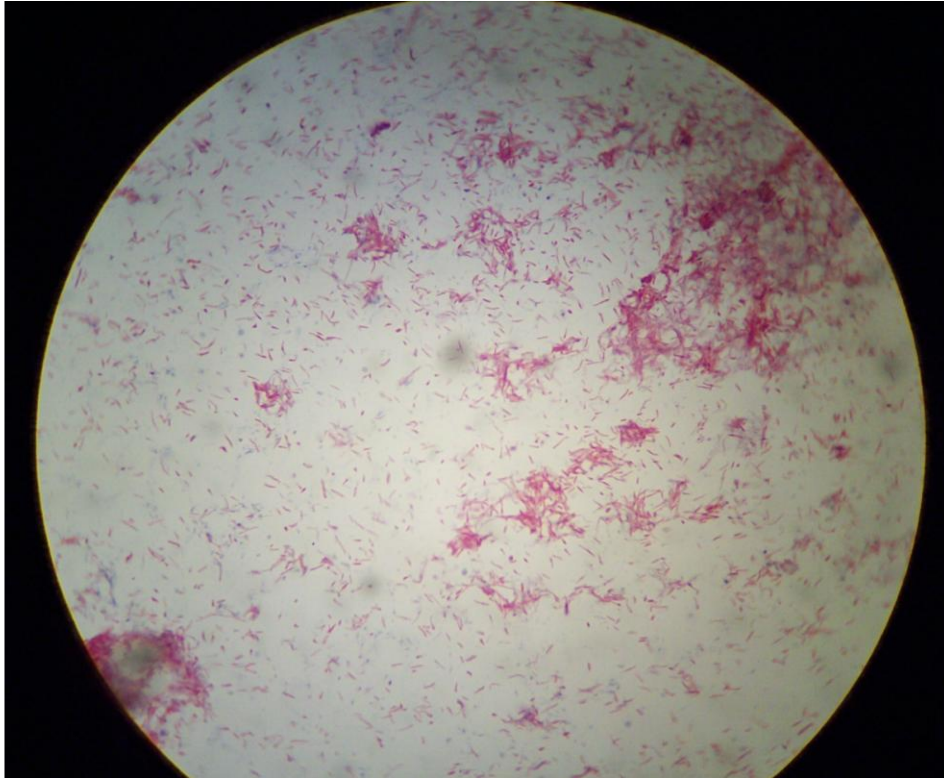
Environmental Mycobacteria

The impact on Mycobacterium avium subspecies paratuberculosis infection in calves



S.J.M. Verkleij
Studentnumber: 3051439
June - August 2010

Supervisors: Karin Orsel (UCVM)
Rienske Mortier (UCVM)
Susanne Eisenberg (UU)
Department: Production Animal Health, of the faculty of Veterinary Medicine, University of Calgary.

Index

Summary 2

Introduction 3

Materials and Methods 6

Results 10

Discussion 19

Acknowledgements 22

References 23

Attachments 25

Summary

Johne's Disease is a debilitating chronic enteritis caused by *Mycobacterium avium subsp. paratuberculosis* (Map), and has great economic impact on affected dairy farms.[1, 2]
Infection occurs in young calves, by exposure to manure from mature Map-shedding cattle. To reduce infection status on farms, prevention plans are in order, because no treatment is available for Johne's Disease. Prevention plans for Johne's disease rely on age-dependent susceptibility, with a cut-off at the age of six months. This cut-off is based on one paper from 1975, where only 9 animals were included in the experiment[3].
In a clinical trial at the University of Calgary this age dependent susceptibility is being investigated with a larger number of animals: 34 male Holstein-Friesian calves are subject of a research to age- and dose dependent susceptibility to infection with Map. They will be infected with a virulent Map strain at 5 different ages (2 weeks, 3-6-9 and 12 months) with a high-(5 x 10^9 CFU) and a low inoculum dose (5 x 10^7 CFU).
Besides this pathogenic mycobacterium, there are also environmental mycobacteria where young calves are exposed to. These mycobacteria can be found in a wide variety of environmental reservoirs, including natural and municipal water, soils, aerosols and protozoans.[4] Because of ingestion and close contact with these reservoirs, environmental mycobacteria can also be found in animals and humans.
Evidence exists for a cross-reaction in immunity between rapid growing environmental mycobacteria and other pathogenic mycobacterial species (tuberculosis and leprosy) in humans[4]. That's why we are interested in the impact of environmental mycobacteria on the infection with Map in calves.
There are different protocols known for isolating mycobacteria from water, soil or milk[5, 6], but there is none for isolating mycobacteria from faeces.
That was the aim of this study: to identify an appropriate protocol to isolate different mycobacteria from faeces. *Mycobacterium smegmatis* was used as a model for rapid growing mycobacteria.
Unfortunately, no perfect protocol was found. An important conclusion of this research is that HPC (Hexadecyl Pyridinium Chloride), frequently used to isolate Map from faeces, is not appropriate to isolate environmental mycobacteria, because *M. smegmatis* does not survive concentrations higher than 0,1%, and other faecal bacteria do.

Introduction

Johne's disease (JD) is a debilitating chronic enteritis in ruminants caused by *M. avium subsp. paratuberculosis* (Map). In infected herds, calves are likely to be exposed to manure from mature cattle that shed the bacteria in their faeces, resulting in contaminating water, feed or even milk. Infection can occur in young calves with a dose which is easily surpassed in 2 grams of heavily infected faeces [7]. The infectious dose is likely to increase with increasing age.
Infection with Map slowly evolves through 4 stages, defined by the severity of clinical signs, the shedding of organisms in the environment and the ease of detection by current laboratory methods. In stage 1 (silent infection) no effects of infection can be observed or detected diagnostically. In stage 2 (subclinical disease) a portion of the animals will start shedding the bacteria in a low dose and alter their immune response. Only 15-25% of infected cattle can be detected by fecal culture. In this stage they mainly have subclinical signs: decrease of milk production, decrease of fertility and a higher mastitis prevalence. Stages 3 (clinical disease) and 4 (advanced clinical disease) are characterized by increasingly severe clinical symptoms. Stage 3 consist of periods of intermitting diarrhea. This stage takes about 3-4 months before they progress to the terminal stage of disease, stage 4. Most animals will have positive diagnostic test results: serum ELISA and fecal culture. Symptoms are chronic waterhose diarrhea, intermandibular edema caused by hypoproteinemia, emaciation. Eventually death will occur as result of dehydration and cachexia, since there is no cure available [8].

JD has a great economic impact on dairy farms, caused by premature culling, reduced carcass value, decreased weight gain and milk production in the subclinical stage and eventually leading to chronic diarrhea, emaciation, debilitation and death. JD costs the US dairy industry 200-250 million dollars annually [1]. The direct cost of JD to the Canadian dairy industry is estimated at $15 million annually [1]. Seroprevalence reported in Canadian dairy cattle ranges at the herd level from 10% in Ontario to 40% in Alberta[9]. An Alberta study with fecal samples suggests that the true herd-level prevalence (seroprevalence cutt-off point >2 animals) ranges from 28 to 57%, estimated to be the highest in the country[10]. The median costs of JD per 50 cow herd is $2556[2]. Beef cattle and beef herds are generally at lower risk of being infected than dairy, although this may vary by geographical region world wide[11].

Recent meta-analyses demonstrate that the association of Map with Crohn's disease in human is specific and cannot be denied[12, 13], although a causal role has not yet been demonstrated. Neither has the transmission from cattle to humans been proven. However, this potential zoonotic implication hangs over the cattle industry as the sword of Damocles, especially since viable Map bacteria have been found in retail pasteurized milk[14]. So, potential losses to

the dairy industry are also those associated with consumer confidence in foods of ruminant origin.

To control the spread of Map, 2 kinds of intervention need to be taken: prevent new infections and remove shedding animals.
To remove shedding animals, a test-based culling intervention is typically recommended. Current diagnostic tests, such as ELISA, fecal culture and fecal PCR have high test sensitivities for detecting infectious animals shedding high levels of Map (in stage 3 and 4), but relatively low test sensitivities for detecting animals shedding low levels [15-18]. They indeed produce many false-negative results in subclinically infected animals (stage 2), making interpretation and utilization in the majority of the animals challenging.
In addition to culling of infectious animals, changes to management practices are effective in reducing the transmission rates due to direct contacts between the susceptible and infectious animals. All existing recommendations[1] for decreasing the risk of new infections of Map in a dairy operation are directly meant to reduce the infection rate in calves by decreasing the contact with manure of adult cows and assume an age-dependent susceptibility to infection with a cut-off at 6 months of age.

