Research project C2001:

Quantification of resistance genes in veal manure by qPCR
"But first we have to have some conditions clear"

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Index

Abstract

The relation with the big therapeutic use of antibiotics in human and veterinary medicine and the growing problem of resistance is getting more and more attention world wide. In the Netherlands, a political agreement has been reached to decrease antibiotic use in veal farming immediately. The aim of this research is to determine the qPCR detection limit of antibiotic resistance genes (tet(S)) in veal feces, to choose between two (absorbing overshoe and plastic cup) sampling methods and to investigate PCR inhibition and its removal by dilution. Also a start will be made in the setup of a protocol for quantification of Integrase genes (intI1) in feces samples. Integrons are intermediates in the pickup and expression of resistance genes and are often found on plasmids.

The sampling with a plastic cup will gave the best qPCR data. Because of inhibition factors, which are present in feces, a ten fold dilution of the DNA was necessary for an optimal qPCR reaction. The detection limit for tet(S) was determined as 10^5 spiked genes per gram feces, which is a workable detection limit for veal feces. Unstable standard dilutions were seen during this research, reasons and solutions for this are discussed.

General introduction

Antibiotic use and resistance

Antibiotic use and resistance is getting more and more attention world wide because of the growing problem of resistance and the relation with the big therapeutic use of antibiotics in human and veterinary medicine.
Since the beginning of the general use of antibiotics, around 1945, resistance has been observed. It has been suggested that for any antibiotics, it is not a matter of "if" but only a matter of "when" resistance can be seen (Walsh 2003).

For a long time the use of antibiotics in food-producing animals was growing in the Netherlands. Probable causes for this are the ban of antibiotics as growth promoters and the persistent growth of farms. In 2008 (and 2009) a small decrease in antibiotic use can be seen (figure 1), which could be, and probably is, the result of the start of a different approach in antibiotic use (Mevius et al 2010).

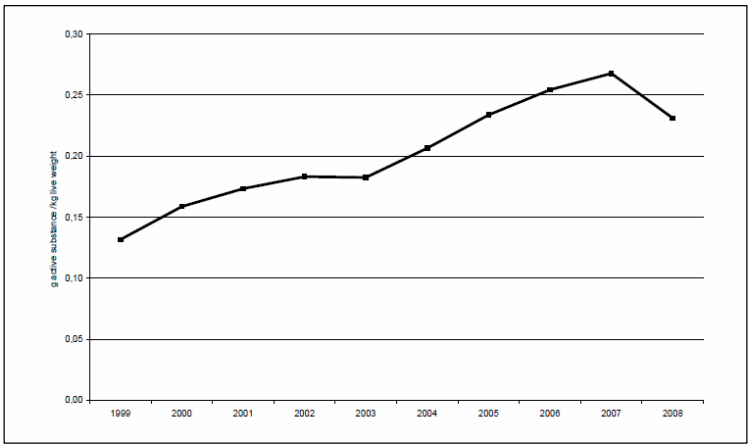

Figure 1 Total sales of therapeutic antibiotics 1998-2008, in g per kg live weight (Mevius 2010)

In 2008 different relevant authorities in the Netherlands made a 'convenant' called 'Antibioticaresistentie dierhouderij'. Authorities like the KNMvD, LTO and PVV (Productschap Vee en Vlees) set up goals and made agreements on three large topics:

- Define responsibilities for the selling and administration of antibiotics
- Increase the transparency of antibiotic use
- Actions to improve responsible antibiotic use

(Convenant Antibioticaresistentie Dierhouderij 2008)

To put more pressure on these agreements a taskforce was formed by the ministries of LNV and VWS in March 2010. They set an ambitious goal of 20% reduction in antibiotic use in 2011 and 50% reduction in 2013, which they

presented in November 2010. To achieve these goals a central registration program is introduced, to monitor all antibiotic sales and use (Bleker 2010).

As mentioned, the decrease in useful antibiotics is a big threat for human and animal health. For a long time, until the 1970s, the pharmaceutical industry provided new antibiotics. After that, only few new antibiotics were brought onto the market, and the most 'new' antibiotics belong to existing antibiotic classes and are often not effective against multidrug resistant bacteria. This big gap between supply and demand is alarming (Norrby et al 2009).

Veal farming

In veal farming the antibiotic use also needs to be decreased immediately (Convenant Antibioticaresistentie Dierhouderij 2008). Veal calves come from dairy farms. Calves that will not be used for milk production are gathered on another farm, a veal farm. There the calves are raised to be slaughtered (www.overkalfsvlees.nl). There are two kinds of veal farming.

There is (white) meat from calves up to 8 months old that are mostly fed with milk or milk products (Algemene voorwaarden IKB Vleeskalveren 2008 blanke Vleeskalveren). Red meat is meat from calves from 8 to 12 months old that are not mostly fed with milk or milk products (Algemene voorwaarden IKB Vleeskalveren 2008 rosé Vleeskalveren).

All regulations are bundled in 'IKB vleeskalveren' (www.ikbkalveren.nl). 'IKB vleeskalveren' is developed by 'Productschap Vee, Vlees en Eieren' in partnership with the farmers and others involved.

In the figures below the antibiotic use on veal calves, the resistance percentage and their evolution in time is shown.

