Master Thesis

Fine-mapping of the F4ab/F4ac receptor locus on pig chromosome 13

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Fine-mapping of the F4ab/F4ac receptor locus on pig chromosome 13

Master thesis
from
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10.03.2014

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Feinkartierung des F4ab/F4ac Rezeptorlocus auf Schweinechromosom 13

Die Arbeit umfasst einleitend eine Darstellung des Kenntnisstandes der enterotoxigenen E.coli Bakterien-erkrankungen beim Schwein (ETEC).


Ein alternatives Typisierungsverfahren mittels Schmelzkurven-Analyse soll für den aussichtsreichsten Marker entwickelt und auf seine Praxistauglichkeit getestet werden.

Referent: PD Dr. S. Neuenschwander
Korreferent: Prof. Dr. M. Kreuzer
Betreuer: Herr A. Rampoldi
Abstract

Enterotoxigenic *Escherichia coli* (*ETEC*) with F4ab/F4ac fimbriae are an important cause of diarrhea in pigs and can therefore be responsible for considerable economic losses and negative impacts on animal welfare. Since some pigs are resistant to adhesion of *ETEC* F4ab/F4ac in the intestine, a genetic approach to implement F4ab/F4ac resistance in breeding schemes is under development. The receptor locus for ETEC F4ab/F4ac has been mapped to a region around the gene *MUC13* on the pig chromosome 13, and recent research gave high evidence that *MUC13* is responsible for resistance/susceptibility against F4ab/F4ac adhesion. Due to technical difficulties in amplification and sequencing in parts of *MUC13*, the causative mutation has not yet been found. Therefore, genotyping of marker single nucleotide polymorphisms (SNPs) is used to predict the receptor genotype for F4ab/F4ac susceptibility/resistance. For efficient and cost-effective SNP genotyping, alternative methods to sequencing and polymerase chain reaction – restriction fragment length polymorphism tests (PCR-RFLP tests) are desired. The methods Kompetitive Allele-Specific PCR (KASP), Melting Temperature (Tm)-Shift, and High Resolution Melting (HRM), which are based on allele-specific PCR or melting curve analysis, are thus tested on reliability and applicability in the present thesis. With the method KASP, we genotyped pigs from different sample groups on the marker SNPs *ALGA5*, *CFCH1* and *CFCH2*.

For a possible replacement of the current PCR-RFLP test for *E. coli* F18 resistance, a KASP assay was developed and successfully tested. The established labour intensive PCR-RFLP test can therefore be replaced for genotyping purposes.

The KASP assays for the different SNPs achieved 100% concordant results with previous genotyping data. Importantly, no samples got a wrong genotype assigned. Some samples did not amplify well, due to poor DNA quality or amount, and remained without assigned genotype.

Similar results were achieved for Tm-shift. The two Tm-shift assays for *ALGA5* and *CFCH2* both genotyped all samples concordant to previous data.

With HRM, we were unable to reliably genotype samples for *ALGA5* and *CFCH2*. It is concluded, that HRM makes higher demands on assay development and result interpretation compared to KASP and Tm-shift. In terms of applicability, KASP and Tm-shift are comparable and both simple to perform. Tm-shift has the advantage to be more flexible due to the primer design being done directly in the lab. Furthermore Tm-shift is more suitable to genotype few samples while KASP may be more suitable to genotype many samples, due to different demands on the minimal number of samples.
and the number of necessary control samples. KASP and Tm-shift are considered both suitable to genotype marker SNPs for ETEC F4ab/F4ac resistance, while HRM is considered less suitable due to extra costs for the analysis software and the suggested higher demands on assay development.

Based on our experiences with KASP assays for CFCH1 and CFCH2, the SUISAG decided to genotype 271 boars in use for artificial insemination (AI) in a routine lab. The two markers were completely concordant in all 196 Large White boars, whereas in the breeds Piétrain, Duroc and Landrace up to 40% showed discordant genotypes. Fifteen recombinant boars were genotyped with additional markers and phenotype information from three Landrace boars revealed the SNP CFCH1 to be prioritized in this breed. Duroc and Piétrain pigs can only be reliably genotyped if CFCH1 and CFCH2 give concordant results. Phenotypic data of discordant genotypes are needed in these breeds in order to select a suitable marker.
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1. Introduction

1.1. *E. coli* diarrhea

Pathogenic *Escherichia (E.) coli* bacteria are an important cause of diarrheal diseases and oedema disease in pigs, often occurring during the neonatal and postweaning period. In both periods enterotoxigenic *E. coli* (*ETEC*) are one of the most important pathotypes (Alexander, 1994; Hampson, 1994). These diseases are relevant wherever pigs are raised, all over the world, and can account for significant economic losses due to mortality and treatment costs (Edfors-Lilja and Wallgren, 2000; Fairbrother and Gyles, 2012).

*ETEC* adhere to the brush borders of intestinal epithelial cells and produce enterotoxins, which can induce diarrhea through changes in the regulation of the ion concentration across the epithelial cell membranes, resulting in the secretion of water into the lumen of the intestine (Nataro and Kaper, 1998; Fairbrother and Gyles, 2012). In case of oedema disease, the enterotoxins enter the bloodstream and damage vessel walls resulting in oedema in various tissues (Fairbrother and Gyles, 2012). Cerebral oedemas lead to neurological injuries and therefore paralysis and similar symptoms typical for the disease.

The adhesion of *ETEC* to the enterocytes is mediated by surface antigens, called fimbriae (Edfors-Lilja and Wallgren, 2000). Several fimbrial antigens have been identified, and it has been recognized that species specificity is governed by them. For example, *ETEC* expressing F5 fimbriae are pathogenic for calves, lambs and pigs, while *ETEC* carrying F4 fimbriae are only pathogenic for pigs (Nataro and Kaper, 1998).

*ETEC* with fimbriae of the types F4, F5, F6 and F41 are important for neonatal diarrhea in pigs. In postweaning diarrhea (PWD), *ETEC* with F18 and/or F4 fimbriae are predominant (Fairbrother and Gyles, 2012). In an examination of *E. coli* isolates from diarrheic pigs submitted for testing in the US in 2007, 57.6% of the strains possessed fimbrial genes, whereof F4 fimbriae accounted for 64.6% and F18 fimbriae for 34.3% respectively, while other fimbrial genes accounted for not more than 1.1% (Zhang et al., 2007). Similar results were also found in a study from Slovakia, but with a slightly higher occurrence of other fimbrial genes (Vu-Khac et al., 2007).

For F4 fimbriae, three antigenic variants have been identified: F4ab, ac and ad (Guinée and Jansen, 1979), with F4ac being the most prevalent (Alexa et al., 2001). F18 has two known variants, ab and ac, with F18ab being associated with oedema disease and F18ac being a possible cause for PWD like *ETEC* F4 (Fairbrother and Gyles, 2012).
The prevalence of ETEC with different fimbriae types and variants can differ between countries (Alexa et al., 2001; Wang et al., 2006; Zhang et al., 2007) and breeds (Michaels et al., 1994; Li et al., 2007).

1.2. Resistance to ETEC

The adhesion of the bacteria with their fimbriae occurs on specific receptors on the brush border of the enterocytes. Some pigs do not express these receptors and are therefore resistant to colonization and diarrhea caused by these bacteria (Bertschinger et al., 1993; Jacobsen et al., 2010). In view of genetic resistance, a breeding strategy as a method of disease control is highly promising. A genetic approach to reduce diarrheal diseases is sustainable, increases animal welfare and reduces the use of antibiotics, leading to less antibiotic resistant microbes.

In case of E. coli F18, research revealed that the FUT1 gene on pig chromosome 6 is responsible for E. coli F18 susceptibility/resistance. A SNP marker inside the FUT1 cDNA was found to co-segregate 100% with the E. coli F18 receptor, and this marker is currently used in the Swiss pig breeding program since several years, to reduce the prevalence of susceptibility to E. coli F18 in the Swiss pig population (Meijerink et al., 2000; Luther 2011).

Efforts are being made to extend the Swiss breeding program with the selection against E. coli F4ac susceptibility. However, the causative mutation has not yet been found. The past research progress can be summarized as follows.

Susceptibility to bacterial F4ab/F4ac adhesion was found to be inherited as a monogenetic trait with the susceptible allele dominant over the resistant allele (Sellwood et al., 1975; Gibbons et al., 1977). The receptor loci for F4ab and F4ac were found to be in strong linkage disequilibrium and several studies proposed either one locus coding for both receptors (Bijlsma and Bouw, 1987; Python et al., 2002; Jørgensen et al., 2003), or two closely linked loci (Guérin et al., 1993; Edfors-Lilja et al., 1995). For the present study, a combined locus for F4ab and F4ac is assumed (F4bcR), although the question of the relation between F4ab and F4ac susceptibility is not finally answered. However, since the focus is on F4ac in the present study, the choice of the model is of minor importance.

The receptor locus for ETEC F4ab/F4ac has been mapped to the pig chromosome 13 (SSC 13) (Edfors-Lilja et al., 1995; Python et al., 2002) (Figure 1). Subsequent studies narrowed down the possible location further. Joller et al. (2009) refined the region to a 5.7 cM interval spanned by the microsatellite markers SW207 and S0075. Rampoldi et al. (2011) found the 620 kb interval between the markers LMLN g.15920 and S0283 to be the most likely region.
Recent research gave high evidence that the gene \textit{MUC13}, inside the 620 kb interval, is responsible for resistance/susceptibility against F4ac adhesion. A Chinese group proposed \textit{MUC13} as the responsible gene (Ren et al., 2012). The most significant SNPs they studied were all inside \textit{MUC13} and \textit{p} values revealed a 1000-fold stronger association with the adhesion phenotype than the other SNPs studied. Furthermore, they found two different transcripts of \textit{MUC13} – \textit{MUC13A} and \textit{MUC13B}, of which \textit{MUC13B} is heavily O-glycosylated while \textit{MUC13A} is not. The O-glycosylation site is considered to be the binding site for the bacterium (Ren et al., 2012). Analysis of the association between the two transcripts and the F4ac adhesion revealed that all homozygous pigs for \textit{MUC13A} were resistant to F4ac adhesion and therefore all susceptible pigs carried at least one \textit{MUC13B} allele. The concordance between the known biochemical properties of \textit{F4acR} and the association results from the two alleles gave again further evidence for the causality of \textit{MUC13}.
However, in the association analyses, there were also 298 animals out of 718 with one (193) or two (105) $MUC13B$ alleles that were resistant to F4ac adhesion, indicating that the mechanism of susceptibility is not yet fully understood.

1.3. Application for breeding?

The chromosomal segment has been narrowed down continuously and good evidence has been found for $MUC13$ being the responsible gene for F4ab/F4ac susceptibility. However, the real causative mutation is still unknown and a diagnostic test using genomic DNA as a template could not be established due to sequence uncertainty and technical problems amplifying the DNA in the polymerase chain reaction (PCR). Therefore efficient and reliable haplotyping is needed to develop reliable tests for F4ab/F4ac susceptibility, and SNP markers continue to be of high relevance for the implementation of F4ac resistance in breeding schemes.

For SNP genotyping, sequencing and PCR-RFLP (restriction fragment length polymorphism) tests are established methods commonly used in the past. However, there are some considerable disadvantages to those methods that make alternative methods desirable. Sequencing for SNP genotyping, while very reliable, is also expensive and slow. PCR-RFLP tests are highly dependent on the existence of a diagnostic recognition site, which excludes PCR-RFLP as a method of choice for some SNPs. Furthermore, the PCR-RFLP method is labour- and time-intensive since several treatments of the DNA in different buffers and a long incubation time are necessary.

1.4. Objectives of the thesis

With the current genotyping methods, sequencing and PCR-RFLP test, being expensive or time consuming, more efficient alternatives are desired. Possible alternatives should allow a reasonable throughput and some automation possibilities while still being suitable to the daily lab use of a smaller lab.

To choose the methods for this thesis, we concentrated on methods that can be run on 96 well plates and came up with methods based on allele-specific PCR or melting curve analysis, namely Kompetitive allele-specific PCR (KASP), Melting temperature (Tm)-shift and High resolution melting (HRM).
Allele-specific PCR uses two different forward primers and a common reverse primer to separately amplify the two alleles. The primers are each complementary to one of the two alleles of the SNP in their last nucleotide at the 3’-end. The primers can be labeled with different methods, allowing the discrimination of the two alleles through their respective primers.