Besides pathogenic mycobacteria like Map, there are also environmental mycobacteria where young calves get in touch with. That is where this side-project will be focusing on within the age-dose susceptibility PhD project: environmental mycobacteria.
There are 91 species identified in the genus Mycobacterium, which are not in the *M. Tuberculosis* complex. But there are still recent reports of mycobacterial isolates from the environment (water, soil, air and patients) that don't belong to the identified species[4].
Environmental opportunistic mycobacteria distinguish themselves by the fact that they are true inhabitants of the environment, and not obligate pathogens, like the members of *M. avium complex, M. tuberculosis* and *M. leprae* are.
There are two groups of environmental mycobacteria: slow-growing (colony formation requires 7 days or more) and rapidly-growing (colony formation requires less than 7 days). But even rapidly-growing mycobacteria have a growth rate which is significant lower than most bacteria.
Besides growth-rate there is more variation between environmental mycobacteria, like colony morphologies, antibiotic- and biocide sensitivities, plasmid carriage and virulence. There are also shared characteristics, like their great hardiness, acid-fast cell wall containing mycolates, and intracellular pathogenicity.
Environmental mycobacteria can be found in a wide variety of environmental reservoirs, including natural and municipal water, soils, aerosols, protozoans, animals and humans [4].
Mycobacterial species have traditionally been classified by growth rate, thermo tolerance, pigmentation, and biochemical and enzymatic tests. More recently these techniques have been supplemented with for example; DNA–

DNA hybridization, high-performance liquid chromatography analysis of mycolic acids, and sequence and/or restriction analysis of the 16S rRNA-gene[19].

Because of the presence of environmental mycobacteria in different reservoirs it's likely that humans and animals infected with pathogenic mycobacteria also have been in touch with them. There are papers which report evidence for a cross-reaction between rapid growing environmental mycobacteria and other mycobacterial species, which may alter the immune status of the patient leading to more or less susceptibility to development of disease due to mycobacterial priming [4].
Human tuberculosis purified protein derivative skin testing shows cross-reactivity with environmental mycobacteria exposure[20-23]. Also a BCG vaccination against tuberculosis in an Indian trial shows cross-reaction with environmental mycobacteria. A poor efficacy of the vaccination shows up after common exposure to environmentals [24]. The interactions of environmental mycobacteria and pathogenenic mycobacteria on the immune system are not yet understood, but it seems to be that environmental mycobacteria do have impact on pathogenic mycobacteria.
Therefore it would be interesting to identify the spectrum of mycobacterial species in the environment of the research animals of the age- dose-susceptibility project, just as the excretion pattern by individual animals. Then there will be an ability to anticipate for possible effects of the environmental mycobacteria on the challenge infection during the trial.

The first aim of this study is to identify an appropriate protocol to isolate different mycobacteria out of faeces, because that's the medium where we suppose all ingested mycobacteria accumulate.
After a good protocol is formulated, another aim will be to identify different Mycobacterial species in faeces of calves of different-age and infection-status.

Materials and Methods

Experimental set up:

For the research to age- and dose dependent susceptibility to infection with Map, 34 Male Holstein-Friesian calves where obtained from Map-unsuspected dairy herds. They are/ will be infected with a virulent Map strain at 5 different ages (2 weeks, 3-6-9 and 12 months) within each group 3 calves receiving a relatively high bacterial inoculum dose (5 x 10^9 CFU) and 3 calves receiving a relatively low dose (5 x 10^7 CFU). 3 control calves will not be experimentally infected.

The calves are individually housed in a biosecurity level 2 housing facility (a former dairy facility, now rented by University of Calgary and linked to the Veterinary Sciences Research Station) with highly trained personnel to take care of the animals on a daily basis. There are 2 barns at the facility. The smaller barn can be heated and was used for housing the newborn calves in the winter. The housing units consists of a pen (6 by 8 feet) containing the calf, and a marked zone in front of the pen where bootdip, boots, coveralls and gloves are provided, to be used when entering the pen. This way no materials will be moved from one calf to another.

The other barn contains larger individual pens (6 by 14 feet), where the steers will be housed from 3-4 months of age until necropsy at 18 months. Also in this barn every pen has a marked zone for boots, gloves and coveralls, so no materials need to be exchanged between cows when entering the pen.

The calves were fed 6L of colostrum (collected from seronegative farms and irradiated with gamma irradiation) within 12 hours after birth. Then they were fed with High Performance Calf Milk Replacer (Grober Nutrition, USA) following a prescribed feeding schedule. From 2 weeks of age calf starter (FeedRite, Canada) without added quinolones and hay was provided. At 7 weeks of age the calves where weaned. At 6 months of age, calf starter pellets were replaced by calf grower pellets, without monensin (Feedrite, Canada).

The pens are bedded with wood shavings (UFA, Canada).

The calves are/will be routinely sampled.

Method of sampling:

The fecal samples were collected by anal massage to stimulate the calf to strain. The feces were captured in a fecal container, refrigerated until transport.

The blood and serum samples were taken using a vacutainer, after cleaning the skin on the Vena Jugularis with chlorhexidine gauzes and alcohol gauzes.

The urine samples in the younger calves (up to 3 months) were achieved by manipulating the preputium, what stimulated them to urinate. After 3 months of age, the urine samples were achieved by gluing a rubber glove to the preputial skin, so the glove caught the urine when they urinated.

Only fresh fecal samples were used for this research on environmental mycobacteria. The other samples were taken for the age- and dose susceptibility project.

M. smegmatis
The model used for rapid-growing environmental mycobacteria is *M. smegmatis.* The reason for this is that there was a M. smegmatis stock in the freezer, it grows on LB-agar and in LB-broth, and it grows relatively fast for mycobacteria, so it's not necessary to wait very long to see the results of the different isolation protocols.
M. smegmatis grows well in LB-broth, except we discovered that clumps will be formed. In some articles Tween 80 is used to avoid that[25, 26]. Different concentrations of Tween 80 have been used to try to let *M. smegmatis* grow in LB-broth without clumping. (0, 0,1, 0,3, 0,5, 0,7, 0,9 and 1%) The bacteria where transferred from a LB-agar plate with a sterile öse into LB-broth with Tween 80. The settings of the shaker (New Brunswick Scientific, Excella E24 Incubator Shaker Series) were: 37°C, 177 RPM.

Protocol 1: Spiking *M. smegmatis* in faeces, different concentrations HPC
The first protocol tried is spiking different amounts of *M. smegmatis* in 2 grams faeces (frozen), using different dilutions of Hexadecyl Pyridinium Chloride (Alfa Aesar, USA) in ½ Brain Heart Infusion (BD, Canada). This is to see how different amounts of *M. smegmatis* react on different concentrations HPC.
See attachment for the entire protocol. Briefly, 2 grams of feaces were mixed with 100 µl different *M. smegmatis* dilutions (None *M. smegmatis* added, undiluted (10^7 CFU/ml expected), 1/10 times diluted, 1/100 times diluted, 1/1000 times diluted and 1/10 000 times diluted). This was solved in 25 ml ½ BHI with X% HPC (0, 0,2, 0,4, 0,6, 0,8 and 1%). This was placed in a stove ((37°C, 5%CO_2) (ThermoScientific Steri-Cycle CO2 incubator, Hepa Class 100) for 24 hours. The upper 5 ml was transferred to a 15 ml Falcontube (VWR, Canada) and centrifuged for 30 minutes at 900G (Thermo Scientific Sorvall legend RT+ centrifuge). The pellet was solved in 5 ml ½ BHI, serial dilutions were made and 100 µl was inoculated on a LB-agar plate, incubated in the stove for several days.

Protocol 2: Only faeces in different concentrations HPC
This second protocol tried is to see what different concentrations HPC do to fresh faeces (from calf 33). For the entire protocol, see attachment. Briefly, 2 grams of faeces were solved in 25 ml ½ BHI with different concentrations HPC (0, 0,1, 0,2, 0,3, 0,4, 0,5, 0,6, 0,7, 0,8, 0,9 and 1%). This mixture was placed in the stove((37°C, 5%CO_2) for 24 hours. The upper 5 ml was transferred into a Falcontube and centrifuged for 30 minutes at 900G. The pellet was solved in 5 ml ½ BHI, 100 µl was inoculated onto LB-agarplates and placed in the stove for several days.

Protocol 3: Only *M. smegmatis* in different concentrations HPC

The third protocol tried is to see if and how *M. smegmatis* survives in different concentrations HPC. For the entire protocol, see attachment. Briefly, 100 µl *M. smegmatis* was solved in 10 ml ½ BHI with different concentrations HPC (0, 0,1, 0,2, 0,3, 0,4, 0,5, 0,6, 0,7, 0,8, 0,9 and 1%). This mixture was placed in the stove((37°C, 5%CO_2) for 24 hours. The upper 5 ml was transferred into a Falcontube and centrifuged for 30 minutes at 900G. The pellet was solved in 5 ml ½ BHI, 100 µl was inoculated onto LB-agarplates and placed in the stove for several days.