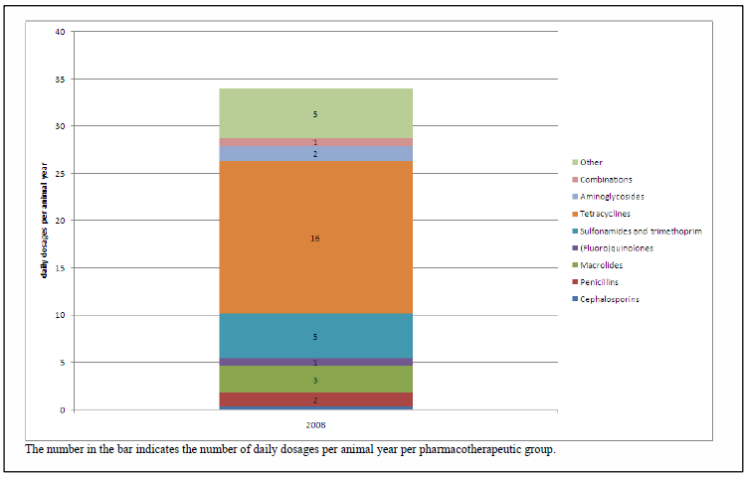

Figure 2 Antibiotic use on veal calf farms in daily dosages per animal per year in 2008 (Mevius 2010).

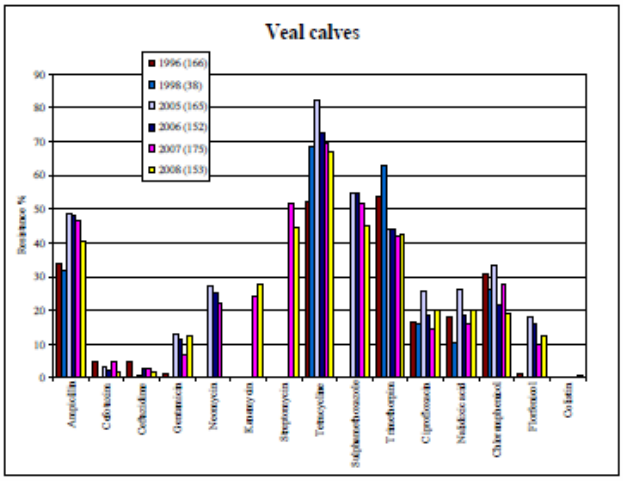

Figure 3 Trends in resistance (in %) of E.coli isolated veal calves in the Netherlands from 1998-2008 (Mevius 2010).

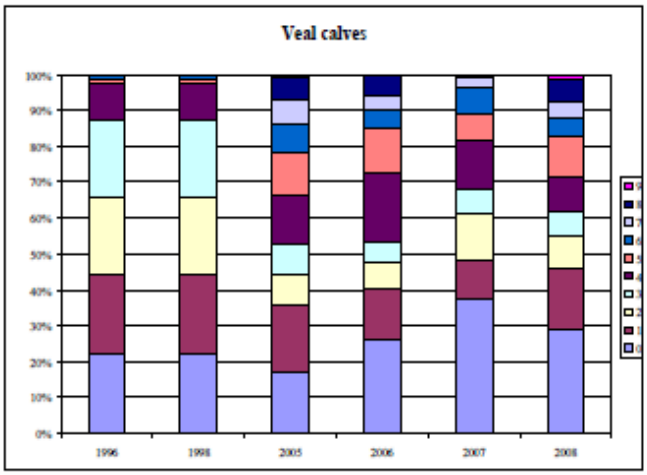

Figure 4 Trends in percentages of E.coli strains fully susceptible, resistant to one to a maximum of nine antimicrobial classes in veal calves in the Netherlands from 1998-2008 (Mevius 2010).

The average veal calf is administered 34 daily dosages of antibiotic per year (figure 2). Assuming 1,5 production periods per year for white and red calves together, a calf gets antibiotics for 23 days of his average 222 day life (Mevius et al 2010). In figure 3 can be seen that there is a little reduction in resistance of several antibiotics in the last years. But striking to us was the growth of multi resistance as can be seen in figure 4.

Quantitative PCR versus bacterial cultivation

PCR is a technique to determine, by DNA amplification, if a sample contains (antibiotic resistance) genes. Quantitative or Real Time Polymerase Chain Reaction (qPCR) not only shows if a certain gene is present in the sample but also demonstrates how many of those genes are in the sample. This means you can qualify and quantify the genes. To be able to do the quantification, adding a fluorescent dye or a fluorescent reporter probe is necessary. When single stranded DNA becomes double stranded, the dye will fluoresce. The probe will fluoresce when it is degraded by the polymerase when single stranded DNA becomes double stranded DNA. By measuring the level of fluorescence with a camera you can determine the number of genes in a sample. The qPCR software program will not give absolute numbers of genes but it gives the threshold cycle when fluorescence is detected. By running standard dilutions (with known gene

concentrations) at the same time, results can be compared and gene concentrations can be determined (Klein 2010, en.wikipedia.org/wiki/Real_time_pcr).

For a long time research has been based on cultivatable bacteria. The aim of bacterial cultivation is to determine which and roughly how many bacteria where in the sample. For this a special growth medium is used to let these (aerobic) bacteria multiply.
The advantage of qPCR compared to bacterial cultivation is that it is less labor intensive. But above that, all genes can be measured and not only from cultivatable bacteria (Smith et al 2004). This is important because the AR gene pool is larger than the genes in cultivatable bacteria, as every bacterium can contain AR genes. Also, because the bacteria have mobile elements they can share their genes with each other so the spread of the AR genes can go very fast.