Melting curve analysis allows differentiation between two different PCR fragments by their specific melting behavior, when temperature is increased and the double-stranded DNA (dsDNA) starts to diverge in single strands (melting). The melting behavior is monitored by a dsDNA binding dye, that emits a fluorescent signal only when being bound to dsDNA and excited with laser light.

Those methods, KASP, Tm-shift and HRM, are believed to possibly fulfill the set of requirements and are thus tested on reliability and applicability to genotype SNP markers for $F4bcR$ in pigs.

The analysis of chosen SNP markers with these genotyping methods shall allow to test pigs for $F4bcR$ in a reliable, cost effective and easy way.

Furthermore, the possibility to adapt the method to test pigs for F18 genotype, as a replacement of the current PCR-RFLP test, is of further interest.
2. Material and Methods

2.1. Genotyping Methods

2.1.1. Sequencing

Sequencing allows to determine the arrangement of the nucleotides of a DNA fragment. Traditional is the Sanger sequencing method. Sanger sequencing is based on dideoxy-nucleotides, which prevent the further elongation of a DNA strand when they are incorporated into a strand during DNA synthesis. To sequence a DNA fragment, four PCR reactions are done, each with one of the four nucleotides as a dideoxy-nucleotide (ddNTP) and all four normal (deoxy) nucleotides (dNTP). In each reaction several fragments of different length are synthesized, that all end with the respective dideoxy-nucleotide, depending on the sequence. For example, in the reaction containing ddATP, all possible fragments are synthesized that end on the nucleotide A, adenosine. The four solutions from the PCR reactions are then run in a gel electrophoresis that is able to separate fragments that differ in size by only one nucleotide. From the resulting picture, the sequence can be determined by following the length of the fragments and checking the respective ddNTP that stopped the synthesis of said fragment.

Over time, the method evolved and instruments improved, but the principle remained the same. Today, capillary sequencers are often used instruments, where only one reaction with four differently labeled ddNTPs is done and the sequencing products are separated in a capillary instead on a gel. At the bottom of the capillary, the labeled ddNTPs of the fragments that flow past are monitored.

2.1.2. PCR-RFLP

In the PCR-RFLP test, a DNA fragment, that contains the SNP, is first amplified with PCR. Then the fragment is digested with an appropriate restriction enzyme. A restriction enzyme recognizes a specific sequence of a few nucleotides, called restriction site, and cuts the DNA strand at a defined position within or close to the restriction site. In case of the alternative allele, the restriction site is mutated and the restriction enzyme does not cut the DNA fragments. A restriction site, which is specific for one allele of a SNP, is called diagnostic restriction site, whereas a non-allele-specific restriction site is called constant and can give an indication of the correct function of the restriction enzyme. After digestion, the resulting fragments are analysed by gel electrophoresis, which allows
determining the number of different fragments, as well as their length. In case of *E. coli* F18, the enzyme *Cfo*I cuts also at a second, constant restriction site in both alleles (Figure 2), which results in an additional fragment that is always present. Depending on the genotype, the following fragments (in bp) are expected in the test: homozygous AA (375, 25), homozygous BB (288, 87, 25) or AB heterozygous (375, 288, 87, 25).

![Figure 2: PCR-RFLP test for *E. coli* F18. The diagnostic restriction site GCGC is present in the maternal chromosome but mutated to ACGC in the paternal chromosome. Numbers indicate the lengths of the resulting fragments [in bp].](image)

### 2.1.3. KASP

The KASP method is based on two allele-specific primers, which are elongated with a tail-sequence at the 5’-end, and two differently fluorescently labeled oligo-nucleotides (oligos) that can bind to the complementary sequence of the tail-sequences. As a third primer, a common reverse primer is used. With quencher oligos that are complementary to the sequence of the fluorescent oligos, the fluorescent signal is quenched until incorporation of the fluorescent oligos during PCR (Figure 3, A).

For the KASP reaction, two components are necessary: a reaction mix, containing PCR reagents and the oligos, and a primer mix, containing the three primers specific for the target sequence.

A two-step touchdown-PCR is performed, in which the tailed primers, are elongated, resulting in longer strands. Each fragment representing an allele variant is amplified by its respective primer, while the other primer does not lead to an elongation (Figure 3, B).

During a touchdown-PCR, the initially high annealing and extension temperature is successively lowered with each cycle (-0.8°C/cycle). This procedure allows more specific amplification in the first cycles, while maintaining sufficient amplification of the product in the later cycles.
Once the complementary strand to the tail-sequences is built, the fluorescent oligos can bind to these complementary sequences. The fluorescent signal is then no longer quenched and therefore each allelic strand is labeled with the corresponding fluorescent dye (Figure 3, C), ready to be analysed on a fluorescence plate reader. To differentiate between genotypes, the strengths of the fluorescent signals of the two dyes are plotted against each other.

Figure 3: Principle of the allele-specific PCR by KASP (A-C) and Tm-shift (D-F). KASP uses two allele-specific primers with different tails that correspond in their sequence to fluorescently labeled oligo-nucleotides (A), and a common reverse primer (light blue). The fluorescent oligos, indicated as H and F in the figure, are quenched by quencher-oligos (indicated with Q) such that no fluorescence can be detected. During PCR, only one primer is elongated for each allele (B) and therefore, the PCR leads to two allele-specific products that are each labeled by their respective fluorescent oligo (C), since the oligos bind to the complementary sequence of the primer tails that is present on one strand of the PCR products. Finally, the two allele-specific PCR products can be detected through their fluorescence label. Tm-shift works similar, but with two allele-specific primers that have a GC-rich tail of different length (D), resulting in two allele-specific PCR products of different length in addition to the nucleotide difference at the SNP (F). Primer elongation during PCR (E) is working just like in KASP. Detection of the two products in Tm-shift is done by melting curve analysis.
2.1.4. Tm-shift

Genotyping with Tm-shift is done with allele-specific PCR primers that are extended with GC-rich tails of different length at the 5’-end. A primer for one allele gets a short tail and the primer for the other allele gets a long tail (Figure 3, D). A common primer is used as reverse primer. PCR with these primers results in two allele-specific products of different length and different sequence (Figure 3, E and F).

To analyse and differentiate the two PCR products, melting curve analysis is performed after PCR. The allele-specific PCR was performed in presence of a dsDNA binding fluorescent dye (e.g. SYBR green, Eva green), that is now used to keep track of the melting of the PCR products. The fluorescent dye is bound to the double-stranded DNA and fluoresces only in this bound state. During melting curve analysis, the temperature is slowly increased, and the dsDNA starts to dissociate into single strands (melting), releasing the dye. Therefore, the fluorescent signal decreases during melting, giving a melting curve when the fluorescent signal is plotted against temperature. The point, where the decrease of the fluorescence is highest, and where the melting curve has the steepest slope, is defined as the melting point.

Melting point and shape of the melting curve depend on fragment length and GC content (GC-rich sequences have a higher melting point compared to AT-rich sequences of the same length). Therefore, a melting curve is specific for a certain DNA fragment.

For clear differences in melting temperatures (Tm), it is also important that the primers are designed to amplify just a short fragment (less than 70 bp, according to the Fluidigm Tm-shift protocol). The PCR and melting curve analysis are carried out on a real time PCR System.

2.1.5. HRM

HRM is based on the amplification of a short DNA fragment (80 to 100 bp; recommendation from the ABI HRM guide) containing the SNP, in presence of a dsDNA binding, fluorescent dye, and DNA melting after PCR as an endpoint analysis (melting curve analysis). The melting curve analysis basically works the same way as in the method Tm-shift. But due to improved dyes, high resolution in acquiring data during the melting stage and sophisticated software, it is possible to differentiate single nucleotide differences, provided that a DNA fragment is short enough.
2.2. Procedures

2.2.1. Pigs

Pigs from four different sample groups were used for the analyses. Group 1 consisted of 46 samples of known F4ab/F4ac phenotypes and known marker genotypes from pigs of a crossbreed between Large White purebred and Large White/Landrace crossbred, originating from a university experimental herd (UEH) at the Department of Farm Animals, Faculty of Veterinary Medicine, University of Zurich. The UEH was studied in several papers in association with E. coli F4 (e.g. Joller et al., 2009, Jacobsen et al., 2010, Rampoldi et al., 2011).

Group 2 consisted of 30 samples taken from a group of 40 representative samples of the Swiss pig population from the Large White and Large White sire-line breeds. The pigs were randomly selected at the Swiss Performing Station of Sempach, the SUISAG, in June and July 2012.

Group 3 consisted of 32 blood samples from pigs from practical farms in Switzerland, taken and analysed for E. coli F18 phenotype in the year 2012.

In addition, 15 boars of the breeds Duroc, Piétrain and Landrace, used for artificial insemination (AI) in Switzerland and originating from the SUISAG, were genotyped with KASP on the SNPs ALGA0072075 (ALGA5), CFCH1 and CFCH2. The samples were previously genotyped in an external lab (Dr. van Haeringen Laboratorium B.V. (VHL), Wageningen, Netherlands) as recombinant between CFCH1 and CFCH2. Genotyping of these boars happened in a process of routine genotyping of 271 AI boars from the SUISAG on the SNPs CFCH1 and CFCH2 as markers for F4ab/ac susceptibility. To confirm the recombinant genotypes, the samples were genotyped again in the present study.

2.2.2. DNA extraction from blood

DNA was extracted from blood samples, stored at -20°C, with the “GenElute™ Mammalian Genomic DNA Miniprep Kit” from Sigma-Aldrich (St. Louis, MO, USA).

After thawing, the blood samples were vortexed and 500 µl blood were pipetted into an Eppendorf tube. To separate and wash the white blood cells, 1400 µl ECL buffer were added, the tube shortly vortexed and then centrifuged for 15 min at 13000 rpm at room temperature. After that, the supernatant was removed with a water-jet vacuum pump. The remaining pellet was resuspended in 1400 µl ECL buffer, again vortexed and centrifuged for 15 min at 13000 rpm. The supernatant was removed a second time.
To the remaining pellet, 200 µl of resuspension solution were added and the cells were resuspended with a pipette, by slowly pipetting up and down for several times. Then, 20 µl of Proteinase K were added and the tube shortly vortexed, before 200 µl of Lysis Solution C were added, again with a short step of vortexing. The sample was put into the water bath for 10 min, which was preheated to 55°C. For the DNA elution step, the binding columns were prepared with 500 µl column preparation solution and a centrifugation step at 13000 rpm for 1 min. The flow-through was discarded. After incubation, 200 µl of 100% ethanol were added to the lysate and mixed by vortexing for 5-10 s. Then, the whole content of the tube was transferred to the binding column and the column was centrifuged for 1 min at 13000 rpm. The tube with the flow-through was then discarded and the column was placed in a new 2 ml collection tube.

500 µl of wash solution, that was previously prepared with ethanol according to the instructions of the manufacturer, were added to the binding column and centrifuged for 1 min at 13000 rpm. The flow-through was discarded and another 500 µl of wash solution were added to the column. Another centrifugation step of 3 min at 13000 rpm was done to dry the binding column. If there was still ethanol on the column, the flow-through was discarded again and the column was centrifuged for another 1 min at 13000 rpm. Finally, the binding column was placed in a new 2 ml collection tube.

To elute the DNA from the column, 200 µl of elution solution were pipetted directly into the center of the binding column. The column was then covered with a paper towel and let for 5 min for incubation of the elution solution at room temperature. Finally, the binding column was centrifuged at 13000 rpm for 1 min.

2.2.3. Measuring the DNA concentration with Qubit™

Measurement of DNA concentration with Qubit™ (Invitrogen, Carlsbad, CA, USA) is based on fluorescence of the sample compared to the fluorescence of a two point standard curve (0 to 100 ng/µl). Before the start of the measurements, two standards are needed to calibrate the Qubit™ fluorometer. In order to prepare the samples and the two standards, a master mix of 1 µl Quant-iT™ reagent and 199 µl Quant-iT™ buffer per sample and standard was prepared. The standards and the samples were each prepared in a clear 0.5 ml tube.