Protocol 4: *M. smegmatis* with 3% SDS, different concentrations NaOH

This fourth protocol is to see if and how *M. smegmatis* survives 3% SDS (Sodium Docedyl Sulfate (Fisher Scientific, Canada), and NaOH (Fisher Scientific, Canada). For the entire protocol, see attachment. Briefly 100µl *M. smegmatis* was added to 10 ml 3% SDS with different concentrations NaOH (1, 2 and 4%). This mixture was shaken for 30 minutes in the shaker (37 °C, 220 RPM) (New Brunswick Scientific, Excella E24 Incubator Shaker Series). H_3PO_4 was added to neutralize the mixture, then it was centrifuged for 20 minutes at 3716G (Thermo Scientific Sorvalllegend RT+ centrifuge). The pellet was solved in 15 ml destilled water, and centrifuged again at 3716G for 20 minutes. The pellet was solved in 1,5 ml destilled water, 100 µl was inoculated onto LB-agarplates, and placed in the stove for several days.

Protocol 5: *M. Smegmatis* in 3%SDS with 0%, 0,2%, 0,4%, 0,6%, 0,8% and 1% NaOH. 3% SDS with 1% and 2% cetrimide

A fifth protocol was tried to see what *M. smegmatis* does in 3% SDS with different concentrations NaOH. Also Cetrimide (Myristyltrimethylammoniumbromide (Sigma Aldrich, Canada)) in combination with SDS was tried. The entire protocol is added in the attachments. Briefly 100µl *M. smegmatis* was added to 10 ml 3% SDS with different concentrations NaOH (0, 0,2, 0,4, 0,6, 0,8,and 1%). This mixture was shaken inoculated at room temperature for 15 minutes. Then it was centrifuged for 15 minutes at 935G (Thermo Scientific Sorvall legend RT+ centrifuge). The pellet was solved in 10 ml X% Cetrimide (1 or 2%) for 5 minutes, and centrifuged again at 935G for 15 minutes. The pellet was solved in 15 ml destilled water, and centrifuged for 15 minutes at 935G. This pellet was solved in 0,5 ml destilled water, 100 µl was inoculated onto LB-agarplates, and placed in the stove for several days.

Protocol 6: Faeces in 3% SDS, 0%, 1%, 2% and 4%NaOH, with 1% cetrimide and 2% cetrimide.

To see what SDS combined with NaOH and cetrimide does with the faecal bacteria, a sixth protocol was done. The entire protocol can be found in the attachments. Briefly, 2 gram faeces was added to 20 ml destilled water and shaken manually for 1 minute. This was placed in the centrifuge for 5 minutes at 600G (Thermo Scientific Sorvall egend RT+ centrifuge). 10 ml supernatant was transferred to another tube and centrifuged for 15 minutes at 935G. The pellet was solved in 15 ml 3% SDS with X% NaOH (0, 1, 2 and

4%). This was incubated at room temperature for 15 minutes, and centrifuged for 15 minutes at 935G. The pellet was solved in 15 ml X% cetrimide (1 and 2%) and incubated at room temperature for 5 minutes. This mixture was centrifuged at 935G for 15 minutes. The pellet was solved in 15 ml destilled water, and centrifuged for 15 minutes at 935G. The pellet was solved in 0,5 ml destilled water, 100 µl was inoculated on LB-agarplate and placed in the stove for several days.

Standard PCR method, based on identification of Heat Shock Protein 65

If any environmental mycobacteria would be isolated out of the faeces by any protocol, a standard PCR method was done, based on amplification of Heat Shock Protein 65, a 441-bp fragment used to identify rapid growing environmental mycobacteria[27]. This gene is encoding a heat shock protein of 65 kiloDalton, which is sequenced in 37 mycobacterium isolates from diverse sources. There are eight *hsp65* alleles identified. DNA sequencing of the *hsp65* gene is a rapid and clear method to identifiy acid-fast mycobacteria for diagnostic purposes.
So, in case a colony would be isolated out of faeces, the first step taken would be a standard PCR, to see if the colony was an environmental mycobacterium. If positive, sequencing would be the next step, to identify which mycobacterium was present.
Template was achieved by touching a colony on a LB-agarplate with a sterile pipette tip, and added to 10µl Q-buffer, 5 µl PCR-buffer, 5 µl coral-load, 2 µl $MgCl_2$, 2 µl dNTPS (10mM), 2 µl (20 picomol) primer 1: Tb11 (ACCAACGATGGTGTGTCCAT) , 2 µl (20 picomol) primer 2: Tb12 (CTTGTCGAACGCATACCCT) , 0,2 µl Taq-polymerase and 21,8 µl H_2O. (The PCR-kit used is from Qiagen, Canada, dNTPS and H_2O was from Invitrogen, USA.)
This mixture was processed in the PCR-machine (BioRadS1000 Thermal cycler), for the program see the entire protocol in the attachment.
The sample was run in a 1% agarosegel, together with a 1kB PCR-ladder (Invitrogen, USA) for 60 minutes, 100V, 400mA, and visualized with a BioDoc it Imaging system (UVP, USA).

Results

Growing *M. smegmatis*
All different concentrations of Tween couldn't avoid clumping of *M. smegmatis*. All the tubes where positive for Ziehl-Neelsen staining (for protocol, see attachment).

Tween concentration	Day 1	Day 2	Day 3	Day 7
0	Floating clumps, no sediment	Floating clumps, no sediment	Sediment	Sediment
0,1	Floating clumps, no sediment	Floating clumps, Sediment	Cloudy, mucous sediment	Mucous sediment
0,3	Cloudy, no sediment	Cloudy, sediment	Cloudy, mucous sediment	Cloudy, mucous sediment
0,5	Very few floating clumps, no sediment	Few floating clumps, no sediment	Cloudy, sediment	Cloudy, a bit mucous sediment
0,7	Floating clumps, no sediment	Floating clumps, bit of sediment	Cloudy, sediment	Cloudy, mucous sediment
0,9	Floating clumps, no sediment	Floating clumps, sediment	Cloudy, sediment	Cloudy, mucous sediment
1	Floating clumps, no sediment	Floating clumps, cloudy, bit of sediment	Cloudy, a lot of mucous sediment	Cloudy, mucous sediment.

Table 1: Different Tween 80 concentrations in LB-broth with *M. smegmatis*

Protocol 1: Spiking *M. Smegmatis* in faeces, different concentrations HPC
Results of this protocol are shown in table 2 in the attachments, a picture taken on day 1 is visible on picture 1, also in the attachments. The colonies on the Agar-plates where judged by appearance (colour, size, shape, smell, growth-rate). If they looked like mycobacteria (compared to the Agar-plate with bacteria grown from the M. smegmatis stock out of the freezer)(also visible on picture 1), they were noted as look-a-like Mycobacteria.
After this protocol the used *M. smegmatis* strain appeared to be not positive for Ziehl-Neelsen staining, so all results of this protocol were useless.

Protocol 2: Only faeces in different concentrations HPC

Results of this protocol can be found in table 3.

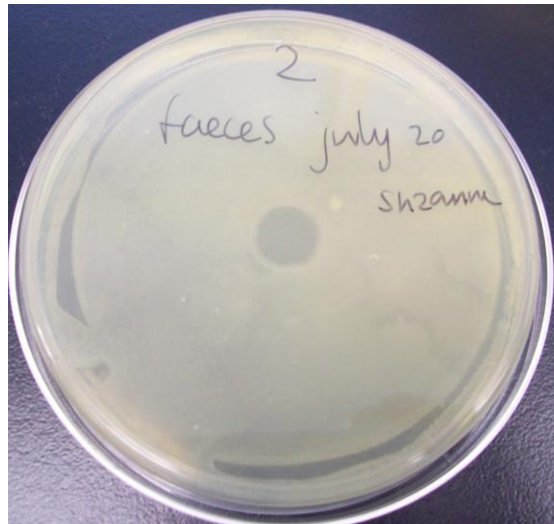
The colonies growing on the agar plates were judged by appearance and amount. The concentration of 0,5% HPC inhibits growth of faecal bacteria for at least 2 days. The concentration of 0,9 % HPC inhibits growth of faecal bacteria for at least 10 days. None of the colony's where positive on Ziehl-Neelsen staining.