Feces as sampling material

Every type of sampling material has its own characteristics to be aware of when working with it. Feces contain enzymes that can inhibit qPCR reactions. This inhibition is not well understood but often seen in biological samples (Oikarinen 2009, Pei 2006, Kreader 1995). It is necessary to get these inhibition factors as much as possible out of the samples.
Collecting feces can be done in different ways, with a main division in internally and externally. We need external mixed samples. Externally there are different options. We investigated two of them. The first one is the absorbing overshoe as sampling method as in Cobbaut 2008. This is a paper overshoe mainly used for salmonella detection in chickens (Heyndrickx 2002, Korsak 2003). The second one is a plastic cup with a little spoon in the lid.
Every type of sampling material has its own qPCR detection limit. By determining the detection limit, we determine the limitation of the technique. It is important to know the minimum absolute gene copies that can be measured in feces and how many cells that mean in the real sample. Gene copies beneath that minimum won't be found in the qPCR data.

Integrons

Integrons are potential mobile elements. Boucher et al 2007 described integrons as followed:
"Integrons are one part of a two-component system that capture disparate individual genes and physically link them in arrays. The first component of this

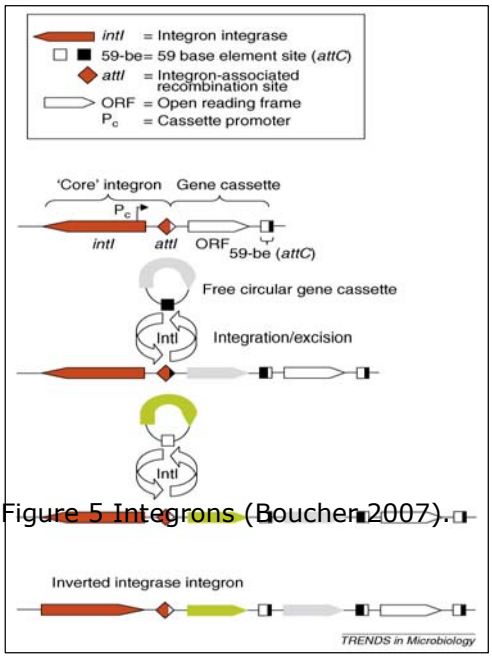

Figure 5 Integrons (Bouchen 2007).

system is the 'core' integron, which includes a gene (intI) encoding a site-specific recombinase (IntI) and a recombination site (attI). The second part of the system comprises a family of mobile elements known as gene cassettes." (Figure 5)

There are two types of integrons; mobile integrons and chromosomal superintegrons. Mobile integrons are integrated in mobile DNA elements as plasmids and transposons and are primarily involved in the spread of AR genes. Chromosomal superintegrons seem to be located on the chromosome and are not associated with mobile elements (Mazel 2006, Leverstein-van Hall 2002).

Integrons are intermediates in the pickup and expression of resistance genes and are the source of the majority of the transferable antibiotic resistance genes found in gram negative bacteria. But they are not restricted to these bacteria, as class 1 integrons (integrase 1) have also been found in gram positive bacteria (Davies 2010, Mazel 2006). Large numbers of integron cassettes have been found in natural environments that do not code for (known) resistance genes.

Many complete integrons including resistance gene cassettes are known, underlining the universal importance of integrons mediated gene pick up in resistance evolution. The origins of integrons are not known, although the similarity of sequence between the integrases and bacteriophage recombinases suggest an evolutionary relationship (Davies 2010).

More than 130 gene cassettes have been described, coding for resistance to almost all antibiotics. There are five classes of integrons described, based on their integrase (IntI). Integrases 1,2 and 3 are most common or at least most described in articles (Barraud 2010). One cassette promoter can promote several cassettes/genes which leads to multi-resistance (Gillings 2008, Alekshun 2007).

The research project

As mentioned earlier, antibiotic resistance is a big threat for human (and animal) health. Food-producing animals are a source of AR genes for humans. But how do these genes come from animal (veal calve) to human? Most resistance genes are located in the intestines, and on the skin and noses of the calves. In the

slaughterhouse the calves are cleaned and cut and contamination can take place. For example one of the steps in this process is cutting out the intestines. In this step of slaughtering there is often contamination from intestines to muscle (meat).

To determine how resistance can be decreased, a research project has been set up at IRAS. The aim of the whole research is to determine in what concentration different antibiotic resistance genes are present in feces of veal calves from farms with different management systems towards hygiene, by using qPCR.
The samples will be from farms with different intervention plans to decrease the antibiotic resistance. By comparing those farms we can see which interventions do and don't contribute.

Aim of the research

The aim of my research is to determine the qPCR detection limit of antibiotic resistance genes (tet(S)) in veal feces, to choose between two (absorbing overshoe and plastic cup) sampling methods and to find the optimal DNA dilution to use for qPCR.
Also, I investigated protocols for quantification of Integrase genes (intI1) in feces samples by using qPCR.

Materials and methods

Sampling method

We used sampled feces from calves from 'proefboerderij De Tolakker'. We collected feces that were on the ground, so we did not have to handle the calves. We collected the feces in two ways.

Method 1: absorbing overshoe

The overshoes are placed over the boots. By walking through the whole pen we collected feces on the absorbing shoes.