For the standards, 190 µl of master mix and 10 µl of standard 1 (0 ng/µl) or standard 2 (10 ng/µl) respectively were pipetted into the tube. The tubes were then vortexed for a few seconds and incubated at room temperature for 2 min. After that, the Qubit™ fluorometer was calibrated with standard 1 and 2, following the instructions on the fluorometer.
For the samples, 199 µl of master mix and 1 µl of DNA sample were pipetted into the tube and vortexed for a few seconds, following an incubation period of 2 min at room temperature, before the sample tube was placed in the fluorometer and measurement started.

2.2.4. Primers

The primers used in this thesis are given in Table 1. Primers were synthesized by Microsynth (Balgach, Switzerland). Primers for the KASP assays were designed by the company LGC Genomics (Teddington, Middlesex, UK) based on the provided sequences. No sequence information about the primers was delivered by the company. The sequences that were sent to LGC Genomics for primer design are shown in Table 2.

Table 1: List of the used primers. Tail-sequences (italics) and annealing sequences are divided by a slash. Underlined is the mismatch nucleotide in the mismatch primers.

<table>
<thead>
<tr>
<th>SNP</th>
<th>Primer name</th>
<th>Sequence 5'-&gt; 3'</th>
<th>Fragment size</th>
<th>SNP position on Sscrofa 10.2</th>
</tr>
</thead>
<tbody>
<tr>
<td>CFCH2</td>
<td>A4s</td>
<td>CGC/GGT AGC TGA GCC CCT</td>
<td>46 bp (A4s/A4r)</td>
<td>SSC13: 145009837</td>
</tr>
<tr>
<td></td>
<td>A4l</td>
<td>TGC CGC CTG CCT GCG/GTA GCT GAG CCC CC</td>
<td>57 bp (A4l/A4r)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>A4sm</td>
<td>CGC/GGT AGC TGA GCC CAT</td>
<td>46 bp (A4sm/A4r)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>A4lm</td>
<td>TGC CGC CTG CCT GCG/GTA GCT GAG CCC AC</td>
<td>57 bp (A4lm/A4r)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>A4c</td>
<td>CCT TTA GGT AGC TGA GCC CC</td>
<td>49 bp (A4c/A4r)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>A4r</td>
<td>ACA CAG ACT TTA AGT ACA ATG GGA A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ALGA5</td>
<td>A5s</td>
<td>CGC/CAT GCG TTG GAG AAT GGT</td>
<td>72 bp (A5s/A5r)</td>
<td>SSC13: 144832256</td>
</tr>
<tr>
<td></td>
<td>A5l</td>
<td>TGC CGC CTG CCT GCG/ATG CGT TGG AGA ATG GC</td>
<td>83 bp (A5l/A5r)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>A5sm</td>
<td>CGC/CAT GCG TTG GAG AAT GAT</td>
<td>72 bp (A5sm/A5r)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>A5lm</td>
<td>TGC CGC CTG CCT GCG/ATG CGT TGG AGA ATG AC</td>
<td>83 bp (A5lm/A5r)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>A5c</td>
<td>CTT TGG AAG CCA TGC GTT GG</td>
<td>79 bp (A5c/A5r)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>A5r</td>
<td>GGT GGC TCT CCC CAA CAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FUT1 M307</td>
<td>Fut1ecF</td>
<td>CCA CCT CTG TCT GAC CTT CC</td>
<td>400 bp (Fut1ecF/R)</td>
<td>SSC6: 49826913</td>
</tr>
<tr>
<td></td>
<td>Fut1ecR</td>
<td>AGG AGC GTG CCT GTC TAC C</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 2: List of sequences that were sent to LGC Genomics for KASP primer design. SNP positions are given in brackets and bold.

<table>
<thead>
<tr>
<th>SNP Identifier</th>
<th>SNP position on Sscrofa 10.2</th>
<th>Sequences sent to LGC Genomics for primer design</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alga 0106330 (=ALGA4 / CFCH2)</td>
<td>SSC13: 145009837</td>
<td>RTGTTGGTTCCATAGCCAGTGGCATCCAGCAACTGTAACACAGACTTTAGTGAGGAGGCAAGAATGGGCTAAGTG</td>
</tr>
<tr>
<td>Alga 0072075 (=ALGA5)</td>
<td>SSC13: 144832256</td>
<td>GATTGGGAGTGTAATTCAGGCCCCTGGGAGAATGG[C/T]AGAGCCACTCAGATGAGACCTGAGTGGCTGTGTTGGGAGAGCCACCCA</td>
</tr>
<tr>
<td>CFCH_1_144944384</td>
<td>SSC13: 144944384</td>
<td>CTAATGGGAGAATTCCAGGCCCCTGGGAGCGGATCCGACCTGGGACATGGTGGGTGTTGGGAGAGCCACCCA</td>
</tr>
<tr>
<td>F18_FUT1_M307</td>
<td>SSC6: 49826913</td>
<td>GGATGGCCTTTTGGGACATGGGACATGAGACCTGCTGGGCCCTG[G/A]CGCAGCTCAACGCGGCGCGCGCCTTCATCCAGGCCTGGCA CGCGTGCTGGGCC</td>
</tr>
</tbody>
</table>

2.2.5. Standard PCR

PCRs were performed in 200 µl single tubes or 8-tube strips, on a PTC100 (MJ Research, Bioconcept, Allschwil, Switzerland) or a TPersonal thermocycler (Biometra, Biolabo, Châtel-St-Denis, Switzerland). Reaction volumes were 25 µl containing 50-250 ng DNA, 200 µM of each dNTP, standard PCR buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM MgCl$_2$, 0.001% gelatine), 10 pmol of each forward and reverse primer, up to 0.5 mM additional MgCl$_2$, and 1.5 U Taq DNA polymerase or 0.75 U Taq DNA JumpStart polymerase.

The PCR conditions were set for an initial denaturation at 94°C for 2 min, followed by 30-38 cycles of the following steps: denaturation at 92°C for 25 s, annealing at 50–65°C for 25 s, and extension at 72°C for 25 s. At the end, the samples were extended at 72°C for 2 min.

2.2.6. Gel electrophoresis

Agarose gel electrophoresis was used to check the result of the PCR before a product was sent for sequencing.

For that purpose, the following procedure was used: Depending on the number of samples that needed to be analysed, a small gel of 50 ml or a big gel of 100 ml was prepared. If not differently stated, gels were prepared with 1.2% agarose. For the small gel, 0.6 g Agarose powder was weighed into an Erlenmeyer flask and 50 ml of 0.5x TBE buffer (Appendix) was filled into it. For a big gel, 1.2 g
Agarose and 100 ml of 0.5x TBE buffer were used. Then, the flask was heated in a microwave oven to completely melt the Agarose. After heating, the flask was placed on a shaker for 10 min, then 0.9 µl (small gel) or 1.2 µl (big gel) ethidium bromide (10 mg/ml) were added and the fluid was poured into a frame containing a comb with eight or 20 slots. Polymerization was normally finished within 20 min.

When the gel was ready, the comb was removed, the frame with the gel was placed in the buffer solution (0.5x TBE buffer) and the gel was loaded with 5 µl of sample DNA, mixed with 1 µl of loading dye (Appendix). In one slot, 3 µl of a 50 bp ladder was loaded.

The voltage was set to 120-160 V, and was stopped when the bromophenol blue dye reached around 2/3 of the gel length. The DNA fragments were visualized on a UV-transilluminator and pictures were done using a photo camera with a red filter.

2.2.7. Sequencing

The SNP markers MUC4-8227, CFCH3, MUC13_G.15376, and MUC13-226 were determined in the recombinant AI boars by sequencing. A DNA fragment containing the SNP was amplified with standard PCR (see chapter 2.2.5), which was performed in three replicates.

After PCR, the result was checked by gel electrophoresis and if the DNA fragment amplified well, the DNA was purified using Microcon centrifugal filter units (Merck Millipore, Billerica, MA, USA). A filter unit was placed on top of an Eppendorf tube and 340 µl of ddH₂O were added onto the filter. From the three tubes with the PCR product, 60 µl of the product were placed in the filter unit containing the ddH₂O. The filled unit was centrifuged for 15 min at 1000x g (3400 rpm). Afterwards, the filter was placed in a new Eppendorf tube and 15 µl of ddH₂O were added. After 5 min, the filter was placed upside down in the tube. With a final step of centrifugation for 2 min at 1000x g, the purified DNA was eluted.

The amount of eluted DNA was measured with Qubit™ (see chapter 2.2.3) and the needed volume of DNA sample for sequencing was calculated. 15 ng of DNA per 100 bp fragment length were needed for sequencing in a final volume of 10 µl, containing 1 µl (20 µM) of the forward or the reverse primer. The tube was sent to the company Microsynth, which performed the sequencing.
2.2.8. PCR-RFLP test

To evaluate the KASP genotyping results for the *E. coli* F18 locus, results from a PCR-RFLP test as described by Meijerink et al. (1997) and Vögeli et al. (1997) of sample group three were used for comparison.

In brief: a 400 bp DNA fragment was amplified with Standard PCR with the primers FUT1ecF/R (Table 1). Correct amplification was checked by gel electrophoresis. Then the PCR product was digested with 1-2 units of the restriction enzyme *Cfo*I according to the manufacturer’s protocol (Promega Corp., Madison, WI, USA). Digestion was carried out in a 30 µl reaction with 10 µl PCR product. Master mix and PCR product were pipetted into a 1.5 ml Eppendorf tube and mixed by briefly vortexing followed by a short spin. The samples were then put into the incubator over night at 37°C. After incubation, the digested samples were then analysed by gel electrophoresis.

2.2.9. KASP

We investigated four SNPs with KASP: ALGA0106330 (*CFCH2*), ALGA0072075 (*ALGA5*), *CFCH1*, and *FUT1-M307* (*ECF18R*). The sequences, shown in Table 2, were sent to LGC Genomics for the primer design by their service KASP-by-design and we received the ready-to-use primer mixes together with the reaction mix. Table 3 shows the allocation of the fluorescent markers HEX and FAM to the alleles of the SNPs.

Table 3: Allocation of the detection markers to the alleles of each SNP for KASP. HEX and FAM are the two fluorescent dyes used in the KASP assays. Susceptible or resistant allele means the allele of the SNP that is associated with F4ab/F4ac (*ALGA5*, *CFCH1*, *CFCH2*) or F18 (*F18 M307*) susceptibility or resistance, respectively.

<table>
<thead>
<tr>
<th>SNP</th>
<th>Susceptible allele</th>
<th>Marker</th>
<th>Resistant allele</th>
<th>Marker</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALGA5</td>
<td>T</td>
<td>HEX</td>
<td>C</td>
<td>FAM</td>
</tr>
<tr>
<td>CFCH2</td>
<td>G</td>
<td>FAM</td>
<td>A</td>
<td>HEX</td>
</tr>
<tr>
<td>CFCH1</td>
<td>A</td>
<td>HEX</td>
<td>C</td>
<td>FAM</td>
</tr>
<tr>
<td>F18 M307</td>
<td>G</td>
<td>HEX</td>
<td>A</td>
<td>FAM</td>
</tr>
</tbody>
</table>

The KASP assays were carried out according to the KASP protocol from Genetic Diversity Centre (GDC) at ETH Zurich.

The setup for one reaction was as follows:
- 5 µl KASP 2x reaction mix
- 0.14 µl primer mix
5 µl genomic DNA sample (0.4 ng/µl to over 120 ng/µl DNA)

The reaction mix and the primer mix were pipetted together into a master mix in a 1.5 ml Eppendorf tube, vortexed and spun briefly before pipetting 5 µl master mix into each well of the Fast RT-PCR (real time PCR) reaction plate. 5µl DNA sample were then added to the wells. The plate was sealed with an optical adhesive film followed by a short spin of the plate in order to remove air bubbles.