% HPC	Day 1	Day 2	Day 6	Day 10
0	PF	PF	PF	PF
0,1	PF	PF	PF	PF
0,2	1 IS, 6 SRC	1 IS, 38 SRC	1 IS, URC	URC
0,3	1 IS, 2 SRC	1 IS, 6 SRC	1 IS, URC	URC
0,4	1 SRC	8 SRC	8 BRC, URC	URC
0,5	Nothing	nothing	7 BRC	PF
0,6	1 SRC	2 IS, 2 SRC	PF	PF
0,7	Nothing	Nothing	1 BRC	1 BRC
0,8	1 SRC	2 IS	2 IS	2 IS
0,9	Nothing	Nothing	Nothing	Nothing
1	Nothing	Nothing	1 SRC	1 SRC

Table 3: faeces in different concentration HPC

PF = Plate Full IS= Irregular Shaped SRC= Small Round Colony

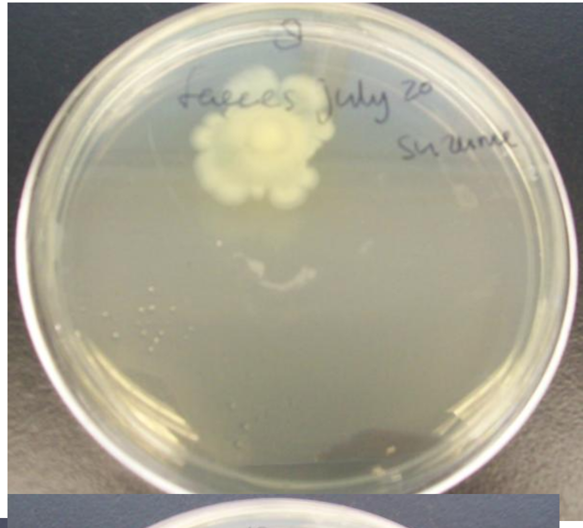
URC= Uncountable Round Colonies BRC= Big Round Colony



5
faeces july 20

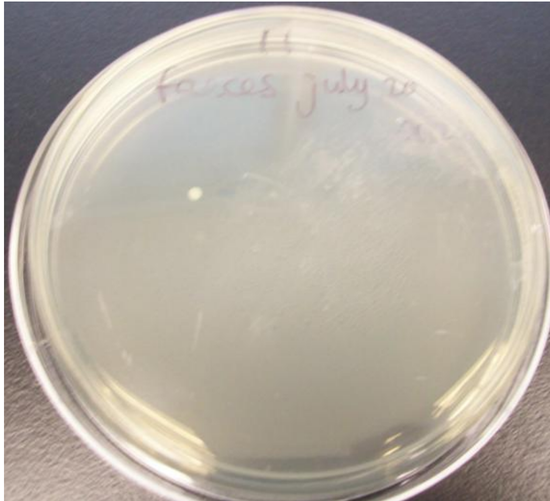
faeces july 20

faeces july 20


faeces july 20

9
faeces july 20

10
faeces july 20



Picture 2: HPC and faeces, taken on day 6.

Protocol 3: Only *M. Smegmatis* in different concentrations HPC
Results of this protocol can be seen in table 4, and on picture 3.
There was no growth of *M. smegmatis* in any concentration of HPC.

%HPC	0%	0,1%	0,2%	0,3%	0,4%	0,5%	0,6%	0,7%	0,8%	0,9%	1%
Day 2	URC	Nothing	Nothing	Nothing	Nothing	Nothing	Nothing	Nothing	Nothing	Nothing	Nothing

Table 4: *M. smegmatis* in different concentrations HPC
URC= Uncountable Round Colonies

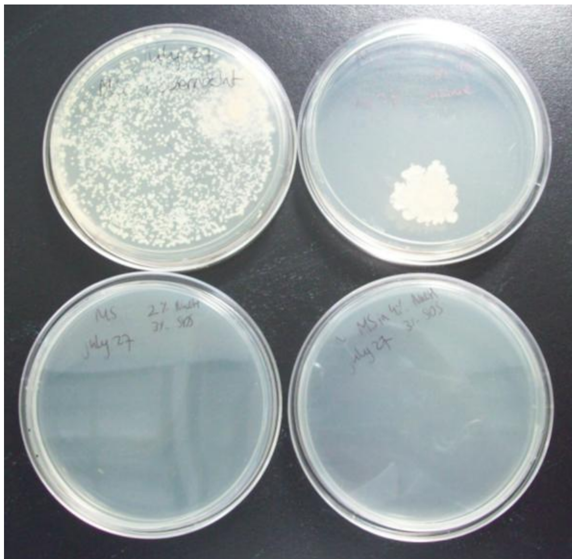
Picture 3:*M. smegmatis* in HPC, taken on day 8

Protocol 4: ***M. Smegmatis*** **with 3% SDS, different concentrations NaOH**.

Results of this protocol can be found in table 5, and picture 4.
There was 1 colony growing in 3% SDS and 1% NaOH, in concentrations higher than 1% NaOH there was no growth.

%NaOH	1%	2%	4%
Day 2	1 IS	Nothing	Nothing

Table 5: *M. Smegmatis* with 3% SDS, different concentrations NaOH



Picture 4: M. smegmatis in 3%SDS, 1, 2 and 4% NaOH, taken on day 8

Protocol 5: ***M. Smegmatis*** **in 3%SDS with 0%, 0,2%, 0,4%, 0,6%, 0,8% and 1% NaOH, 3% SDS with 1% and 2% cetrimide**

Results of this protocol are shown in table 6. There was no growth at all in any of the concentrations NaOH or Cetrimide.

3% SDS with	Day 1	Day 5	Day 8
0% NaOH	Nothing	Nothing	Nothing
0,2 % NaOH	Nothing	Nothing	Nothing
0,4% NaOH	Nothing	Nothing	Nothing
0,6% NaOH	Nothing	Nothing	Nothing
0,8% NaOH	Nothing	Nothing	Nothing
1% NaOH	Nothing	Nothing	Nothing

3% SDS	Day 1	Day 5	Day 8
1% cetrimide	Nothing	Nothing	Nothing
2% cetrimide	Nothing	Nothing	Nothing

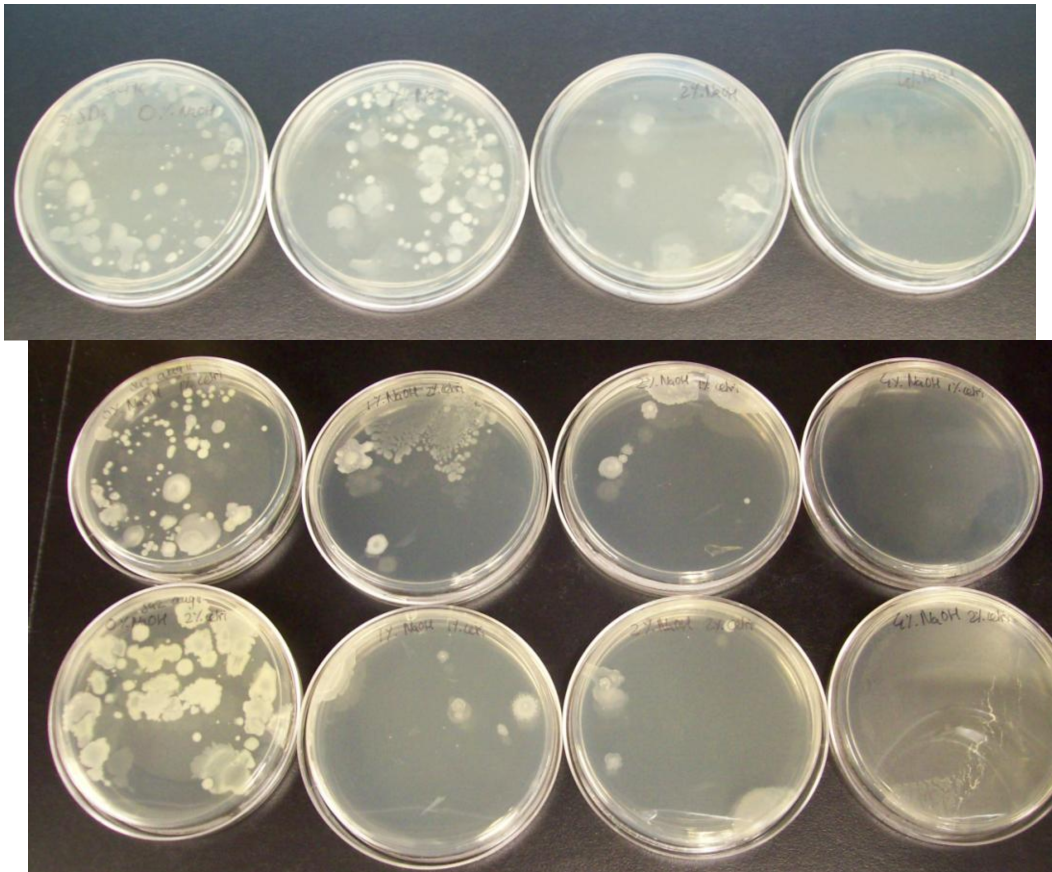
Table 6: M. smegmatis in 3% SDS with different concentrations NaOH and Cetrimide.

Protocol 6: Faeces in 3% SDS, 0%, 1%, 2% and 4%NaOH , with 1% cetrimide and 2% cetrimide

Results of this protocol are shown in table 7, and a picture of the results can be seen in picture 5. There is no growth of any faecal bacteria in 4% NaOH. 2% NaOH inhibits a big amount of faecal bacteria, but not everything. Use of Cetrimide seems to have little influence on faecal bacterial growth. Combined with NaOH there seems to be more inhibition of faecal bacterial growth.