Method 2: plastic cup

The plastic cups are made with a lid that contains a little spoon to pick up feces from the floor. Mixed samples were made, which means feces was collected from all over the floor of the pen.

Inhibition

DNA extraction

For DNA extraction we used the Qaigen QIAamp DNA stool kit and followed the protocol provided by the supplier. The main goals of this kit are to degrade cells to precipitate proteins and exclude inhibitors and to bind all DNA to a filter. Finally a buffer was added to get the DNA out of the filter. We then made dilutions with sterile (MQ) water.

Quantitative PCR (qPCR)

A reaction mixture (without DNA template) was made in a laminar flow cabinet. The qPCR reactions were set in a total volume of 20 µl, containing 5 µl of DNA template extracted from fecal samples with the stool kit as described above, 350 nM of forward and reverse primer, 10 µl of SYBR Green and 3,6 µl of MQ water. Primers for 16S were used as shown in table 1. The 15 µl of mixture with the 5 µl of DNA template was put in a 96 wells plate. The Bio-Rad MyiQ1 Single-Color Real-Time PCR Detection System was used and the amplification conditions were 3 minutes at 95°C, followed by 40 cycles of 15 second at 95°C, then annealing for 45 seconds at 52,5°C.

The amplification of the correct qPCR product was verified by analyzing the melting curves, which showed a peak at melting temperature 82°C, indicating a positive and correct amplification.

The handbook of the extraction kit suggests the option to optimize the reaction and to reduce inhibition by adding BSA (Bovine Serum Albumin) to the reaction mixture, as other articles suggested as well (QIAamp DNA Stool Handbook, Oikarinen 2009, Huijsdens 2004, Jiang 2005). We did this with a concentration of 0,1µg/µl. It proved ineffective.

Standard curve

Positive control preparation was done as follows. DNA is extracted from an overnight culture of a tet(S) positive strain, *Strep. dysagalactiae* (EF 682210), using Qiagen DNaesy Blood and Tissue kit. We measured the concentration by Nanodrop so we could calculate tet(S) gene copies. The standard curve was constructed by performing qPCR of dilutions of the known template.

Detection limit

For determining the detection limit we first set up a protocol. This can be found in annex 1.

Quantitative PCR (qPCR)

The qPCR reactions were set in a total volume of 20 µl, containing 5 µl of DNA template extracted from fecal samples with the stool kit as described above, 350 nM of forward and reverse primer, 10 µl of SYBR Green and 3,6 µl of MQ water. Primers for Tet(S) were used as shown in table 1. The 15 µl of mixture with the 5 µl of DNA template was put in a 96 wells plate. The amplification conditions were 3 minutes at 95°C, followed by 40 cycles of 15 second at 95°C, then annealing for 45 seconds at 50°C.

Gene	Rev/fw	Primer	Reference
tet(S)	fw	GAA AGC TTA CTA TAC AGT AGC	Aminov R 2001
tet(S)	rev	AGG AGT ATC TAC AAT ATT TAC	Aminov R 2001
16S	fw	ATGGYTGTCGTCAGCTCGTG	Amann RI 1995
16S	rv	GGGTTGCGCTCGTTGC	Wilmotte A 1993

Table 1 Reverse and forward primers of tet(S) and 16S.

The amplification of the correct qPCR product was verified again by analyzing the melting curves, which showed a peak at melting temperature 80°C, indicating a positive and correct amplification.

Integron qPCR

To determine integrons in the feces samples we needed to set up a new qPCR protocol for these integron genes. Not many articles are published about quantification of integron genes by qPCR. To be specific we found Zhang et al 2009, Barraud 2010, Hardwick et al 2008 and Laroche 2009.

Results

Sampling method

We examined two sampling methods; the absorbing overshoes method and plastic cup method. With both methods we took feces from the ground, which is suitable for this research because we want to see which genes are present in a group of calves and not in individual calves. We then asked ourselves two

questions: 1) Can we see a difference from the qPCR restults in the amount of DNA we picked up from the pen. This is most important but if there is no difference then: 2) is there a difference in the convenience during the sampling.

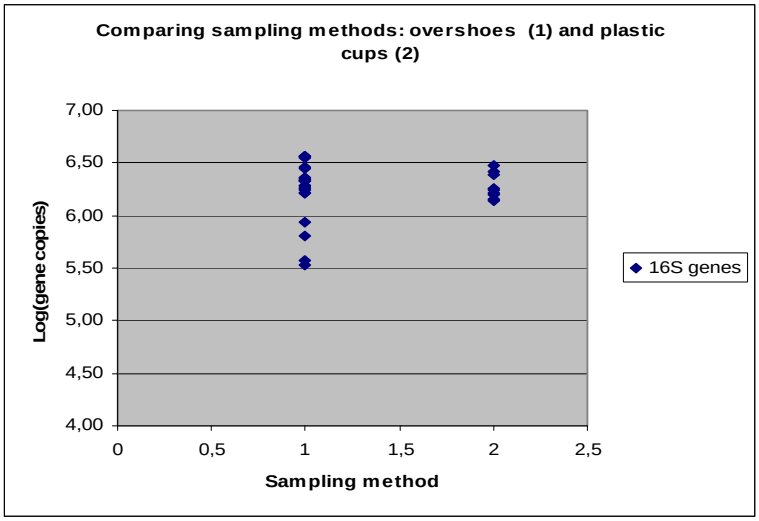

Figure 6 Comparing sampling methods; plastic cups (1) and overshoes (2)