PCR was performed under the following conditions (Table 4) on a Labcycler (Sensoquest, Göttingen, Germany):

<table>
<thead>
<tr>
<th>Stage</th>
<th>Step</th>
<th>Temp.</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Holding</td>
<td>Enzyme activation</td>
<td>94°C</td>
<td>15 min</td>
</tr>
<tr>
<td>Cycling (10 cycles)</td>
<td>Denature</td>
<td>94°C</td>
<td>20 sec</td>
</tr>
<tr>
<td></td>
<td>Anneal/extend</td>
<td>Touchdown 65°C (-0.8°C per cycle)</td>
<td>1 min</td>
</tr>
<tr>
<td>Cycling (30 cycles)</td>
<td>Denature</td>
<td>95°C</td>
<td>10 sec</td>
</tr>
<tr>
<td></td>
<td>Anneal/extend</td>
<td>60°C</td>
<td>1 min</td>
</tr>
<tr>
<td></td>
<td>Final</td>
<td>4°C</td>
<td>∞</td>
</tr>
</tbody>
</table>

The fluorescence analysis was carried out on an ABI 7500 FAST RT-PCR System (Applied Biosystems, Foster City, CA, USA) at 25°C for 30 s with the following settings (Table 5):

<table>
<thead>
<tr>
<th>Document/experiment properties</th>
<th>Instrument: 7500 Fast (96 wells)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Experiment type: genotyping</td>
</tr>
<tr>
<td></td>
<td>Reagents: TaqMan reagents</td>
</tr>
<tr>
<td></td>
<td>Ramp speed: standard (2 hours to complete a run)</td>
</tr>
<tr>
<td></td>
<td>What to include: post PCR read only</td>
</tr>
<tr>
<td>Detector/target and plate layout</td>
<td>Reporter: VIC/FAM (allele 1/allele 2)</td>
</tr>
<tr>
<td></td>
<td>Passive reference: ROX</td>
</tr>
<tr>
<td></td>
<td>Quencher: none</td>
</tr>
<tr>
<td>Thermal profile/run method settings</td>
<td>Reaction volume per well: 10 µl</td>
</tr>
<tr>
<td></td>
<td>Reading temperature: 25°C for 30 sec</td>
</tr>
</tbody>
</table>

If the results were not satisfactory, a re-cycling/re-reading step with 5 or 10 cycles of 94°C for 20 s and 57°C for 1 min was done in the common PCR machine, followed again by fluorescence analysis on the RT-PCR system.
2.2.10. Tm-shift

Derzelle et al. (2011) reported that allele-specificity for Tm-shift is improved, if a destabilizing mismatch is introduced within the 3’-end of the allele-specific primers. This was adapted for our experiments, in order to test if better allele discrimination can be reached with mismatch primers, compared to matching primers. Therefore, a second batch of forward primers was ordered, where the primers had an adenine at the penultimate base of the 3’-end as mismatch (see Table 1).

Tm-shift was tested on the SNPs CFCH2 and ALGA5. The assays were carried out according to the Tm-shift protocol from GDC. The allocation of the short and the long primer tail to the alleles of the SNPs is shown in Table 6.

Table 6: Allocation of the detection markers to the alleles of each SNP for Tm-shift. Susceptible or resistant allele means the allele of the SNP that is associated with F4ab/F4ac susceptibility or resistance, respectively.

<table>
<thead>
<tr>
<th>SNP</th>
<th>Susceptible allele (S)</th>
<th>Primer tail</th>
<th>Resistant allele (s)</th>
<th>Primer tail</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALGA5</td>
<td>T</td>
<td>short</td>
<td>C</td>
<td>long</td>
</tr>
<tr>
<td>CFCH2</td>
<td>G</td>
<td>long</td>
<td>A</td>
<td>short</td>
</tr>
</tbody>
</table>

To perform a Tm-shift assay, a solution of 0.25% Tween 20 in TE buffer was first prepared. This was done in 1.5 ml tubes in two steps, in order to improve the accuracy of pipetting, since the high viscosity of Tween 20 complicates pipetting. In a first step, 10 µl of Tween 20 were pipetted into 90 µl of TE buffer (Appendix), to create a 10% solution of Tween 20 in TE. That solution was diluted 40 times to get the working solution of 0.25% Tween 20 in TE. So 37.5 µl of the 10% solution were pipetted into 1462.5 µl TE buffer. After every step, the tube was vortexed briefly to mix the components.

The allele-specific primers, short and long, and the common reverse primer were combined to a primer mix. Four different primer ratios, short to long primer, were tested for the assays. Mixes A-D, with approximate ratios of 3.5:1, 2:1, 1:1 and 1:2, short to long primer were prepared according to Table 7. The primers were thoroughly mixed through repeated pipetting in the solution.
Table 7: Components for the Tm-shift primer mixes.

<table>
<thead>
<tr>
<th>Components</th>
<th>Mix A (3.5:1)</th>
<th>Mix B (2:1)</th>
<th>Mix C (1:1)</th>
<th>Mix D (1:2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.25% Tween 20 in TE</td>
<td>100 µl</td>
<td>100 µl</td>
<td>100 µl</td>
<td>100 µl</td>
</tr>
<tr>
<td>Short primer 100 µM</td>
<td>4.3 µl</td>
<td>2.1 µl</td>
<td>2.125 µl</td>
<td>2.125 µl</td>
</tr>
<tr>
<td>Long primer 100 µM</td>
<td>1.25 µl</td>
<td>1.05 µl</td>
<td>2.125 µl</td>
<td>4.35 µl</td>
</tr>
<tr>
<td>Reverse primer 100 µM</td>
<td>2.15 µl</td>
<td>2.1 µl</td>
<td>2.125 µl</td>
<td>2.175 µl</td>
</tr>
<tr>
<td>Total volume</td>
<td>107.7 µl</td>
<td>105.25 µl</td>
<td>106.38 µl</td>
<td>108.65 µl</td>
</tr>
</tbody>
</table>

For plate preparation, a master mix with the following reagents was prepared in a 1.5 ml Eppendorf tube:

6 µl of 2x TaqMan gene expression mix (Life Technologies, Carlsbad, CA, USA)
0.6 µl of 20x Eva Green (Biotium, Hayward, CA, USA)
2.4 µl of ddH₂O

The mixing of the components was reached through vortexing and a short spin. The master mix was then pipetted into the Fast RT-PCR reaction plate, previously placed on ice. After the master mix, 1.2 µl of the appropriate primer mix were added to each well, followed by the addition of the DNA samples in a volume of 1.5 µl (7 ng/µl to over 120 ng/µl DNA). Total volume was 11.7 µl.

After pipetting, the plate was sealed with an optical adhesive film, spun briefly and placed again on ice as well as kept protected from light until the run was performed.

The plates were run on an ABI 7500 Fast RT-PCR System, running the 7500 software v2.0, with the following settings and PCR conditions (Tables 8 and 9):

Table 8: Software settings for the Tm-shift assays.

<table>
<thead>
<tr>
<th>Document/experiment properties</th>
<th>Instrument:</th>
<th>Experiment type: Quantitation-Comparative Ct</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagents: SYBR</td>
<td>Ramp speed: standard (2 hours to complete a run)</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Thermal profile/run method settings</th>
<th>Reaction volume per well: 11 µl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Passive reference: ROX</td>
<td>Reporter: SYBR</td>
</tr>
<tr>
<td>Quencher: none</td>
<td>Quencher: none</td>
</tr>
</tbody>
</table>
Table 9: RT-PCR conditions for the Tm-shift assays.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Step</th>
<th>Temp.</th>
<th>Time</th>
<th>Fluorescence reads</th>
</tr>
</thead>
<tbody>
<tr>
<td>Holding</td>
<td>Enzyme activation</td>
<td>95°C</td>
<td>10 min</td>
<td></td>
</tr>
<tr>
<td>Cycling (2 cycles)</td>
<td>Denature</td>
<td>95°C</td>
<td>10 sec</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Anneal/extend</td>
<td>60°C</td>
<td>6 min</td>
<td></td>
</tr>
<tr>
<td>Cycling (38 cycles)</td>
<td>Denature</td>
<td>95°C</td>
<td>10 sec</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Anneal/extend</td>
<td>60°C</td>
<td>1 min</td>
<td>X</td>
</tr>
<tr>
<td>Melt curve stage</td>
<td>Denature</td>
<td>95°C</td>
<td>15 sec</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Anneal</td>
<td>60°C</td>
<td>1 min</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dissociation/HRM</td>
<td>95°C</td>
<td>30 sec</td>
<td>X</td>
</tr>
<tr>
<td></td>
<td>Anneal</td>
<td>60°C</td>
<td>15 sec</td>
<td></td>
</tr>
</tbody>
</table>

To determine the appropriate primer ratio, two samples of each genotype were tested with primer mix C (Table 5), with an equal primer ratio, and the results were checked for differences in peak heights between the peaks for the short and the long primer in heterozygous samples. If the peaks from one primer were smaller, compared to the other primer, an increase of the ratio of that primer to the other should increase the height of those peaks. Therefore, on a second plate, primer mixes with a higher ratio of the respective primer were again tested on two samples per genotype.

### 2.2.11. HRM

We investigated the two SNPs CFCH2 and ALGA5 with HRM, according to the HRM protocol from GDC, and amplified a fragment of 49 bp and 79 bp respectively, containing the SNP. For amplification of CFCH2 primers A4c and A4r, and for ALGA5 the primers A5c and A5r were used (Table 1). Reactions were set with the following volumes:

- 10 µl MeltDoctor HRM master mix (Life Technologies, Carlsbad, CA, USA)
- 1.2 µl of each primer (5 µM), forward and reverse
- 6.6 µl ddH2O
- 1 µl of genomic DNA (7 ng/µl to over 120 ng/µl DNA)

Total volume was 20 µl. The reagents were combined to a master mix and 19 µl of master mix were pipetted into the Fast RT-PCR reaction plate, followed by 1 µl of DNA sample (7 ng/µl - >120 ng/µl DNA). The plate was placed on ice during pipetting. Afterwards, the plate was sealed with an optical adhesive film, spun briefly and stored on ice, as well as protected from light, until the run was performed.
The plates were run on an ABI 7500 Fast RT-PCR System, running the 7500 software v2.0, with the following settings and PCR-conditions (Tables 10 and 11):

Table 10: RT-PCR conditions for the HRM assays.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Step</th>
<th>Temp</th>
<th>Time</th>
<th>Fluorescence reads</th>
</tr>
</thead>
<tbody>
<tr>
<td>Holding</td>
<td>Enzyme activation</td>
<td>95°C</td>
<td>10 min</td>
<td></td>
</tr>
<tr>
<td>Cycling (40 cycles)</td>
<td>Denature</td>
<td>95°C</td>
<td>15 sec</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Anneal/extend</td>
<td>60°C</td>
<td>1 min</td>
<td>X</td>
</tr>
<tr>
<td>Melt curve stage</td>
<td>Denature</td>
<td>95°C</td>
<td>10 sec</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Anneal</td>
<td>60°C</td>
<td>1 min</td>
<td></td>
</tr>
<tr>
<td></td>
<td>High resolution melting</td>
<td>95°C</td>
<td>30 sec</td>
<td>X</td>
</tr>
<tr>
<td></td>
<td>Anneal</td>
<td>60°C</td>
<td>15 sec</td>
<td></td>
</tr>
</tbody>
</table>

Table 11: Software settings for the HRM assays.

<table>
<thead>
<tr>
<th>Document/experiment properties</th>
<th>Instrument: 7500 Fast (96 wells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment type</td>
<td>Quantitation-Standard Curve</td>
</tr>
<tr>
<td>Reagents</td>
<td>Other, include melt curve selected</td>
</tr>
<tr>
<td>Ramp speed</td>
<td>Standard (2 hours to complete a run)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Detector/Target and plate layout</th>
<th>Reporter: MeltDoctor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Passive reference: none</td>
</tr>
<tr>
<td></td>
<td>Mark NTC and positive controls: none</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Thermal profile/run method settings</th>
<th>Reaction volume per well: 20 µl</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Expert mode: Expert mode selected</td>
</tr>
<tr>
<td></td>
<td>Select/View Filters: only Filter-1 selected</td>
</tr>
</tbody>
</table>

The results were finally analysed with the ABI HRM software.
3. Results

Assays were designed for KASP, Tm-shift and HRM, each for the two SNPs **CFCH2** and **ALGA5**, and samples with known genotypes, from the sample group one, were analysed with all three methods. In further experiments with KASP, samples with unknown genotypes, from group two, were analysed on the SNP **CFCH1** and the results were compared with the phenotype and other marker genotype information of the samples. Finally, an assay for the **F18-M307 SNP** was tested on samples from group three with known genotype.