Without Cetrimide	Day 2	Day 6	Day 9
0% NaOH	LIS	LIS	LIS
1% NaOH	LIS	LIS	LIS
2% NaOH	SIS	SIS	SIS
4% NaOH	Nothing	Nothing	Nothing
1% Cetrimide	Day 1	Day 5	Day 8
0% NaOH	70 BRC, 9 IS	LIS	LIS
1% NaOH	2 BRC, 9 IS	LIS	12 IS
2% NaOH	1 BRC, 6 IS	LIS	10 IS
4% NaOH	Nothing	Nothing	Nothing
2% Cetrimide	Day 1	Day 5	Day 8
0% NaOH	3 BRC, 33 IS	SIS	SIS
1% NaOH	4 RC, 32 IS	SIS	SIS
2% NaOH	5 IS	7 IS	8 IS
4% NaOH	Nothing	Nothing	Nothing

Table 7: faeces in 3% SDS, 1,2, and 4% NaOH, 1 and 2% Cetrimide.
LIS = Lots of Irregular Shaped SIS= Several Irregular Shaped
BRC = Big Round Colonies



Picture 5 : faeces in 3% SDS, 1,2, and 4% NaOH, 1 and 2% cetrimide
Left to right is 0, 1, 2 and 4 % NaOH, the upper row is without cetrimide, middle row is 1% cetrimide, the lowest row is 2% cetrimide. Taken on day 5,6

Standard PCR method, based on identification of Heat Shock Protein 65

The colony used for the PCR had a DNA band just a bit smaller than the 1 Kb ladder (range 500 to 1200 bp), shown in picture 6.
The size of HSP65 in *M. Smegmatis* is 441 bp [27].

Picture 6: PCR result

Discussion

The most important aim of this research was to find a good protocol to isolate environmental mycobacteria of calf faeces.
That is an important step in the following research to environmental mycobacteria. If we know which mycobacteria are present in the intestinal environment of the calf, we can investigate how they get there and what they do there.
There are some protocols known to isolate environmental mycobacteria out of water, soil and milk, but none of the protocols has been proven to work in faeces[5, 6, 16, 28].
All kinds of bacteria are present in faeces, that's why it is important to isolate mycobacteria and inhibit all other bacteria, otherwise they will overgrow the slower growing mycobacteria.
Keeping the protocols for isolating mycobacteria out of soil, milk and water in mind, we have tried to create some protocols to use on faecal samples.

Mycobacterium smegmatis was used as model for environmental mycobacteria. To let it grow in LB-broth without clumping, there are reports Tween 80 can be used[25, 26]. In our lab it did not inhibit clump-formation, but in a concentration of 0,5% the least clump-formation is seen. This knowledge can be used in further investigations using *M. Smegmatis*.

A first decontaminant used: HPC, is one often used in isolating *Mycobacterium avium subsp. paratuberculosis* out of faeces [16, 28]. HPC is a quaternary ammonium which increases the recovery of Map and decreases contamination by other microorganisms. That is the reason why it is used in the following protocols: to decrease contamination by other organisms, and isolate all present species of mycobacteria.
The first protocol: "spiking M. Smegmatis in faeces using different concentrations HPC" gave some unexpected results. There seemed to be growth of *M. smegmatis* when the highest HPC concentration was used and where we did not add *M. smegmatis*. After trying this protocol again without logical results, a Ziehl-Neelsen stain was made of the *M. Smegmatis* we grew out of the freezer stock. That stain was negative, meaning the *M. Smegmatis* we spiked the faeces with was not a mycobacterium. The agarplate was probably contaminated, so results of this protocol were not useful.

To split up this first protocol, the impact of different concentrations of HPC to the bacterial overload in faeces is investigated, and the impact of different concentrations of HPC to *M. smegmatis*. The lowest HPC concentration which inhibits most of the faecal bacteria for a couple of days is 0,5%. But *M. smegmatis* did not even grow when the 0,1% HPC was added. Knowing that, HPC is not a good decontaminant to use for isolating environmental mycobacteria.

HPC was not the only bacterial growth-inhibitor known to isolate mycobacteria. Another frequently used bacterial growth-inhibitor for the selection of environmental mycobacteria is SDS, with or without combination with NaOH, sometimes also used with cetrimide[5, 6]. That is what is tried in the following protocols.
Out of protocol 4, it seemed to be 3% SDS combined with 1% NaOH was already too concentrated for the *M. smegmatis* to survive. But, there is not been tried to isolate *M. smegmatis* with 3% SDS without any NaOH, or in lower SDS concentrations. So, we cannot conclude the failure to isolate *M. Smegmatis* all depends on the NaOH-concentration, because it's uncertain if 3% SDS is a good concentration to isolate *M. Smegmatis.*
Also it is not certain if the used concentration *M. Smegmatis* (grown for 24hours in LB-broth) is high enough to survive the many washing steps in this protocol.

Protocol 5 tests the effects of 3% SDS without NaOH, and in combination with 1 or 2% cetrimide on *M. smegmatis*. There is also been tried to investigate if a NaOH concentration between 0 and 1% would make a difference. Again, there was no *M. smegmatis* growing on the agarplates... The expectations of this protocol were that M. Smegmatis would survive the lowest concentrations of NaOH, and at least 1% cetrimide.
Uncertain is the cause of death of *M. smegmatis* is the SDS, the amount of washing steps, or the amount of *M. smegmatis*.

In protocol 6 there is been tried to visualize which effect SDS, NaOH and cetrimide have on faecal bacteria.
The conclusions made out of this protocol are: the higher the concentration NaOH, the less faecal bacteria survive. 4% NaOH is enough to kill all the faecal bacteria. What also is visible is that adding cetrimide in the protocol does not make a big difference, although it seems to have a little effect, especially combined with 1 or 2 % NaOH.
On the agarplate with 1% NaOH and 2% cetrimide a couple colonies where found that had a similar appearance as a mycobacterium. Ziehl-Neelsen staining did not bring a solution twice (there was doubt if the colour was pink enough), so the PCR should confirm the presence of a mycobacterium. Unfortunately, also the PCR failed twice, without known cause.

Looking at all the protocols tried in these 3 months, the best advise is to use 3% SDS, 1% NaOH and 2% cetrimide to isolate environmental Mycobacteria. That seems to give a good balance between inhibiting the unwanted bacteria to grow, but allowing mycobacteria to grow. Although there is more and better research necessary to say that. What I would recommend to try is using different concentrations of SDS with a good control sample, combined with 1 and 2% NaOH, combined with 1, 2 and maybe up to 5% cetrimide.

There are so many uncertainties in this research, even the most fundamental assumption: that M. Smegmatis is a good model for environmental bacteria, is not certain.

It’s a pity there is not one clear answer for the main aim of this research. There still is no appropriate protocol for isolating environmental mycobacteria of faeces. But we do know now that HPC is not very suitable.

Acknowledgements

I want to thank my supervisors, Rienske, Karin and Susanne, for their support! I also want to thank a couple of people of the department of Production Animal Health, of the faculty of Veterinary Medicine, University of Calgary. Without their help in the laboratory I wouldn't have learned so much about doing research. They let me make my mistakes, but helped me when I asked for it! There's no better way to learn than discovering everything by yourself.
Another part of doing research is patience, what I also needed to have, because I didn't got a lot of results. Despite that I really enjoyed my time in Calgary, especially helping in the barn, with the calves for the research about age- and dose susceptibility of infection with Map.