By comparing samples from these two methods we concluded that there was no large difference in mean qPCR results as can be seen in figure 6. To determine this we amplified the 16S gene and compared threshold cycles (Ct), gene copies and log(gene copies) (see figure 6) from the two sampling methods. What can be seen is a bigger spread of results with the overshoe method. By performing an f-test with the different variances of the two methods we concluded that there is a difference in spread and it is significant.

$$\sigma^2 = \frac{\sum (x - \bar{x})^2}{(n-1)}$$

$$Ftest = \frac{\sigma_1^{\,2}}{\sigma_2^{\,2}} = \frac{0{,}085}{0{,}015} = 5{,}78 \text{ with } p < 0{,}05.$$

This is an argument why sampling with plastic cups gives better results. Besides this we looked at the convenience of the two methods. The overshoes were inconvenient but to get a sample one is forced to walk through the whole pen, which is necessary for a mixed sample. Using the plastic cups was more convenient, but sample takers have to pay attention that they will take samples from all over the pen and not from only one or two manure piles.

Inhibition

We wanted to determine if inhibition can be seen in DNA dilutions of our samples. To determine this we performed qPCR for different dilutions of the DNA. We tested this with the 16S gene because this gene is almost always present in high concentrations.

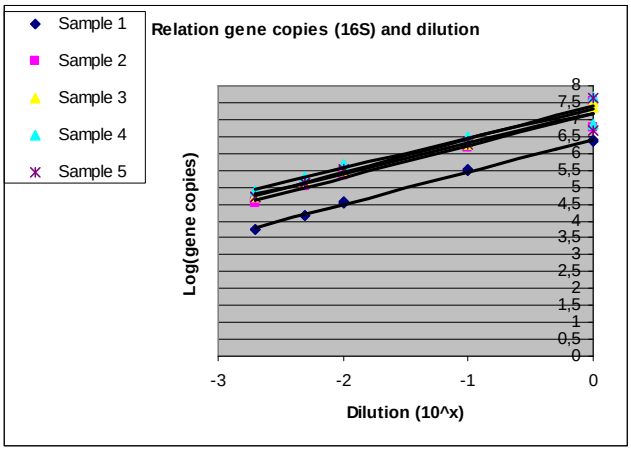

Figure 7 Relation between Log(dilution) and Log(gene copies)

Because inhibition factors will dilute with every higher dilution (and so have less influence), we tested 1, 10, 100, 200 and 500 fold dilutions of DNA. As can be seen in figure 7 there is a linear relation between gene copies and dilution. We choose the dilution which is as low as necessary with a slope of 3,3.

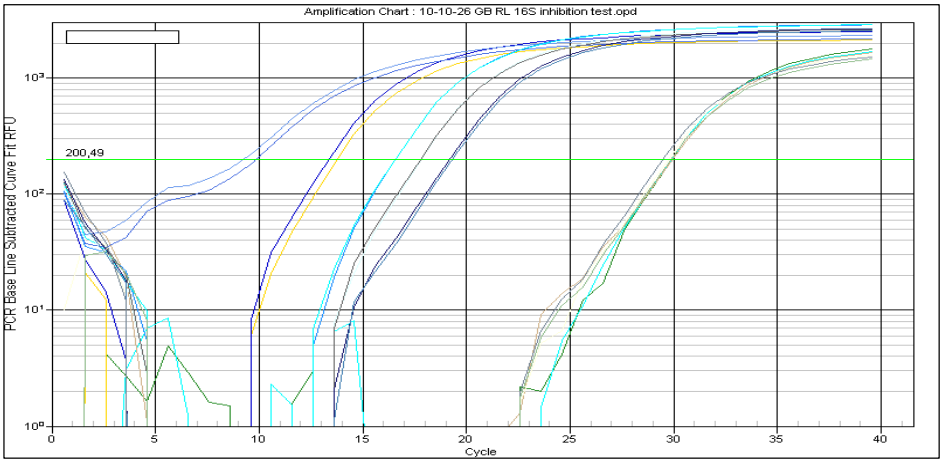

Figure 8 Amplification graph of different dilutions containing 16S genes

As seen in figure 7 (with 5 samples as example), there is a linear relation between the logarithm of the dilution factor and of the gene copies. The starting quantity increases with steps of 10. Besides that there is a 3,3 spread in cycles between dilutions as shown in figure 8. In that same figure we see a deviating first dilution graph but other dilutions give a good graph. This shows the lowest dilution sufficient for our purposes was the 10 fold dilution.

Standard curve

A six-point standard curve for qPCR was produced by nine fold serial dilution of positive controls in duplicate, from 10^6 to 10^2 gene copies per reaction. Our standard had a slope of -4,32 and an efficiency of 70,40% (purple blocked line). The following equations were used:

$$x = \frac{(Ct - b)}{a}$$

$$Eff = \left(10^{(-1/a)} - 1\right) * 100$$

In which **Ct** is the threshold cycle number, **b** is the intercept from the standard curve, **a** is the slope of the curve, **x** is the log gene copy number and **Eff** is efficiency of the slope. The graph is showed in figure 9.