3.1. KASP

3.1.1. KASP assays for **CFCH2** and **ALGA5**

Forty-four samples of known genotype were analysed with KASP on **CFCH2** and **ALGA5**. Results were taken after 0, 10 and 20 additional cycles after the regular PCR program of 40 cycles. For both SNPs, the results after ten additional cycles were chosen as the most valuable to determine the sample’s genotype.

The allelic discrimination plot from **CFCH2** is shown in Figure 4. Of the 44 investigated samples, the assigned genotypes of 40 samples were in accordance with earlier data. Although the samples inside the heterozygous (green) and the homozygous resistant (red) cluster are spread over a wide area, the clusters can be distinguished clearly from each other. Exceptions are the four samples near the bottom end of the heterozygous cluster (red circle) where differentiation between the heterozygous and the homozygous susceptible samples may be difficult. Therefore, two of these samples could not be assigned to a genotype and one sample was determined as heterozygous in opposition to earlier data that showed the sample as homozygous susceptible. The genotype of the fourth sample (homozygous susceptible) was again in accordance with earlier data.

For a better interpretation of the results and optimizing the assay, the DNA concentration of the samples was measured with Qubit after performing the assay. The concentrations in the samples ranged from 0.4 ng/µl to over 120 ng/µl. Of the four circled samples, three had very low concentrations below 4 ng/µl. For the fourth sample (2151), a concentration of 51.5 ng/µl was measured.
The sample B698 was expected to be homozygous susceptible according to earlier data. However, further analyses with KASP and Tm-shift showed that the sample is heterozygous. Another sample was extracted from the blood of that animal and was analysed. That newly extracted sample was determined as homozygous susceptible. Therefore, it is likely that our first sample was contaminated and accurately determined as heterozygous in the analyses although earlier data first suggested otherwise.

Considering the results from B698, the number of accurately detected genotypes in the first assay for CFCH2 is increased to 41 out of 44 samples.

To obtain an improved and more accurate result, a second KASP assay was performed. As optimization steps, DNA concentrations of the samples were balanced through dilution of the samples to 30 ng/µl. Samples with lower concentrations were used undiluted and samples with concentrations lower than 4 ng/µl were excluded from the analyses. Thirty-five samples, previously analysed on the first plate, were used in that assay, with nine of them being analysed twice through duplicates on the same plate. For nine samples, there was not enough material available, therefore we could only analyse 35 samples.

Results of the second KASP assay for CFCH2 (Figure 5), taken after ten additional cycles, showed the expected genotype for 34 of 35 samples. There was again the same sample, 2151, located between the homozygous susceptible and the heterozygous cluster, which could not be assigned to a genotype, as in the first assay. In that second assay, the samples clustered slightly better together, but there were also two outlier samples in the heterozygous group that were located farther away from the cluster.

From the nine duplicates, one did not amplify at all and therefore clustered together with the controls. The other eight duplicates were in line with their counterpart and got the same genotype assigned, clustering near together inside the respective cluster.
The exact same procedure for the analyses on CFCH2 was also applied to ALGA5, as the two KASP assays were run on the same two plates and the sample alignment was copied from one SNP to the other.

The allelic discrimination plot for ALGA5 from the first assay can be seen in Figure 6. Of the 44 samples, the genotypes of 39 samples were in accordance with earlier data. After the result of the sample B698 being contaminated and for that reason heterozygous, it is counted as accurately genotyped into that ratio. The three samples with very low concentrations had also in ALGA5 lower fluorescent values and could not be assigned to a genotype (red circle). The result from one sample, 2151 was not in accordance with earlier data. Instead, it was genotyped as homozygous resistant while earlier data suggested that the sample is heterozygous. Finally, there was one sample without a genotype assigned that is located between the heterozygous and the homozygous resistant cluster in the allelic discrimination plot.
The optimized assay for ALGA5 (Figure 7) resulted in a more clear separation of the heterozygous and the homozygous resistant cluster from each other, while the results were evaluated after five additional cycles. The two questionable samples from the first assay, between these two clusters were now genotyped in accordance with earlier data. However, another sample, which was expected to be homozygous susceptible due to earlier data, got located in the allelic discrimination plot between the homozygous susceptible and the heterozygous cluster and therefore did not get a genotype assigned.

In total, with the optimized assay for ALGA5 it was possible to genotype 34 of 35 samples in accordance to earlier data.

The results from the duplicates were comparable to those of CFCH2. The one duplicate that did not amplify in CFCH2, neither did in ALGA5. The other eight duplicates were again in line with their counterpart and therefore in accordance to earlier data.

<table>
<thead>
<tr>
<th>KASP assay for ALGA5</th>
<th>Optimized assay for ALGA5</th>
</tr>
</thead>
</table>

Figure 6: Allelic discrimination plot from a first KASP assay for ALGA5. The x- and the y-axis show the fluorescence values of the two reporters for allele T and C. Samples are indicated as coloured dots according to the assigned genotype or crosses if no genotype could be assigned. Black squares represent the controls (NTC). Blue dots represent homozygous resistant samples (CC genotype), green means heterozygous (CT) and red dots represent homozygous susceptible samples (TT).

Figure 7: Allelic discrimination plot from the optimized KASP assay for ALGA5. See Figure 6 for further explanation.
3.1.2. Influence of cycle number on the result

The effect of the number of cycles is illustrated in Figure 8. It shows the results from the KASP assay for ALGA5 after 0, 10 and 20 additional cycles after the regular PCR program of 40 cycles. It can clearly be seen that the number of cycles is of great relevance for the genotyping result.

<table>
<thead>
<tr>
<th>Influence of cycle number</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1.png" alt="Allele Discrimination Plot" /></td>
</tr>
</tbody>
</table>

Figure 8: KASP assay for ALGA5 after 0, 10 and 20 additional cycles (from left to right). See Figure 6 for further explanation.

On the left, after 40 cycles, no genotypes were assigned through the software to any of the samples. Several samples positioned in regions on the plot where it is not possible to say whether they are heterozygous or homozygous CC (resistant).

In the middle, on the plot after 50 cycles, the samples clustered better together and the software assigned the genotypes to the samples inside the three clusters. Only four samples remained unassigned. A comparison of the left plot and the plot in the middle shows that with further PCR cycles, some samples moved closer together forming one of the three clusters for the three genotypes.

The plot on the right side shows the situation after 60 cycles. The assay was run for too many cycles resulting in a shift of the No Template Controls to the right, to direction of the fluorescence labeling the T-allele. The same shift can also be seen in the cluster for the genotype CC. Some of these samples are now unassigned because they shifted too much to the right.
3.1.3. CFCH1

In a further experiment, samples were analysed with a KASP assay on CFCH1 (Figure 9). Thirty samples from sample group two (SUISAG pigs) and 13 samples of sample group one (UEH) were used. DNA concentration was measured with Qubit and samples were diluted to a concentration of 25 ng/µl.

![KASP assay for CFCH1](image)

Figure 9: Allelic discrimination plot from the KASP assay for CFCH1. The x- and the y-axis show the fluorescence values of the two reporters for allele A and C. Samples are indicated as coloured dots according to the assigned genotype or crosses if no genotype could be assigned. Black squares represent the controls (NTC). Blue dots represent homozygous resistant samples (CC genotype), green means heterozygous (CA) and red dots represent homozygous susceptible samples (AA).

The results show clearly assigned genotypes for 41 samples out of 43. One sample did not amplify and therefore clustered with the NTCs. Another sample, of sample group one, did not get a genotype assigned, because it was located too far outside the clusters. This same sample also remained unassigned in the optimized assay for ALGA5. Two samples, B167 and 2313, were known to be recombinant between ALGA5 and CFCH2. Their CFCH1 genotype was in accordance with their ALGA5 genotype. The other ten samples from that group were all genotyped as expected from the available phenotype and marker genotype information (from CFCH2 and ALGA5), under the assumption that the F4bcR locus and the markers ALGA5, CFCH1 and CFCH2 are effectively linked. The genotyping results from the other 29 samples (SUISAG pigs) were also in accordance with phenotypes and the other marker genotypes.
3.1.4. \textit{F4bcR} marker genotypes

Genotype information was compiled for the samples that were analysed on all three markers \textit{ALGA5}, \textit{CFCH1} and \textit{CFCH2} (Table 12).

<table>
<thead>
<tr>
<th>Experimental herd</th>
<th>Samples</th>
<th>Phenotype</th>
<th>ALGA 5</th>
<th>CFCH1</th>
<th>CFCH2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B167</td>
<td>R</td>
<td>CC</td>
<td>CC</td>
<td>AG</td>
</tr>
<tr>
<td></td>
<td>2313</td>
<td>R</td>
<td>CC</td>
<td>CC</td>
<td>AG</td>
</tr>
<tr>
<td></td>
<td>1400</td>
<td>R</td>
<td>CC</td>
<td>CC</td>
<td>AA</td>
</tr>
<tr>
<td></td>
<td>B605</td>
<td>R</td>
<td>CC</td>
<td>CC</td>
<td>AA</td>
</tr>
<tr>
<td></td>
<td>2734</td>
<td>S</td>
<td>CT</td>
<td>AC</td>
<td>AG</td>
</tr>
<tr>
<td></td>
<td>2117</td>
<td>S</td>
<td>TT</td>
<td>AA</td>
<td>GG</td>
</tr>
<tr>
<td></td>
<td>2429</td>
<td>R</td>
<td>CC</td>
<td>CC</td>
<td>AA</td>
</tr>
<tr>
<td></td>
<td>3021</td>
<td>S</td>
<td>CT</td>
<td>AC</td>
<td>AG</td>
</tr>
<tr>
<td></td>
<td>2051</td>
<td>S</td>
<td>TT</td>
<td>AA</td>
<td>GG</td>
</tr>
<tr>
<td></td>
<td>8887</td>
<td>R</td>
<td>CC</td>
<td>CC</td>
<td>AA</td>
</tr>
<tr>
<td></td>
<td>2094</td>
<td>S</td>
<td>CT</td>
<td>AC</td>
<td>AG</td>
</tr>
<tr>
<td></td>
<td>B698</td>
<td>S</td>
<td>TT*</td>
<td>AA</td>
<td>GG*</td>
</tr>
<tr>
<td></td>
<td>756</td>
<td>S</td>
<td>TT*</td>
<td>AA</td>
<td>GG*</td>
</tr>
</tbody>
</table>

Legend:
- nd = not determined
- R = resistant
- S = susceptible
- * = Tm-shift results

\textit{ALGA5}: T = susceptible, C = resistant
\textit{CFCH1}: A = susceptible, C = resistant
\textit{CFCH2}: G = susceptible, A = resistant

Phenotype and genotype of all SUISAG samples were 100% consistent. From the experimental herd, there were the two recombinant samples (B167 and 2313), while all the other samples showed a
consistent phenotype and genotype too. The phenotype of the two recombinant samples was consistent with the genotype in ALGA5 and CFCH1, while the genotype in CFCH2 was recombinant.

Based on our experiences with KASP assays for CFCH1 and CFCH2, 271 boars from the SUISAG were genotyped on the markers CFCH1 and CFCH2 by the Dr. van Haeringen Laboratorium B.V. (VHL) (Table 13). The Large White boars were all concordant between the two markers, whereas up to 40% of the boars from the other breeds were revealed to be discordant. In total, 17 discordant boars were found in the breeds Piétrain, Duroc, and Landrace. Fifteen of these recombinant boars were genotyped again in the present study for confirmation. The two remaining boars could not be genotyped again, because DNA material was not available anymore.

Table 13: Number of samples concordant and discordant between the markers CFCH1 and CFCH2, analysed by VHL.

