGCGCAGTTAACGCGATAAGTAGACCGCCTGGGGAGTACGGCCGCAAGGTTAA
AACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGA
TGCAACGCGAAGAACCTTACCTGGTCTTGACATAGTGAGAATCTTTCAGAGATGAGA
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CAGAGGG