References

1. McKenna SL, Keefe GP, Tiwari A, VanLeeuwen J, Barkema HW. Johne's disease in canada part II: Disease impacts, risk factors, and control programs for dairy producers. *Can Vet J*. 2006;47:1089-1099.
2. Chi J, VanLeeuwen JA, Weersink A, Keefe GP. Direct production losses and treatment costs from bovine viral diarrhoea virus, bovine leukosis virus, mycobacterium avium subspecies paratuberculosis, and neospora caninum. *Prev Vet Med*. 2002;55:137-153.
3. Larsen AB, Merkal RS, Cutlip RC. Age of cattle as related to resistance to infection with mycobacterium paratuberculosis. *Am J Vet Res*. 1975;36:255-257.
4. Primm TP, Lucero CA, Falkinham JO,3rd. Health impacts of environmental mycobacteria. *Clin Microbiol Rev*. 2004;17:98-106.
5. Konuk M, Korcan E, Dulgerbaki S, Altindis M. Isolation and identification of mycobacteria from raw milk samples in afyonkarahisar district of turkey. *Int J Food Microbiol*. 2007;115:343-347.
6. Parashar D, Chauhan DS, Sharma VD, Chauhan A, Chauhan SV, Katoch VM. Optimization of procedures for isolation of mycobacteria from soil and water samples obtained in northern india. *Appl Environ Microbiol*. 2004;70:3751-3753.
7. Begg DJ, Whittington RJ. Experimental animal infection models for johne's disease, an infectious enteropathy caused by mycobacterium avium subsp. paratuberculosis. *Vet J*. 2008;176:129-145.
8. Whitlock RH, Buergelt C. Preclinical and clinical manifestations of paratuberculosis (including pathology). *Vet Clin North Am Food Anim Pract*. 1996;12:345-356.
9. Tiwari A, VanLeeuwen JA, McKenna SL, Keefe GP, Barkema HW. Johne's disease in canada part I: Clinical symptoms, pathophysiology, diagnosis, and prevalence in dairy herds. *Can Vet J*. 2006;47:874-882.
10. Scott HM, Sorensen O, Wu JT, Chow EY, Manninen K, VanLeeuwen JA. Seroprevalence of mycobacterium avium subspecies paratuberculosis, neospora caninum, bovine leukemia virus, and bovine viral diarrhea virus infection among dairy cattle and herds in alberta and agroecological risk factors associated with seropositivity. *Can Vet J*. 2006;47:981-991.
11. Scott HM, Sorensen O, Wu JT, Chow EY, Manninen K. Seroprevalence of and agroecological risk factors for mycobacterium avium subspecies paratuberculosis and neospora caninum infection among adult beef cattle in cow-calf herds in alberta, canada. *Can Vet J*. 2007;48:397-406.
12. Abubakar I, Myhill D, Aliyu SH, Hunter PR. Detection of mycobacterium avium subspecies paratuberculosis from patients with crohn's disease using

nucleic acid-based techniques: A systematic review and meta-analysis. *Inflamm Bowel Dis*. 2008;14:401-410.
13. Feller M, Huwiler K, Stephan R, et al. Mycobacterium avium subspecies paratuberculosis and crohn's disease: A systematic review and meta-analysis. *Lancet Infect Dis*. 2007;7:607-613.
14. Ellingson JL, Anderson JL, Koziczkowski JJ, et al. Detection of viable mycobacterium avium subsp. paratuberculosis in retail pasteurized whole milk by two culture methods and PCR. *J Food Prot*. 2005;68:966-972.
15. McKenna SL, Barkema HW, Keefe GP, Sockett DC. Agreement between three ELISAs for mycobacterium avium subsp. paratuberculosis in dairy cattle. *Vet Microbiol*. 2006;114:285-291.
16. McKenna SL, Keefe GP, Barkema HW, Sockett DC. Evaluation of three ELISAs for mycobacterium avium subsp. paratuberculosis using tissue and fecal culture as comparison standards. *Vet Microbiol*. 2005;110:105-111.
17. McKenna SL, Sockett DC, Keefe GP, McClure J, VanLeeuwen JA, Barkema HW. Comparison of two enzyme-linked immunosorbent assays for diagnosis of mycobacterium avium subsp. paratuberculosis. *J Vet Diagn Invest*. 2005;17:463-466.
18. Whitlock RH, Wells SJ, Sweeney RW, Van Tiem J. ELISA and fecal culture for paratuberculosis (johne's disease): Sensitivity and specificity of each method. *Vet Microbiol*. 2000;77:387-398.
19. Howard ST, Byrd TF. The rapidly growing mycobacteria: Saprophytes and parasites. *Microbes Infect*. 2000;2:1845-1853.
20. Albertini M, Haas H, Chiche V, Bourrier T, Dageville C, Mariani R. Non-specific tuberculin reactivity due to sensitization to non-tuberculous mycobacteria (NTM) in children not vaccinated with BCG. diagnostic value of a comparison of intradermal tests with tuberculin and NTM antigens. *Rev Mal Respir*. 1996;13:273-279.
21. Bahrmand AR, Madani H, Samar G, et al. Detection and identification of non-tuberculous mycobacterial infections in 6,472 tuberculosis suspected patients. *Scand J Infect Dis*. 1996;28:275-278.
22. Kwamanga DO, Swai OB, Agwanda R, Githui W. Effect of non-tuberculous mycobacteria infection on tuberculin results among primary school children in kenya. *East Afr Med J*. 1995;72:222-227.
23. von Reyn CF, Horsburgh CR, Olivier KN, et al. Skin test reactions to mycobacterium tuberculosis purified protein derivative and mycobacterium avium sensitin among health care workers and medical students in the united states. *Int J Tuberc Lung Dis*. 2001;5:1122-1128.
24. Smith D, Wiegeshaus E, Balasubramanian V. An analysis of some hypotheses related to the chingelput bacille calmette-guerin trial. *Clin Infect Dis*. 2000;31 Suppl 3:S77-80.

25. Yoichi Maruyama, Tetsu Ono and Tetsuro Sato. Synchronous growth of *mycobacterium smegmatis* as induced by cellular crowding. *The Journal of General and Applied Microbiology*. 1963;9:41-47.
26. van Boxtel RM, Lambrecht RS, Collins MT. Effect of polyoxyethylene sorbate compounds (tweens) on colonial morphology, growth, and ultrastructure of mycobacterium paratuberculosis. *APMIS*. 1990;98:901-908.
27. Ringuet H, Akoua-Koffi C, Honore S, et al. Hsp65 sequencing for identification of rapidly growing mycobacteria. *J Clin Microbiol*. 1999;37:852-857.
28. Clark DL,Jr, Koziczkowski JJ, Radcliff RP, Carlson RA, Ellingson JL. Detection of mycobacterium avium subspecies paratuberculosis: Comparing fecal culture versus serum enzyme-linked immunosorbent assay and direct fecal polymerase chain reaction. *J Dairy Sci*. 2008;91:2620-2627.

Attachments

Growing Mycobacteria smegmatis

Materials:

LB-Agar plates →

Solve 32 gram LB Agarpowder (Invitrogen, USA) in 1 liter destilled water, autoclave for 15 min at 121 °C, pour it under sterile conditions in the empty plates (approximately 50), let them cool and store in the fridge. (4°C)

Luria Broth Base →

25 g LB Broth powder (Invitrogen, USA) in 1 liter destilled water, autoclave for 15 min at 121 °C.

Mycobacterium smegmatis Stock, saved in the freezer (-80°C)

Sterile öse

Stove (37°C, 5%CO_2) (ThermoScientific Steri-Cycle CO2 incubator, Hepa Class 100)

Shaker (New Brunswick Scientific – Excella E24 Incubator Shaker Series

Tween 80 (Fisher Scientific, Canada)→ 0,05% 0,1%, 0,3%, 0,5%, 0,7%, 0,9%, 1%

Method

Inoculate *Mycobacterium smegmatis* (glycerolstock in freezer -80°C) with a sterile öse on a LB-agarplate. Place it in the stove (37 °C, 5% $CO_{2)}$ for 48 hours.

If the bacteria grew on the plate, they were transferred to the LB broth, (with or without Tween 80, different concentrations) under sterile circumstances. Place this solution in the shaker at 37 °C, 220 RPM.

Staining bacteria, Ziehl-Neelsen method

Material:

Objectslides

Öse

Bacteria (on a agarplate or in LB broth)

PBS (Gibco, Invitrogen, USA)(if the colony is not in LB broth)

Bunsen burner

Timer

Carbol Fuchsin (100 ml Basic Fuchsin solution (10 gram Basic Fuchsin powder in 100 ml Ethanol, stir for 3-4 days), 50 ml of Liquefied Phenol and 850 ml deionizedwater, mix thoroughly)

Destilled water

3% acid-alcohol (Add 30 ml concentrated hydrocholoric acid carefully to 970 ml 95% ethanol)

Methylene Blue (Add 900 ml Absolute Alcohol to 9,0 gram Methylene Blue, add 0,3 gram Potassium Hydroxide, add 3000 ml Deionized Water, stir for2-3 days)

Method

1. Use the öse to spread the bacteria on the slide (use a drip of PBS on the slide if the bacteria grow on a plate)
2. Flame the slide to heat fix.
3. Flood the entire slide with Carbol Fuchsin
4. Using a Bunsen burner, heat the slides slowly to let them steam. Maintain steaming for 5 minutes.
5. Rinse the slides with destilled water
6. Flood the slide with 3% acid-alcohol, to decolorize for 2,5 minutes.
7. Rinse the slides with destilled water
8. Flood the slide with Methylene Blue for 1 minute.
9. Rinse the slides with destilled water
10. Let them dry (optional: glue a coverglass on it)
11. View under the microscope.

Staining bacteria, Gram method

Material:

Objectslides
Öse
Bacteria (on a agarplate or in LB broth)
PBS (Gibco,Invirtogen, USA) (if the colony is not in LB broth)
Bunsen burner
Carbol Fuchsin solution
Iodin
Destilled water
Alcohol
Crystal violet

Method

1. Use the öse to spread the bacteria on the slide (use a drip of PBS on the slide if the bacteria grow on a plate)
2. Flame the slide to heat fix.
3. Flood the entire slide with Crystal Violet for 60 sec
4. Flood the entire slide with Iodin for 180 seconds
5. Flood the entire slide with Alcohol to decolorize, rinse with water
6. Flood the entire slide with Carbol fuchsine solution, rinse with water
7. Let the slide dry, and view under the microscope.