<table>
<thead>
<tr>
<th>Breed</th>
<th>discordant</th>
<th>concordant</th>
<th>Total genotyped</th>
<th>% discord.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Piétrain</td>
<td>6</td>
<td>19</td>
<td>25</td>
<td>31.6</td>
</tr>
<tr>
<td>Landrace</td>
<td>6</td>
<td>15</td>
<td>21</td>
<td>40.0</td>
</tr>
<tr>
<td>Large White</td>
<td>0</td>
<td>196</td>
<td>196</td>
<td>0.0</td>
</tr>
<tr>
<td>Duroc</td>
<td>5</td>
<td>24</td>
<td>29</td>
<td>20.8</td>
</tr>
<tr>
<td>Total</td>
<td>17</td>
<td>254</td>
<td>271</td>
<td>6.7</td>
</tr>
</tbody>
</table>

Our genotyping results for CFCH1 and CFCH2 were in agreement with the results from VHL on 14 of the 15 samples. Sample 655 HVE was genotyped as homozygous resistant in all three SNPs, ALGA5, CFCH1, and CFCH2, contrary to VHL that genotyped the sample as heterozygous in CFCH2. A further analysis of additional SNPs gave further evidence that the sample is not recombinant between the tested SNPs. (Table 14)

The genotypes of the recombinant AI boars revealed three different recombinations between the SNPs CFCH1 and CFCH2.

Phenotype information has only been available for three of these boars (personal communication, A. Bratus), as the phenotype is analysed post mortem and most of the boars are either still in use for AI or were already slaughtered without intestine sampling. The three phenotyped Landrace boars were all resistant to E. coli F4ab/F4ac adhesion and the genotypes in ALGA5, CFCH1, and CFCH3 were in accordance with the phenotype in all three boars, and MUC4-8227 only in boar 5676 NO3. The markers MUC13_G.15376, CFCH2, and MUC13-226 were discordant with the phenotype.
Table 14: Marker genotypes from 15 AI boars, considered recombinant between \textit{CFCH1} and \textit{CFCH2} based on results from VHL. R indicates a resistant phenotype, while \textit{r} and \textit{s} indicate the marker genotypes associated with resistance and susceptibility to \textit{E. coli} F4ab/F4ac respectively.

<table>
<thead>
<tr>
<th>Animal-ID</th>
<th>Breed</th>
<th>MUC4-8227</th>
<th>ALGA5</th>
<th>CFCH1</th>
<th>CFCH3</th>
<th>MUC13_G.15376</th>
<th>CFCH2</th>
<th>MUC13-226</th>
<th>Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>1153 HK</td>
<td>Duroc</td>
<td>\textit{r/r}</td>
<td>\textit{r/r}</td>
<td>\textit{r/r}</td>
<td>\textit{r/r}</td>
<td>\textit{r/s}</td>
<td>\textit{r/s}</td>
<td>\textit{r/s}</td>
<td></td>
</tr>
<tr>
<td>1216 HK</td>
<td>Duroc</td>
<td>\textit{r/r}</td>
<td>\textit{r/r}</td>
<td>\textit{r/r}</td>
<td>\textit{r/r}</td>
<td>\textit{r/s}</td>
<td>\textit{r/s}</td>
<td>\textit{r/s}</td>
<td></td>
</tr>
<tr>
<td>1221 HK</td>
<td>Duroc</td>
<td>\textit{r/r}</td>
<td>\textit{r/r}</td>
<td>\textit{r/r}</td>
<td>\textit{r/r}</td>
<td>\textit{r/s}</td>
<td>\textit{r/s}</td>
<td>\textit{r/s}</td>
<td></td>
</tr>
<tr>
<td>1259 HK</td>
<td>Duroc</td>
<td>\textit{r/r}</td>
<td>\textit{r/r}</td>
<td>\textit{r/r}</td>
<td>\textit{r/r}</td>
<td>\textit{r/s}</td>
<td>\textit{r/s}</td>
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<td></td>
</tr>
<tr>
<td>8318 JS3</td>
<td>Landrace</td>
<td>\textit{r/s}</td>
<td>\textit{r/r}</td>
<td>\textit{r/r}</td>
<td>\textit{r/r}</td>
<td>\textit{r/s}</td>
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<td>\textit{r/s}</td>
<td>\textit{R}</td>
</tr>
<tr>
<td>844 JS4</td>
<td>Landrace</td>
<td>\textit{r/s}</td>
<td>\textit{r/r}</td>
<td>\textit{r/r}</td>
<td>\textit{r/r}</td>
<td>\textit{r/s}</td>
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<td>\textit{r/s}</td>
<td>\textit{R}</td>
</tr>
<tr>
<td>7463 JS3</td>
<td>Landrace</td>
<td>\textit{r/r}</td>
<td>\textit{r/s}</td>
<td>\textit{r/r}</td>
<td>\textit{r/r}</td>
<td>\textit{s/s}</td>
<td>\textit{s/s}</td>
<td>\textit{s/s}</td>
<td></td>
</tr>
<tr>
<td>5676 NO3</td>
<td>Landrace</td>
<td>\textit{r/r}</td>
<td>\textit{r/r}</td>
<td>\textit{r/r}</td>
<td>\textit{r/r}</td>
<td>\textit{s/s}</td>
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<td>\textit{s/s}</td>
<td>\textit{R}</td>
</tr>
<tr>
<td>7918 JS3</td>
<td>Landrace</td>
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<td>\textit{r/r}</td>
<td>\textit{r/r}</td>
<td>\textit{r/r}</td>
<td>\textit{s/s}</td>
<td>\textit{s/s}</td>
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</tr>
<tr>
<td>644 PPG</td>
<td>Piétrain</td>
<td>\textit{r/s}</td>
<td>\textit{r/s}</td>
<td>\textit{r/r}</td>
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<td>\textit{s/s}</td>
<td>\textit{s/s}</td>
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<td></td>
</tr>
<tr>
<td>204 BH</td>
<td>Piétrain</td>
<td>\textit{r/r}</td>
<td>\textit{r/r}</td>
<td>\textit{r/r}</td>
<td>\textit{r/r}</td>
<td>\textit{r/s}</td>
<td>\textit{r/s}</td>
<td>\textit{r/s}</td>
<td></td>
</tr>
<tr>
<td>564 HVE</td>
<td>Piétrain</td>
<td>\textit{r/r}</td>
<td>\textit{r/r}</td>
<td>\textit{r/r}</td>
<td>\textit{r/r}</td>
<td>\textit{s/s}</td>
<td>\textit{s/s}</td>
<td>\textit{s/s}</td>
<td></td>
</tr>
<tr>
<td>660 HVE</td>
<td>Piétrain</td>
<td>\textit{r/r}</td>
<td>\textit{r/r}</td>
<td>\textit{r/r}</td>
<td>\textit{r/r}</td>
<td>\textit{r/s}</td>
<td>\textit{r/s}</td>
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<td></td>
</tr>
<tr>
<td>655 HVE</td>
<td>Piétrain</td>
<td>\textit{r/r}</td>
<td>\textit{r/r}</td>
<td>\textit{r/r}</td>
<td>\textit{r/r}</td>
<td>\textit{r/r}</td>
<td>\textit{r/r}</td>
<td>\textit{r/r}</td>
<td></td>
</tr>
</tbody>
</table>

3.1.5. \textit{ECF18 M307}

In the last experiment, the SNP \textit{F18 M307} was analysed on different samples with KASP (Figure 10), to check whether a test with that method would be possible for \textit{E. coli} F18 genotyping instead of a PCR-RFLP test.

For that purpose, 32 samples, all tested with the PCR-RFLP test, were analysed with the KASP assay. The samples were diluted to a concentration of 30 ng/µl and three randomly selected samples were analysed as their undiluted counterpart to have a control over the dilution process.
The results showed clearly assigned genotypes for all 32 samples. Genotypes from 31 samples were in accordance with the results from earlier PCR-RFLP tests. One sample was genotyped as homozygous resistant with genotype AA, while the data from the PCR-RFLP test listed the sample as homozygous susceptible with genotype GG. However, sequencing of that sample gave certainty that the sample was homozygous resistant with genotype AA and that the KASP result was therefore correct. The three undiluted duplicates were genotyped in accordance with their diluted counterpart.

3.2. Tm-shift

For Tm-shift, both variants of primers, with or without a mismatch right before the SNP (see Chapter 2.2.10), were tested. For that purpose, six samples, two of each genotype, were analysed with the primers for CFCH2 and ALGA5. Primer mix C (equal ratios) was used. In order to find the best primer ratio, other primer mixes were tested too as described in the chapter 2.2.10, but those resulted in far more unequal peaks than the equated primer mix C, partially up to the point where the samples from the different genotypes could not be distinguished anymore.

Results are shown in Figures 11 to 14.
Figure 11: Derivative melting curves from the Tm-shift assay for CFCH2 with the matching primers. Samples are represented as coloured curves indicating the fluorescence changes during melting. A rise from a curve means a faster decrease of fluorescence or rather a faster melting from the PCR products. Peaks represent the melting point where melting is fastest. The resistant allele leads to a peak at 75°C and the susceptible allele to a peak at 78°C.

Figure 12: Derivative melting curves from the Tm-shift assay for CFCH2 with the mismatch primers. The shorter primer leads to a peak at 72°C and the longer primer to a peak at 77°C. See Figure 11 for further explanation.

Figure 11 shows the melting curve results for CFCH2 with the matching primers. The different genotypes can clearly be distinguished based on their distinct melting curves. The homozygous resistant samples (ss) were amplified with the shorter primer and showed a peak at a lower temperature (75°C) compared to the homozygous susceptible samples (SS), which were amplified with the longer primer (78°C). The heterozygous samples (Ss) show both peaks, indicating that both alleles were amplified. No amplification took place in the NTCs, so that there is only a slight peak at very low temperatures indicating the unused primers. Overall, the matching primers for CFCH2 amplified well and allele-specificity was good, resulting in a clear and expected picture.

Figure 12 shows the melting curve results using the mismatch primers. All samples have a strong peak at a higher temperature (77°C) and only the homozygous resistant samples have a peak at a lower temperature (72°C) too. This means that the longer primer for the susceptible allele amplified much stronger than the short primer and that the longer primer was poorly allele-specific.
The assay for ALGA5 gave similar results as the assay for CFCH2. Figures 13 and 14 illustrate the melting curve results for ALGA5. With the matching primers, genotypes could be properly detected also in ALGA5. Peaks at a lower temperature (78°C) represent the susceptible allele S, while peaks at a higher temperature (82°C) represent the resistant allele s. The longer primer was not totally allele-specific, leading to a small shoulder in the melting curve of homozygous susceptible samples at a higher temperature. Also for heterozygous samples, the peak at higher temperatures was slightly higher than the peak at lower temperatures, but that was not a problem for the differentiation between the three genotypes.

The mismatch primers for ALGA5 gave worse results compared to the matching primers, just like in the case of CFCH2. Homozygous samples had the peaks in their melting curve as expected and could be distinguished from each other, but the heterozygous samples were almost exclusively amplified with the shorter primer. This means again that the allele-specificity from one of the primers was bad, in this case from the shorter primer.
For a further test of the genotyping ability of the Tm-shift assay, 16 samples of different genotypes were included on the plate and investigated on \( CFCH2 \) with matching primers in mix C. All 16 samples gave distinct melting curves and they could easily be assigned to their expected genotype (Figures 15 and 16). Therefore, the genotyping results of these 16 samples were completely in accordance with earlier data.

<table>
<thead>
<tr>
<th>( CFCH2 ) – Genotypes Samples 1-8</th>
<th>( CFCH2 ) – Genotypes Samples 9-16</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1.png" alt="Melt Curve" /></td>
<td><img src="image2.png" alt="Melt Curve" /></td>
</tr>
</tbody>
</table>

Figure 15: Derivative melting curves from the first eight samples analysed on \( CFCH2 \) with matching primers.

The distinct melting curves from all three genotypes are clearly visible: Homozygous susceptible with just one peak at a higher temperature (78°C), homozygous resistant with just one peak at a lower temperature (75°C) and heterozygous with two peaks, one at each temperature point.

Figure 16: Derivative melting curves from a further eight samples analysed on \( CFCH2 \) with matching primers.
3.3. HRM

Thirty-eight samples of known genotype were analysed with HRM on the markers CFCH2 and ALGA5. Additionally, seven of these samples were analysed as duplicates on the same plate.