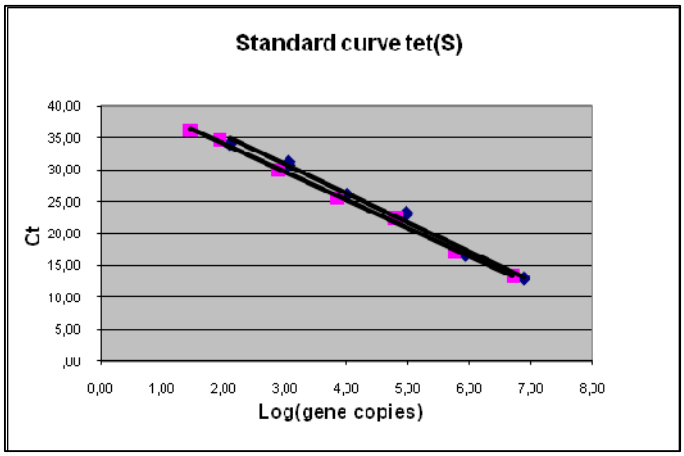

Figure 9 Standard curve tet(S) for detection limit determination.

During the experiments it turned out that the standard solution was not stable. Every time we used it, the efficiency of the reaction decreased. The blue blocked line in figure 9 shows these results. We chose to use the curve with efficiency 70,40% to calculate all results.

Detection limit

With the standard curve we could calculate gene copies of our samples as displayed in the next figures with logarithmic scale, with a threshold at 200 RFU (Larionov 2005).

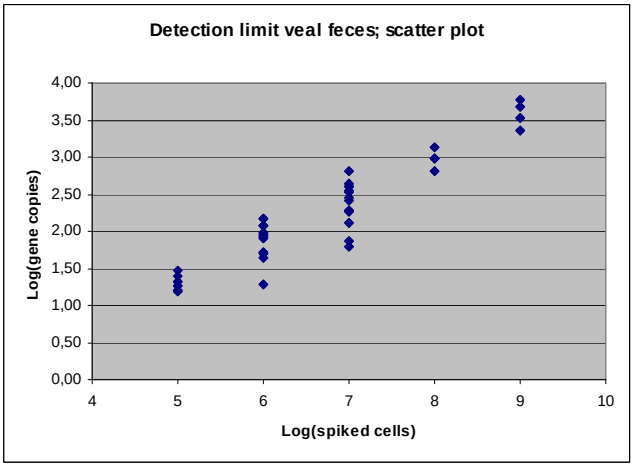

Figure 10 Detection limit veal feces, per gram feces; scatter plot

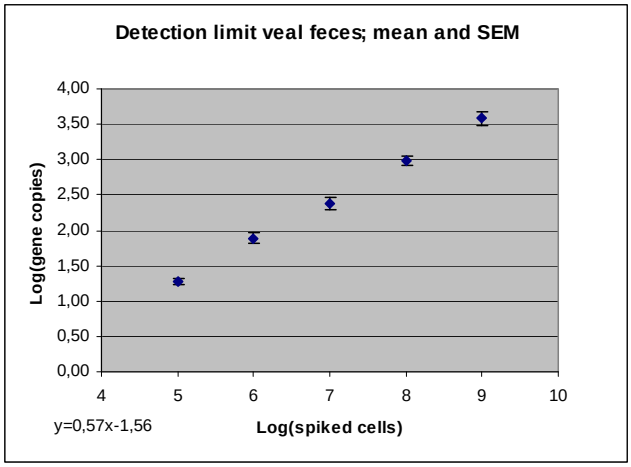

Figure 11 Detection limit veal feces per gram feces; mean and standard error of the

The following equations were used:

$$SD = \sigma = \sqrt{\frac{\sum(x-\bar{x})^2}{(n-1)}}$$

$$SEM = \frac{\sigma}{\sqrt{n}}$$

As can be seen in figures 10 and 11 detection in these fecal samples was shown to be linear between 10^9 and 10^5 cells per gram of feces.

We have an absolute detection limit of 10^1,28 gene copies, determined from samples with 10^5 spiked cells.

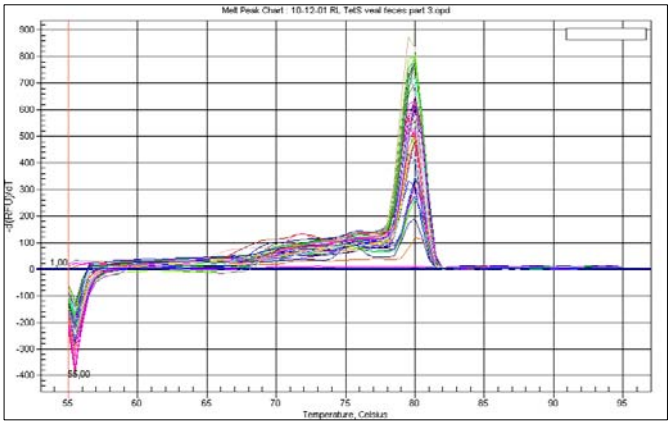

Figure 12 Meltcurve qPCR with good peaks

72% of all duplicates between 10^9 and 10^5 gave a reliable result. Below 10^5 results are not reliable. Those samples didn't show good melt curves (see figure 13) or showed very odd results. In figure 12 the melt curves from all the good samples are shown. As can be seen they all have a peak at 79-80°C just like the standard has.