Protocol 1, spiking M. Smegmatis in faeces, different concentrations HPC.

Material

½ BHI (BD, Canada) (solve 9,25 gram Brain Heart Infusion powder in 491,75 ml destilled water, autoclave for 15 minutes at 121°C)
½ BHI (BD, Canada)with 1% HPC (Alfa Aesar, USA)(Add 10grams Hexadecyl Pyridinium Chloride under sterile conditions to 1 liter sterile ½ BHI)
½ BHI (BD, Canada) with 0,2, 0,4, 0,6 en 0,8% HPC (Alfa Aesar, USA) (mix the right proportions ½ BHI en ½ BHI 1% HPC under sterile conditions)
Faeces (in this protocol I used faeces from cow 118, group 7 AV, freezer -80°C)

M. Smegmatis 2 days grown in LB broth in shaker, diluted in PBS (up to 10^3 bacteria/ml)
Falcon tubes, (VWR, Canada) (36 of 45 ml and 36 of 15 ml)
LB-agar plates
Scale
Pipettes
Stove (37°C, 5% CO_2)
Centrifuge (Thermo Scientific Sorvall legend RT+ centrifuge
Alcohol
turntable
Bacteria-divider tool (to equally divide the bacteria on the agar plate)
Vortex

Method

1. Mix 2 gram faeces with 100µl different M. Smegmatis dilutions
2. Solve 2 gram mixed faeces 25 ml ½ BHI + X% HPC, vortex this
3. Place in the stove for 24 hours
4. Use the pipet to place the upper 5 ml of the mixture in a 15 ml Falcontube
5. Centrifuge for 30 min at 900G
6. Discard supernatant
7. Solve pellet in 5 ml ½ BHI, vortex this
8. make serial dilutions
    - 1-6 and 25-36 not diluted
    - 7-12 3 times diluted
    - 13-18 2 times diluted
    - 19-24 once diluted
9. Inoculate 100 microliter onto LB-agar plates
10. place it in the stove

HPC Faeces	0%	0,2%	0,4%	0,6%	0,8%	1%
0	1	2	3	4	5	6
1	7	8	9	10	11	12
2	13	14	15	16	17	18
3	19	20	21	22	23	24
4	25	26	27	28	29	30
5	31	32	33	34	35	36

Faeces dilutions:
0 = No M. Smegmatis added
1 = No dilution (expected: 10^7 Colony Forming Units per ml)
2 = 1/10 time diluted (expected 10^6 CFU per ml)
3 = 1/100 times diluted (expected 10^5 CFU per ml)
4 = 1/1000 times diluted (expected 10^4 CFU per ml)
5 = 1/10000 times diluted (expected: 10^3 CFU per ml)

To make sure how many CFU's are in the M. Smegmatis LB broth, a 1/100 000, 1/1000 000 and 1/10 000 000dilution are inoculated on an agar plate, to count the colonies after 24 hours in the stove.

Protocol 2, only faeces in different concentrations HPC

Material

½ BHI (BD, Canada)
½ BHI with 1% HPC (Alfa Aesar, USA)
½ BHI with 0,1, 0,2, 0,3, 0,4, 0,5, 0,6, 0,7, 0,8, 0,9% HPC
Faeces (fresh from Calf 33)
Falcontubes (VWR, Canada) (11 of 45 ml, 11 of 15 ml)
Pipettes
Scale
LB-agarplates
Stove at 37°C, 5% CO_2 (Thermo Scientific Steri-cycle CO2 incubator hepa class 100)
Centrifuge (Thermo Scientific Sorvalllegend RT+ centrifuge)
Alcohol
Turntable
Tool to equally divide the bacteria on the Agar plate

Method

1. Solve 2 gram faeces in 25 ml ½ BHI + X% HPC and vortex
2. Place it in the stove for 24 hours
3. Use the pipet to place the upper 5 ml of the mixture in a 15 ml Falcontube.
4. Centrifuge for 30 min at 900G
5. Discard supernatant.
6. Solve pellet in 5 ml ½ BHI oplossen and vortex.
7. Inoculate 100 microliter onto LB-agarplates
8. Place in the stove

Protocol 3, only M. Smegmatis in different concentrations HPC

Material

½ BHI (BD, Canada)
½ BHI with 1% HPC (Alfa Aesar, USA)
½ BHI with 0,1, 0,2, 0,3, 0,4, 0,5, 0,6, 0,7, 0,8, 0,9% HPC
M. Smegmatis, grown for 24 hours in LB broth in shaker
Falcontubes (11 of 45 ml, 11 of 15 ml) (VWR, Canada)
Pipettes
Scale
LB-agarplates
Stove at 37°C, 5% CO_2 (Thermo Scientific, Steri-Cycle CO2 incubator Hepa Class 100)
Centrifuge (Thermo Scientific Sorvall legend RT+ centrifuge
Alcohol
Turntable
Tool to equally divide the bacteria on the Agar plate.

Method

1. Solve 100 µl M. Smegmatis in 10 ml ½ BHI + X% HPC and vortex
2. place in the stove for 24 hours
3. Use the pipette to place the upper 5 ml of the mixture in a 15 ml Falcontube.
4. Centrifuge for 30 min at 900G.
5. Discard supernatant.
6. Solve pellet in 5 ml ½ BHI and vortex.
7. Inoculate 100 microliter onto a LB-agarplate
8. place in the stove

Protocol 4 M. Smegmatis with 3% SDS, different concentrations NaOH.

Material

3% SDS (Fisher Scientific, Canada) (Solve 3 grams Sodium Dodecyl Sulfate in 100 ml destilled water, autoclave for 15 min at 121°C)
3% SDS with 4% NaOH (Fisher Scientific, Canada)(add 2 gram NaOH to 50 ml 3% SDS under sterile circumstances)
3% SDS 1 and 2% NaOH (mix the proper proportions of 3% SDS and 3% SDS 4% NaOH under sterile circumstances)
M. Smegmatis (24 hours grown in LB broth)
H_3PO_4 (8%)
Sterile water
LB-agarplates
Shaker (37 °C, 220 RPM) (New Brunswick Scientific, Excella E24 Incubator Shaker Series
Falcontubes (3 of 15 ml) (VWR, Canada)
pH-meter, (Fisher Scientific, Accumet excel, XL25)
pipettes
centrifuge (Thermo Scientific Sorvall legend RT+ centrifuge
alcohol

turntable
Tool to equally divide the bacteria on the agar plate.

Method
1. Add 100 µl M. Smegmatis to 10 ml 3% SDS and X% NaOH
2. vortex and shake for 30 min
3. Add H_3PO_4 to neutralize the mixture
4. centrifuge at 3716G for 20 minutes
5. discard supernatant
6. solve pellet in 15 ml destilled water and vortex
7. centrifuge at 3716G for 20 minutes
8. discard supernatant
9. solve pellet in 1,5 ml destilled water
10. Inoculate 100 µl onto LB-agarplates
11. place in the stove

Protocol 5: M. Smegmatis in 3%SDS with 0%, 0,2%, 0,4%, 0,6%, 0,8% and 1% NaOH.
3% SDS with 1% and 2% cetrimide

Material:
M. Smegmatis, grown for 24 hours in LB-broth in shaker
3% SDS (Fisher Scientific, Canada) with 0, 0,2, 0,4, 0,6 0,8 and 1% NaOH
1% and 2% Myristyltrimethylammoniumbromide (cetrimide) (Sigma Aldrich, Canada)
Pipettes
Falcon tubes (8 of 15ml) (VWR, Canada)
Vortexer
Centrifuge (Thermo Scientific Sorvall legend RT+ centrifuge)
LB-agar plates (8)
Turntable
Tool to equally divide the bacteria on the agar plate
Alcohol
Bunsen burner

Method
1. Add 0,1 ml M.Smegmatis. to 10 ml 3% SDS, X NaOH
2. vortex and inoculate for 15 minutes at room temperature.
3. Centrifuge for 15 minutes at 935G
4. *Solve pellet in 10 ml X% cetrimide*
5. *Inoculate for 5 minutes at room temperature*
6. *Centrifuge for 15 minutes at 935G*
7. *Solve pellet in 15 ml destilled water and vortex*
8. *Centrifuge for 15 minutes at 935G*
9. Solve pellet in 0,5 ml destilled water
10. Inoculate 0,1 ml on a LB-agarplate

Protocol 6: Faeces in 3% SDS, 0%, 1%, 2% and 4%NaOH
With 1% cetrimide and 2% cetrimide