HRM analysis results for CFCH2 showed that 23 of the 38 analysed samples were in accordance with previous data concerning their genotype, while 15 samples were assigned to another genotype than expected. The five homozygous susceptible samples were all genotyped as homozygous susceptible with HRM and no other samples were genotyped as homozygous susceptible. From the 13 samples previously known as homozygous resistant, one was genotyped as heterozygous with HRM and from the 20 heterozygous samples, 14 were genotyped as homozygous resistant while only 6 were genotyped as expected.

While the homozygous susceptible samples could clearly be distinguished from the other two genotypes, the homozygous resistant and the heterozygous samples could not be distinguished properly from each other. This result can be seen on the plot of the aligned melting curves and on the difference plot (Figures 17 and 18). The curves from samples determined as homozygous resistant (red) and heterozygous (green) are just adjacent to each other and clear differences are not visible, while the curves from homozygous susceptible samples (blue) are clearly different from the other curves.

Figure 17: Aligned melting curves for all 38 samples, analysed on CFCH2. Each curve represents one sample. Blue melting curves represent homozygous susceptible samples. Red curves were determined as homozygous resistant and green curves as heterozygous.
Figure 18: Difference plot for all 38 samples, analysed on CFCH2. Each curve represents one sample. The melting curve from one homozygous susceptible sample was taken as standard, and the difference from the melting curves from all other samples was calculated and illustrated in the plot. Blue melting curves represent homozygous susceptible samples. Red curves were determined as homozygous resistant and green curves as heterozygous.

The seven duplicated samples were all either heterozygous or homozygous resistant. In accordance with the results over all samples, three of them were assigned to the expected genotype and four of them were not genotyped as expected. From the sample pairs that were analysed twice, five sample pairs exhibited the same result and two showed different results.

The HRM assay for ALGA5 did not work as expected. The HRM analysis software showed multiple peaks in the melting curves, large difference in fluorescence between samples, and wavelike curves in general (Figure 20). As a result, it was impossible to differentiate between any genotypes. The difference plot shows also, that the software was not able to detect genotypes (Figure 19). The curves from one sample to the other were so different, that the software determined five different variants. Moreover, even within variants there were still huge differences as the green curves illustrate as an example.
Figure 19: Difference plot for all 38 samples, analysed on ALGA5. Each curve represents one sample. The melting curve from one sample was taken as standard, and the difference from the melting curves from all other samples was calculated and illustrated in the plot. Different colours indicate different variant calls from the software. The samples cannot be associated with any genotype, since the differences between the curves are too chaotic.

Figure 20: Derivative melting curves for all 38 samples analysed on ALGA5. Each curve represents one sample. Colours indicate the different variant calls from the difference plot (Figure 19). Multiple peaks and wavelike curves are shown, while a picture of only two peaks that clearly differ from the basic level of the curves was expected.
4. Discussion

4.1. KASP

4.1.1. Interpretation of the KASP results

For the KASP assays for *CFCH2* and *ALGA5*, a working procedure was found that showed high reliability in genotyping. With the optimized assays, all determined samples had their correct genotype associated. However, there was one sample in each assay that remained undetermined in their genotype. These undetermined samples raised evidence for some critical points for accurate genotyping, as did the differences between the original and the optimized assays.

One sample stood out several times, the heterozygous sample 2151, as described in chapter 3.1.1. A possible reason could be that the sample was of bad or too different quality, compared to the other samples and therefore made it difficult to compare. The samples used for those assays were of different age, different origin (blood or tissue), different extraction times, and were used in different previous studies. In summary, they were quite heterogeneous and relatively old in general. That probably influenced the clustering in the allelic discrimination plot.

In the first assays done for *CFCH2* and *ALGA5*, the three samples of very low DNA concentration indicated that too low concentrations or too big concentration differences between samples might be a problem for genotyping with KASP. Samples of the same genotype cluster were also spread over a wide area, which supported this hypothesis. Therefore, for an optimized assay, samples with high DNA concentrations were diluted to get more equal concentrations and samples with a DNA concentration below 4 ng/µl were excluded from further analyses.

Instead of the exclusion, a DNA precipitation could have been done, which would have been an interesting idea to see if it would have been possible to accurately genotype the concentrated samples. The exclusion of the samples makes it also somewhat complicated to draw conclusions from the comparison of the original and the optimized assays. Therefore, possible conclusions are more limited.

If we compare the original assays for *CFCH2* and *ALGA5* with the optimized assays, there is not an absolute clear improvement beside the excluded samples. In *CFCH2*, the unassigned sample 2151 remained unassigned in the optimized assay. Also in the optimized assay for *ALGA5*, there were still
unassigned samples. The clustering of the samples seems better in the optimized assays, but the scale is also different, so the actual difference is a little smaller. However, there were also some indications for an improvement through the dilution. In ALGA5, the sample 2151 was genotyped incorrect in the original assay and another sample remained unassigned, while these samples were both accurately genotyped in the optimized assay. Furthermore, the results from the optimized assay for ALGA5 could be taken after five additional cycles already, instead of ten additional cycles in the original assay.

In summary, the dilution and balance of the DNA concentration seem to have a positive effect on the results.

It is also debatable whether the influence of the dilution may be based on the possible advantage of more equal concentrations or more likely, if the dilution effect may just have lowered the concentration of PCR inhibitors. Therefore, for the analyses done, possible influences of DNA concentrations and of sample quality cannot really be separated, as there are too much uncertainties and not enough information available about sample quality.

The assays for CFCH1 and F18 M307 gave further evidence for the proposed influences in this chapter and no contradictions were found.

The unassigned sample in the CFCH1 assay may be explained again through sample quality, while the sample that did not amplify was probably because no DNA material was pipetted into that well. The unassigned sample between the heterozygous and the homozygous AA cluster was the same sample that remained unassigned in the optimized assay for ALGA5, therefore it is likely that the same reason applies to both occurrences.

The results from the KASP assays for the different SNPs seem to improve over the different assays, since less PCR cycles were needed for the assays for CFCH1 and F18 M307 than the assays for CFCH2 and ALGA5. The CFCH1 and the F18 samples are considered more equal in quality than the collection of different samples that was used for CFCH2 and ALGA5, which could explain these findings. One reason for this hypothesis is that all samples for CFCH1 and F18 originated only from blood, while the samples for CFCH2 and ALGA5 originated from blood and tissue. Furthermore, the CFCH1 samples were all done in one single extraction.
4.1.2. Cycle number

The results from the first assay for ALGA5 (see Figure 8) showed that the number of PCR cycles has a large effect on the genotyping results and that it is important to find an ideal number of cycles. This can be done through a repetition of a few PCR cycles if the result after the regular 40 cycles is not satisfying. These re-cycling steps need to be small enough, because the cycling is irreversible and the result can get worse, once a certain number of cycles are exceeded.

The results suggest that an increase in the cycle number, through a re-cycling, may help to genotype difficult samples. The original assays for CFCH2 and ALGA5, as well as the optimized assay for CFCH2 were done with 50 cycles (one re-cycling step of 10 cycles), while the optimized assay for ALGA5 was done with 45 cycles (5 cycles of re-cycling) and the assays for CFCH1 and F18 M307 were done with 40 cycles (no re-cycling).

4.1.3. Adaptation of KASP for E. coli F18 test

Since KASP as a method is designed to work identically, independent of the SNP investigated, the adaptation for the E. coli F18 test was simple and without problem at all. The achieved results were good, as all 32 samples were genotyped with 100% concordance to their known genotype, tested with PCR-RFLP or sequencing.

In the discrimination plot, the position of the heterozygous cluster relative to the two homozygous clusters can vary depending on the SNP, as the position indicates the relation of the amplification efficiency between the two allele-specific primers. For a good KASP assay, equal amplification efficiency is needed. Ideally, the heterozygous cluster is located in the middle between the two homozygous clusters in a sufficient distance to both of them.

If the amplification efficiency between the primers is insufficient, there is no possibility to alter the ratio between the two primers, since the primer mix is premixed by the company. However, a success rate of 90% is declared for KASP assays without validation, and the company also offers a validated KASP assay for a SNP at increased costs. This might be considered either in advance or for a specific assay that did not work.

The KASP assay for F18 as well as the assays for the other SNPs did not show problems with amplification differences between the two primers. So, based on the results of the present thesis, the KASP assay can be safely used for the E. coli F18 test.
4.1.4. Reliability of KASP

Over all KASP analyses done with diluted samples (DNA concentrations ranged from 7 ng/µl to 30 ng/µl), the samples were genotyped always in accordance to their known genotype or in case of \textit{CFCH1}, in accordance to the genotypes from other related marker SNPs. Only four samples in total remained unassigned to a genotype and importantly, no samples were genotyped contrary to their known genotype. This should be reasonably reliable so that KASP can be used as a method to test pigs on \textit{F4bcR} marker genotypes.

Further improvements may be achieved with more samples per genotype, which should make the boundaries between genotype clusters more clear, but sample number is not always under full control of an experimenter. Alternatively, more thorough re-cycling in small steps might lead to improved results too, if the best possible cycle number can be found.

If optimizations do not lead to a better result, the uncertain samples can be easily identified and analysed a second time with another method. Although a second analysis would increase the costs, KASP may still be advantageous compared to methods like sequencing, if only a few samples need to be confirmed while the majority can be genotyped faster and at lower costs.

4.1.5. Applicability of KASP

KASP is a relatively simple method, both in its principle and the amount of work needed. For the primer design, the sequence around an SNP is simply sent to the company, which then designs the assay. So no own work is needed for the primer design. The reagents are then delivered in two parts, primer mix and reaction mix. This also allows an easy preparation of a KASP analysis, since the primer and reaction mix are combined to a master mix and pipetted into the 96-well plate, followed by the addition of DNA sample to the wells. Therefore, only two reagents need to be handled and a plate is filled in two steps. In addition, amplification and the result interpretation are simple as well.

Independent of the analysed KASP assay, the same basic PCR protocol is used. This is not only a simplification, but also allows analysing different SNPs on one plate and in the same run. This can be an advantage, especially when a smaller amount of samples needs to be genotyped on multiple SNPs, where one assay does not fill the plate.

Thanks to the allelic discrimination plot, the interpretation of the results is intuitive and simple. Undetermined or uncertain samples can easily be identified, if present.
The KASP assay, since carried out on 96-well plates, also fulfills the requirements described in the introduction of this thesis, such as high throughput and automation possibilities. Pipetting can be done with different levels of automation, using multi-channel pipettes or a pipetting robot. However, a disadvantage of KASP may be that there is no possibility to alter the primer ratios, due to their premixed state. A challenge may be to find the ideal cycle number, if re-cycling steps are meaningful or necessary. To make sure the ideal result is not missed, re-cycling needs multiple small steps, which itself needs time and patience. Therefore, if a lot of re-cycling is needed, the advantages of KASP might be diminished.

KASP poses requirements on the minimal number of samples that need to be analysed on a plate, since multiple samples per genotype are needed to ensure sufficient clustering and allow genotyping. The user manual recommends at least 22 samples, while it is also important that they are equally distributed between the three genotype clusters (KASP genotyping manual). Therefore, KASP as a method is more appropriate if many samples need to be genotyped and the minimal recommended number of samples can be exceeded. Otherwise, multiple control samples could be added to an analysis of just a few samples, but that would increase the costs. In such a case, it could possibly be better to use Tm-shift as genotyping method, which then should certainly be considered.

Overall, KASP can easily be used for genotyping by a smaller or medium lab, provided that a RT-PCR machine such as the ABI 7500 or another kind of fluorescence analyser that can read 96-well plates is available. PCR can be carried out in a common PCR machine, but a possibility to read the fluorescence after amplification is needed. The steps to perform genotyping with KASP are simple and little work is needed to possibly genotype many samples or even multiple assays. However, if just a few samples need to be genotyped, KASP has special disadvantages that need to be considered and in that case, other genotyping methods may be more adequate.
4.2. Tm-shift

4.2.1. Interpretation of the Tm-shift results

4.2.1.1. Matching primers

The Tm-shift results with matching primers showed clear melting curves that could easily be interpreted, and the samples were therefore easily genotyped. All tested samples were correctly genotyped, concordant with previous data. In the results for *ALGA5*, there was a slight predominance from the longer primer, leading to slightly unequal peak heights between the shorter and the longer primer in the heterozygous samples. This is probably because the longer primer was not completely allele-specific, as indicated by a small shoulder around 85°C in the melting curves from the resistant samples. Fortunately, this was not a problem for the genotyping of the tested samples. However, with a slight change to the primer ratios in favour of the short primer, this could possibly have been balanced. But a ratio of 2:1 in favour of the short primer was much too strong in the testing of alternative primer ratios (data not shown), meaning that the best ratio is probably just a wee bit over the 1:1 ratio, so that it would not be worth it to find the perfect ratio with trial and error, since the advantages for genotype discrimination would be too small.