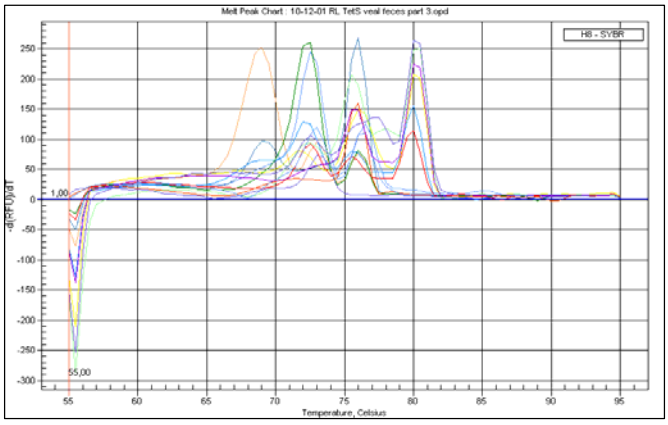

Figure 13 Meltcurves qPCR with different peak(s)

Integron qPCR

Because this research group didn't have experience in quantification of integrase by qPCR, we had to make a new protocol. In literature we found two useful articles; Zhang et al 2009 an Barraud et al 2010.

The difference between these two is that Zhang et al 2009 uses SYBR green as fluorescent dye and Barraud et al 2010 uses a fluorescent probe. We chose to try both methods to see which gives better results. The results will come in next period.

Discussion

Aim of research

This research is the beginning of a two year study into antibiotic resistance genes in veal feces. In the next three years a big reduction of antibiotic resistance in the Netherlands has to be realized. Of course the biggest factor in this decrease will have to be the reduction of antibiotic use. But besides that, other ways are investigated of which this research is an example. During two years changes will be made in management on the selected veal farms. All approaches will combine hygiene with less antibiotic use. By taking samples every six months reduction in AR genes will be monitored. But before the start of a large research, some conditions have to be clear.

Sampling method

For this research we need general fecal samples of the calves. We want to know the status of AR genes on a veal calf farm. In general all animals are treated the same way, have a lot of contact with each otherand get the same care. That is why we don't expect big differences between animals. Besides that, management adjustments will be made per farm, so we expect to see differences per farm not per animal. This is why we want to take mixed samples from different pens, to get representative samples from all farms. Our chosen sample method, the plastic cups method, will provide this and is easy to perform.

Inhibition

Several articles write about qPCR inhibition factors in feces (Kreader 1995, Pei 2006, Oikarinen 2009). We did not find much inhibition at all. We tested 1, 10, 100, 200 and 500 fold dilution. With every DNA dilution, inhibition factors will dilute too. Only the 1 fold dilution sometimes gave deviating results. At a 10 fold dilution we had proper results. One of the reasons for the little inhibition (al least less then expected) could be the 'inhibition factors reduction step', which is included in the DNA extraction ktit. The handbook of the extraction kit suggested the option to optimize the reaction and to reduce inhibition by adding BSA (Bovine Serum Albumin), as other articles suggested as well (handbook, Oikarinen 2009, Huijsdens 2004, Jiang 2005). When we did (0,1µg/µl), the

results were not different. We concluded that our reaction was already optimized and not inhibited (at a 10 fold dilution). It is possible that more BSA does have an affect (Jiang 2005), but we didn't test that.
There are no inhibition factors in the standard dilutions, so that is why it is important to remove them from the samples. Otherwise it's difficult to calculate the gene copies in the samples, from the standard curve (Hardwick 2007).

Standard curve

To interpret qPCR data, a standard (calibration) curve is needed. qPCR results are Ct (threshold cycle) values. Only if we know how many genes are in the standard dilutions we know what those Ct values mean. With that knowledge, we can calculate how many genes are in the samples. By calculating the efficiency, we can determine the quality of the reaction. The higher its efficiency the better (Yu 2005, Manuzon 2007, Auerbach 2006). The following equation was used:

$$Eff = \left(10^{(-1/a)} - 1\right) * 100$$

In which **Eff** stands for efficiency and **a** for the slope of the standard curve
The difficulty with our detection limit qPCR measurements was caused by an instable standard curve. To determine the detection limit we measured the tetracycline resistance gene tet(S). We chose tet(S) because the group of tetracycline's is a wide used antibiotic. And we determined that tet(S) normally is present in a low concentrations in veal feces (results not shown), so it would not interfere with our detection limit measurements.
Almost every time we used this standard it's efficiency has been decreased compared to previous experiments. With different standards it is impossible to compare different qPCR data. Unfortunately, but expected, a few articles write about their standard curve and especially about their possible difficulties with it (Töwe 2010, Bustin 2009, Larionov 2005).

We have thought of following options as possible negative influences on the efficiency:

1. Damaged DNA because of frequently thawing and freezing (Bustin 2009)
2. Damaged DNA because of electronic pipetting
3. Weak binding of primer to target DNA because of primer dimers and secondary structures (Bustin 2009)
4. Wrong reaction mixture used for this qPCR
5. Wrong storage material

The likelihood of these options is explained beneath:

1. If all DNA is equally damaged the slope of the standard curve would not change and that's why the efficiency will not change either. Only the Ct values would be higher because of less gene copies but this causes a parallel shifting of the graph. It is still possible that the freezing and thawing is bad for the DNA dilutions.
2. See explanation 1. In literature we found no articles write about this possibility.
3. Primer dimers are unlikely candidates because that would give a different peak in the melt curve and we did not see this at all. Secondary structures, when present at the DNA part where primers have to bind, can interfere with primer binding. This is unlikely because we didn't see them, while we predict them with the Mfold program (http://mfold.rna.albany.edu/).
4. We used the same mixture for all qPCR reactions and this problem is only seen with this standard, so it would be very unlikely the mixture is the factor of negative influence.
5. BioRad pointed out to us that they get a lot of complaints about the brand 'Bioplastics'. They supply plastic strips, to store DNA dilutions. We did use them for standard dilutions so this is a reasonable explanation.