Material:
Sterile water
3% SDS (Fisher Scientific, Canada)
3% SDS with 1%, 2% and 4% NaOH (Fisher Scientific, Canada
2% Cetrimide → Add 2 grams of Myristyltrimethylammoniumbromide (Sigma Aldrich, Canada) to 100 ml destilled water, autoclave for 15 minutes at 121 °C.
1% Cetrimide → Mix the sterile water with 2% cetrimide in equal proportions under sterile conditions.
Falcon tubes (12 of 45 ml, 12 of 15 ml) (VWR, Canada)
Centrifuge (Thermo Scientific Sorvall Legend RT+ centrifuge)
Vortexer
LB-agar plates (12)
Turntable
Tool to equally divide the bacteria on the agar plate
Alcohol
Bunsen burner
Pipettes

Method

1. Add 2 gram faeces to 20 ml destilled water
2. Shake manually for 1 minute
3. Centrifuge for 5 minutes at 600G n
4. Transfer 10 ml supernatant to another tube
5. Centrifuge for 15 minutes at 935G
6. Discard supernatant, solve pellet in 15 ml 3% SDS X NaOH
7. Incubate at room temperature for 15 minutes.
8. Centrifuge for 15 minutes at 935G
9. *Solve pellet in 15 ml X% cetrimide*
10. *Incubate at room temperature for 5 minutes*
11. *Centrifuge for 15 minutes at 935G*
12. *Solve pellet 15 ml destilled water and vortex*
13. *Centrifuge for 15 minutes at 935G*
14. Solve pellet in 0,5 ml destilled water
15. inoculate 0,1 ml on a LB-agarplate

Standard PCR method, based on identification of Heat Shock Protein 65

Material:
What does the 50µl PCR mixture contain:

- Template (touching a colony with a sterile pipette tip)
- Q-buffer (Qiagen, Canada) 10µl
- PCR-buffer (Qiagen, Canada) 5 µl
- Coral-load (Qiagen, Canada) 5 µl
- $MgCl_2$ (Qiagen, Canada) 2 µl
- dMTPS (nucleotides) (Invitrogen,USA) (10mM) 2 µl
- primer 1: Tb11 (ACCAACGATGGTGTGTCCAT) 2 µl

- primer 2: Tb12 (CTTGTCGAACGCATACCCT) 2 µl
- Taq-polymerase(Qiagen, Canada) 0,2 µl
- H_2O (Invitrogen, USA) fill up to 50 µl = 21,8 µl

Method

In the PCR-machine(BioRadS1000 Thermal cycler):

Temperature 1: 96 °C 5 minutes
Temperature 2: 96 °C 30 seconds
Temperature 3: 60 °C 30 seconds
Temperature 4: 72 °C 30 seconds
Temperature 5: 72 °C 10 minutes
Store forever at 4 °C
Repeat cycle 2 to 4 36 times

Electrophoresis:

To make the 1% agarosegel → solve 0,7 gram agarose(Invitrogen, USA) in 70 ml Tris-acetate-EDTA buffer (for a small gel). Boil this mixture in the microwave and add SYBR safe(Invitrogen, 10 000x concentrate in DMSO) (1/10 000 parts → 7 µl), place a gel-comb and let it solidify, covered in aluminumfoil (no light).

Load 10 µl of 1kB PCR-ladder in blue-juice (both Invitrogen, USA, 1:1) in the gel. Load the positive control, samples and negative controle as well.

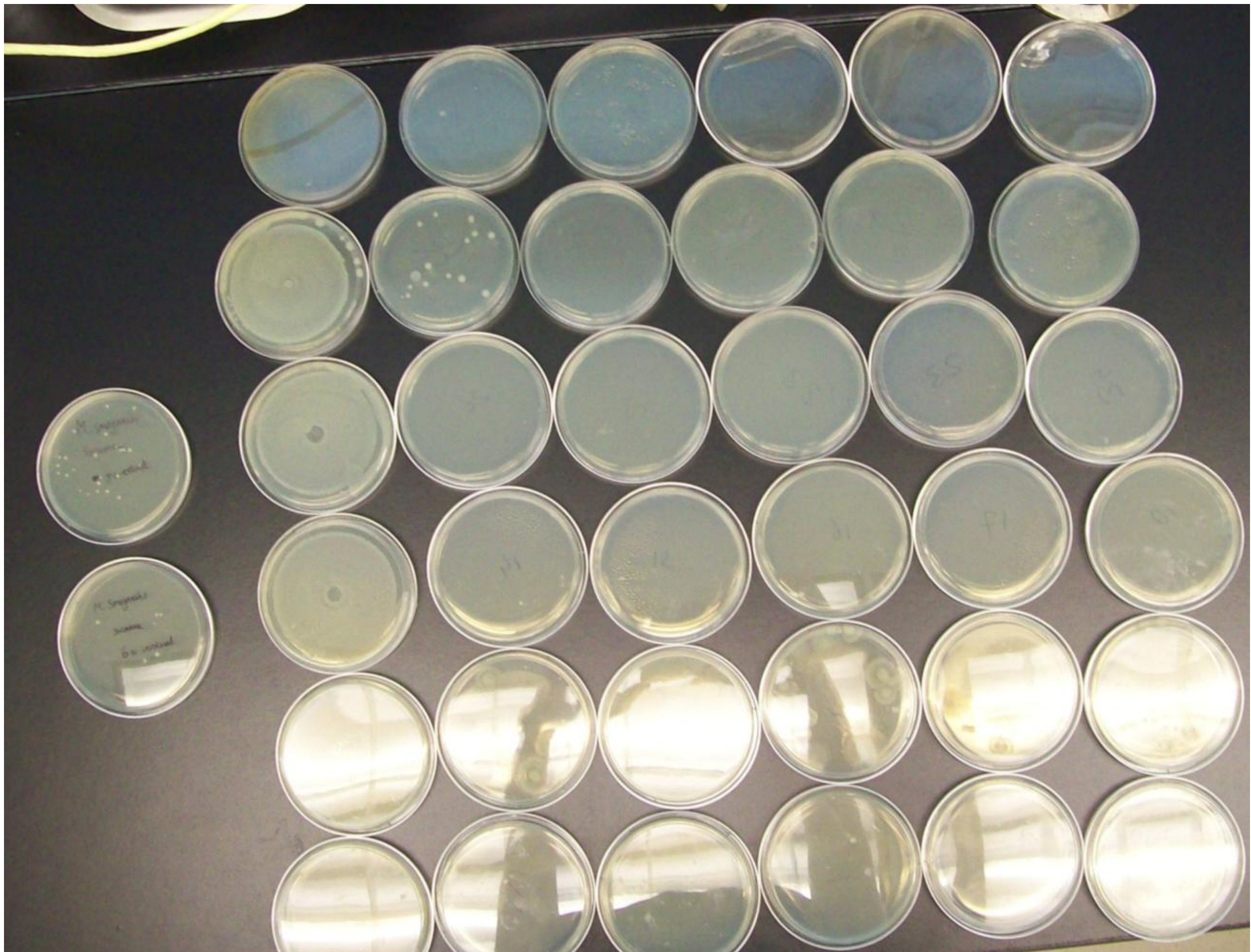
Running conditions are: 100 V, 400 mA, for 60 minutes.

PCR amplification products were visualized with a BioDoc it Imaging system (UVP, Upland, USA).

Smegmatis/HPC	Day 1 **0%**	Day 2	Day 4	Day 5	Day 1 **0.2%**	Day 2	Day 4	Day 5	Day 1 **0.4%**	Day 2	Day 4	Day 5	Day 1 **0.6%**	Day 2	Day 4	Day 5	Day 1 **0.8%**	Day 2	Day 4	Day 5	Day 1 **1%**	Day 2	Day 4	Day 5
0	NM + LM	NM + LM	NM + LM	NM	NM	NM	0	0	0	0	0	0	NM	0	0	0	0	0	26M	36M	0	0	43M	60M
Undiluted	NM	NM	NM	NM	NM	NM	NM	NM	NM	NM	NM	NM	NM	NM	NM	NM	NM	NM	NM	NM	NM	NM	NM	NM
1 x diluted	NM + LM	NM + 67M	NM + 67M	NM + LM	0	1M	1M	1M	0	0	0	0	0	NM	0	0	0	0	0	0	0	0	0	0
2 x	N	N	N	N	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

diluted	M + LM	M + 21M	M + 28M	M + SM																				
3x diluted	NM + LM	NM + LM	NM + LM	NM + LM	NM	NM	NM	NM	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
4 x diluted	NM	NM	NM + 23M	NM + SM	0	NM	NM	NM	0	0	0	0	0	0	0	0	NM	0	0	0	0	0	0	0

Table 2: different amounts of smegmatis, different concentrations of HPC.
NM = Non Mycobacteria LM = Lot of look-a-like Mycobacteria SM = Several look-a-like Mycobacteria



Picture 1: different amounts of smegmatis, different concentrations of HPC, taken on day 1