Allele-specificity is crucial for this method and might be a topic where difficulties can raise in some assays. With *CFCH2* and *ALGA5*, this was not the case for the matching primers. However, alternations to the PCR protocol or the reagents, like manipulation of MgCl₂ concentration or a higher annealing temperature, may be helpful to improve allele-specificity in problematic assays. The use of an enhanced polymerase may also help for improved allele-specificity, as it was done by Wang et al. (2005). However, this could be a very expensive optimization that may not be practicable for most genotyping purposes, especially in smaller labs.

Before heavy investigations with trial and error are done in an attempt to improve allele-specificity, alternative primers should be tested. The primer design according to the Fluidigm Tm-shift protocol results in two alternative primer groups, depending on which DNA single strand the primers are selected. Usually one primer group is chosen over the other, according to the criteria in the protocol. However, if the first group of primers does not satisfy, the alternative group may be tested.
The results for CFCH2 and ALGA5 with matching primers showed some remarkable differences in peak heights between samples, especially the two heterozygous samples in CFCH2. Different peak heights indicate differences in melting speed and may be influenced by the conditions during melting and therefore sample quality. However, for genotyping, the temperature of the peak is more important than the actual level of the curve, and the differences in peak heights did not affect genotyping abilities in the analyses done.

4.2.1.2. Mismatch primers

Analyses with the mismatch primers, for which allele-specificity was expected to be improved, resulted in the opposite with poor or missing allele-specificity for some of the primers. Therefore, the different genotypes of the samples could not be distinguished.

It may be possible that mismatch primers make higher demands on the PCR conditions to reach good allele-specificity and that therefore changes to the PCR conditions, namely MgCl₂ concentration and the PCR program, could have improved the results.

In other studies, mismatch primers for Tm-shift were successfully used in genotyping. Derzelle et al. (2011) used mismatch primers in Tm-shift to discriminate Bacillus anthracis from other related bacterial strains. Their primers had the mismatch right next to the allele-specific nucleotide at the 3’-end of the primer as the second last nucleotide, just like the primers in the present study. Papp et al. (2003) used Tm-shift with mismatch primers for improved allele-specificity, but with a mismatch at the third base from the 3’-end of the primers. Those studies indicate that the location of the mismatch, as well as the mismatch base that was incorporated, give different possibilities that do not necessarily have the same outcome.

So in order to improve the results from the mismatch primers in the present thesis, different mismatch nucleotides and locations could have been tested. However, because the matching primers gave good results, the optimization of the mismatch primers was not followed up. Instead, it was focused on the matching primers.

4.2.2. Reliability of Tm-shift

As previously mentioned, the analysed samples were all in agreement with their previous genotyping data. Therefore, the analyses gave no indications on reliability problems of Tm-shift, as long as allele-
specificity of the primers is good, which can easily be seen on the melting curves from control samples for each genotype.

Control samples are as important for Tm-shift as for KASP, and the melting curves from unknown samples should always be compared to melting curves from control samples of each genotype, even though the shape of the curve may already indicate the corresponding genotype.

4.2.3. Applicability of Tm-shift

The effort for a Tm-shift assay can be compared to a KASP assay in many aspects. Especially plate preparation, pipetting and the amplification through PCR are comparable with only slight differences. Tm-shift as a “self-made” method has a few more small preparation steps, such as mixing the primers to a primer mix or adding fluorescent dye to the master mix.

The big difference between KASP and Tm-shift in regard to method application is the primer design. While primer design for KASP is completely carried out by the company, primer design for Tm-shift is completely under own control and done directly in the lab. This translates to more work for Tm-shift, but gives the advantage of the flexibility to change the primer ratios to the most suitable one. There is also more information available regarding primer properties for Tm-shift than for KASP, which may be an advantage in assay optimization.

KASP and Tm-shift differ also in their requirements regarding number of samples. For KASP, a minimal number of 22 samples is recommended for genotyping (see chapter 4.1.5) while for Tm-shift the results in the present study suggest that two control samples for each genotype are sufficient to assign the samples to the right genotype according to their melting curves. Therefore, Tm-shift may be at an advantage over KASP if only few samples need to be genotyped.

4.3. HRM

4.3.1. Interpretation of the HRM results

4.3.1.1. CFCH2

The HRM assay for CFCH2 had some difficulties to differentiate between homozygous resistant and heterozygous samples. With the earlier discussed influences of low sample quality on the KASP results, low sample quality may have also influenced the HRM results for CFCH2. The small wavelike
structures along the shape of the curves in the difference plot seem to be abnormal. They may be an indication for an interference coming from contaminants or problematic salt concentrations. If that would be the case, a dilution of the samples could have helped to improve the results, due to the dilution effect on contaminants.

To overcome the insufficient differences between the melting curves from homozygous resistant and heterozygous samples, an alternation of the MgCl\textsubscript{2} concentration in the reaction might have helped. As mentioned in the ABI HRM Guide, MgCl\textsubscript{2} can have a great effect on the differences in the melting curves between genotypes and therefore a titration experiment of MgCl\textsubscript{2} could have been appropriate.

Depending on the success of such optimization steps, it could be possible to develop a working HRM assay for CFCH2 based on the assay that was tested for the present study. At least the results suggest that the assay has the potential to accurately detect the CFCH2 genotypes.

4.3.1.2. ALGA5

In contrast to CFCH2, the HRM results for ALGA5 give no indication that genotyping with the tested assay could be successful. The assay failed to accurately genotype any of the 38 tested samples. Instead, it resulted in multiple peaks in the melting curve. The amplification plot showed long flat amplification curves that were hardly exponential and the plateau phase was reached very late, if at all, during the 40 PCR cycles (data not shown). Therefore, the amplification plot suggested that amplification was poor.

The poor amplification might be explained through PCR inhibition due to poor sample quality or contaminants in the primer mix. On the other hand, sample quality might not be enough to explain the findings, since the assay for CFCH2 was done with the same samples, but got a better result. Other explanations for the poor amplification could be that the primers were bad and did not work well, or that the PCR conditions were suboptimal.

Beside the problems in amplification, the multiple peaks in the melting curve indicate that unspecific amplification could have been a problem too. To explain those peaks, there might have been other DNA fragments that were amplified than the target fragment, or the amplification of the target fragment was incomplete due to PCR inhibition as proposed before.

Overall, the results for ALGA5 showed messy amplification and melting curves that prevented successful genotyping. The quality of the samples could have negatively influenced the assay and for further examination of HRM assays for ALGA5, investigation of the sample quality could be
informative. However, with the current state of the results for ALGA5, it would be reasonable to start again with the primer design and develop a new assay for ALGA5 with new primers.

4.3.2. Reliability of HRM

The HRM results for CFCH2 and ALGA5 in the present study showed no reliable genotyping. However, HRM as a method for genotyping is generally in use and other studies developed successful genotyping assays based on HRM, such as Derzelle et al. (2011), who used HRM to discriminate Bacillus anthracis from other related bacterial strains.

From the principles of the methods KASP, Tm-shift and HRM, it seems that HRM might be more susceptible to suboptimal conditions, due to the smaller differences that the method needs to discriminate with high resolution, compared to KASP or Tm-shift.

To reliably genotype pigs from practical farms on markers for E. coli F4ab/F4ac resistance, an assay must be robust and reliable with a broad range of sample quality. Based on the two tested HRM assays, this is not the case for HRM and therefore this method was not further considered.

4.3.3. Applicability of HRM

With the proposed higher susceptibility of HRM to suboptimal conditions compared to KASP or Tm-shift, it would be more difficult to reach the demands of HRM for reliable genotyping than to reach the demands of KASP or Tm-shift. This would be a disadvantage for HRM over KASP and Tm-shift.

In other aspects of method application, HRM can easily compete with the other two methods, as it is a straightforward method. Only two standard primers need to be designed with standard procedures compared to a more sophisticated protocol for Tm-shift primer design. In addition, plate preparation and PCR are as easy as with KASP and Tm-shift, while no allele-specific PCR is needed for HRM.

HRM result interpretation is a more complex topic though, and HRM results are analysed with a software solution specific to this method. The HRM software is an important matter of expense, which makes the method more expensive and less available for smaller labs or smaller genotyping projects.

This, together with lower robustness and extended needs for optimizations, makes HRM less suitable for pig genotyping for E. coli F4ab/F4ac resistance, compared to KASP and Tm-shift.
4.4. Pig genotyping on the markers *CFCH1* and *CFCH2*

While analyses of the SUISAG pigs of the Large White breeding lines showed 100% concordance between the two markers, *CFCH1* and *CFCH2*, and their phenotype as described in chapter 3.1.4 and Neuenschwander et al. (2013), 17 boars with recombinations between the two markers were found in the breeds Duroc, Piétrain and Landrace during routine genotyping of AI boars. From this 17 boars 15 were analysed and 14 were confirmed as recombinant whereas one turned to be resistant. Therefore, the two markers are highly reliable for the Swiss Large White population, since no recombinations were found in Large White pigs.

The adhesion phenotype of the 14 recombinant boars is investigated in an ongoing project and up to now, the phenotype from three Landrace boars has been determined as resistant (see chapter 3.1.4). These results indicate that for Landrace breeds, the marker SNP *CFCH1* is more reliable than *CFCH2*, which was recombinant in the three phenotyped boars.

For the breeds Duroc and Piétrain, it is not yet clear, which of the markers is more reliable, due to missing phenotype information. Pigs from these breeds can so far only be reliably genotyped for *E. coli* F4ab/F4ac adhesion, if they show concordant genotypes for both markers, *CFCH1* and *CFCH2*.

Including more markers such as *CFCH3* in recombinant AI boars could also lead to further information on the exact location of the *F4bcR* locus.
5. Conclusions

We found KASP to be a suitable method to genotype pigs on marker SNPs for \textit{E. coli} F4ab / F4ac resistance. Furthermore, KASP is a simple method and allows genotyping with little work that follows simple steps. Therefore, it is perfectly suitable for a smaller to medium laboratory, provided the availability of a fluorescence plate reader. The analyses in the present study also suggest that KASP can be safely used for an \textit{E. coli} F18 test, since the results showed 100% accurate genotyping. The established PCR-RFLP test can therefore be replaced.

Tm-shift as a genotyping method was found to be comparable with KASP in reliability and applicability. Therefore, Tm-shift seems also to be suitable for marker genotyping for \textit{E. coli} F4ab / F4ac resistance. KASP and Tm-shift have different advantages over each other, depending on the situation. To genotype few samples, Tm-shift may be more suitable, while KASP may be favoured over Tm-shift to genotype many samples.

For HRM, we were unable to develop an assay that would have allowed to reliably genotype the marker SNPs, although the method was successfully used by other studies. It seems that HRM is less robust and needs extended optimizations. Moreover, specific and costly software is needed for HRM analysis.

Genotyping of 271 AI boars on the markers \textit{CFCH1} and \textit{CFCH2} revealed 17 recombinant boars belonging to the breeds Duroc, Piétrain and Landrace. The first phenotyping of three recombinant Landrace boars suggest that marker \textit{CFCH1} is superior over \textit{CFCH2}, whereas in Large White pigs both markers are equally reliable for \textit{E. coli} F4ab/F4ac genotyping. The lack of phenotypes in Duroc and Piétrain recombinant pigs does not allow prioritizing a marker. So far, only Duroc and Piétrain pigs showing concordant \textit{CFCH1} and \textit{CFCH2} markers can be classified in a breeding program for F4ab/F4ac resistant pigs.

Further analysis of the recombinant boars may lead to new evidence on the causative mutation responsible for \textit{E. coli} F4ab/F4ac adhesion.
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(http://www.lgcgenomics.com/genotyping/kasp-genotyping-reagents/, January 2014)
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Appendix

Media and Solutions

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Product information KASP assays

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