To get a decrease in efficiency, the slope of the standard has to get steeper (**a** has to become lower) every time. This means the Ct values of high dilutions are getting relatively higher than the Ct values of low diluted standard DNA. If DNA damaging is the reason for the problem, then the DNA of the high dilutions is relatively more damaged than the DNA of de low dilutions. Finding a reason for that is difficult, but it could be the case. Maybe somehow the DNA in higher concentration is more protected for damaging by freezing and thawing then DNA in low concentration. Or possible present degrading enzymes have more influence on small amounts or DNA. A solution for this could be keeping the standard dilution in the fridge while using it, if not for too long (Bustin 2009).
Besides this we asked Bio-Rad, the company responsible for the machine if they know this 'problem' and if it has something to do with the machine or other material as mentioned above. That is why we are going look into the option that those plastic strips may have bad influence on the DNA dilutions.

Detection limit

We want to determine the detection limit, because we want to know the minimum absolute gene copies that can be measured in feces and how many cells that mean in the real sample. Gene copies beneath that minimum cannot be detected by qPCR, so it could be there but we can't measure it. This represents the limitation of the technique. This knowledge is needed if we want to draw conclusions from the final data.

We found a detection limit of 10^5 spiked cells in 1 gram feces, which is equal to 10^5 genes per gram. The fact that there is a linear relation between gene copies and DNA dilution and that results are repeatable, confirms that 10^5 cells per gram feces is a reliable result as detection limit. Very few articles write about the detection limit, let alone about their procedure to determine it, so comparison is difficult. We expect much more AR cells in the future feces samples, so 10^5 is a workable detection limit.

A curious thing is that the qPCR of the samples with 10^5 spiked cells, should show 10^2 genes per sample, but show an average of 41 gene copies. What also results in a slope of 0,57 instead of 1 which we 'expected' (figure 11). So it looks like we 'lost' some genes between the point where we spiked the sample to the point where the DNA is pipetted in de 96 wells plate. This 'loss' could have different reasons. In the first place we could have really lost DNA during DNA extraction, which is the result of inaccuracy of the extraction kit. Besides that it could (just) be a limitation of the qPCR device with a reason we can't explain. This last explanation does not seem to be the case because we did not see that much inaccuracy with standard dilutions, which where not made with a DNA extraction kit. So the inaccuracy seems to come from the DNA extraction. Which indeed is a complicated process. Because 16S genes will be determined in future samples as well, the inaccuracy of the DNA extraction kit will not be problematic.

Integron qPCR

Integrons can carry and exchange antibiotic resistance genes. Integrons are not mobile themselves but are found on plasmids and transposons which are mobile (Davies 2010, Gillings 2008, Mazel 2006). We will set up a protocol to detect and quantify Integrase 1 (and maybe later 2 and 3) by qPCR. The advantages of detecting integrons are as followed:

First, it is of great interest which genes are linked to which integron, because there seems to be a relation between an integrase and AR genes (Barroud 2010, Leverstein-van Hall 2003). So we can roughly say: if we determine the integron we know the AR genes.

Second, to find integrase implies that there is easy gene transfer (and there are mobile elements) present. Because those integrons can carry AR genes it means that the AR genes probably spread fast among the present bacteria. This is important to be aware of, if a long research is being performed and you want to see concentrations of AR genes vary in time. Besides these reasons, it is good to learn more about integrons and where and in what concentration they are present, because there is still a lot unknown (Zhang 2009).

We couldn't find much literature about quantification of integrons by qPCR. Most groups write about determining integrons with normal PCR (nPCR). The big advantages of qPCR over nPCR are that first, we can see differences in time and secondly, qPCR is more sensitive then nPCR (Hardwick 2007). Because this research group never quantified integrons before, a protocol had to be made.

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Annex 1

Protocol detection limit:

1. Step 1
    a. Culture S*trep. dysgalactiae* (to determine how many tet(S) genes are really in the dilutions):
        i. Make 5 dilutions (from -7 to -3) in duplicate

    b. Autoclave sample (to get the feces DNA free):
        i. 2 times 15 minutes at 121°C

    c. Spike samples:
        i. Spike all samples with 0,01 ml of each dilution tet(S)
        ii. Make triplicates

2. Step 2
    a. Use 10 TSA agar plates (20ml/plate)
    b. Spread 0,1 ml of each dilution over agar plate
    c. Place in the stove (1 night at 37°C)
    d. Next day: count colonies (1 colony = 1 bacterium = 1 tet(S) gene)

    Max count 25 – 250 per plate

3. Step 3
    a. Extract DNA

4. Step 4
    a. Use Qaigen QIAamp DNA Stool kit and follow protocol

5. Step 5
    a. Run qPCR for tet(S)
    b. Make standard curves

Used article: Mathys 2008